THE BRINE SHRIMP
ARTEMIA

volume 2

editors :
g. persoone
p. sorgeloos
o. roels
e. jaspers

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THE BRINE SHRIMP
ARTEMIA

Guido PERSOONE, Patrick SORGELOOS,
Oswald ROELS and Edmonde JASPERS

Editors

Proceedings of the International Symposium on the brine shrimp *Artemia salina*.
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State University of Ghent
J. Plateaustraat 22
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- *The University of Texas*
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- *Hernandorena A. (France)*
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- *Sandifer P. (USA)*
- *Simpson K. L. (USA)*
- *Sorgeloos P. (Belgium)*
- *Warner A. H. (Canada)*

Foreword

The "International Symposium on the brine shrimp Artemia salina*" was convened at the La Quinta Royale Motor Inn in Corpus Christi (Texas, USA) in August 1979 and was attended by approximately 200 participants from 26 countries of the five continents.*

This symposium was entirely devoted to all research and application aspects of an aquatic invertebrate which is used worldwide as a most suitable study object for fundamental research in practically all biological disciplines, and which moreover constitutes an indispensable source of live food for larval crustaceans and fishes mass cultured for commercial purposes.

The purpose of the Convention was three-fold :

- *To bring together all those working or interested in* Artemia *to exchange their findings on this unique crustacean ;*
- *To promote contacts among specialists in different* Artemia *research areas and to stimulate interdisciplinary research on brine shrimp ;*
- *To publish the reviews, the contributed papers and the syntheses and recommendations of the workshops as a reference book giving the state of the art of the present knowledge on* Artemia.

Because the response to the call for papers was overwhelming, the program was divided in concurrent sessions with the following topics :

Morphology – Radiobiology – Genetics
Physiology – Toxicology
Biochemistry – Molecular Biology
Ecology – Culturing – Use in Aquaculture

During these sessions seven reviews covering the major areas of Artemia *research were presented by invited authorities and complemented by approximately 100 contributed papers.*

In order to make a synopsis of the progresses reported at the Symposium and to define particular areas of Artemia *research deserving urgent attention, the last day of the Symposium was devoted to four workshops on the following themes :*

- *Characterization of* Artemia *strains for application in aquaculture ;*
- *Commercial aspects of* Artemia *exploitation ;*
- *Species characterization in* Artemia *;*
- *Proposal for an intercalibration exercise for a standard* Artemia *toxicity test.*


Welcome International Symposium on Brine Shrimp
"ARTEMIA SALINA"

Group picture of participants

Despite all efforts and help we have not been able to identify all the people on the picture. We apologize for the "blanks" and list the names of those we recognized.

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153. Arantxa Hernandorena
154. Joan Mitchell
155. Anthony D'Agostino
157. Pauline Riordan
158. Masotoshi Kondo

List of participants

Abreu-Grobois A., University College of Swansea, Department of Genetics, Singleton Park, Swansea SA2 8PP, UK.

Acey R., Wayne State University, School of Medecine, 540 East Canfield Avenue, Detroit, Michigan 48201, USA.

Allen K. A., Sanders Brine Shrimp Company, 1180 W. 4600 So., Odgen, Utah 84404, USA.

Amat Domenech F., Planta Piloto de Acuicultura, Torre de la Sal, Castellon de la Plaña, Spain.

Anadon A., University of Oviedo, Department of Zoology, Oviedo, Spain.

Anadon E., University of Oviedo, Department of Zoology, Oviedo, Spain.

Anderson S. M., University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373, USA.

Bagshaw J. C., Wayne State University, Department of Biochemistry, 540 East Canfield Avenue, Detroit, Michigan 48201, USA.

Barrera T. C., Universidad Autonoma Metropolitana, Apartado Postal 23-181, Mexico DF, Mexico.

Barigozzi C., Universita di Milano, Istituto di Genetica, Via Celoria 10, I-20133 Milan, Italy.

Barlow S., 9111 Wilshire Boulevard, Beverly Hills, California 90213, USA.

Bates S. S., Aqua Finca de Camarones, Apartado Postal 677, San Pedro Sula, Honduras.

Baudo G., Idroallevamenti SRL, Via B. Crespi 70, I-20121 Milano, Italy.

Baust J. G., University of Houston, Department of Biology, Houston, Texas 77004, USA.

Beck A., US-EPA, Environmental Research Laboratory, South Ferry Road, Narragansett, Rhode Island 02882, USA.

Belk D., University of San Antonio, 840 E. Mulberry Street, San Antonio, Texas 78212, USA.

Bengtson D. A., US-EPA, Environmental Research Laboratory, South Ferry Road, Narragansett, Rhode Island 02882, USA.

Bes J. C., Laboratoire de Biologie Médicale, Faculté de Medecine, 37 Allées Jules Guesde, F-31000 Toulouse, France.

Bilio M., Piazza del Santo 4, I-35100 Padova, Italy.

Boddeke R., Netherlands Institute for Fisheries Research, Haringkade 1, IJmuiden NL-1970 AB, Netherlands.

Bossuyt E., State University of Ghent, Artemia Reference Center, J. Plateaustraat 22, B-9000 Ghent, Belgium.

Bowen S. T., San Francisco State University, Department of Biology, 1600 Holloway, San Francisco, California 94132, USA.

Bruce R. D., Route 2, Box 411, Crystal River, Florida, USA.

Bruggeman E., State University of Ghent, Artemia Reference Center, J. Plateaustraat 22, B-9000 Ghent, Belgium.

Brune D., University of California, Department of Agricultural Engineering, Davis, California 95616, USA.

Cacace G. M., Laboratory of Molecular Embryology, Via Toiano 2, I-80072 Arco Felice (Na), Italy.

Campello S., Instituto De Pesquisas da Marinha, Rua Ipiru s/n, Ilha do Governador, Rio de Janeiro, Brazil.

Cano A., University of Michigan, Division of Biological Science, Natural Science Building, Ann Arbor, Michigan 48109, USA.

Castelli S., Idroallevamenti SRL, Via B. Crespi 70, I-20121 Milano, Italy.

Cervera M., Massachusetts Institute of Technology 56-527, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA.

Chanley M., Fundacion Chile, Casilla 773, Santiago, Chile.

CLAUWAERT J., University of Antwerp UIA, Department of Cell Biology, B-2610 Wilrijk, Belgium.
CLEGG J. C., University of Miami, Department of Zoology, Laboratory for Quantitative Biology, Coral Gables, Florida 33124, USA.
COHLAN B. F., 2850 Moraga Drive, Los Angeles, California 90024, USA.
COLEMAN D., 2209 A Seaview Avenue, Honolulu, Hawaii, 96822, USA.
COLLINS N. C., University of Toronto, Erindale College, 3359 Mississauga RD, Mississauga L5L-1C6, Ontario, Canada.
CONTE F. S., University of California, Rm. 554 Hutchinson Hall, Davis, California 95616, USA.
CONTE F.P., Oregon State University, Department of Zoology, Corvallis, Oregon 97331, USA.
CORAZZA L., Cornell University, Department of Natural Resources, Fernow Hall, Ithaca, New York 14853, USA.
CRIEL G., State University of Ghent, Laboratory for Anatomy, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium.
CROWE J. H., University of California, Department of Zoology, Davis, California 95616, USA.
CUMMINS B., Intensive Culture Systems, P.O. Box 509, Route 2, Summerland, Florida 33042, USA.
D'AGOSTINO A. S., New York Ocean Science Laboratory, Edgemere Road, Montauk, New York 11954, USA.
DANA G., 1388 Sanchez, San Francisco, California 94131, USA.
DAVIS J. S., University of Florida, Department of Botany, Gainesville, Florida 32611, USA.
DE BOER J., University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373, USA.
DECLEIR W., University of Antwerp RUCA, Laboratory for Biochemistry, Slachthuislaan 68, B-2000 Antwerp, Belgium.
DE LOS SANTOS C., Jr., Ceramar Agro Marine Industries, 330 Mac Arthur Drive, Jaro, Iloilo 5931, Philippines.
DEESE H., Weyerhaeuser Company, 1788 NW 8 Avenue, Homestead, Florida 33030, USA.
DOBBELEIR J., State University of Ghent, Artemia Reference Center, J. Plateaustraat 22, B-9000 Ghent, Belgium.
DYE J. E., White Fish Authority, Marine Farming Unit, Ardtoe, Argyll PH36 4LD, UK.
RED EWALD H. J., Red Ewald, Inc., P.O. Box 519, Highway 181, South Karnes City, Texas 78118, USA.
FARLEY K., University of California, Department of Zoology, Davis, California 95616, USA.
FELICETTI L., Laboratorio di Biologia Cellulare, C.N.R., Via Romagnosi 18/A, I-00196 Rome, Italy.
FINKEL A., Technion Israel Institute of Technology, Department of Engineering, Technion City, Haifa, Israel.
FISHER J., Ralston Purina Co., P.O. Box 545, Crystal River, Florida 32629, USA.
FORMAN W., Freeport Sulphur Co., P.O. Box 61520, New Orleans, Louisiana 70161, USA.
GAFFNEY P., University of California, Department of Animal Science, Davis, California 95616, USA.
GAMBLE S., 1663 Dove Creek Road, Tavernier, Florida 33070, USA.
GAUBIN Y., Faculté de Medecine, Laboratoire de Biologie Médicale, 37 Allées Jules Guesde, F-31000 Toulouse, France.
GEDDES M. C., University of Adelaide, Zoology Department, Adelaide 5000, South Australia, Australia.
GLUDE J. B., 2703 W. McGraw, Seattle, Washington 98119, USA.
GOLDSTEIN B., University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373, USA.
GOPHEN M., Oceanographic and Limnological Research Co. Ltd., Kinneret Limnological Laboratory, P.O. Box 345, Tiberias, Israel.
GOY J., 200 Calico Court Apts., Morehead City, North Carolina 28557, USA.
GROSCH D. S., North Carolina State University, Genetics Department, Raleigh, North Carolina 27650, USA.
GUIER M. E. R., Maricultura S.A., P.O. Box 5255, San José, Costa Rica.
GUNTHER D. C., San Francisco State University, Biology Department, 1307 Eureka Avenue, Davis, California 95616, USA.

Halscott K. R., Florida Department of Natural Resources, Marine Research Laboratory, 100 8th Avenue SE, St. Petersburg, Florida, USA.
Hartlins D. K., University of Hawaii, Bekesy Laboratory of Neurobiology, 1993 East-West Road, Honolulu, Hawaii, 96822, USA.
Hastings R., P.O. Box 66, Comfort, Texas 28013, USA.
Haxby R., Morton Salt Company, Morton Bahamas Limited, Matthewtown, Inagua, Bahama Islands.
Hedgecock D., University of California, Bodega Marine Laboratory, Bodega Bay, California 94923, USA.
Herbst D. B., Oregon State University, Department of Zoology, Corvallis, Oregon 97331, USA.
Hernandorena A., Muséum National d'Histoire Naturelle, Plateau de l'Atalaye, F-64200 Biarritz, France.
Hines H. B., University of Houston, Department of Biophysical Sciences, Houston, Texas 77004, USA.
Hoet E., Cultiostras SRL, Apartado 61092, Caracas, Venezuela.
Hoff F., Instant Ocean Hatcheries Inc., Route 2, Box 86, Dade City, Florida 33525, USA.
Hover R. J., 3465 W. Bardot, Tucson, Arizona 85704, USA.
Hultin T., Wenner-Gren Institute, Department of Cell Physiology, Norrtullsgatan 16, S-113 45 Stockholm, Sweden.
Hurley D., Keys Aquaculture Industries, P.O. Box 772, Tavernier, Florida 33070, USA.
Insalata I. A., Aquafauna Inc., P.O. Box 91272, Los Angeles, California 90009, USA.
Ishiyama H., Warner/Lambert Japan Ltd., Kowa, Bldg. No. 31, 19-1, Shiroganedai 3, Chome Minatoku, Tokyo 108, Japan.
Isunza R. M. Alvaro Obregon No. 269-7th Floor, Mexico City, Mexico.
Iwasaki T., Division of Biology, National Institute of Radiological Sciences, Chiba, Japan.
Jaspers E. Institute for Marine Scientific Research, Prinses Elisabethlaan 69, B-8401 Bredene, Belgium.
Javor B., Exportadora de Sal, P.O. Box 150, San Diego, California 92112, USA.
Jayaraman K., Madurai University, Department of Molecular Biology, Madurai 625 021, India.
Johns D. M., US-EPA, Environmental Research Laboratory, South Ferry Road, Narragansett, Rhode Island 02882, USA.
Johnson D. A., US-EPA, Environmental Research Laboratory, South Ferry Road, Narragansett, Rhode Island 02882, USA.
Kerby J., North Carolina State University, Zoology Department, P.O. Box 5577, Raleigh, North Carolina 27650, USA.
Klein-MacPhee G., US-EPA, Environmental Research Laboratory, South Ferry Road, Narragansett, Rhode Island 02882, USA.
Kondo M., University of Antwerp UIA, Department of Cell Biology, Universiteitsplein 1, B-2610 Wilrijk, Belgium.
Koshida Y., Osaka University, Department of Biology, Machikaneyama 1-1, Toyonaka, Osaka 560, Japan.
Kramer G. A., University of Texas at Austin, Clayton Foundation Biochemical Institute, Department of Chemistry, Austin, Texas 78712, USA.
Lai L. Aquafauna Inc., P.O. Box 91272, Los Angeles, California 90009, USA.
Laramore C., Ralston Purina Company, Microbiology Department, Checkerboard Square, St. Louis, Missouri 63188, USA.
Leger P., State University of Ghent, Artemia Reference Center, J. Plateaustraat 22, B-9000 Ghent, Belgium.
Lenz P. H., University of California, Department of Biological Sciences, Santa Barbara, California 93106, USA.
Leonhard S. L., Canada Department of Fisheries and the Environment, Freshwater Institute, 501 University Crescent, Winnipeg R3T 2N6, Manitoba, Canada.
Lytwyn M., Hartz Mountain Corporation, 700 South Fourth Street, Harrison, New Jersey 07029, USA.
MacDonald G. H., Station Biologique de la Tour du Valat, F-13200 Le Sambuc, Arles, France.
Malecha S. R., Anuenue Fisheries and University of Hawaii, Area 4, Sand Island, Honolulu, Hawaii 96819, USA.

Clauwaert J., University of Antwerp UIA, Department of Cell Biology, B-2610 Wilrijk, Belgium.
Clegg J. C., University of Miami, Department of Zoology, Laboratory for Quantitative Biology, Coral Gables, Florida 33124, USA.
Cohlan B. F., 2850 Moraga Drive, Los Angeles, California 90024, USA.
Coleman D., 2209 A Seaview Avenue, Honolulu, Hawaii, 96822, USA.
Collins N. C., University of Toronto, Erindale College, 3359 Mississauga RD, Mississauga L5L-1C6, Ontario, Canada.
Conte F. S., University of California, Rm. 554 Hutchinson Hall, Davis, California 95616, USA.
Conte F.P., Oregon State University, Department of Zoology, Corvallis, Oregon 97331, USA.
Corazza L., Cornell University, Department of Natural Resources, Fernow Hall, Ithaca, New York 14853, USA.
Criel G., State University of Ghent, Laboratory for Anatomy, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium.
Crowe J. H., University of California, Department of Zoology, Davis, California 95616, USA.
Cummins B., Intensive Culture Systems, P.O. Box 509, Route 2, Summerland, Florida 33042, USA.
D'Agostino A. S., New York Ocean Science Laboratory, Edgemere Road, Montauk, New York 11954, USA.
Dana G., 1388 Sanchez, San Francisco, California 94131, USA.
Davis J. S., University of Florida, Department of Botany, Gainesville, Florida 32611, USA.
De Boer J., University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373, USA.
Decleir W., University of Antwerp RUCA, Laboratory for Biochemistry, Slachthuislaan 68, B-2000 Antwerp, Belgium.
de los Santos C., Jr., Ceramar Agro Marine Industries, 330 Mac Arthur Drive, Jaro, Iloilo 5931, Philippines.
Deese H., Weyerhaeuser Company, 1788 NW 8 Avenue, Homestead, Florida 33030, USA.
Dobbeleir J., State University of Ghent, Artemia Reference Center, J. Plateaustraat 22, B-9000 Ghent, Belgium.
Dye J. E., White Fish Authority, Marine Farming Unit, Ardtoe, Argyll PH36 4LD, UK.
Red Ewald H. J., Red Ewald, Inc., P.O. Box 519, Highway 181, South Karnes City, Texas 78118, USA.
Farley K., University of California, Department of Zoology, Davis, California 95616, USA.
Felicetti L., Laboratorio di Biologia Cellulare, C.N.R., Via Romagnosi 18/A, I-00196 Rome, Italy.
Finkel A., Technion Israel Institute of Technology, Department of Engineering, Technion City, Haifa, Israel.
Fisher J., Ralston Purina Co., P.O. Box 545, Crystal River, Florida 32629, USA.
Forman W., Freeport Sulphur Co., P.O. Box 61520, New Orleans, Louisiana 70161, USA.
Gaffney P., University of California, Department of Animal Science, Davis, California 95616, USA.
Gamble S., 1663 Dove Creek Road, Tavernier, Florida 33070, USA.
Gaubin Y., Faculté de Medecine, Laboratoire de Biologie Médicale, 37 Allées Jules Guesde, F-31000 Toulouse, France.
Geddes M. C., University of Adelaide, Zoology Department, Adelaide 5000, South Australia, Australia.
Glude J. B., 2703 W. McGraw, Seattle, Washington 98119, USA.
Goldstein B., University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373, USA.
Gophen M., Oceanographic and Limnological Research Co. Ltd., Kinneret Limnological Laboratory, P.O. Box 345, Tiberias, Israel.
Goy J., 200 Calico Court Apts., Morehead City, North Carolina 28557, USA.
Grosch D. S., North Carolina State University, Genetics Department, Raleigh, North Carolina 27650, USA.
Guier M. E. R., Maricultura S.A., P.O. Box 5255, San José, Costa Rica.
Gunther D. C., San Francisco State University, Biology Department, 1307 Eureka Avenue, Davis, California 95616, USA.

Halscott K. R., Florida Department of Natural Resources, Marine Research Laboratory, 100 8th Avenue SE, St. Petersburg, Florida, USA.
Hartlins D. K., University of Hawaii, Bekesy Laboratory of Neurobiology, 1993 East-West Road, Honolulu, Hawaii, 96822, USA.
Hastings R., P.O. Box 66, Comfort, Texas 28013, USA.
Haxby R., Morton Salt Company, Morton Bahamas Limited, Matthewtown, Inagua, Bahama Islands.
Hedgecock D., University of California, Bodega Marine Laboratory, Bodega Bay, California 94923, USA.
Herbst D. B., Oregon State University, Department of Zoology, Corvallis, Oregon 97331, USA.
Hernandorena A., Muséum National d'Histoire Naturelle, Plateau de l'Atalaye, F-64200 Biarritz, France.
Hines H. B., University of Houston, Department of Biophysical Sciences, Houston, Texas 77004, USA.
Hoet E., Cultiostras SRL, Apartado 61092, Caracas, Venezuela.
Hoff F., Instant Ocean Hatcheries Inc., Route 2, Box 86, Dade City, Florida 33525, USA.
Hover R. J., 3465 W. Bardot, Tucson, Arizona 85704, USA.
Hultin T., Wenner-Gren Institute, Department of Cell Physiology, Norrtullsgatan 16, S-113 45 Stockholm, Sweden.
Hurley D., Keys Aquaculture Industries, P.O. Box 772, Tavernier, Florida 33070, USA.
Insalata I. A., Aquafauna Inc., P.O. Box 91272, Los Angeles, California 90009, USA.
Ishiyama H., Warner/Lambert Japan Ltd., Kowa, Bldg. No. 31, 19-1, Shiroganedai 3, Chome Minatoku, Tokyo 108, Japan.
Isunza R. M. Alvaro Obregon No. 269-7th Floor, Mexico City, Mexico.
Iwasaki T., Division of Biology, National Institute of Radiological Sciences, Chiba, Japan.
Jaspers E. Institute for Marine Scientific Research, Prinses Elisabethlaan 69, B-8401 Bredene, Belgium.
Javor B., Exportadora de Sal, P.O. Box 150, San Diego, California 92112, USA.
Jayaraman K., Madurai University, Department of Molecular Biology, Madurai 625 021, India.
Johns D. M., US-EPA, Environmental Research Laboratory, South Ferry Road, Narragansett, Rhode Island 02882, USA.
Johnson D. A., US-EPA, Environmental Research Laboratory, South Ferry Road, Narragansett, Rhode Island 02882, USA.
Kerby J., North Carolina State University, Zoology Department, P.O. Box 5577, Raleigh, North Carolina 27650, USA.
Klein-MacPhee G., US-EPA, Environmental Research Laboratory, South Ferry Road, Narragansett, Rhode Island 02882, USA.
Kondo M., University of Antwerp UIA, Department of Cell Biology, Universiteitsplein 1, B-2610 Wilrijk, Belgium.
Koshida Y., Osaka University, Department of Biology, Machikaneyama 1-1, Toyonaka, Osaka 560, Japan.
Kramer G. A., University of Texas at Austin, Clayton Foundation Biochemical Institute, Department of Chemistry, Austin, Texas 78712, USA.
Lai L. Aquafauna Inc., P.O. Box 91272, Los Angeles, California 90009, USA.
Laramore C., Ralston Purina Company, Microbiology Department, Checkerboard Square, St. Louis, Missouri 63188, USA.
Leger P., State University of Ghent, Artemia Reference Center, J. Plateaustraat 22, B-9000 Ghent, Belgium.
Lenz P. H., University of California, Department of Biological Sciences, Santa Barbara, California 93106, USA.
Leonhard S. L., Canada Department of Fisheries and the Environment, Freshwater Institute, 501 University Crescent, Winnipeg R3T 2N6, Manitoba, Canada.
Lytwyn M., Hartz Mountain Corporation, 700 South Fourth Street, Harrison, New Jersey 07029, USA.
MacDonald G. H., Station Biologique de la Tour du Valat, F-13200 Le Sambuc, Arles, France.
Malecha S. R., Anuenue Fisheries and University of Hawaii, Area 4, Sand Island, Honolulu, Hawaii 96819, USA.

Mancebo V. J., San Miguel Corporation, 6766 Ayala Avenue, Makati, Metro Manila, Philippines.
Manzi J. J., South Carolina Marine Resources Center, P.O. Box 12559, Charleston, South Carolina 29412, USA.
Marco R., Universidad Autonoma de Madrid, Instituto de Enzimologia del CSIC, Arzobispo Morcillo s/n, Madrid 34, Spain.
McCourt R. P., Hofstra University, Biology Department, 1000 Fulton Avenue, Hempstead, New York 11550, USA.
McCoy P., University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373, USA.
McDonald L., University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373, USA.
McDonald P., University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373, USA.
McMaster M. F., Ocean Farming Systems Inc., P.O. Box 164, Tavernier, Florida 33070, USA.
McTigue K., 1083-A Reez Avenue, Sunnyvale, California 94086, USA.
Medlyn R. A., Florida Department of Natural Resources, Marine Research Laboratory, 100 8th Avenue S.E., St. Petersburg, Florida 33701, USA.
Metalli P., CNEN-CSN Casaccia, Laboratory of Animal Radiation, C.P. 2400, I-00100 Rome A.D., Italy.
Milligan D. J., Dow Chemical USA, Texas Division, 2302 Bio Research, Freeport, Texas, 77541, USA.
Mitchell J. R., National Science Foundation, 1800 G St., N.W., Washington DC 20550, USA.
Mock C. R., National Marine Fisheries Service, Galveston Laboratory, 4700 Avenue U, Galveston, Texas 77550, USA.
Morales J., State University of Ghent, Laboratory for Mariculture, J. Plateaustraat 22, B-9000 Ghent, Belgium.
Mauntain J., El Dorado 6-4600, Panama, Republica de Panama.
Naegel L., Universidad Nacional, Escuela de Ciencias Biologicas, Heredia, Costa Rica.
Newman H. W., MR Biologicals Inc., 25590 Seaboard Ln, Hayward, California 94545, USA.
Okazaki N. J., Bodega Marine Laboratory, University of California, P.O. Box 247, Bodega Bay, California 94923, USA.
Olney C. E., University of Rhode Island, Department of Food Science and Technology, Nutrition and Dietetics, 122, Woodward Hall, Kingston, Rhode Island 02881, USA.
Owens J. D., 23 Cardinal Road, Covington, Louisiana, USA.
Pavillon J. F., Institut Océanograhique, Laboratoire de Physiologie des Etres Marins, 195 rue Saint Jacques, F-75005 Paris, France.
Persoone G., State University of Ghent, Laboratory for Mariculture, J. Plateaustraat 22, B-9000 Ghent, Belgium.
Phillips T. R., US Fish and Wildlife Service, Route 1, Box 159D, San Marcos, Texas 78666, USA.
Planel H. R., Laboratoire de Biologie Médicale, Faculté de Médecine, 37 Allées Jules Guesde, F-31000 Toulouse, France.
Perez Rodriguez J. M., PEMARES, Edificio Casa del Mar, 5a planta, Cadiz, Spain.
Quinones D., Exportadora de Sal, P.O. Box 150, San Diego, California 92112, USA.
Reynolds R. S., Aquakultur, Development and Advice, D-2850 Bremerhaven, Fed. Rep. Germany.
Riazantsev Y. B., VNIRO, 17 V. Krasnoselskaya, Moscow, USSR.
Riordan P. F., Groton BioIndustries, 39 Hollis Street, P.O. Box 878, Groton, Massachusetts 01450, USA.
Robichaux D. M. Smithsonian Institute, National Museum of Natural History, NHB W-310, 10th Constitution, Washington DC 20560, USA.
Roels O. A., University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, Texas 78373, USA.
Samain J. F., Centre Océanologique de Bretagne, B.P. 337, F-29283 Brest Cedex, France.
Sanders G. C., Sanders Brine Shrimp Co., 1255 W. 4600 So. Ogden, Utah 84403, USA

Sandifer P. A., Marine Resources Research Institute, P.O. Box 12559, Charleston, South Carolina 29412, USA.
Schaaf S., Sandtech, 8368 Park Place, Houston, Texas 77017, USA.
Schauer P. S., University of Rhode Island, Department of Food Science and Technology, Nutrition and Dietetics, 122 Woodward Hall, Kingston, Rhode Island 02881, USA.
Schmidt A., San Francisco Bay Brand Company, 8239 Enterprise Drive, Newark, California 94560, USA.
Schurr K. M., Bowling Green State University, Department of Biology, Bowling Green, Ohio 43403, USA.
Scura E. D., Aquatic Farms Ltd., 49-139 Kamehameha Highway, Kaneohe, Hawaii 96744, USA.
Sebastian J., Universidad Autonoma di Madrid, Instituto de Enzimologia del CSIC, Arzobispo Morcillo s/n, Madrid 34, Spain
Seidel C. R., University of Rhode Island, Department of Food Science and Technology, Nutrition and Dietetics, 122 Woodward Hall, Kingston, Rhode Island 02881, USA.
Seitz P. K., University of Texas Medical Branch, Baylor College of Medecine, Hendrix Bldg, Galveston, Texas 77550, USA.
Servin-Massieu M. M., Universidad Autonoma Metropolitana, Apartado Postal 23-181, Mexico DF, Mexico.
Prescott S., New Technology Ltd., The Aquaculture Centre, Hadlow Kent, England.
Sharfstein B. A., University of Texas, St. Croix Marine Station, P.O. Box Z, Kingshill, St. Croix 00850, US Virgin Islands.
Shaw W., 209 Sycamore Avenue, Easton, Maryland 21601, USA.
Shleser R. A., Oceanic Institute, Makapuu Point, Waimanalo, Hawaii 96795, USA.
Sierra C. A., Sea Farms de Honduras, Apartado No. 33, Cholteca, Honduras.
Simpson K. L., University of Rhode Island, Department of Food Sciences and Technology, Nutrition and Dietetics, 122 Woodward Hall, Kingston, Rhode Island 02881, USA.
Sindermann C., Sandy Hook Laboratory, Highlands, New Jersey 07732, USA.
Sissom M. S. L., 904 Indiana, San Marcos, Texas 78666, USA.
Slessor P. J. H., Caribbean Development Bank, Wildey, P.O. Box 408 Bridgetown, Barbados, West Indies.
Slobin L. I., University of Mississippi, Department of Biochemistry Medical Center, 2500 North State Street, Jackson Mississippi 39216, USA.
Soejima T., University of Rhode Island, Department of Food Science and Technology, Nutrition and Dietetics, 122 Woodward Hall, Kingston, Rhode Island 02881, USA.
Spitchak M. K., Ministry of Fisheries, 12 Rozhdestvensky Boulevard, Moscow, USSR.
Sorgeloos P., State University of Ghent, Artemia Reference Center, J. Plateaustraat 22, B-9000 Ghent, Belgium.
Squire R. D., University of Puerto Rico, Biology Department, Mayaguez Campus, Mayaguez, Puerto Rico 00708, USA.
Steffens F., Aquakultur, Development and Advice, D-2850 Bremerhaven, Fed. Rep. Germany.
Strawn K., Texas A & M University, Department of Wildlife and Fisheries Sciences, College Station, Texas 77843, USA.
Szer W. W., New York University, Department of Biochemistry, 550 First Avenue, New York, New York 10016, USA.
Tank Yew R., 96 Western Main Road, St. James, Port-of-Spain, Trinidad, West Indies.
Teruel H. B., Universidad de Oriente, Instituto Oceanografico, Apartado Postal 6613, Caracas, Venezuela.
Tiro L. IDRC Scholar, 5990 Iona Drive, c/o IDRC, Vancouver, British Colombia V6T 1L4, Canada.
Tobias W. J., University of Texas, St. Croix Marine Station, Box Z, Kingshill, St. Croix 00850, US Virgin Islands.
Tyson G. E., Mississippi State University, Electron Microscope Center, Drawer EM, Mississippi 39762, USA.
Vanhaecke P., State University of Ghent, Artemia Reference Center, J. Plateaustraat 22, B-9000 Ghent, Belgium.

Versichele D., State University of Ghent, Artemia Reference Center, J. Plateaustraat 22, B-9000 Ghent, Belgium.

Wahba A. J., University of Mississippi Medical Center, Department of Biochemistry, 2500 North State Street, Jackson, Mississippi 39216, USA.

Warner A. H., University of Windsor, Department of Biology, Windsor, Ontario N9B 3PA, Canada.

Webber H. H., Groton BioIndustries, 39 Hollis Street, P.O. Box 878, Groton, Massachusetts 01450, USA.

Weintraub H., P.O. Box 371, Rockville Center, New York 11571, USA.

Wetherbee K. C., Sanders Associates Inc., 95 Canal St., Nashua, New Hampshire 03061, USA.

Williams D. C., 4264 Livermore P1, Cypress, California 90630, USA.

Wolfe A. F., Lebanon Valley College, Department of Biology, Annville, Pennsylvania 17003, USA.

Woodley C. L., University of Mississippi Medical Center, Department of Biochemistry, 2500 North State Street, Jackson Mississippi 39216, USA.

Yamada S., University of Rhode Island, K-8 Graduate Apartment, Kingston, Rhode Island 02881, USA.

Young J., 9111 Wilshire Boulevard, Beverly Hills, California 90213, USA.

Editorial note on the taxonomy of *Artemia*

The binomen Artemia salina *L. is taxonomically no longer valid (Bowen and Sterling, 1978).*

Crossing experiments of different Artemia *populations revealed reproductive isolation of several groups of populations (Halfer-Cervini* et al., *1968 ; Barigozzi, 1972, 1974 ; Clark and Bowen, 1976) and led to the recognition of sibling species to which different names have been given according to the International Conventions of Taxonomical Nomenclature (Bowen and Sterling, 1978). So far 20 bisexual strains have been classified into five sibling species (Bowen* et al., *1978).*

Theoretically the conventional name Artemia salina *L. can only be used for the original material from salt ponds in Lymington, England employed by Schlosser in 1755 to make the first drawing and by Linnaeus in 1758 to make the first description of the species (Kuenen and Baas-Becking, 1938). Because these salt ponds have disappeared, and* Artemia *no longer occurs in England, the species name* salina *should no longer be used.*

In view of the important genetical differences that exist between parthenogenetical strains of brine shrimp (Abreu-Grobois and Beardmore, 1980) species definition in the genus Artemia *has become confusing.*

It is suggested that unless the exact sibling species of a bisexual Artemia *strain can be identified* (cf. *Bowen* et al., *1978, 1980) and until speciation in brine shrimp is more clearly understood, only the genus designation* Artemia *should be used.*

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Life history of the brine shrimp *Artemia*

Patrick Sorgeloos[1]

Artemia Reference Center, State University of Ghent
J. Plateaustraat 22, B-9000 Ghent, Belgium

The first written record of the existence of the brine shrimp dates back to 1755 (Schlosser in Kuenen and Baas-Becking, 1938). Nonetheless this "filtering animal" was known since much longer times by different ethnic groups who attributed a better salt production in brine pools to the presence of Artemia *; hence its popular names such as brineworm, Salztierchen, verme de sale, sóféreg, Bahar el dud, Fezzanwurm,* etc.

Despite the primitive optical equipment available at that time, Schlosser's drawings were very detailed (Fig. 1) and rightly gave the adult animal 11 pairs of thoracopods. Several other scientists, including Linnaeus (1758), later described adult Artemia *with only 10 thoracopods. This controversy lasted until 1836 when finally Audouin confirmed the observations of Schlosser.*

From the second half of the 19th century on, several studies were published dealing with the morphology and taxonomy of this Anostracan crustacean. Soon Artemia *was used as a most suitable test-object in the most diverse disciplines of biological sciences : histology, genetics, radiobiology, toxicology, biochemistry, molecular biology, ecology,* etc.

Salt lakes and brine ponds with Artemia *populations are found worldwide. The ecological conditions in these biotopes are extreme* (e.g. *the salinity can exceed 300 g salts/l water), and as a result only a small number of bacterial and algal species can survive. As a consequence of the often occurring blooms of monocultures of specific algal species, these waters are colored red, blue or green. One of the very few invertebrates that could adapt to such an extreme habitat is the brine shrimp* Artemia. *Favored by the absence of predators and food competitors,* Artemia *mostly develops into very dense populations in the salinas.*

At certain moments of the year, enormous quantities of minuscule brown particles (200-300 µm in diameter) are floating at the lake's surface and are finally thrown ashore by wind and waves (Fig. 2. 1). These apparently inert particles are in fact the inactive dry cysts of the brine shrimp which remain in diapause as long as they are kept dry or under anaerobic conditions. Upon immersion in seawater, the cysts hydrate, become spherical and within their shell the metabolism of the embryo is activated. A number of hours later, the outer membranes of the cyst burst (= "breaking" or E-1 stage) and the embryo appears, surrounded by the hatching membrane. The only structural feature which can be observed is the nauplius eye (Fig. 2. 2). During the following hours the embryo leaves the cyst's shell (E-2 stage ; Fig. 2. 3). Inside the hatching membrane, the newly differentiated antennae and mandibles start moving ; within a short period of time the hatching membrane is ruptured and the free-swimming nauplius is

[1] "Bevoegdverklaard Navorser" at the Belgian National Foundation for Scientific Research (NFWO).

born. This first instar larva which is colored brownish-orange due to the presence of yolk, has three pairs of appendages : the antennae which have a locomotory function, the sensorial antennulae and the rudimentary mandibles (Fig. 2. 3). An unpaired red ocellus is situated in the head region between the antennulae. The ventral side of the animal is covered by a large labrum.

The larva grows and differentiates through about 15 molts : the trunk and abdomen are elongating ; the digestive tract becomes functional ; food particles are collected from the medium by the setae of the antennae ; paired lobular appendages which will differentiate into the thoracopods are budding in the thrunk-region ; lateral complex eyes are developing on both sides of the ocellus ; etc. (Fig. 2. 4 and 5).

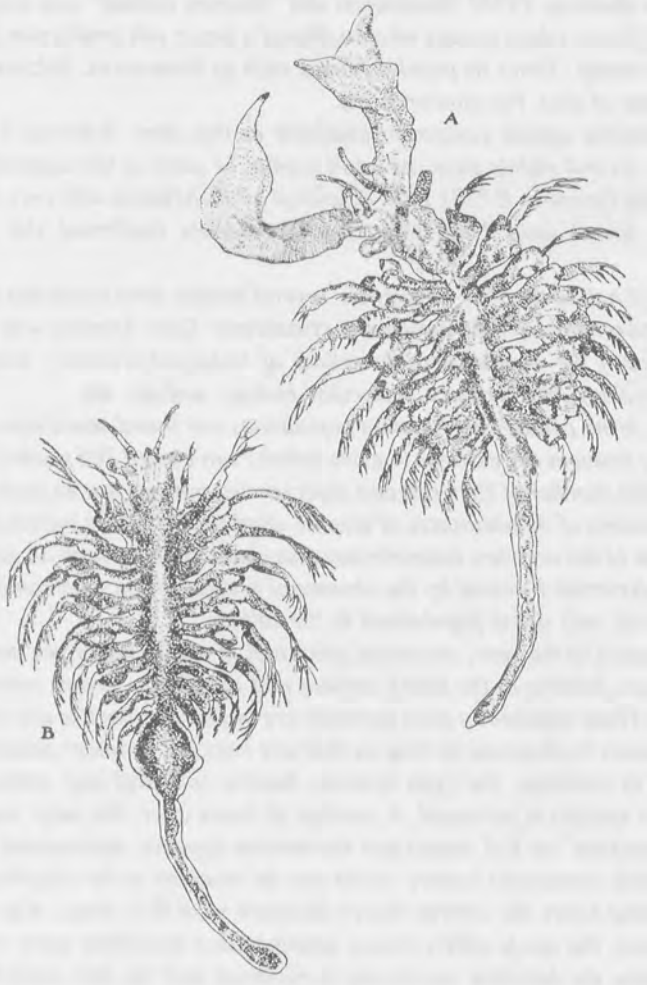

FIG. 1. Schlosser's drawing of a male (A) and a female (B) brine shrimp (From Kuenen and Baas-Becking, 1938).

FIG. 2. 1. Brown layer of brine shrimp cysts accumulated on the shore of a salina ; 2. Pre-nauplius in E-1 stage ; 3. Pre-nauplius in E-2 stage and freshly hatched instar I nauplius ; 4. Instar V larva. a. nauplius eye ; b. lateral complex eye ; c. antennula ; d. antenna ; e. mandible ; f. labrum ; g. budding of thoracopods ; h. digestive tract.

FIG. 3. 5. Head and anterior thoracic region of instar XII ; 6. Head and anterior thoracic region of young male (instar XV) ; 7. Posterior thoracic region and uterus of fertile female ; 8. Head of adult male ; 9. *Artemia*-couple in riding position. a. nauplius eye ; b. lateral complex eye ; c. antennula ; d. antenna ; e. mandible ; h. exopodite ; i. telopodite ; j. endopodite ; k. frontal knob ; m. inactive ovary ; n. ripe eggs in oviduct ; o. uterus ; p. penis.

From the 10th instar on, important morphological changes are taking place : the antennae loose their primitive locomotory function ; i.e. *they loose their long setae and undergo sexual differentiation. In the future males they develop into hooked graspers, while in the females the antennae degenerate into sensorial appendages (Fig. 3. 5, 6, and 8). The thoracopods are now differentiated into three functional parts : the telopodites acting as a filter, the oarlike endopodites having a locomotory activity, and the membranous exopodites functioning as gills (Fig. 3. 5 and 6).*

The adult animal 8-10 mm long, is characterized by the stalked lateral (complex) eye, the sensorial antennulae, the linear digestive tract, and the 11 pairs of functional thoracopods (Fig. 3. 6 and 9). In the male Artemia *the antennae are transformed into muscular graspers which have a frontal knob at their inner side (Fig. 3. 8). In the posterior part of the trunk region a paired penis can be observed (Fig. 3. 9).*

Female Artemia *have very primitive antennae with sensorial function ; their paired ovaries are situated on both sides of the digestive tract behind the thoracopods. The ripe oocytes are transported from the ovaries into the unpaired brood pouch or uterus via two oviducts (Fig. 3. 7).*

Precopulation in adult brine shrimp is initiated by the male in grasping the female with its antennae between the uterus and the last pair of thoracopods. In this "riding position" the couples can swim around for long periods (Fig. 3. 9).

Copulation itself is a very fast reflex : the male abdomen is bent forward and one penis is introduced into the uterus aperture. The fertilized eggs develop into either free-swimming nauplii (ovoviviparous reproduction) which are set free by the mother, or when reaching the gastrula stage, they are surrounded by a thick shell and are deposited as cysts, which are in diapause (oviparous reproduction).

[Morphology of Artemia *after Heath (1924), Nakanishi* et al. *(1962), Anderson (1967), Benesch (1969), and Wolfe (1973)].*

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Biochemistry of *Artemia* development. Report on a symposium held in Toronto (Canada) in July 1979

Joseph C. Bagshaw

Department of Biochemistry, Wayne State University School of Medicine
Detroit, Michigan 48201, USA

On July 7, 1979, a symposium was held in Toronto on the subject of "Biochemistry of *Artemia* Development". This symposium, organized by Dr. Alden Warner and myself, was designed to take advantage of the presence in Toronto of a number of biochemical "Artemiologists" who had come for the XIth International Congress of Biochemistry. The scope of our symposium was, of course, much narrower than that of the International Symposium in Corpus Christi, but within the field of biochemistry, a number of subspecialties were represented. Although the call for papers placed no restrictions on the topics to be presented, most of the papers dealt with a few indentifiable themes.

For a number of years, a subject of major interest in *Artemia* biochemistry has been the mechanisms of protein synthesis, particularly the isolation and characterization of polypeptide initiation and elongation factors. It seemed entirely appropriate to begin the day with a paper on the initiation factors eIF-2 and eIF-3, presented by T. H. MacRae and co-authored by C. L. Woodley and A. J. Wahba. Their work will also be presented at this symposium, so I will not go into it here. However, the major point of the paper should be re-emphasized : contrary to a previous report (Filipowicz *et al.*, 1976), the dormant encysted gastrulae are not deficient in initiation factor eIF-2. MacRae's observations were confirmed by a report given at the Corpus Christi meeting by T. Hultin. Thus synthesis or activation of eIF-2 is not the mechanism whereby encysted embryos "switch on" translation when development resumes.

A paper on the polypeptide elongation factor EF-2 was presented by Dr. Z. Yablonka-Reuveni and co-authored by Dr. Warner. To investigate the distribution of EF-2 in dormant and developing embryos, Dr. Reuveni made use of the facts that this protein can be uniquely radiolabeled *in vitro* by ADP-ribosylation catalyzed by diptheria toxin. She found that in the dormant embryos, EF-2 is about equally divided between "free" and "bound" forms defined operationally by centrifugation at 150 000 × g. The bound form is not complexed with ribosomes, and appears to be identical to the free form after being released from its complex by salt elution. During pre-emergence development there is an apparent decrease in bound EF-2 and a concomittant increase in free EF-2, so that in newly hatched nauplii 75-80% of the EF-2 molecules are in the free form. Dr. Reuveni also found that in extracts from hatched larvae, EF-2 is specifically cleaved to yield a set of smaller polypeptides. The enzyme that carries out this cleavage is present in a "masked" form in dormant embryos. This "EF-2

hydrolase" can be activated *in vitro* by the action of an acid protease present in dormant embryos (A. H. Warner, unpublished).

Having initiated and elongated a discussion of protein synthesis, we continued with a description of a polypeptide release factor from encysted embryos by M. A. Reddington, D. Williamson, and W. P. Tate (presented by Dr. Tate). The factor isolated by Dr. Tate and his colleagues causes the release of completed polypeptide chains *in vitro*. Like its counterpart from rabbit reticulocytes, (Tate *et al.*, 1973), the *Artemia* release factor responds to all three termination codons (UAA, UAG, UGA), provided they are present within a tetranucleotide or longer oligomer. The release factor exhibits a ribosome-dependent GTPase activity that is stimulated by mRNA. Ribosomes from newly hatched nauplii are about 10-fold more efficient in the *in vitro* termination reaction than ribosomes from dormant embryos, and Tate showed that the increasing ability of ribosomes to support polypeptide chain termination parallels the increase in poly (U) translation activity previously observed by Huang and Warner (1974). He presented evidence that this stage-specific difference in the complex termination reaction lies in the interaction of ribosomes with the release factor.

It is well known that dormant encysted embryos do not synthesize proteins, but the mechanism(s) that maintain this quiescent state are not yet defined. One candidate is the translation inhibitor detected by Warner *et al.* (1977), and Dr. Warner reported on the further characterization of this inhibitor. The inhibitory protein co-purifies with elongation factor EF-2 through a variety of fractionation procedures, and appears to be a chemically modified form of EF-2. Several observations support this hypothesis. A slight separation between EF-2 and inhibitor activity can be achieved by chromatography on hydroxyapatite. The inhibitory form of EF-2 can be preferentially released from crude ribosomes (150 000 × g pellet) by 100 mM KCl. The inhibitory activity of the "loosely bound" EF-2 increased 3-fold during storage at 0 °C for 10 days ; under the same conditions to low inhibitor activity of "tightly bound" EF-2 remained unchanged. This suggests a chemical and/or conformational alteration in the protein during storage. A possible role of sulfhydryl groups in this alteration is suggested by the observations that blocking reagents also block inhibitor activity, and that high levels of dithiothreitol prevent the increase in inhibitory activity seen on storage of "loosely bound" EF-2.

Protein synthesis in developing encysted embryos is more than just an interesting subject to study ; it can also be a useful indicator of development. Polyribosomes are not present in dormant embryos, but are formed very rapidly when development resumes (Golub and Clegg, 1968). In experiments performed in my laboratory and reported by C. F. Austerberry, polyribosome formation was used as a diagnostic parameter of normal development. In 1975, we discovered that a then current batch of encysted embryos from the Great Salt Lake in Utah gave less than 1% hatching, and we soon learned that the hatch rate of the Great Salt Lake cysts had been declining since about 1970. We switched to the San Francisco strain for routine use, but we were able to obtain a few small cans of "old" Great Salt Lake cysts (Long Life Fish Food Co., batch no. 414). Since pre-emergence development occurs inside an opaque shell, it is impossible to determine by simple inspection whether the defective embryos have developed at all or are still arrested at the early gastrula stage. Austerberry first determined that about 17% of "OGSL" (old Great Salt Lake) cysts hatched, compared with $< 1\%$ for RGSL (recent Great Salt Lake, vintage 1975) and 76% for "SF" (San Francisco) cysts. He then examined all three populations for polysome formation and showed that RGSL cysts fail to

form polysomes, even after incubation for 8 hr at 30 °C. Under the same incubation conditions, polysomes were readily detectable in 1 hr in OGSL and SF embryos. Austerberry also showed that RGSL cysts do not exhibit the increase in nuclear RNA polymerase activity that occurs in normal developing embryos (D'Alessio and Bagshaw, 1977). This is perhaps not surprising, since the increase in RNA polymerase activity involves the *de novo* synthesis of RNA polymerase molecules (Bagshaw *et al.*, these Proceedings). By these biochemical criteria, the defective RGSL embryos never develop beyond the stage at which they were arrested. Austerberry's measurements of DNA content indicated that the amount of DNA per embryo (and presumably the number of nuclei) was identical in SF and OGSL cysts but significantly lower in RGSL cysts, suggesting that the latter may have been arrested too early in development. The failure of Great Salt Lake embryos to resume development is at least temporally correlated with changes in the ecology and salinity of the lake (Post, 1977). After this review was presented in Corpus Christi, Mr. Gail Sanders informed me that cysts harvested around the Great Salt Lake are again hatching at a more reasonable rate (at least 50 %), possibly due to a partial reversal of salinity changes in the lake.

The immediate onset of protein synthesis that occurs when encysted embryos resume development implies that the dormant embryos already contain messenger RNAs. Several research groups have in fact shown that functional mRNAs can be isolated from dormant cysts and translated *in vitro*, provided that the mRNA molecules are separated from their associated proteins. H. Slegers, from M. Kondo's group in Antwerp, presented a paper on the characterization of ribonucleoprotein particles, isolated from dormant embryos, that contain polyadenylated mRNAs. This work, which deals primarily with the proteins associated with the repressed mRNP particles, was also presented in Corpus Christi (these Proceedings). Another paper from Kondo's group, presented by L. Moens, described studies on the transcription *in vitro* of chromatin by the homologous RNA polymerases, and the effect on this transcription of actinomycin D and aurintricarboxylic acid. This work was also presented in Corpus Christi.

The hemoglobins of *Artemia* have become a focal point for research in several laboratories, and papers presented at the symposium dealt with three diverse aspects of the subject: genetics, gene expression, and physical chemistry. A comparison of hemoglobins from three geographically separated populations – those from San Francisco Bay, the Great Salt Lake, and the Gulf of Kutch – was presented by D. A. W. Bucks and co-authored by S. T. Bowen. Young adults (3 weeks) of all three populations, when raised under 6 % oxygen, contain in their hemolymph four electrophoretically distinct hemoglobins, which Bucks and Bowen designate Hb-1, Hb-2, Hb-x, and Hb-3 in descending order of electrophoretic mobility. Hb-3 is absent in older (8 weeks) adults, and nauplii of al three populations have only Hb-2 and Hb-x. Differences in electrophoretic mobility make the total hemoglobin pattern of each strain distinct from that of each of the other two. Bowen *et al.* (1977) have previously presented evidence that Hb-2 is a heteropolymer of subunits of two types, which they term α and β, and that Hb-1 and Hb-x are homopolymers of the α and β type, respectively. Bucks showed that these hemoglobins, and by implication the constituent subunits, are differentially inducible by low oxygen tension, the α-type being induced at a higher oxygen level than the β-type. This observation makes the hemoglobins an attractive system for studies of gene expression (see below).

A quite different aspect of *Artemia* hemoglobin was presented by J. Clauwaert. He and his co-author, F. De Voeght, used a variety of light scattering techniques to study the physical structure of the hemoglobin molecule. Their work was also presented in Corpus Christi (these Proceedings).

The process of development is characterized, biochemically, by a complex series of changes in the pattern of selective gene expression. Conversely, developing organisms present some of the best model systems for studies of eukaryotic gene expression. It is not surprising, then, that stage-specific gene expression has become a subject of major interest in *Artemia* biochemistry. The most direct approach begins with the identification of genes that are expressed in a stage-specific manner, *i.e.*, expressed at one stage but not at another. This can be accomplished either by measuring enzyme levels at different stages or, more directly, by examining *in vivo* synthesis of specific proteins. Three papers presented at the symposium dealt with this approach to gene expression. J. Sebastian summarized the work of the large and active research group at the Universidad Autonoma in Madrid on developmental changes in a number of enzymes. This work was also presented in Corpus Christi and appears elsewhere in these Proceedings. Another example of a stage-specific enzyme was presented by G. L. Peterson and co-authored by L. E. Hokin, who have studied the appearance of Na, K-activated adenosine triphosphatase in encysted developing embryos and newly hatched larvae. This Na, K-ATPase activity is first detected in emerging pre-nauplius larvae and increases rapidly in hatched nauplii (Ewing *et al.*, 1974 ; Conte *et al.*, 1977). Using $NaH^{14}CO_3$ to radiolabel proteins *in vivo*, Peterson showed that synthesis of the enzyme actually begins prior to emergence and precedes the appearance of enzyme activity of about 4 hr. This lag period may reflect an obligatory assembly of the enzyme into cell membranes. Peterson has also identified two alternative forms of the large subunit of the enzyme and shown that the relative abundance of these two forms changes during early larval development. If these two subunit forms are encoded by different genes, Peterson's results indicate a quantitative shift from expression of one gene to the other.

State-specific gene expression has become a major component of the research in my laboratory, and I described some of our results in a paper co-authored by R. Acey. We have identified two groups of proteins that are synthesized in nauplii but not in developing encysted embryos. DNA synthesis does not occur in developing embryos, but resumes after hatching and continues for about 36 hr (McClean and Warner, 1971). We reasoned that histone synthesis should be temporarily coupled with this wave of DNA synthesis, and *in vivo* radiolabeling confirmed that histones are synthesized in hatched nauplii, but not during at least the first 8 hr of pre-emergence development. We then prepared total RNA from dormant cysts and from nauplii, and hybridized these in RNA excess to cloned sea urchin histone gene DNA radiolabeled *in vitro* by nick translation. The results indicate that cysts do not contain "masked" histone mRNA sequences ; thus, the histone mRNAs present in nauplii must be the result of transcription. In another set of experiments, we fractionated total soluble proteins (150 000 × g supernate) from nauplii of different ages by acrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE). This experiment was designed to detect only proteins present in such quantity that they appear as discrete bands on a gel. During the first 48 hr of larval development, the only change in the SDS-PAGE pattern was the appearance of one polypeptide, beginning about 4 hr after hatching. By combining non-denaturing PAGE with SDS-PAGE in a two-dimensional analysis, we showed that this protein was hemoglobin,

or more accurately two hemoglobins corresponding to Hb-2 and Hb-x (Bowen) or Hb-N and Hb-III (Kondo). Since the subunits of these molecules are identical in size (130 000 daltons), they appear as a single band on SDS-PAGE. We now have two sets of genes to use in further studies of gene expression, and we are presently constructing a complete library of cloned *Artemia* DNA sequences from which we can select histone and hemoglobin genes.

A paper presented by J. R. Tate, and co-authored by T. C. James and C. C. Hentschel, dealt with gene expression in the broader context of total mRNA populations. They showed that the ability of total RNA to support translation *in vitro* increased several-fold during pre-emergence development. This was particularly true of polyadenylated RNAs, which showed a ten-fold increase in apparent messenger content. They also observed qualitative differences in the proteins translated *in vitro*, as visualized by SDS-PAGE : RNA from developing embryos and newly hatched larvae coded for larger polypeptides than RNA from dormant cysts. To investigate further the sequence complexity (nucleotide information content) of the polyadenylated mRNA's, Tate and his colleagues hybridized it to copy DNA reverse transcribed from the same mRNA population. As in most other organisms, they found three abundance classes, *i.e.*, mRNAs present in a very few (low abundance), a few hundred (middle abundance), or several thousand (high abundance), copies per cell. By using RNAs from dormant or developing embryos or newly hatched larvae, they concluded that during pre-emergence development the total sequence complexity increases about ten-fold, then decreases again by hatching to a level comparable to that of dormant cysts. However, cross-hybridization between cyst RNA and cDNA copied from nauplius highly abundant mRNAs indicated substantial qualitative difference between these mRNA populations.

The DNA sequence organization of the *Artemia* genome was described by J. C. Vaughn (co-authored by C. J. Petropoulos). Eukaryotic genomes tend to be organized in one of two patterns : short period interspersion, in which reiterated DNA sequences of a few hundred base pairs are interspersed among single-copy sequences, or long period interspersion, with several thousand base pairs of reiterated DNA followed by equally long single-copy sequences. By studying the kinetics of reassociation of DNA fragments of different lengths, Vaughn showed that the *Artemia* genome is present as single-copy DNA, 16 % as sequences repeated about 2 000 times, and 44 % as sequences repeated 20 000 times or more. This last category includes about 6 % inverted repeat sequences.

After a day of discussing what goes on inside a brine shrimp, we finished with two papers that remined us that what goes on inside is profoundly influenced by what is on the outside. J. S. Clegg presented his work on the hydration dependence of metabolism and the physical state of water in encysted embryos. He showed that limited metabolism, but not true development, can occur in the absence of what we normally think of as "free" water. A. Hernandorena described her studies on the influence of dietary purines and pyrimidines on larval development. In a defined medium, growth rate and morphogenesis are dependent upon the balance of purines and pyrimidines. Both of these papers were also presented in Corpus Christi and appear elsewhere in these Proceedings. In his closing remarks, Dr. Warner brought up a problem that has gone unnoticed for a long time. For biochemical work, cysts are generally sterilized by washing with a solution containing NaOCl. Warner found that subsequent homogenization of these cysts results in inactivation of 50 % of polypeptide elongation factor 2, and the same is very likely true of other enzymes. No amount of washing will remove this residual NaOCl, nor will washing with 1 % urea or 0.1 N HCl. Thus,

experiments showing an apparent increase in an enzyme activity during pre-emergence development must be interpreted with some caution.

The Toronto symposium brought together, for the first time, a representative sample of biochemists working on *Artemia*. I think I can speak for all the participants in saying that it was a real pleasure to get to know people whose papers we have been reading for years. The meeting resulted in a lively exchange of ideas that extended through the following week of the International Congress and beyond, and in several cases potential collaborative research efforts were arranged. The Corpus Christi symposium supplemented these contacts and gave us an opportunity to meet "Artemiologists" working in other fields. In addition, the papers presented at these two symposia comprise an important source of information for all those interested in *Artemia* biochemistry.

The proceedings of the Toronto symposium have been published in 1979 under the title "Biochemistry of *Artemia* Development". Bagshaw J. C. and A. H. Warner (Eds). University Microfilms International, 300 N. Zeeb Road, Ann Arbor, Michigan 48106, USA. The book counts 240 pages and comprises the following papers :

INTRODUCTION
J. C. Bagshaw

CHARACTERIZATION OF POLYPEPTIDE CHAIN INITIATION FACTORS DURING *ARTEMIA SALINA* DEVELOPMENT
T. H. Macrae, C. L. Woodley, and A. J. Wahba

CHARACTERIZATION OF ELONGATION FACTOR 2 IN DORMANT CYSTS AND DEVELOPING EMBRYOS OF *ARTEMIA SALINA*
Z. Yablonka-Reuveni and A. H. Warner

A RELEASE FACTOR FROM THE CYST OF *ARTEMIA SALINA* : CHARACTERIZATION AND ACTIVITY OF STAGE-SPECIFIC RIBOSOMES
M. A. Reddington, D. Williamson, and W. P. Tate

PARTIAL CHARACTERIZATION OF A PROTEIN SYNTHESIS INHIBITOR FROM *ARTEMIA* CYSTS
A. H. Warner, V. Shridhar, and Z. Yablonka-Reuveni

BIOCHEMICAL ANALYSIS OF DEVELOPMENTAL FAILURE IN GREAT SALT LAKE EMBRYOS
C. F. Austerberry and J. C. Bagshaw

CHARACTERIZATION OF REPRESSED POLY (A)-CONTAINING RIBONUCLEOPROTEINS IN CRYPTOBIOTIC GASTRULAE OF *ARTEMIA SALINA*
H. Slegers, E. De Herdt, R. Mettrie, E. Piot, and M. Kondo

THE NAUPLIAR HEMOGLOBINS OF THREE POPULATIONS OF *ARTEMIA*
D. A. W. Bucks and S. T. Bowen

STUDY OF EXTRACELLULAR HEMOGLOBIN FROM *ARTEMIA SALINA* BY SCATTERING TECHNIQUES
F. De Voeght and J. Clauwaert

BIOGENESIS OF THE (Na + K)-ACTIVITED ADENOSINE TRIPHOSPHATASE DURING NAUPLIAR DEVELOPMENT IN *ARTEMIA*
G. L. Peterson and L. E. Hokin

STAGE-SPECIFIC GENE EXPRESSION IN *ARTEMIA*
J. C. Bagshaw and R. Acey

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Golub A. L. and J. S. Clegg. 1968. Protein synthesis in *Artemia salina* embryos. I. Studies on polyribosomes. *Develop. Biol.* 17:644-656.

Huang F. L. and A. H. Warner. 1974. Control of protein synthesis in brine shrimp embryos by repression of ribosomal activity. *Arch. Biochem. Biophys.* 163:716-727.

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A review of the cellular and developmental biology of *Artemia*[1]

James S. Clegg[2] *and Frank P. Conte*[3]

[2] *Laboratory for Quantitative Biology, University of Miami*
Coral Gables, Florida 33124, USA

[3] *Department of Zoology, Oregon State University*
Corvallis, Oregon 97331, USA

Content

[1] We dedicate this review to Dr. Frank J. Finamore of the Oak Ridge National Laboratory in recognition of his important work on the developmental biochemistry of *Artemia*.

Introduction

It has often been the case in experimental biology that a particular organism, by virtue of its peculiarities, provides a singularly useful means by which a specific biological problem can be examined. In this review we will describe a number of properties of this organism which render it very suitable for experimental work in developmental biology. As we extol its virtues we also point out that in some respects, it is not a very good experimental system. Indeed, some of the very same features that render it uniquely useful, also offer frustration and chagrin.

Artemia has attracted the attention of such notable reproductive and developmental biologists as Friedrich Brauer (1886), Franz Leydig (1851), Jacques Loeb (1901), and Joseph and Dorothy Needham (1930). During the last decade or so there has been a revival of interest in research on the developmental biology of *Artemia*, and we believe this will continue to grow in the decade ahead. We hope this attempt of ours to describe what is currently known will facilitate this growth.

Hultin and Nilsson (1980) will cover the molecular biology of *Artemia*, so we will exclude from our coverage the details of gene replication, transcription, and transition. Also, we will be dealing exclusively with the diploid, bisexual populations of *Artemia* from the Great Salt Lake and the San Francisco Bay area unless stated otherwise.

Gametogenesis and fertilization

Spermatogenesis

Fig. 1 illustrates the adult male reproductive system (Wolfe, 1971). All structures are paired and the histology of the testis is similar throughout its length. Supporting cells and germ cells are the two cell types present (Fautrez-Firlefyn, 1949, 1951 ; Fautrez-Firlefyn and Fautrez, 1954), the former constituting an epithelial sheath toward the tubule periphery. The spermatogonia are found just beneath the supporting cells, usually in clusters. As spermatogenesis occurs, the stages within a cluster tend to be the same, and may be connected intercellularly according to Wolfe (1971). Mature sperms, located toward the central lumen, are spherical, non-motile and non-flagellated, but exhibit slender "cytoplasmic arms" (Brown, 1966 and 1970). There is disagreement concerning the presence (Fautrez-Firlefyn and Fautrez, 1955) or absence (Brown, 1966) of an acrosome.

Because the earlier stages (spermatogonia and spermatocytes) are located in close proximity to the hemolymph, Wolfe (1971) has suggested they are nourished in this fashion, whereas the supporting cells presumably perform this function for spermatids and sperm. The mature sperm are moved along the testes and into the vas deferens apparently by non-testicular activity, perhaps chiefly by general body movement (Wolfe, 1971). The vasa deferentia secrete seminal fluid and store sperm. Their structure is the same throughout, consisting of a secretory epithelium surrounded by longitudinal and circular muscles. The sperm are presumably nourished by the seminal fluid which contains a substantial amount of neutral mucopolysaccharide (or mucoprotein) according to Wolfe (1971). The vasa deferentia empty near the tips of the two eversible penes, in close proximity to the openings of the two accessory glands (Fig. 1). The duct of this gland does not join the sperm duct but rather

empties near the tip of the eversible penis. The function of the accessory gland secretions is not known. Several roles have been suggested (Wolfe, 1971) including lubrication of the female genital opening and ovisac, an activation substance for the sperm, and a copulatory plug. These glands are apparently homologous with the shell glands of the female.

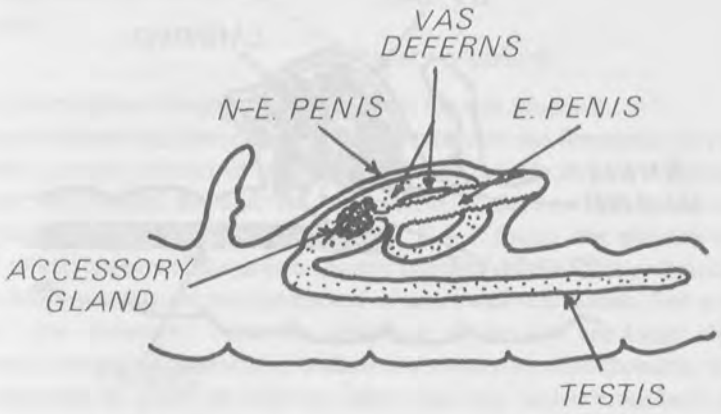


FIG. 1. Diagram of the adult male reproductive system of *Artemia* (after Wolfe, 1971).

The preceding account has been based chiefly upon the works of Wolfe (1971), who appears to have done the definitive study, and Brown (1966 and 1970). Fautrez-Firlefyn (1949, 1951) and Fautrez-Firlefyn and Fautrez (1954, 1955) have described various cytochemical details of spermatogenesis. These papers adequately cover earlier work which extends back to Leydig's (1851) study.

OOGENESIS, VITELLOGENESIS AND FERTILIZATION

The female reproductive system is shown in Fig. 2 based on the description by Lochhead (1941 and 1950). All structures are paired with the exception of the single ovisac, also called a uterus or brood pouch. A great deal of work has been carried out on this subject by Fautrez-Firlefyn, Fautrez, and other colleagues at the University of Ghent in Belgium and it will not prove feasible to review all of it here in any detail. Criel (1980ab) considers the morphology of the genital apparatus, including ultrastructure.

Bands of oogonia are found throughout the ovary length, being most abundant toward the ventral side. Some of these oogonia enlarge and undergo vitellogenesis, during which numerous nurse (nutritive) cells participate (Anteunis *et al.*, 1966ab). As vitellogenesis proceeds the cells enter prophase of the first meiotic division (primary oocyte) and remain at that stage as they approach and enter the opening to the enlarged lateral sacs (oviducts) where they are halted at metaphase of the first meiotic division (see Fautrez-Firlefyn, 1951, 1957; Goldschmidt, 1952). Lochhead (1941) stated that it is only while the oocytes are in the lateral sacs that copulation is effective, but Bowen (1962) was the first, to our knowledge, to provide good evidence for this, and she also showed that sperm are not stored within the females from

one mating to the next. Subsequently, cleavage of the zygote begins and embryonic development proceeds along two general lines as will be discussed later. At present we will consider some events taking place in the ovary and lateral sac.

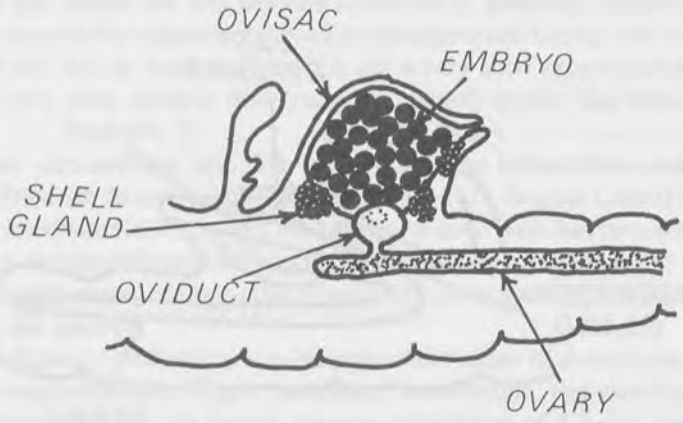
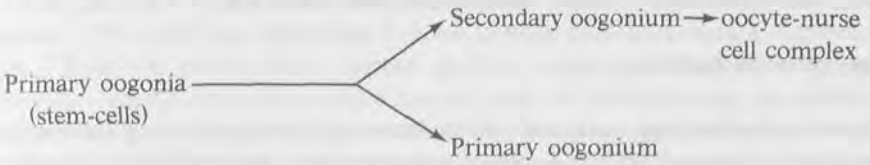


FIG. 2. Diagram of the adult female reproductive system of *Artemia* (after Lochhead, 1941, 1950).

In a brief abstract Lochhead and Lochhead (1967) describe the ovary as a tubular structure in which oogonia occupy in strip along the full length of one side, while "special somatic cells" are similarly disposed along the other side. In between these occur long twisting chains of cells which apparently can be observed only in living teased preparations. Each chain is attached at one end to the oogonial strip where the chain arises by mitosis. Within each chain the cell nearest the "special somatic cell" strip increases markedly in size relative to the others and will eventually give rise to a primary oocyte. The remaining chain cells become nutritive cells which the authors describe as polyploid, multi-nucleolate cells containing relatively small amounts of protein, but very large amounts of cytoplasmic RNA. There are roughly 70 nutritive cells per oocyte, but only two are attached at a given time. These two nutritive cells appear to supply the oocyte with cytoplasm while their nucleus degenerates (see Anteunis *et al.*, 1966b). Presumably, the process repeats when two more nutritive cells are brought close to the growing oocyte. To our knowledge this interesting sequence of events has not been commented on by subsequent workers, presumably because they were not aware of the abstract.

Iwasaki (1970 and 1973a) has described the structure of the ovary as consisting of four cell types : germinal cells (oogonia, we presume), nutritive cells, oocytes, and follicle cells. It is not clear to us whether or not the "follicle cells" represent the "special somatic cells" of Lochhead and Lochhead (1967). Iwasaki (1970) proposes that the germinal cells divide and give rise to both the nutritive cells and the oocytes. Interestingly, at least 12 nutritive cells were observed to provide for the growth of a single oocyte, and the nutritive cells are arranged in "rows", each cell closely pressed to the others. Thus, at least some of Iwasaki's observations appear to be compatible with Lochhead and Lochhead (1967).

Squire (1970) proposes that the oogonia represent a non-exhausting stem-cell population as shown in the following diagram :



Iwasaki's (1973a) irradiation study provides support for this proposal.

One of the more interesting features of oogenesis concerns the formation of yolk platelets which constitute a major portion of the mature oocyte cytoplasm and which are vital to nauplius growth. As we shall see later the yolk platelets should be considered as extremely important developmental organelles. Anteunis *et al.* (1964) found that platelets arise from a "yolk nucleus" which appears in the previtellogenic primary oocyte. This yolk nucleus (YN) is composed of a dense cytoplasmic area composed of microvesicular bodies, free microvesicles, "dense bodies", and ribosomes. Near the periphery of the YN are Golgi elements (the suggested producer of the microvesicular bodies) and numerous mitochondria. Yolk platelet formation is proposed to occur as follows : microvesicular bodies transform into "dense bodies" which leave the YN and take up residence elsewhere in the cytoplasm. Here they become much larger, perhaps by accretion, giving rise to the fully formed yolk platelets. What is remarkable is that the YN persists well beyond vitellogenesis ; indeed, it is maintained through cleavage and eventually ends up in one of the "germinal initiales" (one of the two first cells to invaginate during gastrulation). This observation was made by Fautrez and Fautrez-Firlefyn (1964) who also proposed that the YN could play the role of a "genital determinant".

Another feature of importance in the oocyte is the formation of a tubular-mitochondrial complex composed of elongated mitochondria displayed in a hexagonal array with rough endoplasmic reticulum tubules arranged in between to form an elaborate coiled architecture (Anteunis *et al.*, 1966c). These authors suggested that the complex is involved with the synthesis of proteins destined to be incorporated into the yolk platelets because it appears in very young previtellogenic oocytes and precedes the appearance of the YN.

Detailed analyses of "mature" yolk platelets (*i.e.*, those in encysted gastrula) have been carried out by Warner *et al.*, 1972). We shall cover these organelles later in detail, but mention now in passing that there is good reason to believe the yolk platelets perform a far more important role in development than simply to provide nourishment for the developing embryo and larva.

Although fertilization occurs in the ovisac (Bowen, 1962) it has, to our knowledge, never actually been observed (Brown, 1970 ; Wolfe, 1971). Subsequent events, involving behavior of pro-nuclei, spindle formation and so forth can be found in the massive study of Benesch (1969), and particularly in papers by Fautrez-Firlefyn (1951), Anteunis *et al.* (1967) and the review by Fautrez-Firlefyn and Fautrez (1967). A recent study by De Maeyer-Criel *et al.* (1977) warrants brief description. These workers showed that the mature oocyte is not covered with any structure other than the cell membrane, and no special subcortical organelles exist. However, upon sperm penetration a fertilization membrane is progressively secreted during the first 1.5 hr after fertilization. The precursor of this "membrane" first appears as large granules in the smooth endoplasmic reticulum. These are eventually extruded

in vesicles liberated from the Golgi apparatus, onto the cell exterior where the secretion spreads over the entire egg surface, generating a perivitelline space as well as the fertilization membrane. We shall return to these observations in the following section.

Development to the gastrula

Although the fertilized egg is extremely yolky, cleavage is complete, having apparently been described first by Artom (1907) and subsequently by several others (Gross, 1936 ; Fautrez-Firlefyn, 1951 ; Dutrieu, 1960 ; Benesch, 1969). Fautrez-Firlefyn (1951) describes the cleavage as intermediate between complete and partial, and transitional between spiral and equal non-spiral, but this does not appear to be in complete accord with the description by Benesch (1969). In any event, it is unusual that the blastocoel begins to form during the first cleavage division (Anteunis *et al.*, 1961). Certainly, the clearest and most complete description of cleavage is that given by Benesch (1969) who provides detailed drawings as well as a time-sequence for all cleavage stages. That author's work indicates the nuclei of the blastomeres are always contained within cell membranes, whereas Fautrez-Firlefyn (1951) and Dutrieu (1960) describe the formation of a "periblastula" in which the cell membranes of the blastomeres break down, and the yolk platelets come to lie in what was the blastocoel. Perhaps a detailed ultrastructural study would clarify this point. Gastrulation occurs by invagination initially of the two "germinal initiales", resulting in formation of the primary germ layers, which subsequently will give rise to the larval tissues (Benesch, 1969).

Once more, the group at Ghent has contributed significantly to our knowledge about this phase of *Artemia* development. Fortunately, much of this work has been reviewed by them (Fautrez-Firlefyn and Fautrez, 1967) and we will not repeat that effort here. However, a brief account of some of the work done since then will be given.

Roels (1970, 1971) presented evidence that peroxisomes, first formed in previtellogenic oocytes, release their contents into the perivitelline space during early cleavage, apparently ceasing at the 8-cell stage when the peroxisomes have essentially all but disappeared from the blastomeres. He implicates this secretion in the hardening of the "cell envelope", perhaps through cross-linking of proteins with concomittant decrease in permeability. This process continues through the blastula, at least. It is tempting to suggest that this secretion-induced hardening might play some role in the formation of the embryonic cuticle which de Chaffoy *et al.* (1978a) have described as becoming impermeable at the "second gastrula" stage (Benesch, 1969). However, de Chaffoy *et al.* (1978a) point out that this permeability change occurs only if the embryos are allowed to remain within the ovisac during development to the gastrula. Thus, if peroxisome release is involved, it is not a sufficient condition for the development of impermeability at the second gastrula stage. We will return to this point later.

A number of studies have been carried out on the effects of inhibitors and various other agents on early development (mostly cleavage). Studies prior to 1968 have already been reviewed (Fautrez-Firlefyn and Fautrez, 1967). Since then, the effects of a variety of compounds have been examined : hydroxyurea (Fautrez-Firlefyn and Fautrez, 1968b, 1969, 1970 ; Fautrez and Fautrez-Firlefyn, 1970ab) ; actinomycin-D (Fautrez and Fautrez-Firlefyn, 1973) ; puromycin (Fautrez and Fautrez-Firlefyn, 1976) ; several simple amines (Fautrez-Firlefyn and Fautrez, 1968a) ; cycloheximide (Fautrez and Fautrez-Firlefyn, 1974).

The two alternative developmental paths

It has been known since the earliest published works (reviewed by Mathias, 1937 ; Barigozzi, 1939 ; Dutrieu, 1960) that *Artemia* females can either release encysted embryos ("cysts") from the ovisac (the oviparous path) or retain the embryos and give birth to swimming nauplius larvae (the ovoviviparous path). In the latter case the gastrula simply continues its development to the nauplius. By far, the most complete morphological description of this development has been given by Benesch (1969), a work we consider to be the definitive study on cell differentiation and morphogenesis in *Artemia*. It is the former "oviparous" condition we will concentrate upon, and will refer to the released encysted embryo as a "cyst". According to Benesch (1969) it is at a stage referred to as gastrula-II, an advanced gastrula. This confirms the prior observations of Fautrez-Firlefyn (1951) and Dutrieu (1960).

Some authors (Lochhead, 1940 and 1941 ; Mathias, 1937 ; Dutrieu, 1960) have observed two types of encysted embryos : one type produces nauplii shortly after the cysts are released ; the other type is apparently dormant (in diapause) and must be "activated". While several factors might be involved in terminating dormancy, one of the most effective treatments is dehydration, either by osmotic withdrawal of water from the cysts or through their exposure to air of low relative humidity. Thus, when such dehydrated cysts are placed into an aqueous environment of suitable osmotic pressure, containing sufficient oxygen, the embryos will resume their development. We believe it's fair to say that the precise conditions required to terminate dormancy in freshly released cysts (and the mechanisms involved) are not known. Several interesting suggestions have been advanced (Barigozzi, 1939 ; Mathias, 1937 ; Dutrieu, 1960 ; Morris and Afzelius, 1967).

The other side of the coin has to do with the factors that lead either to the production of dormant cysts or nauplii within the female. We should point out that although a given female can alternate between these conditions, all of the embryos of a given brood (or clutch) follow the same path. There seems to be uniform agreement that the shell gland (Fig. 2) secretion and/or the shell itself play an important role in determining the path taken (Von Siebold, 1873 ; Lochhead and Lochhead, 1940 ; Dutrieu, 1960 ; Stefani, 1961 ; Ballardin and Metalli, 1963 ; Benesch, 1969 ; Bowen *et al.*, 1966 ; Finamore and Clegg, 1969 ; Anderson *et al.*, 1970), but other possibilities exist (Morris and Afzelius, 1967, and de Chaffoy *et al.*, 1978a). Having said this, we point out that little is known concerning the details of this relationship.

The ultrastructure of the shell glands has been described in detail (Anderson *et al.*, 1970 ; De Maeyer-Criel, 1973), and Fautrez and Fautrez-Firlefyn (1971) have performed a number of cytochemical analyses. The origin of the various shell components has been documented by the excellent work of Anderson *et al.* (1970). The ultrastructure of the shell of the encysted dormant gastrula has been the object of several studies (Morris and Afzelius, 1967 ; Humphreys *et al.*, 1967 ; Khalaf *et al.*, 1978, 1980 ; Mazzini, 1978). The comparative study of anostracan egg shells by Gilchrist (1978) should also be consulted.

Fig. 3 is a diagrammatic sketch of the shell of a dormant cyst at the point of being released from the ovisac (after Anderson *et al.*, 1970 ; Morris and Afzelius, 1967). The region from (and including ?) the outer cuticular membrane inward is apparently produced by the embryo whereas the remainder of the shell appears to originate from the maternal shell gland secretion and perhaps other components of the fluid in the ovisac. Secretion by the shell

glands into the ovisac begins as the oocytes pass into the ovisac. After fertilization and the first cleavage division, the shells begin to form at the surface, consisting at this point of a tertiary envelope of irregular thickness. The tertiary envelope is, in general, completed by 30-36 hr after fertilization, at which point the embryo is a late blastula (Anderson *et al.*, 1970). Further alterations occur in the tertiary envelope as gastrulation occurs, until the final structure (Fig. 3) is formed.

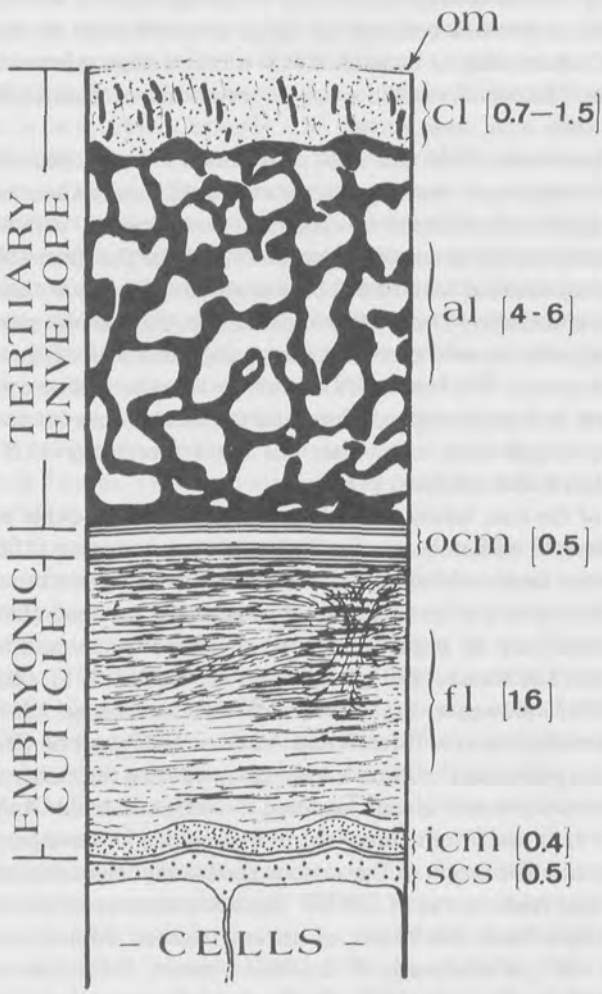


FIG. 3. Diagram of the ultrastructure of the shell of an encysted gastrula (after Morris and Afzelius, 1967 ; Anderson *et al.*, 1970). Abbreviations : (om) outer membrane ; (cl) cortical layer ; (al) alveolar layer ; (ocm) outer cuticular membrane ; (fl) fibrous layer ; (icm) inner cuticular membrane ; (scs) subcuticular space. The numbers given in the brackets represent the approximate thickness in μm given by Khalaf *et al.* (1978).

Of some importance is the observation by Anderson *et al.* (1970) that the embryonic cuticle is not synthesized by the developing embryo until after the tertiary envelope has been formed. The outer cuticular membrane appears to be the permeability barrier characteristic of the encysted gastrula after it has been dried and rehydrated (Morris and Afzelius, 1967). Thus, one would expect the pre-gastrula stages to be generally permeable to a variety of solutes but to lose this characteristic as gastrulation occurs. That is precisely what de Chaffoy *et al.* (1978) have convincingly shown by experiments on developing embryos both *in vitro* and in the ovisac. They also demonstrated specific ultrastructural changes in the outer cuticular membrane (and fibrous layer) that accompany the permeability changes. de Chaffoy, *et al.* (1978) suggest that the outer cuticular membrane might not form correctly unless the embryo is supplied with material from the maternal female. While the origin of the outer cuticular membrane remains an open question, its developmental importance is established, and it should be the object of further study.

The approach used by de Chaffoy *et al.* (1978a) is clearly a very useful one for the investigation of *Artemia* embryogenesis. It is somewhat surprising that so few studies have been performed using early embryonic stages in culture, other than those by Fautrez, Fautrez-Firlefyn, and colleagues, who were first to do so (Fautrez-Firlefyn and Van Dyck, 1961). However, the observations of de Chaffoy *et al.* (1978a) do not indicate that the particular developmental route taken will be determined by the permeability and membrane changes they observed since both kinds of embryos appear to exhibit similar changes during development. In this context it might be significant that shell glands that lack the brownish secretion ("while shell glands") exhibit significant differences in both the structure of the gland cells as well as in the secretory granules as shown by De Maeyer-Criel (1973) and Criel (1980ab). That the secretion of the "white shell glands" might promote the ovoviviparous route seems one reasonable possibility, among several others (De Maeyer-Criel, 1973).

Properties of the dehydrated encysted gastrula (cyst)

The ultrastructure of the dried encysted gastrula has been described by Morris (1968) (also see Humphreys *et al.*, 1967 ; Morris and Afzelius, 1967). There can be no doubt concerning the extreme resistance of the dried cyst to various environmental insults, and several papers presented at the present symposium will add to what we will summarize here (Gaubin *et al.*, 1980 ; Iwasaki *et al.*, 1980 ; MacDonald, 1980 ; Metalli, 1980 ; Planel *et al.*, 1980). However, one fact that must be kept in mind when evaluating tolerance is the difference between the ability of the embryo to emerge from the shell, and even to hatch as a nauplius, compared with the ability of the resulting larvae to give rise to a reproductively competent adult. Since the encysted gastrula gives rise to the newly hatched nauplius without cell division (Nakanishi *et al.*, 1962) this tends to render the emergence process (and often hatching as well) much less sensitive to damage than subsequent growth, molting and development of the nauplius.

Nevertheless, the performance of the dried cyts is impressive. There seems to be no lower temperature limit since the cysts have been placed at -271.7 °C for 2 hours (Iwasaki, 1973b), -190 °C for 24 hr (Whitaker, 1940), and -270.8 °C at reduced pressure for 6 days (Skoultchi and Morowitz, 1964) with negligible or modest decreases in viability (Dutrieu, 1960). With regard to the upper temperature limit, Hinton (1954, 1968) observed negligible change in % hatch when cysts were pre-incubated at 103 ± 0.5 °C for up to 1.5 hr but longer exposures

rapidly reduced viability as judged by hatching assays. Iwasaki (1973b) obtained similar results, but carried out a much more detailed kinetic study. She found no change in viability when cysts were incubated for 9 hr at 60 °C. However, at 70 °C a decrease of about 15% hatchability occurred between 5 and 9 hr. Over this time span, the "critical" temperature would appear to be between 60-70 °C. These observations agree generally with those of Dutrieu (1960) who found the critical temperature to be above 66 °C. Thus, the dried cells of these cysts seem to be capable of exposure to a total temperature range of 330 °C for appreciable times, clearly an impressive ability. Equally impressive is their resistance to low pressure ("vacuum"). Iwasaki (1973b) detected no change in % hatch of cyst populations incubated for 48 hr at 5×10^{-5} torr (the longest time examined). Whitaker (1940) observed the same outcome after 6 months at 10^{-6} torr, and Gaubin-Blanquet *et al.* (1976) exposed cysts to 9×10^{-7} torr for 38 hr with comparable results. In a study designed to remove all "residual" water from the cysts, the following treatment was applied using a B.E.T. volumetric apparatus : evacuation to 10^{-5} torr, cool to -196 °C, bombard with pure N_2 vapor at 650 mm pressure for 0.5-1 hour, warm tube to room temperature ($< 10^{-5}$ torr) and evacuate for one hour with a mercury diffusion pump (Clegg *et al.*, 1978). That treatment was applied for 12 consecutive cycles with no significant decrease in the hatching rate or percentage. This same treatment applied to tendon reduced it to powder. Clearly, the tolerance of *Artemia* cysts to desiccation is remarkable.

Smith and Siegel (1975), Tazawa and Iwanami (1974), and Iwanami *et al.* (1975) have examined the effects of soaking dry cysts in a wide variety of organic solvents. Negligible effects on viability were observed for a 30 day exposure to "absolute" acetone, n-butanol, n-propanol, isopropanol, ethyl ether and xylene. Longer periods of exposure showed temperature dependence in their effects (Tazawa and Iwanami, 1974). Interestingly, absolute ethanol exposure reduced viability to some extent, while absolute methanol rapidly killed the cysts (Smith and Siegel, 1975). The authors propose the latter effect results from the similarity of methanol to water.

A great many studies have been carried out on the resistance of dry cysts to various sorts of radiation treatments. The first detailed studies were performed during the early 1950's (see Bowen, 1963) using X-rays, but γ-rays, fast neutrons, proton beams, and high energy electrons have been studied since then, most extensively by Iwasaki at the Radiological Institute in Chiba, Japan (see Iwasaki, 1973ab, and her article in the present proceedings).

These and other well known attributes of dried cysts have no doubt provided the stimulus to utilize them in several space experiments (NASA's Biosatellite II, Apollo 16 and 17, the Apollo-Soyuz Test Project, and Cosmos 782 and 936). The outcome of these studies is still in progress (for details see Planel *et al.*, 1975, 1980 ; Blanquet, 1977 ; Gaubin *et al.*, 1979, 1980). In some of these experiments the cysts were housed on the outside of the space craft and were exposed directly to the conditions of outer space. Yet, relatively little overall effect on viability was observed for cysts not directly hit by cosmic heavy ions (Gaubin *et al.*, 1979).

It seems clear that these tolerances are a consequence of the ability of the cysts to reversibly lose practically all of their intracellular water. The biochemical and biophysical basis of reversible water loss has been the subject of considerable study (for reviews see Clegg, 1974a, 1978a, 1979). Of particular importance appears to be the accumulation of large amounts of trehalose (a disaccharide) and the polyhydroxy alcohol, glycerol during early embryonic development (Clegg, 1965). It has been proposed that glycerol (and perhaps trehalose) serve as

a "substitute" for water as the cells undergo dehydration, and this mechanism appears to be widespread in a variety of organisms capable of reversible dehydration (Crowe and Clegg, 1973, 1978 ; Crowe, 1971, Clegg, 1978ab).

The tolerance of hydrated cysts is another matter. Iwasaki (1973b) examined the tolerance of cysts pre-hydrated for 1, 2, and 3 hr at 4 °C in 2% NaCl and then exposed to -196 °C. No effect on viability was observed for the 1 hr pre-hydration preparation but a large fraction of the cysts were killed after 0.5 hr at -196 °C using the 3 hr pre-hydration group (maximally hydrated cysts). These findings contrast, however, with those of Dutrieu and Chrestia-Blanchine (1967) who observed no significant decrease in cyst viability after several hours exposure to -196 °C. It is difficult to reconcile these observations. To my knowledge, no published data are available on the exposure of hydrated cysts to higher than ordinary temperatures ; in principle, hydration should decrease thermal tolerance appreciably.

As expected, addition of water also dramatically changes the response of cysts to various kinds of radiations (Engel and Fluke, 1962 ; Snipes and Gordy, 1963 ; Iwasaki, 1964, 1973b, 1980 ; Metalli, 1980). Hydration exerts a profound effect on the penetration of alcohols into cysts as judged by viability decreases (Smith and Siegel, 1975). At this point we should briefly consider the general permeability characteristics of hydrated encysted embryos. We believe the evidence shows clearly that the cysts are essentially impermeable, in the strict sense of that word, to all solutes of low volatility. There is good evidence that CO_2 can penetrate the cells (Clegg, 1966) probably as free CO_2 rather than HCO_3^- (Finamore and Clegg, 1969). Studies using radioactive ions, amino acids, sugars, nucleotides and glycerol show that these substances do not penetrate the cells prior to emergence (Clegg, 1966, 1967 ; Finamore and Clegg, 1969 ; Conte *et al.*, 1977). One must be careful, of course, to consider adsorption of these substances by the shell components and their trapping within the extensive vesicular network of the porous tertiary envelope (Conte *et al.*, 1977). The electron microscopical study of Morris and Afzelius (1967) demonstrated convincingly that the shell is impermeable to lead phosphate, and they identified the outer cuticular membrane as the site of impermeability. That observation was confirmed and extended by the detailed work of de Chaffoy *et al.* (1978a) that we have already discussed. The only exception to this conclusion is the work of Susheela and Jayaraman (1976) who claimed that ^{32}P-inorganic phosphate did penetrate encysted embryos. But, the nature of this incorporation was not what one would expect from what is known about the nucleotide pool, and nucleotide metabilism (Warner and Finamore, 1967 ; Finamore and Clegg, 1969 ; Van Denbos and Finamore, 1974). It could be of importance that the cysts used by Susheela and Jayaraman (1976) were collected from solar salt ponds on the southeastern coast of India, and were not the ones used in the studies cited above. If indeed the Indian cysts are permeable to labelled phosphate they should prove of considerable value in the study of developmental biochemistry during the encysted period.

In the aggregate, the foregoing suggest that the cells of the dried encysted gastrula represent the most resistant of all animal cells to environmental hazards. These abilities reside within the cells themselves, as well as in the complex shell.

A matter of some interest concerns the metabolic status of "dry cysts", and its relationship to cyst longevity. With regard to the latter it seems evident that all storage conditions that increase longevity are those that suppress metabolism : low temperatures, maintenance of low hydration levels, and removal of oxygen (Bowen, 1963 ; Clegg, 1967, 1973). Thus, if a metabolism exists it is apparently not desirable in terms of longevity. The conclusion of one of

us is that the cysts are certainly ametabolic below water contents of 0.1 g H_2O/g dry wt of cysts, and probably below 0.3 g/g, based on a wide variety of experiments (reviewed by Clegg, 1978b, 1979a) and theoretical considerations of macromolecular hydration (Clegg, 1973, 1978b). What can be said then of the observation by Sundnes and Valen (1969) that *Artemia* cysts, containing about 0.15 g/g water consumed oxygen at detectable rates in a temperature dependent fashion? But oxygen consumption need not reflect metabolic participation in dried biological systems, a great many inanimate objects "consume oxygen". (One need only place some "dry" iron shavings in a Warburg respirometer to observe this). Consequently, the uptake of oxygen at low rates in dry biological systems is completely inconclusive in the absence of additional evidence that the oxygen is in fact coupled to ongoing metabolic processes (*i.e.*, the electron transfer chain). We maintain that the encysted gastrula is ametabolic at hydrations less than 0.1 g/g and probably less than 0.3 g/g. As a result, the dry *Artemia* cyst is a true cryptobiotic form, exhibiting properties and characteristics that are neither those of living nor dead cells. Further accounts of these general considerations can be found in works by Keilin (1959), Clegg (1967, 1973, 1978b), Hinton (1968), Crowe (1971) and Crowe and Clegg (1973, 1978).

Resumption of metabolism and development of the encysted gastrula

Granted that the severely dried cyst is ametabolic due to water lack, it follows that rehydration must reinitiate metabolism, and a number of studies have "mapped" this hydration dependence of metabolism (reviewed by Clegg, 1978b, 1979a). A conventional metabolism (*i.e.*, one characteristic of fully hydrated cysts) is initiated at 0.65 g H_2O/g dry wt ($\pm$ 0.07), roughly at one-half the hydration level of fully hydrated cysts ($\approx$ 1.4 g H_2O/g dry wt) achieved by incubation for 3 hr in 0.1 M NaCl, or equivalent water activity (Clegg, 1978a). This minimal hydration reinitiates metabolism, but at a low rate. However, the cyst has biochemical properties that allow it to increase its water content once metabolism is initiated : the cells adaptively synthesize and accumulate glycerol which tends to decrease the osmotic differential between the inside and outside of the cyst, thus allowing for increased water uptake and a more rapid metabolism (Clegg, 1964). Thus, embryonic development is also resumed above the threshold hydration. The bulk of available evidence supports the claim of Dutrieu (1960) that, in addition to hydration at suitable temperature, the only other external requirement for resumption of development is molecular oxygen, at adequate partial pressure. The nature of the requirements that exist for the processes of emergence (splitting of the shell) and hatching (escape of the nauplius) are matters we will consider later in this review.

Certainly, the vast majority of what is known about the developmental biochemistry of *Artemia* comes from the study of the reactivated dried gastrula, its transition into a nauplius larva, and the first three or four larval stages. Before examining the biochemical findings, it is important to describe what is known regarding the morphology of this segment of development. Fig. 4 shows the overall structure of some characteristic landmarks of development. The encysted gastrula consists of 3 000 to 4 000 cells depending upon the geograhical origin (Nakanishi *et al.*, 1962 ; Olson and Clegg, 1976). "Pre-emergence development" (PED) usually requires between 8-16 hr of incubation in optimal conditions – obviously, external conditions will strongly influence its duration. The newly emerged embryo is referred to as an E_1 (Nakanishi *et al.*, 1962) and is a partially formed nauplius larva.

After about 2 hr of incubation the embryo protrudes further from the shell as an E_2 (Fig. 4) and this will hatch as a swimming nauplius larva after an additional 2 hr, depending on the conditions of incubation. Fig. 5 shows the composition of a population as a function of incubation time for cysts from the Great Salt Lake, Utah (Conte *et al.*, 1977 ; Peterson *et al.*, 1978ab). We point out that biochemical studies done as a function of time are often difficult to interpret because of the heterogeneity of these populations.

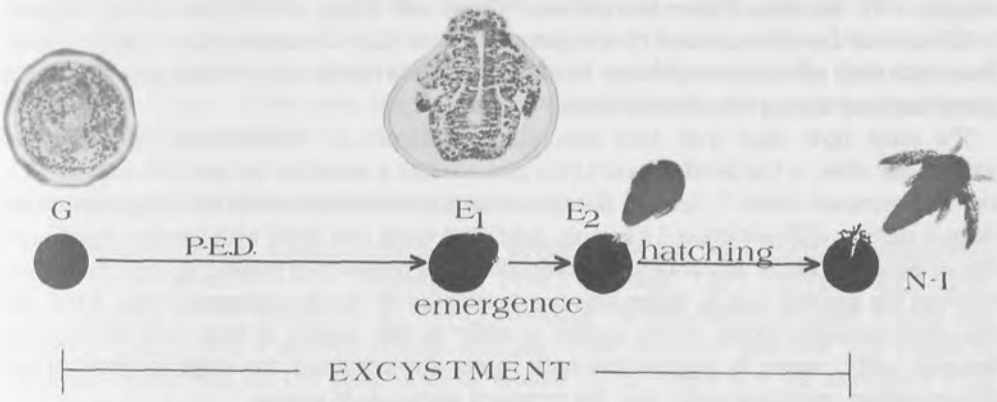


FIG. 4. Development of the encysted gastrula. The photographs given above G (gastrula) and E_1 (just emerged embryo) are cross sections of these stages as seen with the light microscope. The diameter of the encysted gastrula is about 0.2 mm. N-I refers to the first stage nauplius, and P-E.D. means pre-emergence development.

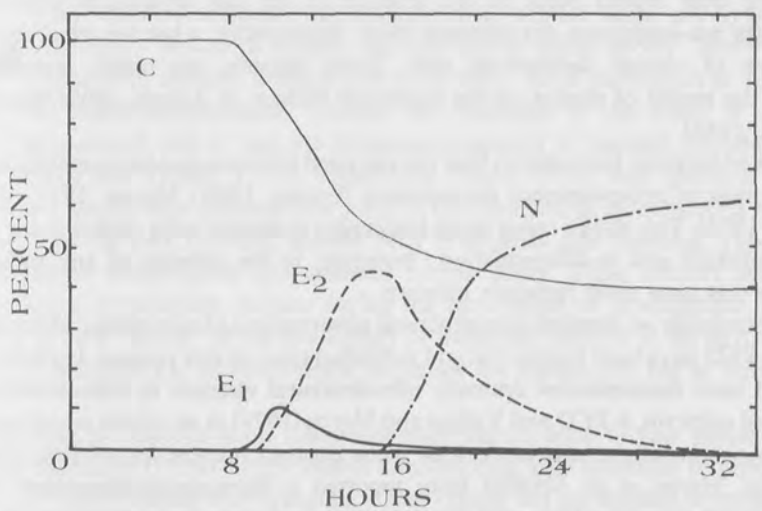


FIG. 5. Relative composition of a developing population with increasing incubation time. Abbreviations : (C) unemerged cysts ; (E_1) just emerged embryo ; (E_2) fully emerged embryo ; (N) stage-I nauplius).

An observation of considerable importance is that the entire process of PED occurs without the participation of DNA synthesis (Bellini, 1960 ; Nakanishi *et al.*, 1963) or the occurrence of cell division (Nakanishi *et al.*, 1962). These findings have been confirmed and extended (Finamore and Clegg, 1969 ; McClean and Warner, 1971 ; and Olson and Clegg, 1978). Furthermore, it appears that most (all ?) of the cell division taking place in the E_1, the E_2, and the first two larval stages is not mandatory to development because the E_1 to nauplius-II transition occurs "normally" even when DNA synthesis and cell division are essentially stopped with the drug 5-flurodeoxyuridine (Olson and Clegg, 1978). Hence, all processes involved with the development of the gastrula into a stage II nauplius can occur with far fewer cells than normally are present. Furthermore, these results indicate that gene replication is not required during this developmental period.

We must now deal with two interrelated questions of considerable developmental importance : first, is the development of the gastrula into a nauplius the same for the dormant and non-dormant routes ? ; second, does pre-emergence development of the encysted embryo involve cellular differentiation ? The only published work that deals with the first question is that of Benesch (1969). His drawings clearly show the presence of mitotic figures in embryos between the gastrula and E_1 stages that are developing by the non-dormant route. Thus, the two developmental routes would appear to differ in this respect at least. But the work of Benesch (1969) seems to suggest that both routes are essentially the same in terms of cell differentiation, morphogenesis, and the temporal sequence of events.

This second question must be asked because of Morris (1971) who hypothesized that no differentiation occurred during PED until just before the embryo (E_1) emerged from the shell. Morris was apparently unaware of Benesch's (1969) study which clearly details the occurrence of differentiation of a variety of cell types, and widespread histogenesis during the entire period between gastrula and nauplius. Thus, we can conclude that cellular differentiation does indeed occur in the absence of any cell division or DNA synthesis throughout the pre-emergence development stage. Presumably, what we are observing is the differentiation of already determined cells. These features are rather important when interpreting the results of studies on the molecular biology of *Artemia* development (Hultin and Nilsson, 1980).

A number of workers have shown that the encysted embryos can be reversibly dehydrated throughout most of pre-emergence development (Ewing, 1968 ; Morris, 1971 ; de Chaffoy and Kondo, 1976). This ability raises some fascinating questions with regard to the possibility of dedifferentiation and re-differentiation ; however, in the absence of any morphological observations this issue must remain a mystery.

To our knowledge no detailed ultrastructural observations of subcellular changes in intact cysts during PED have been carried out and published prior to this volume. However, Schmitt *et al.* (1973) have demonstrated dramatic ultrastructural changes in mitochondria isolated from encysted embryos in PED and Vallejo and Marco (1976) in an article which we have not been able to obtain, might have performed some electron microscopy of yolk platelets and mitochondria. Marco *et al.* (1980a) have reported a development-dependent release of mitochondria-like structures from yolk platelets during pre-emergence development, and have related these observations to various *in vitro* assays of mitochondrial enzymes. We will consider their work and that of Schmitt *et al.* (1973) in more detail later in the review.

Biochemistry of pre-emergence development

Having documented the morphological changes that take place we will now examine the biochemistry that presumably either underlies or is a consequence of these changes. We will exclude coverage of RNA and protein metabolism since these considerations are covered elsewhere (Hultin and Nilsson, 1980). We warn the reader that we have not critically evaluated all of the techniques and methods used in the papers we cite ; rather, we will generally accept the findings stated by the authors. Several reviews dealing with various aspects of the biochemistry of PED have been published (Dutrieu, 1960 ; Urbani, 1959 ; Green, 1965 ; a particularly good review by Huggins and Munday, 1968 ; Finamore and Clegg, 1969 ; Clegg, 1978a ; and the published proceedings of a very recent symposium, Bagshaw and Warner, 1979).

Respiration, mitochondria, and anoxia

Oxygen consumption during hydration and development has been examined extensively (Urbani, 1946 ; Dutrieu, 1960 ; Muramatsu, 1960 ; Emerson, 1963, 1967a ; Clegg, 1964, 1976b ; Morris, 1971 ; Schmitt *et al.*, 1973). Although the conditions of these studies and sources of cysts vary, a general pattern emerges when encysted gastrulae are placed in solutions 0.5 M NaCl (or the equivalent) at around 25 °C : there is a lag associated with hydration of about 15 to 30 min, followed by an increase in the rate of respiration for several hours, then a leveling off as the time for emergence approaches. Another increase in rate occurs at about the time of emergence and hatching. The latter increase has been ascribed by Emerson (1967a) to the increase in surface area that accompanies emergence and hatching,but there is an alternative that we will describe later. The initial increase in respiratory rate seems to be due to dramatic changes in the structure and function of free mitochondria (Schmitt *et al.*, 1973). Those workers showed that mitochondria isolated from pre-hydrated (0 °C) gastrulae are essentially devoid of cristae, and the matrix stains poorly. However, matrix staining and cristae both increase markedly over the first 7 hr of incubation at 30 °C. Nicely correlated with these morphological changes are increases in the levels of cytochrome oxydase, cytochromes b and c, and the respiratory capacity of isolated mitochondria. The findings of Marco and Vallejo (1976), Vallejo and Marco (1976), and Marco *et al.* (1980a) presented at this symposium indicate that some of this increase in mitochondria results from their release from the yolk platelets, notably after emergence and hatching. This is a rather surprising series of observations and deserves further study. These authors also ascribe a critical function to cytochrome oxidase during the resumption of development, considering this molecule to be a "sensor" of the availability of molecular oxygen in the environment : in the absence of adequate O_2, developmental processes are turned off, but in fully reversible fashion.

This suggestion is related to some interesting experiments done 13 year earlier by Dutrieu and Chrestia-Blanchine (1966) which clearly showed that fully hydrated encysted gastrulae responded in a rather unique way to anoxia – they simply did not resume development upon anoxic hydration, even when incubated at room temperature for half a year. However, it was noted that upon restoration of oxygen, the gastrulae resumed development and gave rise to normal nauplii at rates, and percentages, comparable to controls. Ewing (1968) in a

completely independent study made similar observations. Dutrieu and Chrestia-Blanchine (1966) examined the total lipid and carbohydrate levels of anoxic fully-hydrated cysts, but observed no significant change in either of these after 5 months of incubation, the implication being that these metabolic pathways had been reversibly halted by anoxia. Ewing and Clegg (1969) confirmed and extended these observations, concluding that the gastrulae did not carry out a conventional carbohydrate-based anaerobic metabolism. These studies raised the question as to how the cells could maintain their integrity over many months of anoxia, if indeed they did lack an energy-generating anaerobic metabolism. This question was answered by the thorough and detailed studies of Stocco *et al.* (1972). They showed that nucleotide metabolism is not halted by anoxia, and that the stored nucleotide, diguanosine tetraphosphate (Gp_4G) is slowly metabolized, presumably providing the primary source of free energy. Apparently, this free energy is somehow coupled to processes concerned with maintaining the integrity of the gastrula. Ewing and Clegg (1969) had observed that the first stage nauplius, in striking contrast to all stages of unemerged incubated embryos in PED, were killed within the first 5 hr of anoxia, and that a conventional anaerobic metabolism did occur in the nauplius, resulting in lactic acid formation. Stocco *et al.* (1972) confirmed the lethal effect of anoxia on nauplii and also found that changes in the control of guanine nucleotide metabolism take place at hatching. In view of all the foregoing, it seems that the postulated role for cytochrome oxidase advanced by Vallejo *et al.* (1980) is reasonable. Of course, it will be necessary to show precisely how cytochrome oxidase activity controls the totality of metabolic processes and development during anoxia.

Finally, we note that Stocco *et al.* (1972) did observe some changes in developmental rates and % hatch levels of cysts during long term anoxia, in contrast to Dutrieu and Chrestia-Blanchine (1966). These appear to be relatively minor differences and do not alter the general picture described above, but they do indicate something of considerable importance we will consider later : namely, the potentially different behavior and properties of different batches of cysts from the same geographical origin, as well as cysts from different origins. We will also return to the matter of nucleotide metabolism during normal development.

There is uniform agreement that the respiratory quotient of embryos in PED is very nearly unity (Dutrieu, 1960 ; Muramatsu, 1960 ; Emerson, 1963 ; Clegg, 1964) providing good indication that carbohydrate utilization is the substrate for respiration. Those studies also showed that the R.Q. decreases after hatching, usually to values between 0.7-0.8, indicating a switch in respiratory substrates. As we shall see in the next section, these expectations have been confirmed.

Bulk energy and carbon sources for PED

We have documented the impermeability of the embryonic cuticle to non-volatile organic molecules. Hence, it is clear that all energy sources and substrates for metabolism must already be present in the gastrula as it encysts and becomes dormant within the maternal female. The bulk source of energy for PED has been established to be α, α-trehalose, the non-reducing disaccharide of glucose, first demonstrated in these cysts by Dutrieu (1959, 1960). Trehalose utilization not only serves as the major, and likely the only bulk respiratory substrate, it apparently also serves as a source of carbon for glycogen and glycerol synthesis during PED (Dutrieu, 1960 ; Clegg, 1964) and perhaps as the carbon skeleton for a variety of

metabolites (Emerson, 1967b ; Huggins and Munday, 1968 ; Boulton and Huggins, 1977). Although a detailed study of the glycolytic pathway and Krebs' cycle in encysted embryos has apparently not been done, it seems very likely indeed that the overall pathways will be similar to those found in eucaryotic cells in general. What little evidence is available supports this expectation (Huggins and Munday, 1968 ; Huggins, 1969 ; Boulton and Huggins, 1970, 1977). Nevertheless, we must stress that this is an assumption and that a detailed examination of these pathways might reveal unique and interesting features.

At this point we digress briefly to emphasize something of great importance in evaluating published work on these cysts as well as in the design of experiments, and publication of the results. It is imperative to clearly state the geographical origin, the year the cysts were collected, and the commercial supplier, in the published work. The reason is simple : different populations (even from the same area) can exhibit different behavior and properties, and the metabolism of trehalose is an excellent example of this. In 1970, Huggins and Boulton published an abstract in which they stated findings completely at odds with earlier work (Dutrieu, 1960 ; Clegg, 1964 ; Ewing and Clegg, 1969). We successfully repeated our earlier work (Clegg, 1976a) and initiated an exchange of several letters with Dr. Huggins. Eventually, we decided to resolve the matter by exchanging samples of the cysts we each had used. Whereupon, we were able to confirm our respective observations : the seeming conflict in results was due to different behavior of the two cyst populations (ours were from the California population near San Francisco while theirs were from a location in Canada). Details of this work are given by Boulton and Huggins (1977). Other examples of this sort could be given.

In view of the importance of trehalose metabolism during PED one might postulate an important role for the enzyme(s) involved with the first step in its metabolism. Only two papers have been published on the matter. Boulton and Huggins (1977) observed no change in trehalase activity throughout PED, using 20,000 g (1 hour) supernatants as the sole source of enzyme. However, Ballario *et al.* (1978) have shown that almost all of the trehalase activity of whole homogenates of unincubated cysts, sediments at 1 000 g (10 min). Thus, the developmental change in this enzyme activity, if any, remains to be determined. Ballario *et al.* (1978) purified the enzyme 180 fold and carried out the usual characterization. It is highly specific for α, α-trehalose and is, therefore, an α, α-trehalose 1-D-glucohydrolase (E.C.3.21.28). Its K_m is 4.3×10^{-3}M (for trehalose), the pH optimum is close to 6, and its molecular weight is about 75 000. Although its regulation was not examined in detail these workers observed no effect of adding ATP or 3',5'-AMP on enzyme activity. Further study of this enzyme is clearly needed, particularly in terms of developmental changes and possible regulation. It is of some interest that the enzyme appears to be chiefly particulate since Warner *et al.* (1972) have obtained evidence that appreciable amounts of trehalose are associated with the yolk platelets. Perhaps it is worth suggesting that the initial step in trehalose utilization might not be hydrolytic and that future studies might take this into account. It seems quite possible that phosphorylytic cleavage could be involved since this would reduce the free energy requirement for subsequent utilization of the resulting phosphorylated glucose compared with free glucose.

"Balance sheet" studies have indicated that trehalose provides the substrate for glycerol and glycogen synthesis, both of which occur during PED (Dutrieu, 1960 ; Clegg, 1964). In the absence of tracer studies, this conclusion must be tentative. We have no information on the

enzymes involved with glycerol formation although a likely pathway would involve glycolysis to dihydroxyacetone phosphate, with subsequent reduction and phosphate removal. Because of the postulated role of glycerol in the emergence process (Clegg, 1964) and in the desiccation resistance of the encysted gastrula (Clegg, 1974a) a detailed study of the enzymes of glycerol metabolism should prove of interest.

Unpublished work from one of our laboratories (J. S. Clegg) has been directed toward the enzymes of glycogen metabolism and we will briefly summarize our findings (Tables I and II). These studies were performed by Dr. Z. Gunja-Smith. Glycogen synthetase activity is detectable in all stages examined, increases during PED and as the nauplius is formed, and is practically all of the "dependent" form (Table I). In contrast, glycogen phosphorylase shows no significant change during PED but increases very sharply at the E_2 to nauplius transition (Table II). Curiously, the gastrula and nauplius enzyme exhibit strong dependence on AMP, whereas the activity of E_1/E_2 extracts is much less dependent on this nucleotide. These activities can be compared with the net increase in glycogen that occurs during PED (Dutrieu, 1960 ; Clegg, 1964).

TABLE I

Glycogen synthetase in three developmental stages

Stage examined	Activity (μmoles ^{14}C-glucose incorp./mg P/min)[1]	
	Plus G-6-P	Minus G-6-P
Gastrula	0.189	0.009
E_1/E_2	0.380	0.014
Nauplius-I	0.550	0.019

[1] 10 000 g (10 min) supernatant used for assay : Methods in enzymology. Vol. 8, p. 374. Average of two experiments.

TABLE II

Glycogen phosphorylase in three developmental stages of *Artemia salina*

Stage examined	Activity (μmoles Pi/mg P/min)[1]	
	Plus 5'-AMP	Minus 5'-AMP
Gastrula	0.51	0.067
E_1/E_2	0.52	0.145
Nauplius-I	1.28	0.005

[1] 10 000 g (10 min) supernatant used for assay : Methods in enzymology. Vol. 8, p. 526. Average of two experiments.

Except for the analysis by Dutrieu (1960) on total lipid contents we know virtually nothing about changes in lipids during PED, let alone their metabolism ; a fertile area for study.

Fortunately, Schauer *et al.* (1980) have initiated such studies, and have observed differences between the total lipid content and fatty acids of encysted gastrulae compared with nauplii.

The total protein content decreases slightly during PED (Dutrieu, 1960 ; Peterson *et al.*, 1978a). However, there are significant changes with regard to the intracellular distribution of proteins, and important qualitative changes do indeed occur (Hultin and Nilsson, 1980 ; and subsequent sections of our review). Since a large fraction of the total cyst protein is in the yolk platelets (Warner *et al.*, 1972) and since these are degraded to some extent during PED (Olalla *et al.*, 1977) this is to be expected. We will return to these considerations later.

Nucleotide metabolism

Without doubt the most thorough and extensive study of metabolites in *Artemia* concerns the nucleotide pool. Virtually all of this work can be traced to the laboratory of Dr. Frank Finamore of the Oak Ridge National Laboratory. In the early 1960's, he and Dr. Alden Warner discovered an unusual guanine-containing nucleotide in the dormant gastrula (Finamore and Warner, 1963), the first in a series of studies on the nucleotides of *Artemia*. Practically all of the research on this and related subjects since then has been heavily influenced by Dr. Finamore. Indeed, his contributions to the developmental biochemistry of *Artemia*, and his influence on the field, via his students and associates, has been far reaching. It is in recognition of this that we dedicate this review to him.

The nucleotide Finamore and Warner (1963) discovered was shown by them to have the structure shown in Fig. 6. In current nomenclature it is abbreviated as G(5')pppp(5')G, or simply Gp_4G. It is present in very large amounts in the dormant gastrula (about one-half of the total free nucleotide pool) and its study during the last 16 years has revealed its importance to the developmental biochemistry of *Artemia*. In addition to Gp_4G, other unusual dinucleotides were discovered in the gastrula : G(5')ppp(5')G by Warner and Finamore (1965a), G(5')pp(5')G and G(5')ppp(5')A by Gilmour and Warner (1978). In terms of the total acid soluble nucleotide pool the amounts present in the gastrula are Gp_4G (45 %), Gp_3G (7 %), Gp_2G (0.5 %) and Gp_3A (0.5 %) (Gilmour and Warner, 1978). While a variety of potential origins and roles for Gp_2G and Gp_3A have been considered, little is known about these matters at present (Gilmour and Warner, 1978). In contrast, a great deal of information has been obtained on the metabolism and roles of Gp_4G.

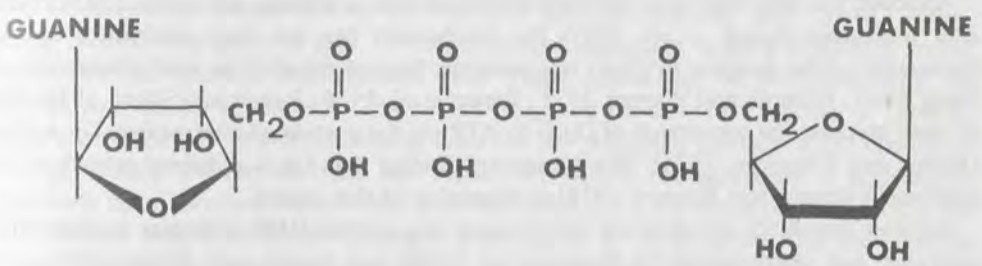


FIG. 6. The structure of guanosine 5'-tetraphospho-5'-guanosine (Gp_4G) discovered in *Artemia* by Finamore and Warner (1963).

We have already described the importance of Gp_4G in anoxia of hydrated gastrula (Stocco *et al.*, 1972). Now we will review the evidence that Gp_4G also functions as a source of purine nucleotides for nucleic acid synthesis, particularly after emergence when a net increase in both RNA and DNA occur (Bellini, 1960 ; Finamore and Clegg, 1969 ; McClean and Warner, 1971). Interesting, *Artemia* lacks the ability to synthesize the purine ring *de novo* (Clegg, *et al.*, 1967 ; Warner and McClean, 1968). Consequently, it must have available an exogenous source of purines to support nucleotide and nucleic acid metabolism. Since the diet of the larvae and adults is rich in purines this poses no problem for these life cycle stages. But the encysted gastrula is impermeable to such solutes and feeding does not begin until the nauplius II stage is reached (Benesch, 1969). This hiatus in the availability of purine is bridged chiefly by Gp_4G. Warner and McClean (1968) demonstrated that Gp_4G is essentially absent from all stages of the life cycle except for the sexually mature female and all developmental stages from oocyte to stage II or III nauplii, in which Gp_4G has essentially disappeared. They also concluded that Gp_4G synthesis was limited to ovarian tissues in the female and that Gp_4G was synthesized by the oocytes themselves during oogenesis. In contrast, Gp_3G, Gp_2G, and Gp_3A are made by the developing embryo prior to the gastrula stage.

Subsequent work by Warner *et al.* (1972) showed rather conclusively that Gp_4G is restricted to the yolk platelets ($\approx$ 90% of the total) of the encysted gastrula. That result was contrary to an earlier claim for a nuclear location (Sillero and Ochoa, 1971), which has apparently not been withdrawn (Renart *et al.*, 1976). Warner *et al.* (1972) found that tritiated GDP and GTP were incorporated into Gp_4G by purified yolk platelets *in vitro* and they proposed that these cell organelles were also the biosynthetic sites during oogenesis (vitellogenesis) as well as being the intracellular storage location in later developmental stages. Further analysis of the system (Warner *et al.*, 1974 ; Warner and Huang, 1974) provided good support for that contention, since yolk platelets were shown to contain a Gp_4G synthetase (GTP:GTP guanylyltransferase) which utilizes two GTP molecules as substrates to reversibly form Gp_4G and inorganic pyrophosphate. They also demonstrated the formation of Gp_3G but at a much slower rate ($GDP + Gp_4G \rightarrow Gp_3G + GTP$), presumably by the same enzyme. However, competition studies showed clearly that two catalytic sites must exist if a single enzyme is involved, and these workers did not rule out the possibility that two different enzymes are involved.

In this fashion then, the encysted gastrula has a supply of purine nucleotide as well as a source of "high energy phosphate" to meet the needs of anoxia (Stocco *et al.*, 1972).

Although it is clear that Gp_4G provides adenine as well as guanine for nucleic acid synthesis after emergence (Clegg *et al.*, 1967) the mechanism has not been established for the conversion of the guanine in Gp_4G into adenine. Several possibilities exist (Finamore and Clegg, 1969 ; Gilmour and Warner, 1978 ; Renart *et al.*, 1976 ; Renart and Sillero, 1974). One of these involves the conversion of Gp_4G to ATP *via* the postulated intermediate Gp_4A (Van Denbos and Finamore, 1974). The subsequent finding that Gp_3A is indeed present in the gastrulae (Gilmour and Warner, 1978) is interesting in that regard.

Another possibility concerns the involvement of a soluble GMP reductase isolated (from gastrulae) and characterized by Renart *et al.* (1976) and Renart and Sillero (1974). This enzyme converts GMP to IMP, requiring NADPH as coenzyme. This group has shown that IMP and XMP competitively inhibit the reductase, notably XMP whose presence produces «hyperbolic kinetics». Gp_4G alters the shape of velocity *versus* (GMP) curves when XMP is

present, essentially reversing inhibition by XMP. On the basis of these and other findings they speculate that the production of adenine nucleotides from Gp_4G could involve IMP as an intermediate : $Gp_4G \rightarrow GTP + GMP$; GMP → IMP ; and (postulated) IMP → AMP. It is perhaps important to point out that Hernandorena (1972) has shown that IMP satisfies the purine requirement that exists for larval growth in axenic culture.

Of course, Gp_4G is likely to play roles in addition to serving as an anoxic energy source and as a precursor of adenine. Warner and Huang (1974) pointed out that Gp_4G synthetase could also be involved in Gp_4G utilization (provided that inorganic pyrophosphate were available) supplying GTP as the product. Other enzymes from gastrulae that are likely to be involved with Gp_4G utilization have also been demonstrated. Warner and Finamore (1965b) purified and characterized a diguanosine-tetraphosphate guanylohydrolase that converts Gp_4G to one mole each of GMP and GTP. It is interesting that this enzyme increases in activity as the level of Gp_4G falls in the developing nauplius (Beers, 1971). Vallejo *et al.* (1976) carried out further studies on what seems to be the same enzyme, showing among other things that it was present largely in the 150 000 g supernatant. They made the interesting observationt that Ap_4 and Gp_4 are both extremely potent inhibitors of Gp_4G-guanylohydrolase as well as another similar enzyme (dinucleosidase tetraphosphatase) that has been studied by this group (Vallejo *et al.*, 1976). Therefore, even though these nucleoside tetraphosphates have not been detected thus far in the cysts the effective concentrations required for inhibition are so low that routine screening might well miss them, and they remain potentially important. In this connection it is worth noting that Gilmour and Warner (1978) resolved over 20 nucleotide components in their thorough work on the gastrulae, and that a number of these have not yet been identified.

From the foregoing it seems quite clear that the guanine nucleotides play a major role in PED and the early naupliar stages. (For details of their participation in protein synthesis see Hultin and Nilsson, 1980). We hope that the laboratories examining these various aspects will continue their work, and that others will join them. A recent review of "unusual" nucleotides in eucaryotic cells clearly indicates their potential importance in metabolism (Silverman and Atherly, 1979), and the work on *Artemia* could prove to be of general applicability.

Free amino acids

The free amino acids have not been studied in any great detail, being limited to a few papers. Dutrieu (1960) did not quantify the free amino acids but did state the order of their apparent relative abundance based on visual inspection of chromatograms. She lists these (in order of decreasing abundance) for the dormant gastrula : aspartic acid, cystein, serine, glycine, alanine, and glutamic acid. In nauplii the corresponding ranking is alanine, serine, glycine, proline, tyrosine, and glutamic acid. These values can be compared with the quantitative estimates of Emerson (1967b) and Clegg and Lovallo (1977) shown in Table III for dormant gastrula (A) and developing embryos (B). Although the incubation conditions were appreciably different in these two studies we believe the agreement is remarkable, particularly since Emerson's cysts were from the Great Salt Lake while ours were from the San Francisco area. Unless this agreement is fortuitous, it would appear that the embryos exercise considerable control over free amino acid levels. Obviously, the net concentrations of the free amino acids will be determined by the balance between supply from yolk platelet metabolism (and any other sources of free amino acids) and utilization of amino acids for protein synthesis, and other metabolic pathways.

TABLE III

Free amino acids in *Artemia* encysted embryos
(μg of amino acid/mg dry weight)

Amino acid	Dormant gastrula		Developing embryo[1]		B/A	
	A. Emerson	A. Clegg and Lovallo	B. Emerson	B. Clegg and Lovallo	Emerson	Clegg and Lovallo
Cys	0.12	0.11	0.46	0.12	3.8	1.1
Asp	1.66	0.65	1.11	0.55	0.7	0.9
Thr	0.14	0.12	1.30	0.62	9.3	5.2
Ser	0.42	0.53	1.25	1.53	3.0	2.9
Glu	1.42	0.93	0.83	0.69	0.6	0.7
Pro	0.43	0.30	1.54	1.10	3.6	3.7
Gly	0.44	0.30	1.28	0.85	2.9	2.8
Ala	1.00	1.00	2.90	2.80	2.9	2.8
Ile		0.03		0.08		2.7
Leu	0.10	0.04	0.21	0.21	2.1	3.0
Tyr	0.62	0.12	0.94	0.29	1.5	2.4
His	0.13	0.05	0.32	0.09	2.5	1.8
Lys	0.19	0.11	0.33	0.21	1.7	1.9
Arg	0.45	0.19	0.69	0.22	1.5	1.2
Val	0.15	–	0.33	–	2.2	–
Phe	0.01	–	0.24	–	24.0	–
Taurine	–	1.66	–	2.31	–	1.4
Totals	7.28	6.14	13.73	9.27	1.9	1.5

[1] Emerson's (1967b) data are from embryos incubated for 44 hr in 1.0 M NaCl. Clegg and Lovallo's (1977) data are from cysts incubated for 48 hr in the vapor phase of 1 M NaCl.

With regard to the total free amino acid pool there is uniform agreement that this gradually increases, starting very soon after hydration is complete and PED is resumed. Reasonably similar trends and relative increases have been observed by Emerson (1967b), Bellini (1960), Dutrieu (1960), Boulton and Huggins (1977) and Clegg and Lovallo (1977). Since the total amino-nitrogen shows relatively little change during PED (Dutrieu, 1960, and references cited therein ; Peterson *et al.*, 1978b) these increases in most of the free amino acids most likely reflect yolk protein breakdown. The yolk platelets are, indeed, rich sources of a variety of proteins (Warner *et al.*, 1972 ; de Chaffoy *et al.*, 1978b ; Olalla *et al.*, 1977) and the yolk platelets are degraded during PED, at least to some extent (Olalla *et al.*, 1977). While protease activity is present in extracts of dormant gastrula and embryos in PED (Bellini, 1957 ; Nagainis and Warner, 1979) the level of activity has been reported to be essentially zero for four different protease activities (Osuna *et al.*, 1977). The situation is complicated by the fact that several protease inhibitors have also been shown to exist and these apparently are involved with protease regulation (Osuna *et al.*, 1977 ; Nagainis and Warner, 1979). It is not at all clear what the substrates for these proteases are, but the work of these two laboratories clearly indicates that such enzymes could play a far more specific role during development

than simply to hydrolyze yolk protein. Further work on *Artemia* proteases will likely provide information of considerable developmental importance.

Enzyme activities

It is beyond the scope of this review to critically examine all of the information on this subject. Much of the earlier work was carried out using very crude extracts and the enzyme activities were poorly characterized, if at all. As a result, one must view the data with some trepidation.

In previous sections we have covered, in a little detail, studies on several enzymes involved with carbohydrate and nucleotide metabolism. A number of the papers presented at this symposium deal, in considerable detail, with various enzymes : aspartate transcarbamylase (Alayse-Danet, 1980) ; RNA polymerases (Bagshaw, 1980 ; de Chaffoy *et al.*, 1980 ; Sastre and Sebastian, 1980 ; Sebastian *et al.*, 1980) ; poly (A) polymerase (Jeyaraj *et al.*, 1980) ; proteases (Marco *et al.*, 1980 ; Sillero *et al.*, 1980 ; Warner and Shridhar, 1980) ; cytochrome oxidase (Vallejo *et al.*, 1980) ; mitochondrial enzymes (Marco *et al.*, 1980a) ; and amylase and trypsin (Samain *et al.*, 1980). In addition, the review by Hultin and Nilsson (1980) considers enzyme activities associated with the protein synthesizing system specifically and transcriptional and translational processes in general. We did believe it would be worthwhile to compile a list of the enzyme activities that have been studied in addition to those in the present volume, and the source of this information. We have also constructed a rather arbitrary classification of these enzymes (Table IV) for the purpose of indicating their presence or absence in the dormant gastrula, and to detect general trends (if any) for changes in enzyme activity during PED and the first few larval stages. We should stress that a major problem in summarizing the data in this fashion is the difficulty of evaluating when emergence and hatching occur in the population studied, and the rates of these events. As stressed previously (Fig. 5) most studies have been carried out on mixed populations. Therefore, when we refer to an enzyme activity as increasing, for example, at "emergence" this decision must be re-examined for each specific case if one is interested in precise relationships. Also we do not imply that an increase in activity should be taken to indicate enzyme synthesis. Clearly, there is good reason to believe that enzyme activation/inhibition is of major importance in the developmental biochemistry of *Artemia*.

Inspection of Table IV reveals a few generalities. First, almost all of the enzymes that have been studied in *Artemia* exhibit some sort of fluctuation in activity during development between the encysted gastrula and the first stage nauplius. The exceptions are given in Class III. Of these, only the N-substituted aa-tRNA hydrolase has been studied in detail, the others represent rather preliminary data.

Only one of the enzymes in Class I has been shown to be synthesized *de novo*, the (Na + K)-activated ATPase (Conte *et al.*, 1977 ; Peterson *et al.*, 1978ab). Furthermore, it seems very likely that this enzyme is absent in the cells of embryos in pre-emergence development. We will return to its important role in the nauplius later in the review. It is difficult to say whether or not the other enzymes assigned to this class are synthesized *de novo* or are held at low activity in the gastrula due to the presence of inhibitors, or are "masked" in some other fashion. The individual papers should be consulted.

TABLE IV

Summary of enzyme activities during development between the encysted gastrula and nauplius

CLASS I. – *Not detectable (or extremely low) in the gastrula ; appears later*

Enzyme	*Comments*	*Reference*
(Na + K)-ATPase	De-novo synthesis at E_1/E_2 ; peaks, falls in nauplius	Conte *et al.*, 1977 ; Peterson *et al.*, 1978ab
RNase	Sharp rise after hatching ; differs from other RNase's	Sebastian and Heredia, 1978
RNA polymerase III	Linear increase thru PED to hatching	Renard and Sebastian, 1976
RNA polymerase I	Barely detectable ; 4-fold increase thru PED (activation ?) ; declines in nauplius	D'Alessio and Bagshaw, 1977 ; Bagshaw *et al.*, 1978
DNase	Sharp rise in activity after hatching	Cervera *et al.*, 1980
Proteases (A,B,C,D)	B & C increase after hatching ; A & D do so later ; activation ?	Osuna *et al.*, 1977
aa-tRNA hydrolase	N-substituted non-specific form, increases after hatching	Miralles *et al.*, 1978

CLASS II. – *Detectable in the gastrula ; fluctuates during development*

(A) *No change during PED*

Enzyme	*Comments*	*Reference*
Dipeptidase and Protease	Reach a peak at the E_1 to N transition, then decreases in nauplius	Urbani *et al.*, 1952 ; Urbani and De Cesaris-Coromaldi, 1953 ; Bellini, 1957
RNase	Increase at E_1, peaks, falls in nauplius	Urbani and Bellini, 1958
Alk. phosphatase	Increase at E_1, continues in nauplius	Bellini, 1959
Acid phosphatase	Increase E_1 to E_2 ; levels off in nauplius	Bellini, 1959
Amylase	Increase at E_1 ; continues in nauplius	Urbani *et al.*, 1953 ; Bellini, 1958
Glycogen phosphorylase	Very sharp increase E_2 to N transition	Gunja-Smith and Clegg (unpublished data)
Lipase	Increase at E_1, peak nauplius, then falls	Bellini and Lavizzari, 1958
Lactate dehydrogenase	Increase at E_1, continues thru nauplius ; one isozymic form	Ewing and Clegg, 1969, 1972
Histone acetyltransferase	Increase at E_1, seems to level off in nauplius ; activity on nuclear fraction basis ; activation ?	Cano and Pestaña, 1976
5'-UMPase, NADH-oxidase, G-6 Pase, Mg-ATPase (?)	Increase during E_1 to nauplius transition, level off or slight decline in nauplius	Peterson *et al.*, 1978b
Sucrase	Increase occurs in nauplius	Boultin and Huggins, 1977

(B) *Significant change during PED*

Enzyme	*Comments*	*Reference*
RNA polymerase-I	4-fold increase PED, falls in nauplius	Renart and Sebastian, 1976 ; Bagshaw *et al.*, 1978
RNA polymerase-II	Falls, then increases PED and nauplius	Renart and Sebastian, 1976
RNA polymerase-II	Increases and levels off PED ; falls in nauplius	D'Alessio and Bagshaw, 1977 ; Bagshaw *et al.*, 1978
Cytochrome oxidase	Steady increase PED, peaks in E_2 and nauplius	Peterson *et al.*, 1978b
Cytochrome oxidase	Sharp increase 1st hr PED, then gradual increase	Schmitt *et al.*, 1973
Glycogen synthetase	Gradual increase PED, continues in nauplius	Gunja-Smith and Clegg (unpublished data)
Mg-activated alk. phosphatase	Seems to increase during PED, does in nauplius	Bellini, 1959
Glu-pyr transaminase	Steady increase PED, peaks E_1, rises in nauplius	Emerson, 1967b
Glu-oxa transaminase	Steady increase PED, levels off in nauplius	Emerson, 1967b

Class III. – *No change in activity, gastrula through nauplius*

Enzyme	*Comments*	*Reference*
Soluble trehalase, maltase, GPDH, PFK, LDH	20 000 g supernatants used ; all activities said not to change during development	Boulton and Huggins, 1977
N-acetylphenylalanyl-tRNA hydrolase	No change during 40 hours of development	Miralles *et al.*, 178

Other enzymes examined : chitin synthetase in extracts from 3-day old nauplii (Carey, 1965) ; phosphoenolpyruvate carboxykinase and malate dehydrogenase in young nauplii (Conte, 1977) ; glutamic acid dehydrogenase in mixed populations (Emerson, 1967b) ; microsomal enzyme preparations from nauplii involved with phosphatidyl choline synthesis (Ewing and Finamore, 1970ab) ; phosphoglucomutase, phosphofructokinase, glyceraldehyde phosphate dehydrogenase, aldolase, pyruvate kinase, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase in adults (Boulton and Huggins, 1970).

There seems to be little doubt that the majority of enzymes examind do exist in the dormant gastrula at appreciable levels (Class II). While the levels of activity of a number of these enzymes change little if at all during PED (Class IIa) they uniformly exhibit an increase at emergence, or shortly afterward. Whether or not this represents synthesis or "activation" remains to be seen. However, since the cells begin to divide at this point, and since increases in RNA and protein synthesis also accompany emergence and hatching (Hultin and Nilsson, 1980) it seems reasonable to us that enzyme synthesis is likely to be occurring. Even for enzymes of Class IIb which appear to exhibit fluctuations in the level of their activity during PED, abrupt changes also usually occur at the time of emergence and/or hatching. The same is true for enzymes of Class I. There seems to be little doubt that these two important

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RNase	Sharp rise after hatching ; differs from other RNase's	Sebastian and Heredia, 1978
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DNase	Sharp rise in activity after hatching	Cervera *et al.*, 1980
Proteases (A,B,C,D)	B & C increase after hatching ; A & D do so later ; activation ?	Osuna *et al.*, 1977
aa-tRNA hydrolase	N-substituted non-specific form, increases after hatching	Miralles *et al.*, 1978

CLASS II. – *Detectable in the gastrula ; fluctuates during development*

(A) *No change during PED*

Enzyme	*Comments*	*Reference*
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RNase	Increase at E_1, peaks, falls in nauplius	Urbani and Bellini, 1958
Alk. phosphatase	Increase at E_1, continues in nauplius	Bellini, 1959
Acid phosphatase	Increase E_1 to E_2 ; levels off in nauplius	Bellini, 1959
Amylase	Increase at E_1 ; continues in nauplius	Urbani *et al.*, 1953 ; Bellini, 1958
Glycogen phosphorylase	Very sharp increase E_2 to N transition	Gunja-Smith and Clegg (unpublished data)
Lipase	Increase at E_1, peak nauplius, then falls	Bellini and Lavizzari, 1958
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5'-UMPase, NADH-oxidase, G-6 Pase, Mg-ATPase (?)	Increase during E_1 to nauplius transition, level off or slight decline in nauplius	Peterson *et al.*, 1978b
Sucrase	Increase occurs in nauplius	Boultin and Huggins, 1977

(B) *Significant change during PED*

Enzyme	*Comments*	*Reference*
RNA polymerase-I	4-fold increase PED, falls in nauplius	Renart and Sebastian, 1976 ; Bagshaw *et al.*, 1978
RNA polymerase-II	Falls, then increases PED and nauplius	Renart and Sebastian, 1976
RNA polymerase-II	Increases and levels off PED ; falls in nauplius	D'Alessio and Bagshaw, 1977 ; Bagshaw *et al.*, 1978
Cytochrome oxidase	Steady increase PED, peaks in E_2 and nauplius	Peterson *et al.*, 1978b
Cytochrome oxidase	Sharp increase 1st hr PED, then gradual increase	Schmitt *et al.*, 1973
Glycogen synthetase	Gradual increase PED, continues in nauplius	Gunja-Smith and Clegg (unpublished data)
Mg-activated alk. phosphatase	Seems to increase during PED, does in nauplius	Bellini, 1959
Glu-pyr transaminase	Steady increase PED, peaks E_1, rises in nauplius	Emerson, 1967b
Glu-oxa transaminase	Steady increase PED, levels off in nauplius	Emerson, 1967b

Class III. – *No change in activity, gastrula through nauplius*

Enzyme	*Comments*	*Reference*
Soluble trehalase, maltase, GPDH, PFK, LDH	20 000 g supernatants used ; all activities said not to change during development	Boulton and Huggins, 1977
N-acetylphenylalanyl-tRNA hydrolase	No change during 40 hours of development	Miralles *et al.*, 178

Other enzymes examined : chitin synthetase in extracts from 3-day old nauplii (Carey, 1965) ; phosphoenolpyruvate carboxykinase and malate dehydrogenase in young nauplii (Conte, 1977) ; glutamic acid dehydrogenase in mixed populations (Emerson, 1967b) ; microsomal enzyme preparations from nauplii involved with phosphatidyl choline synthesis (Ewing and Finamore, 1970ab) ; phosphoglucomutase, phosphofructokinase, glyceraldehyde phosphate dehydrogenase, aldolase, pyruvate kinase, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase in adults (Boulton and Huggins, 1970).

There seems to be little doubt that the majority of enzymes examind do exist in the dormant gastrula at appreciable levels (Class II). While the levels of activity of a number of these enzymes change little if at all during PED (Class IIa) they uniformly exhibit an increase at emergence, or shortly afterward. Whether or not this represents synthesis or "activation" remains to be seen. However, since the cells begin to divide at this point, and since increases in RNA and protein synthesis also accompany emergence and hatching (Hultin and Nilsson, 1980) it seems reasonable to us that enzyme synthesis is likely to be occurring. Even for enzymes of Class IIb which appear to exhibit fluctuations in the level of their activity during PED, abrupt changes also usually occur at the time of emergence and/or hatching. The same is true for enzymes of Class I. There seems to be little doubt that these two important

developmental events are accompanied by significant changes in biochemistry, and that at least some of these can be directly related to morphological or physiological changes associated with differentiation. These will be considered in a later section of this review.

Finally, inspection of Table IV indicates that most of the enzymes involved with hydrolytic activity do not fluctuate during PED, whereas those involved with synthetic processes increase in activity over this period. The relationship is not exact.

Other compounds in the gastrula, emerged embryo and nauplius

A considerable amount of work has been done on the pigments present in these stages. The characteristic brown coloration of the shell is due to hemoglobin-derived haematin localized in the tertiary envelope (Needham and Needham, 1930 ; Gilchrist and Green, 1960). The extent of coloration of the shell (cream to dark brown) is a result of the amount of haematin present and is not apparently due to other pigments. As discussed earlier the origin of the tertiary envelope can be traced to the secretions of the shell glands in the maternal female (Anderson *et al.*, 1970). Presumably, these gland cells process hemoglobin found in the hemolymph (which we will consider later) and release haematin during secretion into the ovisac.

It is well known that the gastrula often contains very large amounts of carotenoids (Needham and Needham, 1930 ; Dutrieu, 1960 ; Gilchrist and Green, 1960). However, there is no substantial evidence that these are synthesized by *Artemia*, of any stage ; rather, they originate from the diet. In nature this usually consists of a wide variety of algae, from which the carotenoids are obtained (Czeczuga, 1980). It is perhaps for this reason that considerable disagreement exists over the kinds of carotenoids present. However, for the gastrula and nauplius, the major carotenoids appear to be canthaxanthin and echinenone (Krinsky, 1965 ; Czygan, 1966 ; Gilchrist, 1968 ; Hsu *et al.*, 1970) ; furthermore, it appears that canthaxanthin is contained within the yolk platelets conjugated to protein (Warner *et al.*, 1972 ; Zagalsky and Gilchrist, 1976 ; de Chaffoy *et al.*, 1978b). These statements appear to hold for the San Francisco *Artemia* population. However, we should note that Czeczuga (1971, 1980) claimed the two most common carotenoids to be β-carotene and astaxanthin ; however, the geographical origin of the cysts was not specified.

In reading these papers we were impressed by the complexity of the situation and the reader would have to do the same to appreciate this. While considerable effort has been devoted to the identification of these various carotenoids, it is fair to say we believe, that absolutely nothing can be said regarding their function(s), at the present time, with any degree of confidence.

Mead and Finamore (1969) isolated a sulfur derivative of ascorbic acid from encysted gastrulae which had the properties of ascorbic acid sulfate. In a subsequent publication from that laboratory (Bond *et al.*, 1972) the compound was proven to be ascorbic acid-2-sulfate,the structure of which is given in Fig. 7. Mead and Finamore (1969) suggested several potential roles that this compound might play (storage of ascorbic acid during embryonic dormancy and/or as a form of active sulfate). Apparently no additional work on this compound in *Artemia* has been published.

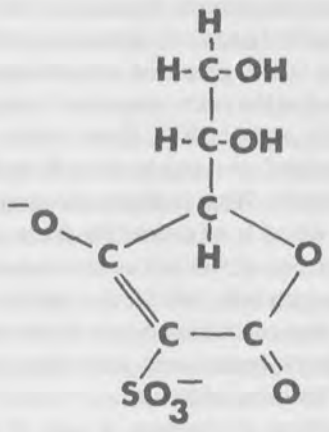

FIG. 7. The structure of ascorbic acid-2 sulfate discovered in *Artemia* by Mead and Finamore (1969). (see Bond *et al.*, 1972 for proof of structure).

Emergence and hatching

If one observes a cyst at the time of emergence it is evident that the embryo, now a partially formed nauplius (Benesch, 1969) or "prenauplius", does not very gradually emerge ; rather, the shell seems to "pop" as the pre-nauplius suddenly protrudes slightly (Fig. 4, E_1). It is as if the cyst contents are under positive pressure prior to emergence. The mechanism of emergence is not established, although some information is available. Clegg (1964) observed an increase in free glycerol during PED and proposed that some of this glycerol accumulates in the extra-embryonic space (beneath the outer cuticular membrane). It was suggested that glycerol accumulation could establish an osmotic pressure gradient which, in turn, might cause (or contribute to) the rupture of the shell. To our knowledge no specialized region of the shell is involved (*i.e.*, there seems to be no morphologically-distinct area that ruptures at emergence). However, it is a fact that the anterior end of the pre-nauplius (E_1) always is the area that protrudes at emergence. Consequently, either the embryo "rotates" to find the opening when the shell does split, or there is another device at work. One possibility is evident from the shape of the E_1, which is roughly pear shaped : the establishment of this morphology could simply result in the pre-nauplius pushing its way out, "head first" as it were. This possibility could be evaluated by calculating the stress exerted on a sphere by internal formation of such a "pear".

A series of studies by Sato (1967) implicates the participation of a "hatching enzyme" in the E_1 to nauplius transition. When emergence occurs, the E_1 is surrounded by two envelopes or membranes, and as the E_2 is formed the outer envelope ruptures (Myint, 1956). Sato (1967) collected the filtrate of media (Herbst's artificial seawater) in which nauplii had previously hatched, and dialyzed it, presumably against artificial seawater although that is not stated. This dialyzed solution was then employed in a variety of studies, the general conclusion being that an enzyme was present which dissolved the outer envelope after the E_1 emerged.

Furthermore, the outer envelope was always dissolved at the anterior part of the E_1. The enzyme was inhibited strongly by Fe^{+3} and Cu^{+2}, and activated by Ca^{++} (which, however, did not reverse Fe^{+3} inhibition). Sato (1967) proposed a mechanism of action for the process.

In other studies, it was shown that the entire process of "excystment" (E_1 through hatching of nauplius) requires NaCl, $CaCl_2$ and $NaHCO_3$ (Sato, 1966a, 1967). Furthermore, several amino acids were found to "stimulate" conversion of the E_1 to E_2 stage, histidine and cysteine being the most effective (Sato, 1966b). What is most interesting is that α-D-amino acids were apparently used, not the L-form which is, of course, the form required for normal amino acid metabolism and protein synthesis. Sato (1966b) does not comment on this, but does point out that his evidence indicates a non-metabolic role for the amino acids, perhaps as a chelator of heavy metal ions. The basis for that conclusion comes from studies with EDTA, glutathione and other compounds. Sato's work poses some interesting possibilities for further study, particularly with regard to the "hatching enzyme".

A comparative review of hatching mechanisms in eggs of aquatic invertebrates includes brief discussion of *Artemia* (Davis, 1968).

With regard to the escape of the nauplius from the inner envelope (hatching) it seems that no elaborate devices are employed : the nauplius becomes motile and essentially "claws its way out" (Myint, 1956).

The transition from the encysted embryo through E_1 and E_2 stages to the free swimming nauplius is obviously a critical one in terms of development. In addition, however, the embryo faces "ionic problems" for the first time in its existence once it leaves the protection of its ion-impermeable shell. It has, so to speak, come out into the real world. We will consider this transition in the next section.

Nauplius development

Having reviewed the developmental changes that take place in the pre-naupliar stages we will now examine the physiology and biochemistry that is involved in nauplius development. We shall separate our presentation into three major sections, one on osmotic and ionic regulation, a second deals with the changes in respiration and respiratory pigments and the third on the primitive sensory organ, the ocellus. Obviously there are other important changes occurring in the nauplius but the lack of information precludes us from making any definitive judgements on their importance.

Formation of the larval salt gland

Osmotic and ionic regulation during naupliar growth have been examined extensively by Conte (1977), Conte *et al.* (1972a ; 1977 ; 1979a), Hootman and Conte (1975), Hootman *et al.* (1972) and Russler and Mangos (1978). These studies have conclusively established that the hemolymph in the naupliar stage can be maintained at concentrations and composition that are considerably lower and different than the external medium. As a consequence, this mode of regulation requires the animal to possess some type of structure to permit water uptake from the medium while eliminating excess ions. The cephalothoracic structure, commonly referred to as the neck organ or neck shield, is now identified as the organ responsible for water and electrolyte equilibrium. Since its ultrastructural, physiological and biochemical

features are similar to many other types of salt extruding organs, Conte *et al.* (1972) has suggested that in the future it be referred to as the larval salt gland.

Conte *et al.* (1977) has reported on the transition of the pre-emergence embryo using the hyperosmotic trehalose-glycerol mechanism to maintain water balance, to one of hypoosmotic regulation that is entirely based upon the regulation of sodium and chloride. It is the ontogeny of sodium regulation in the nauplius that establishes control over its external environment. This system requires the presence and *de novo* synthesis of Na + K-activated ATPase membranes (Peterson *et al.*, 1978ab) and it is the timing of the increase of this enzymatic activity with the concommitant rise of the cytochrome oxidase (Peterson *et al.*, 1978b ; Marco *et al.*, 1980b ; Vallejo *et al.*, 1980) that suggests within the nauplius there is integration of ion transport and cellular respiration. Conte *et al.* (1980) has reported upon the specific effects of ouabain on active ion transport and glycolysis, and suggested that the pivotal point for the control of ion transport lies somewhere in the switch over from anaerobic to aerobic metabolism.

Biogenesis of blood cells and ontogeny of respiratory pigments

Lochhead and Lochhead (1941) reported the existence of specialized nodules that are located at the base of each trunk limb and whose function is to form the free floating blood cells. They established the nature and function of the blood cells by cytological and cytochemical analyses revealing the blood cells to be nucleated and that the nucleus in the forming cell is mitotically active. However, the cytoplasm lacked color suggesting the cells do not contain the heme-containing respiratory pigment, hemoglobin.

The cytoplasm does have soluble proteins and many inclusion bodies, some identified as mitochondria but the remainder being unidentified, yet shown to be composed of acidic and/or basic type complex proteins. Carbohydrates such as glycogen or mucopolysaccharides are absent. With regard to the function of the blood cells, physiological experiments were not performed but direct observation either *in situ* or in fixed preparations showed them to be involved in agglutination or in the nutrition of developing eggs. One function that they do not appear to be involved in is respiration.

Blood plasma (assumed to be free from blood cells) has been reviewed by Kuenen (1939) and the existence of extracellular hemoglobin by spectroscopic analyses has been reported by Lochhead and Lochhead (1941). Thus, the respiratory pigment, hemoglobin, is a soluble plasma protein and is not found packaged in blood cells. The ability to gain or lose extracellular hemoglobin was investigated by Gilchrist (1954) and shown to be dependent upon the oxygen content of the water in which the adults live. As a consequence of this finding, she also examined the effects of salinity upon hemoglobin synthesis. It was found that as salinity increases the dissolved oxygen is lower and hemoglobin synthesis is stimulated. The function of hemoglobin in adults clearly is involved in oxygen transport and is especially important to adults living in concentrated brines. The presence of hemoglobin in the nauplius was not observed by Gilchrist (1954). However, Bowen *et al.* (1969), Waring *et al.* (1970) and Heip *et al.* (1977 ; 1978ab) have demonstrated the existence of three distinct hemoglobins (Hb-1, Hb-2 and Hb-3) synthesized during nauplius growth. Synthesis does not occur until after hatching, with the highest rate of synthesis being observed for Hb-2 and Hb-3 in the 2-3 day old nauplius. Subsequently, on about day 20, Hb-1 synthesis dominates. Physicochemical

characterization of these hemoglobins has been reported by Moens and Kondo (1976 ; 1977) and Heip *et al.* (1980) and shown to consist of two high molecular weight globin subunits (MW $\approx$ 125 000), each containing multiple heme groups. Also, Hb-2 and Hb-3 contain one identical, or at least immunologically indistinguishable, subunit.

Respiratory rates for adults (Kuenen, 1939 ; Eliassen, 1952 ; Gilchrist, 1956 ; 1958) and nauplii (Kratovich, 1964 ; Angelovic and Engel, 1968 ; and Conte *et al.*, 1980) are quite different. Nauplius respiratory rates have also been found to vary, some investigators reporting an increase in respiratory rate with increasing salinity, others a decrease, and a few reporting that there is no significant change. In the adult and the metanauplius stages there is complete agreement that oxygen transport is facilitated by the participation of extracellular hemoglobin. How hemoglobin facilitates cellular and tissue respiration is not clear at this time.

Sensory organs : formation of primitive nauplius eye

The ocellar anlagen appears early in the pre-nauplius stages of development. At the time of hatching these primitive photoreceptor cells, together with accessory neuronal cells, integrate into a nauplius eye. It is apparent from anatomical organization that the nauplius eye does not lend itself to be a photoreceptor organ designed for precise visualization of discrete forms as is the case for the compound eye found in the adult. Rather, the nauplius eye appears to be responsible for the phototactic responses exhibited in the locomotory behavior of the larvae. Specific anatomical and physiological details concerning the nauplius eye as found in the larva have not been investigated, but Anadon and Anadon (1980) have studied the nauplius eye as it exists in the adult. The adult "nauplius eye" is composed of two cell types : (1) large pigmented cells and (2) numerous neuronal cells referred to as retinula cells. Both types of cells are integrated into a neuronal complex which also involves interdigitation with cells comprising the cavity receptor organ. The nature of the cavity receptor organ (also known as the frontal organ) was revealed by the earlier studies of Elofsson (1966), Elofsson and Lake (1971), and Rasmussen (1971), who showed these organs to be comprised of bipolar neurons.

The physiological significance of the innervation between pigment cells, retinular cells and bipolar neurons with the optic neuropiles remains to be revealed.

Metanauplius and juvenile development

Very little information is available on the physiology and biochemistry that accompanies development of the metanauplius and juvenile stages. Nonetheless, we will consider two aspects that we believe to be important, one on the transition between larval and adult mechanism dealing with osmotic and ionic regulation, and a second dealing briefly with neuroendocrine control of moulting.

Destruction of larval salt gland and formation of the adult salt organelles

The segmentation of the post-mandibular region reveals that as each thoracic appendage develops, it becomes specialized through the addition of new structures. For example, as the trunk leg becomes specialized for adult locomotory and feeding behavior the setation of naupliar limb buds is increased along with the final development of the alimentary tract (Hootman and Conte, 1974) preparing the nauplius for gathering and digesting food obtained

from the external environment. In addition, the trunk leg segment also becomes specialized for adult respiration by forming numerous gill leaflets that increase gaseous exchange through increased surface area, allowing for increased oxygen consumption by the tissues. Finally, the trunk leg becomes modified to act as an extrarenal organ responsible for excretion of excess electrolytes. It was shown by the ultrastructural studies of Copeland (1966, 1967) that the epithelial lining of the metepipodite does contain salt organelles.

It is the development of these salt organelles that provides for the transition from a larval renal system to an adult renal system because the salt organelles along with the midgut (and possibly the antennal gland for which there is little direct evidence) become the adult renal system. Since these salt organelles are products of the segmental development of the larvae, they are not physiologically active until completion of the last instar. It is at this precise moment that the destruction of the larval salt gland begins to occur. By the time the mandibles are complete the gland has undergone complete autolysis and is reabsorbed. Apparently, the co-existence of both the adult and larval renal systems within the same individual is biologically incompatible. Alternatively, they may simply require different control mechanisms. Needless to say, this intriguing phenomenon warrants further research.

Development of neuroendocrine system

The morphological features which support nauplius metamorphic development at each successive molting cycle provide sufficient evidence to question whether physiological or biochemical factors are at work in controlling timing of each molt. Since neuroendocrine factors play a major role in insect molt cycles, one would suspect that a similar circumstance might occur in *Artemia*. But, to our knowledge, there is a complete lack of information on the role of neurosecretory hormones controlling embryonic and juvenile development, a highly productive area for future research.

Adult development

We will briefly examine a few developmental and physiological aspects of the adult. D'Agostino (1980) reviews the system's physiology of the adult, so we will exclude the details of gastrointestinal digestion and nutrition, reproduction, cardiovascular and neuronal regulation of locomotion, and sensory physiology. We shall consider in this section primitive mechano-sensory receptors, a comparison between naupliar and adult locomotory mechanisms, the anaerobic and aerobic aspects of respiration, the renal and extrarenal system, and the development of the digestive tract.

Sensory receptors : cuticular receptors

The structure and function of two types of sensory receptors, (1) mechanoreceptors and (2) chemoreceptors have been reported by Tyson and Sullivan (1979 ; 1980) and Wolfe (1980). The cuticular receptors found on the first antennae were shown to be easily stained with a crystal violet solution. This simple cytochemical technique has been quite useful in identifying chemosensory cells. Additional observations by scanning electron microscopy revealed the presence of cuticular pores which supports the idea that the antennular sensilla are primarily chemosensory rather than tactile. The cuticular receptors (frontal knobs) found

on the second antennae, primarily in males, are modified tactile organs and are used in copulatory behavior. Wolfe (1980) describes the frontal knobs as spherical protuberances located along the anteromedial surface of the protopodite which bear two types of cuticular processes, cuticular spines and mechano-sensory setae. The cuticular spines are acellular and non-sensory, whereas the mechano-sensory setae are comprised of a dome-shaped supporting cell, a setal cell and a sensory neuron.

Comparison of naupliar *versus* adult locomotory mechanisms

Recent studies by Barlow and Sleigh (1980) and Miller *et al.* (1979) involving high speed cinematography or video-computer analysis have revealed how the propulsion of water by *Artemia* is involved in swimming and feeding behavior. In the adult, water is drawn in from the ventral side of the animal by action of the power strokes of the eleven pairs of limbs. Compression of the water between the limbs at the end of the power strokes propels it out as a jet of water behind the animal to produce the thrust. The action of drawing the water from the ventral surface enables the animal to feed by filtering the water before it is used for propulsion. Only a small fraction of the water through which the animal passes is filtered. The metachronism exhibited by the limbs improves the efficiency with which they propel water.

In the nauplii the fluid propulsion is much simpler, involving only one pair of limbs. As the limbs develop, metachronism also develops and increases the efficiency of the system and this in turn increases the maneuverability of the organism to the degree found in the adult animal.

Aerobic and anaerobic aspects of respiration

The primary function of the respiratory system in animals is to transport oxygen to, and carbon dioxide from the tissues in order to maintain aerobic metabolism of the cells. As was shown in the preceding section, extracellular hemoglobin is the respiratory pigment that functions as the gas transporting molecule in *Artemia*. Oxygen consumption is a reflection of these basic requirements and past investigations on the respiratory physiology of brine shrimp have been directed toward providing measurements of basal respiratory rates of individual adult and nauplius. Unfortunately, much of this information on oxygen consumption is not in very good agreement (Conte *et al.*, 1980).

A most notable exception has been the early work of Gilchrist (1956, 1958) showing that the oxygen consumption in adult females did not change appreciably in response to increasing salinities but adult males showed a significant difference in the rate of oxygen consumption when reared in the lower salinities (35‰). Additionally, Gilchrist (1954) reported that adults of both sexes respond to low dissolved oxygen content at the higher salinities by synthesizing more hemoglobin. In a similar fashion, Decleir *et al.* (1980) and Vos *et al.* (1979) studied the effects of several environmental factors upon the respiratory physiology of adults. They found, as did Engel and Angelovic (1968), that when temperature increased, the rate of respiration also increased. If the oxygen concentration of the environmental medium diminished to levels below 1.5 ml O_2/l, respiratory rates fell precipitously and the animals showed a marked reduction in locomotory movements. The level of dissolved oxygen which suppressed major functional activities (swimming, feeding, *etc.*) was shown for adult males to be around 2.0 ml O_2/l at 35 ‰ salinity. Below these oxygen levels, anaerobic processes must be invoked to maintain the energetic requirements of the tissues. Decleir *et al.* (1980) have

proposed that in adults the compensatory pathway utilized by the respiratory system is primarily extracellular, namely, the stimulation of hemoglobin synthesis. They report that at oxygen levels of about 1.0 ml/l Hb-3 is the principal form that is manufactured to enrich the blood concentrations and appears to be appropriately fashioned for his purpose since it was shown to have the highest oxygen affinity with the lowest Bohr effect. In this way, the adult adjusts to hypoxic conditions by making a better oxygen trapping system and avoids or lessens the need to utilize less efficient energy producing pathways such as glycolysis.

Does this compensatory mechanism exist in the nauplius ? Several facts suggest that is unlikely. First, nauplii which are less than 36 hr old cannot synthesize large amounts of Hb-3 as was shown in the ontogenetic studies of Heip *et al.* (1980). Second, nauplii utilize large quantities of glycogen at moderate levels of dissolved oxygen (about 1.5-2.0 ml/l) but produce very little lactate ($\approx$ 1 μg) from sizable quantities of glycogen ($\approx$ 80 μg). Lastly, acid secretion from the nauplius (which is not lactate) appears to be salt-dependent but independent of oxygen concentrations. What is the compensatory mechanism for the primitive respiratory system of the nauplius ? Conte (1977) and Conte *et al.* (1980) propose that in the early nauplius simple passive diffusion of the respiratory gases is an adequate transport mechanism for supplying oxygen to the tissues. When dissolved oxygen concentrations fall below 1.0 ml/l, however, an intracellular facultative C-4 anaerobic pathway is stimulated in contrast to the stimulation and production of extracellular hemoglobin in the adult stage.

Renal and extrarenal system

Studies by Croghan (1958abcde), Thuet *et al.* (1968), Smith (1969ab), and Geddes (1975abc) on the adult brine shrimp have established that the hemolymph can be maintained at concentrations lower than the external medium. The compensatory mechanism that has been postulated for water and electrolyte homeostasis is that diffusive water loss incurred by the external osmotic gradient is balanced by ingesting the external brine followed by an active uptake of NaCl. The water which accompanies NaCl uptake maintains the fluid volume while excess hemolymph NaCl is removed by active extrusion against a steep electrochemical gradient via an effector organ. In the adult, the effector organs are the gill leaflets located on the middle leg segments. They have been shown to be covered with a highly permeable cuticle (Croghan, 1958b). From studies involving measurement of ionic fluxes (Smith, 1969b) and the transepithelial electrical gradient across the body wall (Smith, 1969a), the underlying gill epithelium was shown to be actively secreting ions. Thus, the alimentary tract together with the gill leaflets appear to be the organs responsible for the renal and extrarenal homeostatic mechanisms involved with water and electrolytes.

Alimentary tract : water absorption, digestion and nutrition

As previously mentioned, Croghan (1958c) showed the midgut of the adult was the most probable site of water absorption. Bayly (1972) disagrees with this hypothesis and bases his argument upon the fact that the gut fluids have a low NaCl concentration despite having been derived from ingested brine. Also, this small amount of NaCl accounts for less than 30 % of the total osmotically active material found in the gut fluid. Furthermore, animals ligated at the neck and anus appear capable of resisting osmotic desiccation despite the fact that they are prevented from drinking. Dall (1967) suggests that the gill leaflets are the site of water

reabsorption but there is no evidence to support this idea. It is apparent that the role of the alimentary tract in water and electrolyte balance is still unresolved.

Developmental studies by Weisz (1947), Anderson (1967), and Benesch (1969) have shown that the alimentary tract is not complete prior to the first larval ecdysis. Ultrastructural studies by Hootman and Conte (1974) showed several structural specializations suggestive of a solute (*i.e.*, NaCl) absorptive role. Chief among these is the amplification of the plasmalemma along the apical and basal cell surfaces with a concomittant rise in the level of (Na + K)-ATPase. Second instar nauplii have a cellular morphology which is consistent with the finding that the foregut, midgut and hindgut are complete, thereby permitting food and ingested fluids to be acted upon by the digestive processes.

The mechanisms of digestion and nutritional aspects of the alimentary tract will be described in the review by D'Agostino (1980).

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The vital requirements of *Artemia* : physiology and nutrition

Anthony D'Agostino

New York Ocean Science Laboratory
P.O. Box 542, Montauk, New York 11954, USA

Content

Abstract

The vital requirements of *Artemia* are reviewed from a nutritional point of view. *Artemia* is one of a very select few species of Crustacea that have been reared bacteria-free, feeding on unicellular algae and/or chemically defined media under aseptic and standardized culture conditions. Survival, growth, and reproduction were found to be dependent on assimilation efficiencies which, in turn, varied with the quality and quantities of the food. At food concentrations suboptimal for maximum ingestion and assimilation, *Artemia* does not appear to have the metabolic plasticity to compensate for changes in the nutritional value of the algal food. The metabolic activities of *Artemia* change with age : carbohydrate appears to be the principle energy source during emergence ; a lipid-protein metabolism predominates during hatching and early post-embryonic life ; and a mixed carbohydrate-lipid-protein metabolism is possible in juveniles and adult *Artemia*. Several aspects of intermediary metabolism in *Artemia* need clarification. Under axenic conditions in chemically defined media, *Artemia* requires a particulate source of carbohydrates, (starch), protein (albumin), nucleic acids (of which adenylic acid is the most important), eight water soluble vitamins, and cholesterol. Studies on the requirements for exogenous sources of fatty acids, exotic lipids, fat soluble vitamins, and fertility factors need to be carried out. Maturation, sex differentation and reproduction are nutrient dependent for *Artemia* reared on algae or in chemical defined media.

Introduction

After nearly a century of studies on the genetics and physiology of different strains of *Artemia*, the systematics of the genus remain unsettled primarily because there is surprisingly few gross morphological differences between the widely dispersed and geographically isolated strains (Barigozzi, 1974). With few exceptions, all salt lakes and salines, which do not have predatory forms, carry sizable populations of *Artemia*. The habitats are extraordinarily harsh and diverse in many of their environmental parameters. Accordingly, the lack of morphological diversity is suspect of masking subtle physiological and reproductive differences. In the past, most experimental work on nutrition and physiology was done with *Artemia* of the Great Salt Lake, Utah, USA or the San Francisco Bay, California, USA. In recent times with the advant of better culture methods, more of the lesser known strains have been brought into laboratory cultures and some of the consequences of evolutionary adaptation may be forthcoming.

Cyst diameter

For example, measurable differences occur in several characters of the wintering eggs of *Artemia* from different locations. The size frequency distribution of maximally hydrated wintering eggs of the San Francisco Bay and the Great Salt Lake strains are distinctly different. The modal and the median classes of eggs of the same strains, *i.e.*, Great Salt Lake eggs collected in the wild and produced in the laboratory under culture conditions simulating the natural habitat, were identical, and measurably different for eggs of different strains (Fig. 1, from D'Agostino, 1965). Similarly, a comparative study of the rate of hydration of the eggs of three North American strains collected in different years were identical within each strain but significantly divergent between strains (Fig. 2, from D'Agostino, 1965) *i.e.*, the Great Salt Lake eggs required less than 60 min for all to become turgid but, after only 30 min lysis was

conspicuous. The eggs from Little Manitou Lake, Canada, employed well over 195 min to become turgid with no incidence of lysis at 300 min. These observations suggested that there were functional differences in either the chorion or the embryonic membranes protecting the dormant bastula. Presumably, environmental conditions may have been the causative factor but, in the absence of evidence to the contrary, a genetic influence could not be ruled out. For corroborative data, see Vanhaecke and Sorgeloos (1980).

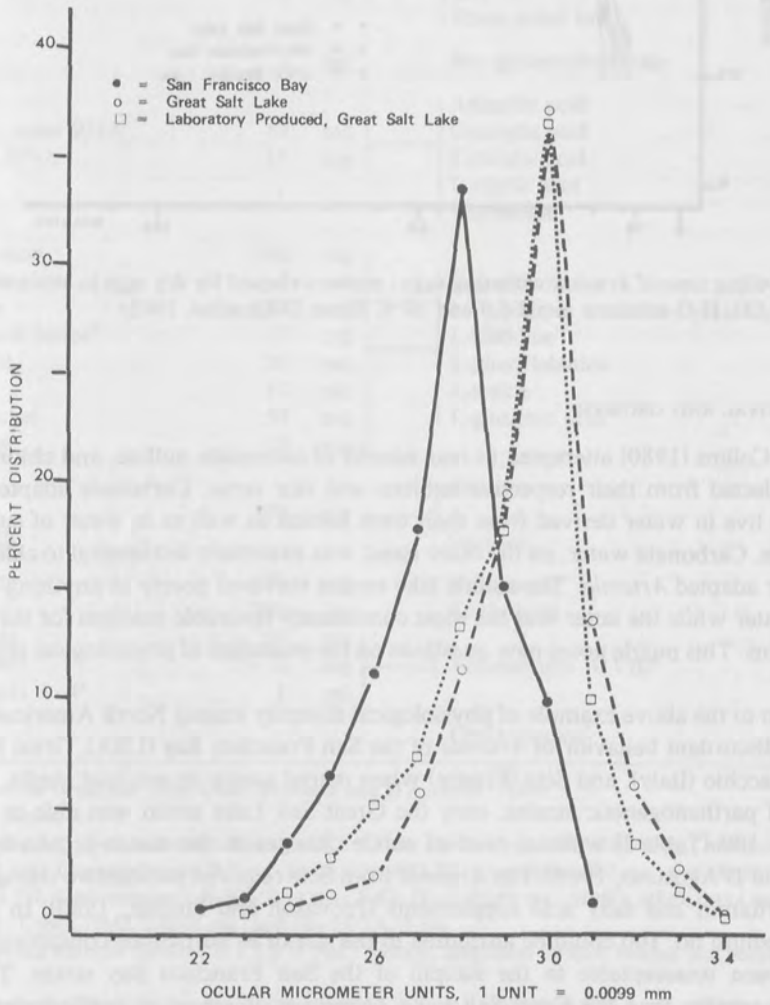

FIG. 1. Size distribution of hydrated eggs (San Francisco Bay, Great Salt Lake, and laboratory produced, Great Salt Lake) (from D'Agostino, 1965).

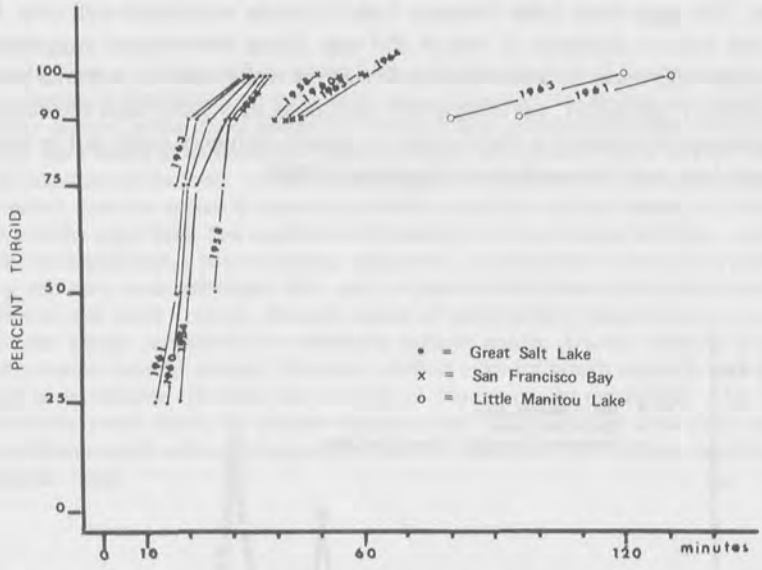

FIG. 2. Swelling time of *Artemia* wintering eggs ; minutes elapsed for dry eggs to attain turgidity in 10 mg.% $Na_2CO_3.H_2O$ solutions at pH 8.0 and 20 °C (from D'Agostino, 1965).

NAUPLII SURVIVAL AND GROWTH

Recently, Collins (1980) attempted to rear nauplii of carbonate, sulfate, and chloride lakes in water collected from their respective habitats and *vice versa*. Carbonate adapted strains were able to live in water derived from their own habitat as well as in water of sulfate and chloride lakes. Carbonate water, on the other hand, was extremely detrimental to chloride and sulfate water adapted *Artemia*. The sulfate lake strains survived poorly in anything but their own type water while the latter was the most consistently favorable medium for the chloride adapted strains. This puzzle poses new questions on the evolution of physiological plasticity of *Artemia*.

In addition to the above example of physiological diversity among North American strains, there is the discordant behavior of *Artemia* of the San Francisco Bay (USA), Great Salt Lake (USA), Comacchio (Italy), and Sète (France) when reared axenic in artificial media. Of these bisexual and parthenogenetic strains, only the Great Salt Lake strain was able to grow in medium no. 100 (Table I) without need of subtle changes in the starch-protein-lipid ratio (Provasoli and D'Agostino, 1969). The *Artemia* from Sète required substantive changes in the fat soluble vitamin and fatty acid supplements (Provasoli and Pintner, 1980). In part, the failure of medium no. 100 could be attributed to the size of its particulate constituents which may have been unacceptable to the nauplii of the San Francisco Bay strain. These are considerably smaller than the Great Salt Lake *Artemia* at all stages of development (Fig. 3, from D'Agostino, 1965). In fact, the starch albumin gel preparation had to be modified in order to have finer particles acceptable to the *Artemia* nauplii of Sète. The latter, and nauplii of the San Francisco Bay strain are of comparable size.

TABLE I

Artificial media for *Artemia*[1]

1959 old medium (% w, v/v)			1969 new medium no. 100 (% w, v/v)		
			NaCl	2.4	g
			$MgSO_4.7H_2O$	0.6	g
Seawater	80	ml	$MgCl_2.6H_2O$	0.4	g
H_2O	15	ml	$CaCl_2.6H_2O$	0.22	g
Soil extract	5	ml	KCl	60	mg
			Trace metal mix[4]	3	ml
K_2HPO_4	1	mg	Na_2 glycerophosphate	50	mg
			Adenylic acid	60	mg
Alk. hydr. yeast RNA	40	mg	Guanylic acid	2.5	mg
Ac. hydr. DNA	10	mg	Cytidylic acid	5.0	mg
			Uridylic acid	5.0	mg
			Thymidine	2.5	mg
Liver infusion	100	mg			
Horse serum	5	ml	Egg albumin[5]	10-20	mg
Trypticase	320	mg	L-threonine	20	mg
Paramecium factor[2]	5	mg	L-histidine	20	mg
Yeast autol.	20	mg	L-phenylalanine	10	mg
Glycine	10	mg	L-serine	40	mg
L-glutamic ac.	50	mg	L-glutamic acid	100	mg
DL-alanine	10	mg			
			Glucose	300	mg
Sucrose	300	mg			
			Sucrose	300	mg
Rice starch	500	mg	Rice starch	100	mg
Cholesterol	200	µg	Cholesterol	800	µg
Glutathione	30	mg			
Ascorbic ac.	3	mg	Vitamin mix A·VIII[6]	2	ml
Vitamin mix A·II[3]	1	ml			
			Glycylglycine[7]	100	mg

[1] *Artemia* of the Great Salt Lake, Utah, (Provasoli and D'Agostino, 1969).

[2] Lilly and Klosek (1961).

[3] Vitamin mix A·II, 1 ml contained : thiamine HCl, 0.1 mg ; biotin, 5 µg ; folic acid, 70 µg ; nicotinic acid, 0.5 mg ; choline, 5 mg ; Ca-pantothenate, 0.7 mg ; pyridoxine, HCl 80 µg ; carnitine, 0.2 mg ; and riboflavin, 1 µg.

[4] Metal mix PII, 1 ml contained : H_3BO_3, 1.14 mg ; $FeCl_3.6H_2O$, 0.049 mg ; $MnSO_4.4H_2O$, 0.164 mg ; $ZnSO_4.H_2O$, 0.022 mg ; $CoSO_4.7H_2O$, 0.0048 mg ; and Na_2-EDTA, 1 mg.

[5] Crystalline egg albumin dissolved in a 3 g % NaCl solution, coagulated by slow heating, and homogenized before use.

[6] Vitamin mix A·VIII, 1 ml contained : thiamine HCl, 1.2 mg ; nicotinic acid, 2.4 mg ; Ca-pantothenate, 4 mg ; pyridoxine HCl, 100 µg ; riboflavin, 300 µg ; folic acid, 0.7 mg ; biotin, 60 µg ; and putrescine, 200 µg.

[7] The pH was adjusted at 7.4-7.6.

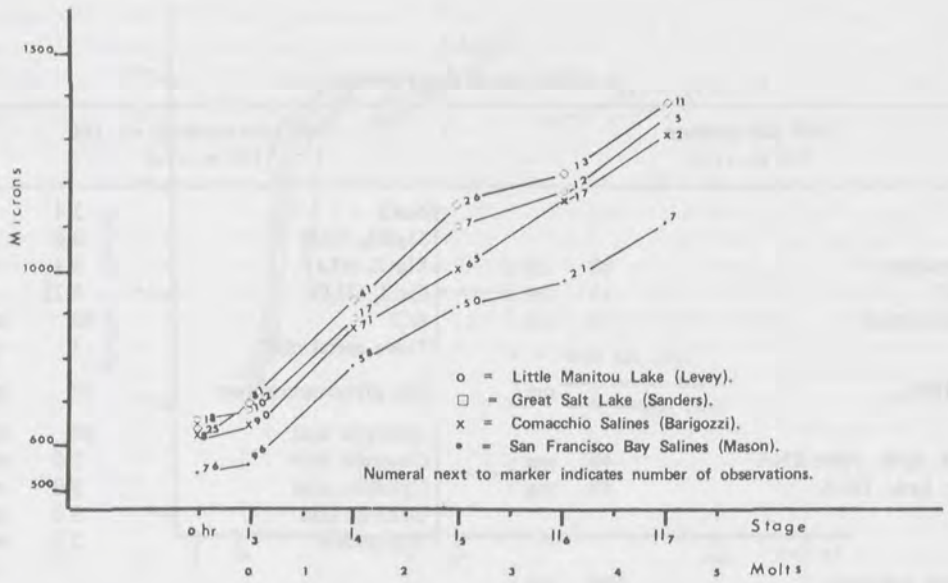

FIG. 3. Mean length of *Artemia* from Little Manitou Lake (Levey); Great Salt Lake (Sanders); Comacchio Salines (Barigozzi); San Francisco Bay Salines (Mason) (from D'Agostino, 1965).

LIFE HISTORY

The typical life cycle of *Artemia* in nature, in a lake, in a temperate zone, begins in the early spring and is completed before winter. Vorhies (1917) described it as follows: In the Great Salt Lake, *Artemia* nauplii emerged from the wintering eggs, referred to as cysts, in early spring. The water of the lake had a specific gravity of 1.036 (~1.9% NaCl) and the temperature was between 15 and 25 °C. The nauplii grew quickly into adults without remarkable metamorphosis and were most abundant through the summer months. In October, they became less conspicuous and in December were no longer found. At this time the specific gravity was 1.158 (~ >24% NaCl) and the temperature about 1 °C. In the interval, May to December, considerable numbers of encysted eggs were deposited. These, however remained unhatched and were found the following spring to have drifted onto shore, only to be washed into the lake with the first abundant rainfall of the season.

From this very brief account it is possible to preview two of the most important functional characteristics that distinguish *Artemia* from other metozoans. Namely, 1) all life stages are tolerant of wide fluctuations of salinity, temperature, and dissolved oxygen; and 2) in preparation for adverse conditions the adult may deposit overwintering eggs, encysted blastula in cryptobiosis, to safeguard the continuity of the species.

TOLERANCE

Croghan (1958abcde) effectively explained the osmoregulatory mechanism which allows the *Artemia* to tolerate salinities ranging from 1 to 200 ‰. Briefly, *Artemia* maintain the

haemolymph hypotonic with respect to the medium in high salinity media, and hypertonic in low salinity media. This it does by continuously swallowing the medium from which, while in the gut, the Na^+, Cl^-, and H_2O are absorbed by active transport into the haemolymph. The Na^+ and Cl^- from the haemolymph are then actively excreted out of the organisms through permeable portions of 10 pair of branchiae, again, against a concentration gradient. This type of osmoregulation is common with freshwater forms and because of this similarity the osmoregulation of *Artemia* is often mentioned in support of the notion that the brine shrimp has freshwater ancestry. In the nauplius osmoregulation is mediated by the neck organ as suggested by Croghan (1958b) and verified by Conte *et al.* (1972). In the adult, turnover rates for Na^+ and Cl^- exceed 50% of the in-animal sodium concentration per hour. More significantly, the drinking rate, measured in seawater adapted adults, amounted to 3% of the body weight per hour (Thuet *et al.*, 1968).

The necessity of having to continuously and actively transport ions (Smith, 1959) through the gut epithelium and again through the epithelium of the branchiae against large concentration gradients implies the expenditure of energy and the obvious participation of ATP. Surprisingly, there is evidence to suggest that the metabolism of the adults (Gilchrist, 1954) is not appreciably affected by salinity stress (Huggins, 1969). But, Emerson (1967) found correlation between O_2 consumption and the metabolic activity of the emerging and hatching nauplii. He interpreted the RQ value, ~ 1, of the emerging nauplii to be typical of carbohydrate metabolisms, probably trehalose (Dutrieu, 1960 ; Clegg, 1964) while the lower RQ of the newly hatched nauplii seem to indicate the onset of lipid metabolism. While the lipid stores of the nauplius may be the substrate of choice during early post embryonic development (Urbani, 1946, 1959) there is no reason to discard the possibility that, in adult *Artemia* and Crustacea in general, metabolism is fueled opportunistically with carbohydrates, proteins or lipids depending on their availability (Wolvekamp and Waterman, 1960).

Respiration

Reports on the rate of oxygen consumption of *Artemia* exposed to different salinities are generally contradictory (Kuenen, 1939 ; Eliassen, 1952 ; Dutrieu, 1960 ; Clegg, 1964 ; Emerson, 1967). Gilchrist (1956) reported the average consumption of 236-693 μl O_2/g wet wt/hr, for adult females in 195 ‰ at 23.5 °C when oxygen concentration of the media varied from 0.51-2.00 ml/l. Subsequently, contrary to expectation, adult females reared in brines of different salinities but similar oxygen saturations were found to have identical rates of oxygen consumption (Gilchrist, 1958). This led Gilchrist to suggest that the available internal oxygen, probably in deficiency for animals under salinity stress, was being partitioned preferentially to energy using pathways at the expense of metabolism for growth. The possibility of anaerobic glycolysis was not considered. Recently, Conte *et al.* (1973), while considering the same problem with respect to nauplii speculated that a feedback type mechanism could be simultaneously regulating ion transport and protein synthesis by way of the "energy charge control postulate" of Atkinson (1968). But Vos *et al.* (1979) and Decleir *et al.* (1980) found that, at least with regard to adult males, *Artemia* acclimated to different partial O_2 pressures and showed that : 1) the animal is a respiratory regulator above a critical oxygen tension of ~ 2 ml O_2/l ; 2) under hypoxic conditions *Artemia* may produce three types of hemoglobin ; Hb I, II, III, with distinctly different oxygen affinities which in turn permit it to cope with low

environmental pO_2 values ; 3) under anaerobiosis glycogen was consumed with the production of lactic acid, *i.e.*, anaerobic glycolysis. Many factors may be involved in the regulation of metabolic rates. Clearly, there is room for speculation.

Nutrition

The nutrition of *Artemia* has been studied in 1) agnotobiotic or xenic conditions, *i.e.* with none or only partial knowledge of the microorganisms and the reaction of nonliving artificial feeds in the cultures ; and in 2) gnotobiotic or axenic conditions, *i.e.* with all associated food organisms known and/or in aseptic chemically defined media. Dougherty (1959) gave precise definitions to the terminology employed above and identified the limitations of the various conditions of cultures for the study of the trophic requirements of invertebrate species.

Xenic

Agnotobiotic culture

Artemia was reared in crude cultures in a wide variety of salt solutions, preferably of > 30 ‰, at 20°-30 °C, with slight aeration and fed algae or particulate nonliving matter such as brewer's yeast. For many years, as succinctly summarized by Bond (1937), the medium of choice for rearing vigorous and fecund *Artemia* consisted of seawater supplemented with 5-8 g % (w/v) of NaCl, and frequent additions of *Dunaliella salina, D. viridis* or *Platymonas subcordaeformis*. Many researchers have exploited this method, with slight modifications, to keep stocks of *Artemia* in laboratory cultures not necessarily intended for nutritional studies : *Chlamydomonas* sp. in seawater (Gross, 1932), phytoflagellates of uncertain origin in a standard synthetic seawater enriched with nitrates and phosphates (Barigozzi, 1939), several flagellates common to salines in nutrient-rich salt solutions (Gibor, 1956), different species of Chlorophyceae, Chrysophyceae and Chryptophyceae in synthetic and natural seawater (Provasoli *et al.*, 1959), *Chlamydomonas engametos* in seawater (Dutrieu, 1960), *Phaeodactylum* sp. and *Dunaliella tertiolecta* in seawater (Gilchrist, 1960), *Chlamydomonas marina* in a standard artificial seawater (Ballardin and Metalli, 1963), *Chlamydomonas* sp. in seawater (Nimura, 1963), *Phaeodactylum tricornutum* in seawater (Reeve, 1963) and *Cyclotella nana* in seawater (Takano, 1967).

Attempts at rearing *Artemia* on live bacteria have generally given negative results unless some small protists were interposed in the food chain (Seki, 1964).

Alternatively, in order to avoid the cumbersome task of growing large quantities of food algae, *Artemia* was reared feeding solely on suspension of ordinary Fleishmann's yeast (Bond, 1937 ; Weisz, 1946). Bowen (1962) kept genetic stocks of *Artemia* spp. in seawater supplemented with 5 g % NaCl and fed on a diet of brewer's yeast. A standard yeast suspension was prepared (= SYS) by dispensing 1 ml of dry powder brewer's yeast in 9 ml of medium. The SYS was then added to the cultures in aliquotes of 0.05 ml per three animals in 5 ml of medium on the 1st and 8th day. From the 15th day onward, 0.05 ml was added per surviving animal irrespective of volume.

Artemia, in all stages of development, have become routine food organisms for a large variety of species in aquaculture. Consequently, in recent years various attempts have been made to rear *Artemia* in batch cultures using inexpensive powdered natural products.

Teramoto and Kinoshita (1961) experienced some partial success with a commercially available vitamin tablet, "Wakamoto", composed of a mixture of *Aspergillus, Eremothecium*, and Lactobacteriaceae. The tablets were powdered and fed daily at the rate of 100 mg/100 nauplii. But, while growth rates were satisfactory, survival was < 50% and the medium had to be changed every 2 days to avoid excessive mortality following contamination with competing adventitious microbes. From a practical point of view, the experiences of Teramoto and Kinoshita (1961) with "acetone-butanol fermentation waste" were most promising. Although survival did not exceed 65%, the new-born *Artemia* grew to adults within 6-7 days. Growth of the *Artemia* was attributable to the chancy occurrence of a suitable microflora rather than a balance of nutrients in the fermentation waste. Samples of the waste offered to *Artemia* under axenic condition supported no growth unless contaminated with non-acidifying bacteria (D'Agostino and Provasoli, unpublished). Nevertheless, the cultures could be scaled to 200 l vessels with resulting population densities of 1 000-1 500/l.

"Juvenile" *Artemia* fed wheat flour alone in combination with soybean powder and with *Nitzschia closterium* gave doubtful results (Takano, 1967). Wheat flour alone was not a satisfactory food, presumably because of its high carbohydrate and low protein content. Mixtures of wheat flour and soybean powder and or wheat flour and live *N. closterium* appeared acceptable to the *Artemia*, but the longevity of the cultures was doubtful, either because of subtle nutritional deficiencies of the diet or the unpredictable onset of putrefaction.

Recently, several new products derived from mass culture of algae, heated-drum dried *Scenedesmus* (Clement and Van Landeghem, 1970), and freeze-dried *Spirulina* (Feldheim, 1973) were proposed and tested for intensive culture of *Artemia*. The dried *Scenedesmus* cells fed to *Artemia* in high-density culture (Sorgeloos, 1973) gave growth rates comparable to those reported by Teramoto and Kinoshita (1961) *i.e.* 4 mm animal in 7 days growth. However, the long term rearing on *Scenedesmus* powder was not studied, hence the ultimate effect of subtle deficiencies that could impair reproduction remain untested. Subsequent trials with another dry algal powder, *Spirulina maxima* (Cognie, 1976 ; Person-Le Ruyet, 1976), showed that under optimal culture conditions, it was possible to rear *Artemia* to 3.75 mm in 6 days. Automated high-density culture techniques are under continuous development. Several alternate natural products of potential value for rearing *Artemia* as feed for other species in aquaculture are reported at this symposium (Bossuyt and Sorgeloos, 1980 ; Sorgeloos, 1980 ; Dobbeleir *et al.*, 1980 ; Johnson, 1980).

The technical feasibility of short-term batch rearing of massive quantities of juvenile *Artemia* using relatively inexpensive natural products such as wholewheat flour (Jahnig, 1977) rice bran, soybean flour, and lactoserum (Sorgeloos *et al.*, 1979) has been demonstrated. The success of such cultures is strictly dependent on the instantaneous establishment in the reaction vessel of a microflora fortuitously harmless to the *Artemia* and supplemental to the nutrient deficient natural products. Neither the latter nor the microbes alone can provide adequate sustenance for *Artemia*. Furthermore, there is good reason to suspect that the microbial flora, resulting from the incubation of organic matter at the elevated temperature typically employed for rearing *Artemia*, although often harmless to the latter, may prove pathogenic to the organisms for which the *Artemia* is intended (D'Agostino and Dobbeleir, 1980). Exclusive of the possible pathogenicity of the associated flora, *Artemia* grown in fermenting natural products may also prove nutritionally alien to the predator with respect to its amino acids and lipid content. Such diversity in chemical composition have been noted

both in newly hatched nauplii of different strains of *Artemia* (Fujita *et al.*, 1980) and adults reared on different feeds (Sorgeloos *et al.*, 1979).

Axenic

Gnotobiotic culture

The successful crude cultures of *Artemia*, referred above, depend upon the occurrence of fortuitously favorable bacteria, flagellates and protozoa. Such cultures last as long as the biotic balance is not upset by changes in temperature, salinity, and the accumulation of metabolic byproducts. Ultimately, the most troublesome member of the biota, species of bacteria, fungi or Protozoa, overwhelm the culture system and in the process kill the *Artemia*. Because of this eventuality and the influence that microbes exercise on the culture medium by releasing essential nutrients and/or inhibiting substances, agnotobiotic cultures are only a first step toward the establishment of gnotobiotic (axenic) cultures. The latter permit more exacting studies of the trophic exchanges between species (D'Agostino, 1975).

Nutritional value of algae in monoxenic culture

The few studies that were carried out with *Artemia* in monoxenic cultures (Gibor, 1956 ; Provasoli *et al.*, 1959 ; Provasoli and Shiraishi, 1959 ; D'Agostino and Provasoli, 1968) had at least two objectives in common : 1) the definition of the role played by algae, bacteria and detritus in the nutrition of a filter feeding herbivore, and 2) the identification of algal species that could function as the primary source of nutrients for *Artemia* in a bacteria-free environment.

The methods employed, although different in certain details, focused on the axenization and feeding of *Artemia* with single species or combination of species of algae grown bacteria-free in differently enriched media.

Aseptic nauplii

Bacteria-free nauplii were obtained routinely by disinfecting the wintering eggs with strong germicides, such as aquaeous solution of Merthiolate 1:1 000, $AgNO_3$ 1:1 000, phenol 1:50 and hypochlorite 1:100. Immersions of 1 to 10 min were sufficient to kill all accompanying micro-organisms. The eggs, rinsed of the germicide, were incubated and hatched in sterile test media (Gibor, 1956 ; Provasoli *et al.*, 1959). For the ovoviviparous strains of *Artemia*, which do not produce wintering eggs with hard shell, aseptic nauplii were obtained by subjecting gravid females to periodic washes through sterile media and antibiotics (D'Agostino and Provasoli, 1968). Rapid transfer, every few hours through successive 30 ml portions of sterile medium containing antibiotics and food organisms, effectively prevented accumulation of fecal matter and bacteria. The nauplii emerging from the ovisac were collected sterilly and in turn washed through successive baths of sterile medium containing antibiotics. The food organism *Dunaliella tertiolecta* was added to stimulate ingestion and defecation. The nauplii were transferred to the experimental cultures after passage through a sterility test medium.

Media

The media reported by different authors for the bacteriafree culture of the algae was enriched to accomodate the requirements of the algae and the experimental design.

Accordingly, the nutritional history of the algae that were fed to *Artemia* differed considerably. These differences may have affected the growth and reproductive behavior of the *Artemia* (D'Agostino, 1965 ; D'Agostino and Provasoli, 1968). In fact, the chemical composition of the algae has been shown to change as a function of salinity, the concentration of dissolved nutrients and photoperiod (Soeder, 1965 ; Strickland, 1966 ; Werner, 1970). Results of different investigators, therefore, must be viewed with knowledge of the culture history of the algae, and the medium in which the algae were being grazed by the *Artemia*.

Monoxenic cultures

Gibor (1956) found that *Stephanoptera gracilis* and *Dunaliella viridis* grown in seawater enriched with only NO_3, PO_4, and trace metals gave larger adults, in 6 days growth, than *D. salina* and *Platymonas* sp.. *Stichococcus* sp. grown in enriched seawater was inadequate apparently because, though voraciously ingested, its cells were not completely digestable. The fecal pellets of adult *Artemia* contained viable *Stichococcus*. *Artemia* fed on a mixture of *Stichococcus* and *D. viridis* reached adulthood, although they were smaller than those grown on *D. viridis* alone. *Stichococcus*, aside from being less easily digested, may have been nutritionally inadequate because in the presence of bacteria no *Artemia* adults were obtained. In contrast, algae such as *Stephanoptera*, *Dunaliella*, and *Platymonas* accompanied by bacteria allowed growth to maturity.

Provasoli *et al.* (1959) enriched the seawater with NO_3, PO_4, 2 ml % soil extract and 5 ml % STP medium. The STP brought in (as % w/v or v/v of the final medium) Na-H-glutamate 2.5 mg ; glycine and DL-alanine 0.5 mg each ; trypticase and yeast autolysate 1 mg each ; sucrose 5 mg ; vitamin mix 8a 0.005 ml. The algae were inoculated 3 to 10 days before introducing the nauplii of *Artemia* hatched separately, bacteria free, from cysts from the Great Salt Lake. Under these conditions *Dunaliella tertiolecta*, *Tetraselmis maculata*, *Stephanoptera* sp., *Brachiomonas pulsifera*, *Isochrysis galbana*, *Stichochrysis immobilis*, *Syracosphaera elongata*, *Chroomonas* sp., *Hemiselmis virescens*, and *Rhodomonas lens* produced adults. Other algae were nutritionally inadequate.

A larger array of algae was screened with *Artemia* nauplii and metanauplii of Great Salt Lake. The seawater was enriched with NO_3, PO_4, Si, trace metals and vitamins (Table II). Species such as *Amphora coffaeiformis*, *Cyclotella nana* (3H), *Nitzschia acicularis*, *N. closterium*, *N. frustulum* (53M), and *Phaeodactylum tricornutum* allowed growth to sexually mature adults, but seldom production of eggs unless supplemented with a bacterium. Other diatoms were inadequate food. The *Artemia* grew to late metanauplii or early juvenile stages.

None of the dinoflagellates were satisfactory food though ingested. Several species were obviously toxic ; *Amphidinium klebsii*, *Gonyaulax monilata*, and *Peridinium trochoideum*. Nauplii, metanauplii and juvenile *Artemia* exposed to suspensions of dinoflagellates survive fewer days in these cultures than in sterile sea water. This observation has since found confirmation in several bioassays (Trieff *et al.*, 1973).

Twelve unidentified isolates of red-pigmented cryptomonads (*Chryptophyceae*, Table II) supported growth to adulthood in 7 to 10 days. However, *Artemia* grown exclusively on cryptomonads were often affected by a "black disease", readily visible black spots developed from the accumulation of fine particles (~ 1 μm, soluble in hot alkali, probably melanine) underneath the exoskeleton. The disease invariably interfered with molting and lead to early death. Similar spots developed in *Artemia* grown on artificial media containing high protein,

TABLE II

A qualitative measure of the nutritional value of food organisms fed to *Artemia*[1] in monoxenic culture

Food Organisms	N	*Artemia*, Great Salt Lake				
		Survival (days)	Developmental stages reached			
			Juvenile	Young	M-F	F-eggs
Chlorophyceae						
Brachiomonas sp. (no. 7)	12				20-24	
Brachiomonas pulsifera	12				10-15	
Brachiomonas submarina	12			6-7		
Carterii sp. (no. 1)	11	10-13	NG			
Carterii chui	11	6-8	NG			
Carterii convaluta	12	6-8	NG			
Chlorella marina (no. 580)	2			16-18		
Dunaliella sp. (brine)	12	9-10	NG			
Dunaliella sp. (marine)	14					14-18
Dunaliella parva	14	8-9	NG			
Dunaliella salina	13					16-20
Dunaliella tertiolecta	12					15-20
Dunaliella viridis	12					18-20
Nanochloris oculata	11				15-40	
Nanochloris thomas	11				16-30	
Platymonas tetrahele	11				18-32	
Prasinocladus lubricus	12			25-28		
Pyramimonas incostans	15	8-10	NG			
Stephanoptera gracilis	11				20-28	
Stichococcus fragilis	10	4-5	NG			
Chrysophyceae						
Chrysochromulina brevifilum (no. 39)	8	2-4	NG			
Chrysochromulina strobilum (no. 43)	7	3-6	NG			
Chrysochromulina kappa	9	2-7	NG			
Coccolithus huxleii (no. 92A)	7	9-10	NG			
Hymenomomas sp. (no. 156)	12	1-7	NG			
Isochrysis galbana	12				10-14	
Monochrysis lutherii	12			20-30		
Pavlova gyrans	14	3-13	NG			
Stichochrysis immobilis	1					28-30
Syrachosphera sp. (no. 181)	9	2-7	NG			
Syrachosphera elongata	4				20-30	
Cryptophyceae						
Chroomonas sp.	2					20-23
Chroomonas pauciplastida	2	6-12	NG			
Cryptomonas sp.	20					12-14
Hemiselmis rufescens	4					13-15
Hemiselmis virescens	4		9-10			
Rhodomonas sp.	8					14-15
Rhodomonas lens	4					11-14
Dinophyceae						
Amphidinium carteri (no. 1)	6	1-2 T	NG			
Glenodinium montanum (no. 1120)	6	1-2 T	NG			

Food Organisms	*Artemia*, Great Salt Lake					
	N	Survival (days)	Developmental stages reached			
			Juvenile	Young	M-F	F-eggs
Gonyaulax catenella	17	1-3 T	NG			
Gonyaulax monilata	16	1-3 T	NG			
Gonyaulax tamarensis	19	1-2 T	NG			
Gymnodinium sp. (Thomas)	12	6-12	NG			
Gymnodinium brevis	2	1-2 T				
Gyrodinium cohnii	11	5-8	NG			
Peridinium sp.	5	3-10	NG			
Peridinium triquetum	26	3-7	NG			
Peridinium trochoideum	18	2-4	NG			
Prorocentrum micans	4	2-9	NG			
Bacillariophyceae						
Achnanthes brevipes	3		8-12			
Amphora coffaeiformis	20				20-21	
Amphora paludosa	3			10-14		
Amphora perpusilla	15				18-25	
Chaetoceros lorenzianus	9	2-8	NG			
Coscinodiscus asteromphalus	16	3-8	NG			
Cyclotella sp.	7				14-18	
Cyclotella nana (no. 131)	12	2-8	NG			
Cyclotella nana (no. 3H)	6				15-19	
Navicula incerta	6			8-13		
Nitzschia sp.	8				14-28	
Nitzschia acicularis	2				15-16	
Nitzschia closterium (no. 640)	2				16-17	
Nitzschia frustulum (no. 53M)	2			19-20		
Nitzschia frustulum (no. 13M)	12	3-24	NG			
Nitzschia ovalis (no. 49M)	2			13-20		
Phaeodactylum tricornutum	2		13-15			
Rhizosolenia sp.	8	2-4	NG			
Skeletonema costatum	13	2-6	NG			
Stephanopyxis turis	12	2-12	NG			
Thallasiosira fluviatilis	14	2-6	NG			
Cyanophyceae						
Coccochloris sp.	17		8-13			
Plectonema roseolum	4	3-11	NG			
Rhodophyceae						
Rhodosarus marina	10		4-14			
Euglenophyceae						
Eutreptia sp.	2	4-13	NG			

[1] *Artemia* cysts from the Great Salt Lake, Utah.

N = number of trials ; numerals indicate number of days survived or days employed to reach indicated developmental stage ; M-F = sexually differentiated individuals (M = male, F = female) ; F eggs = adult female with eggs. NG = no growth ; T = toxic.

low carbohydrates and low vitamin concentrations, suggesting that the cryptomonads differed from other algae in their protein/carbohydrate/fat ratios. Nevertheless, the rate of growth of *Artemia* fed cryptomonads was much greater than those fed other species of algae. The incidence of "black disease" appeared to be linked to the age of the algal culture, indicating that the detrimental changes in cell composition were associated with senescence.

These data extended the list of nutritionally acceptable food algae for the *Artemia* of the Great Salt Lake and confirmed previous impressions of the trophic exchange occurring between the prey and predator algae under changing environmental conditions.

Moreover, the relationships became more ambiguous when the algae were fed to *Artemia* of different geographical provenance. Thus, *Artemia* of the Great Salt Lake and Comacchio salines maintained in seawater (32 ‰) enriched with NO_3, PO_4, trace metals, and vitamins produced fertile adults when feeding on *D. salina* but not when feeding on *D. viridis*. The opposite was true for Gibor's (1956) findings with the same algae fed to *Artemia* of San Francisco Bay, suggesting that there were subtle differences in the nutritional requirements of *Artemia* of different races.

Artemia, *dixenic in artificial seawater, variable salinity and nutrients*

Differences between Great Salt Lake and the Comacchio strains of *Artemia* became obvious when the nauplii were reared in an artificial salt solution instead of seawater and the salinity was varied widely. *D. salina* and *D. viridis* were adapted to grow at the various salinities by repetitive serial transfer in the experimental media and were fed to nauplii of the two strains of *Artemia*. The Great Salt Lake adults become sexually mature and fertile only at salinities of 3 g % or greater, while the Comacchio adults remained nonfunctional females at the same elevated salinities and failed to develop past the juvenile stages at salinities lower than 3 g %, Table III.

It was noted previously that the *Artemia* from Comacchio produced adults and nauplii when fed *D. salina* in seawater enriched with NO_3, PO_4, trace metals and vitamins, but that it did not grow to adulthood when fed on *D. viridis* in the same medium unless an organically rich algal medium such as STP was added. Evidently an organic enrichment was needed. The active components of the organic enrichment were : 20 μg each of liver Oxoid L-25, yeast extract and urea. Considering the minuteness of the additions, the effects were dramatic. The Great Salt Lake females become fertile even at 0.5% total salts, and the Comacchio females produced viable broods at 3 g % salinity or above. At lower salinities, the effect of the organic supplement was not sufficiently adequate to support reproduction in the Comacchio females (D'Agostino and Provasoli, 1968).

These findings were difficult to interpret without recourse to the known prerequisites of *Artemia* reared in totally artificial media (Provasoli and Shiraishi, 1959 ; Provasoli and D'Agostino, 1969) and culture experiments with *Daphnia* (D'Agostino and Provasoli, 1970 ; Provasoli *et al.*, 1970).

The Great Salt Lake strain utilized dissolved amino acids very poorly. The amino acid mixtures, designed to simulate various proteins, were needed in excess of 600 mg % in order to substitute, but only partially, the precipitated proteins normally used as a source of nitrogenous matter. Similarly, the levels of the vitamins required by the Great Salt Lake *Artemia* in artificial media were in the mg % range for riboflavin, pyridoxamine, and folic acid (Provasoli and D'Agostino, 1962). Purines and pyrimidines were needed at levels of 50

mg % or above. No less than 0.1 % of liver Oxoid was needed to obtain sexually differentiated females of the Comacchio *Artemia* reared in artificial media while in the dixenic culture only 20 μg % was sufficient to promote ovogenesis. These data seem to exclude the possibility that the dramatic effect obtained with both strains of *Artemia* feeding on algae in low salinity media fortified with organics could be ascribed to direct uptake of 20 μg % each of liver Oxoid, yeast extract, urea, and 1 mg % glucose. They prompted instead the hypothesis that the organic enrichment acted primarily on the algae cells by affecting cellular metabolism. Alternatively, the organic enrichment may have reversed the algae abnormal metabolism resulting from hyposalinity stress and/or senescence. These alternatives are plausible since ill-defined minor variations of the culture were noted to affect protein, carbohydrate, and fat content of *Phaeodactylum tricornutum* (Collier, in Ansell *et al.*, 1964). Salinity influenced carbon fixation and glycerol formation in *D. tertiolecta* (Craigie and McLachlan, 1964) ; and the ratio protein : fat changed dramatically in *Chlorella* grown in nitrogen-deficient media (Spoehr and Milner, 1949).

TABLE III

Effect of salinities and organic enrichment on the nutritiousness of *D. salina* and *D. viridis* fed to two strains of *Artemia*

Salinity (g % w + /v)	Great Salt Lake		Comacchio	
	Synthetic base[1] medium		Synthetic base[1] medium	
	"E"	"E + org."	"E"	"E + org."
5	M + n.f. F	M + F + eggs	juv.	n.f. F
1	M + n.f. F	M + F + eggs	young	n.f. F
3	M + F	M + F + eggs	n.f. F	F + nauplii
4 or more	M + F + eggs	M + F + eggs	n.f. F	F + nauplii

[1] A concentrated solution containing 20.5 g % total salts was diluted with H_2O to obtain the desired salinities. The concentrated solution in % (w/v) had NaCl, 17.3 ; KCl, 0.64 ; $MgSO_4.7H_2O$, 3.4 ; $MgCl_2.6H_2O$, 1.9 ; $CaCl_2$ anhydrous, 0.088 ; and $Na_2CO_3.H_2O$, 0.037.

"E" = Enrichment brought in final medium per 100 ml of medium : $NaNO_3$, 0.6 mg ; commercial $\alpha + \beta$ Na-glycerophosphates, 0.2 mg % ; $Na_2SiO_3.9H_2O$, 0.2 mg % ; Vitamins 8a, 0.002 ml ; Metals PII, 0.01 ml ; UII Metals, 0.002 ml ;, Fe (as EDTA) 2 μg ; and glycylglycine, 1 mg.

"E + org." = enrichment brought in final medium per 100 ml of medium : enrichment E + liver (Oxoid L-25), 20 μg ; yeast extract (Difco), 20 g ; glucose, 1 mg.

n.f. = non-functional female, *i.e.* primitive bursa, no eggs in ovaries or ovisac.

F = female.

M = male.

These observations have been corroborated with algae fed to a phyllopod, one harpacticoid, and two copepods which live respectively in hypersaline, euryhaline, and freshwater, suggesting that a very basic phenomenon may be subtly regulating the productivity of some phyto-zooplankton associations.

Artificial media

The absolute nutritional requirements of an organism can be defined only by growing it aseptically on a chemically defined medium. Filter-feeding aquatic organisms may be facultative phagotrophs but *Artemia* is an obligate particle feeder at least with respect to the fulfillment of its requirement for carbohydrate and protein. This behavior necessitated the use of complex biphasic media in which the nutrients were provided in part as suspended particulates (carbohydrates, proteins and lipid-protein complexes) and in part as solutes (peptones, amino acids, nucleic acids, vitamins and minerals). A fairly well defined medium was evolved after years of painstaking trial and error replacement of rich organic crudes (Provasoli and Shiraishi, 1959) with chemically defined components (Provasoli and D'Agostino, 1969).

Culture on artificial diets

The stepwise substitution of crude organic compounds of the 1959-medium with chemically defined compounds of the new 1969-medium no. 100 is illustrated in Table I. Seawater and soil extract were replaced with an artificial salt solution and a trace metal mix. Glycylglycine was used as the buffering agent since others, such as Tris, (hydroxymethyl)-amino-methane, were inhibitory to *Artemia*. Eventually, as the medium became more efficient, glutathione and "*Paramecium* factor" (Lilly and Klosek, 1961) became superfluous and were eliminated.

Replacement of liver infusion, horse serum, and yeast autolysate presented the greatest difficulties since these brought into the medium a great array of nutrients : proteins, amino acids, polysaccharides, vitamins, fats, and sterols. Each time such crude products were substituted with simpler compounds, the concentration of the remaining components had to be readjusted. Elimination of serum, yeast autolysate, and liver extract necessitated not only the addition of heat precipitated albumin, as a source of amino acids, but also adjustment of the concentrations of vitamins, cholesterol and nucleic acids. The liver seemed richest in substances supporting sexual differentiation and oogenesis. None of the many mixtures of amino acids, vitamins and fats employed in place of the liver improved fecundity.

Unexpectedly, the substitution of horse serum, as the main source of amino acids, with heat precipitated albumin, accelerated the rate of growth considerably once the total concentration and ratios of particles, insoluble starch/precipitated albumin, was adjusted to suit the filter feeding mechanism of the nauplius and adults. The optimal starch : albumin ratios were between 5:1 and 2.5:1 (Fig. 4). Obviously, development and growth in the suboptimal range, protein 1-10 mg %, depended chiefly on the availability of particles.

In this 1969-medium no. 100, all nutrients aside from starch and albumin were chemically defined. Mortality was less than 10%, 15 instars or better were obtained in 23 days with an average consumption of 5 ml of medium per animal. The medium still lacked factors, supplied by liver, for oogenesis. Without the addition of liver, females invariably carried aborted eggs and died at the 45th day. Addition of 75 mg % of liver induced rapid sexual differentiation and egg deposition on the 25th day.

Partial substitution of liver (Oxoid L-25) was accomplished recently with the introduction into the medium of a complex mixture of fat soluble vitamins and fatty acids absorbed on particles of precipitated albumin fraction V (Provasoli and Pintner, 1980).

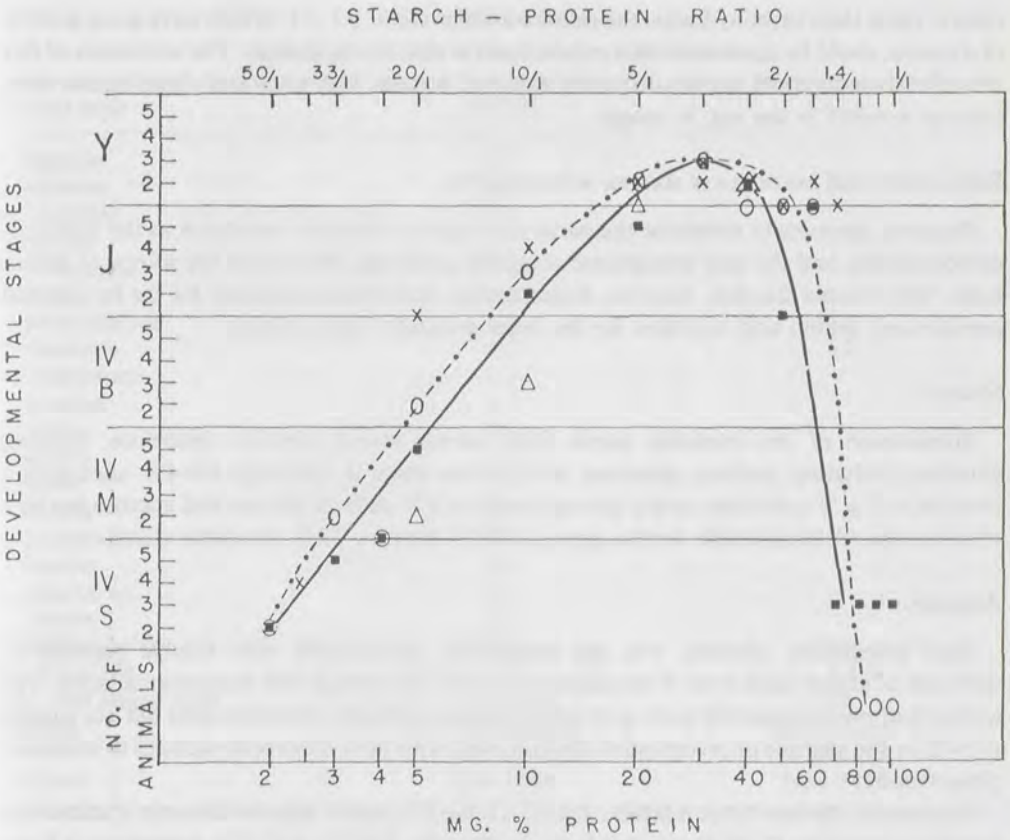

FIG. 4. Relationships between constant particulate starch (100 mg%) and varying quantities of precipitated proteins. • : results at 11 days, 0 = at 13 days, growth in 10 ml of medium with mixture of precipitated protein : albumin 6p : γ globulin 2p : β-lactoglobulin 2p ; X : results at 12 days, growth in 10 ml of medium with protein = albumin only ; △ : same as above ; Y = "young" ; J = "juvenile" ; IV B = "IV big" metanauplius ; IV M = "IV medium" metanauplius ; IV S = "IV small" metanauplius. This arbitary designation of *Artemia* developmental stages was described in detail by Provasoli and D'Agostino (1969).

Reared in medium no. 100 the *Artemia* appeared to require more carbohydrates than proteins and little if any fatty acids, suggesting that it was not utilizing the latter and the amino acids as energy sources. This contradicted expectations based on the experiences with insect nutrition. *Aedes aegypti* in chemical defined media needed no carbohydrates, casein sufficed both as N and C source (Akov, 1962). The preference for a high carbohydrate diet seemed also anomalous because the flagellates eaten by *Artemia* : *T. maculata, D. salina, M. galbana, P. tricornutum* and others contained more protein than carbohydrates. Parsons *et al.* (1961) showed that the composition of these algae, grown under similar physical and chemical conditions was : proteins 35-57% ; carbohydrates 15-30%, and fats 3-10%. If the fat and carbohydrates were to be considered as a single energy source, the ratio would be 1:1, a borderline ratio for *Artemia* reared in artificial media. However, since fats have a much higher

caloric value than carbohydrates, the ratios starch/protein, 5:1-2:1, which gave good growth of *Artemia*, could be equivalent on a caloric basis to that found in algae. The utilization of fats in artificial media could not be adequately explored because fatty acids and phosphatides were toxic to *Artemia* in the mg % range.

Replacement of particulate matter with solutes

Repeated attempts to substitute the particulate starch, insoluble rice starch as the source of carbohydrates, and the heat precipitated crystallin grade egg albumin as the source of amino acids, with soluble starches, dextrins, disaccharides, and monosaccharides for the former and peptides and amino acid mixtures for the latter generally led to failure.

Starch

Substitution of the insoluble starch with soluble starch, dextrin, cellobiose, sucrose, galactose, trehalose, maltose, mannose, and glucose tested in the range 0.4-4% once gave a juvenile in 1 g % cellobiose, and a young female in 2% each of glucose and sucrose but in 3 months time. A comparable female grew in 24-27 days on 0.1% insoluble starch.

Albumin

Heat precipitated albumin was not completely substitutable with soluble peptides or mixtures of amino acids even if containing all of the 10 essential and non-essential acids. The amino acid mixture became toxic at 0.4-0.6% levels. Subtoxic concentrations did not sustain growth in the absence of precipitated albumin even with inert adsorbent particles to stimulate phagotrophy.

Eventually, the least toxic mixture, AA19L (Table IV), partly replaced albumin if added to a medium containing 0.1% starch and 5 mg % albumin. AA19L at 0.33% substituted 5-8 mg % of albumin, *i.e.* gave growth comparable to that of a medium carrying albumin 10-13 mg %. Therefore, 0.3% of soluble amino acids corresponded to 5 mg of precipitated albumin. Or, the particulate source of amino acids was ~60 times more effective than solutes. A concentration of 2.5 mg % of precipitated albumin was the absolute minimum for marginal survival and growth with or without the addition of amino acids. Supplementation of the precipitated protein with a few amino acids, mix AA1A as indicated in Table IV, gave better survival, thus, indicating that albumin may be deficient in some amino acids.

Substitution of particulate organic matter with inert particles

Several kinds of finely ground (1-2 μm) particles were tested in an artificial medium without particulates but rich in amino acids and sugars. All were ingested as evidenced by abundant fecal pellet formation.

Early mortality, 10 days in artificial media, ensued with glass, vegetable charcoal, Celite, montmorillite, graphite, Alfacell and Celloflour ; maximal survival, 20 days, occurred with kaoline, Carbose, and rice starch. Although 2-3 molts occurred in artificial media with the more favorable particles, sexual differentiation was evident only in tubes containing rice starch. Absorbant particles such as Norit A, Sephadex, and mixed weekly cationic and anionic resins were inhibitory.

TABLE IV
Amino acids mixtures (mg/ml) for artificial media

Amino acids	APP19L	AA1A
L-arginine	5.7	
L-histidine	2.4	6.0
L-isoleucine	7.0	
L-leucine	9.2	
L-methionine	5.2	
L-lysine	6.3	
L-phenylalanine	7.7	3.0
L-threonine	4.0	6.0
L-tryptophane	1.2	
L-tyrosine	3.7	
L-valine	7.1	
L-cystine	2.4	
L-serine	8.2	2.0
L-proline	3.6	
L-glutamic ac.	16.5	30.0
L-alanine	6.7	
L-aspartic ac.	9.3	
glycine	3.1	
Total weight/ml	109.3	57.0
Used ml/100	3-6	3.3
Mg. total amino acids	328-656	188
Purpose	Partial substitute of ppt. albumin	Supplement to albumin
Medium	New 1969	New 1969

Celkate at < 10 mg % in an artificial medium containing a total of 0.72 % of amino acids, 0.05 % starch and 5 mg % of precipitated albumin favored growth, giving in general one to two more stages than the controls (Provasoli and D'Agostino, 1969).

SOLUBLE NUTRIENTS

The availability of an artificial medium which was chemically defined, with the possible exception of the particulate starch and albumin, greatly facilitated delineation of the absolute nutritional requirements of *Artemia*.

The B-vitamins, nucleic acids, fats and trace metals were in solution, yet available to the *Artemia*, an obligate phagotroph. This paradox was resolved with a series of experiments in which the particulate phase of the medium was prepared with and without vitamins, the resulting particles were separated and recombined with the liquid phase prepared with and without vitamins. Satisfactory growth ensued only in the media with the vitamins in the liquid phase, indicating that the *Artemia* could utilize micronutrients as solutes. Absorption of micronutrients on the particle of starch and precipitated albumin played a minor role in the uptake of nucleic acids, vitamins, trace metals and other soluble growth factors in the context of medium no. 100 (Table I).

Evidently, while the needs for amino acids and carbohydrates could not be satisfied with compounds in solution, most other nutritional requirements could, provided the nutrients were present in high concentrations often very near toxic levels. The experiences confirmed the notion that *Artemia* was capable of limited osmotrophy as suggested by Croghan's (1958b) observations of the swallowing behavior of *Artemia* and the absorption of vital dyes on the epithelium of the gut wall. But, osmotrophy was limited, substantiating the view of Stephens and Schinske (1961) according to which marine Crustacea differed from all soft-bodied invertebrates in showing no significant uptake of radioactive dissolved organic compounds from seawater. Under the extreme conditions prevailing in laboratory cultures, *Artemia* may profit from its modest osmotrophy (Provasoli and D'Agostino, 1969) but in nature where the dissolved organic matter is extremely dilute, it must be considered an obligate phagotroph. The contrary view is currently under intensive exploration (Pavillon, 1977).

Vitamins

The vitamins needed for optimal growth were determined by the usual dilution subtraction tests, *i.e.* one vitamin was left out of the mixture at a time (Provasoli and D'Agostino, 1962). The list of vitamins (Table V) is remarkable only in that putrescine appears as an absolute requirement. Omission of the latter impeded sexual differentiation and reproduction. This requirement was unique of *Artemia* and the beetle *Oryxaephilus surinamenses* (Davis, 1966). Its function is yet to be determined. Dadd (1970) speculated that the polyamines, putricine, and spermidine, may act as nonspecific metabolic stimulants and are probably not essential in the usual nutritional sense. Their effect then would be detectable only under marginal conditions.

Table V

Vitamin requirements of *Artemia*, Great Salt Lake, Utah

Vitamin omitted	Stage reached	Needed for growth to adult	
		Requirement	Concentration range (wt %)[1]
None	Adult		
Thiamine-HCl	M.IV small	R	0.6-2.4 mg
Nicotinamide	M.IV small	R	1.2-4.8 mg
Ca-pantothenate	M.IV small	R	2-8 mg
Pyridoxamine	M.IV medium	R	50-200 μg
Riboflavin	M.IV medium	R	0.1-0.6 mg
Folic acid	Juvenile	R	0.3-1.4 mg
p-aminobenzoic ac.	Juvenile	R	0.1-0.6 mg
Biotin	Young	S[2]	30-120 μg
Putrescine	Young adult	S[2]	0.1-0.4 mg
Inositol	Adult	0	(0.3-10 mg)[3]
Choline	Adult	0	(0.3-10 mg)[3]
B_{12}	Adult	0	(1-90 μg)[3]
Carnitine	Adult	0	(0.1-1 mg)[3]

[1] Weight in final medium.

[2] Increases survival = longevity.

[3] Range tried – nonessential vitamin, no toxicity.

Inositol, choline, B_{12} and carnitine stimulated fecundity in adults but had not effect on growth rates. Thiamine, nicotinamide, Ca-pantothenate, pyridoxine, riboflavin, and folic acid were the essential vitamins for *Artemia* as they were also for *Moina*, the only other crustacea which to date has been reared in a chemical defined media (Conklin and Provasoli, 1977). Otherwise the *Artemia* shared all other vitamin requirements with the insects.

Surprinsingly, the fat soluble vitamins such as A, D, and E did not seem to influence growth of first generation nauplii of Great Salt Lake *Artemia* reared in basal media no. 100 (Table I). Consequently, they were neglected in the search for substitutes of liver oxoid, although there was some evidence of their influence on growth and reproduction of insects (House, 1966). Subsequently, Conklin and Provasoli (1977) established that omission of carotene, vitamins D and E affected the fertility of *Moina*. Recently, a mixture of fat-soluble vitamins + fatty acids adsorbed on particles of heat precipitated albumin fraction V added to medium no. 100 has permitted partial replacement of liver and cultivation of several serial generations of the parthogenetic *Artemia* of Sète (Provasoli and Pintner, 1980). This suggested that the requirements for fat-soluble vitamins and fatty acids, which were undemonstrable with the one generation growth studies of the bisexual *Artemia* from the Great Salt Lake, may have been due to the presence of ample supplies maternally endowed to the eggs during ovogenesis. Unquestionably, cultivation through several serial generations in deficient media would be essential in order to affect depletion of stores and consequently demonstrate the essentiality of those growth factors for which there is limited cellular use.

Fatty acids

The water soluble liver infusion, Oxoid L-25, which promoted sexual maturation of the *Artemia* of the Great Salt Lake and Comacchio, was contaminated with 12.4% of mixed lipids. The fatty acids were : oleic, palmitic, stearic, myristic, linoleic, and linolenic. All of these had been tested on previous occasions, as additions to medium no. 100, without significant enhancement of growth or fertility. Oleic acid, added to media carrying near toxic concentration of amino acids had a detoxification and a sparing effect on carbohydrate requirements. These fatty acids, adsorbed on particles of heat precipitated albumin fraction V, *i.e.* preparation SFA of Provasoli and Pintner (1980) appeared to have enhanced fertility of the parthenogenetic *Artemia* of Sète. Earlier, Conklin (1973) had reported a definite response of *Moina* to lecithen, palmitic, oleic, linoleic, and linolenic acids. In insect nutrition, only linolenic and linoleic acids are essential and radioisotopic studies have indicated that insects may synthesize most other fatty acids (Dadd, 1970). *Artemia* may be expected to act similarly although the possibility needs to be explored.

Sterols

Dietary cholesterol was required by *Artemia*, 0.8 mg % (w/v) was sufficient ; higher concentrations tended to be inhibitory. The need for cholesterol concurs with Kanazawa *et al.* (1971) finding of no evidence of sterol synthesis in *Artemia*. Similar results with radioisotope studies of *Astacus*, *Homarus*, and *Cancer* suggest that perhaps all Crustacea may require exogenous sources of dietary sterols (in Dadd, 1970). For *Artemia* dietary cholesterol could be substituted in part by ergosterol, stigmasterol and beta-sitosterol among other sterols (Provasoli and D'Agostino, 1969). This agrees with the findings of Teshima and Kanazawa

(1971) on the metabolic conversion of the latter and other sterols into cholesterol. Sterols are essential for most insects and to the extent tested, for all Crustacea.

Nucleic acids

Artemia in chemically defined media required exogenous sources of nucleic acid. Alkali and acid digests of RNA and DNA were substituted with mixtures of adenylic, guanilic, uridilylic, cytidilic acids and thymidine with sustained growth and metamorphosis (Provasoli and D'Agostino, 1969). Hernandorena (1974, 1975, 1976) has studied in some detail the interrelationship of various exogenous sources of the purines and pyrimidines for *Artemia*. Omission of purines results in stasis of metamorphosis. Alteration of the relative quantities of dietary adenylic/cytidylic acids induces aberrant post embryonic development. Surprisingly, the needs for adenylic acid changed with changes in the quality of calorigenic nutrients, temperature and salinity, indicating that the purine was utilized for ATP formation.

Reproduction

The reproductive strategy of *Artemia* is typical of invertebrates inhabiting stressful and unstable environments. It consists of the investiture of energy for the production of numerous offspring (Grosch, 1973) and in the option of shunting whole broods of embryos into an encysted dormant state, cryptobiosis (Keilin, 1959), in response to the onset of unfavorable environmental conditions (Lochhead, 1941).

There is extensive literature on the distribution (Kellog, 1906 ; Daday, 1910 ; Barigozzi, 1957), mode of reproduction and genetics (Artom, 1931 ; Gross, 1932 ; Stella, 1933 ; Goldschmidt, 1952 ; Stefani, 1961 ; Bowen, 1964) of the various geographically isolated strains of *Artemia*. Nevertheless, the systematics of the genus remain in a state of flux (Barigozzi, 1974 ; see also editorial note on *Artemia* taxonomy, this book).

Generally, there are parthenogenetic and bisexual strains. In either case, the females may deposit offspring ovoviviparously or oviparously (Lochhead, 1941). Apart from the questions generated by Ballardin and Metalli (1963) concerning the unpredictability of oviparity in the different strains reared in nature and in the laboratory, several environmental factors have been suggested as probable causative factors in prompting the female to shunt offspring into cryptobiosis (Dutrieu, 1962).

Ovoviviparity

In the wild and in laboratory cultures, the first brood of offspring is invariably ovoviviparous. Following ovulation the eggs pass from the ovaries into the oviducts, the lateral sacs of the ovisac, there to remain from 24-48 hr. In the bisexual strains copulation and fertilization occur at this time and is obligatory with each successive ovulation (Bowen, 1962). From the ovisacs the eggs descend into the uterus, the central sac of the ovisac, where they complete embryonic development in 4 to 5 days, and are expelled as free-swimming larvae, the nauplii (Lochhead and Lochhead, 1940). In the parthogenetic strains such as those from Sète and Comacchio, the eggs develop into free-swimming nauplii without fertilization (Artom, 1931 ; Ballardin and Metalli, 1963 ; Bowen, 1964). The full reproductive cycle from ovulation to the release of the nauplii is usually from 4 to 5 days. Soon after the release of the

brood, the female molts, a new batch of eggs descends into the oviducts and the reproductive cycle is repeated.

Oviparity

Reproduction by dormant encysted blastulae is elective. *Artemia* kept under optimal culture conditions feeding on phytoflagellates *ad libitum* may postpone reproduction by encystment of embryos indefinitely. In the laboratory adult *Artemia* subjected to periods of uncontrolled hypo-alimentation may alternate from ovoviviparity to oviparity without apparent reason. Nevertheless, there is sufficient evidence to indicate that the females are predisposed to oviparity by nutritional factors prevailing at the time of ovulation.

Dutrieu (1960) suggested that oviparity was stimulated by exposure of the adult to low dissolved oxygen tensions, < 2 ppm, but in culture situations that provided *Artemia* with a source of chlorophyl and carotene-proteins. The presence of hemolymph hemoglobin, which was produced during exposure to low oxygen tension, was implicated as well, since the shell which characterizes the encysted egg of *Artemia* is of maternal origin and rich in haematin. Furthermore, Ballardin and Metalli (1963) observed that parthenogenetic diploid *Artemia* of S. Gilla (Cagliari, Italy) feeding on *Chlamydomonas* and on unknown microflora, when subjected to hypo-alimentation, reproduced ovoviviparously on the first deposition and oviparously on the subsequent depositions. The incidence of oviparity could not be correlated with type of food, overcrowding, higher salinity, temperature, and illumination, but it was proportional to the duration of successive periods of undernourishment. These experiences suggested that most abiotic factors previously implicated seemed to have affected oviparity through their influence on the quality and availability of the food organisms. This notion was verified with *Artemia* of the Great Salt Lake and Comacchio reared under controlled conditions in media whose salinity ranged from 0.5% to 20.5% (w/v) (D'Agostino and Provasoli, 1968). The incidence of encysted broods varied widely at all salinities more so at the higher salinities, but invariably it correlated with brief periods of starvation which coincided with ovulation. Mature females of the parthogenetic strain of *Artemia* from Comacchio exposed to an abundant food supply reproduced ovoviviparously as long as the food supply lasted; females of the same cohort starved during the period of ovulation deposited oviparously. In fact, any one female in the course of a long life cycle, > 6 months, could be stimulated to deposit offspring ovoviviparously or oviparously simply by withdrawing the food supply during 3 to 5 days of the reproductive cycle, commencing with the day preceding ovulation. It was surmized that in the absence of gross nutritional deficiencies such as those occurring with *Artemia* fed chemically defined media (Provasoli and D'Agostino, 1969) fecundity and the mode of reproduction was dependent on the quality and availability of nutrients in the algal cell more so than on fluxes of abiotic factors. This notion found confirmation in the work of McDermott (1974) who was able to replicate the phenomenon with *Artemia* of San Francisco Bay and further in the field studies by Lenz (1980) on the reproductive characteristic of the *Artemia* of Mono Lake, California. The data indicated unequivocally that oviparity in this alkali-adapted strain was triggered by a low food supply.

The encysted embryos

The product of oviparity lends extraordinary survival advantage to the species. In cryptobiosis the embryos are enclosed in a chitinous shell heavily impregnated with lipoproteins and

haematin of maternal origin (Dutrieu, 1960). Carbohydrate metabolism seems to play a significant role both in the process of encystment and excystment.

A study of the carbohydrates in *Artemia* indicated that the stores of glycogen of adult females in ovulation were rapidly depleted shortly after onset of suboptimal feeding regimens imposed to stimulate oviparity (McDermott, 1974). The glycogen stores of similarly ovulating females kept fed *ad libitum* remained unchanged. This rapid depletion of glycogen suggested that glycogenolysis may be providing haemolymph glucose, the major blood sugar of adult females, or speculatively sustenance for the nurse (nutritive) cells of the eggs in the oviducts. In either instance, the resulting lower levels of glycogen could have served to trigger alternate bio-synthetic pathways in the embryo leading to the synthesis and storage of trehalose for the cryptobiotic embryo instead of glycogen as in the ovoviviparous larvae. For a complete review of the roles of trehalose, glycogen, and glycerol in dormancy, emergency and hatching of *Artemia* we refer to Clegg (1962, 1964, 1965, 1974).

Following rehydration, the embryo resumes development, swells, ruptures the outer shell and emerges still enclosed in a diaphanous embryonic membrane (Whitaker, 1940). Hatching of the free-swimming nauplii follows the breaking of this last remaining membrane by the mechanical action of the twitching embryo, hydrostatic stress, and/or a hatching enzyme (Sato, 1967).

In *Artemia*, as in most arthropods, ingested nutrients are metabolized to provide energy and molecular species for the synthesis of functional, storage and structural components. *Artemia* seem to utilize carbohydrates extremely well in the context of the artificial and chemically defined medium no. 100, even though carbohydrates are poorly represented in its diet in nature. While glucose is the major sugar in the haemolymph of most crustacea, glycogen appears to be central to intermediary metabolism (Adiyodi, 1969). Glycogen may play a central role in *Artemia* as indicated by the rapid depletion of stores during hypo-alimentation in the adult (McDermott, 1974) and during early post-embryonic development of the nauplius (Clegg, 1964). The utilization of glycogen implies the participation of phosphorylases and adenosine 5-monophosphate (Sagardia, 1969). Accordingly, Hernandorena's (1975) studies on the AMP requirement of *Artemia* may provide indirect evidence of the importance of glycogen in the metabolism of *Artemia* as well as in the synthesis of nucleic acids.

With the exception of linolenic, linoleic acids, and cholesterol, other lipids were not considered essential for growth and reproduction in most arthropods. Nevertheless, the participation and significance of lipid soluble vitamins A, D, and E ; the fatty acids linoleic, linolenic ; the sterols cholesterol and ergosterol ; the nucleic acids (of which AMP appears to be the most important) and the unidentified fertility factor of the water soluble fraction of liver infusion Oxoid L-25 in the reproductive cycle of *Artemia* demand to be explored (Provasoli, 1975).

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The molecular biology of *Artemia*

T. Hultin and M. O. Nilsson

Department of Cell Physiology, Wenner-Gren Institute, University of Stockholm
Norrtullsgatan 16, S-113 45 Stockholm, Sweden

Content

Introduction

The molecular biology of eukaryotes was in the beginning mainly concerned with higher vertebrates. It was gradually felt that experiments on simpler or differently specialized organisms might be fruitful as a complement, and might afford advantages and raise problems

of considerable importance. The encysted embryos of *Artemia* have several remarkable features which make them attractive as research material in molecular biology. They are available in large amounts and can be preserved without special attention until needed. They have relatively low activities of nucleolytic and proteolytic enzymes (Bellini, 1957 ; Urbani and Bellini, 1958). The encysted embryos are in a state of deep dormancy, a remarkable physiological situation never observed in mammalian cells and tissues. After rehydration the embryos resume their anabolic activities, differentiate without cell division, and develop into swimming larvae (nauplii).

The practical convenience of the encysted embryos as a research material and their interesting physiological properties explain why the molecular biology of *Artemia* is mainly confined to the dormant and developing cysts and the early larval stages. In essential respects the situation reminds of that in sea urchin eggs and embryos (Hultin and Bergstrand, 1960 ; Gross and Cousineau, 1963). However, in comparison with the sea urchin material *Artemia* embryos have certain inherent limitations : 1) The important initial stages of development prior to gastrulation and encystment take place in the ovisac, unavailable to large-scale experiments. 2) The cysts are impermeable to specific anabolic inhibitors and most labeled precursors (Clegg, 1966 ; de Chaffoy *et al.*, 1978). Little is therefore known about the basic anabolic events associated with primary cell differentiation. 3) The development is asynchronous. Alterations on the subcellular level are therefore difficult to correlate with well-defined morphogenetic stages. In addition, the newly hatched nauplius is already a relatively complex organism, even on the molecular level (Ewing *et al.*, 1972 ; Moens and Kondo, 1976).

Irrespective of these limitations, *Artemia* embryos have unique and still only partially exploited potentialities as research material in molecular biology. The cysts are eminently suitable for studies of cryptobiosis and mechanisms related to the reactivation of cells after deep repression. Within molecular embryology their potential value seems to be more in the direction of highly specialized cell differentiation (Peterson *et al.*, 1978) than of early morphogenetic events. The molecular genetics of *Artemia* are still in an introductory stage (Bowen *et al.*, 1978). Basic to these specific applications, the embryos have a wide range of general applicability as will appear from the following sections.

DNA and DNA metabolism

Nuclear DNA

The early cleavages of *Artemia* eggs take place in the ovisac, and have so far been studied only by microscopic methods. The experiments include micro-autoradiography, using [^{3}H] thymidine as DNA precursor (Iwasaki, 1970), and cytological analysis of the effects of anabolic inhibitors (Fautrez-Firlefyn and Fautrez, 1966 ; Fautrez and Fautrez-Firlefyn, 1974). As with sea urchin eggs (Hultin, 1961b), cleavage is blocked by the specific inhibitor of protein synthesis, puromycin. It is also prevented by low concentrations of actinomycin D. The increase in DNA content during cleavage is less than proportional to the increase in cell number, suggesting that a part of the total DNA of the eggs is extranuclear (Roels-De Schrijver and Fautrez-Firlefyn, 1966).

The further development has been described in detail by Benesch (1969). Under conditions when the development is diverted into a cryptobiotic phase the mitotic activity including DNA

synthesis is blocked until the rehydrated embryos reach the pre-nauplius stage (Bellini, 1960 ; Nakanishi *et al.*, 1962 ; Emerson, 1963). Although the nauplius stage is characterized by DNA synthesis (McClean and Warner, 1971) and cell divisions (Olson and Clegg, 1978) the increase in cell number is moderate (25 %), and is not strictly needed for normal differentiation.

Evidence obtained by microspectroscopic analysis of Feulgen stained embryos suggests that the cells of developing cysts and nauplii fall into two classes, which differ in DNA content (Nakanishi *et al.*, 1963). Using different methods a DNA content of 6.37 pg per nucleus (Roels-De Schrijver and Fautrez-Firlefyn, 1966) and a haploid DNA value of 3.0 pg (Rheinsmith *et al.*, 1974) have been measured. According to Vaughn (1977) these data may indicate some form of polyploidy in the investigated material since DNA reassociation kinetic data indicate a unit genome size of only 1.5 pg. Single-copy DNA sequences comprise 45 % of the genome. The average repetition frequency for the intermediately redundant DNA component is about 5 000 copies. Sequences with a repetition frequency of 2-90 are rare.

The base composition of *Artemia* DNA is somewhat controversial. According to Antonov *et al.* (1963) and Vaughn (1977) the G + C content is 40-41 %, while Schmitt *et al.* (1974ab) arrived at a value of only 34.7 % based on a buoyant density in CsCl of 1.694 g/cm^3. The content of 5-methylcytosine is very low (Antonov *et al.*, 1963).

Pure nuclear DNA is most easily prepared from nauplii (Cruces and Sebastián, 1978), since at earlier stages nuclei are difficult to separate from heavy, DNA-containing yolk platelets (Warner *et al.*, 1972). A direct relationship between yolk platelets and mitochondria has been suggested (Vallejo and Marco, 1976).

Mitochondrial DNA

The mitochondrial DNA of *Artemia* is circular, with a length of 5.1 μm, a molecular weight of about 10^7, and a buoyant density in CsCl of 1.697 g/cm^3, corresponding to a G + C content of 37.8 % (Schmitt *et al.*, 1974ab). Supercoiled mitochondrial DNA is transcribed *in vitro* by *Escherichia coli* RNA polymerase to products which hybridize with mitochondrial rRNA. It would seem that under these conditions the mitochondrial DNA is mainly transcribed on the anti-rDNA strand.

Repair functions

Although *Artemia* has been used extensively in radiobiological and toxicological studies, there is to our knowledge no investigation of DNA repair mechanisms in this organism.

RNA metabolism

Quantitative aspects of RNA

The total RNA content of encysted *Artemia* embryos increases very little during development, but after emergence there is a marked rise (Bellini, 1960). Although no systematic analyses seem to have been performed, there are probably no pronounced changes in the proportions of the major RNA fractions. However, certain alterations within individual RNA species have been reported.

By reversed-phase chromatography Bagshaw *et al.* (1970) demonstrated quantitative changes in isoaccepting tRNAs for 9 amino acids between the encysted gastrula stage and the nauplius stage.

The nuclear fraction of dormant cysts is devoid of poly(A)$^+$ RNA (Nilsson, 1978). At the stage of 50 % emergence the electrophoretic pattern of nuclear poly(A)$^+$ RNA is dominated by a species with an apparent molecular weight of 500 000. As will be discussed in the section "Messenger RNA" there are also modifications in the electrophoretic pattern and messenger specificity of cytoplasmic poly(A)$^+$ RNA during development.

RNA METABOLISM *IN VIVO*

The possibilities of studying the RNA metabolism of developing *Artemia* cysts *in vivo* by use of labeled precursors have two limitations: 1) The low permeability of the embryonic cuticle which prevents or seriously hampers the uptake of labelled precursors except ^{14}C-bicarbonate (Clegg, 1966); 2) The inability of *Artemia* embryos to utilize simle C_1 precursors for the synthesis of purines (Warner and McClean, 1968). Although the incorporation of ^{14}C-labelled bicarbonate into the nucleic acids of developing cysts is much lower than into protein, there is a gradual increase from the first hours of development. The incorporation involves all classes of RNA (Clegg, 1966; Clegg and Golub, 1969).

After hatching the embryos become permeable to a wider range of RNA precursors including nucleosides, and a period of intense synthesis of all classes of RNA is observed in isotope experiments (McClean and Warner, 1971; Susheela and Jayaraman, 1976). In the unfed nauplii this activity gradually declines.

RNA POLYMERASES

Nuclei prepared from *Artemia* embryos contain the three DNA-dependent RNA polymerases (I, II, III) characteristic of eukaryotic cells (Renart and Sebastián, 1974, 1976; Birndorf *et al.*, 1975; Swennen *et al.*, 1976). Polymerase II has been purified and characterized with respect to subunit composition by SDS gel electrophoresis (Bagshaw, 1976).

D'Alessio and Bagshaw (1977) observed that crude nuclear pellets from undeveloped cysts yielded only traces of DNA-dependent RNA polymerase activity when solubilized by sonication at high ionic strength. Using cysts which had been incubated for one hour in seawater RNA polymerases I and II were detectable under the same conditions. Preparations from nauplii were highly active. Observations of a similar kind had been reported in brief by Swennen *et al.* (1976).

According to Hentschel and Tata (1977) the crude nuclear fraction of dormant cysts contains very little template-engaged RNA polymerases I and II, but relatively large amounts of endogenously inactive, free polymerases. These activities, which were easily destroyed by sonication, were measured by use of poly[d(A-T)] as exogenous template, and in the presence of actinomycin D for blocking the transcription of endogenous, deoxyguanosine-containing templates (Yu, 1974). Inhibition by α-amanitin was used to discriminate between RNA polymerases I and II. During early development there was a striking increase in the template-engaged polymerase I and II activities. The activities of the free enzymes were only moderately increased.

The high activities of RNA polymerases I and II in nuclei from early nauplii decline during the further development of the unfed larvae in parallel with the RNA synthesis *in vivo* (McClean and Warner, 1971 ; Bagshaw *et al.*, 1978). The decrease in polymerase I activity is most striking (Birndorf *et al.*, 1975). At least in the case of polymerase II the effect is parallelled by a loss of enzyme molecules as determined by SDS gel electrophoresis (Bagshaw *et al.*, 1978).

In nuclear extracts from nauplii a modified form of RNA polymerase I has been observed, which is not present in developing cysts (Osuna *et al.*, 1977b). The modification is proteolytic, caused by proteases which appear in the nauplii after hatching (Osuna *et al.*, 1977a).

The endogenous activity of RNA polymerases in isolated nuclei is under the restrictive influence of histones. This may explain the observation by Swennen *et al.* (1978) that aurintricarboxylic acid stimulates the activity of the α-amanitin-sensitive polymerase II in isolated nuclei of *Artemia* larvae. Aurintricarboxylic acid is known to bind indiscriminately to basic proteins (Hultin and Nika, 1975). The nuclei of *Artemia* embryos contain histone acetyltransferase(s) (Cano and Pestaña, 1976). The enzyme may be involved in the regulation of gene activity during differentiation.

The cytoplasm of *Artemia* cysts contains an RNA-dependent poly(A) polymerase, supposed to participate in the processing or activation of mRNA in connection with the resumption of development (Sastre and Sebastián, 1978).

Messenger RNA

Latent mRNA in dormant cysts

The observation of a rapid increase in protein synthesis in rehydrated cysts (Hultin and Morris, 1967, 1968 ; Golub and Clegg, 1968) suggested that the cysts might contain a store of latent mRNA, either of maternal origin, or synthesized by the embryo itself before encystment. Evidence in favour of this assumption was obtained by translation experiments using a heterologous system derived from messenger-depleted bacteria (Nilsson and Hultin, 1972, 1974a). Substantial amounts of RNA with template function were found in the cytoplasm of dormant cysts, while RNA from the crude nuclear fraction was nearly devoid of activity. Within the cytoplasm a small, membrane-associated RNA fraction, accounting for at least 25% of the total template activity, was characterized by a particularly high specific activity in the system. The purified membranes (buoyant density 1.17 g/cm^3) included smooth and rough endoplasmic vesicles together with mitochondria. The same RNA fraction was rich in poly(A)$^+$ RNA with a sedimentation coefficient of about 16 S (Nilsson and Hultin, 1974ab, 1975 ; Sierra *et al.*, 1976 ; Susheela and Jayaraman, 1976). Poly(A)$^+$ RNA was also present in slowly sedimenting cytoplasmic particles (Kondo *et al.*, 1974).

With the introduction of the highly efficient wheat germ system for mRNA translation (Roberts and Paterson, 1973) it became possible to analyze the latent mRNA of *Artemia* cysts in detail, and to compare different fractions of stored mRNA by means of their translation products (Felicetti *et al.*, 1975 ; Grosfeld and Littauer, 1975, 1976 ; Sierra *et al.*, 1976). The translation pattern of poly(A)$^+$ RNA from dormant cysts was very complex as analyzed by SDS gel electrophoresis. It was dominated by 2-3 still unidentified polypeptides with molecular weight around 25 000, which could be further resolved by gel isoelectric focussing

(Grosfeld and Littauer, 1975, 1976 ; Sierra *et al.*, 1976 ; Felicetti *et al.*, 1977). These products were also predominant in a reticulocyte translation system using membrane-associated poly(A)$^+$ RNA as messenger (Hultin *et al.*, 1977).

The poly(A)$^+$ RNA in dormant cysts is bound to proteins, forming particles of different complexity. The postmitochondrial poly(A)$^+$ RNP particles are heterodisperse (Kondo *et al.*, 1974 ; Grosfeld and Littauer, 1975 ; Felicetti *et al.*, 1975) with a bimodal distribution in sucrose density gradients (buoyant densities 1.27-1.30 and 1.20-1.23 g/cm^3) (Slegers and Kondo, 1977 ; Simons *et al.*, 1978). The intact particles are almost inactive in the wheat germ translation system, but RNA with messenger activity can be isolated (Felicetti *et al.*, 1975 ; Grosfeld and Littauer, 1975 ; Slegers *et al.*, 1977). Among the postribosomal particles the messenger activity is particularly high in the 40 S region. Some poly(A)$^+$ RNA-containing particles heavier than 50 S (density in sucrose gradient 1.30) are disaggregated by EDTA, and probably represent ribosome-associated mRNP particles (Slegers and Kondo, 1977). Poly(A)$^+$ RNA isolated from particles heavier than 80 S is translated very effectively in the wheat germ system (Grosfeld and Littauer, 1975 ; Slegers and Kondo, 1977). The activity of the light mRNP particles will be discussed further in the section "Control of translation, inhibition of translation".

The mRNA-containing membrane fraction is in great part sedimentable at 15 000 × g, and can be purified by banding in a sucrose density gradient at 1.17 g/cm^3 (Nilsson and Hultin, 1974a, 1975). Poly(A)-containing particles are easily detached by mild detergents and form a distinct 40 S fraction at sucrose gradient centrifugation. The 40 S particles contain poly(A)$^+$ RNA with an average sedimentation coefficient of 14 S, and are not associated with ribosomes. After glutaraldehyde fixation the poly(A)-containing particles band in CsCl gradients at 1.31 g/cm^3. Without fixation the same material forms a distinct band in Cs_2SO_4 gradients at 1.26 g/cm^3. Electron microscopy of the banding material shows a fairly homogeneous population of spherical to bullet-shaped particles measuring 17-26 nm. The protein composition is complex, and includes lipoprotein (Hultin *et al.*, 1977, 1980 ; Lake and Hultin, 1978). Apparently the latent mRNA-containing particles are intimately associated with some membrane components. In addition to the membranes banding at 1.17 g/cm^3 in sucrose Simons *et al.* (1978) observed poly(A)-containing membranes with a density of 1.25 g/cm^3. After detergent treatment 36 S particles were obtained which banded in sucrose density gradients at 1.3 g/cm^3.

Poly(A)$^+$ RNA from membranes (including membranes purified by banding in sucrose at 1.17 g/cm^3) contain two distinct populations of molecules (360 000 and 520 000 daltons) which can be separated by gel electrophoresis (Grosfeld *et al.*, 1977 ; Nilsson, 1978). Although the membrane fraction contains mitochondria, hybridization experiments with mitochondrial (Grosfeld *et al.*, 1977) as well as nuclear (Nilsson, 1978) DNA indicate that the membrane-associated poly(A)$^+$ RNA is of nuclear origin.

Characteristics of mRNA

Artemia embryos contain both poly(A)$^+$ and poly(A)$^-$ RNA with messenger activity (Sierra *et al.*, 1976 ; Amaldi *et al.*, 1978). For membrane-associated mRNA from dormant cysts the length of the poly(A) sequences after correction (Morrison *et al.*, 1973 ; Burness *et al.*, 1975) has been estimated at 45-65 nucleotides (Nilsson and Hultin, 1975). For developed cysts and nauplii appreciably longer poly(A) chains have been reported (Grosfeld *et al.*, 1977).

The mRNA from undeveloped as well as developing embryos is provided with 5'-terminal "cap" structures, in which a 7-methyl-guanosine is linked to the rest of the polynucleotide chain by a 5', 5'-pyrophosphate bridge (Muthukrishnan *et al.*, 1975 ; Groner *et al.*, 1976). Removal of the cap prevents the translation of the mRNA *in vitro* (Muthukrishnan *et al.*, 1975 ; Zan-Kowalczewska *et al.*, 1977). Translation of poly(A)$^+$ RNA from dormant or developing embryos in the wheat germ system is inhibited by the cap analogue, 7-methyl-guanylic acid, and by synthetic cap structures (Groner *et al.*, 1976).

Alterations during development

At early stages of development there is a flow of informational RNA from cytoplasmic poly(A)-containing particles to polyribosomes without much increase in the total content of poly(A)$^+$ RNA (Amaldi *et al.*, 1977). Later, both poly(A)$^+$ and poly(A)$^-$ mRNA accumulate, particularly in polyribosomes (Sierra *et al.*, 1976). Within the membrane fraction the poly(A)$^+$ RNA population with smaller molecular weight (360 000) gradually disappears (Grosfeld *et al.*, 1977 ; Nilsson, 1978).

The translation pattern of isolated poly(A)$^+$ RNA is only moderately altered during development (Grosfeld and Littauer, 1975, 1976 ; Sierra *et al.*, 1976 ; Amaldi *et al.*, 1977). The 25 000 M_r polypeptides, characteristic of the latent mRNA from dormant cysts, become less prominent. There is also some decrease in the proportion of tubulin among the translation products, while actin shows a marked increase (Grosfeld and Littauer, 1976).

Hatching is associated with the appearance of new mRNA species. Amaldi *et al.* (1978) have shown that poly(A)$^-$ RNA from nauplii (but not from cysts) can be translated to acid soluble products identified as histones. Moens *et al.* (1978b) have demonstrated that globin mRNA is absent from developing cysts, but appears after hatching, bound to large polyribosomes (section "Protein synthesis, Endogenous activity of polyribosomes").

Ribosomes

General properties

The ribosomes of dormant cysts occur almost exclusively in the form of uncomplexed monoribosomes (Hultin and Morris, 1967, 1968 ; Golub and Clegg, 1968 ; Finamore and Clegg, 1969). They are easily dissociated into functional subunits without puromycin treatment. In K^+-deficient media they are rapidly inactivated, and dissociate irreversibly (Hultin *et al.*, 1969). Inactivation and irreversible dissociation are also observed when monoribosomes, exposed to low concentrations of oxidants (NaOCl, H_2O_2), are treated with cationic detergents (Golub and Clegg, 1969). This should be kept in mind when hypochlorite-washed cysts (Nakanishi *et al.*, 1962) are used for the preparation of ribosomes by deoxycholate treatment. Dissociation is furthermore induced by high hydrostatic pressure, as analyzed by light scattering (Nieuwenhuysen *et al.*, 1975, 1978b).

The physical properties of *Artemia* ribosomes, purified by centrifugation in high density sucrose gradients (Nieuwenhuysen and Slegers, 1978ab), have been studied in great detail by Nieuwenhuysen and Clauwaert (1978). The measurements indicate a sedimentation coefficient of 81 S, a specific volume of 0.63 cm^3/g and a molecular weight of 3.8×10^6. Boublik and Hellman (1978) studied the structure of the ribosomes and ribosomal subunits by

electron microscopy. The particles were distinctly asymmetric, with a knob-like protrusion in the base of the 60 S subunit.

Ribosomes from dormant and developing cysts have been found equally active in poly(U)-directed polyphenylalanine synthesis (Hultin and Morris, 1968) as well as in initiation factor-dependent, reticulocyte mRNA-directed globin synthesis (Sierra *et al.*, 1974 ; Hultin *et al.*, 1977). Ribosomes from dormant cysts have been used as effective research tools in the study of selective globin mRNA translation into α and β globin chains (Kramer *et al.*, 1975). Observations of repressed 80 S ribosomes in undeveloped cysts (Huang and Warner, 1974) may be related to soluble inhibitors loosely associated with the particles (Warner *et al.*, 1977). However, Reddington and Tate (1979) have reported that ribosomes from dormant cysts are inferior to those from 18 hr developed embryos, in their response to purified release factor and termination codons (section "Protein synthesis, Peptide chain termination). In addition, Snoeks *et al.* (1975) reported a lower Mg^{2+} optimum for poly(U)-directed polyphenylalanine synthesis using ribosomal subunits from dormant as compared with 16 hr developed cysts.

It has been found in our own and other laboratories that *Artemia* ribosomal subunits recombine with other eukaryotic subunits to give effective polypeptide-synthesizing systems. Klein and Ochoa (1972) reported that *Artemia* 40 S subunits combine with bacterial 50 S subunits to form hybride couples capable of limited, messenger and factor-dependent synthesis of peptides (fMet-puromycin, N-acetyl-Phe-Phe).

Cytoplasmic and mitochondrial rRNA

Cytoplasmic ribosomes from *Artemia* cysts have a density in CsCl of 1.57 g/cm^3, corresponding to an RNA content of 49 % (Nieuwenhuysen *et al.*, 1978a). Chemical analysis indicated an RNA content of 49.5 %.

The two cytoplasmic rRNA species have apparent molecular weights of $0.62\text{-}0.66 \times 10^6$ and $1.3\text{-}1.4 \times 10^6$, as calculated from their electrophoretic mobilities (Schmitt *et al.*, 1974b). As in insects (Shine and Dalgarno, 1973) the larger species is dissociated into two 18 S components by thermal denaturation (Ishikawa, 1977).

As has been observed in other species, the mitochondrial rRNAs in *Artemia* have relatively unstable secondary structures (Schmitt *et al.*, 1974b). This is indicated by the marked dependence of their electrophoretic mobilities on the temperature and ionic composition of the buffer. Nevertheless, the G + C content is relatively high (43 %).

Ribosomal proteins

The proteins of the isolated ribosomal subunits have been analyzed by one- and two-dimensional polyacrylamide gel electrophoresis (Von der Decken and Hultin, 1971 ; Möller *et al.*, 1975).

Möller *et al.* (1975) and Van Agthoven *et al.* (1977, 1978) described two structurally related acid proteins, in the 60 S subunits, designated eL12 and eL12'. The former protein, which was slightly shorter, was also found in the cytosol. The two proteins were immunologically identical and partially phosphorylated. Evidence was presented for the analogy between these proteins and proteins L7/L12 in bacterial ribosomes. Their functional importance for elongation factor EF-1 dependent Phe-tRNA binding was demonstrated by reversible ethanol-salt extraction. Antibodies against eL12 inhibited polyphenylalanine synthesis and EF-2

dependent GTPase. Thus, the involvement of the two proteins in factor-dependent GTP hydrolysis seems to be firmly established. The N-terminal amino acid sequences and the primary structures of an alanine-rich region in the two proteins have been determined, and have suggested far-reaching homologies (Amons *et al.*, 1977, 1978). The sequence similarity between the acid proteins from *Artemia* and rat liver ribosomes was particularly evident. The *Artemia* and rat liver proteins had also similar isoelectric points (about 4.6) and roughly similar apparent molecular weights as based on SDS gel electrophoresis (13 000 for eL12 from *Artemia*).

Protein synthesis

Endogenous activity of polyribosomes

In homogenates of dormant cysts polyribosomes are not clearly demonstrable by sedimentation analysis (Golub and Clegg, 1968 ; Hultin and Morris, 1968). However, mRNA translation experiments and poly(A)$^+$ RNA analyses suggest that a small number of ribosomes are mRNA-associated (Grosfeld and Littauer, 1975 ; Slegers and Kondo, 1977). The proportion of active polyribosomes increases with development. Although the development of *Artemia* cysts is inherently asynchronous, the translation pattern of isolated polyribosomes is clearly stage-dependent as analyzed by SDS polyacrylamide gel electrophoresis (Moens and Kondo, 1976). This is particularly striking in the case of hemoglobin-synthesizing polyribosomes. No hemoglobin is synthesized before hatching. During the early nauplius stage the synthesis of hemoglobins II and III account for approximately 20% of the total protein synthesis (Moens *et al.*, 1978ab). Hemoglobin-synthesizing polyribosomes (250-300 S) can be isolated by immuno-precipitation. Poly(A)$^+$ RNA from the isolated polyribosomes is capable of globin synthesis in a heterologous *in vitro* translation system (Moens *et al.*, 1978a).

Peptide chain initiation

Zasloff and Ochoa (1971, 1972, 1973, 1974) and McCroskey *et al.* (1972) purified a factor from the soluble fraction of *Artemia* cysts which stimulated the AUG-directed binding of bacterial fMet-tRNA, and the poly(U)-directed binding of N-acetyl-Phe-tRNA, to 40 S ribosomal subunits. GTP was not required. In the presence of 60 S subunits the bound acylaminoacids became puromycin reactive. Like the non-enzymic binding of fMet-tRNA to *Artemia* ribosomes (Zasloff, 1973) the factor-stimulated binding was inhibited by edeine. The factor in these studies was only functional in system with artificial messengers (Filipowicz *et al.*, 1976b).

Factor preparations active in the translation of physiological mRNA have been obtained from the cytosol and ribosomal 0.5 M KCl wash of developing embryos (Sierra *et al.*, 1974 ; Filipowicz *et al.*, 1976b). Ribosomal KCl wash from dormant cysts was reported to be inactive.

Of individual initiation factors eIF-2 has been studied in particular detail. This factor acts by forming a ternary initiation complex with GTP and Met-tRNA$_f^{Met}$. In the presence of 40 S ribosomal subunits a quaternary complex (eIF-2 . Met-tRNA$_f^{Met}$ · GTP · 40 S subunit) is formed. Under experimental conditions the two functions can be separated and analyzed sequentially (Levin *et al.*, 1973). The catalytic function of eIF-2 is greatly enhanced by an eIF-

2 stimulating factor, first prepared from reticulocyte ribosomal salt wash by Dasgupta *et al.* (1976).

Filipowicz *et al.* (1975) prepared eIF-2 from the cytosol and ribosomal KCl wash from developing embryos, and reported that the corresponding fractions from dormant cysts had much lower activity. However, ribosomes from unincubated cysts have been used for eIF-2 preparation (Warner *et al.*, 1980), and the whole cytoplasmic fractions of dormant and developing embryos have been found to contain about the same total eIF-2 activities (Hultin *et al.*, 1980). Initiation factor eIF-2 has been further purified by Ochiai-Yanagi and Mazumder (1976), and the nucleotide specificity of the ternary complex has been investigated. The interference of polynucleotides, including uncharged tRNA, with the functions of eIF-2 has also been studied (Ochiai-Yanagi *et al.*, 1977). De Haro *et al.* (1978) and Malathi and Mazumder (1978) prepared a factor, CO-eIF-2, from ribosomal salt wash from developing embryos, which enhanced eIF-2 dependent ternary complex formation in a similar way to the eIF-2 stimulating factor from rabbit reticulocytes described by Dasgupta *et al.* (1976).

A factor catalyzing GTP hydrolysis in the presence of isolated 40 S ribosomal subunits has been partially purified from ribosomal 0.5 M KCl wash by Mazumder (1975). The activity was inhibited by fusidic acid. The relation of this factor to peptide chain initiation is not clear (*cf.* Odom *et al.*, 1978).

Supernatant fractions from dormant and developing *Artemia* cysts transfer methyl groups from [^{3}H]methyl-S-adenosylmethionine to non-methylated reovirus mRNA (Muthukrishnan *et al.*, 1975). The methylation mainly involves the cap region. *Artemia* extracts translate unmethylated mRNA only after methylation, which can be achieved in the extracts by use of endogenous methyl donor(s). Filipowicz *et al.* (1976a) and Szczesna and Filipowicz (1977) prepared a cap-binding protein from the ribosomal KCl wash from developing embryos. They suggested that the cap-binding protein might facilitate the binding of cap-containing mRNA to the ribosomes.

The binding of globin mRNA to *Artemia* 40 S ribosomal subunits in the presence of reticulocyte initiation factors requires ATP in addition to GTP (Kramer *et al.*, 1976). The ATP analogue, Ado PP(CH_2)P, is inactive. The mRNA binding is not inhibited by edeine. However, edeine interacts with 40 S subunits and prevents the formation of the eIF-2 and GTP dependent quaternary initiation complex (Odom *et al.*, 1978). Further aspects of initiation complex formation using *Artemia* ribosomes in combination with reticulocyte initiation factors have been discussed by Nombela *et al.* (1975, 1976a).

Peptide chain elongation

The aminoacyl-tRNA synthetases of *Artemia* embryos do not seem to change in specificity during development (Bagshaw *et al.*, 1970).

Slobin and Möller (1975) found that elongation factor 1 in dormant cysts occurs mainly in a high molecular weight form (EF-1_H) which disaggregates during hatching. EF-1_H has a molecular weight of about 200 000. It contains three different polypeptide chains (A,B,C) and some lipid (Slobin and Möller, 1976ab ; Nombela *et al.*, 1976b). The low molecular weight form (EF-1_L) has an apparent molecular weight of 53 000, identical with that of subunit A in EF-1_H (Slobin and Möller, 1976a). Both forms are comparable in poly(U)-directed polyphenylalanine synthesis and Phe-tRNA binding to ribosomes as well as in ribosome-

dependent GTP hydrolysis (Slobin and Möller, 1976b). However, EF-1_L binds GTP and GDP more effectively than EF-1_H, and also differs from EF-1_H by forming a stable ternary complex with GTP and aminoacyl-tRNA (Slobin and Möller, 1976b). The interaction of EF-1 with ribosomes is inhibited by heparin (Slobin, 1976).

Elongation factor EF-1_H from *Artemia* cysts disaggregates *in vitro* under certain conditions *e.g.* under the influence of GTP or GDP, or by incubation with elastase or carboxypeptidase A (Nombela *et al.*, 1976b ; *cf.* Twardowski *et al.*, 1977). Reticulocyte EF-1_H is disaggregated by incubation with moderate, non-inactivating amounts of extracts or purified preparations from nauplii (cf. Twardowski *et al.*, 1976, 1977 ; Slobin and Möller, 1975).The disaggregating activity of these preparations was sensitive to the protease inhibitor, phenylmethylsulfonylfluoride. Since there is a marked increase in the protease content of the embryos after hatching (Osuna *et al.*, 1977a), the disaggregation of EF-1_H at the same developmental period may possibly be related to these enzymes. Whether the increase in EF-1_L in extracts from nauplii represents a true *in vivo* situation or results from limited proteolysis during preparation is therefore not perfectly clear at present (Twardowski *et al.*, 1977).

Nagata *et al.* (1976ab) observed that the stability of low molecular weight EF-1 was markedly enhanced by use of glycerol-containing buffers. They reported that under these conditions the light form of EF-1 occurred in homogenates from *Artemia* cysts as well as from other materials.

Nagata *et al.* (1976c) were able to resolve mammalian EF-1 into two complementary components, EF-1_α and EF-1_β, analogous to the bacterial elongation factors EF-T_u and EF-T_s. While EF-1_α was responsible for the GTP (or GuoPP(CH_2)P) dependent binding of Phe-tRNA to poly(U)-programmed ribosomes, the more stable EF-1_β enhanced the catalytic function of EF-1_α by stimulating the exchange of free GTP with the EF-1_α . GDP complex. EF-1_β was mainly associated with high molecular weight forms of EF-1 (Nagata *et al.*, 1976b). Slobin and Möller (1977, 1978) purified EF-1_β from EF-1_H preparations from *Artemia* cysts. It consisted of aggregated 26 000 dalton polypeptides identical with the C-chain of EF-1_H, and could form a functional complex with the complementary factor, EF-1_α (EF-1_L).

Experiments by Nombela and Ochoa (1973) indicated that *Artemia* elongation factors 1 and 2 interact with *Artemia* ribosomes at alternating steps of chain elongation, *i.e.* with more or less overlapping ribosomal sites. An alternative model for the function of EF-1 in peptide chain elongation was suggested by Grasmuk *et al.* (1976). According to this model EF-1-bound aminoacyl-tRNA (rather than EF-1 proper) competes with EF-2 for a ribosomal binding site. EF-1 would remain bound to the ribosome during peptide chain elongation, being repeatedly recharged *in situ* with aminoacyl-tRNA and GTP. The model was essentially based on analyses of the binding of [^{3}H]EF-1 to ribosomes under various conditions. With the recognition of EF-1_β as an EF-1 . GTP regenerating factor (Nagata *et al.*, 1976c) it became questionable whether the experimental data behind Grasmuk's model reflected the integrated, cyclic function of EF-1 in continual peptide elongation. To elucidate this point, the ability of EF-1_β from *Artemia* to release bound [^{3}H]EF-1 from mouse tumor ribosomes was investigated by Grasmuk *et al.* (1977). No releasing activity was observed. However, comparable experiments with a homologous *Artemia* system (Roobol and Möller, 1978ab) indicated that the aminoacyl-tRNA independent (uncoupled) binding of [^{3}H]EF-1_L to ribosomes was significantly reduced in the presence of EF-1_β. With GuoPP(CH_2)P as nucleotide component there was a nearly stoechiometric aminoacyl-tRNA-coupled [^{3}H]EF-1_L binding in this system.

With GTP no coupled binding was observed, apparently because the aminoacyl-tRNA-transferring EF-1 had been detached after GTP hydrolysis. The authors concluded that, in the presence of EF-1_{β}, EF-1_{α} acted in a catalytic fashion and that its interactions with the aminoacyl-tRNA accepting ribosomes had a transient character.

Peptide chain termination

Reddington *et al.* (1978) reported that an enzyme fraction (S-100) from dormant *Artemia* cysts had the same activity as a comparable fraction from developed embryos in catalyzing the release of terminated globin chains from reticulocyte polyribosomes. Recently, Reddington and Tate (1979) have purified a release factor from the S-100 fraction. The factor released fMet from ribosome-bound fMet-$tRNA_f^{Met}$ in response either to poly(U,G,A), which contains all termination codons, or to termination codon-containing tetranucleotides.

Control of translation

Inhibition of translation

The remarkable physiological properties of dormant and newly activated *Artemia* embryos, and their low ribonuclease contents (Urbani and Bellini, 1958; Lee-Huang *et al.*, 1977; Sebastián and Heredia, 1978) have encouraged the use of this material for studies of endogenous translation inhibitors.

Lee-Huang *et al.* (1977) prepared an oligonucleotide from the 12% trichloroacetic acid extract of heat-denatured supernatant fractions, which strongly inhibited the translation of artificial and physiological messengers. Both initiation and peptide elongation were affected. The purified inhibitor had an apparent molecular weight of 6 000, corresponding to a chain length of about 20 nucleotides. It was rich in pyrimidines, particularly uracil, and was resistant to ribonuclease T_1. The inhibitor was also found in ribosomal salt wash, and occurred in both dormant and developing embryos.

Slegers and Kondo (1977) reported that poly(A)$^+$ RNP particles from the postmitochondrial fraction of dormant cysts contained a polynucleotide, which could be separated from the poly(A)$^+$ RNA by chromatography on Sepharose-poly(U) in the presence of EDTA. The polynucleotide strongly inhibited the translation of poly(A)$^+$ RNA in the wheat germ system.

According to Ochoa (1977) and Sierra *et al.* (1977) protein chain initiation in *Artemia* is susceptible to inhibition by an eIF-2 phosphorylating protein kinase, analogous to the hemin-controlled repressor of rabbit reticulocytes. The eIF-2 kinase itself could be activated by phosphorylation by means of a cAMP-dependent protein kinase in the same preparation. The phosphorylated eIF-2 was still capable of forming a GTP-dependent ternary initiation complex *in vitro*, and of binding this complex to 40 S ribosomal subunits (section "Protein synthesis, Peptide chain initiation"). However, the reaction was no longer catalyzed by the complementary eIF-2 stimulating factor (De Haro *et al.*, 1978).

Warner *et al.* (1977) purified an elongation-inhibiting protein (M_r 130 000) from the cytosol and ribosomal fraction of *Artemia* cysts. A 75% inhibition of poly(U)-directed polyphenylalanine synthesis was obtained. Translation inhibitors from the supernatant fraction have also been reported by Felicetti *et al.* (1977).

Artemia ribosomes have been used in translation systems for analyzing the mechanisms of action of heterogenous initiation and elongation inhibitors (Cimadevilla *et al.*, 1975 ; Irvin, 1975 ; Suits and Irvin, 1976 ; Dallal and Irvin, 1978).

Translation activators

Lee-Huang *et al.* (1977) prepared a polynucleotide (M_r 9 000) from the heat-denatured supernatant and ribosomal salt wash fractions of developing cysts which complexed with the uracil-rich oligonucleotide inhibitor and inactivated it. The polynucleotide activator was rich in guanine and resistant to ribonuclease A.

RNA and protein metabolism in relation to cryptobiosis and differentiation

Induction of dormancy

The cryptobiotic phase in the development of *Artemia* is induced after a period of active development, a situation analogous to that in plant seeds and avian blastoderms. The encystment takes place at the early gastrula stage, apparently after the determination of the main embryonic regions (Fautrez-Firlefyn, 1951 ; Benesch, 1969). This suggests that the latent mRNA in the cysts is at least in part non-maternal, in contrast to the situation in the sea urchin egg (Hultin, 1961a ; Gross and Cousineau, 1963). It would be of interest to identify the postulated embryo-transcribed mRNA and to determine its intracellular distribution in the cysts. Despite the restricted permeability of the ovisac, this could possibly be done by RNA labelling *in vivo* during the early cleavage stages, followed by recovery of labelled poly(A)$^+$ RNA from different cell fractions after encystment. There is evidence that some of the latent mRNA in dormant cysts is ribosome-associated (Grosfeld and Littauer, 1975 ; Slegers and Kondo, 1977). This material may represent previously active, disaggregated polyribosomes. The structural complexity of the membrane-bound poly(A)$^+$ RNA-containing particles (Hultin *et al.*, 1977 ; Lake and Hultin, 1978) suggests that these structures contain sequestered mRNA with different physiological background, although the translation patterns are essentially similar (Sierra *et al.*, 1976 ; Grosfeld *et al.*, 1977 ; Hultin *et al.*, 1977).

The translation-blocking oligonucleotides in the encysted embryos (Lee-Huang *et al.*, 1977 ; Slegers *et al.*, 1977), may be involved in the induction of dormancy as inhibitors of protein synthesis. A model of their mode of action and of the reversal of inhibition by an activator oligonucleotide has been suggested by Lee-Huang *et al.*, 1977.

Several other mechanisms have been proposed or are conceivable for explaining the almost complete polyribosome disaggregation in the dormant cyst. The cysts contain large amounts of diguanosine nucleotides, including the cap-resembling diguanosinetriphosphate, G5'ppp5'G, synthesized during the cleavage stages prior to encystment (Warner and McClean, 1968). By methylation in the N-7 position (*cf.* Muthukrishnan *et al.*, 1975) this compound would be transformed into a potent initiation inhibitor (Groner *et al.*, 1976). Whether or not such methylation occurs is not known.

The dormant cysts have morphologically degenerate and functionally inefficient mitochondria (Schmitt *et al.*, 1973), and their ATP content is correspondingly low (Warner and Finamore, 1967). Since energy depletion may lead to a rapid, reversible polyribosome disaggregation (Hultin, 1964), this may be an immediate cause of monoribosome ac-

cumulation during the induction of dormancy. In respect to ATP content *Artemia* cysts differ strikingly from sea urchin eggs, which have a rich supply of nucleoside triphosphates (Hultin, 1957). Nevertheless, the activation of protein synthesis in newly fertilized sea urchin eggs fails to occur as long as mitochondrial oxidative phosphorylation is blocked by inhibitors (Hultin, 1964). By analogy, the resumption of mitochondrial functions in rehydrated *Artemia* cysts (Schmitt *et al.*, 1973) may be a precondition of polyribosome formation irrespective of other pathways of nucleoside triphosphate generation (Van Denbos and Finamore, 1974).

Preservation of macromolecules

The remarkable stability of easily inactivated molecules in the dormant cysts raises another set of interesting problems. In the case of mRNA the oligonucleotides reported to be associated with poly(A)$^+$ RNA (Slegers *et al.*, 1977) may not only have an inhibitory but also a stabilizing function. Further protection of the latent mRNA may be provided by proteins. The protein complement of the membrane-bound poly(A)$^+$ RNA-containing particles differs markedly from that of mRNP particles isolated from active polyribosomes (Lake and Hultin, 1978).

Proteins may also participate in the stabilization of easily inactivated enzymes during cryptobiosis, as suggested by Slobin and Möller (1976a) for EF-1_H. The remarkable resistance of the nuclear RNA-synthesizing system against repeated desiccation of the developing cysts has been studied by de Chaffoy and Kondo (1976). The recent work of D'Alessio and Bagshaw (1979) is of interest in this context. They were able to purify a 15 S protein complex from the sonicated nuclei of dormant cysts, which specifically stimulated RNA polymerase II. Although the physiological function of the protein complex remains to be elucidated, it is notable that it gradually disappeared during the pre-emergence development. It may therefore have some relationship to the cryptobiotic state, including the resistance of the RNA-synthesizing system to desiccation. The possible role of glycerol (Clegg, 1964) as a protective agent for nuclear activity against dehydration has been discussed by de Chaffoy and Kondo (1976). The efficiency of glycerol as a stabilizer of EF-1 was demonstrated *in vitro* by Nagata *et al.* (1976ab).

Gene expression

During the further development of the rehydrated embryos there are modifications in the mRNA and protein patterns which may reflect stage-specific alterations in gene expression (Grosfeld and Littauer, 1976 ; Grosfeld *et al.*, 1977). The most striking alterations appear during hatching. They probably represent the manifest final stages of far in advance predetermined sequences of cell differentiation. The new proteins synthesized by the early nauplii include hemoglobins and various hydrolases, some of which may have digestive functions (Moens and Kondo, 1976 ; Osuna *et al.*, 1977a ; Miralles *et al.*, 1978 ; Sebastián and Heredia, 1978). It is of interest from the point of view of membrane differentiation that a number of specific, membrane-associated enzymes increase during the same period (Peterson *et al.*, 1978). These include the ($Na^+ + K^+$)-activated ATPase of the salt secretory cells, which are of vital importance for the survival of the larvae (Ewing *et al.*, 1972).

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The biosynthesis, metabolism and function of dinucleoside polyphosphates in *Artemia* embryos : a compendium

A. H. Warner

Department of Biology, University of Windsor
Windsor, Ontario, Canada N9B 3P4

Content

Introduction

The dinucleoside polyphosphates in *Artemia* embryos have received considerable attention recently and they have become the subject of extensive reviews by Warner (1979), Silverman and Atherly (1979) and Clegg and Conte (1980). The primary purpose of this paper is to summarize the salient aspects of dinucleoside polyphosphate metabolism in *Artemia* embryos to provide the readers of this first comprehensive treatise on *Artemia* with an overview of the biochemistry and physiology of these compounds during *Artemia* development. The reviews cited above and references given herein should be consulted for methodology and additional details on this subject.

The content and distribution of dinucleoside compounds in *Artemia*

At the present time four dinucleoside compounds have been isolated from *Artemia* cysts and/or developing embryos and their concentration and distribution within the embryo have been studied (Finamore and Warner, 1963 ; Warner and Finamore, 1965a ; Gilmour and Warner, 1978). Of these compounds the most abundant are guanosine 5′-tetraphospho-5′-guanosine (Gp_4G) and guanosine 5′-triphospho-5′-guanosine (Gp_3G) which comprise about 1.8% and 0.18%, respectively, of the dormant cyst dry weight. The least abundant dinucleoside compounds are guanosine 5′-diphospho-5′-guanosine (Gp_2G) and guanosine 5′-triphospho-5′-adenosine (Gp_3A) and they represent about 0.013% and 0.008%, respectively, of the cyst dry weight (Warner, 1979).

In *Artemia* these nucleotides have been found in embryonic tissues only ; none has been found in somatic tissue of adult male or female brine shrimp (Warner and McClean, 1968). In the encysted gastrula of *Artemia* Gp_2G, Gp_3G and Gp_4G are localized primarily in the yolk platelets, whereas Gp_3A is found exclusively in the post-mitochondrial supernatant fraction (Warner *et al.*, 1972 ; Warner, 1979).

Biosynthesis and metabolism of Gp_3G and Gp_4G in *Artemia* embryos

The biosynthesis of Gp_3G and Gp_4G is catalyzed by the enzyme GTP : GTP guanylyltransferase or Gp_4G synthetase (Warner *et al.*, 1974 ; Warner and Huang, 1974). In dormant cysts about 80% of the Gp_4G synthetase is firmly associated with the yolk platelets, whereas the remainder is present in the post-ribosomal fraction. Chromatographic analysis of Gp_4G synthetase from both fractions indicates them to be identical. The yolk platelet associated enzyme has been purified about 20-fold and studied *in vitro* using various radiolabeled guanosine nucleotides. These studies have shown unequivocally that Gp_4G and Gp_3G are synthesized in *Artemia* embryos according to equations 1 and 2, respectively.

$$(1)\quad 2GTP \rightleftharpoons Gp_4G + PP_i$$
$$(2)\quad GDP + Gp_4G \rightleftharpoons Gp_3G + GTP$$

During the *in vitro* studies it was observed that the ratio of rates of synthesis of Gp_4G to Gp_3G *in vitro* is similar to their relative concentration in isolated yolk platelets (about 10 to 1). Notwithstanding the above, these data are difficult to interpret in view of the fact that the initiation of Gp_3G and Gp_4G synthesis is separated temporally in *Artemia*. Thus Gp_4G synthesis occurs in the developing oocyte during vitellogenesis whereas Gp_3G synthesis occurs in the embryo sometime between fertilization and the onset of dormancy (Warner and McClean, 1968).

During development of *Artemia* Gp_4G utilization appears to be under the control of at least three enzyme-catalyzed reactions. One catabolic reaction is believed to occur within the yolk platelets whereas the others occur in the cytosol. The scheme shown in Fig. 1 has been proposed to describe the biosynthesis and metabolism of Gp_3G and Gp_4G in *Artemia*. Although additional data are needed on the role of Gp_4G synthetase in *Artemia*, results from this laboratory (both published and unpublished) are consistent with the view that this enzyme is active in the utilization of Gp_4G in both the yolk platelets and cytosol during prenaupliar development. During early development yolk platelet-associated Gp_4G is utilized (in yolk platelets) in accordance with equation 2 and as shown in Fig. 1. This process appears to be regulated in part by the GDP concentration in the cytosol and yolk platelets. As the GDP level increases yolk platelet-associated Gp_4G is utilized to give yolk platelet-associated Gp_3G and GTP ; Gp_3G then appears to serve as a "shuttle" for the movement of high-energy phosphate and guanosine moieties from the yolk platelets to the cytosol. The GTP generated in this reaction is thought to be conserved within the yolk platelets and converted back to Gp_4G as shown in equation 1. Once in the cytosol Gp_3G is metabolized to generate cytosol Gp_4G. Cytosol Gp_4G may then be utilized to provide two equivalents of GTP as described in equation 1 or hydrolyzed asymmetrically to equimolar amounts of GMP and GTP. This latter reaction is catalyzed by the enzyme Gp_4G guanylohydrolase which is present exclusively in the post-ribosomal fraction (Warner and Finamore, 1965 ; Vallejo *et al.*, 1974). Thus far no other

enzymes (except those indicated above) have been described or isolated from *Artemia* embryos which are active in the metabolism of the dinucleoside compounds. However, some investigators have proposed that *Artemia* nauplii utilize Gp_4G in other reactions leading to the synthesis of ATP and dATP (Finamore and Clegg, 1969 ; Van Denbos and Finamore, 1974). These proposals were based on radioisotope incorporation data only and their confirmation will require additional experimentation.

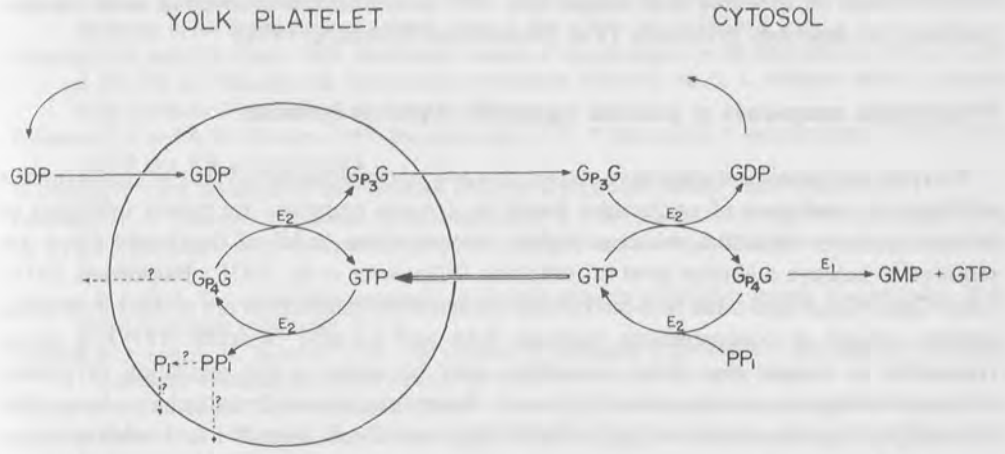
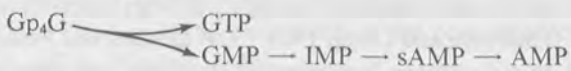


FIG. 1. Proposed cycle for the biosynthesis and metabolism of Gp_4G and Gp_3G in *Artemia* prior to hatching. E_1 and E_2 are Gp_4G guanylohydrolase and Gp_4G synthetase, respectively. From Warner (1979).

Gp_4G as the source of purines for development

In the dormant encysted embryo of *Artemia* Gp_4G accounts for about 55% of the acid-soluble purine-containing nucleotides (Warner and Finamore, 1967 ; Warner, 1979). Following termination of dormancy, Gp_4G utilization commences at a rate of 0.2-0.4 pmol/h/embryo then increases to 1.3-1.8 pmol/h/embryo following hatching (Warner, 1979). In developing nauplii there is a marked increase in the concentration of ATP, and since *Artemia* embryos are unable to synthesize purines *de novo*, Clegg and his coworkers (1967) have suggested that the appearance of ATP is closely linked to the utilization of Gp_4G. This view is supported by the fact that the total purine content of *Artemia* embryos remains constant for at least 4-5 days following the termination of dormancy. The constant level of purines in *Artemia* embryos during development is due primarily to the utilization of Gp_4G for the synthesis of other purine-containing nucleotides. The major unresolved problem concerns the mechanism by which Gp_4G is utilized for the synthesis of ATP and dATP. From the work of Renart *et al.* (1976) who demonstrated the presence of GMP reductase in *Artemia* embryos, Gilmour (1978) who provided evidence for the presence of IMP and adenylosuccinic acid (sAMP) in *Artemia*, Warner and Finamore (1965) who identified Gp_4G guanylohydrolase and Finamore and Clegg (1969) who studied the radioisotope labeling pattern of various

nucleotides in *Artemia* embryos, it appears certain that cytosol Gp_4G is converted to AMP according to the following scheme.



Although this pathway appears to be operational in the cytosol of *Artemia* embryos, it may not be active or utilized in these embryos for the synthesis of ATP (Finamore and Clegg, 1969). In fact, all available data suggest that ATP is synthesized in *Artemia* embryos by a pathway not described previously (Van Denbos and Finamore, 1974).

Dinucleoside compounds as potential regulators of protein synthesis

Previous biochemical studies have shown that low concentrations (μM) of $m^7GpppGm$ and $m^7GpppAm$, analogues of nucleotides found in *Artemia* embryos, are potent inhibitors of protein synthesis initiation, whereas higher concentrations (mM) of Gp_3G and Gp_3A are required to achieve a similar level of inhibition (Filipowicz *et al.*, 1976 ; Filipowicz, 1978). Since Gp_3G, Gp_3A and other non-methylated dinucleoside compounds are present in *Artemia* embryo cytosol at concentrations between 0.04 and 1.2 mM (Warner, 1979), it seems reasonable to suggest that these nucleotides may be active in the regulation of protein synthesis during early development in *Artemia*. Recent experimental results have shown that physiological concentrations of Gp_2G, Gp_3G, Gp_3A and Gp_4G (from 0.1 to 1 mM) stimulate the rate of polypeptide chain elongation on preformed polysomes, and that Gp_2G and Gp_3A are the most active of those dinucleoside compounds studied (Warner, 1979). Furthermore, the stimulatory effect of these compounds was found to be greater at 3.4 mM $MgCl_2$ than at 8.1 mM $MgCl_2$ concentrations.

In contrast to the above results, we have observed that the dinucleoside compounds as a group inhibit the overall rate of aminoacylation of tRNA *in vitro* under otherwise physiological conditions. However, the effect of these nucleotides on individual aminoacylation reactions is complex and additional work will be needed to elucidate their role in regulating aminoacylation of various tRNA's (Warner, 1979).

Although methylated nucleotides have not been detected in acid extracts of *Artemia* embryos, it has now been established that the post-ribosomal fraction from *Artemia* cysts has the capacity to catalyze the methylation of the dinucleoside compounds found in *Artemia* embryos (Warner, 1979). These findings together with those presented above support the view that the dinucleoside compounds in *Artemia* embryos, especially Gp_3G and Gp_3A, play an important role in the regulation of protein synthesis in *Artemia* embryos following the termination of dormancy.

Conclusions

Although diguanosine tetraphosphate (Gp_4G) appears to function mainly as a source of purines and high energy phosphate for early development in *Artemia* and other Anostraca, other dinucleoside compounds such as Gp_2G, Gp_3G and Gp_3A appear to serve mainly as regulatory molecules in nucleotide metabolism and/or protein synthesis. However, additional

work must be undertaken to clearly establish the role of these unusual nucleotides in macromolecular processes.

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PAPERS

Influence of environmental factors on the metabolism of cysts and larvae

The Brine Shrimp Artemia. *1970. Vol. 2. Physiology, Biochemistry, Molecular Biology*
G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 664 p.

Freezing tolerance in larval *Artemia*

John G. Baust[1] *and Addison L. Lawrence*[2]

[1] *Department of Biology, University of Houston*
Houston, Texas 77004, USA

[2] *Texas Agricultural Experiment Station*
P.O. Box 10608, Corpus Christi, Texas 78410, USA
and
Department of Wildlife and Fisheries Science, Texas A & M University
Port Aransas, Texas 78373, USA

Abstract

Preliminary aspects of freezing tolerance in *Artemia* larvae were determined in the presence of single and dual cryoprotectant solutions. Synchronous cultures of larvae were harvested at post-hatch intervals (0, 4, 8, 12, and 24 hr) and instantaneously exposed to respective protectants for 20 min at -1 °C. Following seeding with ice nuclei, specimens were frozen to -30 °C at differential rates ranging between 0.1 and 10 °C/min. Freezing tolerance was decidedly age-specific. 0-4 hr post-hatch larvae were equivalently freeze-tolerant under optimal conditions. Survival precipitously decreased in older nauplii. A freezing rate of 0.3 °C/min was optimal in the presence of 2-4 M glycerol. Freezing in the presence of dual cryoprotectants (glycerol-sucrose) or hypersaline solutions resulted in diminished survival. These results are discussed in light of the development of a successful cryogenic "seed stock" banking procedure for mariculture.

Introduction

The successful development of various mariculture programs will require extensive and integrated knowledge of each species nutritional physiology, behavior and population dynamics under high density conditions. Prior to the acquisition of appropriate information, the prime requisite of species (seed stock) availability cannot be overlooked. Both commercial and research-oriented shrimp mariculture programs are handicapped by their dependency on the acquisition of zygotes from wild caught, gravid females. The problems with sourcing are multiple and include high cost-benefit ratios, unpredictability, restricted season and limited natural ranges of ideal species. On-site maturation programs should minimize the dependency on sourcing and provide a locally reliable seed stock source. However, maturation programs alone may only provide local benefits. The success of such programs demand a more extensive knowledge of the integrative biology of each species, will require costly facilities and specialized personnel.

The maximization of shrimp mariculture technology will be accomplished when select species of nauplii are made available year round and in a form tolerant to long distance shipment. A zygote or nauplii "bank" modeled after mammalian sperm, embryo and blood

banks (Silbert, 1972 ; Smith 1961 ; Moore and Bilton, 1977 ; Whittingham *et al.*, 1972), will provide the desirable link between sourcing, maturation and grow-out facilities.

Preliminary attempts to induce freezing tolerance in marine crustaceans have been reported (Baust and Lawrence, 1977). This study demonstrated that various Penaeid species could survive exposure to -196 °C. However, the limited availability of these species coupled with their stressed condition following long shipment intervals at sub-optimal temperatures led to the selection of *Artemia* as a model system. The development of a banking program is multi-faceted. First, a pre-freeze, chill tolerance must be demonstrated in the presence of molar levels of cryoprotective agents. Baust and Lawrence (1980) have described the survival of various aged nauplii following -1 °C exposure in the presence of multi-molar levels of glycerol, glucose, sucrose, DMSO and glycerol-sucrose combinations. Second, the parameters of solid phase (ice) encounters must be demonstrated. These include freezing rates, hold and storage temperatures, cryoprotectant type and concentration and thawing conditions. This study provides a preliminary report of freezing tolerance in *Artemia*.

Materials and methods

A single source of *Artemia* (San Francisco Bay) was used throughout this study. Synchronous cultures of nauplii were obtained by hatching cysts in 40‰ artificial seawater at 28 °C (20 nauplii/ml). Specimens were harvested following 24-48 hr to yield 0, 4, 8, 12, and 24 hr post-hatch nauplii. Experimental animals were concentrated following passage through a series of light trap baffels thereby eliminating weak swimmers and unhatched cysts. Nauplii were harvested by filtration through appropriate sized screen and instantly rinsed into a -1 °C bath containing one of the potentially cryoprotective agents. Following 20 min incubation, a 2 ml sample was inoculated with ice and frozen in a rate programmable low temperature bath to -30 °C. Specimens were frozen at rates between 0.1 and 10°/min. Samples were removed at each 2 °C interval between -10° and -30 °C Following thawing at a constant rate, specimens were again filtered and rinsed into 50 ml flasks containing 40‰ artificial seawater at 25 °C. Flasks were lightly aerated for 24 hr prior to evaluation. Each recovery flask contained 100-150 nauplii. Nauplii were judged to survive if they were positively phototactic and strong swimmers. Weak or intermittent swimmers were judged "dead".

Results

Pre-freeze survival

Fig. 1 illustrates the pre-freeze, dual controls used in the experimental protocol. Over the 24 hr experimental period, 25 °C controls maintained a mean survival rate of 98 %. Exposure to -1 °C without cryoprotectants was harmful to all age groups. 0 hr post hatch nauplii were most sensitive to chilling with survival decreasing to 35 % after 90 min. Older nauplii were nearly twice as tolerant to prolonged chilling as were 0 hr posthatch specimens. Baust and Lawrence (1980) have reported on the enhancement of chill tolerance in this species when incubated with glycerol and glycerol-sucrose combinations at -1 °C as compared to cryoprotectant-free conditions. Fig. 2 is a composite summary of *Artemia* age dependent survival responses in the presence of various cryoprotectants following up to 90 min incubation at

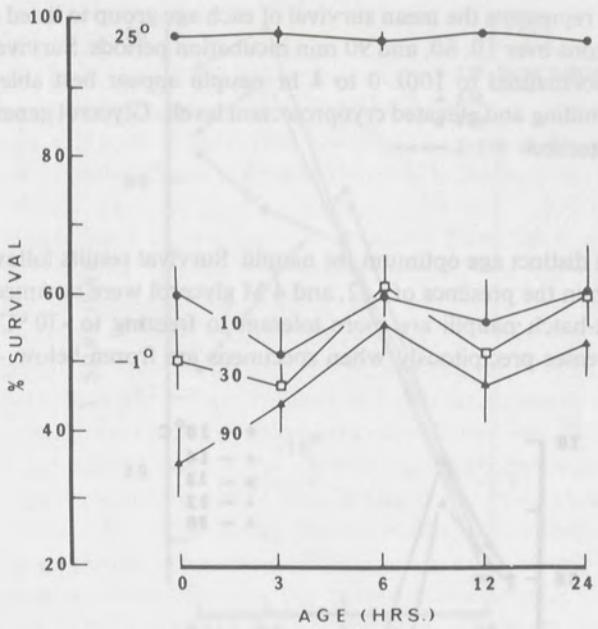

FIG. 1. Percent survival of posthatch larvae of *Artemia* maintained at 25 °C and -1 °C (± SEM).

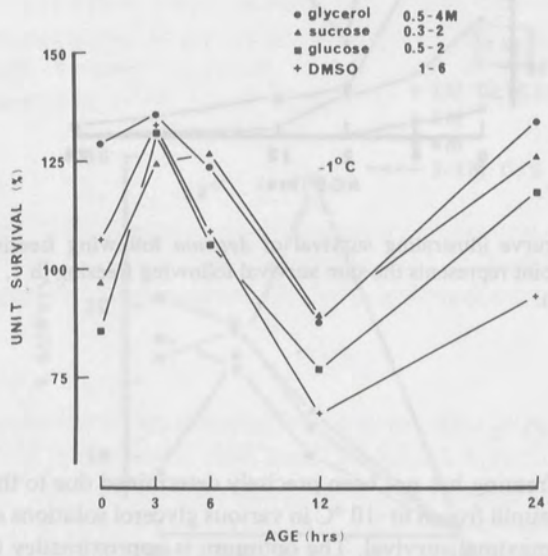

FIG. 2. Unit survival curve illustrating *Artemia* survival enhancement by various cryoprotectant types. Each data point represents the sum survival after 10, 30, and 90 min exposure at -1 °C in the presence of the indicated cryoprotectants.

-1 °C. Each data point represents the mean survival of each age group to listed cryoprotectants and varied concentrations over 10, 60, and 90 min incubation periods. Survival is indicated as percent of controls (normalized to 100). 0 to 4 hr nauplii appear best able to tolerate the combined stresses of chilling and elevated cryoprotectant levels. Glycerol generally affords the greatest degree of protection.

Age optimum

Fig. 3 illustrates the distinct age optimum for nauplii. Survival results following freezing at 0.1, 0.3, and 1 °C/min in the presence of 1, 2, and 4 M glycerol were summed for illustrative purposes. 0-4 hr post-hatch nauplii are more tolerant to freezing to -30 °C than are older groups. Lethality increases precipitously when specimens are frozen below -14 °C.

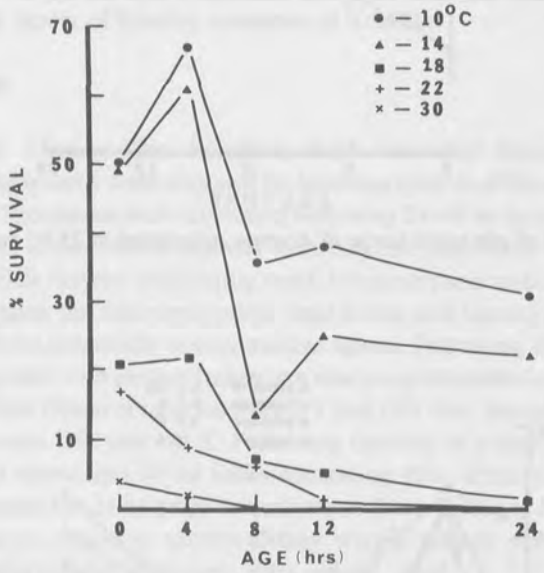

Fig. 3. Unit survival curve illustrating survival of *Artemia* following freezing to the indicated temperatures. Each data point represents the sum survival following freezing in 1, 2, and 4 M glycerol at 0.1, 0.3 and 1.0 °C/min.

Freezing rate optimum

An optimum rate of freezing has not been precisely determined due to the unusually sharp optimum (Fig. 4). 4 hr nauplii frozen to -10 °C in various glycerol solutions appear to require a single freezing rate for maximal survival. The optimum is approximatley 0.3 °C/min and is independent of cryoprotectant concentration. The optimum does however appear to be cryoprotectant-type dependent. When the identical freezing procedure is repeated in the presence of a 3-1 M glycerol-sucrose solution, the optimum clearly shifts to 1.0 °C/min (Fig. 5).

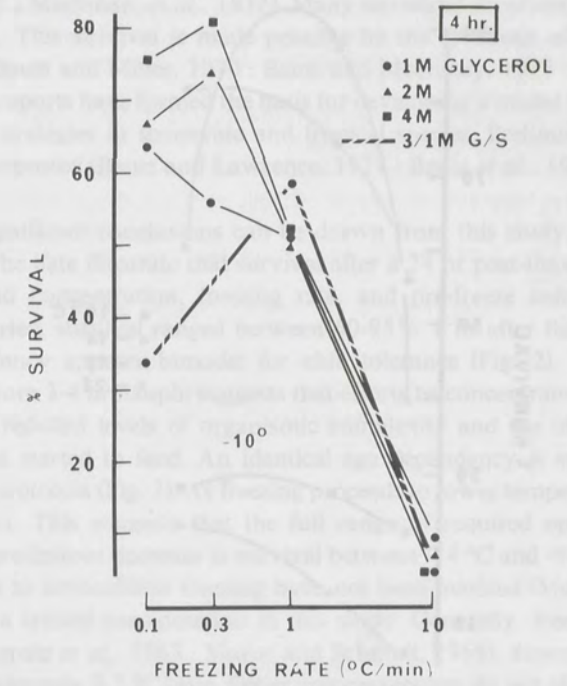

FIG. 4. Freezing rate curves for *Artemia* (4 hr post-hatch nauplii) frozen to -10 °C.

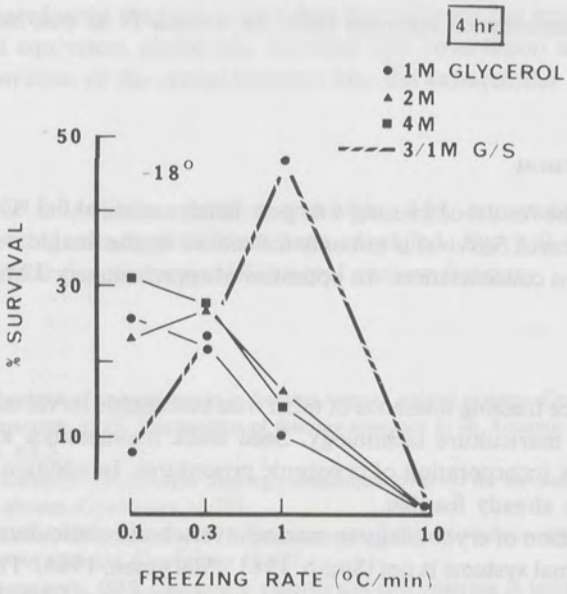

FIG. 5. Freezing rate curves for *Artemia* (4 hr post-hatch nauplii) frozen to -18 °C.

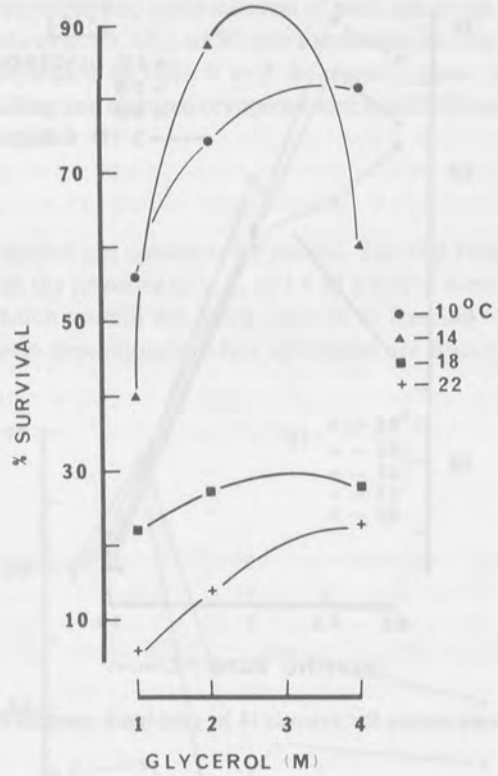

FIG. 6. Glycerol concentration optimum curve for *Artemia* (4 hr post-hatch nauplii) frozen at 0.30 °C/min.

CONCENTRATION OPTIMUM

Fig. 6 illustrates the results of freezing 4 hr post-hatch nauplii at 0.3 °C/min in the presence of 1, 2, and 4 M glycerol. Survival is not only influenced by the final low temperature but also by the cryoprotectant concentration. An optimum of approximately 3 M glycerol is indicated.

Discussion

Attempts to induce freezing tolerance in otherwise susceptible larval marine invertebrates is a novel adjunct to mariculture technology. Seed stock maintenance and transport can be greatly facilitated by incorporation of cryogenic procedures. In addition, the maintenance of live food supplies is already feasible.

While the application of cryobiology to marine invertebrate mariculture is new, the maintenance of frozen animal systems is not (Smith, 1961 ; Meryman, 1966). The role of cryoprotective agents such as glycerol, low molecular weight carbohydrates, DMSO and others has been well documented (Mazur, 1970). The protective mechanisms are, however, unknown (Baust,

1973 ; Mazur, 1977 ; Meryman, *et al.*, 1977). Many terrestrial invertebrates naturally endure prolonged freezing. This survival is made possible by the synthesis of high levels of cryoprotective agents (Baust and Miller, 1970 ; Baust and Morrissey, 1977 ; Baust and Edwards, 1979). These latter reports have formed the basis for developing a model for inducing chill and freezing tolerance strategies in temperate and tropical species. Preliminary aspects of these studies have been reported (Baust and Lawrence, 1977 ; Baust *et al.*, 1978 ; Lawrence *et al.*, 1978).

A number of significant conclusions can be drawn from this study. *Artemia* nauplii are freezing tolerant. The data illustrate that survival after a 24 hr post-thaw period is age, cryoprotectant type and concentration, freezing rate, and pre-freeze conditioning dependent. Although not reported, survival ranged between 60-95% 1 hr after thawing.

The age dependency appears bimodal for chill tolerance (Fig. 2). However, the sharp optimum derived from 3-4 hr nauplii suggests that efforts be concentrated in this range due to the comparatively reduced levels of organismic complexity and the observation that these stages have not yet started to feed. An identical age dependency is suggested following a variety of freezing protocols (Fig. 3). As freezing proceeds to lower temperatures, the optimum became less distinct. This suggests that the full range of required optima have yet to be defined. Also, the precipitous decrease in survival between -14 °C and -18 °C suggests that the problems attendent to intracellular freezing have not been avoided (Mazur, 1977).

Freezing rate is a critical consideration in this study. Generally, freezing rate optima are relatively broad (Rapatz *et al.*, 1963 ; Mazur and Schmidt, 1968). *Artemia* requires a distinct optimum of approximately 0.3 °C/min. Other cryoprotectants do not afford protection equal to glycerol alone at the same osmolality. Glycerol-sucrose combinations do however shift the optimum to a high rate (1 °C/min) (Fig. 4 and 5).

It would be too speculative at this time to attempt a mechanistic evaluation of the results. It may be suggested based upon the preliminary data that differing but equiosmolar cryoprotectants do not afford equivalent protection. Whether this observation is due to biochemical parameters or penetration of the cryoprotectants into the extracellular milieu is unknown.

Summary

Freezing tolerance in *Artemia* nauplii is inducible. Age, cryoprotectant type and concentration, and freezing rate optima have been identified. Survival is maximized in the presence of multi-molar concentrations of glycerol at slow freezing.

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Comparison of *Artemia* strains : survival and growth of nauplii as a function of ionic composition, osmosity and temperature of the medium

Nicholas C. Collins

Department of Zoology and Erindale College, University of Toronto
3359 Mississauga Road, Mississauga, Ontario, L5L-1C6, Canada

Abstract

Median survival times of a variety of North American *Artemia* strains were measured when newly hatched larvae were placed without food in source waters of other strains. Toxicity was assessed as one measure of the barriers to natural genetic interchange among populations. In a second study, naupliar growth rates of seven strains were measured in their own source water under a range of temperatures (15-22 °C) and osmosities (0.25-1.88 S), to assess the degree of differential adaptation to local conditions.

For the toxicity tests sources with seasonally variable concentrations were diluted to osmosities of 0.18-0.5 S, where cysts from all sources hatch well. Waters from the Great Salt Lake and Mono Lake, whose concentrations are much more constant, were not diluted. Carbonate sources from Nebraska and Mono Lake waters were strongly toxic to all other strains tested. Chloride-carbonate waters from Arizona were strongly toxic to sulphate strains from Washington and Saskatchewan, and had milder negative effects on carbonate strains and chloride strains from New Mexico and Utah. Chloride waters from these latter sites reduced survival times of non-chloride strains by 25-80%, depending directly on the osmosity. Water from a sulphate source in Washington was the most consistently suitable medium, producing no significant reductions in survival time for any strain tested. Interestingly, the Washington strain's nauplii were the most sensitive to changes in ionic composition ; the carbonate strains from Nebraska were least sensitive. The Mono Lake strain was not tested.

For the 5-day naupliar growth rate measurements, a *Chlamydomonas-Ankistrodesmus* powder was used as food. The pattern of response of each strain to different source concentrations could be only partially predicted from knowledge of the normal concentration of the source lake. As expected, the Nebraska strains, from dilute sources, showed reductions in growth rate as concentrations were increased, and strains with intermediate source concentrations (Arizona) showed peak growth at intermediate concentrations. However, growth rates of strains adapted to highly concentrated NaCl sources in Utah and New Mexico were less responsive to the concentration range than the other strains. At room temperature they grew faster than most other strains, even at low osmosities ; at high osmosities they always had higher growth rates than the other strains. For other strains and concentrations, the ranking of the growth rates depended on the temperature. Thus, it appears that the nauplii of each strain are adapted to grow at a particular combination of temperature and osmosity.

Details of this work will be published in the near future.

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Aerobic and anaerobic metabolism of *Artemia* nauplii as a function of salinity

Frank P. Conte, Joel Lowry, John Carpenter,
Ann Edwards, Richard Smith, and Richard D. Ewing

Department of Zoology, Oregon State University,
Corvallis, Oregon 97331, USA

Abstract

The recent finding of decreased levels of ATP in nauplii living in concentrated NaCl brines initiated our investigation of the role that aerobic and anaerobic metabolism have in the regulation on ion transport by the larval salt gland. The reduction in the ATP pool occurred despite the fact that naupliar oxygen consumption was high (>10 μl/hr/mg protein) and independent of the salinity gradient. Results from the present study show ion transport to be primarily coupled to metabolic mechanisms which regulate the rate of glycolosis and not upon oxidative phosphorylation. Lactate acid production is slightly stimulated by the steep NaCl gradient and does not account for the major part of glycogen breackdown. Acidification of the external medium, either by lactic acid transport or other acid sources, is salt-dependent. Addition of ouabain to the external medium inhibits glycolysis but lactic acid production remains unaffected.

Introduction

The osmotic and ionic regulatory mechanism(s) in the naupliar brine shrimp *Artemia* has recently been investigated by Russler and Mangos (1978) and Conte *et al.* (1980). Hemolymph Na was found to be in the range of values that are normally exhibited by many osmoregulating marine vertebrates (100-200 mM) while hemolymph Cl values are somewhat lower. Concentrations of these same ions in the external fluids bathing the secretory epithelium is much higher ranging between 100 mM to 5000 mM NaCl, creating a chemical gradient that is some five to ten times greater than that found in the luminal fluids of typical vertebrate salt extruding organs, *i.e.* piscine gill, avian and reptilian salt glands (Kirschner, 1980). Insight into the mechanism of maintenance of this gradient can be realized from electrical measurements made across the larval salt gland epithelium. We have recently accomplished this task and have shown that the transepithelial PD to be significantly different in comparison to the calculated sodium or chloride equilibrium potential. The measurements for unidirectional Na-efflux, Na-influx and flux ratios as calculated from Nernst and Goldman equations, show that neither Na nor Cl are in electrochemical equilibrium. Therefore, some fraction of the movement of these two ions across the glandular epithelium is via active transport.

Since active transport implies the need for metabolic energy, we became interested in the role that cellular bioenergetics play in the regulation of ion transport out of this larval structure. In the adult brine shrimp, Croghan (1958) calculated from the reported respiratory rates and ion flux values that the minimum thermodynamic expenditure to maintain ionic equilibrium under the maximum rate of ion exchange was only 6% of the total available metabolic energy. This small amount of energy expenditure would suggest that ion transport is not a major ATP utilizing reaction even though transport is a vital process. We have measured the levels of ATP in the naupliar cytoplasm as a function of salinity (Ewing *et al.*, 1980) and found that there is a large decrease in the ATP pool during increasing NaCl concentrations. This reduction in ATP concentration occurred despite the fact that oxygen consumption was high and independent of the salinity gradient. Unfortunately, reported values of naupliar respiratory rates are not in very good agreement. Some investigations (Kuenen, 1939) have found an increase in respiration rates with increasing salinity while others have shown a decrease (Eliassen, 1952 ; Engel and Angelovic, 1968). Still others have found very little, if any, effect (Kratovich, 1964 ; Gilchrist, 1956, 1958). Clearly, a situation exists in the nauplius where aerobic oxidation-reductions rx's are either (1) being uncoupled by the ionic gradient or (2) maintained at a maximal rate which is still insufficient to yield levels of cellular ATP necessary to meet the metabolic demands at the higher salinities. When a situation of this nature occurs, it is usually assumed that anaerobic pathways are stimulated in order to compensate for the declining ATP levels. One such situation is most illustrative in this regard, and that is when the environmental oxygen levels either diminish to low values (hypoxia) or become absent (anoxia). Then, glycolysis with all of its attendant end-products becomes the predominate bioenergetic pathway. Ewing and Clegg (1969) have shown this to be the case for the newly hatched nauplius in which it was reported that in anaerobic conditions high cytoplasmic levels of lactate were produced with a concomitant increase in lactic acid excretion into the external medium. There are no reported investigations which show the relationship of lactic acid production being either (1) independent of the external NaCl gradient or (2) coupled to the maintenance of ionic regulation. The present study attempts to determine what shared role, if any, that aerobic metabolism and anaerobic metabolism have when the nauplius encounters a steep chemical gradient.

Materials and methods

Source and treatment of nauplii

Source

Dried brine shrimp cysts, *Artemia* from Great Salt Lake, Utah, were purchased from Longlife Products, Harrison, New Jersey, and stored *in vacuo* at -20 °C since 1974. Hatching rates and viability are in excess of 70%. Additional cysts have been obtained from the San Francisco Bay salterns and were purchased from San Francisco Bay Brand, a division of Metaframe Corp., Newark, California, and have shown an excellent hatchability. Ten to 20 g of either type of dried cysts were hydrated in cold tap water for 4 hr at 4 °C in the refrigerator. After hydration, cysts were transferred to Fernback flasks in ratio of 10 g cysts to 500 ml of 0.5 M Instant Ocean saline (NaCl) and incubated at 30 °C with gentle swirling on a modified shaker. After 18 hr incubation, free swimming nauplii were isolated by the method of Fina-

more and Clegg (1969) and discarded. Residual embryos were transferred to flasks containing fresh incubation medium and incubated for an additional 6 hr. Nauplii (24 hr) were harvested and used in all of the experiments described in the present study.

Salt acclimation of nauplii

24 hr nauplii isolated from the rearing flasks were filtered on Miracloth filters to remove excess incubation medium. Nauplii were then weighed and placed in flasks containing acclimation medium of varying salinities (0.05 M — 2.5 M NaCl) in the ratio of 1 g nauplii to 20 ml of acclimation medium. The acclimation media was prepared using Instant Ocean Salt mixture (I.O.-saline), and either fortifying it with added NaCl or diluting it with distilled water to the desired concentration. The I.O.-saline had contant pH of approximately 8.0.

Oxygen consumption

Respiration rates of nauplii were determined manometrically in a Gilson metabolic respirometer at 30 °C by incubating 0.1 g of acclimated nauplii in respiratory flasks containing 3.0 ml of acclimation medium. Oxygen uptake was measured at 5-min intervals and respiratory rates derived from the slopes of curves generated in a one-hour period. To obtain respiration rates in pure oxygen, the acclimation medium was flushed with pure oxygen for 10 min before the nauplii were added to the flasks. After addition of the nauplii, the flasks were flushed for an additional 10 min before measurement of oxygen uptake were initiated. After the respiratory rates were determined, the nauplii were washed onto pre-weighed millipore filters, and dried in an oven (100 °C) to constant weight. Results are expressed as μl O_2 consumed/hr/mg dry wt.

Determination of acidity and dissolved oxygen in conditioned-acclimation media

Dried brine shrimp cysts were prepared as outlined earlier, with the exception of an antiformin treatment as described by Finamore and Clegg (1969). Sterile synchronized nauplii were placed in a modified acclimation medium where ionic concentrations have been adjusted to approach those found in the Instant Ocean Salt mixture but a bicarbonate buffer (10 mM) or tris buffer (5 mM) was added. Final ionic concentrations were : Na, 500 mM ; K^+, 1-10 mM ; Cl^-, 500 mM ; Ca^{++}, 6 mM ; and Mg^{++}, $SO_4^=$, *etc.* Media were fortified with solid NaCl to reach concentrations greater than 0.5 M. Following chemical preparation, all acclimation media were sterilized by millipore filtration to remove bacteria and other debris prior to the addition of nauplii. After the desired period of incubation had been reached, nauplii were quickly removed by millipore filtration under aseptic conditions and weighed. The condition media were placed in a beaker for acid analysis. The acidity (pH) was measured potentiometrically using a calibrated glass electrode. Allowing 10-20 min for the electrode to reach stability, the acidity of the solution was determined and the results expressed in Δ pH/hr/mg of nauplii.

During the period of exposure to the various acclimation media, several aliquots of individual media were removed from the flasks in such a manner as to prevent entrapment of gas or nauplii and were stored in a gas-tight 5.0 ml syringe containing a small glass bead. The initial and final dissolved oxygen contents were determined by the syringe pipette modification of the Winkler method (Mancy and Jaffe, 1966). The reagents used were a

modification of those prepared for standard Winkler assay. In this microtechnique the reagents are identical but phenylarsine oxide standard (Hach Chemical Co., Ames, Iowa) has been substituted for sodium thiosulfate as the titrant. Appropriate amounts of reagents were added through the top of the syringe tip using a one ml syringe and a narrow gauge needle and mixed by agitation of the glass bead. Samples were titrated immediately in small white ceramic crucibles using a Micrometer Instruments Co. microtitrator to reach the end-point. Results are expressed in ml O_2/l.

Isolation and analysis of glycogen

After incubation, 1.0 g of nauplii were removed from 200 ml of acclimation medium by filtration and washed into a Dounce type glass-teflon homogenizer with cold 0.05 M phosphate buffer. One ml of 50% TCA was quickly added to the naupliar suspension and homogenized thoroughly. The homogenate was transferred to a 15 ml glass centrifuged tube and spun at 10 000 × g for 30 min. The precipitate was removed and set aside for protein analysis by method of Lowry *et al.* (1951). To each ml of supernatant, four volumes of 100% ethanol were added. The ethanolic mixture was allowed to stand for 15 min and then recentrifuged in a 15 ml glass centrifuge tube for 5 min at 10 000 × g. To the precipitate, 1 ml of 30% KOH was added with vigorous stirring. The alkaline solution was transferred to a water bath and heated for 10 min at 100 °C. Following heating, the solution was cooled in an ice bath. To each 1 ml of solution, four volumes of 95% ethanol were added. The solution was capped, shaken vigorously and then placed in a refrigerator overnight to allow precipitation to occur. The precipitate was collected by centrifugation and washed with 3.0 ml of 95% ethanol and then allowed to settle for 15 min before the final centrifugation. After centrifugation, the precipitate was dried and then taken up in 1.0 ml distilled water and assayed by the anthrone method of Seifert *et al.* (1950). Results are expressed as μg/mg naupliar protein.

Isolation and analysis of lactate

In a fashion similar to that described for isolation of glycogen, nauplii were removed from incubation salines and homogenized with cold 50% TCA. After centrifugation, the supernatant was removed and analyzed for lactate as described according to the method of Marbach and Weil (1967) utilizing lactic acid dehydrogenase contained in a glycine buffer. Results are expressed as μg/mg naupliar protein.

Isolation and analysis of lipid

In a similar fashion to that described for lactate and glycogen, nauplii were removed from the incubation salines by filtration and homogenized with cold 50% TCA. After homogenization, an aliquot was removed for protein analysis as previously described and the remaining homogenate transferred to a 15 ml glass centrifuged tube and spun for 30 min at 10 000 × g. The supernatant was removed, and to the remaining pellet a 5 ml aliquot of chloroform-methanol (2:1 v/v) was added, capped and shaken vigorously to extract the lipids. The methanolic-chloroform lipid extract was removed and placed into a 50 ml ground glass graduated cylinder. The pellet was washed 3 times with the chloroform-methanol mixture

until there was little orange color remaining. After each washing the extracts were pooled. Five ml of 0.05 M $MgCl_2$ was added to the pooled methanolic-chloroform lipid extract and shaken vigorously. After shaking, the solution was allowed to settle, and the aqueous phase was removed and discarded. The washed lipid extract was air dried and then taken up in 1 ml of ethanol-diethyl ether (v/v) and assayed. Results are expressed as μg/mg of naupliar protein.

Isolation and quantification of adenine nucleotides (AMP, ADP, and ATP)

Nauplii were removed from the incubation medium by filtration through a Miracloth disc, and then washed into a glass-teflon homogenizer with three 5.0 ml aliquots of perchloric acid. Nauplii were homogenized and the homogenate was transferred to ice-cold 15 ml centrifuge tubes. The perchloric acid brei was centrifuged at 10 000 × g for 30 min. The pellet was saved for protein analysis, and the supernatant was transferred to a 50 ml graduate cylinder. Supernatants were adjusted to between pH of 8.8-9.0 with 5.0 N KOH and let stand at 4 °C. After 30 min the supernatants were centrifuged to remove insoluble $KClO_4$. The pH of the supernatant was adjusted to pH 10 with KOH and applied to 1 × 12 columns of washed Dowex-1-chloride ion exchange resin. The columns were washed with water until neutral, then eluted stepwise with 0.01 M HCl ; 0.01 M HCl + 0.02 M NaCl ; 0.01 m HCl + 0.05 M NaCl ; 0.01 M HCl + 0.1 M NaCl ; and 0.01 M HCl + 0.15 M NaCl. Eluates were collected with a fraction collector and optical densities at 260 nm and 280 nm were obtained for each fraction. Fractions corresponding to the ADP and ATP peaks were pooled and evaporated by boiling to volumes less than 100 ml. Optical densities at 260 nm and 280 nm for the reduced volumes were derived from extinction coefficients at 260 nm for adenine in acid conditions.

Results

Table I contains the results of the effects of salinity upon the acid production by the cultured nauplii. As can be seen, the nauplii are stimulated in high NaCl concentrations to release large quantities of H^+ ions. The maximal rate of acid production as shown in Fig. 1, lies somewhere between 1.5 M to 2.5 M NaCl and also appears possibly to be dependent upon the amount of dissolved oxygen in the medium. We have reported that these low oxygen levels constitute a hypoxic condition (Conte, 1977) but they do not appear to affect oxygen consumption except at the extreme salinity of 2.5 M NaCl. Oxygen consumption is constant, ranging from 12-16 μl O_2/hr/mg protein (Table II), and independent of salinity with the one exception, that being 2.5 M NaCl (Table III). If pure oxygen is substituted for air, then there is no significant differences in respiratory rates. Fig. 2 shows the respiratory rates of 24 hr-nauplii that were hatched from two different sources of cysts ; Great Salt Lake in Utah and the salterns in San Francisco Bay, California. Each value represents a mean of four different populations reared from cysts incubated at different times of year by different staff members. Interestingly, the standard deviation from the mean value for all of the observations taken at a given salinity was always less than 5%. The conclusion arrived at from these results is that salinity has no effect upon respiration provided that the saline media is fully saturated with oxygen.

What possibly could be the reason for the reported differences in naupliar respiration? Table II shows the effects of development on naupliar respiratory rates and it can be seen that between 24-30 hr post-incubation starting from the time of hydration, there is little difference.

TABLE I

The effect of a NaCl gradient upon the acidification of the environmental medium

Salinity of medium (M NaCl)	Density[3] of nauplii (mg/ml medium)	Incubation period in medium (hr)	Dissolved oxygen[1] in medium		Acidity of medium[2]			Δ pH/hr/mg nauplii
			Initial (ml O_2/l)	Final (ml O_2/l)	Initial pH	Final pH	Diff. in pH	
0.1	100	5.0	NA	NA	7.20	7.10	0.10	0.0010
0.5	60	4.0	2.9	3.0	7.82	6.94	0.87	0.0145
1.0	60	4.0	NA	NA	7.81	6.72	1.09	0.0182
1.5	60	4.0	NA	NA	7.80	6.46	1.34	0.0223
2.0	60	2.0	NA	NA	7.81	6.50	1.31	0.0218
2.5	50	5.0	1.5	1.4	7.20	6.20	1.00	0.0200

[1] Dissolved oxygen in fully saturated medium was 3.8 ml O_2/l for 0.5 M salinity and 2.6 ml O_2/l for 2.5 M salinity. Density of naupliar population is 10 mg/ml.

[2] Buffer capacity of medium was between 8 mM to 13 mM.

[3] Density of naupliar population is expressed as wet weight (mg)/unit volume.

NA = Data not available.

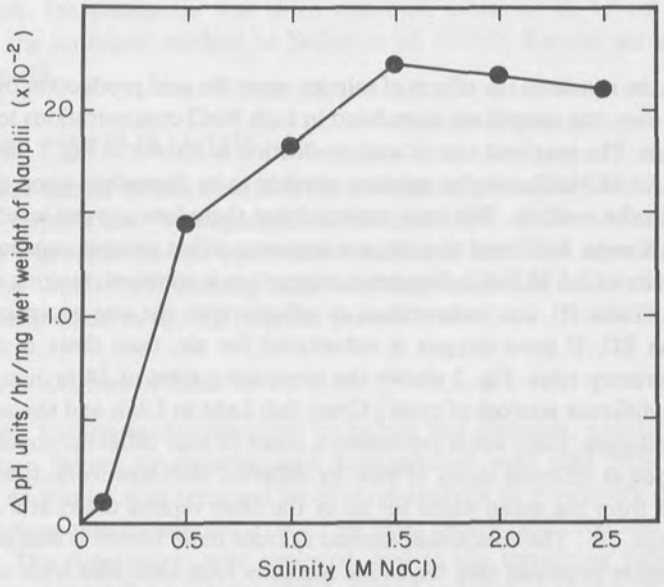

FIG. 1. Effects of salinity on rate of acid production in 24 hr old nauplii.

TABLE II

Ontogenetic relationship of oxygen consumption[1] and acclimation to salinity

Gas[2] phase	Age of nauplii[3] (hr)	Salinity (M NaCl)			
		0.05	0.50	1.0	2.5
Pure O_2	24	11.6	12.4	12.3	11.6
"	30	13.8	14.0	12.7	12.3
"	42	18.3	19.5	19.1	18.7
"	48	22.1	23.0	21.1	22.6
Air	24	11.6	11.2	11.2	8.7
"	26	10.1	11.8	NA	9.3
"	32	12.8	14.3	NA	13.7
"	42	16.5	16.5	NA	17.9
"	48	19.4	23.1	NA	27.2

[1] Values of respiratory rates are means with standard deviation of less than 1.0 μl/hr.

[2] Those flasks equilibrated to air received no special treatment, while those equilibrated to pure oxygen had gas flowing through the flasks for 10 min prior to closing the values to initiate the experiment.

[3] Age of the nauplii are relative to the time when cysts were hydrated. Hatching occurred between 20-22 hr post-hydration.

NA = Data not available.

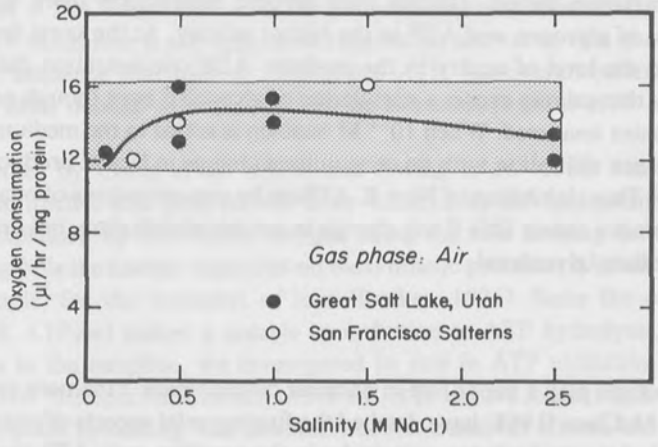

FIG. 2. Oxygen consumption as a function of salinity.

TABLE III

The effect of salinity on carbohydrate, lipid and intermediary metabolism end-products

Salinity (M, NaCl)	Glycolysis[1] (μg glycogen /hr/mg protein)	Acidification of media (Δ pH/hr/mg)	Rate of respiration (μl O_2/hr/mg)	Lactate[1] production (ng/hr/mg)	Lipolysis[1] esterified fatty acids (ng/hr/mg)	ATP content (μmoles/g)	ADP content (μmoles/g)
0.5	* 8.3 ± 0.1	*.0012	< 11.2 (air) 12.4 (oxygen)	*1.03 ± 0.06	1.02 ± 0.01	*1.12 ± 0.01	0.24 ± .001
2.5	*13.3 ± 0.1	*.0040	* 8.7 (air) 11.6 (oxygen)	*4.33 ± 0.02	1.01 ± 0.01	*0.88 ± 0.02	0.22 ± 0.01

[1] Rate of production or breakdown was derived from measurements taken over a 6 hr time period ; 24 hr-old nauplii were used in all experiments.

* Significant differences exist between the mean values using Student's "t" test ($P < 0.001$).

At 42 hr post-incubation there is marked increase in oxygen consumption, but it too is independent of salinity. Our interpretation of this data is that the oxygen consumption in the nauplius is substantial and does not reflect a state of hypoxia. Usually when hypoxia is quite extreme, or if oxygen is entirely absent, it leads to the formation of anaerobic end-products. Lactic acid is by far the most common end product and glycogen is the most common substrate exclusively used for anaerobic energy metabolism. The results in Table III were taken from animals that were acclimated in fully oxygenated salines and each population assayed for glycogen, lactate, pH of medium, respiratory rate, fatty acids, ATP and ADP after a six-hour acclimatation period. Despite high aerobic metabolism there were significant decreases in levels of glycogen and ATP in the higher salinity. At the same time, the level of lactate rose as did the level of acidity in the medium. ADP concentration did not appear to change. It appears that salinity causes a stimulation of glycolysis even though naupliar oxygen consumption remains unaltered. When 10^{-4} M ouabain is added to the medium, there is 40% inhibition of glycogen utilization with no concomitant change in lactate production or oxygen uptake (Table IV). Thus, inhibition of Na + K-ATPase by concentrations of ouabain known to effect the NaCl balance causes little if any change in aerobic metabolism, but drastically effects anaerobic metabolism (glycolysis).

Discussion

Adenine nucleotides play a major role in naupliar development. Finamore and Clegg (1969) and Warner and McClean (1968) have detailed the fundamental aspects of purine metabolism and report that of all the acid soluble nucleotides in the nauplius, only ATP shows an increase in proportion to the decrease in the high energy storage compound diguanosine tetraphosphate (diGDP). This increase in ATP appears to be quite variable and dependent upon certain culture conditions. Insofar as we are aware, there has been no report given on the effects of salinity either upon the utilization of Gp_3G or diGDP or the interconversion of

TABLE IV

Comparison of glycogen utilization and lactic acid production in nauplii during ouabain inhibition under aerobic conditions[1]

Salinity (M NaCl)	Hours of incubation	μg/mg naupliar protein	
		Glycogen decrease	Lactic acid increase
0.5	6	44	< 1
0.5 plus 1×10^{-4} ouabain	6	35	< 1
0.5 plus 4×10^{-4} ouabain	6	25	< 1
2.5	6	80	< 1
2.5 plus 1×10^{-4} ouabain	6	69	< 1
2.5 plus 4×10^{-4} ouabain	6	48	< 1

[1] Pure oxygen was used to saturate the media. Oxygen consumption for nauplii in both salinities was between 14-16 μl/hr/mg. Dissolved oxygen in media was equal to or greater than 2.5 ml O_2/l.

diGDP to ATP. This is a very critical point relative to the finding in the present study which shows a marked decresed in levels of ATP as a function of the NaCl gradient. In those nauplii supplied pure oxygen, to prevent any measurable degree of hypoxia, the ATP levels were identical to those incubated in salines containing air. This suggests that aerobic metabolism is occurring at a maximal rate but is insufficient to maintain adequate ATP concentrations and acts independent to the NaCl gradient. We do not know at this time if the ATP:diGDP interconversion mechanism is salt-dependent and therefore cannot arrive at an answer to the question : "Does a NaCl-regulated ATP biosynthetic pathway exist in the nauplius?" Our finding that ATP utilization is salt dependent requires an answer to that question because for each metabolic sequence that uses or regenerates ATP, the overall balance of the cellular energy system must depend in large part on the stoichiometries of ATP consumption and production.

Previous studies by Conte *et al.* (1973) and Ewing *et al.* (1980) have shown protein biosynthesis and nucleic acid biosynthesis to be inhibited by elevated salinities. These effects could not be explained by insufficient oxygen being the rate limiting factor. Moreover, in rapidly growing cells the energy expended on biosynthetic processes is small compared to that used, for example, for the transport of ions (Racker, 1976). Since the cationic transport enzyme (Na + K-ATPase) makes a notable contribution to ATP hydrolysis, and is found in large quantities in the nauplius, we investigated its role in ATP utilization and production. Using ouabain (10^{-4}), a specific inhibitor of Na + K-ATPase, we found a lack of an effect upon oxygen consumption indicating that inhibition of ion transport (Conte and Edwards, 1972) did not release aerobic metabolism from any metabolic constraints. Surprisingly, we found an inhibition of the salt induced aerobic glycolysis (Table IV), but without a large concomitant stimulation of lactic acid formation. These findings are similar to that found by Scholnick *et al.* (1973) for the effects of Na^+, K^+ and ouabain on glycolysis of Ehrlich ascites cells except they differed in that increased lactate production occurred in Ehrlich ascites cells where in our

embryonic cells it did not. Soulinna *et al.* (1975) have made a survey on several cultured cells observing for the effects of ATPase inhibitors on glycolysis and have found that ouabain produced marked inhibition of cell line L-1210 and P-388 suggesting that the Na + K-ATPase of the plasma membrane sustained glycolysis whereas inhibitors of mitochondrial ATPase were without effect.

At the present time we have little knowledge concerning which of the many types of ATPases present in the crustacean cell might be participating in the control of glycolysis. Table V is a partial list of the several types of cellular ATPases that have been postulated by Racker (1976) as potential participants in glycolysis regulation. It appears that one of the major functions of the Na + K-ATPase in the nauplius is to sustain glucose breakdown. Additionally, we have shown that this enzyme is the major regulator for water and electrolyte balance (Conte, 1977). Now we must provide for the mechanism whereby the joint relationship between ion transport and respiration resides in the linking of energy production with the control of glycolysis. Our finding that lactate formation in the nauplius is not stimulated by ouabain supports an earlier hypothesis (Conte, 1977) that an alternate pathway to lactate formation must exist. In order that continuous breakdown of glucose occurs, an adequate supply of the cofactor NAD^+ is required. A C-4 dicarboxylic acid pathway serving as a facultative anaerobic shunt has been proposed. This mechanism required fixation of CO_2 from exogenous bicarbonate that can form oxaloacetate resulting in (1) transamination to aspartate followed by excretion into the medium or (2) reduction to malate ($NADH \rightarrow NAD^+$) and shuttled to the mitochondrion. Several reports dealing with the details of the pathway and enzymatic components are being prepared (Hand *et al.*, 1978).

TABLE V

Potential ATPase participating in glycolysis[1]

ATPase	Activator(s)
Mitochondrial ATPase	Dissociation of inhibitor ; fatty acids as uncouplers
Na + K-ATPase	Loss of control proteolipid
Ca-ATPase	Loss of control proteolipid
Biosynthetic processes (*i.e.* protein syntheses)	Hormonal
Futile cycles	Hormonal
Lysosomal [Ca-ATPase]	Unknown

[1] Taken from Racker (1976).

Summary

1. Oxygen consumption in newly hatched nauplii appears to be independent to the NaCl concentration in the incubation medium.
2. The effects of development on naupliar respiratory rates indicate that between 24-30 hr post-incubation there is very little difference in oxygen consumption. After 42 hr post-incubation there is a marked increase in oxygen consumption that is also independent of salinity.

3. Significant decreases in levels of glycogen and ATP occurred at the higher salinities despite having high levels of oxygen in the medium.
4. Lactate concentrations found in the naupliar cytosol did not increase with elevated levels of salinity. In contrast, the acidity of the incubation medium increased markedly under the identical conditions.
5. Ouabain, a specific inhibitor of Na + K-ATPase, caused a 40-50 % reduction in the rate of salt-induced glycolysis. Lactate production remained the same either in the presence or absence of ouabain.

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The respiratory physiology of *Artemia*

W. Decleir, J. Vos[1], *F. Bernaerts*[2], *and C. Van den Branden*

Laboratory of Biochemistry, University of Antwerp (RUCA)
Slachthuislaan 68, B-2000 Antwerp, Belgium

Abstract

The respiratory physiology of adult male *Artemia* in 35 ‰ artificial seawater has been studied.

The scope for activity and the potential activity range were studied by measuring the respiratory rate of actively swimming and anaesthetized animals and the respiration of total homogenates. This was carried out between temperatures of 5 °C and 40 °C, which were the lower and higher lethal values.

Artemia is a respiratory regulator above a critical oxygen tension of about 2 ml O_2/l. The anaerobic metabolism consists essentially of lactic acid production, but also alanine, succinate, propionate and butyrate are formed. The accumulation of these anaerobic end products may be considered as a short term – or stress response of animals which are not acclimated to hypoxic conditions.

A long term adaptational mechanism consists of the production of new hemoglobin and especially of hemoglobin type 3, which shows oxygen binding properties that are very suited for survival in a low oxygen or high temperature environment.

Introduction

The brine shrimp *Artemia* inhabits salt lakes and ponds which show a great diversity in environmental parameters such as salinity, temperature, partial oxygen pressure, etc. Therefore the animals must be equipped with a variety of adaptational mechanisms to cope with the changing environment. Adaptation to hypoxic conditions includes a change in reproductive behaviour (Dutrieu, 1960 ; Sorgeloos, 1975), the development of a high concentration of respiratory pigment in the blood (Gilchrist, 1954) and a modified energy balance (Gilchrist, 1956). Bowen *et al.* (1969) have shown the existence in *Artemia* of three types of hemoglobin, which have been called Hb 1, 2, and 3, according to their electrophoretic mobility. Van den Branden *et al.* (1978) demonstrated that the three types of hemoglobin are differently adapted to different environmental factors.

Although several papers treat different aspects of respiratory physiology of adult *Artemia* (Conover, 1960 ; Dutrieu, 1960 ; Gilchrist, 1956, 1959), a more detailed and systematic study of the whole field of respiratory physiology has never been attempted.

This paper is an extension of our first paper on the respiratory physiology of *Artemia* (Vos *et al.*, 1979). It describes our first results in defining the scope for activity and the potential

[1] "Wetenschappelijk medewerker" at the Foundation for Collective Fundamental Research (FKFO).
[2] "Aspirant" at the Belgian National Foundation for Scientific Research (NFWO).

activity range at one salinity (35 ‰) and at different temperatures. It furthermore extends the study of anaerobic end products to molecules other than lactic acid. Finally it describes and discusses the very complex problem of interaction between acclimation and adaptation.

Materials and methods

Animals

We only used adult male specimens of *Artemia* (San Francisco Bay, California). The cysts were hatched under continuous illumination and aeration. The nauplii were grown in 60 l air-water lift operated raceways according to Sorgeloos (1975). They were fed twice a day with a suspension of *Spirulina maxima*. From the adults only 5 weeks-old male specimens with a length of 9 mm were selected for our experiments. The dry weight of one animal was 0.77 mg ± 0.09 mg.

Seawater

For all our experiments we used artificial seawater (HW-Wimex seasalt) with a salinity of 35 ‰.

Respirometry

Respiration was measured in a Warburg constant volume respirometer. For each measurement we used a series of 15 ml flasks each containing six individuals, incubated in 3 ml seawater, filtered through a 0.5 μm Millipore filter. For CO_2 absorption, 0.3 ml KOH 20 % and a small filter-paper were put in the center vial. To obtain specific oxygen concentrations in the incubating medium, the flasks were flushed with O_2-N_2 gas mixtures according to Umbreit *et al.* (1972). The flasks were equilibrated for 30 min at 25 °C. Oxygen consumption was measured every 30 min over a period of 3 hr. The flasks were continuously shaken. Total oxygen consumption was calculated by regression analysis of the oxygen decrease as a function of time. Oxygen consumption was expressed as respiratory rate (μl O_2/ind./hr). In some circumstances mortality occurred in which case O_2 consumption was always corrected accordingly.

Anaesthesia was obtained by adding 0.25 ml of a 0.5 % chloroform solution in seawater to the Warburg vessels, including the control vessel.

Animal homogenates for respiration studies were made with a Potter homogenizer in a buffer solution containing 0.5 M glucose, 0.05 M Tris, 0.005 M $MgCl_2$ and 0.025 M KCl (final pH = 7.5). In this case oxygen consumption was measured every 10 min for a period of 90 min.

Anaerobic metabolism

The animals were incubated for 1 or 2 hr in anaerobic seawater which was obtained by bubbling with N_2 for 15 min at 25 °C. After the experiment the animals were immediately frozen at -25 °C until needed for the determination of glycogen and different anaerobic end products.

The homogenates for glycogen and volatile fatty acid determinations were made by homogenizing 200 animals in 2.5 ml ice-cold bicarbonate buffer (pH = 9.5) and 20 μl of 0.2 M valeric acid. For the glycogen determination the homogenate was subsequently saponified in 30% KOH, neutralized with 5N HCl, then trichloracetic acid was added to induce protein precipitation according to De Zwaan *et al.* (1976).

The homogenates for L-alanine, L-lactate and succinate determination were made by homogenizing 200 animals in 2.5 ml 6% perchloric acid, and subsequent centrifugation for 15 min at 20 000 g. 2 ml of the supernatant were neutralized with 5.0 M K_2CO_3 to pH 8.0 and cooled for 1 hr in ice. Excess potassium perchlorate was removed by centrifugation at 12 000 g for 15 min. Alanine and lactate were determined directly in the perchlorate-free supernatant. For succinate determination 1 ml of the supernatant was passed through a 2.5 ml Dowex 1×8 (200/400 mesh) column in the OH^- form. The column was washed with 8 ml of distilled water after which the organic acids were eluted with 8 ml formic acid (2 N).

Glycogen

Glycogen was determined by precipitation with ethanol and Na_2SO_4, centrifugation and analysis of the pellet (glycogen + Na_2SO_4) according to Handel (1965). Extinction at 620 nm after addition of the anthrone reagent was measured in a Beckman DB spectrophotometer.

Volatile fatty acids

Volatile fatty acids were determined by extraction of the homogenate with diaethyl-ether, followed by gas-liquid chromatography according to Kluytmans *et al.* (1975).

L-alanine

L-alanine was determined spectrophotometrically according to Williamson (1970). Alanine dehydrogenase was obtained from Boehringer (Mannheim-Germany).

Succinate

Succinate was determined by spectrophotometry at 540 nm in the presence of succinate dehydrogenase (purified from *Ascaris lumbricoides*) and with INT (2p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazoliumchloride) as electron acceptor, as described by Kmetec (1966).

Lactic acid

Lactic acid was determined in the acclimation experiments by the method of Barker (1957), modified by Barnes *et al.* (1963) and Umbreit *et al.* (1972). In the anaerobic metabolism study lactate was determined spectrophotometrically at 340 nm by measuring the reduction of NAD^+ in the presence of L-lactate dehydrogenase obtained from Boehringer (Hohorst, 1970).

ACCLIMATION EXPERIMENTS

Acclimation was carried out in oxygen acclimation jars as described earlier (Vos *et al.*, 1978). At a temperature of 25 °C and at 35 ‰ salinity five different acclimations were performed. The acclimation periods were 3 days in 100%, 50%, and 30% air saturation. For acclimations to lower oxygen levels we carried out a stepwise decrease in order to avoid mortality. Acclimation to 20% saturation was obtained by a 3 day incubation period in 30%,

followed by a 3 day period in 20% air saturation. Acclimation to 10% air saturation was done by incubating the animals successively for 3 days in 30%, 3 days in 20%, and finally 3 days in 10% air saturation. For respirometry the animals were always starved for 48 hr.

Preparation of the hemoglobins

For the oxygen equilibrium studies the three hemoglobins (Waring *et al.*, 1970) were isolated from total homogenates as explained earlier (Van den Branden *et al.*, 1978). The three separated hemoglobins were dialyzed against the appropriate buffers. When triphosphates were used, ATP or GTP were added until a final concentration of respectively 0.002 M and 0.01 M was reached.

Oxygen dissociation curves

These were determined with the diffusion chamber technique of Sick and Gersonde (1969). The diffusion chamber used, was constructed by Eschweiler and Co., Kiel.

Results

Experiments with animals acclimated to 100% air saturated seawater

Fig. 1 shows the rate of oxygen consumption (μl/ind./hr) at temperatures ranging from 5 °C to 35 °C of adult male *Artemia*. In this graph we have compared the oxygen uptake of actively swimming animals with animals anaesthetized with chloroform and with total homogenates. The animals for this experiment have been acclimated in fully aerated seawater at 25 °C. In Fig. 2 the respiratory rate is plotted against the environmental oxygen concentration. We obtain a curve with a critical oxygen tension of about 2.0 ml O_2/l. Below this value the respiration decreases quickly to a limiting level of 0.7 ml O_2/l which corresponds to an oxygen consumption of 0.7 μl O_2/ind./hr. At this point the animals become non-motile but they recover when transferred to aerated seawater.

The anaerobic metabolism was studied by experimentally creating hypoxic and anoxic conditions. The animals were put in anoxic seawater for 1 or 2 hr and the consumption of glycogen and accumulation of several possible end products were studied. This experiment was carried out with fed and starved animals. The results are shown in Table I. During the first hour about half of the glycogen is consumed and an important amount of lactic acid is accumulated. Small amounts of alanine, succinate, propionate and butyrate are also formed. During the second hour the accumulation of lactate and most other molecules slow down with the exception of succinate.

Experiments with animals acclimated to different oxygen concentrations

We have acclimated the animals to five different oxygen concentrations. These were 100%, 50%, 30%, 20%, and 10% air saturation corresponding with concentrations of respectively 4.82, 2.42, 1.45, 0.97, and 0.48 ml O_2/l. Table II gives the percent-ratio of the hemoglobins in each of the acclimation groups and the respiratory rate and lactic acid production of the differently acclimated animals in the various experimental oxygen concentrations. Table II shows that adaptation to oxygen concentrations below 30% air saturation in our ex-

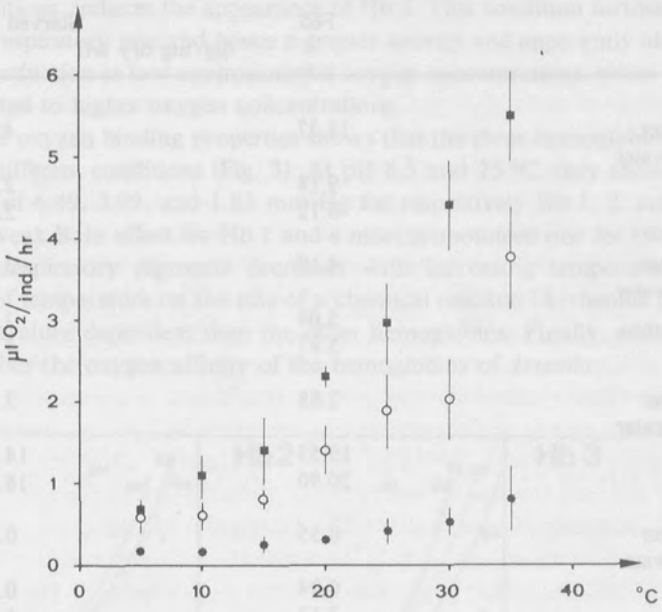

FIG. 1. The active and standard rate of respiration of *Artemia*. The respiratory rate is plotted against temperature. (■) Actively swimming animals ; (○) anaesthetized animals ; (●) whole animal homogenates.

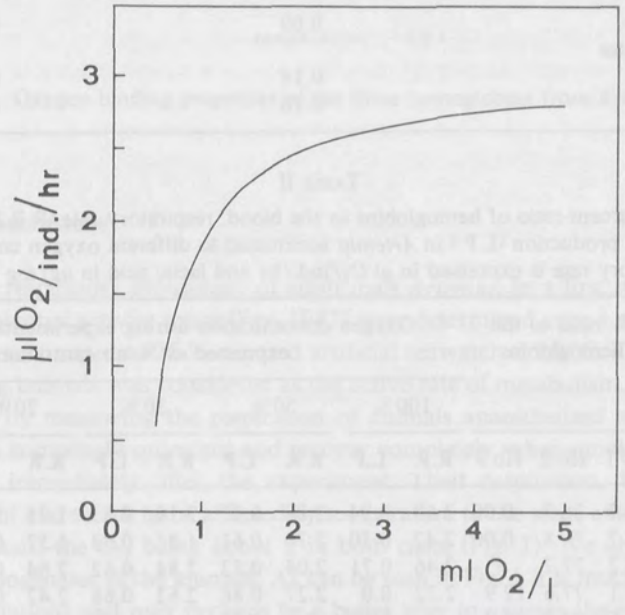

FIG. 2. Relation between respiratory rate and oxygen concentration in *Artemia*.

TABLE I

Determination of glycogen and some possible anaerobic end products in *Artemia*

	Fed	Starved for 48 hr
	(μg/mg dry wt)	
Glycogen		
Aerated seawater	18.37	4.13
Anaerobic seawater		
– after 1 hr	9.18	2.97
– after 2 hr	8.12	2.12
L-alanine		
Aerated seawater	4.19	3.47
Anaerobic seawater		
– after 1 hr	5.08	3.56
– after 2 hr	5.97	4.10
L-lactate		
Aerated seawater	2.88	3.33
Anaerobic seawater		
– after 1 hr	19.55	14.41
– after 2 hr	20.90	18.11
Succinate		
Aerated seawater	0.55	0.69
Anaerobic seawater		
– after 1 hr	0.94	0.86
– after 2 hr	2.12	1.67
Propionate		
Aerated seawater	0.00	0.38
Anaerobic seawater		
– after 1 hr	0.30	0.49
– after 2 hr	0.38	0.42
Butyrate		
Aerated seawater	0.09	0.00
Anaerobic seawater		
– after 1 hr	0.14	0.00
– after 2 hr	0.16	0.00

TABLE II

Percent-ratio of hemoglobins in the blood, respiratory rate (R.R.) and lactic acid production (L.P.) in *Artemia* acclimated to different oxygen concentrations. The respiratory rate is expressed in μl O_2/ind./hr and lactic acid in μg/mg dry weight

Oxygen concentration during acclimation (% saturation)	% ratio of the hemoglobins			Oxygen concentration during experimental incubation, expressed as % air saturation									
				100%		50%		30%		20%		10%	
	Hb 1	Hb 2	Hb 3	R.R	L.P	R.R	L.P	R.R	L.P	R.R	L.P	R.R	L.P
100%	25.7	73.7	0.00	2.69	0.94	2.88	0.97	2.10	0.83	1.75	3.27	0.66	4.03
50%	22.7	76.8	0.00	2.42	0.50	2.70	0.61	1.81	0.63	1.57	0.29	0.93	4.17
30%	22.2	77.2	+	2.46	0.71	2.04	0.73	2.84	0.62	2.64	0.82	1.40	3.22
20%	9.1	77.6	12.9	2.32	0.0	2.27	0.86	2.62	0.68	2.42	0.89	1.62	0.78
10%	5.6	70.3	23.0	–	0.68	–	0.47	–	–	–	–	–	0.72

perimental conditions, induces the appearance of Hb 3. This condition furthermore assures a more adequate respiratory rate and hence a greater activity and apparently also a lesser need for lactic acid production in low environmental oxygen concentrations, when compared with animals acclimated to higher oxygen concentrations.

A study of the oxygen binding properties shows that the three hemoglobins from *Artemia* are adapted to different conditions (Fig. 3). At pH 8.5 and 25 °C, they show high affinities with P_{50} values of 6.49, 3.99, and 1.83 mm Hg for respectively Hb 1, 2, and 3. We find a positive rather weak Bohr effect for Hb 1 and a most pronounced one for Hb 2. The oxygen affinity of the respiratory pigments decreases with increasing temperature. This is the expected effect of temperature on the rate of a chemical reaction (Arrhenius theory). Hb 3 is much less temperature dependent than the other hemoglobins. Finally, addition of ATP or GTP does not alter the oxygen affinity of the hemoglobins of *Artemia*.

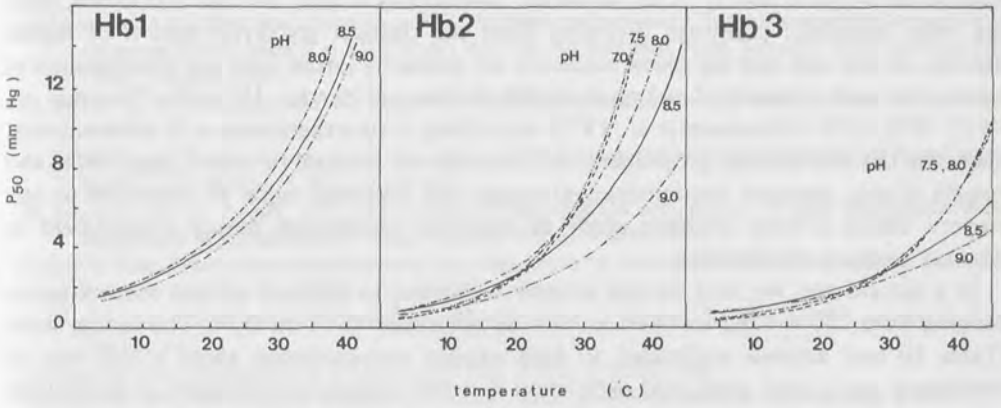

FIG. 3. Oxygen binding properties of the three hemoglobins from *Artemia*.

Discussion and conclusions

We studied the respiratory physiology of adult male *Artemia*. In a first step the scope for activity and the potential activity range (Fry, 1947) were determined over a wide temperature range for animals acclimated to 35 ‰ aerated artificial seawater at 25 °C. The respiration of actively swimming animals was considered as the active rate of metabolism. We tried to find the standard rate by measuring the respiration of animals anaesthetized with chloroform. These animals are completely quiescent and recover completely when quickly transferred to aerated seawater immediately after the experiment. Their respiration, however, is still relatively important and seems to be affected by temperature to the same extent as in the case of the active animals, the Q_{10} being about 2 in both cases (Fig. 1). We also measured the respiration of homogenates of the animals. As can be seen in Fig. 1 it is much less affected by temperature fluctuations and may perhaps be a better way to express the standard metabolism. When we consider the relation between the rate of oxygen consumption and the

ambient oxygen concentration (Fig. 2) we find a large zone of almost independent respiration above the critical oxygen concentration of approximately 2.0 ml O_2/l. Below this value the rate of oxygen uptake depends upon the ambient oxygen concentration and diminishes quickly to a value of about 0.7 μl O_2/ind./hr. This decrease is accompanied by a corresponding reduction in the level of locomotive activity. At an oxygen concentration of 0.5 ml/l (Fig. 2) and at a temperature of 5 °C (Fig. 1), the animals become quiescent and only some respiratory movements of the thoracopods or some very weak swimming movements can be observed. In anaerobic seawater this is obtained after about 1 hr. After 2 hours the animals have become completely inactive and after another 30 min the animals start dying. During the first hour of anaerobiosis, glycogen is consumed and lactic acid is produced. Small amounts of alanine, succinate, proprionate, and butyrate are also formed. During the second hour lactic acid production slows down in favor of a more pronounced formation of succinate (Table I). These results prove that in anoxic conditions *Artemia* shows an anaerobic metabolism which is mainly of the vertebrate skeletal-muscle type, but that next to this, there are other metabolic pathways diverging from the classical glycolytic pattern of higher animals. In this last case the redox balance is not primarily linked with the accumulation of lactate, but with a variety of end products (De Zwaan and Zandee, 1972 ; De Zwaan *et al.*, 1973, 1975, 1976 ; Kluytmans *et al.*, 1975). According to our experiments with adult animals, succinate but also alanine, proprionate, and butyrate are formed. In cysts (Clegg, 1976) and nauplii (Conte, personal communication) malate and aspartate might be important in this respect. Hence a more intensive study of anaerobic metabolism during development in *Artemia* seems to be indicated.

In a second step we have studied animals acclimated to different oxygen concentrations ranging from 100% (4.82 ml O_2/l) to 10% air saturation (0.48 ml O_2/l). The results show (Table II) that animals acclimated to high oxygen concentrations show a low rate of respiration and a high lactic acid production in a low oxygen environment, while animals acclimated to low oxygen concentrations show a much higher respiratory rate and a much lower lactic acid production in the same low oxygen environment. This means that *Artemia* can adapt to different oxygen concentrations. As shown in Table III this adaptation consists in the formation of new hemoglobin, especially Hb 3. We studied the oxygen dissociation curves of the three types of hemoglobin, which may occur in *Artemia*. The effect of pH and temperature on the half-saturation values of these curves (P_{50}) is illustrated in Fig. 3. It shows that Hb 3 has the highest oxygen affinity and at physiological pH the lowest Bohr effect and the lowest temperature sensitivity. Therefor this hemoglobin must be physiologically adapted to cope with high temperatures and low concentrations of oxygen in CO_2-rich water. The most striking phenomenon in the respiratory physiology of *Artemia* is the formation or loss of differently adapted respiratory pigments according to environmental conditions (Van den Branden *et al.*, 1978). When a sudden change in oxygen concentration occurs, the animals have a time to acclimate. In these conditions they show a stress response which consists in the production of lactate and lateron of succinate.

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Effect of different glycerol concentrations upon the hatching of *Artemia*

Remzi Geldiay, Tufan Koray and Baha Büyükişik

Department of Biological Oceanography, Institute of Hydrobiology
Faculty of Sciences, Ege University
Bornova, Izmir, Turkey

Abstract

Embryos of the brine shrimp *Artemia* were incubated in increasing concentrations of glycerol. The time required for the rehydration of the cysts and the prehatching period increased with increasing glycerol concentrations in the external medium. After the rupture of the chitinous membrane, it was observed that the internal sodium reserve decreased continuously while it increased in the external medium. In low glycerol concentrations the continuous loss of sodium led to the formation of non-motile nauplii ; in 0.8 M-1.0 M glycerol concentrations development past the late pre-nauplii (E-2) stage was precluded. These results prove the importance of the embryonic sodium ion reserve for a normal development of incubated cysts. A decrease in the level of this Na-stock probably has a negative effect upon naupliar halocytogenesis and, as the transition from a trehalose-glycerol hyperosmotic regulatory system to a hypoosmotic regulatory sytem is prevented, naupliar protein synthesis is obstructed indirectly.

Introduction

Glycerol has been used as a preservative for living organisms placed in an artificial cryptobiotic (frozen) state. The properties of this water-like solvent, (low volatility, antioxidant, non-toxic, protective against protein denaturation) make it most suitable for this purpose. Interestingly, free glycerol has been found in living organisms which undergo natural cryptobiosis such as insects during diapause. In earlier studies free glycerol has been defined as the major carbohydrate component in dormant *Artemia* embryos and its physiological importance during the hatching process has been established (Clegg, 1962, 1964). Quantitative investigations on the dry cysts and postembryonic stages of the brine shrimp *Artemia*, have shown that the trehalose-glycerol + glycogen transition of the hatching process is dependent upon glycerol and forms the hyperosmotic regulatory system leading to the rupture of the chitinous membrane.

The ecological importance of this feedback type mechanism is known to protect the ability of the embryo to rehydrate in solutions with a wide range of external osmotic pressures. Recent research suggests that this first type of osmotic adaptive mechanism may be short-lived. It appears to be replaced by a second type of osmotic adaptive mechanism, the sodium

hypoosmotic regulatory system, which has a major role in the maintenance of the larval water balance (Conte *et al.*, 1972 ; Ewing *et al.*, 1972 ; Conte *et al.*, 1977).

Within the framework of the latest developments on the matter it appears that embryos incubated in a non-toxic medium at a suitable osmotic pressure and temperature are impermeable to many solvents excluding water. The permeability of the chitinous membrane to water permits the accumulation of intracellular liquid required to initiate the cellular metabolism. Extracellular water is further stored after the complete saturation of the intracellular compartment. When both compartments are saturated, tissue and organ formation begins. The internal osmotic pressure required to rupture the chitinous membrane is supplied by the accumulation of glycerol between the embryo and the chitinous membrane during a period of dependency on the external osmotic pressure. The hatching membrane which, upon rupture of the chitinous membrane, comes into direct contact with the solutes and solvents of the external medium, possesses an ionic selectivity and some special osmotic characteristics required to maintain the salt balance (Morris and Afzelius, 1967).

One of the most important changes observed in the external medium during the transition from the latent period to the differentiation period is the appearance of free glycerol (Clegg, 1964 ; Morris, 1971). At this time, there is a decline in the internal glycerol concentration correlated with a rise in external glycerol levels. Glycerol can be found in early pre-nauplii (E-1), late pre-nauplii (E-2) and even in nauplii, at low concentrations, during the first 72 hr after incubation.

The gradual decrease in the internal glycerol concentrations from the early pre-nauplii (E-1) stage onwards probably results from the diffusion of this compound through the hatching membrane (Clegg, 1964 ; Conte *et al.*, 1977). Therefore the first type of osmotic adaptive mechanism – the trehalose-glycerol hyperosmotic regulatory system – observed in the post-embryonic development of *Artemia* gradually loses its effectiveness after the rupture of the chitinous membrane. In the subsequent post-embryonic development stages, the sodium hypoosmotic regulatory system begins to control the larval water balance.

The results of research on incubation of *Artemia* cysts in ionic and non-toxic solutions are corroborated almost exactly by earlier investigations with non-ionic fluids, (Boone and Baas-Becking, 1930 ; Clegg, 1964). Although glycerol is ecologically and physiologically important for *Artemia* cysts, its effects on incubation of the latter in glycerol solutions have not been investigated in detail because the effects of non-ionic substances are less important. However, an investigation of this type could provide preliminary information on the useful duration of the trehalose-glycerol hyperosmoregulatory system and more definitive knowledge on the behavior of the second type of osmotic adaptation mechanism (the sodium hypoosmotic regulatory system) when the gradual decrease of internal glycerol levels is prevented in the early pre-nauplii (E-1) and late pre-nauplii (E-2) stages.

This paper reports on our research efforts to answer these questions.

Materials and methods

The dried *Artemia* cysts used in this study were collected with a 100 μm plankton net in November 1977 from the pools of the Çamalti Saltern, Izmir. The cysts were washed in tap water, air dried, and stored in nylon bags prior to use.

PROCEDURE A-INCUBATION EXPERIMENTS

Two-year old dried cysts were washed twice with distilled water, and hydrated in distilled water, at 18 °C, for 1 hr, in a 50 cm water column in a graduated cylinder. This procedure separated empty shells and various other debris from the encysted embryos which settled on the bottom and could be collected and redried in an oven at 47 °C for 2 days.

Glycerol solutions were prepared in the range of 0.2 mol/l to 2.0 mol/l at 0.2 mol/l intervals. In order to control bacterial growth, 150 μg/ml penicillin and 200 μg/ml streptomycin were added to the solutions (D'Agostino, 1975).

Each concentration was studied in five duplicates. Fifty mg of dried cysts were placed in Pyrex petri dishes (18 cm diameter) and 5 ml of the appropriate glycerol concentrations added to each dish. With this procedure the cysts were kept in contact with the surface film and directly exposed to the gaseous phase. Controls in increasing concentrations of NaCl were placed under the same conditions in an incubation room at 25 $\pm$ 0.5 °C and illuminated by a 40 W daylight fluorescent lamp at a height of 50 cm, following Sorgeloos (1973). Evaporation which could lead to major changes in the concentrations of the solutions was prevented by sealing the petri dishes with parafilm. At the end of a 96 hr incubation period all samples were fixed with 1-2 drops of Lugol's solution. The number of empty shells, late pre-nauplii (E-2) and nauplii of the fixed samples was counted in a Sedgwick-Rafter chamber and expressed as percentage of the total number of cysts (Nakanishi *et al.*, 1962).

PROCEDURE B-DETERMINATION OF SODIUM IN THE CYSTS AND EXTERNAL MEDIA

For this experiment dry cysts were incubated in 0.25 M, 0.50 M, 0.75 M, and 1.0 M glycerol solutions as described above. The tests were carried out in plastic petri dishes without antibiotic mixture. Samples were taken every 6 hr in each concentration group. The total number of cysts, early pre-nauplii (E-1), late pre-nauplii (E-2) was determined in five separate drops placed on the lid of a petri dish according to the procedure used by Morris (1971). After counting, the material was put back into the dishes. Samples of the incubation medium were also taken every 6 hr with a pipette provided with a 100 μm plankton net to avoid sucking up of cysts or nauplii. These samples were transferred to plastic tubes and stored at -5 °C till analysis. Samples of the solid material [cysts, early pre-nauplii (E-1); late pre-nauplii (E-2), nauplii and empty chitinous membranes] were quickly washed with distilled deionized water (less than 30 sec), dried at 100-105 °C in an oven for 24 hr following Conte *et al.* (1977) and stored.

At the end of the 36 hr experimental period, the Na^+ content of the liquid samples was determined with the standard addition method, in a Carl Zeiss Jena flame photometer. The solid samples were first dissolved in a HNO_3-30% H_2O_2-H_2O (v/v/v) oxidative solution at 100 °C. The Na^+ content of this solution was then measured. Corrections were made for the sodium present in the reactives (calculated from test samples). Sodium in the external medium is expressed in μmol/ml and internal sodium in cysts, pre-nauplii and nauplii in μmol/mg. Counts of cysts, early pre-nauplii (E-1), late pre-nauplii (E-2), and nauplii taken during the sampling periods are calculated as percentages of the total number of cysts. The developmental stages of the cysts incubated in the 0.25 M, 0.50 M, 0.75 M, and 1.0 M NaCl test solutions were noted at each sampling period.

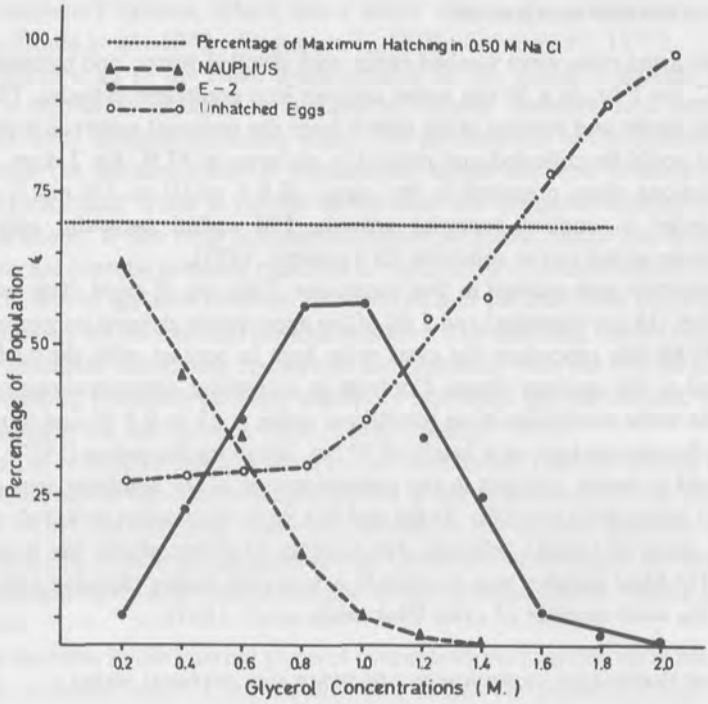

FIG. 1. Effects of increasing glycerol concentrations on the hatching of *Artemia* embryos after a 96 hr incubation period at 25 °C.

Results

THE EFFECTS OF GLYCEROL SOLUTIONS ON NAUPLIAR MORPHOGENESIS (Fig. 1)

In Procedure A, free-swimming nauplii were found in NaCl concentrations from 0.2 M to 1.8 M at the end of the 96 hr experimental period. The hatching success was 68-70%. This figure decreases to 57% in 1.8 M NaCl and 15-27% in 2.0 M NaCl. Nauplii survived for a period of 48-72 hr in all concentrations. The 30% nonhatching of the cysts is most probably due to the two year conservation period under atmospheric circumstances.

At the end of the same incubation period the results from the cysts kept in glycerol differed to an important degree from those in the NaCl solutions. Although nauplii formation in 0.2 M glycerol was almost normal (60.7% hatching success), all the larvae were non-motile. The percentage of hatched nauplii decreases gradually with an increase in glycerol concentration ; in concentrations above 1.4 M no nauplius formation was observed.

The quantity of late prenauplii (E-2) in different glycerol concentrations at the end of the 96 hr incubation period is interesting. From 0.2 M to 1.0 M, the percentage of late prenauplii (E-2) increases with increasing glycerol concentration and a (logical) decrease in the percentage of nauplii in the medium. In 0.8 M and 1.0 M glycerol late prenauplii (E-2) make up 57% on the average of the population. In concentrations above 1.0 M the percentage late

prenauplii decreases in functio of the glycerol concentration ; they make up but 0.1 % of the population in the 2.0 M glycerol medium. The relative quantity of early prenauplii (E-1) in glycerol solutions could not be determined accurately because of the observed separation of late prenauplii (E-2) from the chitinous membranes and the disturbing presence of empty shells with these developmental stages.

The nonhatching rate of the cysts in glycerol media was not greater than 30-40 % in concentrations up to 0.8 M. In concentrations above the value, the nonhatching rate increased, resulting in a smaller number of late pre-nauplii (E-2) and nauplii in the media.

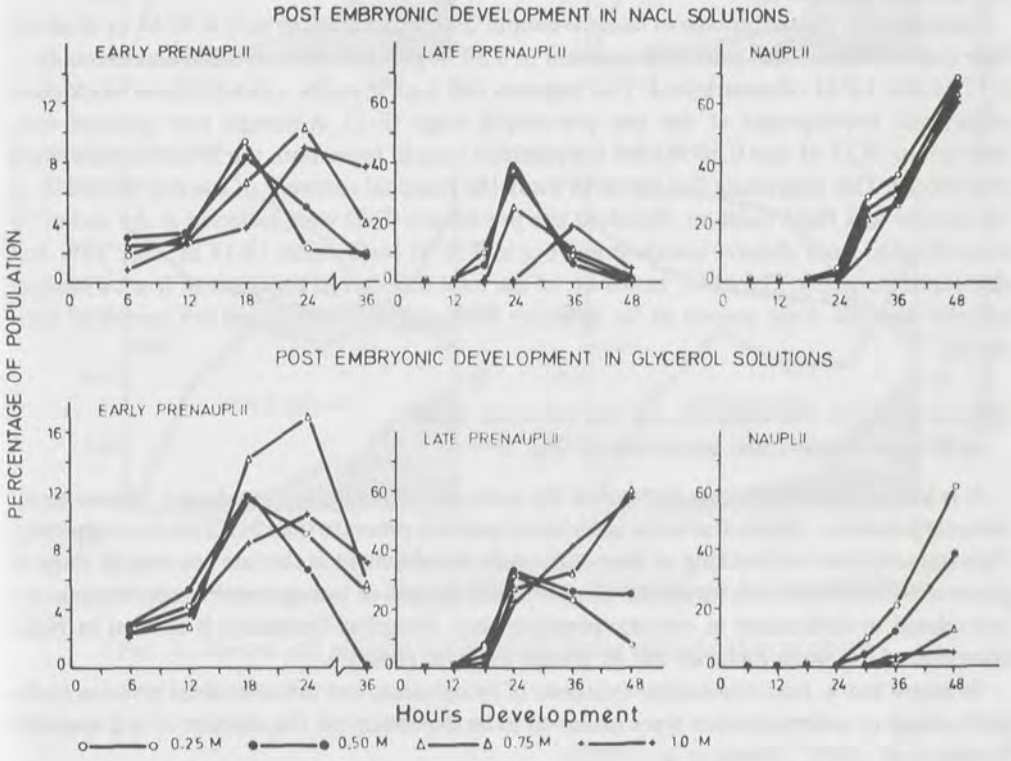

FIG. 2. Percentage of different post-embryonic developmental stages in NaCl and glycerol solutions at 6 hr time intervals during incubation.

EFFECTS OF GLYCEROL SOLUTIONS ON THE RATE OF DEVELOPMENT (Fig. 2)

The relationship between the rate of development and glycerol concentration is expressed as percentage of the total number of cysts for the post-embryonic developmental stages observed at 6 hr intervals during a 36 hr period. In the control NaCl solutions, the post-embryonic rate of development decreases with the increase in salt concentration. The time required for the formation of early pre-nauplii (E-1), late pre-nauplii (E-2) and free-swimming

nauplii increases with increasing NaCl concentrations ; a greater percentage of free-swimming nauplii is thus found in the lower concentrations (0.25 M, 0.50 M) than in the higher concentrations, after a same period of time.

As observed for NaCl solutions, the rate of development of cysts incubated in glycerol solutions decreases in relation to the concentration of this compound in the external medium. Dried cysts were rehydrated normally in glycerol solutions and the first cracks in the shells were observed after 6 hr. In the 12 hr samples, the period of transition to the pre-nauplii (E-1) stage is prolonged with an increase in glycerol concentration. During subsequent observations it was noted that the time required for late pre-nauplii and nauplii formation was a function of the glycerol concentration.

Interestingly, the percentage of late pre-nauplii (E-2) did not fall to zero at 48 hr in all of the four concentrations, but remained constant in 0.50 M glycerol and increased continuously in 0.75 M and 1.0 M concentrations. This suggests that high glycerol concentrations block post-embryonic development at the late pre-nauplii stage (E-2). Although low glycerol concentrations (0.25 M and 0.50 M) did not preclude nauplii formation, the larvae formed were non-motile. This interesting fact led us to study the potential recovery of late pre-nauplii (E-2) by transfer to a NaCl medium. Hundred late pre-nauplii (E-2) were collected at the end of 36 hr incubation from glycerol solutions and put in 0.50 M NaCl. After 14-18 hr, only 32% had developed normally. Only 6%, however, of the total transferred consisted of free-swimming nauplii : the rest were normal as far as organ formation was concerned but remained non-motile.

Sodium levels in the embryos and the external media during post-embryonic development (Fig. 3)

It is known that NaCl and glycerol of the same molar concentration do not possess equal osmotic pressures. Glycerol always has a lower osmotic pressure than NaCl, as it is non-ionic. This suggests that the blocking of post-embryonic development at the late pre-nauplii stage in glycerol solutions and the formation of non-motile nauplii in low glycerol concentrations are not related to differences in osmotic pressure only. Nauplius formation is normal in NaCl solutions of the same molarity but of greater osmotic pressure.

In recent work, macromolecular synthesis of cytoplasmic and mitochondrial proteins in the early stages of embryogenesis were observed to be dependent on the amount of salt available (Conte *et al.*, 1972 ; Ewing *et al.*, 1972).

According to the results of this research, an increase in the NaCl concentration of the external medium leads to a decrease in the rate of protein biosynthesis in nauplii. On the other hand, Conte *et al.* (1977) showed that the continuous loss of glycerol from the cellular and hatching membranes of the excysting embryo transformed the trehalose-glycerol hyperosmotic mechanism into the sodium-mediated transport required to maintain larval water balance. They defined the larval salt gland in the late pre-nauplii (E-2).

The consecutiveness of these two kinds of osmotic adaptive mechanisms and the production of non-motile nauplii after normal organogenesis in low concentrations were investigated by the determination of Na^+ in cysts, early pre-nauplii (E-1), late pre-nauplii (E-2), nauplii and external glycerol solutions according to the methods described for Procedure B.

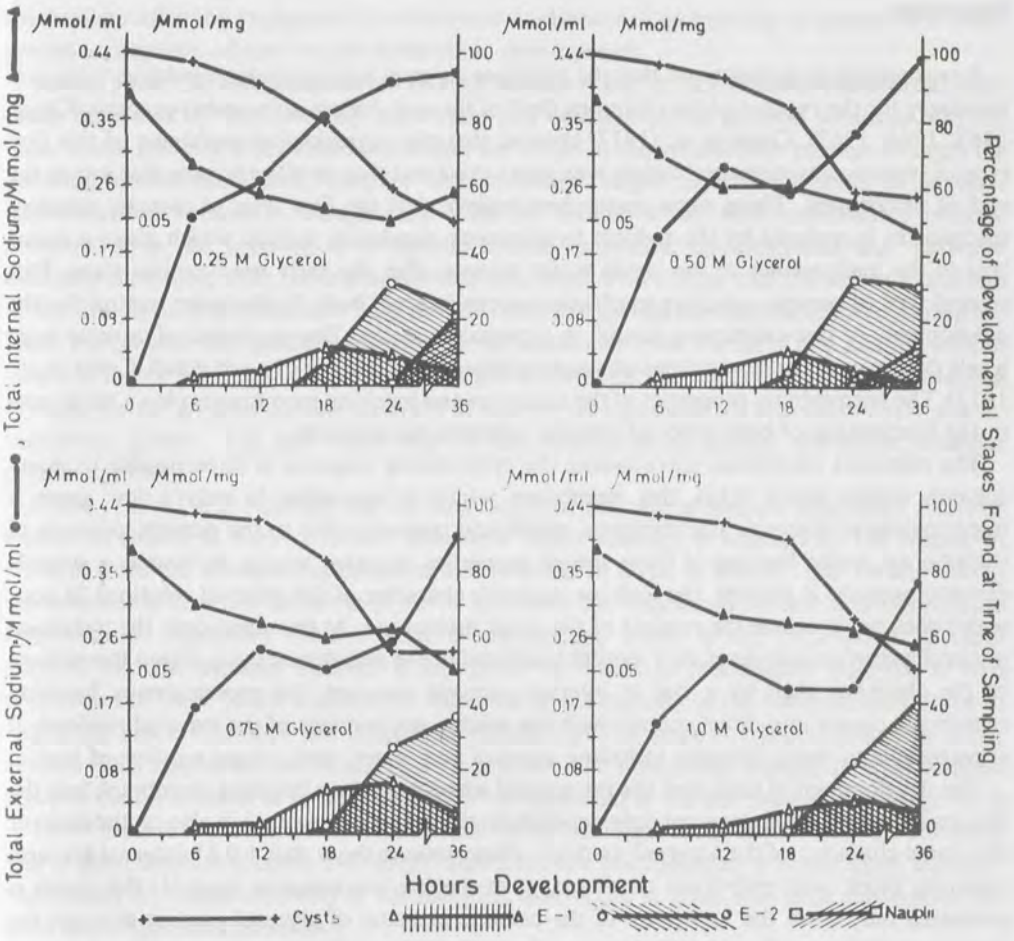

FIG. 3. Internal and external sodium levels during the incubation of cysts.

The quantity of sodium in the dry cysts ranged from a minimum of 0.070 μmol/mg to a maximum of 0.104 μmol/mg (average value 0.087 μmol/mg). This amount of sodium gradually decreased during post-embryonic development while it increased continuously in the external medium. It was interesting to note that the amount of *in vivo* sodium lost during post-embryonic development decreased with an increase in the concentration of external glycerol. An important point of time for changes in the levels of sodium was the period immediately after the rupture of the chitinous membrane and right before the transition to the late pre-nauplii (E-2) stage. Unfortunately, the incubation of the cysts in glycerol solutions precluded the determination of changes in internal and external glycerol levels. Viscous glycerol fills the empty and ruptured chitinous membranes resulting in inaccurate calculations.

Discussion

It was proven in earlier work that the trehalose glycerol hyperosmotic regulatory system is necessary for the rupture of the chitinous shell of the cyst during early embryogenesis (Clegg, 1962, 1964, 1967). Conte *et al.* (1977) showed that the physiological usefulness of this first type of osmotic adaptive mechanism was short-lived and that its effectiveness was lost at the end of excystment. These same researchers believe that the first type of osmotic adaptive mechanism is replaced by the sodium hypoosmotic regulatory system which plays a major role in the maintenance of the larval water balance after the early pre-nauplius stage. This second type of osmotic adaptive mechanism keeps internal body fluids under control for the development of free-swimming nauplii in hypersaline media. The regulation of internal ionic levels during naupliar developmental stages indicates the presence of a salt gland (Conte *et al.*, 1972). The permeability properties of the chitinous and hatching membranes play a major role in the functioning of both types of osmotic adaptive mechanisms.

The chitinous membrane surrounding the cysts during diapause is impermeable to many solvents except water. Also, this membrane which is permeable to only a few gases is impermeable to glycerol. The chitinous membrane responds only to the osmotic pressure of the external media because of these special properties. In other words, as long as a suitable osmotic pressure is present, the ionic or nonionic character of the external solutions (if non-toxic) does not preclude the rupture of the outer membrane. At this time, only the trehalose-glycerol hyperosmotic regulatory system is utilized in the rehydrated cysts. Upon the rupture of the chitinous shell by a rise in internal osmotic pressure, the proteinaceous hatching membrane comes into direct contact with the solutes and solvents of the external medium. It is permeable to many solvents including glycerol and water, and a large number of ions.

The development of early and late pre-nauplii surrounded by a hatching membrane into the free-swimming nauplii stage not only depends on the osmotic pressure but also on the ionic or non-ionic character of the external medium. Observations show that 1.0 M external glycerol solutions block post-embryonic development at the late pre-nauplius stage. If this result is evaluated only from the viewpoint of the osmotic pressure of glycerol passing through the hatching membrane, then the production of non-motile nauplii in low concentrations cannot be explained. If, however, the relationship between the ionic internal body fluids and the non-ionic external medium is considered only with the osmotic pressure factor, then it can be seen that an ionic balance is not established resulting in a continuous loss of internal sodium (and possibly chloride) which finally precludes the post-embryonic development.

Emerson (1967) observed a continuous decrease in the free amino acids of *Artemia* embryos incubated in distilled water. According to this scientist this is related to he absence of the sodium ion along with the ionic composition and osmotic pressure of the incubation medium. The absence of the sodium ion most probably affects the ability of the cell membranes to hold on to amino acids. Sodium ions are necessary for the normal development and survival capacity of *Artemia* embryos.

In addition to sodium, Conte *et al.* (1973) stated the role of the chloride ion as an organizer of protein synthesis in nauplii from *in vitro* studies. At the same time, Conte *et al.* (1977) provided evidence for the relationship between the moment of increase in Na + K- activated ATPase activity and the uptake of sodium during the incubation of the prenauplius. Therefore, the internal embryonic sodium stock must be protected by an appropriate

mechanism after the rupture of the chitinous membrane. The inability to protect this stock results in negative effects on post-embryonic development.

At this point, the following should be emphasized. Water is the solvent of the internal body fluids separated by the hatching membrane and of the external glycerol solutions. On the other hand, glycerol is both the solvent and the solute in the system after passage through the hatching membrane. Early naupliar developmental stages in different external glycerol concentrations behave as though they were in distilled water with controllable osmotic pressure. Also, the production of normal nauplii in natural circumstances (*e.g.* seawater medium) is possible after rupture of the chitinous shell with release into the external medium of the free glycerol accumulated between the chitinous membrane and the embryo during the latent period and with gradual diffusion of the remainder from the early and late pre-nauplii stages to the external environment without glycerol. If this does not occur, then the presence of high internal glycerol concentrations will hamper the transition to the sodium hypoosmotic regulatory system. The end result will be the obstruction of salinity dependent naupliar protein synthesis.

Conte *et al.* (1973) reported the slowing down of the rate of salinity dependent naupliar protein synthesis in high salinities. This slow down reaction is affected by the release of glycerol from the transporting epithelium in function of a rise in salinity. Our results lead to the conclusion that the trehalose-glycerol hyperosmotic regulatory system and the sodium hyperosmotic regulatory system work in consecutive order. If the diffusion of glycerol released into the external medium is hampered by whatever mechanism, then the *in vivo* production of Na-R bonds is impeded. This leads to the inability of the hatching membrane to retain the stock Na^+ and the functional removal of the sodium hypoosmotic regulatory system. This results in the production of normal, but non-motile nauplii in low external glycerol concentrations or to the blocking of development at the late pre-nauplius stage (E-2) in 1.0 M glycerol concentrations. In embryos left at this stage, death occurs after the complete loss of total internal sodium. No doubt, this observed phenomenon is much more complex and studies on the permeability of the hatching membrane to other non-ionic substances will shed more light on this subject.

Although it is difficult to be more precise at this stage, it seems probable that the effect of all non-ionic substances upon the early naupliar phases surrounded by a hatching membrane is similar. For example, the mortality, within a short period of time, of adults placed in mannitol solutions isotonic to hemolymph was explained by Croghan (1958) as the loss of ions, in analogy to the situation in distilled water. The importance of glycerol is that it is not a foreign compound for *Artemia* embryos. Since ionic loss from late pre-nauplii (E-2) is prevented in glycerol solutions, this method will permit the investigation of the metabolism of post-embryonic developmental stages in a more synchronized manner.

Summary

1. The effects of external glycerol concentrations on *Artemia* embryos were investigated.

2. The period required for the rehydration of cysts and the rupture of the chitinous membrane increased in correlation with an increase in glycerol concentrations. The chitinous membrane responded only to the osmotic pressure of the external media.

3. After the rupture of the chitinous membrane there was a rise in external sodium with a consequent decrease in internal sodium levels.

4. The continuous loss of *in vivo* sodium led to the formation of non-motile nauplii in low glycerol concentrations and to the blocking of post-embryonic development at the late pre-nauplii stage (E-2) in 1.0 M glycerol. Possibly, the loss of the sodium stock led to the disappearance of the sodium hypoosmotic regulatory system. In turn, salt-dependent protein synthesis did not occur resulting in the abnormalities mentioned above.

5. Although glycerol is interchanged with water in various intracellular phases and protected from molecular inactivation, it can prevent post-embryonic development at high concentrations. For this reason, after the rupture of the chitinous membrane during the normal incubation period, the external medium must consist of sea water in order to lower the internal embryonic concentration.

Acknowledgements

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Environmental physiology of salt tolerance in an alkaline salt lake population of *Artemia* from Mono Lake, California, U.S.A.

David B. Herbst[1] *and Gayle L. Dana*[2]

[1] *Department of Zoology, Oregon State University*
Corvallis, Oregon 97330, USA

[2] *Department of Biological Sciences, San Francisco State University*
1600 Holloway Avenue, San Francisco, California 94132, USA

Abstract

Artemia monica was subjected to varying concentrations of Mono Lake water, ranging from diluted lake water (25%) through unconcentrated lake water (100%) to three times concentrated lake water (300%). Survival limits, respiration rate and internal solute regulation were monitored as salt concentrations increased. The results indicate the following :

1. For both sexes, mortality increased significantly at and above 200% Mono Lake water. Males experienced higher mortality than females in dilute lake water, while the reverse was true at concentrations above 200%.

2. Change in respiration rate with increased concentrations differed between females and males. Respiration rate of female shrimp remained fairly stable until 200% whereas male respiration rate was maintained at 200%. In both females and males, respiratory rate functions were lost at 300%.

3. *Artemia* demonstrated hyposmotic regulatory abilities at lower concentrations. Above 200% lake water internal solute homeostasis breaks down and concentrations increased 20-fold by 300% salinity.

Other studies on increased salt effects have used media where Na^+ and Cl^- are the only ions increased in concentration. Responses observed in our study may differ due to the inclusion of the lesser ions which may act synergistically with other major and minor ions when increased. The concentration of all ions more closely approximates what shrimp experience in environmental salinity changes.

We present here data which demonstrate potential physiological responses to a real situation. Mono Lake is an endangered ecosystem due to diversion of its influent fresh water to Los Angeles, California for municipal use. Declining water levels over the past 40 years has caused an almost two-fold increase in the ionic concentration of the lake. Limnological data suggest that concentrations in Mono Lake may reach the physiological limits (as defined in this research) of the *Artemia* in the next 25 years. The extinction of *Artemia* from Mono Lake would have devastating effects on many species of birds that use the lake as a breeding ground and migratory fuel stop with the shrimp as their primary food source. Due to habitat isolation, the Mono Lake population (*Artemia monica*) is reproductively isolated from other *Artemia* populations (reviewed by Bowen *et al.*, 1978). This research provides some of the first information on the physiology of this sibling species.

Introduction

The variety of aquatic habitats in which *Artemia* occurs may play a significant role in the expression of phenotype related to salt adaptation in different chemical environments. The following experiments were thus initiated to determine the effects of increased salinity on the physiology of brine shrimp (*Artemia monica*) found in the alkaline Mono Lake, California. Acute toxicity LC-50 bioassays provided information on survival in lake water of different salinities. Oxygen consumption and water-solute regulation were measured after a period of acclimation and indicate the effect of salinity on metabolic activity and osmotic homeostasis. This information may in addition provide a basis for predicting the ability of this population to survive and develop in an environment subject to increasing salinity.

Mono Lake is an unusual body of inland salt water in that it is a deep lake (most high desert lakes are characteristically shallow) and thus relatively stable to changes in elevation and salinity which might arise from short term fluctuations in water budget. However, due to diversion of influent freshwater streams to the Los Angeles aqueduct, the salinity of the lake has gradually increased for the past 40 years to approximately twice that of the lake prior to diversions (salinity continues to increase at a rate of about 2-5 % per year). The high alkalinity of Mono Lake is imparted by carbonates and borates which buffer the lake water at a pH of around 10. In addition, this is a rare «triple-water» lake, having as its main anionic constituents Cl^-, HCO_3^-/CO_3^{--} and SO_4^{--}, combined as mixed sodium salts to around 1.3 M. The current total dissolved solids content of the lake is 96.4 g/l.

Croghan (1958a) and Conte (1972) have shown that both the adult and naupliar stages of *Artemia* are effective hyposmotic regulators over a wide range of salinities. Croghan (1958b) has been able to adapt brine shrimp to a crystallizing seawater brine in the laboratory and field collections have been made in the salt-saturated waters at the Great Salt Lake, Utah. The nauplii, however, appear to be less tolerant of salinity increase as mortality increases sharply above 2.0 M NaCl.

Among the changes in *Artemia* induced by salinity increase are failure of emergence from the cyst above 2.0 M NaCl external concentration (Clegg, 1964), decreased protein biosynthesis (Conte, 1973), and an activation of the salt-regulating Na-K ATPase (Augenfeld, 1969). The demand for ATP in a salt-stressed shrimp would be expected to be reflected in an increased respiration rate. However, Gilchrist (1958) has reported no change for females ; males appear to consume more oxygen at low salinity than at high due to morphological differences.

Of particular interest is the work of Bowen (1964, 1978) and Bowen *et al.* (1980) showing the reproductive isolation by habitat of the Mono Lake population of *Artemia*. Upon being transferred to the media in which other populations of brine shrimp under study were cultured, Mono brine shrimp died. Conversely, the other groups of brine shrimp died upon being transferred to Mono Lake water. This work suggests that both the lake and the organism are unique and that salt adaptation has become specialized to Mono's peculiar conditions.

Previous work by Herbst and Dana (1977) on the physiology of Mono Lake brine shrimp show high mortality at and above 200 % (of 1976) salinity accompanied by declining respiration and increasing tissue chloride content.

Methods

Acute toxicity salinity tolerance bioassays

Animals were collected the same day as they were used to begin LC-50 bioassays from shallow, well-mixed water off the south shore of the lake. Only the most active adult individuals were chosen. Two replicates of approximately 75 mixed male and female brine shrimp were followed at each salinity, in 150 ml of the test media at 15 °C ± 3 °C aerated each 24 hr. Water used in all experiments was prepared from coarse filtered lake wate diluted with distilled water or evaporated by boiling to the following salinities by volume : 25% (600 mOsM), 50% (1 300 mOsM), 100% (2 500 mOsM = filtered 1979 lake water), 150% (3 800 mOsM), 200% (4 900 mOsM) and 300% (7 000 mOsM). These are presented in the data as osmotic concentration (mOsM), determined by previous calibration with specific gravity. In addition to the above lake water salinities, one bioassay was done in distilled water and another, used to control for the effect of boiling, was prepared by reconstituting 300% lake water to the specific gravity of 100% lake water with distilled water. Studies of the effect of boiling on ionic proportions in solution show no enrichment or depletion of chloride and insignificant change in pH (little change in CO_3^{--}/HCO_3^- ratio), though the effect on sulphate and borate is unknown (Herbst, unpublished observations). The criterion for death in *Artemia* was cessation of the rhythmic swimming motion of the phyllopods.

Respiration

Following 48 hr exposure to each salinity, separate groups of 20 male and 20 female brine shrimp were placed in 250 ml closed-system respirometer flasks containing well-aerated water at 600, 1 300, 2 500, 3 800, and 4 900 mOsM. Dissolved oxygen content was then measured before and after a 4 hr period of exposure by the Miller method (Walker *et al.*, 1970). Control flasks without brine shrimp were also run at each salinity. Tissue wet weight and dry weight were determined to ± 5 mg following each experiment and the data used to calculate oxygen uptake on a dry weight basis ($Q_{O_2} = \mu lO_2/min/g$ dry wt). Due to high mortality within 48 hr at 300% salinity, Q_{O_2} at 300% was calculated from the 24 hr acclimated data of Herbst and Dana (1977).

Water and solute regulation

The proportion of water constituting the total body weight and the chloride content of the tissues were used in this study to determine the extent of osmotic homeostasis at different salinities. The assumptions of this method are that chloride is entirely extracellular (*i.e.* in the haemolymph) and that the entire water content of the tissues represents extracellular fluid volume. As the former assumption is probably more valid than the latter, this method will thus tend to underestimate actual haemolymph chloride content. Croghan (1958a) has shown that NaCl is the principal osmotic solute of *Artemia* and that haemolymph volume accounts for most of the total body water, thus the assumptions made here are justifiable.

After a 48 hr period of acclimation at salinities of 600, 1 300, 2 500, 3 800, and 4 900 mOsM (not done at 7 000 mOsM since few survive to 48 hr), the brine shrimp were rinsed thoroughly with distilled water and after blot-drying on tissue paper for 5-10 min wet weight

was measured to ± 5 mg. Following oven drying for 12 hr at 70 °C the dry weight was determined and the difference from wet weight taken as total body water (assuming 1 g weight = 1 000 μl volume). To determine tissue chloride the dried tissue was homogenized in 1 ml of 3% hydrogen peroxide and centrifuged at low speed for 10 min. Aliquots of the supernatant were then titrated using a Beckman/Spinco microtitrator to deliver and mix microliter quantities of mercuric titrant by the method of Schales and Schales (1941).

Results

LC-50 BIOASSAY

Conversion of percent mortality data to probits and estimation of LC-50 times from these statistical conversions are presented in Fig. 1 and 2 and Table I. These data indicate a salt tolerance threshold somewhere between 4 900 and 7 000 mOsM salinity as the onset of mortality is rapid at 7 000 mOsM and occurs only more gradually at 4 900 mOsM. It is clear from LC-50 data in Table I that females succumb earlier at these higher salinities than males. The lower lethal limit to survival is apparent in distilled water, with males showing a greater sensitivity to conditions which doubtless dilute the body fluids and make osmotic homeostasis virtually impossible.

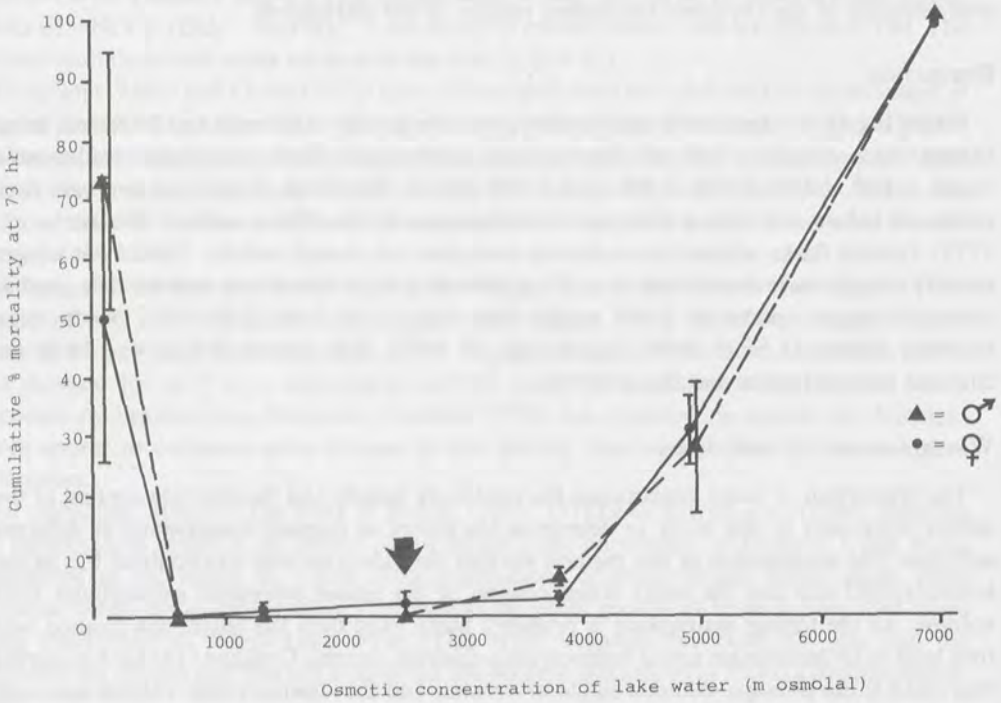

FIG. 1. Cumulative mortality after 73 hr. Tolerance limits of Mono Lake *Artemia* are clearly displayed in this plot. Error bars indicate standard error between two replicate bioassays at each salinity (error bars shifted slighty to right for males when overlap occurs). Arrow on osmotic concentration axis refers to the 1979 salinity of Mono Lake water, as the control standard of these experiments.

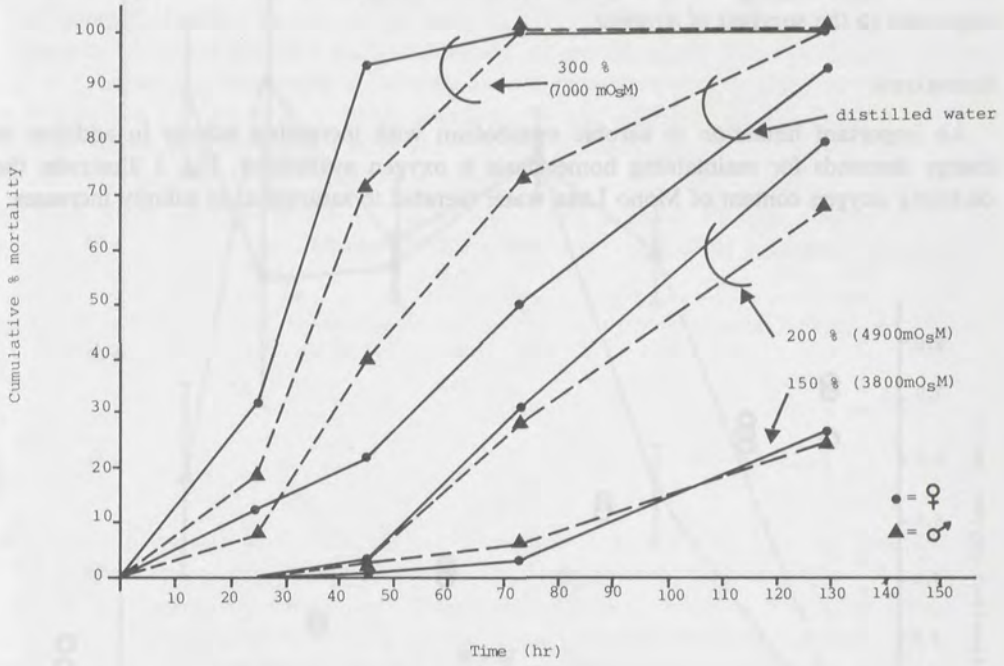

FIG. 2. Mortality rates at tolerance limits. Differences in death rate between the sexes is emphasized in this plot of upper and lower lethal limits. Cumulative percent mortality is averaged for two replicates at each salinity shown. Insignificant rates of mortality at all other salinities (25%, 50%, 100%, and 100% boiling control).

TABLE I
LC-50 levels

Osmolality	% salinity	LC-50 times (hr)	
		Females	Males
0	Distilled water	73	56
3 800	150	156	152
4 900	200	104	115
7 000	300	29	37

Values listed in this table determined from statistical transformation of percent mortality to probits. Salinities between distilled water and 150% (including 100% boiling control) had insignificant mortality levels, survival being > 95% in all cases.

The close similarity of the boiling control to untreated lake water indicates that boiling has no effect on changing the ionic proportions and composition of lake water which are important to the survival of *Artemia*.

Respiration

An important limitation to aerobic metabolism with increasing salinity in addition to energy demands for maintaining homeostasis is oxygen availability. Fig. 3 illustrates the declining oxygen content of Mono Lake water (aerated to saturation) as salinity increases.

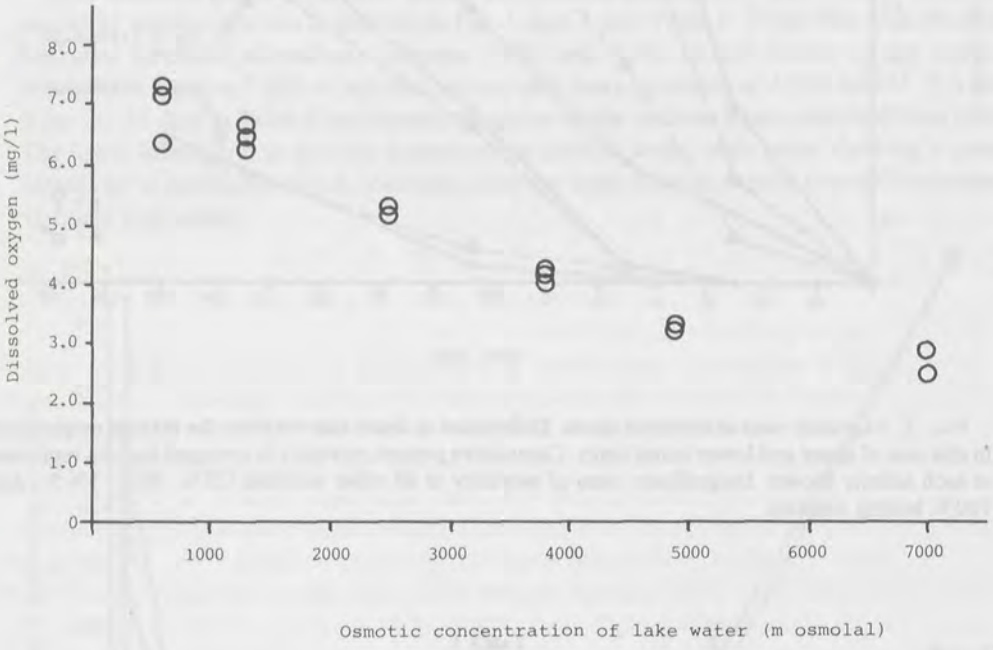

Fig. 3. Dissolved oxygen content of lake water at different salinities. Decreasing oxygen availability with increased salinity is shown in this graph. Lake water of different salinities was aerated thoroughly (metaframe air pump) for 15-30 min and aliquots taken for dissolved oxygen determination by titration using the Miller method (see text).

The rates of oxygen consumption at different salinities are graphed in Fig. 4 and indicate that female shrimp respire at a consistantly high, active rate from 600-3 800 mOsM salinity but decline rapidly at and above 4 900 mOsM salinity. Male shrimp seem to show a mirror image of this relationship in that at the low salinity of 600 mOsM, respiratory activity is low, increasing thereafter to a high level which is maintained at 4 900 mOsM, though again metabolic activity is all but lost at 7 000 mOsM.

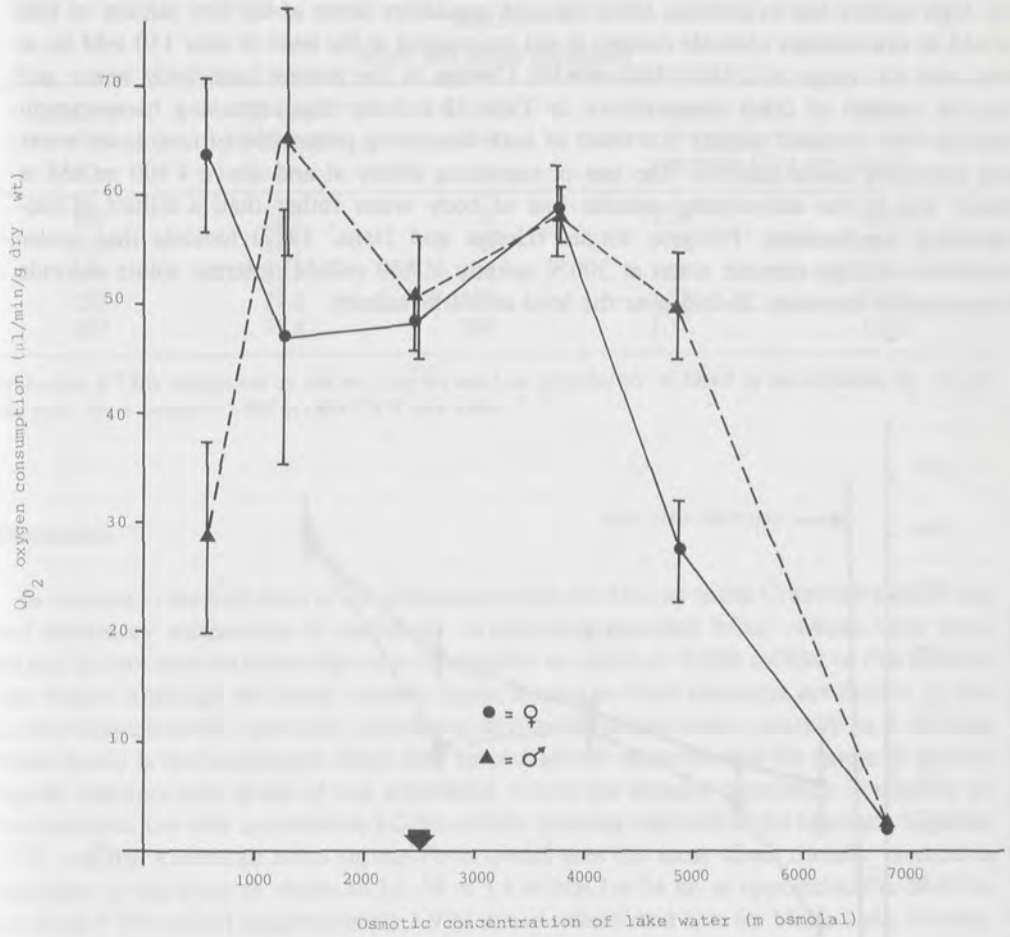

FIG. 4. Respiration at different salinities. Oxygen consumption of male and female brine shrimp after 48 hr acclimation at each salinity as measured by the difference in dissolved oxygen of a closed volume of water after 4 hr exposure. Present day Mono Lake water at 2 500 mOsmolal concentration. Error bars represent pooled standard deviation for three titration replicates at each before and after D.O. determination of the 4 hr period. The final respiration points at 6 800 mOsmolal represent 24 hr acclimation of brine shrimp (converted from data of Herbst and Dana, 1977). Arrow on osmotic concentration axis refers to the 1979 salinity of Mono Lake water.

SOLUTE REGULATION

Fig. 5 illustrates the ability of both male and female brine shrimp to hyposmotically regulate chloride content of body fluids over a wide range of salinity at around 150 mM Cl^-. Females show a gradual increase in haemolymph chloride from 600 mOsM to 3 800 mOsM, increasing more rapidly at 4 900 mOsM and perhaps indicating the beginning of the loss of effective salt regulation. Male brine shrimp also show a loss of solute-chloride homeostasis at

this high salinity but in addition show signs of regulatory stress at the low salinity of 600 mOsM as extracellular chloride content is not maintained at the level of near 150 mM (as is seen over the range of 1 300-3 800 mOsM). Change in the percent total body water and chloride content of dried tissue shown in Table II indicate that increasing haemolymph chloride with incrased salinity is a result of both decreasing proportion of total body water and increasing tissue chloride. The loss of regulating ability at and above 4 900 mOsM is clearly due to the dehydrating osmotic loss of body water rather than a failure of ion-regulating mechanisms. Previous studies (Herbst and Dana, 1977) indicate that under conditions of high osmotic stress at 300% salinity (6 800 mOsM) internal solute chloride concentration increases 20-fold over the level at 100% salinity.

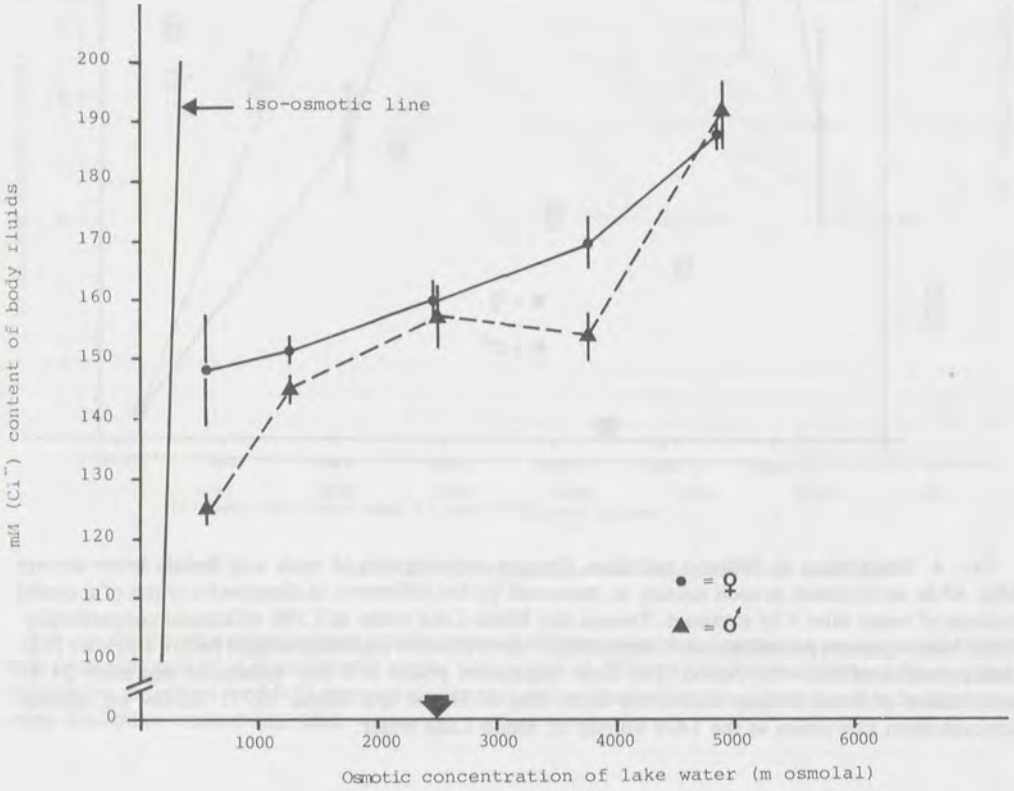

FIG. 5. Chloride-solute regulation. Osmotic homeostasis as measured by the major extracellular solute chloride ion. Iso-osmotic line assumes all haemolymph solutes as Na^+/Cl^-. Error bars indicate the standard deviation in four replicate titrations for chloride (see text) from a single brine shrimp homogenate (of each sex) at each salinity. Arrow on osmotic concentration axis refers to the 1979 salinity of Mono Lake water.

TABLE II

Water and solute regulation

Salinity (%)	% total body water	Salinity (%)	Females	Males
			(mmoles Cl/g dry tissue)	
100	84.7	25	1.04	0.88
150	84.7	50	1.10	0.91
200	81.8	100	1.24	0.94
250	79.2	150	1.27	1.00
300	75.4	200	1.12	1.00

Proportion of TBW determined by wet wt – dry wt and total chloride/dry wt found by microtitration for chloride (see text). 100% salinity = 2 500 m OsM (1979 lake water).

Discussion

In contrast to the high level of salt tolerance shown by *Artemia* in the Great Salt Lake, Utah and laboratory acclimation of shrimp to "crystallizing seawater brine", Mono Lake brine shrimp do not survive acute exposure to salinities in excess of 5 000 mOsM of this alkaline lake water. Although the direct transfer methods used in these bioassays are limited in that genetic adaptation to a gradually increasing salinity (as would occur naturally in a receding Mono Lake) is not considered, these data do provide an assessment of the range of physiological tolerance and limits of salt adaptation which the present population is capable of. Furthermore, the only quantitative LC-50 salinity bioassay reported in the literature (Geddes, 1975 ; on the Australian brine shrimp *Parartemia*) uses the same direct transfer method as employed in this study to obtain an LC-50 of 3.4 M NaCl at 54 hr, as opposed to the 29-37 hr LC-50 at 7 000 mOsM (approximately 3.9 M mixed salts) found here for Mono Lake *Artemia*.

The sub-saturation salt tolerance limits shown by *Artemia* from Mono Lake may be a result of osmotic stress imposed by the diverse composition of dissolved solutes in this lake. In light of the work of Bowen (1964) and of Bowen *et al.* (1980) which seems to identify Mono Lake water as essential to the prolonged survival of Mono Lake brine shrimp and lethal to shrimp from other source populations, it is important to consider that survival in this complex chemical medium may be quite different from the NaCl/seawater and artificially prepared media used in other salt tolerance studies.

The response of oxygen uptake to increased salinity should reflect energy required to maintain homeostasis and outside this zone of tolerance respiratory rate functions should fall and eventually be lost entirely. Much of this aerobically obtained energy will probably be channeled into power for the operation of salt regulating enzyme pumps. Indeed, the work of Reeves (1963) indicates that optimum growth efficiency for *Artemia* occurs at salinities below those considered presently. Decreased growth efficiency at higher salinities may be due to an "increased proportion of assimilated food being appropriated for osmotic work".

Respiration data reported in the present study are consistant with the same pattern as obtained in earlier work (Herbst and Dana, 1977) of a substantially lowered rate of oxygen

consumption at high salinity (4 900 mOsM) among females with males maintaining a high rate. The higher death rates of females in this upper salinity range is probably associated with this observed loss of respiratory function. As Gilchrist (1958) has pointed out, the ability of males to consume oxygen at a greater rate than that of females is probably related to the presence of the large, flattened prehensile antennae which give the males a greater relative surface area over which respiratory gas exchange can occur. However, at low salinity (600 mOsM), aerobic metabolism of males is impaired, which may account for the earlier mortality seen for males in distilled water. A greater sensitivity of males to these hypertonic conditions is further substantiated by their loss of osmotic homeostasis at 600 mOsM (Fig. 5).

An important physical change in the lake water which may accompany a receding lake level is an increase in the temperature of the epilimnion of a shallower lake. In addition to further decreasing the solubility of oxygen, increased temperature acts synergistically with increased salinity to lower the lethal limit of salt tolerance in *Parartemia* (Geddes, 1975). Preliminary studies with Mono Lake *Artemia* in water which varied from 18 °C-27 °C produced within 18 hr 100 % mortality in 300 % salinity and approximately 50 % mortality at 200 % salinity. This substantiates a similar lethal synergism between salinity and temperature for Mono Lake shrimp and also accounts for the somewhat higher mortality rates seen in earlier studies (Herbst and Dana, 1977) as these were conducted at a room temperature of 20 °C-23 °C (rather than the 15 °C of the present study).

In conclusion, the high mortality noted at the upper limit of salt tolerance can probably be attributed to a loss of osmotic homeostasis and decreased consumption and availability of oxygen. This work suggests that physiological constraints on survival and development of *Artemia* will accompany an increasingly saline Mono Lake. The extent to which the productivity of this population will be affected can only be predicted through an experimental consideration of how a changing bioenergetic budget to accomodate increasing osmoregulatory costs will limit growth and reproductive development.

Acknowledgements

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The effect of temperature on the biochemistry of the brine shrimp *Artemia* during development

Harry B. Hines[1,3], *Brian S. Middleditch*[1], *and Addison L. Lawrence*[2]

[1] *Department of Biophysical Sciences, University of Houston*
Houston, Texas 77004, USA

[2] *Texas A & M University Agricultural Research and Extension Center*
P.O. Box 10607, Corpus Christi, Texas 78410, USA

Abstract

Brine shrimp cysts were incubated at different temperatures (5, 10, 15, 18, 25, and 28 °C) to determine if the biochemistry is altered by temperature. The relative fatty acid composition, % protein, carbohydrate, and lipid levels were measured for cysts and starved larvae.

Samples of cysts and larvae were collected from prior to hatching up to 5 days after hatching. No food was available to the hatched larvae. Dry, unincubated cysts were also analysed to yield "control" values. All samples for fatty acid analyses were from the same batch. The relative percentages of the fatty acids were calculated from the peak heights of the components.

There were few changes in the relative percent levels of fatty acids extracted from dry cysts and those from incubated cysts. Variations in levels were observed in starved larvae, but these changes appear to be the result of starvation rather than temperature. Small differences were observed in the % protein, carbohydrate, and lipid levels for incubated cysts and starved larvae incubated at 28 °C and 18 °C.

Introduction

One of the key requirements for the culture of aquatic organisms (*e.g.* fish and crustaceans) is a manageable and nutritionally adequate source of food. The nauplii of the brine shrimp *Artemia* satisfy these requirements and have been used for the culture of fish and crustacean larvae (May, 1970 ; Mootz and Epifanio, 1974 ; Schleser and Tchobanoglous, 1974 ; Weaver, 1974). However, the effectiveness of this approach, especially for marine organisms, has been questioned due to nutritional deficiencies in the cultured organism (Dannevig and Hansen, 1952 ; Morris, 1956 ; Watanabe *et al.*, 1978).

To overcome these problems, efforts have been made to improve the nutritional quality of *Artemia* larvae for marine organisms by feeding them various types of algae. Wickins (1972) fed larvae of the Great Salt Lake strain of brine shrimp with live algae (*Isochyris galbana*) for a maximum of 4 days to convert the larvae to a more useful form for the larvae of a prawn (*Palaemon serratus*). Watanabe *et al.* (1978) demonstrated that the essential fatty acids of

[3] Present address : Biomedical and Environmental Mass Spectrometry Resource, Space Sciences Laboratory, University of California, Berkeley, California 94720, USA.

Artemia are the principal factor in the food value of fish, both marine and freshwater. This is in agreement with the findings of Wickins (1972).

Another possible method for altering the fatty acid composition of *Artemia* would involve culture at low temperatures. Chapelle (1978) found that lowering acclimation temperatures increased the amount of monounsaturated and polyunsaturated fatty acid constituents in a marine crab (*Carcinus maenas*) when starved for 2 weeks. The fatty acids of the phospholipids were especially affected. Similar results were obtained by Ueda (1974) with the short neck clam (*Tapes philippinarum*). However, the clams remained in their natural environment, and the observed effects upon the fatty acids could not be attributed solely to temperature.

In the present study, the effect of temperature on the biochemistry of San Francisco Bay brine shrimp was investigated to note any changes which might occur at different temperatures. Such changes, if favorable, might enhance the nutritional value of brine shrimp larvae without requiring algal feeds.

Materials and methods

Dry San Francisco Bay cysts were purchased from commercial suppliers (Metaframe and Living World). In all instances, artificial seawater (Instant Ocean) of 28 ‰ salinity was used. All solvents were "Nanograde" quality (Mallinckrodt) with the exception of diethyl ether (USP), sodium hydroxide, and hydrochloric acid (Fisher, reagent grade). Samples were exposed to only PFTE, glass, and aluminum.

General biochemical analyses

Cysts were incubated in duplicate at 18 °C and 28 °C. Samples were collected at 12, 24, and 36 hr for the incubations at 18 °C and at 6, 12, and 18 hr for those at 28 °C. Larvae incubated at 28 °C were collected at 3 hr intervals up to 24 hr after hatching, and every 6 hr up to 48 hr after hatching when incubated at 18 °C. Harvesting was accomplished by filtration through fine mesh cloth.

Samples were thoroughly rinsed and dehydrated in a frostless freezer and then over anhydrous calcium sulfate under a vacuum until a constant weight was achieved. Percentage water was then calculated from the initial and final weights.

Carbohydrate levels were measured by the method of Dubois *et al.* (1956). Ten mg of dry tissue was extracted with 10 ml of 5 % trichloroacetic acid by refluxing for 1 hr. Two ml were taken for analysis by spectrophotometry (490 nm, Bausch and Lomb Spectronic 20). Values were obtained by comparing against a standard curve of D-glucose.

Protein levels were determined by the method of Lowry *et al.* (1951). Five mg of dry tissue was digested in 5 ml of 1 M sodium hydroxide for 48 hr. An aliquot of 0.05 ml was subjected to spectrophotometric measurement at 660 nm. The protein content was obtained by comparison with a reference curve using bovine serum albumin as the standard.

Total lipid content was evaluated by a modification of the method of Bligh and Dyer (1959). Approximately 120 mg of dry tissue was extracted with 25 ml of chloroform-methanol (2:1) at 60 °C for 15 min. Twenty ml of the supernatant was filtered and the filtrate mixed with 4 ml of distilled water. After separating for 24 hr, the aqueous phase was removed and the remaining phase heated to dryness at 60 °C under a constant stream of air. The residue was weighed on a Mettler analytical balance.

FATTY ACID ANALYSES

Dry cysts were incubated a 5, 10, 15, and 25 °C. In all cases, these cysts were from the same batch (no. 2987, Living World). Experiments were performed in triplicate at each temperature with the exception of the duplicate experiments at 10 °C. Triplicate analyses of dry cysts were also done. Two fiberglass tanks (schematically represented in Fig. 1) were used to culture larvae. No food was made available to the nauplii.

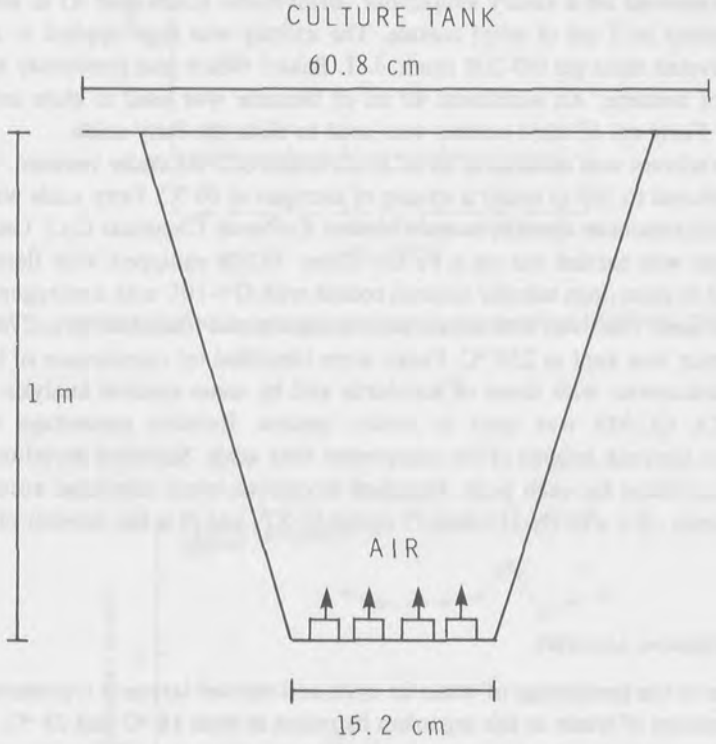

FIG. 1. Schematic representation of the incubation tanks.

Fifteen to 20 g of dry cysts were placed in each tank containing 32 l artificial seawater. Lighting regimens of 24 hr dark/24 hr light or 16 hr dark/8 hr light were used. Twenty-four hr following addition of cysts to the tanks, 5 to 10 g of dry cysts were placed in 6 l Erlenmeyer flasks containing 6 l of seawater and incubated under the same conditions as the tanks. Samples of incubated cysts were obtained from these flasks by noting the time of first hatching in the tanks and harvesting the flasks approximately 22 hr later. Samples of larvae were taken from the tanks on days 3 and 5 following hatching.

Harvesting the larvae was accomplished by bringing a light source near the bottom of the tank after darkening the room and stopping the aeration. Larvae were moving to the bottom of the tank by their positive phototactic behavior while unhatched cysts and hatching debris floated to the surface of the water. Approximately 12 l of water was then drawn from the

bottom of the tank through a valve. Harvested larvae were filtered as described, rinsed, dried by blotting, and finally weighed.

Samples (4 to 5 g wet weight) were homogenized and then saponified using sodium hydroxide (4 ml, 4 M) and heating at 110 °C for 2 hr. Nonsaponifiables were removed by extracting twice with 20 ml of diethyl ether. Fatty acids were removed by acidifying the aqueous phase with concentrated hydrochloric acid (3 ml) and again extracting with diethyl ether (2 × 20 ml).

Ether was removed on a rotary evaporator (Brinkmann Rotovapor R) at 60 °C and the sample redissolved in 2 ml of ethyl acetate. The extract was then applied to a 20 × 1 cm column of activated silica gel (60-200 mesh, J. T. Baker) which had previously been washed with 100 ml of benzene. An additional 40 ml of benzene was used to elute any non-polar contaminants. Forty ml of ethyl acetate was used to elute the fatty acids.

The organic solvent was reduced at 60 °C to a volume of 1 ml under vacuum. This volume was further reduced to 100 μl under a stream of nitrogen at 60 °C. Fatty acids were esterified using dimethylformamide dimethylacetal (Methyl 8, Pierce Chemical Co.). Gas chromatographic analysis was carried out on a Perkin-Elmer 3920B equipped with flame ionization detectors. A 20 m glass open tubular column coated with OV-101 with a nitrogen flow of 1 to 2 ml/min was used. The oven was temperature-programmed from 100 °C to 270 °C at 4 °C/min. The injector was kept at 250 °C. Peaks were identified by comparison of the retention times of the unknowns with those of standards and by mass spectral analysis. A Hewlett-Packard 5992A GC-MS was used to obtain spectra. Relative percentage values were calculated from the peak heights of the component fatty acids. Standard deviations and mean values were calculated for each peak. Standard deviations were calculated according to the following formula : $S = \sqrt{D/(N-1)}$ where D equals $(x-\bar{X})^2$, and N is the number of data points.

Results

General biochemical analyses

The increase in the percentage of water in cysts and starved larvae is represented in Fig. 2. Overall, the amount of water in the organism increases at both 18 °C and 28 °C. However, it requires twice the time for the organism at 18 °C to reach a point (87%) only sightly below that of the animals incubated at 28 °C (90%) for the entire experiment. The sharp drop in the percentage of water at hatching (0 hours) is presumably due to loss of the shell after which the amount of water continues to increase in the larvae.

Fig. 3 through 6 represent the biochemical data obtained for cysts and starved larvae at 28 °C and 18 °C. Fig. 3 contains data at 28 °C as a percentage of the sample taken. Total lipids and carbohydrates decrease throughout the incubation period, whereas total proteins remain relatively constant. Furthermore, there appears to be an increase in protein levels immediately preceeding hatching. On a individual animal basis, the decreases in total lipids and carbohydrates are more evident (Fig. 4). In contrast, the decline in lipid and carbohydrate levels at 18 °C is not as rapid as that at 28 °C on a percentage basis (Fig. 5) or on an individual basis (Fig. 6). As with hydration, the rate of decrease is slower at 18 °C, requiring twice the time to reach approximately the same levels as those obtained at 28 °C. Also total protein levels exhibit a sharp decline at 18 °C on an individual animal basis but appear to be only slightly reduced on a percentage basis.

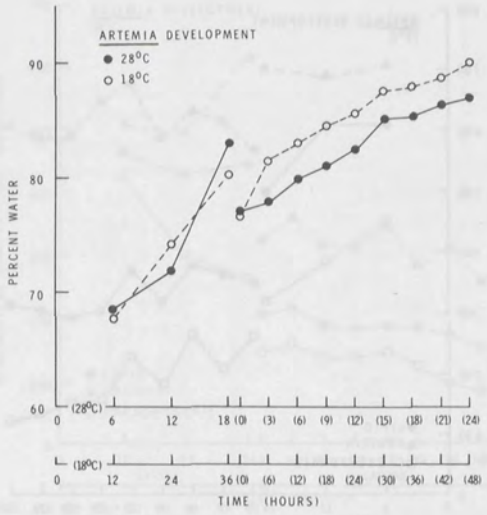

FIG. 2. Percentage of water of cysts and larvae incubated at 18 °C and 28 °C.

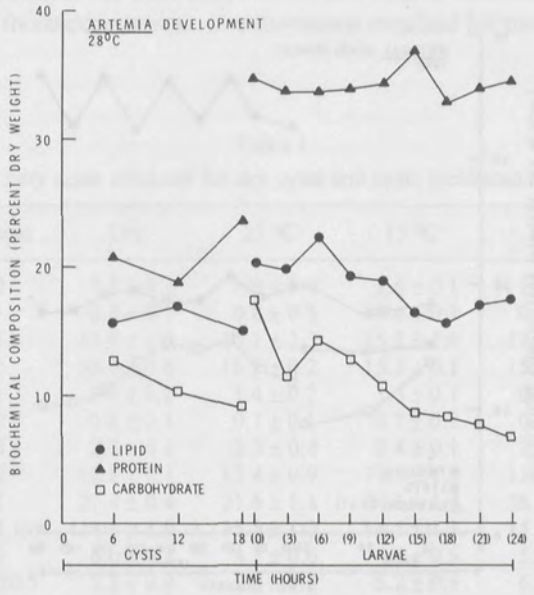

FIG. 3. Percentage levels of the biochemical constituents of cysts and larvae incubated at 28 °C.

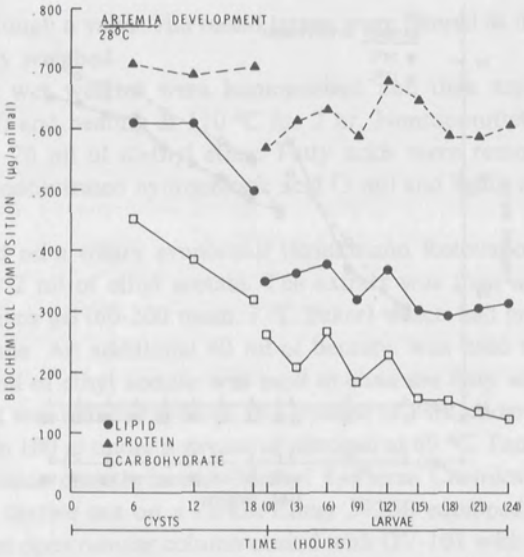

FIG. 4. Biochemical constituents of cysts and larvae presented on an individual animal basis (28 °C).

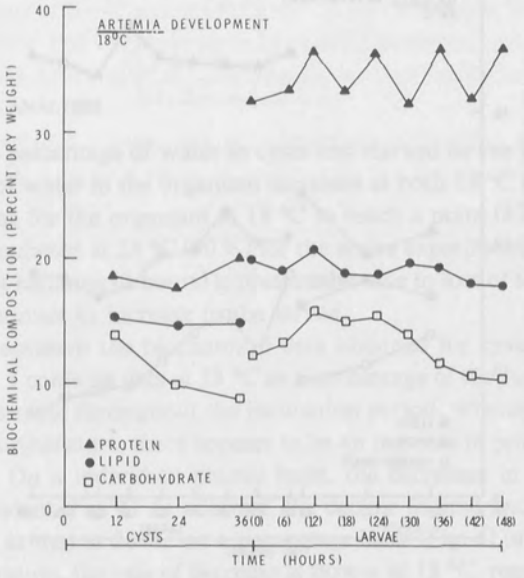

FIG. 5. Percentage levels of the biochemical constituents of cysts and larvae incubated at 18 °C.

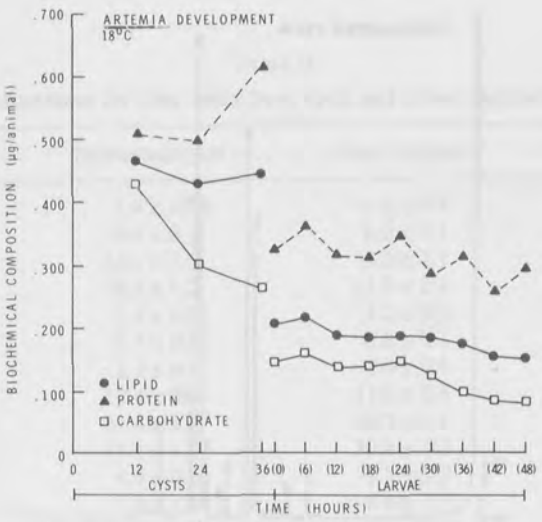

FIG. 6. Biochemical constituents of cysts and larvae presented on an individual animal basis (18 °C).

TOTAL FATTY ACID ANALYSES

Fig. 7 is a gas chromatogram of the fatty acid methyl esters (FAME) of the dry, unincubated cysts. The numbered peaks are identified in Table I. This chromatogram is representative of all gas chromatograms obtained for each temperature and time of collection. Tables I through V contain the results of those experiments, and the values obtained for the dry cysts are included in Table I.

TABLE I

Relative percentages of fatty acids obtained for dry cysts and cysts incubated at different temperatures

Peak no.	Fatty acid	Dry	25 °C	15 °C	10 °C	5 °C
1	14:0	1.5 ± 0.1[a]	1.9 ± 0.3	1.6 ± 0.1	1.3 ± 0.1[b]	1.6 ± 0.1
2	15:1	0.5 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.0
3	16:1	11.9 ± 1.3	15.1 ± 2.3	15.2 ± 1.0	13.3 ± 1.3	16.3 ± 0.4
4	16:0	15.5 ± 0.6	16.3 ± 0.2	15.3 ± 0.1	15.6 ± 0.1	15.5 ± 0.6
5	17:1	1.1 ± 0.0	1.4 ± 0.2	1.3 ± 0.1	0.7 ± 0.6	1.3 ± 0.1
6	17:0	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.0
7	18:3	2.5 ± 0.1	2.3 ± 0.4	2.4 ± 0.1	2.4 ± 0.0	2.5 ± 0.3
8	18:2	11.8 ± 0.1	12.4 ± 0.9	13.3 ± 1.0	11.5 ± 1.4	12.9 ± 2.3
9	18:1	27.4 ± 0.4	21.6 ± 1.1	24.1 ± 1.4	26.6 ± 0.4	23.5 ± 0.8
10	18:1 iso	14.9 ± 1.0	15.1 ± 1.0	14.5 ± 1.1	15.7 ± 0.6	14.7 ± 1.7
11	18:0	6.0 ± 0.2	4.5 ± 0.6	4.5 ± 0.6	5.2 ± 0.8	4.3 ± 1.0
12	20:4 + 20:5	5.8 ± 0.0	5.0 ± 1.2	6.2 ± 0.6	6.3 ± 0.1	5.8 ± 0.2

[a] Mean ± standard deviation : three observations.

[b] Two observations (n = 2).

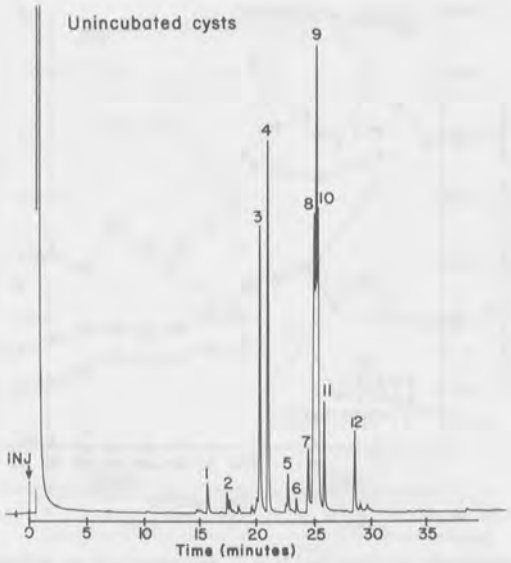

FIG. 7. Gas chromatogram of the fatty acid methyl esters obtained from extraction of dry cysts.

Table I contains the relative percentage values for the fatty acids of dry cysts and those cysts incubated at four different temperatures (25, 15, 10, and 5 °C). In most instances, the values remain nearly constant for dry and incubated cysts, despite the difference in temperature. There are some variations in 16:1 and 18:1. For 16:1, the relative percentage values of incubated cysts are greater than those for the dry cysts. The highest value occurred when the cysts were incubated at 5 °C. Conversely, the relative percentage levels of 18:1 decrease when the cysts are incubated. The lowest value occurred when cysts were incubated at 25 °C. Levels of 18:0 also decreased slightly upon incubation.

When the cysts are incubated at 25 °C and allowed to hatch and the larvae grow for 5 days afterward, the fatty acids undergo changes (Table II). Most notably the levels of 16:1, 16:0, 18:3, and 18:2 decrease in day 3 starved larvae and rise again in day 5 larvae, but the levels remain below those of the incubated cysts. On the other hand 18:1 iso 18:1, 18:0, and 20:4 + 20:5 exhibit increases in day 3 larvae and are only slightly reduced in day 5 larvae. These levels remain above the initial levels found in the incubated cysts. Similar results are seen in Table III for the experiment conducted at 15 °C. Again, 16:1 and 16:0 decrease during the time period. However, the levels of 18:3, 18:2, and 18:1 remain steady throughout the incubation period. Alterations of values occur in 18:1 iso 18:0, and 20:4 + 20:5 ; these values increase from cysts to day 5 larvae.

The values contained in Table IV are nearly the same for each collection time for the incubation at 10 °C. Only slight variations occur in relation to collection times. The greatest differences between incubated cysts and day 5 larvae occur in 18:2 and 20:4 + 20:5. By the same comparison, only 18:0 of Table V demonstrates an increase over the values obtained for the incubated cysts.

TABLE II

Relative percentages for fatty acids from cysts and larvae incubated at 25 °C

Fatty acid	Incubated cysts	Day 3 larvae	Day 5 larvae
14:0	1.9 ± 0.3[a]	0.6 ± 0.4	1.1 ± 0.1
15:1	0.8 ± 0.1	0.3 ± 0.1	1.2 ± 0.7
16:1	15.1 ± 2.3	9.3 ± 2.1	11.5 ± 2.5
16:0	16.3 ± 0.2	11.5 ± 1.4	12.6 ± 2.0
17:1	1.4 ± 0.2	1.2 ± 0.2	1.4 ± 0.3
17:0	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.3
18:3	2.3 ± 0.4	1.0 ± 0.1	1.2 ± 0.6
18:2	12.4 ± 0.9	11.0 ± 0.6	11.6 ± 3.0
18:1	21.6 ± 1.1	25.7 ± 0.1	23.6 ± 0.7
18:1 iso	15.1 ± 1.7	20.8 ± 1.3	19.9 ± 3.6
18:0	4.5 ± 0.6	8.9 ± 1.3	6.9 ± 2.6
20:4 + 20:5	5.0 ± 1.2	8.6 ± 1.7	7.8 ± 1.1

[a] Mean ± standard deviation : three observations.

TABLE III

Relative percentages for fatty acids obtained from cysts and larvae incubated at 15 °C

Fatty acid	Incubated cysts	Day 3 larvae	Day 5 larvae
14:0	1.6 ± 0.1[a]	1.2 ± 0.0	1.0 ± 0.2
15:1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
16:1	15.2 ± 1.0	13.3 ± 1.3	13.2 ± 1.0
16:0	15.3 ± 0.1	12.8 ± 0.5	13.3 ± 0.6
17.1	1.3 ± 0.1	1.3 ± 0.2	1.2 ± 0.2
17.0	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
18:3	2.4 ± 0.1	2.2 ± 0.2	1.8 ± 0.6
18:2	13.3 ± 1.0	13.5 ± 1.8	13.7 ± 2.3
18:1	24.1 ± 1.4	22.1 ± 2.2	23.0 ± 2.1
18:1 iso	14.5 ± 1.1	17.2 ± 1.1	17.5 ± 1.1
18:0	4.5 ± 0.6	5.7 ± 1.2	5.6 ± 0.4
20:4 + 20:5	6.2 ± 0.6	8.7 ± 1.7	8.2 ± 1.3

[a] Mean ± standard deviation : three observations.

For the sake of completenesss the data in Tables I to V (relative percentage values) are also given in absolute values (mg) in Tables VI to X.

TABLE IV

Relative percentages of fatty acids obtained from cysts and larvae incubated at 10 °C

Fatty acid	Incubated cysts	Day 3 larvae	Day 5 larvae
14:0	1.3 ± 0.1[a]	1.2 ± 0.2	1.3 ± 0.0
15:1	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.0
16:1	13.3 ± 1.3	12.4 ± 0.8	13.0 ± 0.1
16:0	15.6 ± 0.1	16.4 ± 0.9	15.1 ± 0.0
17:1	0.7 ± 0.6	1.1 ± 0.1	1.2 ± 0.1
17:0	0.8 ± 0.1	0.6 ± 0.2	0.7 ± 0.1
18:3	2.4 ± 0.0	2.4 ± 0.1	2.4 ± 0.1
18:2	11.5 ± 1.4	11.6 ± 0.6	12.6 ± 1.7
18:1	26.6 ± 0.4	25.9 ± 3.1	25.9 ± 0.9
18:1 iso	15.7 ± 0.6	15.6 ± 2.8	15.1 ± 1.5
18:0	5.2 ± 0.8	5.7 ± 0.6	5.1 ± 0.8
20:4 + 20:5	6.3 ± 0.1	6.8 ± 1.2	7.0 ± 0.8

[a] Mean ± standard deviation : two observations.

TABLE V

Relative percentages for fatty acids obtained from cysts and larvae incubated at 5 °C

Fatty acids	Incubated cysts	Day 3 larvae	Day 5 larvae
14:0	1.6 ± 0.1[a]	1.3 ± 0.1	1.6 ± 0.1
15:1	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.1
16:1	16.3 ± 0.4	13.3 ± 1.5	15.7 ± 0.6
16:0	15.5 ± 0.6	16.8 ± 1.3	15.5 ± 0.3
17:1	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.2
17:0	0.7 ± 0.0	0.8 ± 0.2	0.6 ± 0.1
18:3	2.5 ± 0.3	2.3 ± 0.1	2.3 ± 0.2
18:2	12.9 ± 2.3	11.9 ± 1.0	12.9 ± 1.2
18:1	23.5 ± 0.8	24.2 ± 0.6	23.8 ± 2.2
18:1 iso	14.7 ± 1.7	15.0 ± 0.4	14.8 ± 1.9
18:0	4.3 ± 1.0	6.9 ± 1.5	5.1 ± 0.2
20:4 + 20:5	5.8 ± 0.2	5.7 ± 0.1	5.8 ± 0.6

[a] Mean ± standard deviation : three observations.

TABLE VI

Fatty acids (mg) of *Artemia* dry cysts
and those incubated in artificial seawater at different temperatures
(incubated from 20 hr at 25 °C to 96 hr at 5 °C)

Peak no.	Fatty acid	Dry[a]	25 °C	15 °C	10 °C	5 °C
1	14:0	0.6 ± 0.2	1.2 ± 0.4[b]	1.1 ± 0.1[b]	0.7 ± 0.5[b]	1.9 ± 0.7[b]
2	15:1	0.2 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.3 ± 0.2	0.7 ± 0.3
3	16:1	5.1 ± 1.0	12.0 ± 4.6	11.2 ± 1.0	6.6 ± 4.4	18.8 ± 6.2
4	16:0	6.8 ± 2.0	11.9 ± 3.8	11.7 ± 0.5	7.6 ± 4.4	17.6 ± 5.9
5	17:1	0.5 ± 0.1	1.0 ± 0.3	1.0 ± 0.1	0.3 ± 0.1	1.4 ± 0.5
6	17:0	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.4	0.3 ± 0.2	0.8 ± 0.3
7	18:3	1.1 ± 0.3	1.5 ± 0.6	1.8 ± 0.1	1.2 ± 0.7	3.1 ± 1.5
8	18:2	5.1 ± 1.3	8.9 ± 3.6	10.0 ± 1.4	5.6 ± 3.9	16.7 ± 7.7
9	18:1	12.0 ± 3.1	16.2 ± 5.5	19.0 ± 1.1	12.9 ± 7.3	27.8 ± 11.0
10	18:1 iso	6.5 ± 1.9	11.6 ± 3.4	11.0 ± 0.7	7.6 ± 4.1	15.9 ± 4.2
11	18:0	2.6 ± 0.8	3.1 ± 0.6	3.6 ± 0.4	2.4 ± 1.1	4.4 ± 1.3
12	20:4 + 20:5	2.5 ± 0.6	4.1 ± 1.3	4.5 ± 0.2	3.0 ± 1.7	6.7 ± 2.4

[a] Mean of three observations (mean ± standard deviation).
[b] Mean of two observations.

TABLE VII

Fatty acids (mg) of *Artemia* cysts and larvae incubated at 25 °C

Fatty acid	Incubated cysts[a]	Day 3 larvae	Day 5 larvae
14:0	1.2 ± 0.4[b]	0.2 ± 0.1	0.3 ± 0.0
15:1	0.5 ± 0.2	0.1 ± 0.0	0.2 ± 0.1
16:1	12.0 ± 4.6	1.9 ± 1.2	3.4 ± 0.2
16:0	11.9 ± 3.8	2.2 ± 1.1	3.6 ± 0.3
17:1	1.0 ± 0.3	0.2 ± 0.1	0.3 ± 0.1
17:0	0.5 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
18:3	1.5 ± 0.6	0.2 ± 0.1	0.4 ± 0.0
18:2	8.9 ± 3.6	2.0 ± 1.0	3.4 ± 0.3
18:1	16.2 ± 5.5	4.6 ± 1.8	6.4 ± 0.4
18:1 iso	1.6 ± 5.5	3.6 ± 1.2	4.7 ± 0.4
18:0	3.1 ± 0.6	1.5 ± 0.4	1.4 ± 0.2
20:4 + 20:5	4.1 ± 1.3	1.3 ± 0.2	1.9 ± 0.2

[a] Approximately 20 hr of incubation : collected prior to hatching.
[b] Mean ± standard deviation : two observations.

TABLE VIII

Fatty acids (mg) of *Artemia* cysts and larvae incubated at 15 °C

Fatty acid	Incubated cysts[a]	Day 3 larvae	Day 5 larvae
14:0	1.1 ± 0.1[b]	1.0 ± 0.2	0.8 ± 0.1
15:1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
16:1	11.2 ± 1.0	11.2 ± 3.4	11.3 ± 1.0
16:0	11.7 ± 0.5	10.7 ± 2.4	11.0 ± 0.6
17:1	1.0 ± 0.1	1.0 ± 0.2	1.1 ± 0.1
17:0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
18:3	1.8 ± 0.1	1.7 ± 0.3	1.5 ± 0.2
18:2	10.0 ± 1.4	11.9 ± 2.8	10.4 ± 2.9
18:1	19.0 ± 1.1	19.3 ± 3.3	17.9 ± 2.9
18:1 iso	11.0 ± 0.7	13.8 ± 1.4	14.7 ± 1.3
18:0	3.6 ± 0.4	4.2 ± 0.2	4.7 ± 0.1
20:4 + 20:5	4.5 ± 0.2	7.9 ± 0.6	7.3 ± 0.6

[a] Approximatey 48 hr of incubation : collected prior to hatching.

[b] Mean ± standard deviation : two observations.

TABLE IX

Fatty acids (mg) of *Artemia* cysts and larvae cultured at 10 °C

Fatty acid	Incubated cysts[a]	Day 3 larvae	Day 5 larvae[b]
14:0	0.7 ± 0.5[c]	0.4 ± 0.1	0.8 ± 0.2
15:1	0.3 ± 0.2	0.1 ± 0.1	0.3 ± 0.1
16:1	6.6 ± 4.4	4.3 ± 2.4	7.5 ± 1.6
16:0	7.6 ± 4.4	5.4 ± 2.1	8.8 ± 1.8
17:1	0.3 ± 0.1	0.4 ± 0.2	0.7 ± 0.2
17:0	0.3 ± 0.2	0.2 ± 0.2	0.4 ± 0.2
18:3	1.2 ± 0.7	0.8 ± 0.4	1.4 ± 0.4
18:2	5.6 ± 3.9	3.9 ± 1.2	7.4 ± 2.5
18:1	12.9 ± 7.3	8.6 ± 3.4	15.1 ± 3.5
18:1 iso	7.6 ± 4.1	5.5 ± 3.6	8.7 ± 1.0
18:0	2.4 ± 1.1	2.0 ± 1.2	2.9 ± 0.1
20:4 + 20:5	3.0 ± 1.7	2.4 ± 1.6	4.0 ± 0.3

[a] Approximately 72 hr of incubation : collected prior to hatching.

[b] Larvae + eggs.

[c] Mean ± standard deviation : two observations.

TABLE X

Fatty acids (mg) of *Artemia* cysts and larvae incubated at 5 °C

Fatty acid	Incubated cysts[a]	Day 3 larvae	Day 5 larvae[b]
14:0	1.9 ± 0.7[c]	0.5 ± 0.2	1.2 ± 0.1
15:1	0.7 ± 0.3	0.2 ± 0.1	0.4 ± 0.0
16:1	18.8 ± 6.2	4.3 ± 1.6	11.9 ± 1.6
16:0	17.6 ± 5.9	6.0 ± 2.2	11.9 ± 1.6
17:1	1.4 ± 0.5	0.4 ± 0.2	0.9 ± 0.1
17:0	0.8 ± 0.3	0.3 ± 0.1	0.5 ± 0.1
18:3	3.1 ± 1.5	0.8 ± 0.3	1.9 ± 0.3
18:2	16.7 ± 7.7	3.9 ± 1.4	10.5 ± 1.2
18:1	27.8 ± 11.0	8.2 ± 2.6	19.3 ± 2.0
18:1 iso	15.9 ± 4.2	5.1 ± 1.9	10.7 ± 2.2
18:0	4.4 ± 1.3	2.6 ± 0.8	3.9 ± 0.4
20:4 + 20:5	6.7 ± 2.4	2.0 ± 0.6	4.2 ± 0.7

[a] Approximately 96 hr of incubation : collected prior to hatching.
[b] Larvae + eggs.
[c] Mean ± standard deviation : two observations.

Discussion and conclusions

In the cysts and starved larvae, the extent of hydration is slightly greater for incubations at 28 °C that at 18 °C. The rate of hydration is also increased at 28 °C over 18 °C which required twice the time to reach approximately the same point (90 % *versus* 87 %). This may be due to the influence of temperature on the rate of utilization on the carbohydrates and lipids. These total levels were found to decrease with temperature in both cysts and larvae. The greater decreases were noted in the starved larvae because of no intake of food. Again, the rate of disappearance was affected by temperature. However, initial and final percentage values for each temperature were approximately the same. These results are comparable to those of Claus *et al.* (1979) who also used the San Francisco Bay strain. They also found a decrease of total carbohydrates and lipids with time. In their experiments, they sampled larvae immediately after hatching and again at 48 hr. Their control animals were starved for this period of time, and it is these results which corresponds to those of the present study. However, they did not report values for cysts. Furthermore, they showed that the ash content of the starved larvae was increased over first instar nauplii which is a consequence of decreased organic content. This may be similar to the increasing water content of the starved larvae in the present study. In addition, protein levels remained virtually constant during the 48 hr period used by Claus *et al.* (1979) which is corroborated by similar findings in the present study, regardless of temperature.

Temperature appears to have little effect upon the relative fatty acid composition of the incubated cysts. For cysts (Table I), changes in the levels were observed for certain fatty acids (16:0, 16:1, and 18:1), but these changes seem to be independent of temperature. Instead,

incubation which initiates the metabolism of the cysts appears primarily responsible for the changes over the dry cysts.

A similar situation exists for the starved larvae cultured at different temperature. Variations do occur in the relative percentage levels through time which yield increases in 18:1, 18:0, 20:4 + 20:5, and 18:1 iso (Tables II and III). Decreases were observed in 16:1, 16:0, 18:3, and 18:2. These changes appear to be the result of starvation rather than temperature. This observation is supported by Claus *et al.* (1979) who reported comparable changes in the same fatty acids when the larvae were starved. Culkin and Morris (1969) also reported decreases in palmitic acid for eight different crustaceans which had been starved. The levels of palmitic acid dropped from an average of 16-19% to 12% in those organisms. Conversely, Watanabe *et al.* (1978) found the percentage of 16:0 in the total lipid fraction to decrease only slightly from nauplii to 72 hr larvae when starved. They also used San Francisco Bay brine shrimp cultured at room temperature. Furthermore, 16:1 increased from 3.7% to 5.4% over the 72 hr period. Additionally, increases in the levels of 18:1, 18:0, 20:5, and 20:4 were observed, whereas 18:2 and 18:3 levels decreased. In general, the findings of Watanabe and his co-workers support those previously reported and those of the present study.

Incubations at 10° and 5 °C did not greatly alter the fatty acid patterns of the cysts of larvae. The values remain practically unchanged with time for both temperatures. This may be due to the low rate of hatching observed at these temperatures. Approximately 20 to 22% hatching was noted at 10 °C and less than 3% at 5 °C. Hence, the contributions from the cysts would become increasingly important, especially if metabolism in the cysts slowed. On the other hand, other studies have reported increased levels of unsaturated fatty acids in other crustaceans. Chapelle (1978) reported phospholipids to be the most affected by lowered acclimation temperatures. The percentage levels of unsaturated acids were found to increase sharply with decreased temperature in the phospholipids. These changes were also manifested in the total fatty acid fraction. However, the effects of starvation on the crabs (*Carcinus maenas*) used by Chapelle (1978) were not considered even though the animals were starved for two weeks. Similar results were obtained by Crossins (1975) using a freshwater crayfish (*Austropotamobius pallipes*). In the present study, some shift toward unsaturation with temperature was noted, but this may be attributed to the decreased metabolic activity of the cysts and larvae. The same may be true for the findings of Chapelle (1978) and Crossins (1976).

From these results, it was concluded that temperature affects the metabolic activity of the cysts and larvae when incubated at lower than normal temperatures. There also appears to be no effect upon protein synthesis in the incubated cysts. Furthermore, there seems to be little or no temperature effect on fatty acid composition. Instead, starvation appears to have the greatest impact upon the fatty acid constitution of the animals.

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The role of cytochrome oxidase in the resumption of the development of *Artemia* dormant cysts

C. G. Vallejo[1], *F. de Luchi*[1], *J. Laynez*[2], *and R. Marco*[1]

[1] *Instituto de Enzimología y Patología Molecular del CSIC*
Facultad de Medicina de la Universidad Autonóma
Arzobispo Morcillo s/n, Madrid-34, Spain

[2] *Instituto Rocasolano del CSIC*
Serrano 118, Madrid-6, Spain

Abstract

Besides hydration, we have studied different environmental factors crucial for the resumption of development in *Artemia*. These include oxygen whose molecular sensor has been found to be cytochrome oxydase, temperature which finally acts on cytochrome oxidase through (an)other not characterized sensor(s), and an additional seasonal factor of unknown nature, which functions in a cyclical fashion. All these factors work in a reversible way, allowing the animal to cope with the biological consequences of its changing environment.

Introduction

When the environmental conditions are hostile, *Artemia* reproduces by encysted eggs which in the dry state are able to remain viable for years in their cryptobiotic state. When the habitat becomes suitable for the animal, the eggs are able to resume development.

Since we are interested in the molecular mechanisms that control the expression of the stored information in developmental systems, it was important to establish which are the environmental parameters which influence the resumption of the brine shrimp development. Moreover, it was also important to see whether the parameters act independently or if they are strictly coordinated. The hydration effect on the switching on of the cysts' metabolism is obvious and well characterized thanks to the extensive and careful research of Clegg (1974, 1976).

In the work reported in this paper we have focused mainly on the mode of action of two of the potentially important parameters crucial for the resumption of development : oxygenation and temperature. We have studied the effect of these factors on hatching and respiration of the cysts as well as on isolated mitochondria. In addition, a seasonal cyclical factor of unknown nature has been found to affect the hatchability of the encysted gastrulae in the laboratory.

In order to determine if the action of these environmental parameters was at the level of the initiation of resumption of development or if it simply kills the embryo, it was important to establish the reversibility of the action of these parameters.

Materials and methods

Hatching of cysts

Thirty mg of dry *Artemia* cysts (from San Francisco Bay, California, USA) or 50 mg of cysts hydrated overnight at 4 °C, were added to a flask containing 150 ml of hatching medium (Vallejo *et al.*, 1979) and incubated with continuous agitation at 30 °C for 24 hr, unless stated otherwise. The percent hatching was determined by counting the nauplii and prenauplii *versus* the unhatched cysts in a homogenous 10 ml sample under the dissection microscope.

Isolation of mitochondria

Subcellular fractionation of cysts in the medium 15% Ficoll 400, 0.3 M sucrose, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 25 mM Hepes (pH 7.5) was carried out as described by Vallejo *et al.* (1979). The fraction sedimenting within 30 min at 27 000 × g (after having discarded the centrifugate obtained at 7 500 × g in 20 min) was used as source of mitochondria. Electron microscopical analyses showed that this fraction was quite pure, and mostly free of yolk material.

Enzymatic assay

Cytochrome *c* oxydase was determined as described by Vallejo *et al.* (1979).

Respiration measurements

Respiration was measured with a Clark electrode as described by Vallejo *et al.* (1979) and a Warburg apparatus.

Microcalorimetric determination

Five mg of dry cysts and 4 ml of hatching medium (Vallejo *et al.*, 1979) were brought in the chamber of a Batch microcalorimeter LKB, and the heat production or absorption was recorded during hydration and hatching.

Results

The different factors required for the resumption of development

Oxygen

Oxygen has been found to be a critical factor for the resumption of development. The inhibition by cyanide of the hatching (Fig. 1A) and respiration (Fig. 1B) of the cysts as well as of the cytochrome *c* oxidase activity of isolated mitochondria (Fig. 1C) is shown. As can be seen, cyanide inhibits the three levels at the same concentration. This inhibition is also reversible at the three levels. If cyanide is removed or left for self-degradation (since it is an unstable chemical with a half life of about 4.5 hr-Vallejo, unpublished results), the inhibition is also reversible. In Table I, the reversibility of the inhibition of cytochrome oxidase by cyanide is shown. As can be seen, the reversion is complete if compared to the results of

Fig. 1C. Therefore, it can be concluded from these effects and their reversibility that cytochrome oxidase behaves probably as the molecular sensor for oxygen.

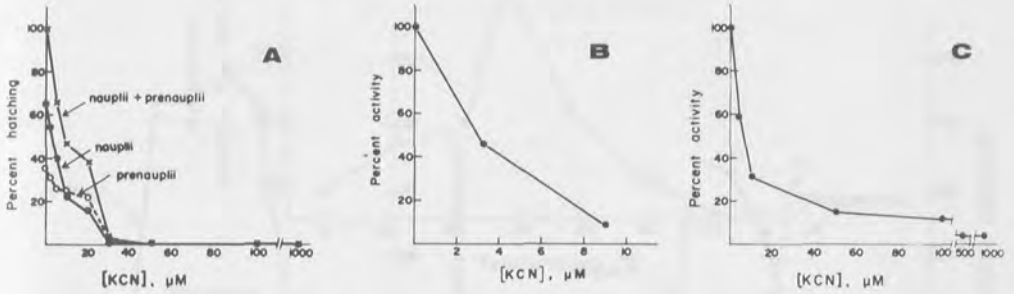

FIG. 1. A. Inhibition of the hatching of *Artemia* cysts, by cyanide, after 24 hr of incubation. B. Effect of cyanide on cyst respiration. C. Cyanide inhibition of cytochrome *c* oxidase.

TABLE I

Reversibility of the inhibition of cytochrome oxidase by cyanide

	Rate
Cytochrome oxidase	100
Cytochrome oxidase + 10μM KCN	20
↓	
diluted to 1μM	59
Cytochrome oxidase + 50μM KCN	14
↓	
diluted to 5μM	33

Temperature

Temperature is the second critical factor for hatching. In Fig. 2, the effect of temperature on the hatchability of the encysted gastrulae is shown. In our experimental conditions, the optimum hatching temperature has been found between 30-35 °C.

At 20 °C there is almost no hatching after 24 hr of incubation but an optimal hatching is found after 48 hr. The hatching time increases exponentially when the cysts are incubated at lower temperatures. For example, when cysts are incubated at 10 °C, 7 days are required to obtain a hatching of 73% in comparison to the control (24 hr at 30 °C). Under these conditions nauplii seem, however, to behave and survive as well as those obtained at 30 °C.

At 40 °C the hatching is completely blocked. The temperature control seems to act in a very narrow range at high temperature since 35 °C is the optimum and at 40 °C no hatching occurs even if the incubation is continued for several days. This blockage is, however, reversible if the temperature is lowered to the optimum. The time required for hatching becomes nevertheless, somewhat longer, depending on how long the cysts were exposed to the high temperature.

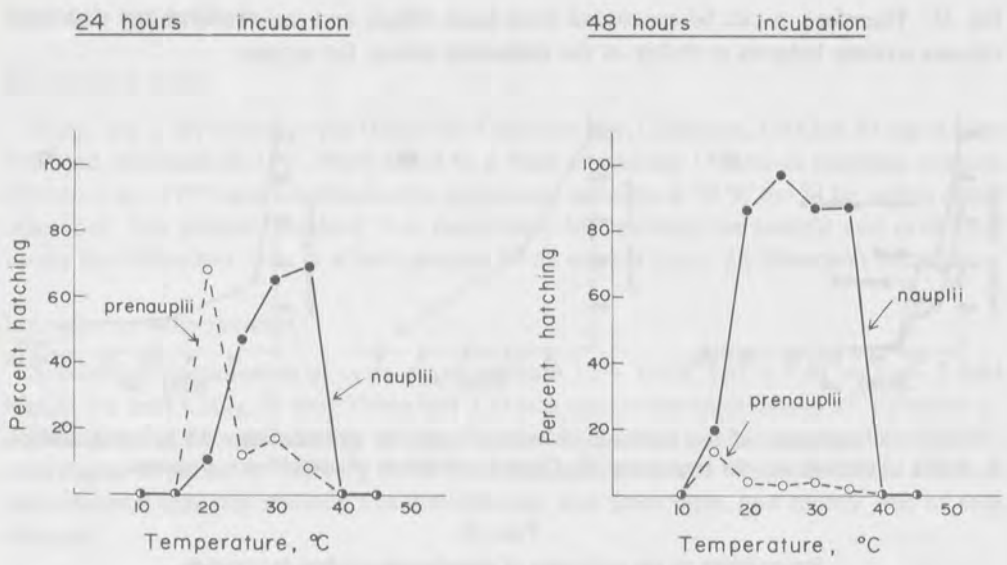

FIG. 2. Hatching of *Artemia* in function of temperature.

If the cysts are incubated at 45 °C for the same length of time as at 40 °C the lag is even longer. For instance, no significant difference in hatching was found, in comparison to the control (24 hr at 30 °C), when the cysts were incubated under the same conditions after having being pre-incubated at 40 °C for 8 hr or 20 hr. But if the pre-incubation was extended up to 48 hr, the hatching was only 11%, when the cysts were brought back to 30 °C for 24 hr. When the cysts were pre-incubated at 45 °C for 8 hr, only 64% hatching was found when incubated further at 30 °C for 24 hr. If the pre-incubation was extended to 20 hr, hatching was only 11%. This does not mean, however, that the remaining cysts are not viable. When the incubation at 30 °C is extended after pre-incubation at 45 °C, the hatching percentage increases (a 40% hatching was obtained after 55 hr at 30 °C in the case of 20 hr pre-incubation). When the pre-incubation was 48 hr at 45 °C no hatching was observed at 30 °C even after 115 hr of incubation. This may point to the possibility that a very long pre-incubation at high temperature results in a loss of viability.

The effect of temperature on the respiration of cysts and on the activity of cytochrome oxidase of isolated mitochondria in shown in Fig. 3A and B, respectively. As can be seen the effect is very similar. The inhibition observed shows, however, a lag (Fig. 3B) which is also observed in respiration. The lag extends over 1 hr in the experiment represented in Fig. 3A. These results seem to implicate cytochrome oxidase in the molecular mechanism of the action of temperature on the initiation of development. Nevertheless, with another batch of cysts, the respiration rate was only significantly decreased after 24 hr of incubation, while development was completely stopped in a reversible manner as described in the previous paragraph. Even with this batch the initial respiration was always lower than the one attained at 30-35 °C. These results have led us to conclude that temperature may act on other sensor(s) of unknown molecular nature which finally control the activity of cytochrome oxidase.

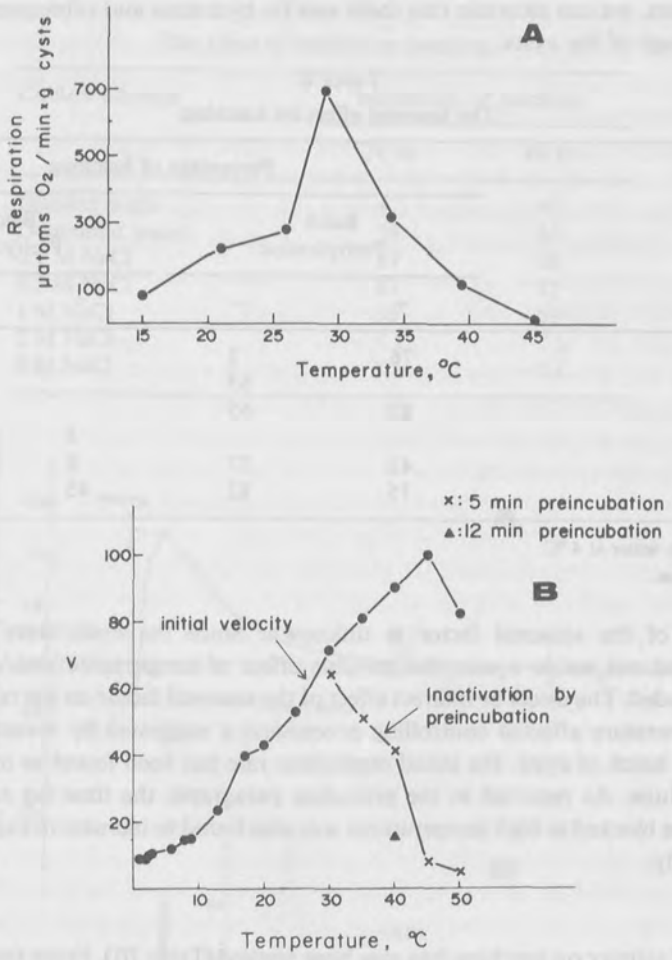

FIG. 3. A. Effect of temperature on respiration of *Artemia* cysts. B. Cytochrome *c* oxidase *versus* temperature.

Seasonal factor

A seasonal factor has been observed which influences the hatchability of the cysts. In Table II, the data show the fluctuations in hatchability depending on the season. This factor seems also to be reversible in the same way as the oxygen and temperature ones described above. The data of Batch 1 have been observed with a batch of cysts (kept in conditions of anaerobiosis and dehydration) which we could not hatch initially without prehydration. This requirement disappeared gradually from winter to spring. Another batch (Batch 2) could not be hatched in winter even with a prehydration treatment. It showed, however, a normal hatching in summer both with cysts from a package which had been kept open during winter in a dessicator and with those from another package of the same batch opened in summer. For

these experiments, we can ascertain that there was no hydration and subsequent oxygenation during the storage of the cysts.

TABLE II

The seasonal effect on hatching

	Percentage of hatching			
	Batch 1 Prehydration		Batch 2 Prehydration	
	+	−	+	−
December 1977	76	3		
April 1978		63		
June 1978	82	65		
January 1979			3	3
April 1979	43	37	8	3
July 1979	75	82	45	61

+ Prehydration in water at 4 °C.
− No prehydration.

The nature of the seasonal factor is unknown. Since the cysts were kept at room temperature and not inside a can, the possible effect of temperature and/or cosmic rays cannot be excluded. The direct or indirect effect of the seasonal factor on the respiration of the cysts and temperature affected controlling process(es) is suggested by several experiments. With the same batch of cysts, the initial respiration rate has been found to increase a 6-fold from May to June. As reported in the preceding paragraph, the time lag required for the respiration to be blocked at high temperatures was also found to increase in experiments made in May and July.

Salinity

The effect of salinity on hatching has also been studied (Table III). From the data, it can be concluded that for the cysts to develop, salt is not a necessary factor in the environment. In its absence, however, the nauplii die immediately after hatching. The difference in hatching observed when the cysts were incubated either in distilled or desionized water may be due to some ions still present in the desionized water that help the animal to break their membranes. As reported before (Morris, 1971 ; Clegg, 1974), the salt optimum seems to be between 0.1 M-0.5 M NaCl. At 1 M NaCl, hatching is decreased by 50% and at 2 M NaCl (satured solution) no hatching is observed even after 48 hr of incubation. Once transferred to the salinity of a normal culture medium, the effect of 2 M salt pre-incubation is completely reversed while for incubation at 5 M it is only partially (50%) reversible.

MICROCALORIMETRIC STUDY OF *ARTEMIA* DEVELOPMENT

When dry cysts were brought into the hatching medium, an instantaneous heat production was observed that lasted for a few seconds followed by a higher heat absorption for about 4 min (Fig. 4A). These observations seem to be related to the hydration phenomenon. The

TABLE III
The effect of salinity on hatching

Culture medium	Percentage of hatching	
	24 hr	48 hr
Distilled water	47	67
Desionized water	56	65
0.1 M NaCl	69	69
0.5 M NaCl	67	72
1 M NaCl	35	59
2 M NaCl	< 1	< 1
5 M NaCl	< 1	< 1

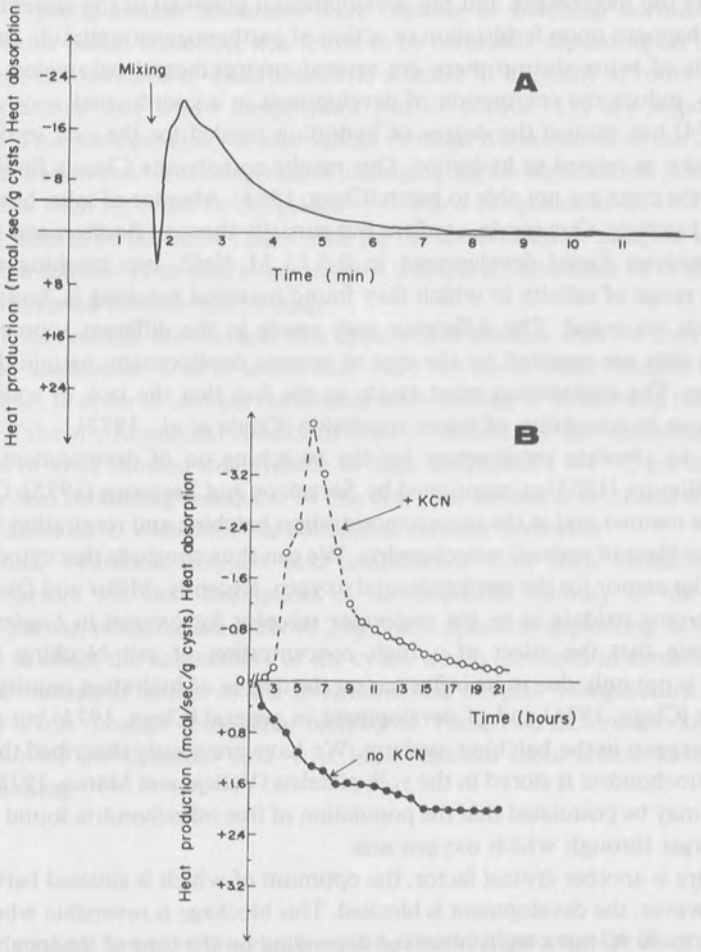

FIG. 4. Microcalorimetric study of *Artemia* development. A. Heat production/absorption during hydration of cysts. B. Heat production/absorption during hatching of cysts.

heat absorption process is also observed when the hydration process occurs with water in the vapor phase. The heat production process results from a sudden addition of water to the dried cysts. The cysts were allowed to hatch inside the chamber and the heat production observed during this period of time is presented in Fig. 4B.

When cyanide was added to the culture medium there was no hatching in the chamber and the second heat production wave was blocked. (Fig. 4B). A high temperature (44 °C) also blocked hatching and heat production reactions. This is in agreement with the hypothesis that oxygen and temperature can control the initiation of the resumption of the development.

Discussion and conclusions

In a certain way the dormant cysts of *Artemia* have several features in common with other developmental systems that are also maintained inactive until some external event occurs which triggers the metabolism and the developmental potential of the system. In the case of oocytes, this happens upon fertilization or action of parthenogenetic stimuli. In the case of the dormant cysts of brine shrimp there are several environmental parameters which, within certain limits, induce the resumption of development in a coordinated way.

Clegg (1974) has studied the degree of hydration needed for the cyst to develop and the effect of salinity as related to hydration. Our results corroborate Clegg's findings that above 2.0 M NaCl, the cysts are not able to hatch (Clegg, 1964). Absence of salts, however, does not preclude the hatching. Our results confirm but partially those of Boulton and Huggins (1977) since these authors found development in 0-0.15 M NaCl, but hatching appeared to be blocked. The range of salinity in which they found maximal hatching is, however, similar to the one which we noted. The difference may reside in the different source of cysts used. Although no salts are required for the cyst to resume development, nauplii die immediately after hatching. The explanation must reside in the fact that the lack of sodium causes the nauplius to lose its possibility of water regulation (Conte *et al.*, 1977).

Oxygen is an absolute requirement for the switching on of development, as previously reported by Nimura (1968) as mentioned by Sorgeloos and Persoone (1975). Cyanide blocks, in a reversible manner and at the same concentration hatching and respiration of the cysts and cytochrome oxidase of isolated mitochondria. We can thus conclude that cytochrome oxidase is the molecular sensor for the environmental oxygen. Recently, Miller and Diehn (1978) have found cytochrome oxidase to be the molecular receptor for oxygen in *Euglena*.

It is possible that the effect of a high concentration of salt blocking the embryonic development is not only due to its influence on the degree of hydration required for the onset of respiration (Clegg, 1976) and of development in general (Clegg, 1974) but also to the low solubility of oxygen in the hatching medium. We have previously described that the majority of the cyst mitochondria is stored in the yolk platelets (Vallejo and Marco, 1976 ; Marco *et al.*, 1980), and it may be postulated that the population of free mitochondria found in the dormant cyst is the target through which oxygen acts.

Temperature is another critical factor, the optimum of which is situated between 30-35 °C. At 40 °C, however, the development is blocked. This blockage is reversible when the cysts are brought back to 30 °C but a lag is observed depending on the time of the incubation at higher temperature. A similar observation has already been made by Sorgeloos *et al.* (1975) and Benijts *et al.* (1977). The lag is much more pronounced when this previous incubation is done

at 45 °C instead of at 40 °C. This points to a higher repression of the cyst metabolism as temperature increases above a certain limit. At low temperatures (down to 7 °C), development can occur but it takes several days to obtain a poor hatching. At these low temperatures, the small differences in hatching synchrony observed at optimal temperatures are very much enlarged : part of the population of nauplii already dies from starvation before another subpopulation hatches. The effect of temperature on hatching is similar to that found for cytochrome oxidase activity and respiration of the cysts. Nevertheless, at both levels, the inhibition by high temperatures presents a lag. For respiration this lag has been found to vary from 1-24 hr, probably depending on the season. Therefore, it seems that cytochrome *c* oxidase is also affected by temperature but likely through its action upon other process(es).

A seasonal effect has been observed on stored cysts. In summer, the hatching success is clearly improved and the immediate respiration of "zero time" cysts was found to increase from May to June. As can be seen in Table II, batches of cysts unable to hatch at all in winter or requiring a pre-hydration treatment were capable of hatching normally in summer. Studying the same batch, this effect was found to be reversible depending on the season. The cysts were kept in conditions of anaerobisis and absence of humidity at room temperature. It can not be excluded that warm temperature and/or cosmic rays are responsible for this phenomenon. This seasonal effect is also visible through the response of the cysts to oxygen (change in respiration rate) and temperature (changing lag on respiration). A triggering factor due to light has been reported by Sorgeloos (1973) and Sorgeloos *et al.* (1975). Light does, however, not seem to be the factor responsible for the seasonal effect since the same result was observed with a batch of cysts kept either inside a transparent container or in the original non-transparent unopened commercial package.

Although until recently we thought that cytochrome oxidase was the only sensor for the environmental conditions, it now seems that there are some other receptors involved that probably interact in order to prevent the animal from starting or continuing the resumption of development if the environmental conditions are not suitable. In this connection, microcalorimetric studies of cysts showed that cyanide or high temperature (44 °C) are also able to stop nearly all the heat producing reactions. In this way, the animal is protected from wasting its reserves and allowed to wait until the conditions become favorable.

In conclusion, hydration, oxygen, and temperature have been found to be the main determining factors for the resumption of development. Salinity is not necessary for development but can block the latter above 2 M NaCl. A season-depending factor of unknown nature seems to affect the hatchability of the cysts, which increases in summer. Cytochrome oxidase is the molecular sensor of the environmental oxygen. Temperature, however, also seems to play a role through some other receptor(s). These two factors also appear to control the metabolism of the hydrated cyst. It is likely that all these effects have an important ecological meaning.

Summary

Hydration, oxygen, temperature plus a season-controlled process are the main factors that determine the resumption of development of *Artemia* cysts. These factors also control the onset of metabolism. Cytochrome oxidase is the molecular sensor of the environmental

oxygen ; it is also affected by unsuitable temperatures but in this case probably through some other receptors.

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Nutrition and digestion

oxygen and is also affected by unsuitable temperatures but in this case probably through some other receptors.

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The feeding of *Artemia* on *Phaeodactylum tricornutum*

J. G. Braun

Laboratorio Oceanográfico de Canarias, Instituto Español de Oceanografía
Avenida José Antonio 3, Santa Cruz de Tenerife, Islas Canarias, Spain

Abstract

The filtering rate of *Artemia* has been measured in experiments carried out under laboratory conditions with the diatom *Phaeodactylum tricornutum* as food ; all but one test are performed at the same temperature (20 °C). The rate at which the animals consumed food was measured using the C^{14} and chlorophyll methods. The filtering rate varied in function of different factors such as food concentration, volume of the vessels used, different developmental stages of the animals, temperature and length of the experiments. Some evidence was obtained that the assimilation of *P. tricornutum* by *Artemia* was near 90 %. Values for gross and net growth efficiencies ranged from 6-38 % for K_1, and from 6-41 % for K_2. The factors that may be responsible for these variations are discussed.

Introduction

One of the factors affecting the control of the biomass of the first trophic level in aquatic systems is grazing by zooplankton. In order to understand the filtering rate and the ingestion rate of zooplankton in general, experiments have been conducted in the laboratory to find out the rates thought to exist in the natural environment.

It is difficult to give a satisfactory explanation for the process happening under natural conditions because of the large number of factors which influence changes of the algal food concentration (Steele, 1956).

A modern approach of the filtering-rate concept was developed by Harvey (1937) and Gauld (1951) by cell counting techniques in *in vitro* experiments with algae and zooplankton. Marshall and Orr (1955ab, 1956, 1962) expanded this field of study, by using radioactive isotopes of carbon and phosphorus, a technique which, since then, has found many adepts.

More recently Adams and Steele (1966) incorporated the C^{14} with the chlorophyll method. Many investigators, in the last decade, have used Coulter Counter equipment for grazing experiments (a.o. Parsons and Le Brasseur, 1970 ; Poulet, 1973 ; Rassoulzadegan, 1978).

A total of 32 experiments on the filtration of *Phaeodactylum tricornutum* by *Artemia* were conducted in this study. *Artemia* was selected because of its relative easiness to cultivate and its consequent importance as a species to refine certain laboratory techniques and measures.

Materials and methods

Phytoplankton cultures were inoculated in two glass bottles of equal volume. One of the bottles served as a control ; animals were introduced in the other for a variable period of time, in most cases 24 hr. The bottles (covered with a black cloth to avoid photosynthesis) were placed on a rotating wheel at a speed of one revolution per minute.

Calculations of filtering rates are based on the equation give by Adams and Steele (1966) :

$$F = \frac{1}{n.t} \log_e \frac{C_o}{C_t}$$

where n is the number of animals per liter, t the duration of the experiment in days, C_o the chlorophyll *a* concentration or counts per minute of the control, and C_t the chlorophyll *a* concentration or counts per minute, of the medium.

During the experiments three types of filtering rates were measured following the equation given above :

1) when C^{14} is used, the term C_t is the radioactivity found in the medium plus that found in the faecal pellets. This filtering rate is called F ;
2) if C_t is the only chlorophyll measured in the experimental bottle the filtering rate is called F_1, and
3) if C_t is the total chlorophyll found in the experimental bottle together with the faecal pellets, the filtering rate is called F_2.

The determination of chlorophyll and phaeophytin were carried out following the basic spectrophotometric equations given by SCOR/UNESCO (1966) and Lorenzen (1967), as well as the fluorescence method of Yentsch and Menzel (1963) and Yentsch (1965).

The cultures of *Phaeodactylum tricornutum* used in the C^{14} test were incubated under artificial light for half a day before the experiment.

Results and discussion

The first experiments were carried out with *Artemia* juveniles and pre-adults (Experiment 2 with juveniles ; Experiments 1,3, and 4 with pre-adults) in order to determine the respective filtering rates in ml/animal/day. The results were 17 ml/animal/day for juveniles and 35-37 ml/animal/day for pre-adults ; this means ingestions of 61 % and 82-87 % respectively (Table I).

A second series of experiments was carried out in triplicate. For F_1 values, a range of 64-70 ml/animal/day was noted with an average value of 66.6 ml/animal/day ; for F_2 the range amounted to 56-57 ml/animal/day with an average value of 61.6 ml/animal/day (Table II). The ingestions were high (78-84 %) and it was noted that the chlorophyll concentration which was 100 % at the start, was reduced to 20-24 % in 24hr. In contrast the phaeophytin concentration, which initially had a value of near 0 % went up to 76-80 %. Fig. 1 shows the average values of the changes in the chlorophyll and the phaeophytin concentrations for the experimental bottle and the faecal pellets respectively.

TABLE I

Filering rates of *Artemia* (juveniles and preadults)

Experiment number	Number of animals/l	Technique	Filtering rate (F) (ml/animal/day)	Ingestion (%)
1	54	C^{14}	36	86
2	54	C^{14}	17	61
3	49	C^{14}	35	82
4	54	C^{14}	37	87

TABLE II

Filtering rates of *Artemia*.
Changes in chlorophyll *a* and phaeophytin in the experimental vessel and in the faecal pellets

Experiment number	Number of animals/ml	Technique	Filtering rate (ml/animal/day)		Ingestion (%)	Percent pigments at the end	
			F_1	F_2		Chlor.	Phaeo.
4b	27	chloro.	70	67	84	24	76
5	27	chloro.	66	62	81	20	80
6	27	chloro.	64	56	78	23	77

The variation in the filtering or ingestion rates with the increase in cell or food concentration has been studied by Reeve (1963a,b), Anraku (1963), Mullin (1963), and more recently by, among others, Adams and Steele (1966), Mc Allister (1970), Corner *et al.* (1972), Steele (1974), Reeve and Walter (1977). Reeve's values for *Artemia* suggest that the filtering rate is constant till a certain (high) food concentration is reached (between 10^4 and 10^6 cells/cc with an average of 10^5 cells/cc) ; from this value on it begins to decrease. Anraku's values for *Calanus finmarchicus* and *Pseudocalanus minutus* show a maximum filtering rate (110 ml/animal/day for *Calanus* and 40 ml/animal/day for *Pseudocalanus*) in the lowest concentration used (10^3 cells/cc) and a decrease in the filtering rate as the food concentration increases.

Bainbridge (1957) on the other hand, suggests that the richest concentration of diatoms in the sea averages around 5 600 cells/cc. The concentration can, however, reach as high as 5.10^4 cells/cc in the case of red tides.

The maximum phytoplankton density is usually around 500 cells/cc for diatoms and 2 500 cells/cc for flagellates. Therefore, Reeve's values are derived from an extremely high concentration in comparison to the natural average. On the contrary the maximum value for the filtering rate mentioned by Anraku (1963) was obtained with algal densities, similar to the richest cell concentrations which can be expected in the marine environment.

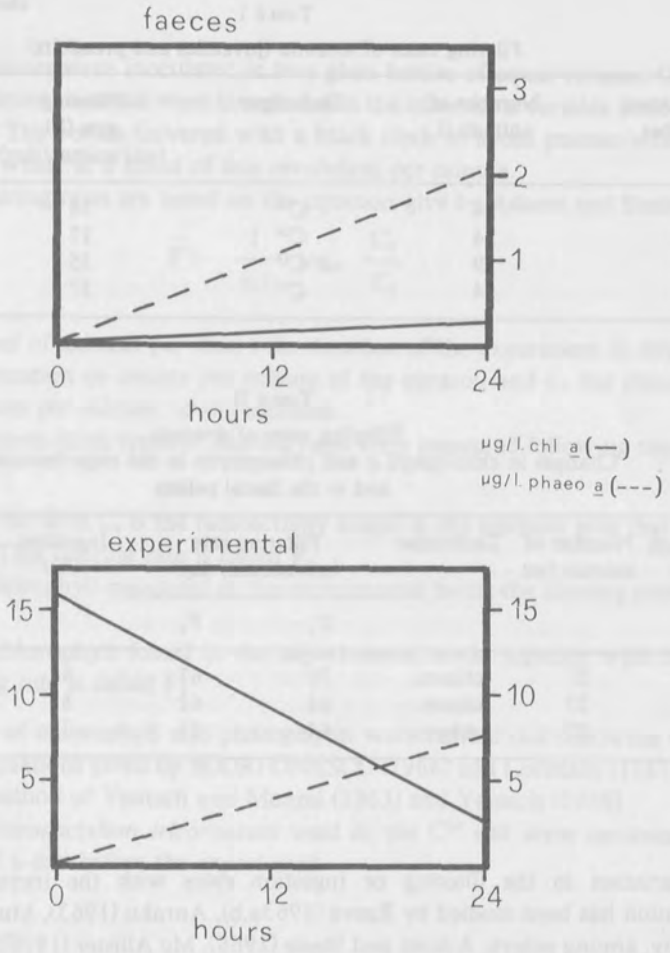

FIG. 1. Changes in chlorophyll and phaeophytin concentration in the experimental vessel and in the faecal pellets in 24 hr.

In our experiments with *Artemia* (Table III) a maximum filtering rate of 17.32 µg/l chlorophyll *a* (Experiment 7-11) and 19.53 µg/l chlorophyll *a* (Experiment 12-16) were noted. Beyond the average of these two results – *i.e.* 18.45 µg/l chlorophyll – the filtering rate decreased as the food concentration increased ; below this point the filtering rate seemed to decrease as the food concentration decreased.

A very high figure for the chlorophyll concentration in the sea would be around 20 µg/l, sometimes even as high as 30 µg/l (Atkins and Jenkins, 1953). Our figure of 18.45 µg/l chlorophyll *a* (the highest result found in our filtering data) corresponds roughly to a very rich phytoplankton population in the sea. This finding corroborates the one mentioned by Anraku (1963) and obtained with the cell counting technique.

TABLE III

Changes in the filtering rate in relation to food concentration

Experiment number	Number of animals/l	Food concentration expressed as chlorophyll (μg/l)	Filtering rate (ml/animal/day)	
			F_1	F_2
7	40	3.99	25	25
8	26-44	11.24	45	12
9	31-44	17.32	92	79
10	47	49.29	56	56
11	40-45	59.07	52	52
12	18-22	4.71	44	44
13	22	11.23	44	44
14	22	19.59	159	109
15	19-23	30.24	33	23
16	18-22	42.59	38	26

In the aforementioned articles by Reeve (1963a,b) and Anraku (1963), there are no results for filtering rates in cases of small food concentrations, *i.e.* between 0-500 cells/cc (or 0-20 μg/l chlorophyll *a*). Under these conditions, Adams and Steele (1966) concluded that the filtering rate seemed to increase, along with the food concentration, from 0-4 μg/l chlorophyll *a*, followed by a slight drop in the average filtering rate. Our results showed that the increase in filtering rate with food concentration takes place from 3.9 μg/l chlorophyll *a* up to 18.45 μg/l. No experiments were conducted at concentrations below 3.9 μg/l. In the recent study published by Reeve and Walter (1977) it can be observed that *Acartia tonsa* has a maximum grazing rate of 10 μg/l, decreasing to zero below 1 μg/l.

The filtering-rate values for *Artemia* nauplii were, as expected, very low, in comparison to the values for the adults. The results range from 0.8-3.8 ml/animal/day for F_2 (Table IV). It can be observed that low filtering rates cause little reduction in chlorophyll concentration and as a consequence, little production of phaeophytin. This problem has been studied elsewhere (Braun, 1975).

TABLE IV

Filtering rates of the nauplius stage

Experiment number	Number of animals/l	Filtering rate (ml/animal/day)		Percent pigments at the end	
		F_1	F_2	Chlor.	Phaeo.
17	50	2	0.8	97	3
18	59	4	3.8	93	7

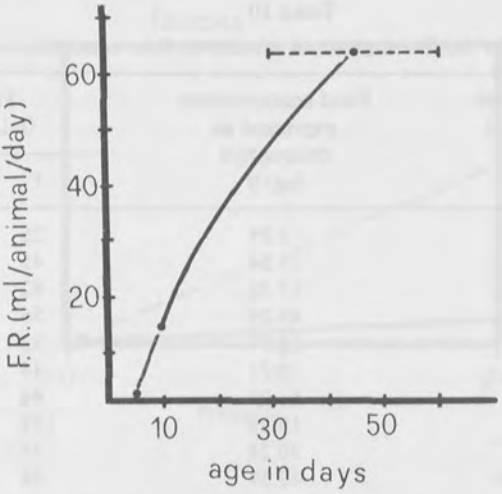

FIG. 2. Relationship between the filtering rate and the age of the animals.

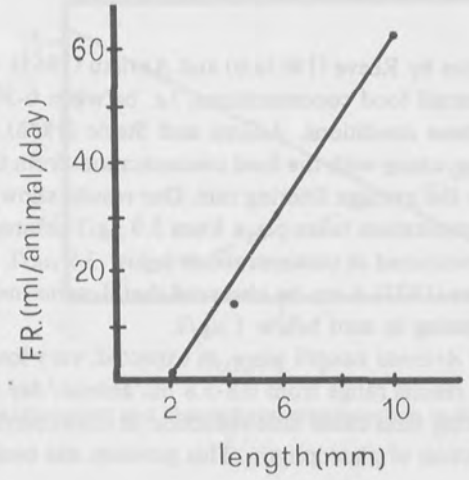

FIG. 3. Relationship between the filtering rate and the length of the animals.

Fig. 2 and 3 show changes in the filtering rate as related to the developmental stage of the animal. In Fig. 2 the filtering rate is shown in relation to the age of the animals and Fig. 3 illustrates the filtering rate in relation to the length of the brine shrimp.

There appears to be a linear relationship between the length and the filtering rate, which is similar to what was found by Cushing (1959a) for copepods in general (Raymont, 1963). Gauld (1951) has shown that the filtering rate is approximately proportional to the square of the length of the copepods.

Three series of animals (Table V) were placed in bottles with seawater of 17 ‰, 35 ‰, and 61 ‰ salinity respectively. Our results indicate that at low salinity the filtering rate decreases whereas at high salinity it does not (Table V).

TABLE V

Changes in the filtering rate in relation to salinity

Experiment number	Number of animals/l	Salinity (‰)	Filtering rate (ml/animal/day)	
			F_1	F_2
19	57	17	58	55
20	34-57	35	73	67
21	51-57	61	78	69

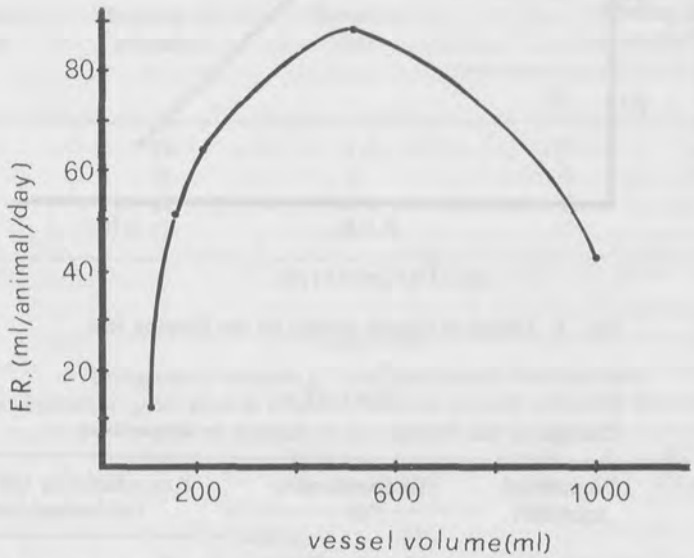

FIG. 4. Changes in the filtering rate in relation to the volume of the experimental vessel.

The contenance of the experimental bottle has an important effect on the filtering rate. Cushing (1959a,b) found that the filtering rate increases along with the volume of the medium in the experimental vessel.

Anraku (1963) came to the same conclusion but observed that, from a certain volume on, the filtering rate begins to decrease.

Our results confirm Anraku's observations (1963), as can be seen in Fig. 4.

Since the volume of the experimental bottle is proportional to the number of animals in the vessel, we have plotted the filtering rate against the number of animals per ml. (Fig. 5). If we plot Anraku's data for the 18 July and the 28 November in the same way the same effect is observed. Around 20 animals/l appears to be the optimum concentration to achieve a maximum filtering rate in *Artemia*. Anraku's graphs for *Acartia tonsa* varied between 70-190 animals/l. This difference might be due to by the different size of the two species. In an

artificial environment the zooplankton population may thus reach several hundreds of animals per litre at high phytoplankton concentrations ; in the natural marine environment the maximum figure is in the order to 50 animals/l (Raymont, 1963).

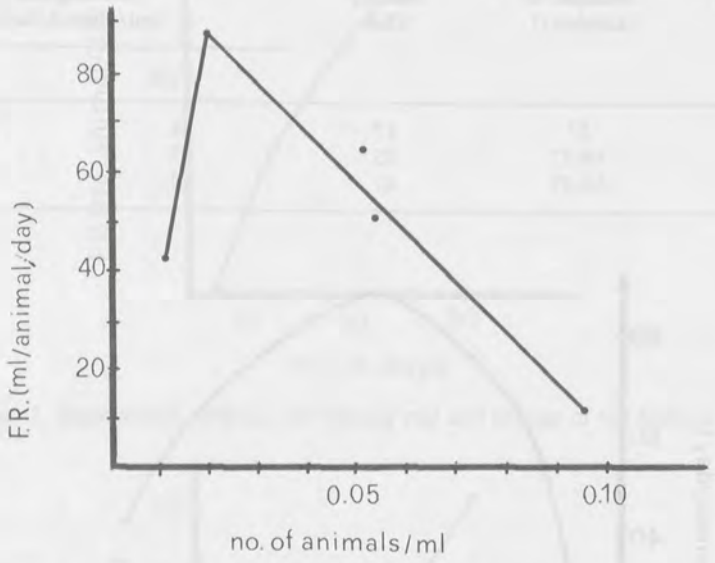

FIG. 5. Effects of animal density on the filtering rate.

TABLE VI

Changes in the filtering rate in relation to temperature

Experiment number	Number of animals/l	Temperature (°C)	Filtering rate (ml/animal/day)	
			F_1	F_2
22	66	4	11	2
23	66	10	33	31
24	40-66	24	61	50

As could be expected the filtering rate increased directly with temperature (Table VI) ; it was found by Gauld and Raymont (1953) that respiration follows an analogous pattern with respect to temperature, parallel to the food intake.

Anraku and Omori (1963) found the same effect in *Centropages typicus* although they state that there was a decrease in the filtering rate with a temperature rise for *Calanus finmarchicus*, a fact also observed by Adams and Steele (1966) for the same species.

The above would seem to indicate that filtering rates decrease when the animals are placed, for some reason, at a temperature different from its normal environment. In our experiments the *Artemia* were kept from the egg to the adult stage at 24 °C. The temperatures of 4 °C and

10 °C used in the experiments of the authors mentioned above, are rather low (at least in comprison to the prevailing temperatures during most of the year); the metabolism of the animals was reduced resulting in a subsequent decrease in the filtering rate.

The highest filtering rate was obtained in the short-term experiment (1.5 hr) (Table VII). Raymont (1963) reviewed the problem of the high values found by Harvey (1937) during his studies on the copepod *Calanus finmarchicus*. Some of the values mentioned by the latter author indeed reached 200 ml/animal/day in short term experiments (7 hr). The general conclusion is that the filtering rate increases with a decrease in the length of the experiment (Mullin, 1963).

TABLE VII

Changes in filtering rate in relation to the length of the experiments

Experiment number	Number of animals/l	Duration (hr)	Filtering rate (ml/animal/day)	
			F_1	F_2
25	28	1.5	119	59
26	28	3	3	–
27	28	21	5	1.7
28	28	26	15	13

TABLE VIII

Comparison between C^{14} and chlorophyll filtering rates.
Assimilation, gross growth efficiency and net growth efficiency for *Artemia*

Experiment number	Number of animals/l	Filtering rate (ml/animal/day)		Assimilation (%)	K_1 (%)	K_2 (%)	Respiration (by subtraction) (%)
		Chlorophyll F_2	C^{14}-F				
29 (adult)	54	40	38	92	38	41	59
30 (adult)	54	38	29	91	37	40	60
31 (nauplii)	171	1		91	6	6	94
32 (nauplii)	161	2.5-3.0	1.5	56	21	38	62

The assimilation values averaged 90 % (Table VIII). Marshall and Orr (1955a,b) found high values as well for *Calanus* using P^{32} and C^{14} methods (50-90 %). Conover (1964) indicated that the percentage assimilation for zooplankton is variable and is situated within the range 6-99 %. Recently Conover (1966), however, found 54.1-84.6 % for *C. hyperboreus*. Lasker (1966) working with *Euphausia pacifica* reports a range of 62-87 % with an average of 84 % assimilation. In more recent studies Ikeda (1971) mentions a 68 % assimilation for *C. crisatus*. The average value determined by Fowler *et al.* (1971) for euphausiids feeding on *Artemia* nauplii in Mediterranean waters is 84.8 %. Harris (1973) found 75.4 % for the assimilation in

harpacticoids. Recent values by Corner *et al.* (1976) yielded 79.5-99.9 % for *C. helgolandicus* feeding as a carnivore.

The findings of values close to 90 % or even higher for assimilation has been commented by Marshall and Orr (1955b), Lasker (1966), Fischer (1970) and Fowler *et al.* (1971) ; these authors suggest that the variabilty of the results reported originated from the different methods of experimentation used or from different environmental factors. Particularly the use of C^{14}, as shown by our work, seems to increase the percentage of assimilation somewhat.

The values for K_1 (gross growth efficiency) are relatively consistent (38 %, 37 %, 21 %) with the exception of experiment 31 were it was only 6 % (Table VIII).

Conover (1968), however, mentions a much wider range of values for K_1 and K_2 (net growth efficiency) and Reeve (1963c) for instance a 20-60 % range. Mason (1963), using the C^{14} technique, determined a range from 2.8-8.2 %. Gibor (1957), using dry weight, obtained 11-53 %. Suschenya (1964) with the caloric method, obtained a 9.02-19.52 % range. The values we observed with the C^{14} technique range from 6-38 %. Our values are higher than those mentioned by Mason (1963) who used the same technique and the same species ; they are of the same order, however, as those found by Lasker (1960) with the same technique but *Euphausia pacifica* as experimental animal ; Corner *et al.* (1967) reported K_1 values in the order of 34 % for *Calanus finmarchicus*.

On the basis of the nitrogen "budget" Harris (1973) found a value of 13 % for K_1 ; this author considers, however, a complete life cycle, from the egg to the adult stage. Corner *et al.* (1976) using the same parameter (nitrogen) find 49.2 % for the gross growth efficiency, although it is pointed out that this is variable and depends on the food ration.

The values give by Suschenya (1964) for *Artemia* lay between 23.65 and 27.44 %. Lasker (1966) obtained 17.1-33.6 % with the C^{14} technique on *Euphausia pacifica*. In this study, K_2 values range between 6-41 %. In the experiments 29, 30, and 32 the results are 41 %, 40 % and 38 % respectively, which is close to the values 42.3 and 30.3 % obtained by Ivlev (1938) (as reported by Conover, 1968) for *Daphnia pulex*, and to the values given by Richman (1958) for the same species in the pre-adult stage.

By subtracting, our values show the respiration percentage to be in the order of 60 % in experiments 29, 30, and 32.

Richman (1958), Reeve (1963c) and Pavlova (1964) found that the values of K_1 and K_2 are related to the age of the animal and that the values are at a maximum in the early stages. Butler *et al.* (1969) report K_2 values of 33.1 % in terms of nitrogen, and 28.3 % in terms of phosphorus. Recently Mullin and Brooks (1970) studied two marine copepods and suggested values for K_2 between 30-45 %. Finally Harris (1973) gives a value of 17.2 % for the harpacticoid copepod *Tigriopus brevicornis*.

Conclusions

From the results obtained we conclude that :

1) quantitatively speaking the filtering rate varies with many factors (food concentration, volume of the experimental vessel, duration of the experiment, *etc.*) ;
2) 24 hr experiments seem to underestimate the filtering rate ; experiments lasting for only 8 hr may give a value up to four times larger ;

3) values for assimilation, gross growth efficiency, and net growth efficiency are variable, but the majority of them give 90 % for assimilation, 6-38 % for gross growth efficiency and 6-41 % for net growth efficiency.

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Programmation of postembryonic development in *Artemia* by dietary supplies of purine and pyrimidine

A. Hernandorena

Laboratoire du Museum National d'Histoire Naturelle, CERS
F-64200 Biarritz, France

Abstract

The availability of an artificial medium for axenic culture of the Utah strain of *Artemia* offers a promising approach to the study of developmental biology in this species. While the complete omission of a nutrient generally leads to death, less than adequate levels lead to prolonged development. In *Artemia*, simple nutritional manipulations of purine and pyrimidine supplies alter the timing of developmental stages and the expression of genetic information. The unique advantages of studying morphogenesis in this primitive crustacean are emphasized and the data presented should encourage geneticists and biochemists to adopt *Artemia* as an experimental tool in the field of developmental biology.

Introduction

At the Toronto Symposium on the biochemistry of *Artemia* development, we presented data concerning the induction of abortive appendicular morphogenesis (Hernandorena, 1979b) (Fig. 1 and 2). This precocious metamorphosis occurs before the end of appendicular morphogenesis in a culture medium lacking folic acid and thymidine. In this paper we present nutritional data gathered on the induction of a supernumerary gonopode morphogenesis (Fig. 3 and 4).

Material and methods

The method developed by Provasoli and d'Agostino (1969) for the axenic culture of the Utah strain is used. The purine and pyrimidine mixture of our standard medium was developed by Provasoli and d'Agostino before their 1969 publication (Table I). We have omitted guanylic acid. Deficiencies and excesses are defined by reference to the standard concentrations. Standard rearing temperature and salinity are 25 °C ± 0.5 °C and 24 ‰ respectively.

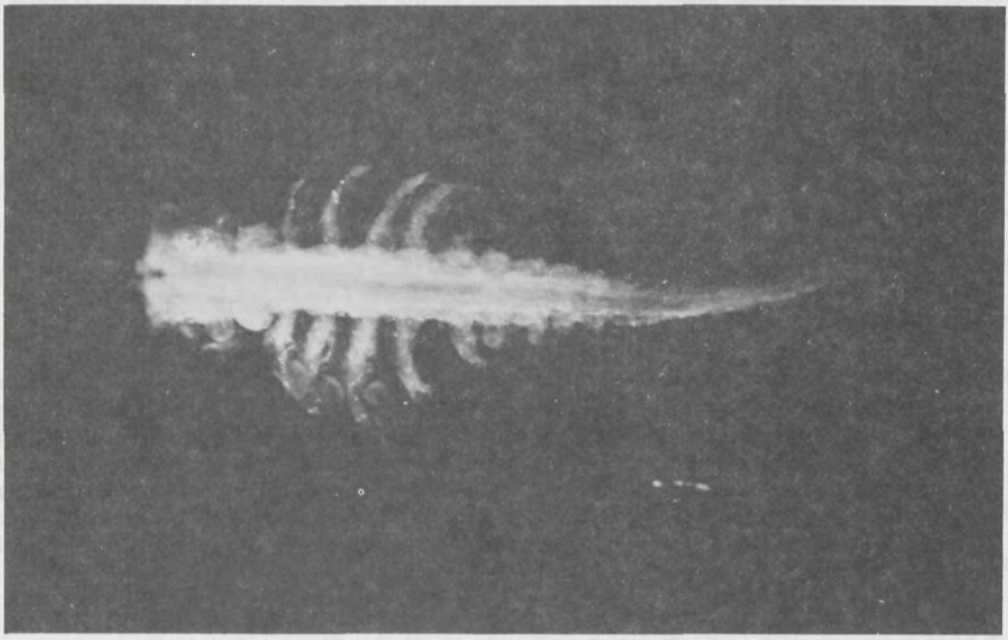

FIG. 1. Abortive appendicular morphogenesis (dorsal view).

TABLE I

Concentrations (mg %) of purine and pyrimidine in standard media

	Provasoli and d'Agostino (1969)	Hernandorena
Adenylic acid	60	60
Guanylic acid	2.5	
Cytidylic acid	5	10
Uridylic acid	5	10
Thymidine	2.5	5

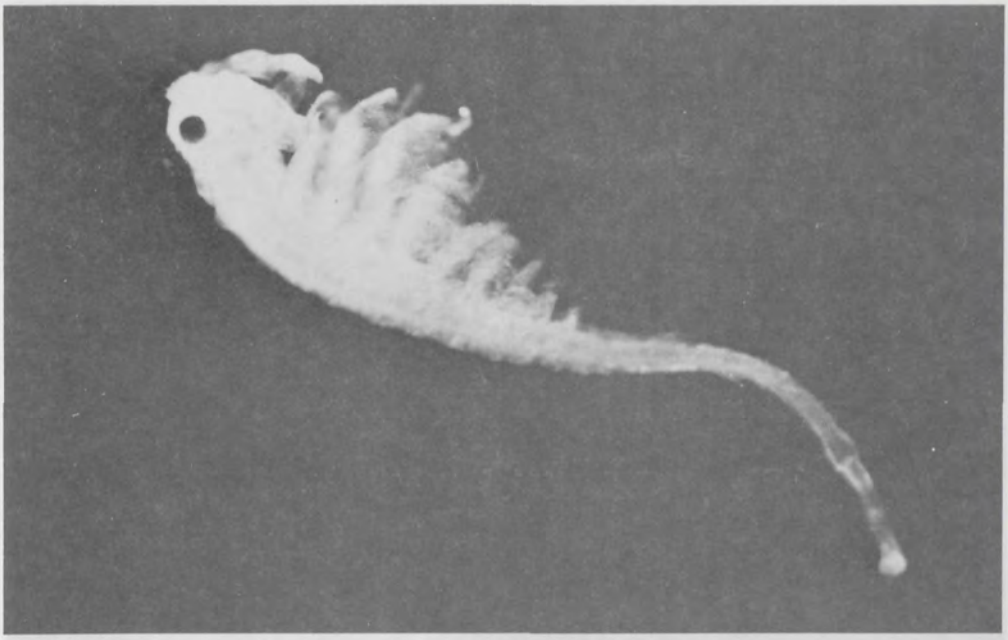

FIG. 2. Abortive appendicular morphogenesis (lateral view).

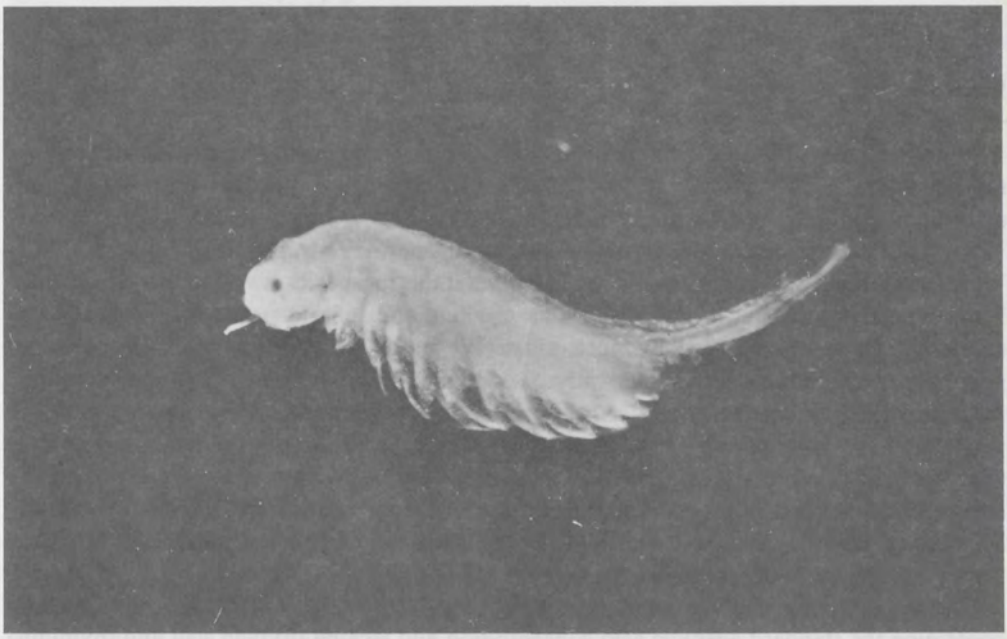

FIG. 3. Supernumerary gonopode morphogenesis.

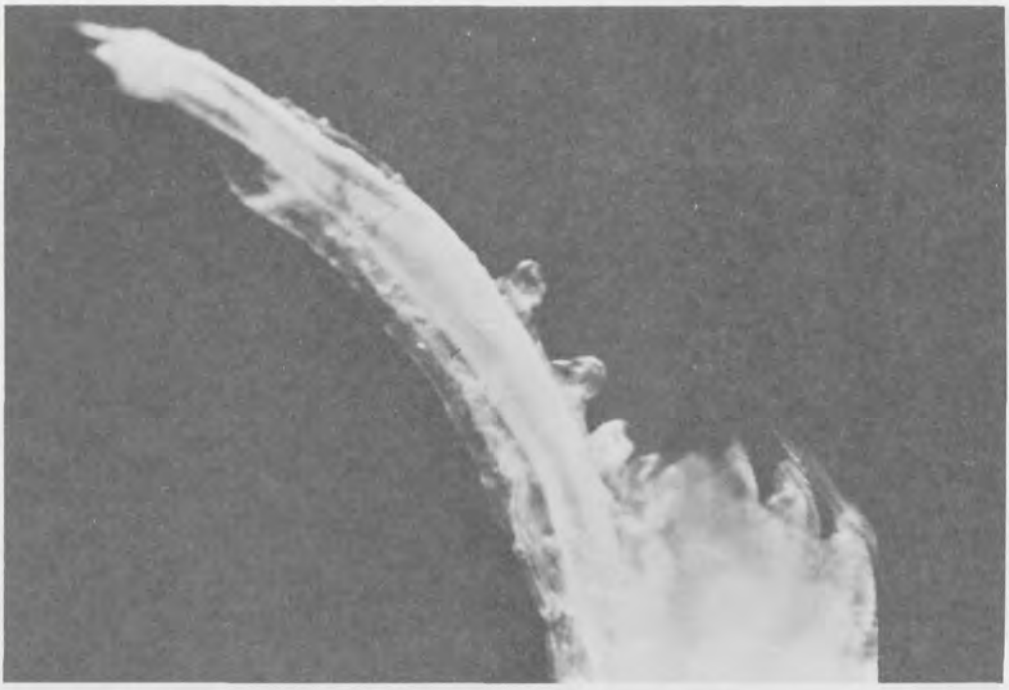

FIG. 4. Supernumerary gonopode morphogenesis (detail of abdominal ovisacs).

Results

Morphogenesis of the supernumerary gonopodes on abdominal segments is induced by a reduced adenylic acid/cytidylic acid ratio. The first abdominal gonopodes were induced by an adenylic acid deficiency in a medium containing the standard pyrimidines (Hernandorena, 1970). The Utah strain is incapable of synthesizing the purine ring *de novo* (Clegg *et al.*, 1967 ; Warner and McClean, 1968). We do not know yet if this incapacity is shared by other strains. It is expressed at the nutritional level by an absolute and specific requirement for a purine source which is best fulfilled by nucleotides. We used adenylic acid but the abdominal gonopodes were induced by any derivative used to meet the requirement. Nauplii reared in a medium lacking a purine source survive for a long time without growing. Growth rate and percentage survival increase with increasing adenylic acid concentration (Fig. 5 and 6) (Hernandorena, 1972). An adenylic acid deficiency restricted to the early larval life induces supernumerary gonopodes, thus suggesting the existence of a critical period. An understanding of what is going on at the end of larval life supposes an understanding of what has been going on at its beginning (Hernandorena, 1974a).

Supernumerary gonopodes also were induced by a cytidylic acid excess in a medium containing the standard adenylic acid concentration and lacking uridylic acid and thymidine (Hernandorena, 1977). It became evident that a reduction of the adenylic/cytidylic acid ratio

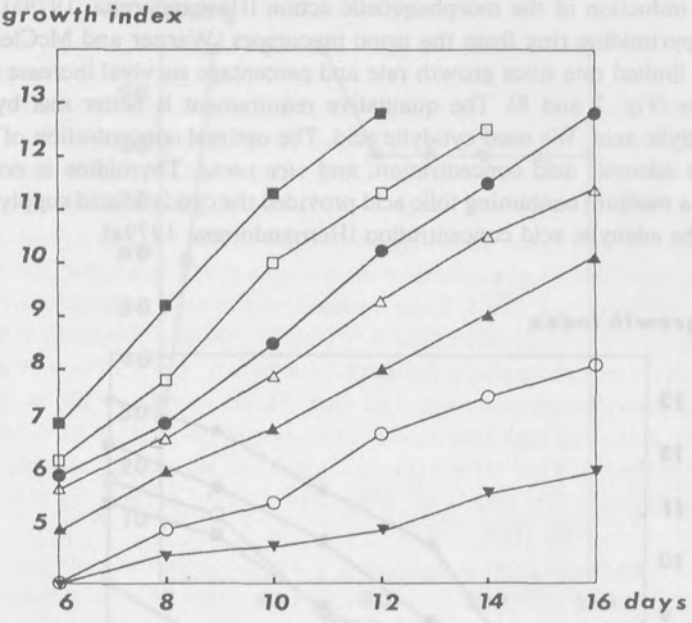

FIG. 5. Effect of the adenylic acid concentration on growth. Standard pyrimidines. Adenylic acid mg% ▼ = 10, ○ = 20, ▲ = 30, △ = 40, ● = 60, □ = 100, ■ = 140.

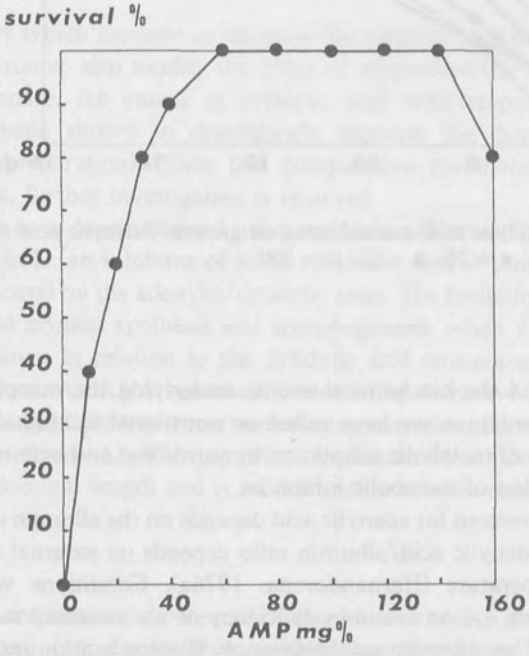

FIG. 6. Effect of the adenylic acid concentration on percent survival. Standard pyrimidines.

is the cause of induction of the morphogenetic action (Hernandorena, 1979a). *Artemia* can synthesize the pyrimidine ring from the usual precursors (Warner and McClean, 1968) but apparently at a limited rate since growth rate and percentage survival increase as the dietary supply increases (Fig. 7 and 8). The qualitative requirement is better met by nucleotides, cytidylic or uridylic acid. We used cytidylic acid. The optimal concentration of cytidylic acid depends on the adenylic acid concentration, and *vice versa*. Thymidine is not an absolute requirement in a medium containing folic acid provided the cytidylic acid supply is not limited in relation to the adenylic acid concentration (Hernandorena, 1979a).

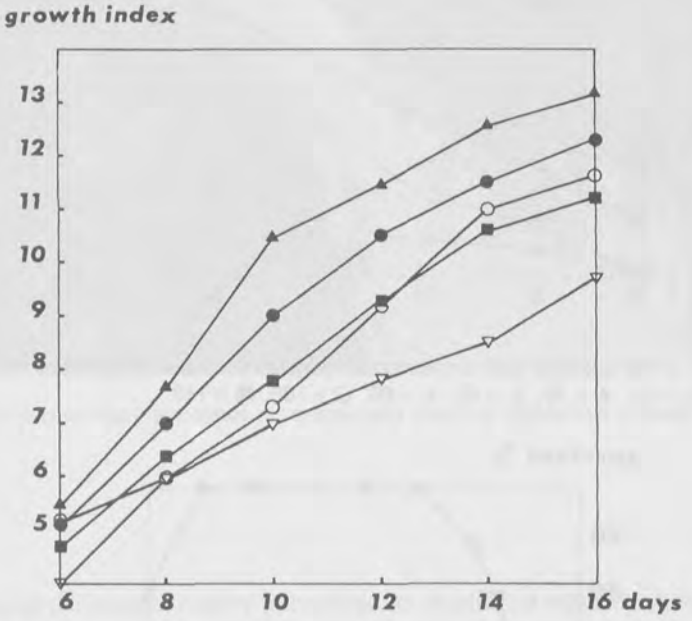

FIG. 7. Effect of the cytidylic acid concentration on growth. Adenylic acid at 60 mg%. Cytidylic acid mg% ▽ = 0, ■ = 10, ● = 20, ▲ = 60, ○ = 100.

In order to understand the biochemical events underlying the morphogenetic action of dietary purines and pyrimidines, we have called on nutritional techniques. Two approaches have been used : a study of metabolic adaptation to nutritional and external conditions and a study of the administration of metabolic inhibitors.

The quantitative requirement for adenylic acid depends on the albumin concentration of the diet while the optimal adenylic acid/albumin ratio depends on external salinity (Hernandorena, 1974b) and temperature (Hernandorena, 1976a). Conditions which decrease the adenylic acid requirement, *i.e.* an albumin deficiency or an increased salinity, suppress the morphogenetic action of an adenylic acid deficiency. Dietary lecithin increases the adenylic acid concentration treshold inducing supernumerary gonopodes (Hernandorena, 1976b).

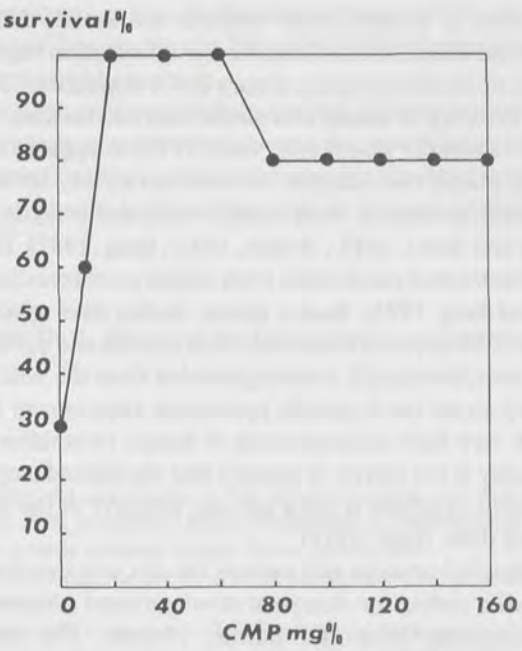

FIG. 8. Effect of the cytidylic acid concentration on percent survival. Adenylic acid at 60 mg%.

Nutritional factors which increase or decrease the adenylic acid requirement, *i.e.* albumin or lecithin concentrations, also modify the effect of allopurinol (HPP) a specific inhibitor of xanthine dehydrogenase. An excess of cytidylic acid with respect to the adenylic acid concentration has been shown to dramatically increase the detrimental effect of HPP (Hernandorena, in preparation). Since few comparative metabolic data are available to support these results, further investigation is required.

Interesting results have been obtained using antibiotics (Hernandorena, 1975, 1980). The effects of actinomycin D, an inhibitor of RNA synthesis, and of puromycin, an inhibitor of protein synthesis, depend on the adenylic/cytidylic ratio. The limitation of RNA synthesis has an opposite effect on protein synthesis and morphogenesis when it results from a relative adenylic acid deficiency in relation to the cytidylic acid concentration or from a relative adenylic acid deficiency. An RNA synthesis limitation by a relative adenylic acid deficiency reduces abdominal length, induces supernumerary gonopodes and is related with a relative increased protein synthesis. An RNA synthesis limitation by a relative cytidylic acid deficiency increases abdominal length and is related with a relative reduced protein synthesis.

Discussion

We would like to emphasize the potential of *Artemia* as a tool in the field of developmental biology by comparing our results with selected nutritional and physiological data on *Drosophila*.

In *Drosophila* the extent to which a strain requires and utilizes dietary RNA is a unique characteristic of that strain (Geer, 1963). Considerable information regarding the adequacy of specific nucleic acids components to replace dietary RNA is available. The data are difficult to analyze because of the diversity of strains and media used and because of the different criteria chosen to evaluate the results (El Kouni and Nash, 1977). It appears that the RNA requirement is essential for the purine base adenine, but satisfied equally by adenosine and adenylic acid, and for a pyrimidine nucleotide, with cytidylic acid and uridylic acid being completely interchangeable (Villee and Bissel, 1948 ; Hinton, 1956 ; Sang, 1957). The need arises because the rate of synthesis of individual nucleotides from simple precursors falls short of the growth requirement (Burnet and Sang, 1963). Recent genetic studies have selected many auxotrophic mutant strains. Mutant 1308 requires a source of both purines and pyrimidines, but unlike the rudimentary strain, is morphologically indistinguishable from the wild type (Vyse and Sang, 1971). The rudimentary strain has a specific pyrimidine requirement (Norby, 1970) but the enigma as to why such very high concentrations of dietary pyrimidine are required to eliminate the wing abnormality is not solved. It appears that the dependency of wing development on endogenous pyrimidine synthesis is not a peculiar property of the wing disk but reflects a property of all imaginal disks (Falk, 1977).

In *Drosophila* the adenylic/cytidylic acid ratio of the diet acts specifically on the expression of particular mutant genes coding for abnormal developmental processes, namely the eyeless phenotype and tumorigenesis (Sang and Burnet, 1963ab). The interesting point which suggests that we are dealing with fundamental processes is that the expression of these same mutant genes is readily affected by juvenile hormone mimics : a farnesoate derivative increases tumor expression in several tumorous strains and farnesyl methylether increases the penetrance of the eyeless gene (Bryant and Sang, 1968). Normalizing effects of farnesol on the Bar eye size, although not confirmed by Bryant and Sang (1968), are noteworthy since uracil has been shown to increase the number of facets of the Bar eye (Demarinis, 1967).

As stressed by Sang and Burnet (1967), it is unlikely that nutritional techniques alone can unravel precisely which process or processes are defective in these mutants. Hunt (1971), working on the nutritional control of gene expression in the eyeless mutant of *Drosophila*, concluded that definitive information about the underlying block in biosynthesis should not be expected by the study of dietary treatments. Yet *Drosophila* is considered to be a good experimental organism to study interactions between genotype and nutritional environment (Collins *et al.*, 1970). In spite of much effort "we are still a long way from realizing the ultimate aim of a biochemical study of growth in any organism not least in *Drosophila*" (Robertson, 1978). We think *Artemia* should become a useful rival to *Drosophila* in the field of developmental biology.

In *Artemia*, the adenylic/cytidylic acid ratio does not act merely on the expression of specific mutant genes but modifies the programmation of postembryonic development. The genetic messages coding for the morphogenesis of abdominal gonopodes which are not translated during "normal" development are translated in larvae reared in a medium containing a lowered adenylic/cytidylic acid ratio. Genetic messages coding for the morphogenesis of posterior thoracic appendages which are translated during "normal" development are not translated in larvae reared in a medium lacking folic acid and thymidine. Thus, completion of "normal" development of *Artemia* requires a proper balance between adenylic acid and cytidylic acid plus folic acid. Since in *Artemia* folic acid is necessary for thymidine synthesis

but not for purine biosynthesis as it is in *Drosophila* (Sang, 1959), the nutritional approach could be more promising in *Artemia* than in *Drosophila*, determining as a result the nutritional basis for a rigorous biochemical approach.

Further work may depend on controlled production of cysts since *Artemia* mutant stocks might be conveniently stored as cysts without need for repeated subculture, (Bowen, 1962). The nutritional requirements of *Moina* for continuous fertility have been formulated (Conklin and Provasoli, 1977) and should facilitate the study of *Artemia* requirements.

Acknowledgement

It is a pleasure to thank Dr. L. Provasoli for his continuous encouragement since my stay in his laboratory.

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One aspect of the nutrition of *Artemia* : the utilization of dissolved amino acids

J. F. Pavillon, Nguyen Thuong Dao, and Vu Tan Tue

Institut Océanographique
195 rue Saint Jacques, Paris, F-75005, France

Abstract

Dissolved organic substances constitute an environmental biological parameter which is important for the development of species. Some of these substances, and in particular those with a low molecular weight, such as amino acids, have been found concentrated in numerous marine invertebrates (Stephens, 1968 ; Stephens, 1972). Their concentration in eggs and larvae of *Artemia* (Metaframe-San Francisco strain) has been studied during the very first stages of development. Twenty amino acids (L) labeled with ^{14}C have been tested and the quantities absorbed measured after combustion of the samples, taken 1, 3, 6, 24, and 48 hr after incubation, before feeding the animals.

During the feeding phase an attempt was made to determine the role of dissolved amino acids on the growth of brine shrimp over a period of 8 to 11 days. Tests were made in the presence of inert food (*Spirulina* powder) in 1 l carboys at a concentration of three *Artemia*/ml ; the results were compared with those obtained with live food (*Phaeodactylum tricornutum*). In order to determine the balance between live food and dissolved amino acids in function of time, a mixture of ^{14}C labeled amino acids was used. Food concentrations were measured with a Coulter Counter and an analyser.

The results show that in certain cases the addition of amino acids can contribute to enrich the inert feed, leading to a positive effect on growth (increase in weight). In the case of live feed, the same addition can turn out to be indirectly harmful to the growth of *Artemia*, in so far as it induces mortality in *Phaeodactylum tricornutum*. Hence the need to control this biological factor in laboratory breeding.

Introduction

The use by marine animals, whether at the egg, larval or adult stage, of dissolved organic substances has been the subject of numerous studies. Indeed the mass of non-organized dissolved organic matter in the environment is generally greater than the biomass of a community and constitutes an important source of energy available under certain conditions for use by living beings. A survey of this problem was made recently by Barker-Jorgensen (1976).

Whereas Putter (1908abc) considered that the dissolved organic substrate was of fundamental importance from the energetic point of view, Krogh (1929) expressed doubts about the analysis techniques employed by Putter and believed that the nutritional value of the dissolved organic substrate was limited.

More recently Stephens (1968) has shown that many marine invertebrates used simple dissolved organic molecules. Some of these molecules can accelerate or inhibit filtration and hence, the capture of nutritive particles in lamellibranchs.

In 1969, Provasoli and d'Agostino, when studying different artifical media for breeding *Artemia* in axenic conditions, came to the conclusion that the amino acids present in the medium were scarcely used and that toxicity thresholds existed. The nutritional contribution was considered insignificant.

Stephens (1972) pointed out that the uptake of dissolved amino acids by marine invertebrates may be compulsory, optional or occasional. In Crustacea he noted that the uptake of dissolved amino acids as food was slight or non-existent.

We have observed for many years that simple organic molecules, such as amino acids, vitamins and fatty acids, present in seawater, could be absorbed by a large number of eggs and larvae of marine invertebrates, according to the metabolic dynamics proper to each species. This observation provided an interesting insight into the biology of development. Indeed, until now, it had always been thought that during the so-called endotrophic phase, the vitelline reserves constituted the larvae's unique source of energy.

Studies on the upake of dissolved amino acids by *Artemia* have two aims : firstly to estimate the cinetics of absorption during the first 48 hr of development and the evolution of the absorption when the animals are feeding, and secondly the impact of the absorption in breeding conditions similar to those applied in aquaculture.

The experiments reported here are divided into three sections :

- a 48 hr study of the absorption of 20 L-amino acids by *Artemia* eggs and larvae ;
- the impact on the growth of *Artemia* of a mixture of dissolved amino acids in the presence of inert pulverised *Spirulina* feed ;
- a study of the absorption of a mixture of ^{14}C labeled amino acids in the presence of live food (*Phaeodactylum tricornutum*).

Materials and methods

Absorption of dissolved amino acids in the medium by eggs and larvae of *Artemia*

Initial solutions are prepared from ^{14}C labeled amino acids in a medium of artificial seawater. The substances are tested separetely and added to the culture medium. They all have a radioactivity of 1 mCi/l which, taking into account all the specific activities, corresponds to the different concentrations of the substances. For example 30 ng of glycine correspond to 1 mCi. Culturing is carried out according to the method described by Sorgeloos and Persoone (1975). 500 mg of dried eggs (San Francisco, Metaframe strain) are added to 500 ml medium contained in pyrex glass siphon tubes. Compressed air bubbling from the bottom keeps the cysts in continuous suspension. The tubes are placed in thermostatic baths at a temperature of 27 °C ± 0.5 °C, in front of a row of fluorescent tubes.

The photoperiod is 12:12 and light intensity at the level of the tubes is 2 000 lux. All the glassware is sterilised before the experiment and no antibiotic is added to the medium, since it can stop the incorporation of amino acids in certain organisms. Prior to inoculation with cysts the medium is filtered through a 0.45 μm porosity membrane. Each test is repeated several times.

Samples

Ten ml samples of eggs and larvae are taken 1, 4, 6, 24, and 48 hr after the start of the experiment ; each sample contains approximately 2 500 individuals. The suspension is passed through a 100 μm mesh strainer to eliminate the radioactive medium, bacteria in suspension and those particles loaded with labeled amino acid. The eggs and larvae are recuperated on a no. 1 Whatman filter paper and rinsed four times in 30 ml of clean medium. After four rinsings we noticed no radioactivity in the waters used for washing. After dessication, the samples collected on the filter are treated by combustion (Packard Tricarb model 306). The combustion products, and in particular $^{14}CO_2$ are trapped by appropriate solvents containing a scintillant mixture (Carbosorb and Permafluor). With this treatment, which under normal conditions produces a 99% yield, it is possible to obtain a homogeneous counting, thus eliminating the problem of autoabsorption.

ABSORPTION OF A MIXTURE OF ^{14}C LABELED AMINO ACIDS IN THE PRESENCE OF LIVE ALGAE (*PHAEODACTYLUM TRICORNUTUM*)

The conditions are the same as for the previous experiments. The ^{14}C labeled amino acid mixture[1] is added at the start of the incubation and every other day. A culture of *Phaeodactylum tricornutum* is added every day as food to the water in which the animals are reared and the particle concentration is measured each time with an analyser (Coultronic's) giving the spectrum of the food concentration (Fig. 1). The difference between the initial spectrum and that obtained after 24 hr corresponds to the gross consumption. The consumption of particles in both media was determined per animal and per 24 hr, after 5×2 experiments. Measurements were made the 3rd, 4th, 5th, 6th, and 9th day of rearing.

The mean filtration rate T which represents the volume filtered per animal within the time unit can be calculated from :

$$T = \frac{V}{N \times t} \times \log \frac{C^o}{C^1} \times 24$$

V = experimental volume
N = number of individuals per unit of experimental volume
t = time in hours
C^o = number of cells introduced per ml in the medium at time t = 0
C^1 = number of cells present in the medium 24 hours later.

[1] Product of CEA, radiochemical grade, 1 mCi of mixture contained :

L-alanine	80 μ Ci	L-isoleucine	50 μ Ci
L-arginine	79 μ Ci	L-phenylalanine	80 μ Ci
L-aspartic acid	80 μ Ci	L-proline	50 μ Ci
L-glutamic acid	125 μ Ci	L-serine	40 μ Ci
glycine	40 μ Ci	L-threonine	50 μ Ci
L-histidine	15 μ Ci	L-tyrosine	40 μ Ci
L-leucine	140 μ Ci	L-valine	80 μ Ci
		L-lysine 60 μ Ci	

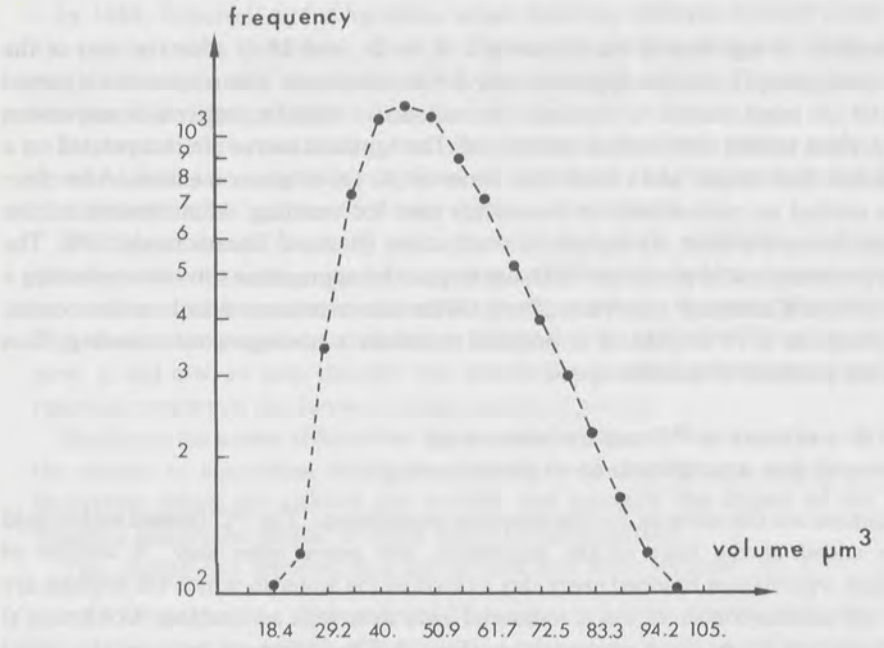

FIG. 1. Volume size distribution (spectrum) of a culture of *Phaeodactylum tricornutum* obtained with a C1000 Analyser (Coultronics). The principal mode is about 45 μm.

Consumption results are adjusted for the mortality rate of *Artemia* in each medium.

Samples of 100 *Artemia* are taken every day for liquid scintillation counting. In the same way the heterotrophy of the algae was estimated by sampling 15 ml of algae and particles and filtering on a membrane.

Mortality is evaluated at the beginning and at the end of the experiments.

Every other day one quarter of the medium is siphoned out and replaced by a fresh medium.

Measurements are carried out on 3×2 strains (three in a filtered natural medium and three in an UV-treated filtered natural medium according to the method described by Manny *et al.*, 1971).

GROWTH OF *ARTEMIA*, FED ON *SPIRULINA* MAXIMA POWDER, IN THE PRESENCE OF A MIXTURE OF DISSOLVED AMINO ACIDS

As a follow-up of the work done by Person-Le Ruyet (1976), who used *Spirulina maxima* (a unicellular blue-green alga) as feed for the development of *Artemia*, several tests have been carried out on the growth of brine shrimp on a mixture consisting of 20 amino acids of the natural series at a concentration of 1 mg/l of the culture medium, *i.e.* an average of 50 μg/l of each amino acid.

the eggs are incubated in the medium containing the mixture of dissolved amino acids. After 24 hr the empty shells, dead eggs and unhatched larvae are separated on a 200 μm mesh strainer. *Artemia* nauplii pass through the strainer whereas the shells are retained. The nauplii are recuperated on a 100 μm mesh strainer and put back in suspension in a well defined volume of seawater in order to determine the concentration of the larvae. The nauplii are then transfered to carboys containing 750 ml of the medium at a concentration of three larvae/ml, *i.e.* a total of 2 000 to 3 000 larvae per carboy. Aeration is provided by a slight bubbling of air through a glass tube connected to an airpump.

The experimental carboys are placed in thermostatic baths at 27 °C ± 0.5 °C in front of rows of projectors (intensity circa 2 000 lux at level of the carboy).

Each experiment lasts for nine days and is repeated several times. The series consists of two test media and two control media.

Before adding the pulverised *Spirulina* powder (100 mg/day) is passed through a 100 μm strainer. Only particles with a diameter smaller than 100 μm are used.

At the end of each experiment the mortality rate is estimated by comparing the number of live *Artemia* in the container with the number introduced originally into the medium (Fig. 2).

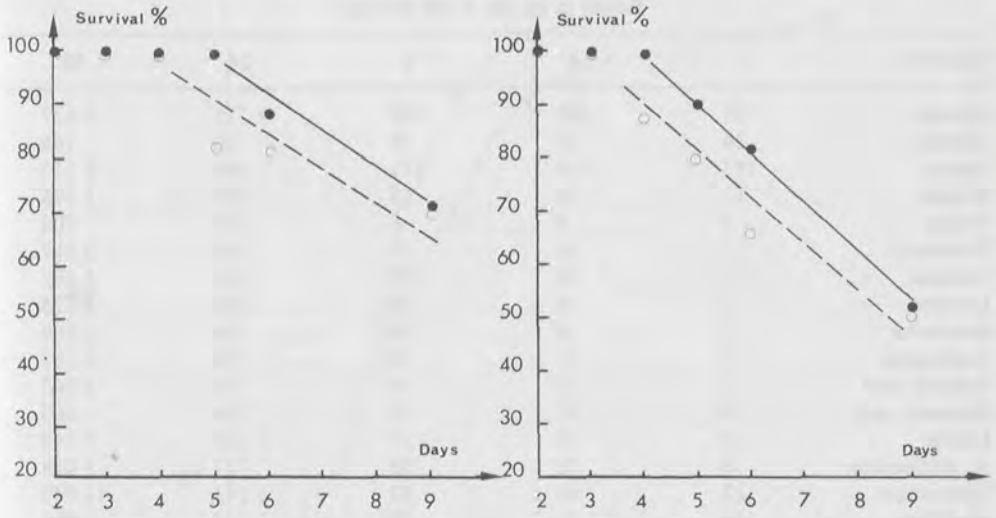

FIG. 2. Percentage survival in natural filtered seawater (●) and natural filtered and UV-treated seawater (○), both in the presence or absence of dissolved aminoacids.

The length of thirty individuals is measured and the dry weight of 500 individuals is determined with a 10% error (the error is of three digits on the microscale or about 3 μg). The samples to be weighed are collected on small strainers previously weighed, rinsed in freshwater and oven-dried for 24 hr at a temperature of 60 °C.

An attempt was also made to compare the growth effects in filtered seawater versus UV-treated seawater.

7×2 tests were run with a concentration of 3 000 nauplii in 750 ml of natural filtered medium (Table III).

4×2 tests were carried out with an initial concentration of 3 000 nauplii in 750 ml of natural filtered medium (Table IV).

3×2 tests were carried out with an intitial concentration of 3 000 nauplii in 750 ml of natural UV treated medium (Table V).

Results

Absorption of dissolved amino acids (Table I)

A 30% adjustment of the resulsts has been made for the eggs, since in previous work we found out that the radioactivity of empty shells could attain this value. The gross radioactivity measure corresponds to the radioactivity of the shell, the radioactivity of the internal medium in which the embryo develops and the radioactivity of the embryo.

Table I

Absorption of ^{14}C-labeled amino acids by *Artemia* eggs (1, 4, and 6 hr) and larvae. Result in pg per 2 500 animals

Time (hr)	1	4	6	24	48
Glycine	80	105	120	1 775	5 627
Alanine	26	53	39	83	166
Serine	187	419	211	600	1 173
Proline	11	15	18	252	1 196
Valine	4	8	8	179	704
Threonine	17	16	18	352	3 317
Cysteine	31	55	99	132	1 102
Leucine	5	6	10	298	1 725
Isoleucine	7	8	10	124	2 669
Asparagine	14	11	14	750	4 163
Aspartic acid	10	23	16	56	1 047
Glutamic acid	10	17	17	214	665
Lysine	8	18	27	1 424	7 743
A. glutamique	8	22	38	727	1 058
Methionine	22	40	42	1 143	11 049
Histidine	19	25	29	153	681
Phenylalanine	12	13	16	168	2 053
Arginine	36	22	23	888	3 691
Tyrosine	15	22	18	127	1 362
Tryptophan	511	365	393	343	1 053

The quantities absorbed by the larvae are relatively low. Only glycine serine and tryptophan are absorbed in fairly large quantities during the first 6 hr.

After 24 hr the absorption in a decreasing quantitative order is the following : glycine, lysine, methionine, arginine, glutamic acid, asparagine, serine, threonine, tryptophan, *etc.* 48 hr later the uptake of methionine is the greatest, followed by lysine, glycine, asparagine, arginine *etc.* The quantities absorbed by 2 500 eggs in 1 hr range from 4 pg (valine) to 411 pg

(tryptophan). For the larvae, the quantities after 48 hr range from 11 ng (methionine) to 166 pg (alanine).

Absorption of a mixture of ^{14}C labeled amino acids in the presence of *Phaeodactylum tricornutum*. Consequence on the grazing

The measurements carried out on a filtered natural medium and a UV treated filtered natural medium, show an absorption in terms of time evolving towards saturation, in accordance with the Michaelis-Menten Law.

Significant differences between the two media are not evident until the end of the 3rd day. On the 9th day, fixation of the amino acids is twice as great in the natural medium as in the UV treated medium (Fig. 3).

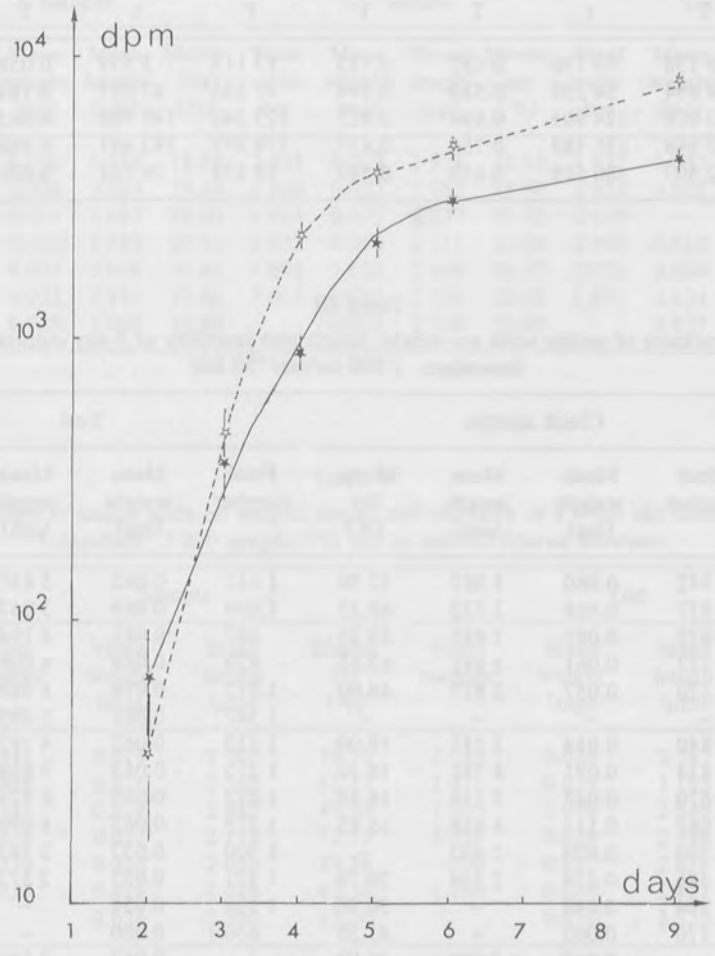

Fig. 3. Absorption of a mixture of ^{14}C-labeled amino acids by 100 *Artemia* during 9 days in natural filtered seawater (☆) and in natural, filtered and UV-treated seawater (★).

The results of Table II show that on the 3rd and 4th day of the experiments the filtration rate is lower in the UV-treated medium. This filtration rate, however, tends to accelerate later. The differences between the medium containing amino acids and the control medium are insignificant.

TABLE II

Mean uptake of *Phaeodactylum tricornutum* by *Artemia* per 24 hr and daily filtration rate in the absence (T) or presence (t) of dissolves amino acids

Days	Seawater				UV-treated seawater			
	Mean food upake per 24 hr		Daily filtration rate in ml^{-1}		Mean food uptake per 24 hr		Daily filtration rate in ml^{-1}	
	T	t	T	t	T	t	T	t
3	39 138	35 790	0.187	0.185	13 113	5 539	0.058	0.028
4	84 854	54 358	0.580	0.294	41 836	47 093	0.194	0.250
5	123 076	124 905	0.694	0.627	123 340	140 996	0.682	0.700
6	137 948	131 185	0.786	0.637	170 952	143 491	0.960	0.695
9	92 503	80 519	0.650	0.540	88 878	76 768	0.600	0.570

TABLE III

Effect of a mixture of amino acids on weight, length and mortality of 9-day culture of *Artemia* (inoculum : 2 000 larvae/750 ml)

Control	Check sample				Test			
	Final number	Mean weight (mg)	Mean length (μm)	Mortality (%)	Final number	Mean weight (mg)	Mean length (μm)	Mortality (%)
1	942	0.060	3 567	52.90	1 131	0.062	3 657	43.45
	1 037	0.068	3 732	48.15	1 109	0.069	3 967	44.55
2	935	0.067	3 885	53.25	687	0.081	4 194	65.65
	727	0.061	3 491	63.65	820	0.068	4 000	59.00
3	1 120	0.057	2 927	44.00	1 012	0.074	3 969	49.40
	–	–	–	–	1 347	0.061	3 595	32.65
4	840	0.054	3 751	58.00	1 110	0.067	4 192	44.50
	834	0.091	4 741	58.30	1 235	0.063	3 658	38.25
5	1 670	0.047	3 210	16.50	1 072	0.069	4 379	46.40
	683	0.113	4 488	65.85	1 175	0.062	3 990	41.25
6	2 104	0.024	2 402	–	1 500	0.037	2 742	25.00
	1 586	0.034	2 534	20.70	1 421	0.032	2 572	28.95
7	1 394	0.049	–	30.30	1 108	0.058	–	44.60
	1 170	0.063	–	41.50	650	0.080	–	67.50
Mean		0.060	3 520	46.09		0.063	3 750	44.33

GROWTH OF *ARTEMIA* FED ON *SPIRULINA MAXIMA* POWDER IN THE PRESENCE OF A MIXTURE OF DISSOLVED AMINO ACIDS

Growth appears to be somewhat higher in the medium to which amino acids have been added. The slight differences (less than 10%), when submitted statistically to a t test, are however not significant. The response expressed by a small increase in weight is nearly identical whatever the medium. (Tables III, IV, V).

TABLE IV

Effect of a mixture of amino acids on weight, length and mortality of a 9-day old population of *Artemia* (inoculum : 3 000 larvae/750) in natural, filtered and UV-treated seawater

Control	Sample				UV sample				UV test			
	Final number	Mean weight (mg)	Mean length (μm)	Mortality (%)	Final number	Mean weight (mg)	Mean length (μg)	Mortality (%)	Final number	Mean weight (mg)	Mean length (μm)	Mortality (%)
1	2 436	0.026	2 733	18.80	2 014	0.027	2 978	32.86	1 932	0.031	3 032	35.60
	2 127	0.026	3 023	29.10	1 960	0.027	3 082	34 66	1 933	0.033	3 293	35.56
2	2 190	0.011	2 097	27.00	1 889	0.015	2 337	37.03	2 634	–	2 122	12 20
	2 396	0.010	2 398	20.13	2 032	0.014	2 111	32.26	2 468	0.012	2 174	17.73
3	2 376	0.021	3 036	20.80	1 892	0.028	2 900	36.93	1 776	0.034	2 759	40.80
	2 174	0.022	2 931	27.50	2 015	0.023	2 818	32.83	1 951	0.024	2 888	34.96
Mean		0.019	2 700	23.88		0.023	2 700	39.90		0.027	2 710	29.45

TABLE V

Effect of a mixture of amino acids on weight, length and mortality of a 9-day old culture of *Artemia* (inoculum : 3 000 nauplii/750 ml) in natural, filtered seawater

Control	Sample				Test			
	Final number	Mean weight (mg)	Mean length (μm)	Mortality (%)	Final number	Mean weight (mg)	Mean length (μm)	Mortality (%)
1	2 441	0.019	2 207	19.70	2 146	0.036	2 347	29.40
2	1 466	0.036	2 565	51.13	1 471	0.039	3 195	50.96
	1 581	0.028	2 642	47.30	1 702	0.038	2 846	43.26
3	836	0.021	2 173	72.13	1 188	0.020	2 194	60.40
	1 339	0.020	2 099	55.36	760	0.027	2 411	74.66
4	1 137	0.029	2 426	62.10	1 190	0.026	2 457	60.33
Mean		0.025	2 350	51.20		0.031	2 570	53.16

Discussion and conclusions

The use of radiotracers in experimental marine ecology makes it possible to follow the pathway of marked substances introduced into the medium. Thanks to current methods of liquid scintillation counting, we are now able to test concentrations compatible with those existing in natural seawater. Preliminary work started a few years ago has shown that an active absorption of dissolved elements takes place in the egg. Comparative tests with dead eggs have revealed that, following the cyst rehydratation stage, radioactivity remains constant even after 48 hr (Pavillon, 1976).

Tests have been carried out on the whole range of amino acids present at varying though usually low concentrations in all types of natural waters. The origin of the amino acids can differ ; either they are metabolic products from living organisms or they result from the degradation of dead organisms or of more complex molecules. The presence of certains amino acids (such as alanine) in the marine environment is doubtful (Daumas, 1971). This theoretical absence may be due to extremely low (undetectable) concentrations or to a rapid uptake or degradation by certains organisms.

There is no doubt that an active absorption of amino acids dissolved in the medium by the eggs and nauplii of *Artemia* occurs during the first 48 hr. The first autoradiographic studies made on the eggs show on the one hand a contamination of the egg membranes, through which the substances pumped from the medium pass, and on the other hand the presence of these substances in embryonic tissues. On the 10th day, a longitudinal cross section of *Artemia* shows that marked substances have penetrated into the intestinal wall.

Quantitatively the notion of a low absorption of amino acids must be defined ; considering the scale at which the phenomenon takes place and the yields obtained by treating the samples by combustion (a treatment after which counting reveals the total of the marked elements absorbed), this phenomenon indeed seems to be important.

Interactions with the pool of free intracellular amino acids are likely. These absorbed amino acids take part in the anabolic and respiration processes of the organisms. It seems that at fairly low concentrations certain amino acids slow down development. The impact of amino acids on the development of bacteria must be taken further into consideration.

In the presence of inert food particles such as *Spirulina maxima* the absorption or adsorption of amino acids helps to increase the nutritive value of the element provided.

This contribution may however, be unfavorable with some amino acids by causing a flight behavior in the animal or a refusal of the food. This has already been demonstrated in fish, particularly salmonids. Furthermore the presence of high concentrations (several hundred μg/l) of amino acids, such as glutamic acid or glycine, may have a toxic effect (slow-down of growth, increased mortality in the larvae).

Dissolved amino acids are still assimilated in the presence of a living food. The heterotrophy of *Phaeodactylum tricornutum* with regard to the amino acids is very slight, as we have been able to demonstrate, and does not account for the radioactivity of *Artemia*. The radioactivity measured corresponds to about 95% of that of the dissolved amino acids absorbed.

Furthermore, because of the toxic effect of the amino acids on the algal culture, the daily particle food may have been overestimated by at least 5 to 10%.

It is rather difficult to compare the two types of food available, since the mixtures of amino acids used differ. The few measurements of size and weight that we have made in the

experiment with food consisting of living particles seem to point to better results with food based on inert particles. But the algal concentration used was relatively low and never exceeded 5.10^5 cells/ml.

Moreover *Spirulina* offers a more varied size range of particles. The use of *Spirulina* in powder as a support of organic components with high nutritive value in aquaculture must be the subject of more precise investigations ; in particular the search for acceptable concentrations and the most interesting substances in different categories of soluble organic compounds (certain fatty acids, vitamins, *etc.*) should receive detailed attention.

At high concentration the impact of amino acids in the environment must be considered as inhibitory for the development mof organisms, because of its inherent toxicity, or because it reduces the quantity of living food available to organisms. In small quantities, on the contrary, dissolved amino acids favor development.

Acknowledgement

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Biphasic particulate media for the parthenogenetic *Artemia* of Sète

L. Provasoli and I. J. Pintner

Haskins Laboratories, Biology Department, Yale University
New Haven, Connecticut 06520, USA

Abstract

The artificial medium for the axenic cultivation of the amphigonic Utah strain of *Atemia* supports good growth to adults, but fails to do so for the parthenogenetic *Artemia*.

The addition to this medium of fatty acids and fat-soluble vitamins supported nine uninterrupted generations of the parthenogenetic *Artemia* of Sète, indicating that lipids are essential for the continued fertility of *Artemia*. The lipids were provided as emulsions and as lipid-containing particles ; the latter, favoring phagotrophy, were more efficiently utilized by *Artemia*.

In these media growth in darkness was slower and mortality higher than in continuous light. Light favored viviparity and darkness oviparity, but occasional ovideposition occured in light and occasional viviparity in darkness.

The life cycle of the Sète *Artemia* is completed in 45 to 60 days indicating that this strain, though voracious, utilizes poorly both the living algae and the artificial food.

Introduction

Biphasic particulate media meet the special phagotrophic needs of the filter feeders *Artemia* and *Moina macrocopa*.

The nutrients in these media are supplied as solutes and particulates. The soluble phase comprises minerals, vitamins, amino acids, peptones and nucleic acids. The particulate phase supplies carbohydrates, proteins and when necessary a protein-lipid particle.

The first of these media was developed for the amphigonic Utah strain of *Artemia* (Provasoli and D'Agostino, 1969). It allowed good survival and rapid growth from newborn to adult. No attempt was made to determine whether or not the adults were fertile and produced durable cysts. When this medium was used on the parthenogenetic Comacchio strain of *Artemia* it allowed, at best, growth from newborn to juvenile ; if young females were inoculated they produced one or two broods of newborn which died very soon after birth. Evidently the medium failed to supply some essential nutrients for continued growth, the only valid criterion for determining whether or not all the nutritional requirements were satisfied.

Since the dixenic culture of the Comacchio *Artemia* on *Dunaliella* was lost a few years ago and we did not have any viable cysts, the parthenogenetic race of Sète (France) was axenized and grown synthrophically on *Dunaliella parva*. A parthenogenetic strain was selected for this study to avoid additional aseptic manipulation such as required for copulation and hatching of the durable cysts.

Because continued reproduction of *M. macrocopa* (Conklin and Provasoli, 1977) depended on the availability of lipids and fat soluble vitamins we suspected that the failure in obtaining reproduction by the Comacchio strain was due to a lack of these fecundity factors. Indeed the addition of suitable lipid particles allowed axenic generations of the Sète strain.

Material and methods

Durable cysts of the Sète strain were kindly supplied by Dr. A. Hernandorena of the Centre d'Etudes et de Recherches, Biarritz, France.

Axenization was accomplished by using the technique of Provasoli and Shiraishi (1959). A few hundred cysts were put at the bottom of a sterile test tube, and the tube was filled with sterile seawater and shaken vigorously to break up cysts clumps. Broken or empty cysts were eliminated by two flotations. When the light cysts were eliminated, the seawater was drained and replaced by a Merthiolate solution (1:1000) to which was added 0.2 ml/100 of a 10% solution of Aerosol OT to favor wetting. The Merthiolate was changed three times while shaking vigorously during the 10 min exposure to the disinfectant, after which the Methiolate was eliminated by draining and filling the test tube four times with sterile seawater. After the last wash a few eggs were transferred into several test tubes of the sterility test medium STP (Provasoli and Shiraishi, 1959). After hatching, single metanauplii from non-infected tubes were transferred to test tubes of sterile seawater enriched with 2 ml/100 ESI (Provasoli, 1966) and inoculated with species of *Dunaliella*. Contrary to the Comacchio strain which preferred a mixture of *D. salina* and *D. viridis* (D'Agostino and Provasoli, 1968) the Sète strain grew best when fed on *D. parva* (Dr. K. Gold isolation). The sterility of the dixenic culture on *D. parva* was tested periodically.

Particle preparation

Starch-albumin 5:1 co-gel

Dissolve 250 mg of two times crystallized egg albumin (ICN Co.) in 30 ml distilled H_2O then add 1.25 g of rice starch. The suspension is autoclaved in a beaker for 20 min at 9,072 Kg pressure. The coagulum and water are transferred to a Virtis container and homogenized at top speed for 5 min (use two straight blades as a cross). Autoclave and homogenize the slurry again and bring to 50 ml by adding filtered seawater.

Carotene mix 2

It is a mixture of two suspensions. Part A : dissolve in 50 ml distilled H_2O 0.1 ml Tween 80 ; heat and disperse in it 50 mg of soy lecithin. Part B : mix well in a small tube 50 mg of β carotene and 50 mg of butylated hydroxytoluene in 0.3 ml of Tween 80 ; add 1 ml of a 50% propylene glycol solution in H_2O, mix and bring the mixture by slow addition of water to 50 ml. Add slowly Part B to Part A, mix well ; it should become a well dispersed suspension ; add preservative, flush with N and store at -20 °C in a tightly closed bottle.

Retinol palmitate suspension

Dissolve in 2.5 ml ethanol 50 μl of retinol palmitate ; add and mix 1 ml of a 5% solution of Tween 80 in H_2O. Disperse 25 mg of egg lecithin in 10 ml H_2O and add to the retinol palmitate

– Tween mix ; stir well and bring to 45 ml with H_2O. Boil to about 20 ml to eliminate the ethanol and bring to 50 ml with H_2O.

TABLE I

Composition of lipid particles (1 ml contains)

	SFA	FV13
Egg albumin	8 mg	8 mg
Egg lecithin	0.75 mg	0.75 mg
Butylated hydroxytoluene	1 mg	1 mg
Dl-α-tocopherol	2 µl	2 µl
Ergocalciferol	660 µg	660 µg
Stearic acid	1.2 mg	—
Palmitic acid	1.2 mg	2.0 mg
Oleic acid	0.2 µl	0.6 µl
Linoleic acid	0.2 µl	1.4 µl
Linolenic acid	0.2 µl	2.0 µl

SFA and FV13 particles

These are more complex mixtures of fatty acids and lipid soluble vitamins. Disolve 300 mg of two times crystalline egg albumin in 25 ml H_2O and disperse into it 75 mg of egg lecithin in a Virtis flask (No. 16-115). This flask has an enlarged bottom with small fluting and a side arm capped with a small rubber cap. The lipid solutions are prepared separately in a short small test tube, by dissolving them in 1.5 ml acetone in the specified order. Stirring with a glass rod and the use of the Vortex Genie helps to dissolve the mixture completely. All of this mixture is drawn in a small hypodermic syringe with a thin needle. The albumin-lecithin dispersion is homogenized thoroughly for 3 min. at top speed (with two straight blades) before the lipid solution is slowly squirted into the Virtis container through the rubber cap covering the side arm. Homogenize for 5 min at top speed, then add 500 mg of egg albumin and homogenize for 5 additional min. The emulsion from the Virtis container is transferred to a 600 ml beaker and coagulated in a boiling water bath with continuous stirring. Following this rapid coagulation the mixture is autoclaved and homogenized twice for 5 min and the final volume brought to 100 ml. Homogenization is done under a continuous flow of nitrogen from a hypodermic needle inserted in the side arm cap of the homogenizer vessel. The lipid particle mixes are stored refrigerated at 5 °C in glass stoppered bottles which have been flushed with N_2 ; N_2 flushing is done after any opening of the bottle. A few drops of a volatile preservative (1 part of each of 0-fluorotoluene and 1,2 dichloroethane and 3 parts of chlorobutane ; Eastman Organic Chemicals) are added to prevent microbial growth. Several lipid formulae were used, the variation being mainly in the quantitative and qualitative content in fatty acids which mimicked the content of fatty acids in the different algal divisions. The more successful were SFA and FV13, a modification of the lipid mix used by Conklin and Provasoli (1978) for *Moina macrocopa americana*. The lipid mix for the SFA particle is as follows : dissolve in 1.5 ml of acetone in the following order : butylated hydroxytoluene (BHT) 100 mg ; dl -α-tocopherol 200 µl ; ergocalciferol (D_2) 66 mg ; retinol palmitate 25 µl ; palmitic acid 120 mg ; stearic acid 120 mg ; oleic acid 20 µl ; linoleic and linolenic acids 20 µl each. The lipid mix for

the FV13 particle is as follows : dissolve in 1.5 ml acetone in the following order, BHT 100 mg ; dl -α- tocopherol 200 µl ; ergocalciferol (D_2) 66 mg ; retinol palmitate 25 µl ; palmitic acid 200 mg ; oleic acid 60 µl ; linoleic 140 µl and linolenic 200 µl.

ARTIFICIAL MEDIA

The media are biphasic and comprise a liquid and particulate part. All media have in common the following base ; they differ only in the lipids additions. Common basal medium : charcoal-treated filtered seawater to 100 ml ; CP3 metals 1 ml (1 ml cotnains Na_2EDTA 1 mg ; Fe 40 µg ; Mn 10 µg ; Zn 10 µg ; Cu 5 µg ; Co 5 µg ; V 5 µg) ; AP3 metals 1 ml (1 ml contains Br (as Na) 3 mg ; B (as H_3BO_3) 200 µg ; Mo (as Na Mo $O_4.2H_2O$) 5 µg ; I (as NaI) 3 µg) ; glycyl-glycine 70 mg ; Na_2 glycerophosphate 50 mg ; glucose 300 mg ; sucrose 300 mg ; amino acid mix 3 ml (3 ml contains histidine base 18 mg ; 1-phenylalanine 9 mg ; 1-threonine 18 mg ; 1-serine 36 mg ; glutamic acid 90 mg) ; nucleic acid mix 2 ml (2 ml contains adenylic acid 120 mg ; cytidylic acid 60 mg ; thymidine 5 mg) ; Vitamin mix 2 ml (2 ml contains thiamine 2.4 mg ; nicotinic acid 4.8 mg ; Ca pantothenate 8 mg ; pyridoxine 0.2 mg ; riboflavin 0.6 mg ; folic acid 1.4 mg ; biotin 0.06 mg ; putrescine 0.4 mg) ; cholesterol 0.5 mg ; Oxoid liver L-25 70 mg ; 6 ml of starch-albumin co-gel ; pH 7.8-7.0.

Media used

Minimal medium 1C = 100 ml common base.

Medium 1B = 100 ml common base + 2 ml of carotene mix 2 + 0.5 mg of retinol palmitate.

Medium 1BS = 100 ml medium 1B + 1 ml % rabbit serum added aseptically.

Medium SFA = 100 ml common base + 1 ml SFA particles slurry.

Medium FV13 = 100 ml common base but using 70 mg % defatted Oxoid liver + 1 ml/100 of FV13 particles slurry.

Results

The Sète strain is a slow grower. In the best artificial media, as well as when it is fed on *D. parva*, the complete cycle from newborn nauplius to fertile female takes from 45-60 days. By contrast, the Utah amphigonic race grows to fertile adults in about 17-20 days. As in the Utah strain the adults are extremely voracious and in 1-2 days free of particles a dense deep green culture of flagellates or an artificial medium having ~30% transmission. Longevity may reach 3 or 4 months. Healthy females may deposit up to a maximum of six to ten broods, either viviparous or oviparous, and the period of deposition may last over a month. The deposition interval varies from 3 to 6 days and is in general shorter in young females ; the size of a brood varies between 30 and 50 eggs. Not all of them hatch viviparously ; the best broods are of 30-40 nauplii even in the best conditions of light and diet.

EFFECT OF LIGHT AND DARKNESS ON VIVIPARITY

In general light favors viviparity and darkness oviparity. However, in darkness occasional viviparous broods may be deposited. In light occasonal depositions are completely viviparous ; hatching, varies from 90% to 10%. The action of darkness or light is more pronounced when the shift to either dark or light occurs at or after the stage of immature

females (*i.e.* with no or very small ovisac). Even mature females which have already deposited several oviparous broods in the dark change to viviparity after a delay of 10-12 days from the day in which the shift to light occurs. Similarly a delay in depositing oviparous progeny occurs when mature females which had already produced viviparous broods in light are transferred to darkness. This delayed effect of light and darkness may indicate that the stimulus acts on the early stages of development of the eggs being produced in the ovaries *i.e.* long before they migrate to the ovisac.

The intensity of light has a general influence ; a dim light (~400-600 lux) especially if alternated with an equal period of darkness (12/12) favors oviparity more than either continuous dim or bright light (6 000 lux). Red light (~650-700 nm) acts very much like dim white fluorescent light even though the crustacean eye is supposed to be insensitive to this wave length.

It has been possible by nurturing the few viable nauplii produced in darkness or in continuous red light to obtain at best two generations in darkness and three to four generations in continuous red light. Under these conditions growth is quite slow and the mature females are far less voracious. Survival under these conditions is also drastically reduced. In general death occurs more often at certain stages of growth : when the meta-nauplii start to ingest, when they become small juveniles or when they are young females.

The sensitivity of these stages to external physical or nutritional conditions is also felt in continous bright light. Not all the viviparous broods result in a new generation even though the females were kept in optimal feeding conditions, *i.e.* they were transferred to new media rich in particles to avoid even a short period of starvation. Despite these precautions only one or two broods out of four to eight viviparous broods reached the stage of fertile females. The viable broods are generally the most numerous ones and show an overall greater vitality in their swimming. Obviously survival and number of fertile broods depends on media completeness.

Media

The failure of medium 100 designed for the amphigonic Utah race to support, the growth of the Comacchio strain indicates that the parthenogenetic strains have higher nutritional demands. Since continued fertility of *Moina*, a freshwater cladoceran, depends on the supply of fatty acids and fat-soluble vitamins, the lack of such substances in medium 100 may be the cause of the unsuitability of the medium (Conklin and Provasoli, 1977).

Assuming that the other nutritional requirements might be identical, the components of medium 100 were added to seawater instead of an artificial mineral base. The particulate phase was 5/1 cogel of starch/albumin. It soon became evident that this medium was inadequate, indicating that the Sète strain was as exacting as the Comacchio strain. Oxoid liver L-25, one of the main components of the undefined medium for the Utah *Artemia* (Provasoli and Shiraishi, 1959) was added at 70 mg % (1C medium) and proved satisfactory. This medium is the simplest and did support, in continuous light, four parthenogenetic generations.

The Oxoid liver extract is a rich source of unknown nutrients and has a total lipid content of 12.4 % (as kindly analysed by Dr. G. Holz). Holz found that most of the lipids were neutral ; the phospholipids and the polar lipids were present only in traces. Gas chromatographic analysis yielded the following fatty acid ratios : 4.2 % myristic ; 29.3 % palmitic ; 3.3 %

palmitoleic ; 19.8 % stearic ; 31 % oleic ; 2.7 % linoleic ; 1.4 % linolenic. This liver preparation does not dissolve completely in water ; it leaves a red-brown precipitate. The fatty acids apparently complex with bile salts forming an emulsion or a colloidal solution. Since these particles may be too fine to be effectively trapped by the filter feeding apparatus and since the medium supports growth, it is probable that the emulsions are adsorbed onto the cogel particles thus allowing an efficient ingestion of the fatty acids.

TABLE II

Effects on *Artemia* of Sète of additions of the basal medium

Medium	Type of addition	Number of succesive generations
1C	Common basal medium only	4
1B	+ carotene and retinol palmitate	4
1BS	+ serum, carotene and retinol palmitate	8
SFA	+ SFA lipid particles	9
FV13	+ defatted Oxoid Liver and FV13 particles	6

Because algae, the usual food for *Artemia*, are rich in carotenoids which are essential for the nutrition of phytophagus insects, medium 1C was supplemented by a β-carotene and retinol palmitate suspension in Tween 80 and lecithin (Medium 1B). Since ascorbic acid is often an additional requirement for phytophagous insects, ascorbylpalmitate was also tried alone and in combination with various carotene – containing mixes (Medum 1A). Media 1A and 1B proved to be not substantially better than medium 1C. Only the aseptic addition of 1 ml percent of rabbit serum to medium 1B(= 1BS) increased substantially the brood size, survival, and speed of growth. Eight generations were obtained in 1BS but started to decline in fertility after the fifth generation.

To insure phagotrophic efficiency the lipids were added to medium 1C in particulate form, *i.e.* lipid/albumin coagulated complexes. Several types of these particules were so formulated as to supply the fatty acid components and ratios present in diatoms, cryptomonads, chrysomonads and chlorophytes. The mixtures mimicking qualitatively and quantitatively the chlorophytes (FV13) and SFA replaced serum ; the others mixtures were partially inhibitory and caused a notable delay in growth.

Various attempts were made to substitute the Oxoid liver. It was possible to grow *Artemia* on defatted liver by adding FV13 particles. These particles are quantitatively rich in unsaturated fatty acids and contain double the amount of the fatty acid mix (*i.e.* 6 mg instead of the 3 mg used for the SFA particles). Medium FV13 is the most defined minimal medium allowing continued fertility for six generations. Attempts made to substitute the water soluble portion of liver were unfruitful. Hemin, chelated ferric iron, a rich trace metal mix, glycogen, high folic acid and B_{12}, bile acids alone or in combination were insufficient to support development of the ovisac and deposition of eggs in young females. Only the combination of all of these led in one case to ovisac-bearing females but no eggs were produced. The search was interupted because the Sète *Artemia* is a slow grower and also because detection of nutritional requirements was outside the scope of this work.

Removal of the ergosterol and tocopherol from the SFA particles resulted in poorly viable nauplii indicating that these fat-soluble vitamins might be important for continued fertility.

Removal of glucose and sucrose from the basal medium as well as substitution of them with glycogen showed that a soluble energy source is not needed or is poorly utilized ; the media are already rich in fatty acids and particulate starch.

Discussion and conclusion

The scope was to find media that allowed continuous generations of *Artemia*. This was achieved. The number of continuous generations which each medium supplied allow some tentative conclusions. The media, 1C, 1A and 1B permitted only four generations, the FV13 six generations, the 1BS eight generations and the SFA nine generations. These media differ mainly in the way the fats are presented to the animals. The main source of fat factors for the poor media (1C, 1A, 1B) is the Oxoid liver which brings in assorted fatty acids in a form which is appartently poorly utilizable. The fats are probably a fine emulsion or a colloidal complex with bile. The size of the fat particles could be therefore too small for efficient filter feeding of *Artemia*. This seems to be confirmed by the ineffectiveness of the addition of fine emulsions of the lecithin-Tween-carotene mixes in media 1A and 1B. An improvement was the addition of 1-2 ml % of rabbit serum (1BS) which increases substantially the quantity of fatty acid added to the medium. This higher fat supply apparently compensates in part for the poor acceptability of an emulsion.

Media SFA and FV13 supply the fats as large particles (2-10 μm) which are efficiently and completely ingested by *Artemia*. The media are cleared of all particles rapidly. The superiority of the SFA medium over the FV13 can be due to several factors. Regarding the quality of fats, the SFA is rich in saturated fatty acids while FV13 is high in unsaturated, hence the nutritional need may be better satisfied by the composition of the SFA particles. The other major difference is that the FV13 medium uses a defatted Oxoid liver, hence the quantity of fats is significantly less despite the fact that FV13 particles have double the amount in fats of the SFA particles. The elimination, however, of the fats coming from the liver may cause a nutritional deficiency in some needed fatty acid.

Despite these uncertainties as to why SFA is better, some conclusions may be reached. It is clear that the Sète parthenogenetic strain is highly inefficient ; it eats an enormous amount of food and grows very slowly in comparison with the Utah strain. Another major difference is that the Utah strain can grow to adults on media devoid of lipids. This indicates that growth up to adults can be sustained by the high reserves in lipids present in the durable cysts. The evident need for lipids at all stages of growth of the Sète strain may depend either on quantitative higher requirements in fats or in lower reserves in lipids of the parthenogenetic eggs. In either case growth and fertility of the Sète strain depends upon a continuous supply of exogenous lipids of the right kind and quantity, making it more sensitive to the algal supply from the environment.

As occurred for *Moina*, particles containing fats are far more efficient than fats presented as emulsions in collodial form ; their greater efficiency depending on stimulating phagothrophy besides supplying the quantitative and qualitative requirements for growth and reproduction.

Summary

The *Artemia* parthenogenetic strains of Comacchio and Sète can not be grown on the artificial medium which supports good growth to adults of the amphigonic Utah strain. Since fatty acids and fat-soluble vitamins are required for the continuous fertility of *Moina macrocopa*, a freshwater cladoceran, liver Oxoid and lipids were added to the defined artificial medium for the Utah strain ; up to nine uninterruppted generations were obtained in the best medium.

Lived Oxoid and lipids are both necessary for continued fertility of the Sète strain. Liver Oxoid supplies unknown essential water-soluble growth factors as well as 12% of emulsified fats, but alone allows only four consecutive generations. The aseptic addition of 1 ml % of rabbit serum resulted in eight generations, though vigor and fertility declined after the fourth generation. Serum can be substituted with advantage by the addition of albumin particles containing fatty acids and vitamins D_2 and E (SFA and FV13 particles). Nine consecutive generations were obtained in liver Oxoid + SFA particles ; vigor declined at the eighth generation.

The different efficiency of the media depends largely upon the way the lipids are presented. The Oxoid liver and serum being fat emulsions are less efficiently utilized than the lipid particles since *Artemia* is a voracious phagotroph. The apparent discrepancy that the amphigonic Utah strain can grow to adults in fat-free media may be due to the high fat reserves of the cysts. The evident need for lipids of the Sète strain indicates that parthenogenetic strains may need a continuous supply of fats for growth.

In these media growth in darkness was slower and mortality higher than in continuous light. Light favored viviparity and darkness oviparity, but occasional ovideposition occured in light and occasional viviparity in darkness.

The life cycle of the Sète *Artemia* is completed in 45 to 60 days indicating that this strain, though voracious, utilizes poorly both the living algae and the artificial food.

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The digestive enzymes amylase and trypsin during the development of *Artemia* : effect of food conditions

J. F. Samain, J. Moal, J. Y. Daniel, J. R. Le Coz, and M. Jezequel

Centre Océanologique de Bretagne
B.P. 337, F-29273 Brest Cedex, France

Abstract

Activities of the digestive enzymes amylase and trypsin have been studied during development of *Artemia* (San Francisco, USA) cultured at different concentrations of phytoplankton (*Tetraselmis suecica*).

The synthesis of the two enzymes varies independently throughout the development. Activity levels are controlled by food levels as well as by food composition. To obtain an optimum assimilation yield at a given food level, an adaptive mechanism would involve 1) modulation of ingestion rates based on physicochemical properties of food particles and 2) regulation of synthesis of digestive enzymes by ingestion and chemical composition of the food. Rates of ingestion and assimilation vary more during development than does the yield. Maximum yield is achieved during the exponential growth phase and is related to food requirements. As such, regulation of digestive enzymes during development would optimize the use of the food available, as a function of the food requirements.

Introduction

Many authors have suggested that Crustacea such as *Artemia* (Bellini, 1957ab), the shrimp *Palaemon serratus* (Van Wormhoudt, 1973), and *Penaeus japonicus* (Laubier-Bonichon *et al.*, 1977) show wide variations in protease and amylase activity during the larval development. They described the influence of developmental stages on these variations, as have other authors for mammals (Corring and Aumaitre, 1970 ; Lebas *et al.*, 1971) and Amphibia (Urbani, 1957). Experiments on pre-feeding stages (developing embryos) demonstrated the existence of periods for genetic expression of amylase and protease synthesis (Bellini, 1957ab ; Kulka and Duksin, 1964). Experiments on the dependence of the period of larval development on food, were generally performed with a progressively adapted diet, either natural or modified by the experimenter. It was impossible to conclude to an adaptation of the digestive equipment resulting from the new food, or to a new particular behavior of the animals corresponding to a time or stage dependent expression of the genetic information (Corring and Aumaitre, 1970 ; Lebas *et al.*, 1971). In order to try to solve this question, we have used a uniform diet throughout the experiments reported in this paper; Since the appearance of digestive enzymes is thought to be an indicator of a nutritional requirement (Cuzon, 1970), relationships between ingestion, assimilation, and digestive enzyme synthesis were investigated.

Materials and methods

Dry *Artemia* cysts (San Francisco strain from Metaframe California, USA) were rehydrated in filtered seawater at 22 °C. Nauplii were collected after 24 hr and incubated in 20 l tanks (at the same temperature) under continuous artificial illumination.

Food was provided daily from the second day following hatching at different concentrations and various ratios (k) of algae/*Artemia* protein.

Daily the cultures were cleaned of moults and dead bodies while the seawater medium was regularly replaced and regulated as a function of the k values.

Monospecific cultures of the phytoplanktont *Tetraselmis suecica*, grown by the aquaculture team of the Oceanologic Center of Brittany, were used as food. Batch cultures were started with a single addition of nutrients in 60 l polyethylene bags. The cultures were harvested after 7-10 days at a concentration of 2.10^6 cells/l. Continuous cultures were maintained in 20 l tanks. Each day 1/4 of the culture was removed for use, followed by the addition of a corresponding volume of seawater enriched with nutrients and vitamins (Flassch and Normant, 1974).

The phytoplankton concentration was estimated by microscopic counting in a Malassez cell or electronic counting with a Coulter Counter. Oxygen, pH, and temperature were regularly controlled ; NH_4, NO_2 and the number of bacteria were followed in experiment number 3.

Artemia were sampled at random ; 100 freshly hatched nauplii and three to four adults were necessary for one analysis. The whole organism was ground up in a Thomas blender with distilled water. Digestive enzymes and proteins were analyzed on Technicon autoanalysers using an adapted method (Samain *et al.*, 1977).

The body length was measured under a binocular lens with an ocular micrometer. The stage of development was determined according to the methodology described by Provasoli and D'Agostino (1969).

Estimation of ingestion and assimilation rates by C^{14} were based on a new method (Samain, in preparation) which takes the criticisms expressed by Conover and Francis (1973) into account. C^{14} respiratory- and faecal excretion was measured simultaneously with the apparent C^{14} ingestion rate ; possibilities of recycling of the labeled material were eliminated by an adapted washing procedure. Prior to use, the phytoplankton was uniformly labeled with sodium C^{14}-carbonate.

The first experiment, carried out in duplicate, was performed with a routine phytoplankton culture at a concentration of 300.10^6 cells/l ; $k = 300.10^2/\mu g$. Samples were taken every 2 hr from 8.00 am to 6.00 pm for 25 days after hatching.

In the second experiment three different algal food concentrations were compared (calculated as number of cells per *Artemia* protein weight : $k = 200.10^2/\mu g$, respectively $300.10^2/\mu g$ and $400.10^2/\mu g$) at a constant concentration of 300.10^6 cells/l of routine phytoplankton culture and a duplication of the $300.10^2/\mu g$ conditions. Samples were taken once a day at 10.00 am for 29 days after hatching. Growth rate data were collected during the first twelve days.

The third experiment was carried out with four different phytoplankton concentrations (75, 150, 300, and 600.10^6 cells/l) at a constant k ratio = $600.10^2/\mu g$. Samples were taken once a day at 10.00 am for 24 days after hatching.

The last experiment was performed with 230.10^6 algal cells/l and $k = 330.10^2/\mu g$; the phytoplankton originated from continuous cultures with high nutrient and vitamin concentration. Samples were taken once a day at 10.00 am for 33 days.

For the labeling experiments, part of the *Artemia* population was incubated with phytoplankton uniformly labelled with C^{14}.

Results

Experiment 1

The results (Fig. 1 and 5.1) show that reproducible peaks of amylase and trypsin appear at different times during growth. This phenomenon is distinct from individual and diurnal variations.

Amylase

A major peak appears from the 1st day to the 11th day. It consists of two possible peaks at day 2 and day 6 respectively (L = 0.68 and 1.00 mm). A secondary peak appears from the 12th to the 15th day (L = 2.24 mm). A tertiary peak begining at 23 days (L = 6.3 mm). Levels of specific amylase activity decrease from the first to the third peak (1.1, 0.6, 0.25 units).

Trypsin

A major peak appears from the 1st to the 20th day, consisting of two possible peaks at days 7 and 14 respectively (L = 1.1 and 2.3 mm). A secondary peak appears on the 22nd day (L = 6.3 mm). Levels are 240 units for the first bimodal peak and 60 units for the secondary peak.

Experiment 2

The results (Fig. 2 and 5.2) show that growth of the $k = 300.10^2/\mu g$ duplicates is nearly identical and only slightly inferior to that at $k = 400.10^2/\mu g$; $k = 200.10^2/\mu g$ conditions give a reduced growth.

These various k conditions do not markedly affect the enzyme profiles except for a distinctly lower amylase level at $k = 400.10^2/\mu g$ conditions and lower trypsin level under the $k = 200.10^2/\mu g$ conditions.

Amylase

A major bimodal peak occurs from the 1st to the 7th day (L = 0.6 to 1.0 mm), a secondary peak occurs from the 7th to the 12th (L = 1.0-1.4 mm), a tertiary one from the 19th to more than 29 days (L > 3.2 mm). Highest levels of specific amylase activity are 0.75-0.47-0.70 units. With the $k = 400.10^2/\mu g$ conditions, lowest levels are 0.5-0.20 units.

Trypsin

A continuous low level (maximum : 80) activity decreases from the 1st day to the 24th. This broad and low peak is perhaps bimodal : 1-13 days (L = 0.6-2.0 mm) and extends beyond the 19th day (L > 5.0 mm). The levels of these two possible peaks are similar (70 units). In comparison with the first experiment, the mean activity of amylase and trypsin is generally

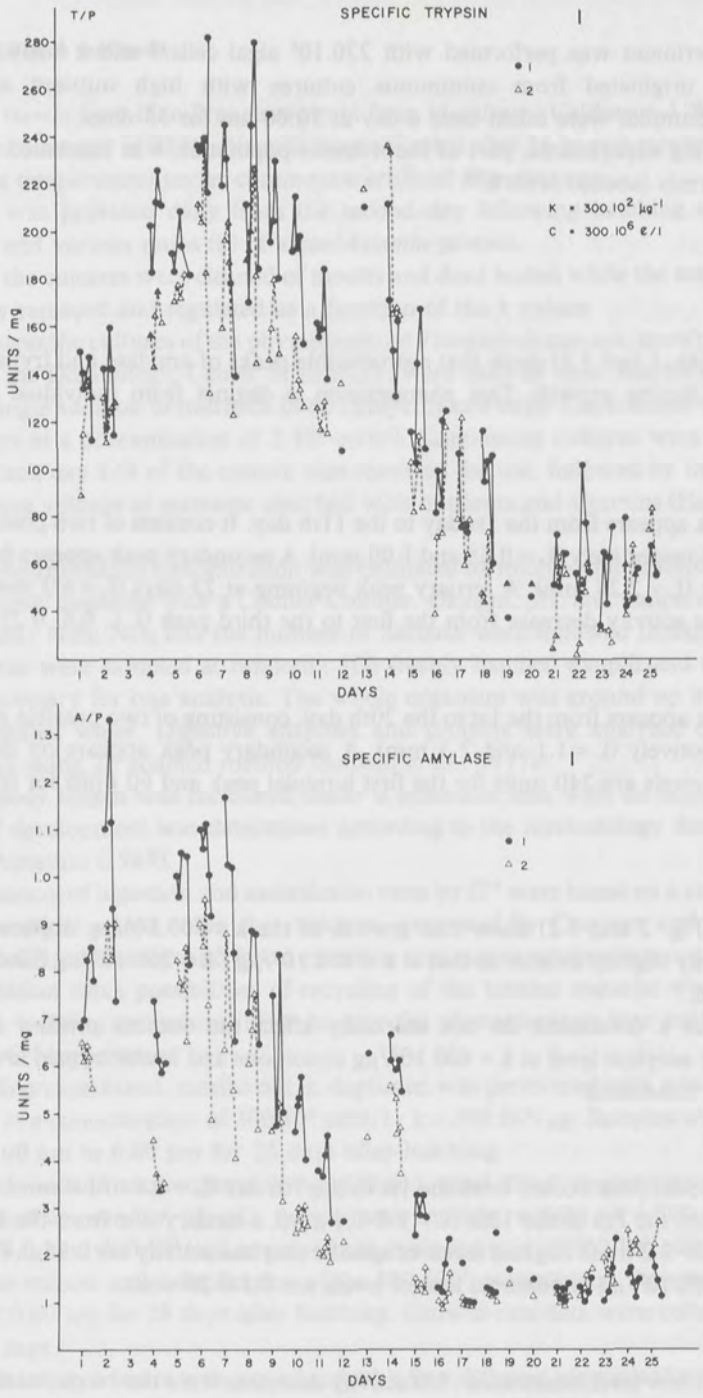

FIG. 1. Experiment 1. Specific trypsin and specific amylase activity during the first 25 days after hatching. Experiment carried out in duplicate. Phytoplankton concentration $C = 300.10^6$ cells/l; $k = 300.10^2/\mu g$.

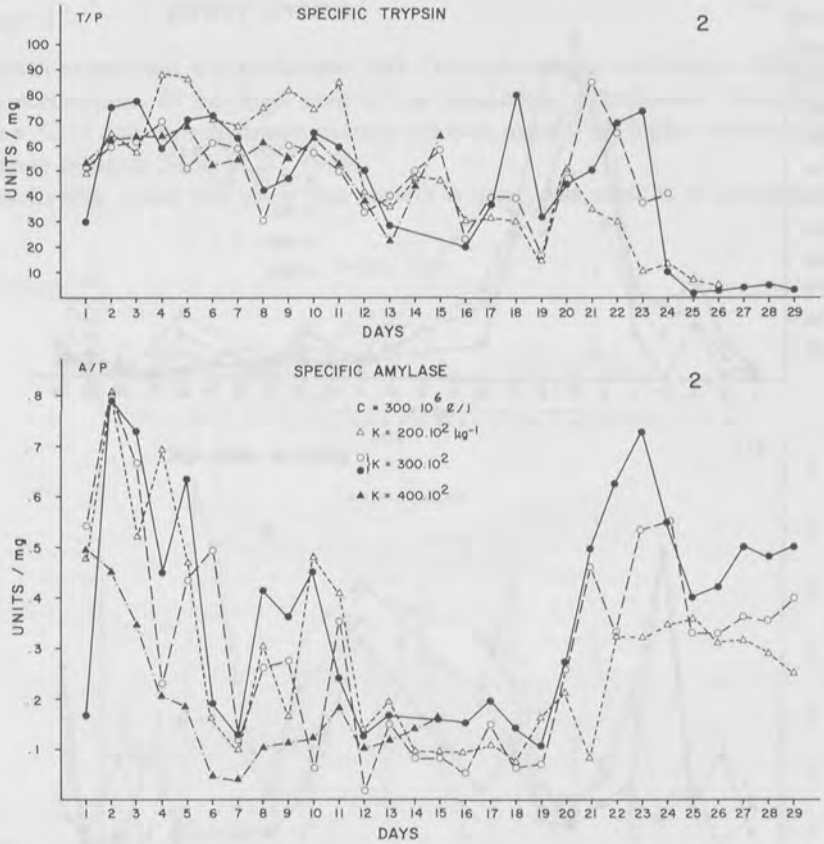

FIG. 2. Experiment 2. Specific trypsin and specific amylase activity during the first 23 days after hatching. Phytoplankton concentration : $C = 300.10^6$ cells/l ; k variable $\triangle k = 200.10^2/\mu g$; $\circ \bullet$ $k = 300.10^2/\mu g$; $\blacktriangle k = 400.10^2/\mu g$.

lower in this experiment (amylase maximum = 0.70 unit/1.1 unit ; trypsin maximum : 70/260 units). The $k = 200.10^2/\mu g$ conditions show a broad bimodal peak reaching 80 units and a lower secondary peak of 50 units.

Observations on developmental stages show that the trypsin peak occurs at the metanauplius stage and decreases slowly until the appearance of the first adults where trypsin levels increase again. The first amylase peak appears at the metanauplius stage, decreases rapidly, while the second one coïncides with a majority of the population at stage V. Subsequently, the levels are depressed from stage VI to X, and the third peak appears as the first animals reach the adult stage.

EXPERIMENT 3

The results (Fig. 3 and 5.3) show that growth and enzyme levels are identical at these four conditions. However, growth is higher than in experiment 1 or 2.

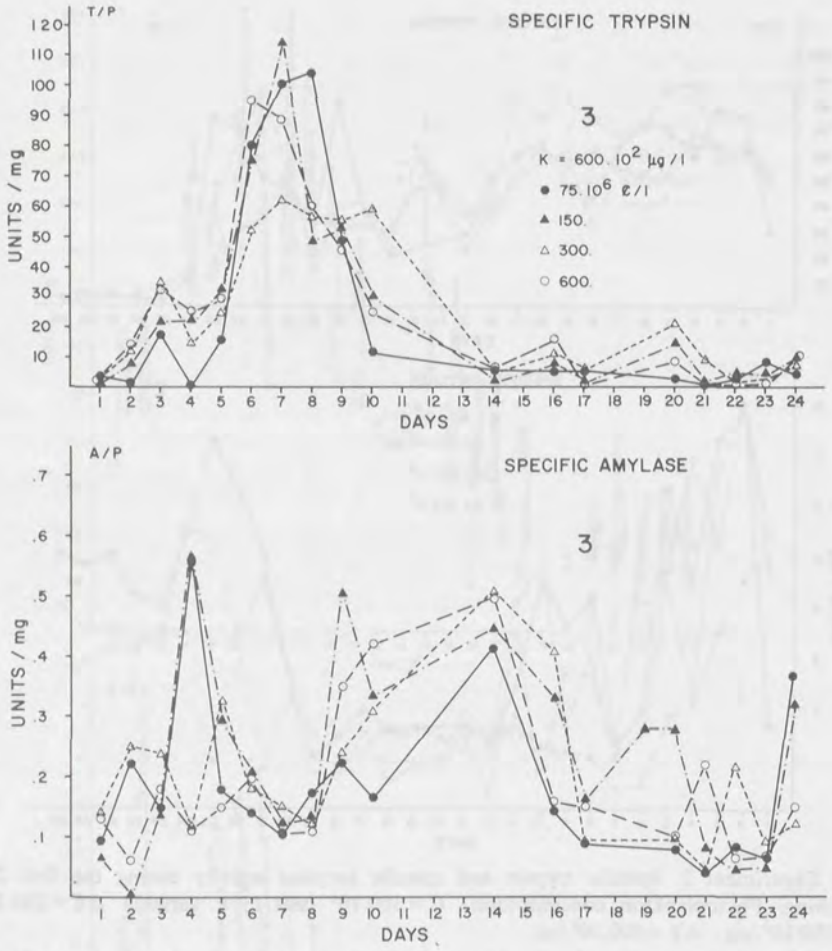

FIG. 3. Experiment 3. Specific trypsin and specific amylase activity during the first 24 days after hatching. Phytoplankton concentrations : ● C = 75.10⁶ cells/l ; ▲C = 150.10⁶ cells/l ; △C = 300.10⁶ cells/l ; ○ C = 600.10⁶ cells/l ; k constant = 600.10²/µg.

Amylase

Two main peaks of amylase occur during the first week (L = 0.60-2.80 mm) and from the 8th to the 17th day (L = 3.5 to 8.9 mm) highest levels are 0.57 and 0.50 units. The first peak is possibly bimodal (1-3, 3-7 days ; L = 0.6-1, 1-2.8 mm).

Trypsin

A sharp peak of trypsin (maximum value = 110 units) is visible between 4 and 10 days (L = 1,4 to 4.5 mm) preceeded by a small peak on the 3rd day (L = 1.1 mm). After this, the activity is at a very low level. Mean activities of these two enzyme levels are lower than in the preceeding experiments.

EXPERIMENT 4

The fourth experiment was performed with *Tetraselmis* from continuous cultures having higher concentrations of nutrients than in the preceeding experiments. These conditions induce low C/N and carbohydrates/protein ratios in algae with higher protein and lower carbohydrate contents (Moal *et al.*, 1978).

The results (Fig. 4 and 5.4) show that growth is nearly the same as in experiment 1.

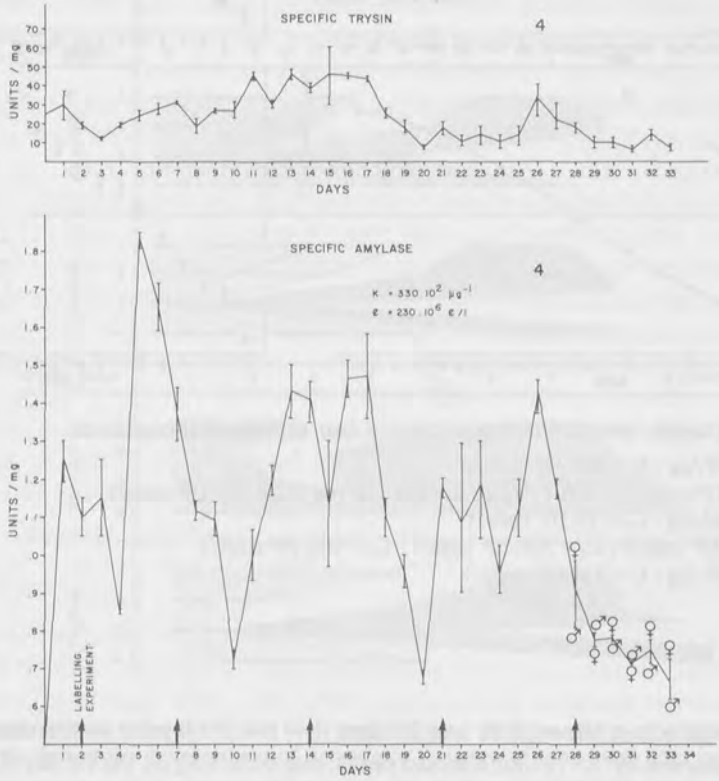

FIG. 4. Experiment 4. Specific trypsin and specific amylase activity during the first 26 days after hatching. Phytoplankton concentration : $C = 230.10^6$ cells/l ; $k = 330.10^2/\mu g$. Experiment carried out in duplicate. Males (♂) and females (♀) are separated when sexual dimorphism is visible. Labeling is performed at five different moments during the development.

Amylase

Three bimodal peaks of equal magnitude occur during growth, the first one appearing between days 1 and 10 with a maximum to 1.25 and 1.65 units (L = 0.6 to 1.0 mm). The second peak appears between 10 to 20 days, with a maximum at 1.4 and 1.45 units (L = 2 to 4 mm), while the third occurs between 20-28 days, with maximum values at 1.2 and 1.4 units (L = 6.3 to 9.0 mm). These maximum values are higher than in the preceeding experiments.

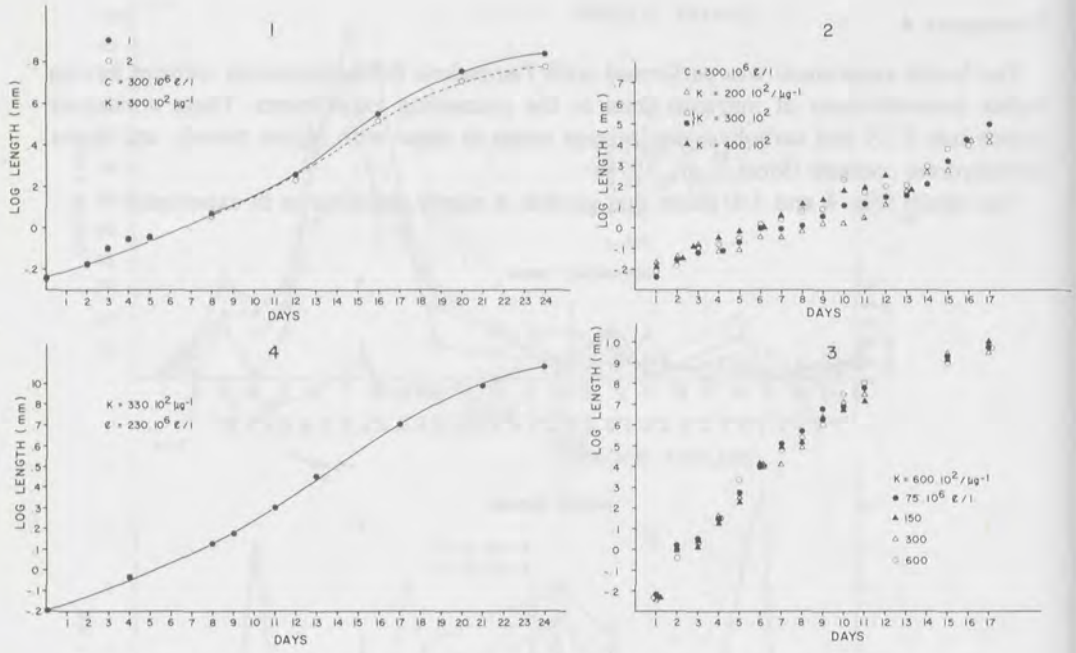

FIG. 5. Log length (mm) in function of time in four experimental conditions.

5.1. $k = 300.10^2/\mu g$; $C = 300.10^6$ cells/l.
5.2. $k_1 = 200.10^2/\mu g$; $k_2 = 300.10^2/\mu g$; $k_3 = 400.10^2/\mu g$; $C = 300.10^6$ cells/l.
5.3. $k = 600.10^2/\mu g$; $C_1 = 75.10^6$ cells/l ;
$C_2 = 150.10^6$ cells/l ; $C_3 = 300.10^6$ cells/l ; $C_4 = 600.10^6$ cells/l.
5.4. $k = 300.10^2/\mu g$; $C = 230.10^6$ cells/l.

Trypsin

A major peak occurs between 10 and 20 days (L = 1.4 to 5.0 mm) with a maximum at 45 units. It is preceeded by 30-32 unit bimodal peaks, one occurring on the 1st day (L = 0.63 mm) and one on the 7th day ($L \simeq 1$ mm). A sharp peak of 32 units occurs between 25-29 days ($L \simeq 9$ mm). These maximum values are lower than in the preceeding experiments.

Occurrence of amylase and trypsin peaks as a function of time or length of the animals are summarized in Fig. 6. Three main peaks of amylase and two main peaks of trypsin generally occur during the development, except in experiment 3, where growth has been considerably higher.

Amylase

Considering the time scale in the three other experiments, the first peak occurs between the 1st and the 10th day, the second between the 8th and the 20th, and the third after the 20th day. On the length scale, the first occurs between 0.6 to 0.9 mm, the second one between 1 to 6 mm, the third one between 3 to 9.5 mm.

Trypsin

Again, on the time scale, the first peak occurs between 1-20 days, and more usually between 1-12 days, while the second is after 19-20 days. In terms of length, the first peak occurs between 0.6-5.0 mm and more usually between 0.6-2.0 mm.

A scheme summarizing the four experiments is shown in Fig. 6.

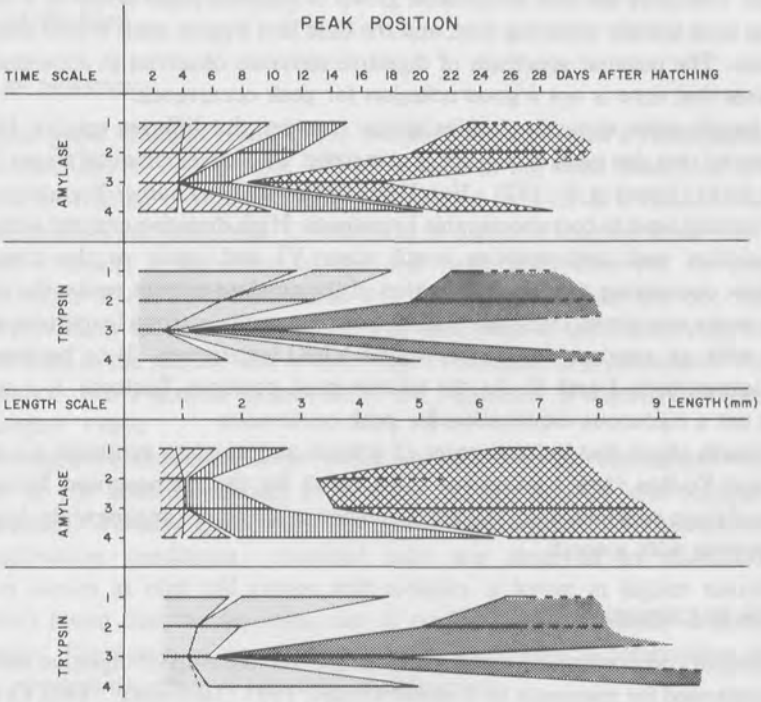

FIG. 6. Summarizing scheme of the four experiments: peak positions of amylase and trypsin specific activity during the development of *Artemia* in function of time (days after hatching) or body length (mm).

Discussion

Very distinct variations of amylase and trypsin activity are observed during the development of *Artemia*. This phenomenon is independent from diurnal and individual variations.

Regulation by growth

When comparing the four experiments, it is obvious that the high growth rate in experiment 3 has restricted this phenomenon to a reduced time scale. On the other hand a low rate of growth spreads the phenomena along the time scale (experiment 4). Two major groups of amylase and trypsin peaks are discernable. The first group is apparent during the early

stages of development, and the second at the beginning of adult stages. Amylase peaks are more distinct from one another than trypsin peaks. Thus, three possible amylase peaks are visible in the first group, a bimodal peak and a more distinct single peak. A possibly bimodal trypsin peak is suggested in the first group (experiments 1, 2, 4). The second group seems to be quite homogenous. In the third experiment, the second broad amylase peak relates to the second group peak in experiment 1, 2, and 4 and so does the major trypsin peak of this experiment. Therefore the first discernable group of amylase peaks could be a surimposition of the three ones usually occuring first, and the little first trypsin peak would contain the first bimodal one. The peculiar spectrum of digestive enzymes observed in experiment 3 clearly demonstrates that time is not a good criterion for peak occurrence.

On the length scale, some similarities appear between the different spectra. Other authors have suggested that this peak occurence is correlated with developmental stages (Corring and Aumaitre, 1970 ; Lebas *et al.*, 1971 ; Van Wormhoudt, 1973 ; Laubier-Bonichon *et al.*, 1977). Our observations tend to corroborate this hypothesis. High digestive enzyme activity is visible during nauplius and metanauplius (until stage V) and again at the preadult stages. Nevertheless, comparing the relative position of the amylase-trypsin peaks, the second group of trypsin peaks sometimes coincides with the second amylase group (experiment 1) (preadult stage), or with an amylase depression (stages VI-X) (experiment 3) or between these two positions (experiment 2 and 4). As the relative peak positions fluctuate, it is apparent that stages are not a rigourous explanation for peak occurrence.

These results show that enhancement of trypsin and amylase synthesis are independent. Howard and Yudkin (1963) found the same result for the rat pancreas. Nevertheless, the occurence of these enzymes is not strictly correlated to a length – or time scale, but to a general scheme varying with growth.

Regulation by chemistry of the food

The chemical composition of the diet influences the make-up of digestive enzymes as has been demonstrated for mammals by Pavlov (Vasilev, 1893 ; Jablonsky, 1895), Grossman *et al.* (1942), Desnuelle and colleagues (Reboud *et al.*, 1962, 1964, 1966 ; Ben Abdeljlil *et al.*, 1963 ; Ben Abdeljlil and Desnuelle, 1964 ; Marchis-Mouren *et al.*, 1963), Corring and Aumaitre (1970), O. Mack *et al.* (1975), and Bucko *et al.* (1976). The same has been suggested for insects by Shambaugh (1954), Langley (1966), Engelmann (1969), Yang and Davies (1968), Hosbach *et al.* (1972) and for Crustacea by Van Weel (1960). In each experiment reported here, *Artemia* were fed a single species of food, therefore the occurrence of peaks should be independent of the nature of the food. The nature of food can be characterized by different species of phytoplankton or particles, but also by the chemical composition of the food. Chemical composition of *Tetraselmis* cultures had been systematically monitored and it was demonstrated that the chemical composition of these algae was highly influenced by nutrients and stages of the culture (Moal *et al.*, 1978). Nevertheless no correlation was found with peak occurrence. The intensity of peaks is so variable that they sometimes disappear (first bimodal peak of amylase experiment 3, second amylase peak of k $400.10^2/\mu g$ in experiment 2, first mode of the trypsin bimodal peak in experiment 4). The influence of chemical composition of the food on the intensity of enzyme synthesis can be extrapolated from the present study. The intensity of digestive enzyme systems varies more from one experiment to another than with

concentrations or k ratio variations within an experiment. Experiments 1, 2, and 3 have indeed been performed with cultures from uncontrolled batch production (high and varying C/N and carbohydrates/protein ratio), whereas experiment 4 was carried out with controlled continuous cultures under conditions of constant saturated nutrient conditions (low C/N and carbohydrates/proteins ratio). The larger differences in the two enzyme levels observed between experiments with batch cultured algae and continuously cultured algae, corroborate the hypothesis that digestive enzyme levels are related to fluctuations in the chemical composition of the food.

Regulation by ingestion

In a previous paper (Samain *et al.*, 1975), we have demonstrated a relationship between various concentrations (0, 35, 75, 150, 300.10^6 cells/l) of the same food, and amylase and protease levels of *Artemia* of different length categories, as well as a relationship between ingestion and digestive enzyme levels. Correlations of digestive enzyme levels with ingestion processes and chemical composition of the diet have been observed for zooplankton organisms (Boucher *et al.*, 1975 ; Mayzaud and Poulet, 1978). Last but not least a short starvation of previously fed *Artemia* results in a *de novo* peak of specific amylase and trypsin which we attribute to the sudden lack of food (Fig. 7) (Samain, in preparation). These results are in accordance with a general hypothesis of the regulation of digestive enzyme level by ingestion (Langley, 1966).

Indeed a changed chemical composition of the phytoplankton can correspond with physical modifications of the cells influencing in turn the grazing activity and consequently the ingestion processes. We have noticed that the general appearance of *Tetraselmis* cells varies with the cultivation conditions : clustered cells are observed in stationary phases, sedimentation occurs at high pH values and mobility is lower at higher nutrient levels. Conover (1966) found that the ingestion rate of copepods was markedly depressed in old cultures of algae. Regulation of digestive enzymes by the chemical composition of particles could be the result of the effect of ingestion modifications.

Regulation by food requirements

In the four experiments reported above only the k ratio significantly influences the growth of *Artemia* and seems to be the limiting factor during the entire study. When k is constant, modification of algal concentrations probably induces few variations in ingestion and, as a consequence in digestive enzyme levels (experiment 3). When k varies, some differences can be observed (experiment 2) at a constant high concentration ($c = 300.10^6$ cells/l). Resultant growths are quite modified and some differences in enzyme levels are significant ($k = 400.10^2$ amylase, $k = 200.10^2$ trypsin (experiment 2), $k = 600.10^2/\mu g$ (experiment 3)). During his study on the influence of quantity and concentration of food on *Artemia* growth, Mason (1963) found the same phenomena. The k factor corresponds to the food available. We have to compare it to the food requirement. For an identical and limiting k (experiments 1, 2, 4) the results in terms of growth are quite the same. As digestive enzyme levels are very different from one experiment to another, it is possible that an adaptive mechanism is operating by way of digestive enzyme synthesis to modulate the assimilation efficiency in relation to the varied ingestion levels (Sushchenya, 1970). This adaptive mechanism would consist of the

modulation of ingestion rates induced by the physicochemistry of particles (Provasoli and d'Agostino, 1969 ; Poulet and Marsot, 1978) in the regulation of digestive enzyme synthesis, as a function of these ingestion levels, and as a function of the chemical composition of particles, to obtain an optimum assimilation yield in relation to food requirement.

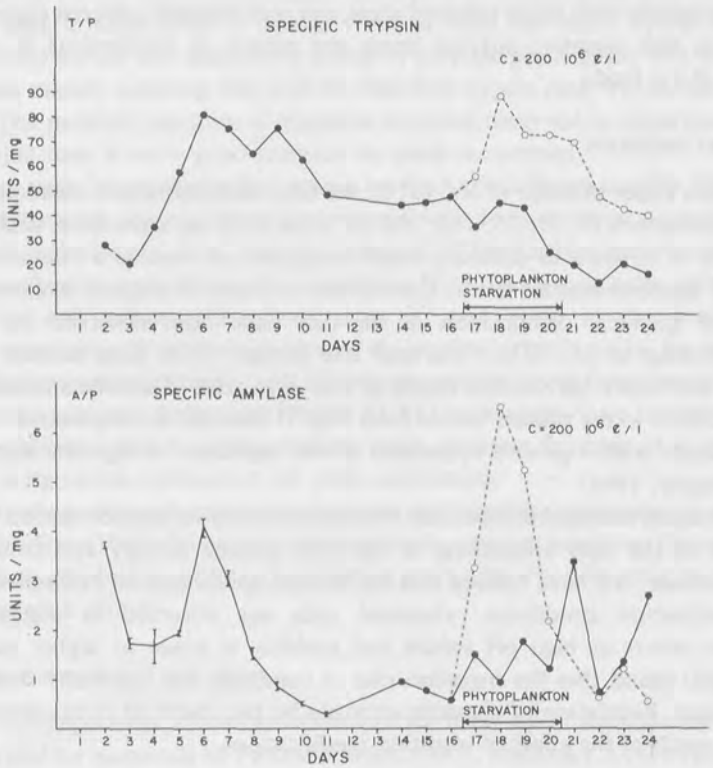

FIG. 7. Experiment on short term starvation during *Artemia* development. Effect on 4 days starvation on specific amylase and trypsin activity of *Artemia* usually fed phytoplankton at a concentration of 200.10^6 cells/l.

Many authors suggested that the variations of digestive enzymes during developmental processes can be attributed to food requirements (Van Wormhoudt, 1973 ; Laubier-Bonichon *et al.*, 1977). Nutritionists take into account observations of the digestive equipment for the determination of food requirements (Cuzon, 1970). Some observations on varying growth efficiency at different developmental stages have already been reported for *Artemia* (Mason, 1963 ; Reeve, 1963) and copepods (Mullin and Brooks, 1967 ; Päffenhöfer, 1976) ; varying ingestion levels have also been mentioned (Marshall and Orr, 1956 ; Paffenhöfer, 1971). The results are in accordance with the hypothesis suggested above.

Our fourth experiment has been performed with five determinations of ingestion and assimilation rate during growth by a new C^{14} method (Samain, in preparation). Results show

that digestive enzymes vary with assimilation and ingestion in a well defined pattern which can be mathematically expressed as follows (Samain *et al.*, in preparation):

$$\frac{\text{Ass}}{k(E)} = \frac{\text{Ass max}(I)}{K + (I)}$$

where Ass = Assimilation rate
Ass max = maximum assimilation rate
I = ingestion rate
E = digestive enzyme specific activity
k = proportional constant
K = ingestion rate corresponding to $\frac{\text{Ass max}}{2}$

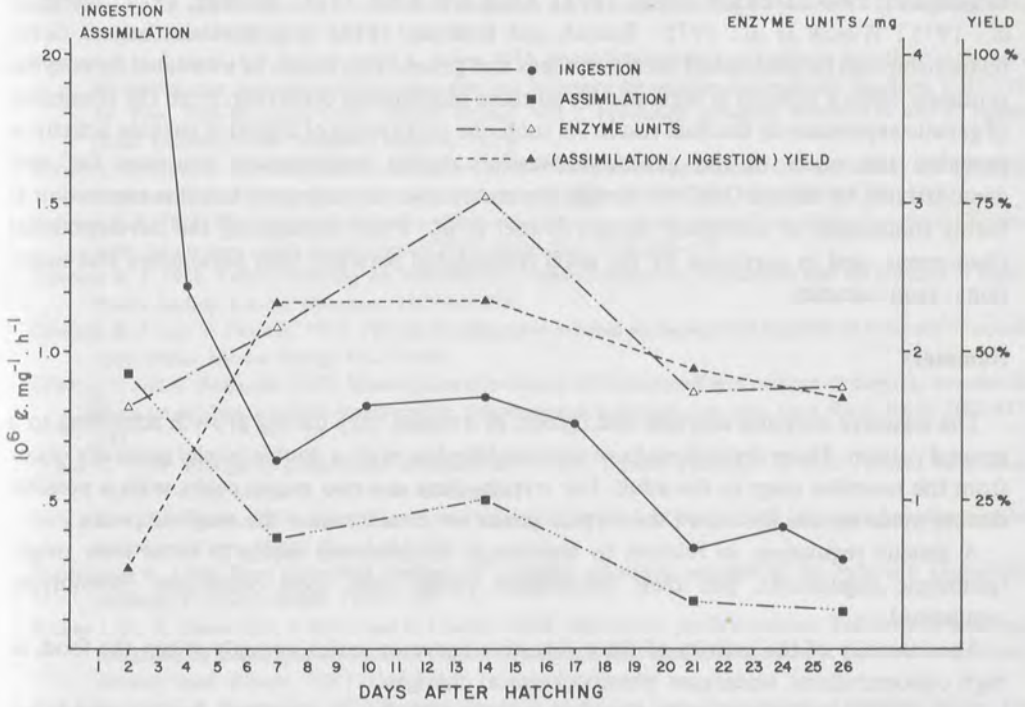

FIG. 8. Experiment 4. Ingestion and assimilation rates, enzyme units = total (amylase + 0.045 trypsin) specific units; mean value m of 18 daily samples, s = 0.1 m (assimilation/ingestion) × 100 yield, in function of time.

Furthermore ingestion and assimilation rates vary during the growth when food parameters (concentration and k ratio) are constant (Fig. 8). These latter results demonstrate the existence of an adaptive mechanism involving ingestion rates and digestive enzyme synthesis. They corroborate the hypothesis of specific periods of nutritional requirements in relation to

development. In the fourth experiment, two periods of higher assimilation are visible at the first stages of development (nauplius, metanauplius) and at the preadult stages, with an optimum of the assimilation/ingestion yield during the exponential phase and a lower yield during an apparent latent and stationnary phase. The occurrence of a latent phase depends on the experiment, and could appear when the assimilation yield is low, or when ingestion and enzyme-stage dependent syntheses are not in good accordance with food characteristics. The specific activity of amylase and trypsin has been summed in the mathematical expression. From all the experiments presented here, it appears that the relative amylase/trypsin ratios vary from one expriment to the other. This could point to mechanisms of optimisation in the use of food components.

As moulting or developmental phenomena are the result of complex interactions between genetic expression, hormonal regulation, and ecological conditions such as food, temperature, light, *etc.* (Fisk and Shambaugh, 1952 ; Van Weel, 1960 ; Langley 1966 ; Rutter *et al.*, 1968 ; Engelmann, 1969 ; Dickard Felber, 1974 ; Adler and Kern, 1975 ; Butcher, 1975 ; Felber *et al.*, 1975 ; Wojcik *et al.*, 1975 ; Rudich and Butcher, 1976), it is obvious that no direct relationship can be established between stages and genetic expression as translated by enzyme synthesis. Such a relation is regulated by all these phenomena occurring, from the possibility of genetic expression to the final result. As such, the occurrence of digestive enzyme activity is probably the result of the genetic expression during development processes (as was demonstrated by Bellini (1957ab) during the embryonic development) but this expression is highly modulated by ecological factors (Rutter *et al.*, 1968) influencing the developmental phenomena, and in particular by the good relationship between food parameters and nutritional requirements.

Summary

The digestive enzymes amylase and trypsin in *Artemia* vary during growth according to a general pattern. Three distinct peaks of amylase (the first with a double mode) generally occur from the nauplius stage to the adult. For trypsin there are two major peaks with a possible double mode on the first one ; the trypsin peaks are broader than the amylase peaks.

A genetic regulation, in relation to nutritional requirements seems to be at their origin (genotypic regulation), but their occurrence varies with food conditions (phenotypic regulation).

The intensity of the activity of these digestive enzymes varies strongly when the food, at high concentrations, undergoes physicochemical changes.

Assimilation, resulting from digestive enzyme activity on ingested food, depends on developmental stages. In our experimental conditions, two periods of higher activity are visible : metanauplius stage and preadults stage ; assimilation/ingestion yield is optimum during the exponential phase. These results corroborate the hypothesis of specific periods for food requirement during the development.

The two digestive enzymes studied are synthesized independently. Their respective intensity is dependent on the food characteristics and seems to result from an adaptive mechanism to particular requirements of *Artemia* at every stage. These results confirm that *Artemia* is very adaptable with respect to food requirements.

Acknowledgements

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Analysis, role and activity of enzymes in cysts and larvae

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Aspartate transcarbamylase in *Artemia* during early stages of development[1]

Anne-Marie Alayse-Danet

Centre Océanologique de Bretagne
B.P. 337, F-29273 Brest Cédex, France

Abstract

Kinetic properties of aspartate transcarbamylase (ATC) in crude extracts of *Artemia* were studied. The enzymatic kinetic is linear with respect to time for at least 1 hr with crude extract from cysts or the earliest development stages, but not with crude extract from older stages. ATC activity was assayed during early stages of development of two *Artemia* strains ; a relation has been sought between ATC activities and growth rates.

Introduction

Aspartate transcarbamylase (ATC) catalyses the first specific step to *de novo* synthesis of pyrimidine bases. Studies on this enzyme have indicated that its activity is correlated with growth in plant and animal tissues. ATC is particularly active in tissues which are growing rapidly (Calva *et al.*, 1959 ; Nordmann *et al.*, 1964 ; Kim and Cohen, 1965 ; Stein and Cohen, 1965 ; Herzfeld and Knox, 1972 ; Waymire and Nishikawara, 1972 ; Weichsel *et al.*, 1972). Because of the apparent relationship between rate of cell growth or cell division and ATC activity it seemed interesting to investigate the possibility to use this enzyme activity as indicator of growth rate in *Artemia*. This paper presents data on the enzymatic properties of ATC in different *Artemia* strains and preliminary results on the variation of ATC activity during the first stages of development in *Artemia*.

Material and methods

Origin of material

Experiments were performed with *Artemia* strains obtained from Metaframe (San Francisco Bay Brand Division Newark, California, USA), CIRNE (CIA Industrial Do Rio Grande Do Norte, Macau, Brazil), World Ocean (Shark Bay, Australia), Ege University (Bornova Izmir, Turkey), Ministry of Agriculture and National Resources of Cyprus (Nicosia, Cyprus) and the Great Salt Lake (Utah, USA).

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At day 0, the cysts were incubated in filtered natural seawater at 25, 22, or 16 °C. The hatched larvae were harvested and separated from the hatchery debris. Approximately 200 000 nauplii were put into 20 l of filtered natural seawater. Every day the water was changed and food was added (*Tetraselmis suecica*, 2.10^5 cells/ml of *Artemia* culture medium).

Preparation of the enzyme extract

ATC activity was assayed on cysts or animals homogenized in a Potter glass homogenizer with H_2O or different buffers under freezing conditions. Homogenates were made just before the ATC assay. All the assays were performed on crude extracts.

Estimation of enzyme activity

The assay for ATC was based on the radiochemical method of Bresnick and Mosse (1966). The assay medium had the following composition : 0.2 ml homogenate, 9 μmoles of L. aspartate (brougth to pH 9.5), 6.3 μmoles of carbamyl phosphate (brought to pH 9.5), and 120 μmoles of Tris HCl buffer in a total volume of 0.5 ml. Incubation was performed at 35 °C and the exact pH was measured in the assay medium at the incubation temperature. The reaction was stopped by the addition of 0.1 ml 1 N HCl after which the assay medium was cooled. After centrifugation, 0.5 ml of the supernatant layer was passed through a column of Merck Lewadit H^+ resin (60-150 mesh ASTM) in a glass tube (6 mm internal diameter and 6 cm length). After collection of the eluate, the column was washed with 3.5 ml distilled H_2O. The eluate and washings were combined, and a 1.0 ml aliquot was transferred to counting vials containing 10 ml Packard picofluor. The samples were counted in an Intertechnique liquid scintillation spectrometer (Model SL 33). A blank was run for each experiment, consisting of the components mentioned above, except that the enzyme preparation was replaced by distilled H_2O.

Estimation of proteins

Proteins were determined on samples using the automatic method of Samain and Boucher (1974).

Results

Preparation of the enzyme

Different buffers were tested to prepare homogenates ; the results are presented in Table I. The most active homogenate was the one in which phosphate buffer pH 7, had been used ; the activity was, however, more stable with distilled H_2O when the homogenates were kept at 18 °C. The activity of homogenates prepared with 0.15 M KCl had the same intensity as that of the homogenates prepared with distilled H_2O but it was less stable. Homogenates with distilled H_2O were therefore chosen for the following experiments. Cysts were incubated in filtered seawater (Millipore 0.3 μm) containing streptomycine (50 mg/l), penicillin (50 000 UI/l) and chloramphenicol (8 mg/l) ; the latter precaution was taken to ensure that the activity measured was the ATC of *Artemia* and not ATC of bacteria. The activity measured

TABLE I

Effect of different buffers used to prepare *Artemia* crude extracts on the activity and the stability of aspartate transcarbamylase (cysts incubated at 25 °C for 1 day)

Buffer	Activity cpm/mg/90 min	% Activity after freezing at -18 °C	
		24 hr	48 hr
Distilled H_2O	1.51×10^{-5}	99	99
	1.41×10^{-5}	96	99
	1.40×10^{-5}	97	98
Buffer Tris 0,2M pH 8,5	1.09×10^{-5}	86	84
	1.12×10^{-5}	87	80
	1.08×10^{-5}	80	81
Buffer Tris 0,2M pH 9	1.01×10^{-5}	76	72
	9.93×10^{-5}	76	78
	9.20×10^{-5}	83	76
Buffer Tris KH_2PO_4 0,04M pH 7	1.62×10^{-5}	83	69
	1.63×10^{-5}	74	71
	1.60×10^{-5}	81	77

after 24 hr incubation was similar to that measured on homogenate prepared from *Artemia* cultured in parallel but without antibiotics.

KINETIC PROPERTIES OF ATC IN *ARTEMIA* CYSTS AND LARVAE

With the cysts of the different strains the rate of formation of carbamylaspartate was linear with respect to time for at least 1 hr. The effect of pH on the enzymatic velocity is presented in Fig. 1. Maximum velocity was observed at around pH 9. At this pH the temperature optimum was 42 °C for a 30 min incubation (Fig. 2). With San Francisco larvae maximum velocity was also observed around pH 9 but the rate of formation of carbamylaspartate was not always linear. This non linear kinetic was also observed with the larvae of the other strains (Fig. 3) ; this was not due to too small concentrations of substrate because the reaction was proportional with the concentrations of the homogenate up to formation of about 1.5 μmoles of carbamylaspartate (Fig. 4). This quantity of carbamylaspartate corresponds to the utilization of 16% aspartate and 24% carbamylphosphate. Fig. 4 shows the result obtained with an homogenate of larvae of the San Francisco strain which exhibited a linear kinetic ; the same result was, however, also obtained with an homogenate of larvae of this strain which exhibited a non-linear kinetic.

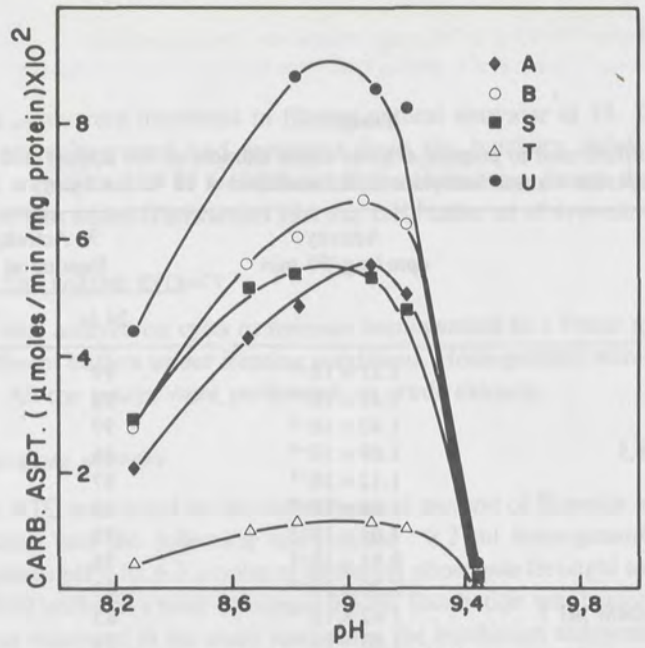

FIG. 1. Dependence of the enzymatic velocity upon pH. The reaction mixture was incubated for 30 min at 35 °C and contained the following compounds : carbamyl phosphate, 6.6 μmoles ; L. aspartate, 9 μmoles ; homogenate of cysts, 0.2 ml and buffers in a total volume of 0.5 ml. $NaHCO_3$ 0.2 M was used for pH 10 and tris 0.4 M for all other pH's to prepare carbamyl phosphate and L. aspartate pH of the extract was measured in the reaction mixture ♦ Australia, ○ Brazil, ■ San Francisco, △ Turkey and ● Utah strain.

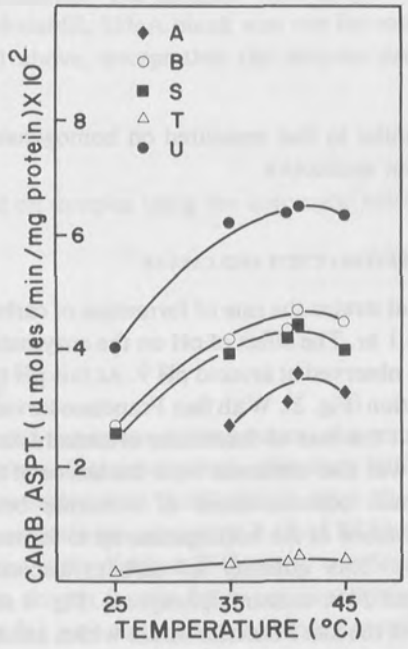

FIG. 2. Dependence of the enzymatic velocity upon temperature. The incubation conditions were similar to those indicated in the legend of Fig. 1. Tris 0.4 M pH 9.5 was used to prepare carbamyl phosphate and L. aspartate.

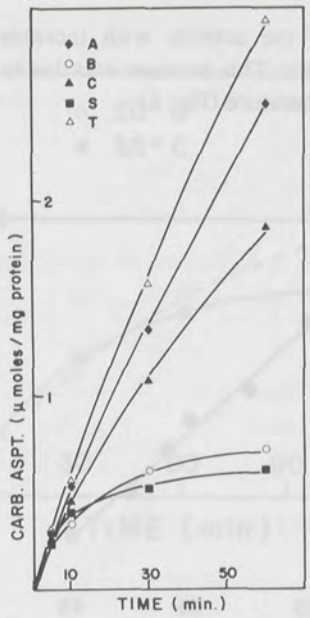

FIG. 3. Dependence of the velocity upon the time of incubation with homogenate of *Artemia* larvae of ♦ Australia, ○ Brazil, ▲ Cyprus, ■ San Francisco, and △ Turkey strains (cysts incubated for 48 hr at 25 °C). The reaction mixtures were similar to those indicated in the legend of Fig. 1. Tris 0.4 M pH 9.5 was used to prepare carbamyl phosphate and L. aspartate.

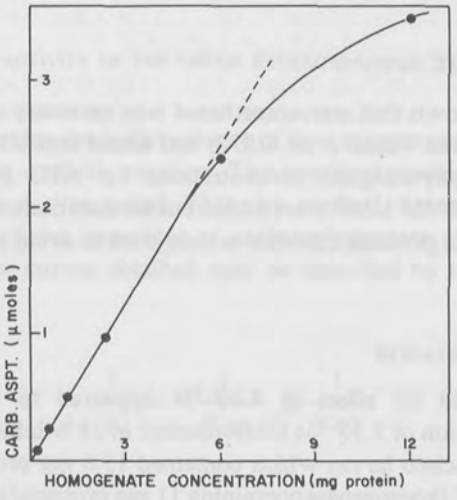

FIG. 4. Dependence of the velocity upon the homogenate concentration for larvae of the San Francisco strain (cysts incubated for 24 hr at 25 °C). The reaction mixture was similar to that described in the legend of Fig. 3 with the exception that the incubation time was 90 min and the temperature was 30 °C.

Fig. 5. shows a decrease of the activity with increase of the temperature with an homogenate with non-linear kinetic. This decrease was due to the increase of the instability of the activity with rise of the temperature (Fig. 6).

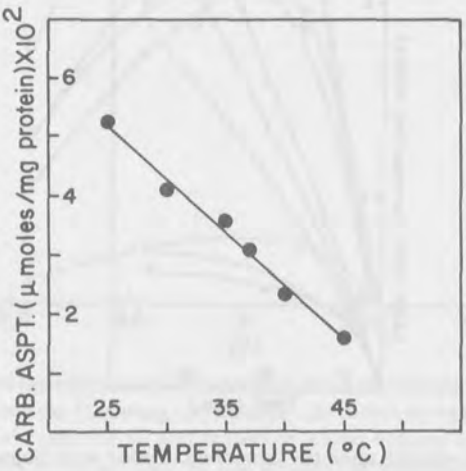

FIG. 5. Dependence of the enzymatic velocity upon temperature for an homogenate of larvae of the San Francisco strain (cysts incubated for 3 days at 25 °C). The reaction mixture was similar to that described in the legend of Fig. 2 with the exception that the incubation time was 90 min.

TESTS TO STABILIZE THE ATC ACTIVITY

Bresnick (1962) has shown that mercaptoethanol was necessary to stabilize the ATC from rat liver ; on the other hand Vassef *et al.* (1973) had found that UMP was effective as an *in vitro* stabilizer within physiological concentrations for ATC from *Chlorella*. 2.10^{-3}M mercaptoethanol and 4.10^{-3}M UMP were added but no stabilization was observed. 8.10^{-4}M EDTA and 1mM PMSF, a protease inhibitor widely used to avoid proteolytic artefacts, were without effect.

EFFECTS OF DIFFERENT SUBSTANCES

UMP which exhibited no effect at 4.10^{-3}M appeared to be inhibitory at higher concentrations. The addition of 5.10^{-2}M UMP resulted in 28 % inhibition of the activity of an homogenate of San Francisco larvae which contained 15.8 mg proteins/ml. The activity of San Francisco strain ATC (homogenate containing 11 mg proteins/ml) was inhibited for 90 % in the presence of 4.8 10^{-3}M p. hydroxymercuribenzoate. The heavy metals were also effective as inhibitor. A 26 % reduction in enzymatic activity was observed in presence of 10^{-4} M Cu^{2+} in a crude extract containing 2.5 mg proteins/ml and 18 % at the same concentration with Zn^{2+}.

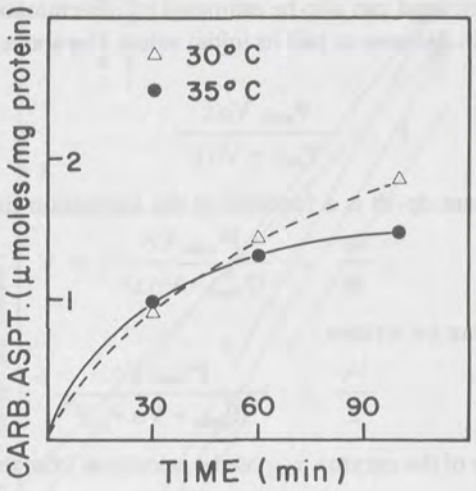

FIG. 6. Dependence of the velocity upon the time of incubation for an homogenate of larvae of the San Francisco strain (cysts incubated for 48 hr at 25 °C). The reaction mixture was similar to that described in the legend of Fig. 3. with the exception that the two temperatures tested were 30 °C and 35 °C.

ESTIMATION OF THE ATC ACTIVITY OF THE CRUDE EXTRACTS WHICH EXHIBIT NON-LINEAR KINETICS

It was difficult to determine the initial velocity of the enzyme reaction with the inactivation which occured during its catalytic reaction. The graphical method described by Stinshoff (1972) for arylsulphatase A was tested. With this method, linear relations were obtained between the reciprocal of the quantities of carbamylaspartate synthetised (1/P) and the indubation time (1/t). The curves obtained may be described by the following equation of Stinshoff (1972):

$$\frac{1}{P} = \frac{1}{P_{max}} + \frac{1}{Vo.t}$$

where P is the quantity of carbamylaspartate formed.

P_{max} is the maximum quantity of carbamylaspartate that the enzyme can synthetise under the conditions of incubation at infinite time.

Vo is the initial velocity of the enzymatic reaction.

It was therefore possible to estimate graphicaly P_{max} and Vo from this equation. The rate at which the enzyme is inactivated can also be estimated by determination of the time necessary for the reaction velocity to decrease to half its initial value. The above equation can be written in this manner :

$$P = \frac{P_{max}\ Vo.t}{P_{max} + Vo.t}$$

The differentiation quotient dp/dt is a function of the incubation time :

$$\frac{dp}{dt} = \frac{P^2_{max}.Vo}{(P_{max} + Vo.t)^2} = V$$

if $V = \frac{Vo}{2}$, the equation can be written :

$$\frac{Vo}{2} = \frac{P^2_{max}.Vo}{(P_{max} + Vo.\tau_{1/2})^2}$$

where $\tau_{1/2}$ is the half time of the enzyme. $\tau_{1/2}$ can be calculated from the following equation :

$$\tau_{1/2} = 0.414 \quad \frac{P_{max}}{Vo}$$

Fig. 7 shows the application of the graphical method outlined above to calculate the activity of the larvae of different *Artemia* strains, the kinetics of which are presented in Fig. 3. The characteristics of the straight lines, slope (Vo) and the point of intersection with the reciprocal activity axis (P_{max}) were calculated by the method of the least squares. The maximum quantities of carbamyl aspartate that the homogenates could synthetize per mg of proteins (P_{max}), the specific initial velocities (Vo) and the half-lives ($\tau_{1/2}$) are listed in Table II. Differences appeared between the strains though the cysts had been incubated at the same time and under the same conditions (for 48 hr at 25 °C). The initial velocity was approximately equal for the Australia, Cyprus, and Turkey strains and higher with the Brazil and San Francisco strains. The crude extract from the San Francisco strain was about 1.7 times more active than that of the Australia strain. The differences were more important between the half-lives. ATC activity was more stable in crude extracts from the Turkey, Australia, and Cyprus strains than in those from the Brazil and San Francisco strains. In particular ATC of the Turkey strain had a $\tau_{1/2}$ 19 times longer than ATC from the San Francisco strain.

Influence of substrate concentrations

The influence of the two substrates was studied on the activity of a crude extract of San Francisco larvae which exhibited linear kinetics (cysts incubated for 1 day at 22 °C). Lineweaver-Burk plots obtained by varying the concentration of aspartate at a constant carbamylphosphate concentration, and vice versa, were linear. A system of parallel straight lines was obtained in each case (Fig. 8A,B). A replot of intercepts on the reciprocal activity axis versus the reciprocal of the substrate concentration gave a straight line. Figures insert in Fig. 8A,B show that the V_{max} is similar ($1.33.10^{-3}$ μmoles/min/mg of protein) for each curve and indicate the Km values of 7 mM and 1.6 mM for aspartate and carbamylphosphate respectively.

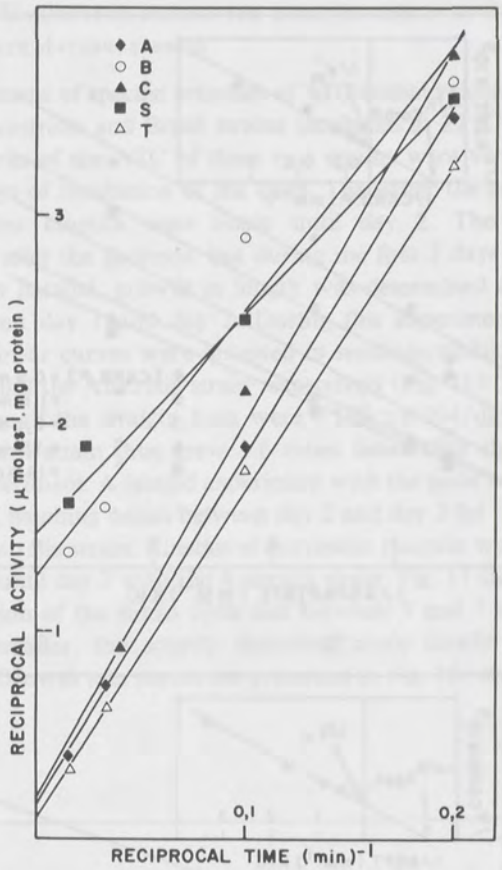

FIG. 7. Reciprocal plot corresponding to Fig. 3.

TABLE II

Parameters of the enzymatic reaction of aspartate transcarbamylase for different *Artemia* strains (cysts incubated at 25 °C for 2 days)

Strain	Vo Initial velocity (μmoles/min/mg protein)	Maximum P_{max} amount of carbamyl aspartate that can be formed (μmoles/min/mg protein)	$\tau_{1/2}$ Half life (min)
Australia	0.059	6.02	41.6
Brazil	0.079	0.78	14.0
Cyprus	0.055	4.80	36.3
San Francisco	0.096	0.68	2.9
Turkey	0.063	8.79	57.6

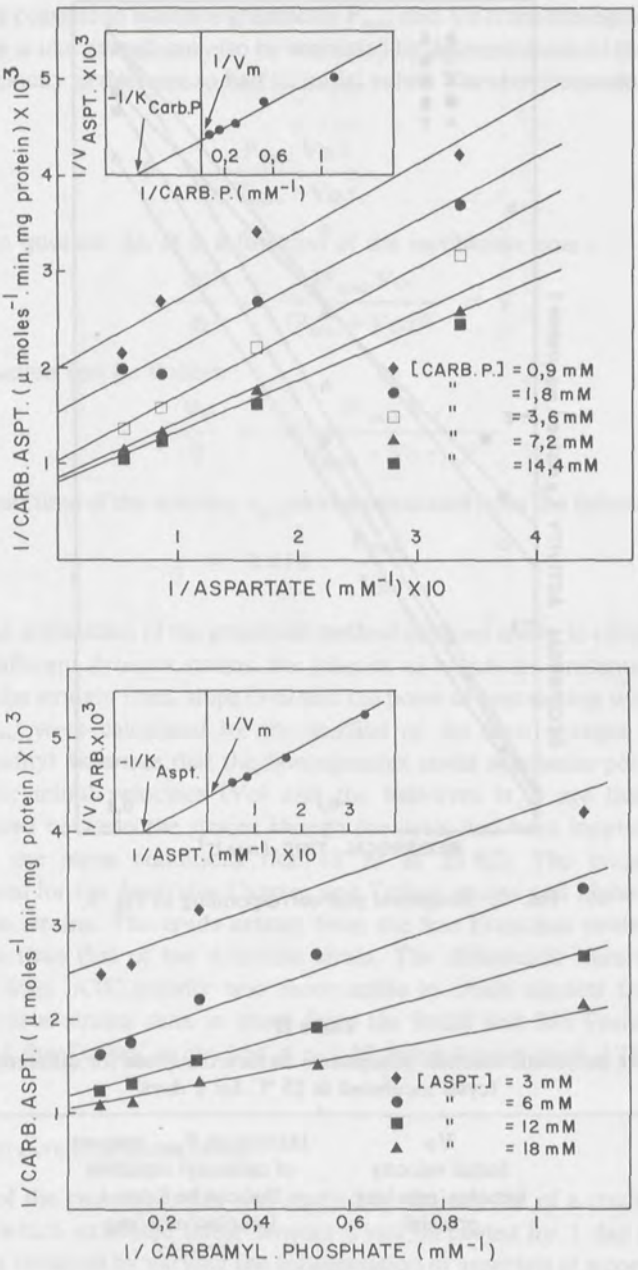

FIG. 8. Reciprocal plot corresponding to the dependence of the velocity upon the concentration of carbamyl phosphate. (A) and of L. aspartate (B) with larvae of the San Francisco strain (cysts incubated at 25 °C for 1 day). The reaction mixture was incubated for 90 min at 30 °C and contained the following : carbamyl phosphate, L. aspartate (the concentration of the two substrates varied as indicated in the figure), in a total volume of 0.5 ml-0.2 M $NaHCO_3$; carbamyl phosphate and L. aspartate were prepared at pH 9.5.

VARIATION OF THE ATC ACTIVITIES DURING THE EARLY STAGES OF DEVELOPMENT OF AUSTRALIA AND BRAZIL *ARTEMIA* STRAINS

Developmental patterns of specific activities of ATC (initial velocities per mg of proteins) in crude extract from Australia and Brazil strains incubated at 22 °C are compared in Fig. 9. Developmental patterns of the ATC of these two strains were very similar, with maximal activity after two days of incubation of the cysts. Thereafter the activity decreased rapidly. With the two strains kinetics were linear until day 2. The specific activities were approximately equal after the decrease, but during the first 2 days the activity in the Brazil strain was higher. In parallel, growth in length was determined after the hatching period which started between day 1 and day 2. During this experiment, the two strains grew exponentially since linear curves were obtained in semilogarithmic plot with $r = 0.996$ and 0.974 for the Brazil and the Australia strain respectively (Fig. 10). The specific growth rates estimated as the slope of the straight lines were 0.150 ± 0.004/day and 0.091 ± 0.007/day respectively. The Brazil strain thus grew 1.6 times faster than the Australia strain in the conditions of this experiment. A second experiment with the same two strains was performed at 15 °C. In this case, hatching began between day 2 and day 3 for the Brazil strain and day 3 and day 4 for the Australia strain. Kinetics of enzymatic reaction were linear until day 5 with the Brazil strain and until day 7 with the Australia strain. Fig. 11 shows the maximal activity at 5 days of incubation of the Brazil cysts and between 5 and 7 days of incubation of the Australia cysts. Thereafter, the activity decreased more slowly than in the experiment performed at 22 °C. Growth rate curves are presented in Fig. 12 ; straight lines were obtained

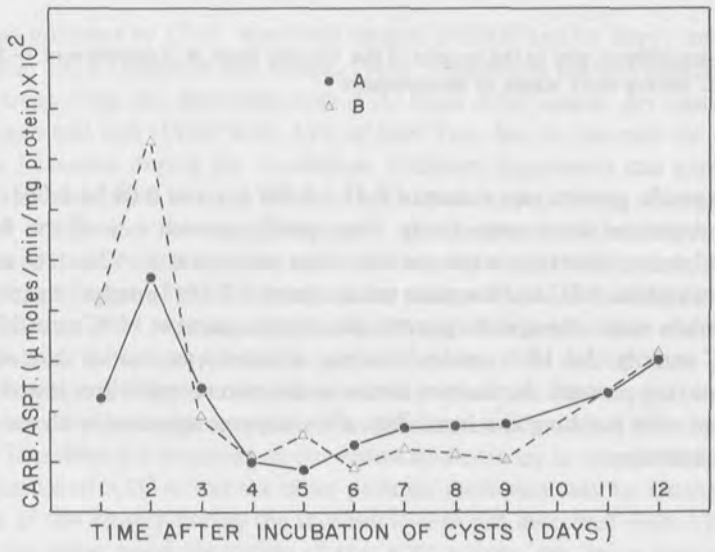

FIG. 9. Patterns of aspartate transcarbamylase activity during early stages of development of *Artemia* from ● Australia and △ Brazil strains incubated at 22 °C.

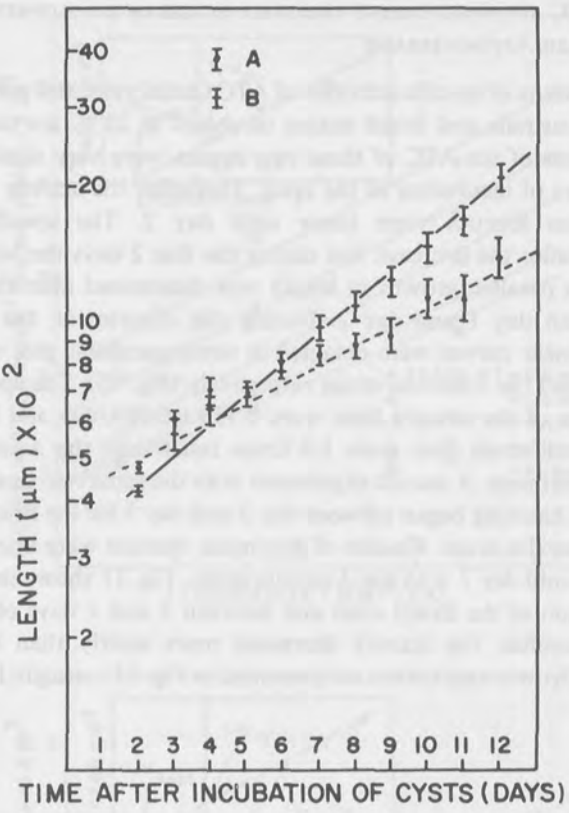

FIG. 10. Semi-logarithmic plot of the lengths of the *Artemia* from • Australia and — Brazil strains incubated at 22 °C during early stages of development.

which suggest specific growth rate values of 0.11 ± 0.03/day and 0.093 ± 0.003/day for the Brazil and the Australia strain respectively. The specific growth rate of the Brazil larvae cultured at 15 °C was smaller than when the strain was cultured at 22 °C but the maximum of the specific activity of the ATC had the same value around 7.2 10^{-2} μmoles/mg protein. In the case of the Australia strain, the specific growth rate was the same at 15 °C as at 22 °C and the maximum ATC activity (3.4 10^{-2} μmoles/min/mg protein) was smaller than at 22 °C (4.6 10^{-2} μmoles/min/mg protein). In the two strains at the two temperatures tested the specific activity increased after hatching and instability of the enzyme appeared with the subsequent decrease of the activity.

Discussion

Aspartate transcarbamylase from *Artemia* shows similarities with the enzyme from rat liver. Its pH optimum near 9 is similar to the 9.2 reported for rat liver enzyme. The *Artemia*

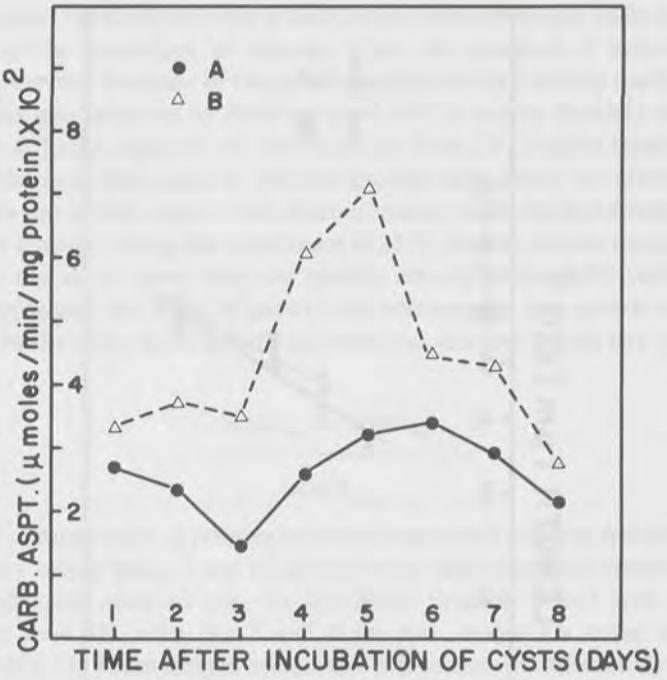

FIG. 11. Patterns of aspartate transcarbamylase activity during the early stages of development of *Artemia* from • Australia and △ Brazil strains incubated at 15 °C.

enzyme is also inhibited by UMP, mercurial reagent (PHMB) and by heavy metals (Cu^{2+} and Zn^{2+}) (Bresnick, 1962 ; Bresnick and Mosse, 1966). Anomalous kinetics observed with crude extracts of larvae were not described with ATC from other origin. An unusual kinetic as found by Cygan and Zak (1966) with ATC of beef liver, but in this case the activity of the homogenates increased during the incubation. Different hypotheses can explain this non-linear kinetic obtained with *Artemia* larvae : degradation by proteolytic enzyme(s), enzymatic release of an inhibitor or inversely enzymatic disappearance of an activator during the incubation, or the substitution of the stable enzyme by an instable isoenzyme in *Artemia* during the earliest stages of development or the disappearance of a stabilizer. Osuna *et al.* (1977) showed that after hatching of *Artemia* nauplii a high increase of four proteolytic activities appeared in extracts from the larvae and that only one of these proteases was strongly inhibited by PMSF. Also it is not possible to eliminate that hypothesis of an artefact due to the presence of proteases to explain the appearance of instable ATC activity in extracts from larvae. Therefore it is necessary in our future studies to try to determine the origin of the non-linear kinetics of ATC to test the other protease inhibitors used by Osuna *et al.* (1977).

If decrease of the activity during the incubation was not described with ATC from other sources, on the other hand instability of the ATC activity has been observed in diluted homogenates of *Chlorella*. Vassef *et al.* (1973) could demonstrate that UMP was effective as an *in vitro* stabilizer within physiological concentrations for ATC of *Chlorella*.

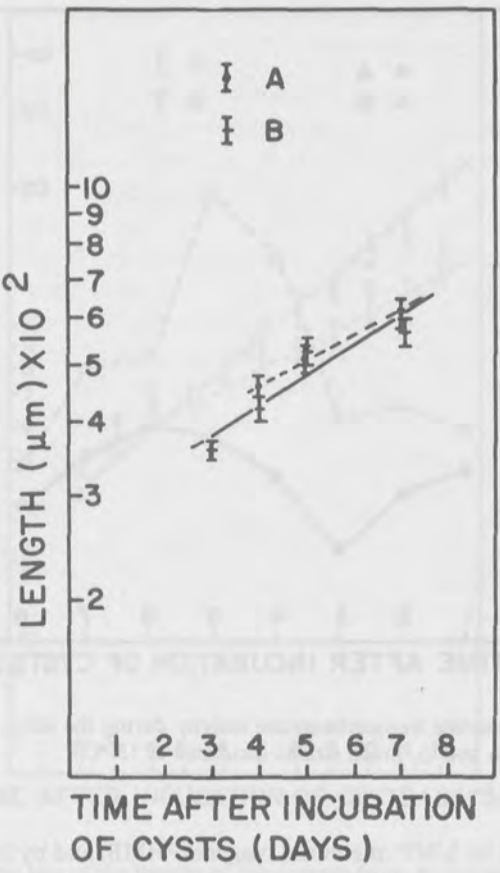

FIG. 12. Semilogarithmic plot of the lengths of *Artemia* from • Australia and — Brazil strains incubated at 15 °C during early stages of development.

The system of parallel straight lines obtained by varying the concentration of the two substrates with homogenate which exhibit linear kinetic suggest a non sequential ping-pong mechanism. This same system was obtained by Lue *et al.* (1976) with ATC from Baker's yeast, by Ong and Jackson (1972) with ATC from *Phaseolus aureus* and in our laboratory with ATC from mussel and scallop (Bergeron and Alayse-Danet, in preparation). Lue *et al.* (1976) and Ong and Jackson (1972) could not opt unequivocally for a ping-pong mechanism because the inhibitor effects obtained with the products of the reaction (phosphate and carbamylaspartate) on bakers' yeast ATC were consistant with an ordered BiBi mechanism where carbamylphosphate and then aspartate bind to the enzyme followed by release of carbamylaspartate and phosphate, in that order. Ordered BiBi mechanism had been also suggested in the case of the ATC from mouse spleen (Hoogenraad *et al.*, 1971) and the catalytic subunit of *Escherichia coli* ATC (Porter *et al.*, 1969).

Clegg and Golub (1969) showed that a fast *de novo* RNA synthesis could be detected from the beginning of the incubation of *Artemia* cysts ; the presence of active ATC in cysts corroborates this result. Decrease of the specific activity of ATC during the first stages of the development was also observed by Weichsel *et al.* (1972) and by Herzfeld and Knox (1972) with ATC from different organs of rat. Herzfeld and Knox (1972) could establish an equation for tumors exhibiting a wide range of different growth rates. From our results a relation has been sought between growth rates of two *Artemia* strains (Australia and Brazil) and their ATC activities. On an average during the experiment at 22 °C the two strains seemed to grow with a constant rate but at the same time the specific activity of the ATC varied. No relation appears since apparently one value of growth rate corresponds with several values of specific ATC activity. On the other hand specific growth rates calculated each day by the following formula :

$$\frac{\text{length}_{\text{day}_n} - \text{length}_{\text{day}_{n-1}}}{\text{length}_{\text{day}_{n-1}}}$$

were taken into consideration. A positive correlation appeared with the specific activity values measured the day before (day_{n-1}) and the growth rates determinations mentioned above. The correlation coefficients obtained are : for the Brazil strain $r = 0.809$ with $df = 6$; for the Australia strain $r = 0.626$ with $df = 7$ and if the two strains are taken in consideration $r = 0.624$ with $df = 15$. These sample correlation coefficients are different from zero respectively at the 2, 10, and 1 % level of significance. The differences which appear between the specific growth rates each day are important since they may explain the variation of the specific ATC activity.

In the experiment performed at 15 °C larvae of the Australia strain seemed to grow with the same average specific growth rate as at 22 °C which is certainly an artefact due to the small number of length values especially as the development in the cysts had slackened (hatching started between day 3 and 4 instead of day 1 and 2 at 22 °C). With this experiment it is not possible to look for a correlation because only one pair of values can be used with the Australia strain and two with the Brazil strain. The specific activity of ATC at its maximum level-particularly in the Brazil strain – was not significantly different from that at 22 °C thought the growth rate was weaker. These results are not inconsistent because at 15 °C the same quantity of enzyme is certainly less active.

A relation seems to exist between ATC activity and growth rate in *Artemia*. This interpretation of our experiments is based on the hypothesis that, on one hand the ATC activities were not significantly influenced during homogenization and, on the other hand the daily ATC and protein determinations were reproducible. New experiments have to be made to confirm such a relation with a wider range of growth rates and, if possible, after a way to stabilize the ATC activity during the experiments will have been found.

Summary

Aspartate transcarbamylase (ATC) of homogenates of cysts of different *Artemia* strains – Australia, Brazil, San Francisco (USA), Cyprus, and Utah (USA) – has a pH optimum around

9 and exhibits linear kinetics. Non-linear kinetics were observed in larvae and were dependent of the temperature of the culture medium.

The mathematical correlation between the reaction velocity and the incubation time, as used by Stinshoff (1972), could be applied and the initial velocity of the anomalous kinetics could be calculated.

The rate of decreasing of the enzymatic activity depends of the temperature of incubation. Initial velocity studies with homogenate containing stable ATC activity, and variation of the concentration of aspartate at a constant carbamyl phosphate concentration and *vice-versa*, revealed a set of parallel reciprocal plots suggesting a non sequential ping-pong mechanism.

The levels of ATC activity were found to be highest in the earliest stages of development. The specific ATC activity was significantly correlated with the specific growth rate calculated each day.

Acknowledgements

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RNA polymerases and transcriptional switches in developing *Artemia*

Joseph C. Bagshaw, Roger Acey, and Judith C. Helder

Department of Biochemistry, Wayne State University School of Medicine
Detroit, Michigan 48201, USA

Abstract

RNA synthesis increases rapidly upon resumption of development in encysted embryos and declines by 80-90% during development of nauplius larvae. We refer to these events as the transcriptional "on" and "off" switches. Nuclear RNA polymerases increase in embryos and decrease in nauplii in parallel with the rate of RNA synthesis. Similar quantitative changes have been found in activities measured in intact isolated nuclei and in soluble enzyme preparations. The number of RNA polymerase II molecules per nucleus declines during development of nauplii. All stages contain substantial amounts of RNA polymerases in the cytoplasmic fraction. *In vivo* radiolabeling studies have shown that RNA polymerase II is synthesized at all stages, including pre-emergence embryos during the on switch and nauplius larvae after the off switch.

Introduction

Embryonic and early larval development of *Artemia* provide some excellent opportunities for biochemical studies of development, including regulation of RNA synthesis. Stage-specific selective gene expression is characteristic of early development, and is regulated at least in part at the level of transcription. Eukaryotic cells contain multiple forms of RNA polymerase grouped into three classes which differ in structure and apparent function (reviewed by Roeder, 1976). By definition, RNA polymerases must be involved in all forms of transcriptional regulation.

Major quantitative changes in RNA synthesis in *Artemia* provide useful systems for studies of transcriptional regulation. When dormant embryos resume development, the rate of RNA synthesis increases during the pre-emergence period. Shortly after hatching, the rate of RNA synthesis in nauplius larvae begins to decline, reaching a level of $\sim$ 20% of the maximal rate about 48 hr later (McClean and Warner, 1971). We refer to these events as the transcriptional "on" and "off" switches, and for several years this laboratory has been studying the behavior of RNA polymerases during this period of development. Isolation and characterization of RNA polymerases I and II from nauplius larvae were first reported in 1975, along with the observation that late nauplius larvae (72 hr) yielded less RNA polymerase than earlier (36 hr) larvae (Birndorf *et al.*, 1975). We subsequently showed that the transcriptional "on" switch is accompanied by an increase in nuclear RNA polymerase activity (D'Alessio and Bagshaw, 1977), and that the "off" switch involves parallel decreases in the RNA synthetic capacity of isolated nuclei, the amount of soluble RNA polymerase activity obtained from the nuclei, and

the number of RNA polymerase II molecules per nucleus (Bagshaw *et al.*, 1978). More recently we have turned to the question of how the amount of RNA polymerase II is regulated. It is an obvious but underappreciated fact that RNA polymerase molecules are also protein, and therefor they may be the subject as well as the agent of regulation. Results presented here indicate that RNA polymerase II is synthesized *de novo* at all stages, including pre-emergence embryos and late nauplii after the "off" switch.

Materials and methods

Dormant encysted embryos of the San Francisco strain (Metaframe brand) were purchased from a local supplier and stored at -20 °C. Cysts were sterilized by washing in dilute antiformin solution (Nakanishi *et al.*, 1962) and incubated in sterile artificial sea water as described previously (Bagshaw *et al.*, 1978). Isolation of nuclei, solubilization and fractionation of RNA polymerases, and assays for RNA polymerase activity in isolated nuclei and in soluble preparations have been described in detail. One unit of RNA polymerase is defined as the amount of activity that incorporates one nanomole of UMP into RNA in 10 min at 30 °C with denatured calf thymus DNA as template. For studies of in vivo synthesis, 1 g of cysts or nauplii was incubated in 50 ml of seawater containing $NaH^{14}CO_3$ (50 μCi/ml, 50-60 mCi/mmole). RNA polymerase II was partially purified and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) and subsequent fluorography as described elsewhere (Bagshaw and Acey, 1979).

Results and discussion

The transcriptional "off" switch

During the first 48 hr of development of hatched nauplius larvae, the rate of RNA synthesis in vivo declines by 80-90 % (McClean and Warner, 1971). We wished to determine whether the levels of RNA polymerase activity also declined ; this is the expected, but not always the observed, result (Roeder, 1976). Total RNA polymerase activity in isolated nuclei, and activities of partially purified RNA polymerases I and II were measured as a function of developmental age. The results (Bagshaw *et al.*, 1978) showed that indeed the transcriptional off switch involves a decline of 80-90 % in RNA polymerase activities measured both in intact nuclei and in soluble preparations. The decrease was quite abrupt ; for example, between 24 and 48 hr after immersion of cysts, the yield of RNA polymerase I dropped from 5.53 ± 0.65 (mean $\pm$ standard deviation) enzyme units per 10^9 nuclei (from which the enzymes were derived) to 1.72 ± 0.25 units/10^9 nuclei ; at the same time, RNA polymerase II decreased from 21.46 ± 1.70 to 5.83 ± 1.37 units /10^9 nuclei. Mixing experiments have shown that this decrease can not be attributed to any deleterious effect (*e.g.*, proteolytic degradation) on the solubilized enzyme preparations from older larvae. Moreover, the enzymes as we isolate them are functionally and structurally stable at this stage of purification ; they can be stored at 4 °C for several days with no loss of activity or alteration of subunit structure. Unpublished studies of R. Acey show that the composition of the homogenization medium is critical for stability of

soluble proteins from nauplii, and that homogenization is isotonic sucrose mitigates against proteolysis of soluble proteins, whereas hypotonic media activate and/or release proteases. Thus we believe that the decrease in RNA polymerases is not due to proteolysis *in vitro*, and that it is real and biologically meaningful.

It is generally assumed that the activity of soluble eukaryotic RNA polymerases on a non-selective template such as denatured calf thymus DNA is a measure of the concentration of enzyme molecules (Roeder, 1976). We were able to perform a more direct measurement of this important parameter because of the relative ease with which the two major subunits of *Artemia* RNA polymerase II (Bagshaw, 1976) can be visualized by SDS-PAGE. RNA polymerase II preparations, partially purified by DEAE-cellulose chromatography and sucrose gradient centrifugation, were subjected to SDS-PAGE, and the amount of subunit IIb was determined by scanning the gels (Bagshaw *et al.*, 1978). Knowing the number of nuclei from which the enzyme was solubilized, we were able to calculate that the level of RNA polymerase II decreased from $20\,000 \pm 2\,300$ molecules/nucleus at 24 hr to $3\,800 \pm 200$ molecules/nucleus at 48 hr. Thus the decline in RNA polymerase II activity is accounted for by a loss of enzyme molecules.

A simple mechanism could account for the observed decrease in RNA polymerase II. If the synthesis of the enzyme were shut off at around 24 hr, subsequent degradation would reduce the number of molecules. Then at a later stage, after a new and lower level of enzyme had been reached, synthesis would resume. To test this possibility, we have been studying the synthesis in vivo of RNA polymerase II. Nauplii were incubated with $NaH^{14}CO_3$ for periods of 8 hr at various stages, and RNA polymerase II was examined by SDS-PAGE and fluorography to determine whether the enzyme was radiolabeled. The results of one such experiment are shown in Fig. 1. In this case the labeling periods were 16-24 hr (newly hatched nauplii), 24-32 hr and 40-48 hr ; these times were chosen to span the most dramatic decline in polymerase II. We were not surprised to find that the enzyme was synthesized in early nauplii ; however, it is quite clear that some RNA polymerase II was also synthesized between 40 and 48 hr, even though the net number of polymerase molecules is decreasing at this time. Clearly, the simple hypothesis will not do. It now appears that the change in RNA polymerase II requires a shift in the balance of synthesis and degradation, not merely cessation of synthesis. To characterize this shift more sophisticated measurement of the rate of accumulation of radioactivity in RNA polymerase II will be needed, and these experiments are now in progress.

The suggestion has been made (Hentschel and Tata, 1976 ; Sebastian *et al.*, 1980) that the decline in RNA synthesis and RNA polymerases in nauplius larvae is the result of starvation, but whether the condition of the larvae at this stage can properly be called starvation is debatable. Prior to the first larval ecdysis, which in our cultures does not occur before 36 hr of development, the nauplii do not have a functional gut and can not utilize food (Hootman and Conte, 1974). The supply of yolk platelets is not nearly depleted even as late as 72 hr. Moreover, our protein labeling experiments show that the rate of total protein synthesis decreases very little if at all between 24 and 48 hr, and that there is no general catabolism of proteins as would be expected in starvation. Nevertheless, the results reported by Sebastian and his colleagues (1980) indicate that feeding a blue-green alga to the nauplii has a definite effect on the RNA polymerase levels. It would be interesting to know whether we can alter the rate of synthesis of RNA polymerases by feeding our nauplii.

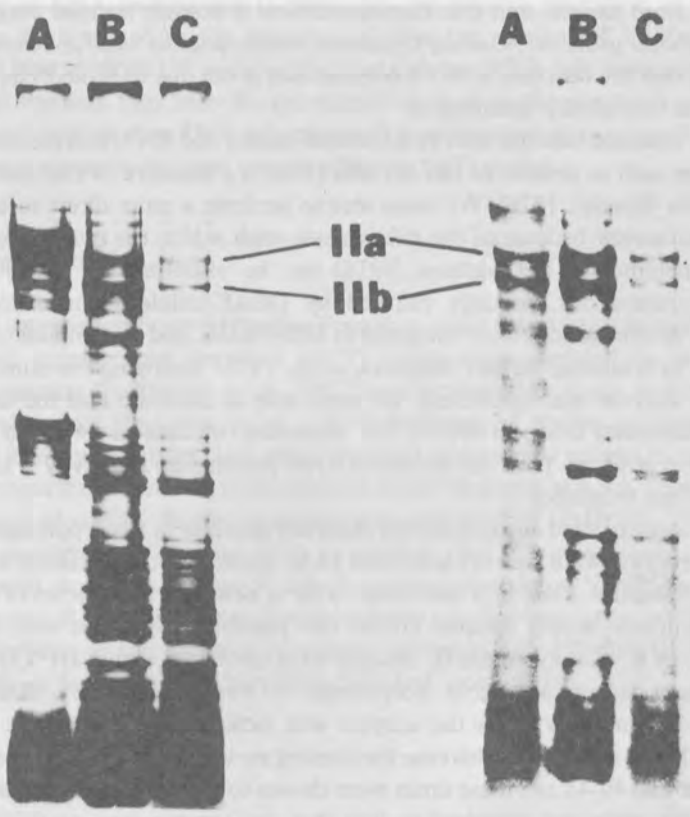

FIG. 1. Synthesis *in vivo* of RNA polymerase II in nauplius larvae. Nauplii were incubated with $NaH^{14}CO_3$ at (A) 16-24 hr (newly hatched), (B) 24-32 hr, or (C) 40-48 hr. Partially purified RNA polymerase II was analyzed by SDS-PAGE and fluorography ad described in the text. Left panel : gel stained with Coomassie blue ; right panel : fluorogram of the same gel. The positions of RNA polymerase II subunits IIa and IIb are indicated.

THE TRANSCRIPTIONAL "ON" SWITCH

RNA synthesis *in vivo* begins immediately upon resumption of development of dormant embryos and increases during the pre-emergence period (Clegg and Golub, 1969). An earlier report from this laboratory showed that the amount of RNA polymerase activity that can be solubilized from nuclei increases during the first 8 hr of development (D'Alessio and Bagshaw, 1977). A similar increase has been observed in the RNA synthetic capacity of intact isolated nuclei (unpublished data of Bagshaw). Although we obtained only very small amounts of nuclear RNA polymerases from dormant cysts, Renart and Sebastian (1976) reported finding substantial amounts of these enzymes in homogenates of whole cysts. Experiments in this laboratory subsequently showed that 70-80% of this activity is in the cytoplasmic (post-ribosomal supernatant) fraction of the cell when cysts are homogenized in isotonic sucrose (D'Alessio, 1977). The proportion of RNA polymerase activity found in the nuclei and in the

cytoplasm is unaffected by repeated re-homogenization or complete mechanical disruption of the nuclei (unpublished data of Bagshaw); thus the enzymes found in the post-ribosomal supernate appear to be truly cytoplasmic in origin and not the result of leakage from the nuclei. We have quantitated the amount of partially purified RNA polymerases I and II as a function of development by separating the enzymes on DEAE-cellulose, and the results of such an experiment are shown in Table I. Normalization of these data presents some tactical problems. Total enzyme activity is not a completely satisfactory parameter, because we deliberately sacrifice some yield of nuclei in order to obtain cleaner preparations, whereas the recovery of the cytoplasmic fraction is virtually complete. Specific enzyme activity (units/mg protein) is clearly unsatisfactory because nuclear preparations from early embryos contain large amounts of a few proteins that co-chromatograph with RNA polymerases and disappear during development; thus the specific activity is spuriously high at later stages compared with earlier ones. As an alternative, we have measured the amount of DNA in both the total homogenate and the suspension of nuclei just before solubilization of RNA polymerases. These values should reflect the number of cells and nuclei from which the cytoplasmic and nuclear enzyme were derived, and the data in Table I indicate the relative concentration, at various stages, of RNA polymerases in the nuclei and the cytoplasm. Moreover, values for nuclear and cytoplasmic enzymes can be directly compared within one preparation, as long as we remember that we are looking at the amount of enzyme per cell (assuming that all cells are monunucleated). The results show that, as expected, the amounts of nuclear RNA polymerases I and II both increase during pre-emergence development, reaching levels in hatched larvae that are 15- to 20-fold greater than in dormant cysts. The cytoplasmic RNA polymerases also increase, although less dramatically. Given that we recover about half the nuclei in the initial homogenate, estimates of total activity (subject to the reservation cited above) clearly show that nuclear RNA polymerases do not accumulate at the expense of cytoplasmic polymerases, *i.e.*, the cytoplasmic enzymes are not merely storage forms of the molecules waiting to get into the nucleus. Polymerase molecules in the cytoplasm of dormant cysts may certainly enter the nucleus when development resumes, but the pool of cytoplasmic enzymes is certainly not depleted.

TABLE I

Nuclear and cytoplasmic RNA polymerases from developing embryos and nauplius larvae[a]

	Nuclear RNA polymerase[b]		Cytoplasmic RNA polymerase[c]	
Age[d]	I	II	I	II
0 hr	0.04	0.12	0.20	0.18
4 hr	0.22	0.64	0.31	0.72
8 hr	0.29	1.07	0.35	1.04
20 hr	0.56	2.63	1.10	1.54

[a] RNA polymerase activity was assayed after fractionation on DEAE-cellulose.
[b] Enzyme units per mg DNA in the nuclei from which the enzymes were solubilized.
[c] Enzyme units per mg DNA in the homogenate from which the cytoplasmic fraction was derived.
[d] Hours after immersion of cysts in seawater at 30 °C; nauplii hatch at 16-20 hr.

As mentioned above, partially purified RNA polymerase II from cysts and early embryos contains large amounts of certain contaminating proteins that disappear as development progresses. Unfortunately, some of these polypeptides co-sediment with RNA polymerase II in sucrose gradients and nearly co-electrophorese with subunits IIa and IIb in SDS-PAGE. Thus we have not yet been able to perform accurate quantitation of the number of enzyme molecules, and we can only presume that this number increases during pre-emergence development. We have, however, incubated the developing embryos with $NaH^{14}CO_3$ and looked for labeling of RNA polymerase II. The results, shown in Fig. 2 show that subunits IIa and IIb were radiolabeled in both nuclear and cytoplasmic RNA polymerase II. Thus both of these enzymes were being synthesized during the pre-emergence period. In the future, we plan to quantitate the specific radioactivity of these polypeptides, and the rate of accumulation of radioactivity in them. We hope that these measurements will tell us whether the cytoplasmic polymerases are newly synthesized molecules not yet transported into the nuclei, "old" molecules ready to be degraded, or a mixture of both. In any case, these experiments represent the first study of the synthesis and turnover of RNA polymerases in a developing eukaryote.

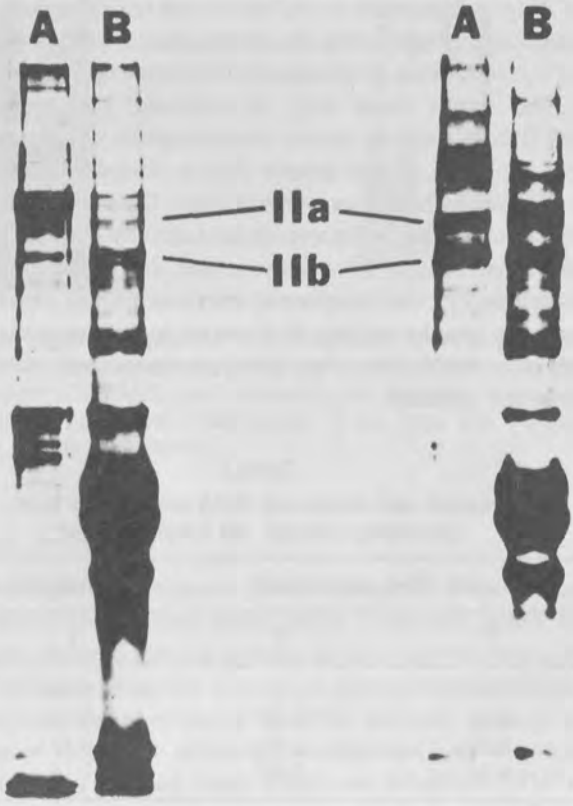

FIG. 2. Synthesis *in vivo* of RNA polymerase II in pre-emergence embryos. Embryos were incubated with $NaH^{14}CO_3$ at 0-12 hr. Experimental details are given in the legend for Fig. 1. (A) nuclear RNA polymerase II ; (B) cytoplasmic RNA polymerase II.

Acknowledgement

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DNA polymerase in *Artemia* embryos

Marcello G. Cacace[1], *Paola Ballario*[2], *Claudio Colapicchioni*[2], *and Mario Bergami*[2]

[1] *Laboratory of Molecular Embryology, CNR*
Via Toiano 2, I-80072 Arco Felice, Naples, Italy

[2] *Chair of Molecular Biology, University of Rome*
Piazzale delle Scienze 2, I-00100 Rome, Italy

Abstract

Dormant embryos of *Artemia* after a period of prolonged dormancy, may resume development upon rehydration. Development from the gastrula stage to swimming nauplius is complete in 18-24 hr. During this period DNA synthesis and mitosis are absent up to the prenauplius stage. DNA-dependent DNA polymerase was isolated from nuclei of dormant embryos, which present a measurable level of enzyme activity. It is possible to prepare nuclei directly from dehydrated cysts with an extraction method involving non-aqueous solvents. The purification procedure included sonication at high ionic strength, ammonium sulphate precipitation, DEAE-cellulose and phosphocellulose chromatography. The partially purified enzyme had a sedimentation coefficient $\geqslant 7S$ in 0.5 M KCl and was completely inhibited by 2 mM N-ethylmaleimide. pH optimum was 7.8 in Tris-HCl buffer. Enzymic activity was 50% inhibited by 0.1 M KCl ; on the other hand, it was dependent from Mn^{++} and Mg^{++} which were optimally active at a concentration of 1.6 mM and 8 mM, respectively.

Introduction

Cryptobiotic embryos of *Artemia* can resume development and reach a nauplius stage in 18-24 hr depending on environmental conditions. During this period of development, DNA synthesis is practically absent event though extensive morphological changes take place (Nakanishi *et al.*, 1962 ; McClean and Warner, 1971 ; Olson and Clegg, 1978). In order to clarify the mechanism underlying this rather unusual aspect of the development in *Artemia* we have undertaken the study of some properties of DNA polymerase from dormant embryos.

Materials and methods

Artemia cysts were from San Francisco Bay Brand, Newark, California, nucleotides from Boehringer, Mannheim ; (Me-^{3}H)dTTP (42 Ci/mmol, 1 μCi/μl) from the Radiochemical Centre, Amersham. Salmon testes DNA, Type III, from Sigma, was activated (Aposhian and Kornberg, 1962). Soybean trypsin inhibitor was from Sigma. DEAE cellulose was from Whatman. All other chemicals were reagent grade.

Artemia cysts were washed and treated with hypochlorite as described elsewhere (Ballario *et al.*, 1978). Nuclei were prepared from dried cysts with the non-aqueous method of Warner *et al.*, (1972). Nuclei pelletted in the last centrifugation step were dried in a dessicator under vacuum. No activity loss was observed in nuclei stored at -20 °C in the presence of silica gel even after several months.

DNA polymerase activity was assayed by incubating the enzyme preparations in a 300 μl incubation mixture containing 67 mM Tris-Hcl pH 7.8, 0.5 mM each of dATP, dCTP and dGTP, 0.2 μM (Me-^{3}H)dTTP, 0.25 mg/ml activated DNA, 0.1 mM EDTA, 0.1 mM dithiothreitol and 9 mM $MgCl_2$. The reaction was allowed to proceed for 30 min at 35 °C and then terminated by the addition of 10% TCA. The precipitate was collected on Whatman GF/C glass filters. After prolonged washing with 5% TCA, the filters were dried and counted in a toluene-based scintillator.

Results and discussion

Enzyme extraction

All operations were performed at 0-4 °C. One gram of dried nuclei was suspended in 5 ml of an homogenization buffer containing 50 mM phosphate buffer pH 6.5, 1 mM EDTA, 1 mM dithiothreitol, and 2 μg/ml soybean trypsin inhibitor. Solid KCl was then added to 0.5 M final concentration and the viscous suspension obtained was sonicated until viscosity decreased and drops were readily obtained on the tip of a Pasteur pipette. After centrifugation for 60 min at 30 000 rpm in a Spinco 50 Ti rotor, the supernatant was dialyzed against the buffer used for the subsequent chromatography step.

DNA polymerase chromatography

Partial purification of DNA polymerase has been carried out by ion exchange chromatography on a phosphocellulose column. Fig. 1 shows the elution pattern of a nuclear fraction prepared by the non-aqueous method. Elution was performed with a linear (0-0.6) M KCl gradient. Only one peak of activity was detectable in the eluate at an ionic strength of 0.36 M KCl. The metal requirements and pH optimum of such a partially purified nuclear enzyme are shown in Fig. 2 and 3. Optimal concentrations for Mg^{++} and Mn^{++} were 8 mM and 1.6 mM, respectively. The pH dependence of polymerase activity was investigated in the range 6-10 using three appropriate buffer systems. With Tris-HCl buffer optimal activity was found at pH 7.8. The higher range was studied with glycine buffer and a broad plateau was found in between pH 8.6 and pH 9.2.

Thermal inactivation of nuclear enzyme

Fig. 4 shows the thermal inactivation of the nuclear enzyme pre-incubated at various temperatures. The pattern of inactivation indicates a biphasic mode of decay of activity with time. A fairly stable activity level was observed within at least 30 min when the nuclear preparation was incubated at 45 °C.

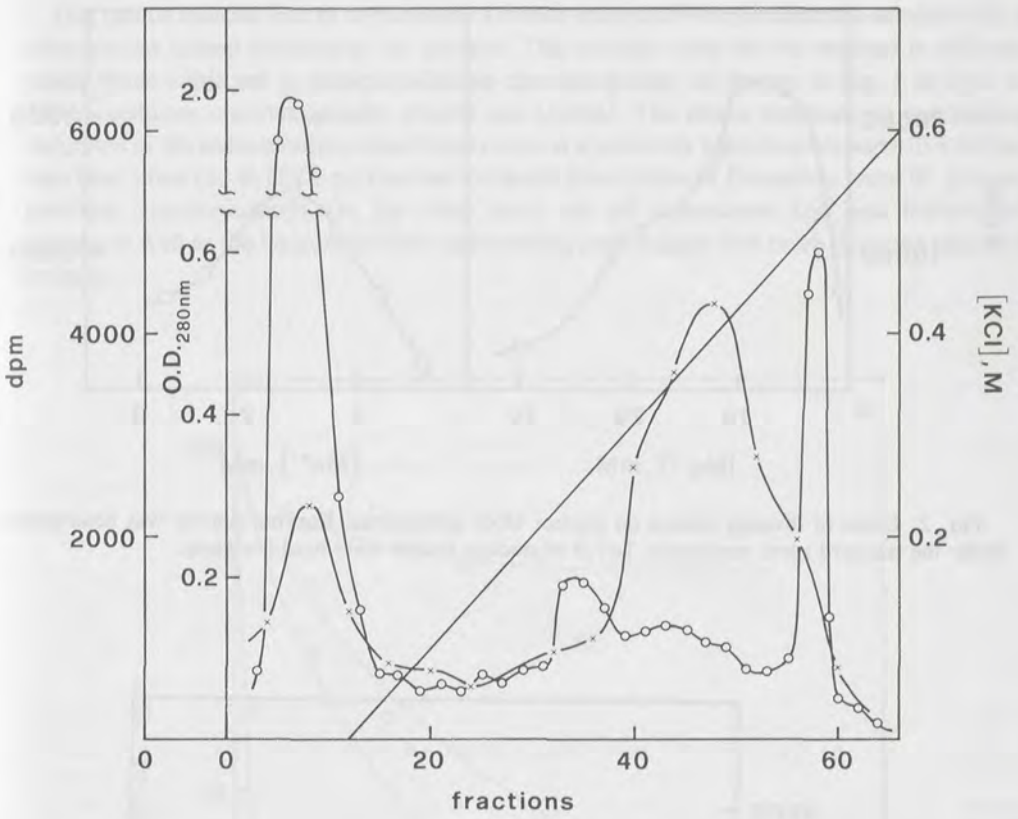

FIG. 1. Phosphocellulose chromatography of nuclear DNA polymerase. Two ml of the nuclear enzyme extract were loaded onto a 1 × 8 cm column equilibrated with 10 mM phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol and eluted with a (0-0.6 M) KCl gradient and 2 ml fractions were collected. (○ – – – ○), OD_{280nm} ; (x – – – x), enzyme activity.

SEDIMENTATION ANALYSIS OF ENZYMES EXTRACTED FROM NUCLEI AND FROM TOTAL EMBRYO

The pattern of sedimentation is shown in Fig. 5. The gradient was run in the presence of 0.5 M KCl. A heavy sedimenting species was observed with a sedimentation coefficient $\geqslant 7$. The activity was totally inhibited by 10 mM N-ethylmaleimide, thus indicating the absence of β-like activity in the embryo.

The intracellular localization of different DNA polymerase activity has been controversial mainly because of possible artifacts during the extraction procedure. It has been clearly demonstrated, in a few cases, that DNA polymerase α is a nuclear enzyme (Martini *et al.*, 1976).

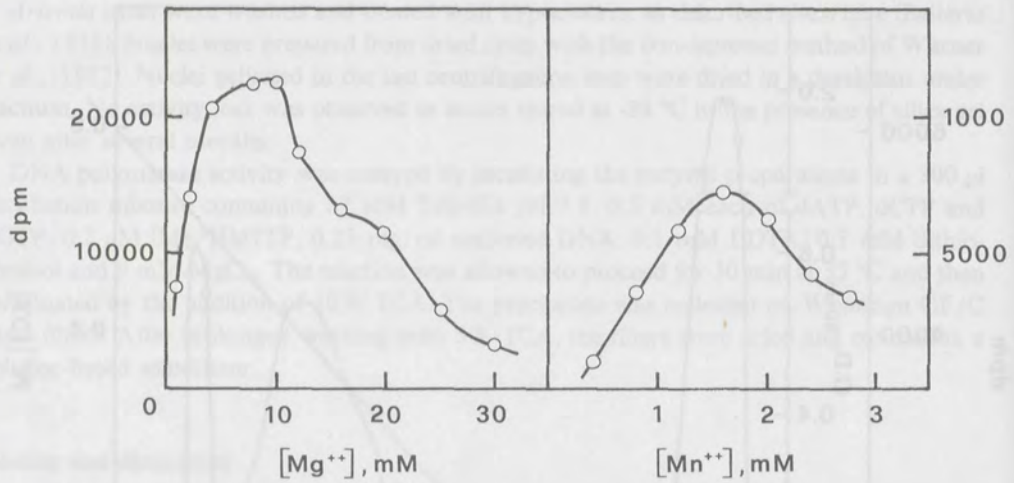

FIG. 2. Effect of divalent cations on nuclear DNA polymerase. Enzyme activity was determined under the standard assay conditions. Ten μl of nuclear extract were used per assay.

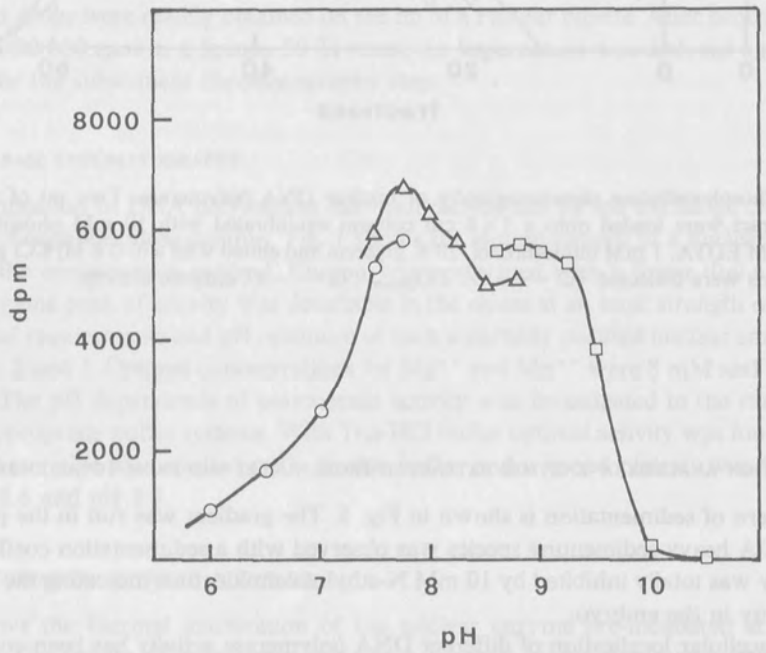

FIG. 3. pH dependence of nuclear DNA polymerase. The assay conditions were standard except that three different buffer systems were used : potassium phosphate, 35 mM ; Tris-HCl, 67 mM and glycine-NaOH 67 mM.

Our results indicate that in cryptobiotic *Artemia* embryos N-ethylmaleimide-sensitive DNA polymerases indeed localized in the nucleus. This activity could not be resolved in different peaks when subjected to phosphocellulose chromatography as shown in Fig. 1 or even to DEAE-cellulose chromatography (results not shown). The strong tendency to aggregation exhibited in the sedimentation experiments even at a relatively high ionic strength (0.5 M) has also been observed in DNA polymerase extracted from nuclei of *Drosophila* testes (P. Grippo, personal communication). On the other hand, the pH dependence and heat inactivation pattern as well as the broadness of the sedimenting peak suggest that more than one species is present.

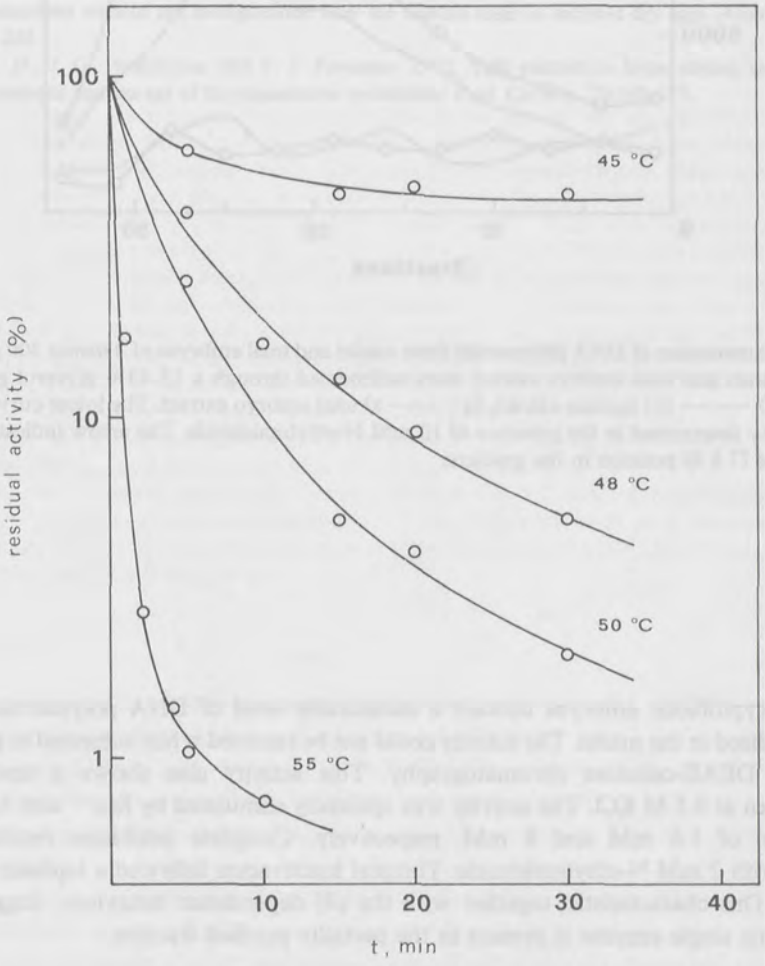

FIG. 4. Thermal inactivation of nuclear DNA polymerase. Aliquots of nuclear enzyme were incubated at the indicated temperatures for various lengths of time. Residual activity was then measured under standard assay conditions.

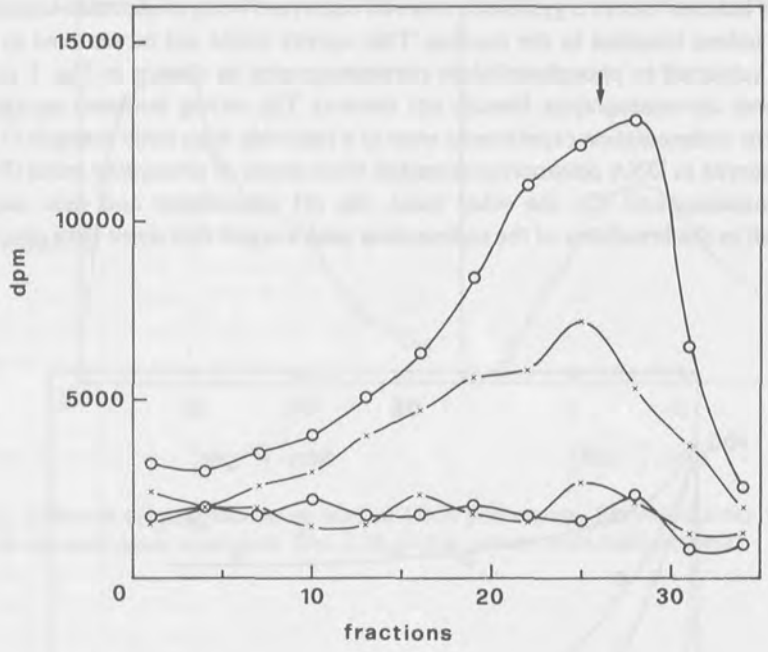

FIG. 5. Sedimentation of DNA polymerase from nuclei and total embryos of *Artemia* 300 μl aliquots of nuclear extract and total embryo extract were sedimented through a 15-40% glycerol gradient in 0.5 M KCl. (○ – – – – ○) nuclear extract, (x – – – – x) total embryo extract. The lower curves refer to enzyme activity determined in the presence of 10 mM N-ethylmaleimide. The arrow indicates alcohol dehydrogenase (7.6 S) position in the gradient.

Summary

Artemia cryptobiotic embryos contain a measurable level of DNA polymerase activity which is localized in the nuclei. The activity could not be resolved when subjected to phosphocellulose or DEAE-cellulose chromatography. This activity also shows a tendency to aggregate even at 0.5 M KCl. The activity was optimally stimulated by Mn^{++} and Mg^{++} at a concentration of 1.6 mM and 8 mM, respectively. Complete inhibition resulted from incubation with 2 mM N-ethylmaleimide. Thermal inactivation followed a biphasic mode of time decay. This characteristic, together with the pH dependence behaviour, suggests that more than one single enzyme is present in the partially purified fraction.

Acknowledgement

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DNase activity during early development in *Artemia*

M. Cervera[1,2], *E. Martín*[1], *A. Domingo*[1], *C. G. Vallejo*[1], *and R. Marco*[1]

[1] *Instituto de Enzimología y Patología Molecular del CSIC*
Facultad de Medicina de la Universidad Autónoma
Arzobispo Morcillo, s/n. Madrid-34, Spain

Abstract

The dormant gastrulae of *Artemia* have a very low DNase activity that can only be detected using highly sensitive methods. Upon resumption of development, the activity increases markedly (over 30 fold) after hatching. This DNase has been partially purified by hydroxyapatite chromatography from dormant cysts and swimming nauplii. A new peak of activity that appeared after hatching has been shown to be the result of the *in vitro* proteolytic modification of the DNase found in the cysts. The majority of the catalytic properties of the enzyme indicate that it is a DNase I-like activity. It has a pH optimum in the neutral range. It degrades double stranded DNA preferentially over single stranded DNA, primarily by a haplotomic mechanism and secondarily by a diplotomic one. The major catalytic difference is that the *Artemia* enzyme is not stimulated by Ca^{++} in a synergistic way with Mg^{++} as it is in the case of DNase I. When yolk platelets are disrupted during homogenization, the enzyme activity is recovered mostly in the cytosolic fraction. On the other hand, when the integrity of yolk platelets is preserved, the DNase activity is found associated with the yolk platelets or with their partial degradation products. The evidence suggests that the DNase activity is associated with the yolk platelets in an inactive form that becomes active when yolk platelets are metabolized.

We advance the hypothesis that the role of the DNase in the early development of *Artemia* may be related to the presence of important quantities of DNA associated with yolk platelets. In such a hypothesis, the activation of the DNase will be one of the factors required for the physiological control of processes metabolizing yolk platelets.

Introduction

In contrast to the situation in procaryotic organisms, where the role of DNase activities in many crucial physiological processes like genome replication, recombination and repair are well substantiated (Kornberg, 1980), eukaryotic DNases have as yet to be ascribed defined functions (Sierakowska and Shugar, 1976). The particular characteristics of the early development of *Artemia* seemed to provide an interesting situation to study the presence and the role of DNases. In addition, the knowledge acquired would help to prevent the potential deleterious effects of these enzymes in studies of possible genome modifications during differentiation of *Artemia*. In this paper, the initial characterization of the enzymatic properties and subcellular localization of an endonuclease active on DNA are presented.

[2] Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

Materials and methods

The dormant cysts of *Artemia* which were utilized are from San Francisco Bay ("Metaframe" brand), California, USA.

Treatment of the cysts and culture conditions were as described in Vallejo *et al.* (1979); the homogenization media and the fractionation procedure were as described in Marco *et al.* (1980).

Hydroxyapatite was prepared as described by Bernardi (1971). Soy bean trypsin inhibitor (STI) is from Sigma Chemical company.

The high molecular weight calf thymus DNA was from Worthington and the tRNA from Boehringer. The denatured DNA was prepared by heating a preparation of native DNA for 5 min in a boiling water bath followed by immediate cooling in ice. DNA free of endogenous divalent cations was pretreated with 0.25 M EDTA at pH 7 for 3 to 5 hr followed by a thorough dialysis against 10 mM Tris at pH 7.5 for 48 hr. Other reagents and chemicals used were of analytical grade.

The highly sensitive clot assay for DNases described by Smith (1974) was used. This assay can be made quantitative and is specific for monitoring endonucleolytic attack of double stranded DNA (Cervera, 1978; Cervera and Marco, in preparation). One unit of DNase is the amount of enzyme necessary to cleave the double stranded DNA into a uniformly clear suspension in 20 min at 37 °C. Cytochrome oxidase was assayed as described in Vallejo *et al.* (1979).

The analysis by velocity sedimentation of the mechanism of attack of the DNase was carried out in a SW56 rotor in a Sorvall OTD-2 ultracentrifuge in neutral (10mM Tris pH 7.5) 5 to 20% sucrose gradients in the presence of 1 M salt; The 5 to 20% alkaline sucrose gradients had 0.9 M NaCl and 0.1 M NaOH. Neutral gradients were run at 50 000 rpm for 5.5 hr while alkaline gradients were run at 40 000 rpm for 14 hr.

The concentration of DNA in the gradients was measured by the fluorometric method of Abdel-Rahman *et al.* (1975). The sedimentation coefficients were determined as described by Prunell and Bernardi (1973) and the corresponding molecular weights calculated with the formula of Studier (1965). Proteins in gradients were measured by the method of Kalb and Bernlohr (1977).

Results

The detection of a DNase activity during *Artemia* development

Dormant gastrulae of *Artemia* have very low DNase activity that can only be detected using very sensitive methods. A simple, inexpensive and highly sensitive method specific for endonucleases is the clot assay (Smith, 1974). When the activity of endonucleases was measured during development, a marked increase in activity is detected when hatching. A peak in activity is reached after approximately 48 hr of development (Fig. 1). The levels detected are independent of the inclusion of STI in the homogenization medium. Therefore, the decrease in assayable activity after 61 hr of development seems unrelated to an *in vitro* proteolytic degradation of the enzyme in the homogenates.

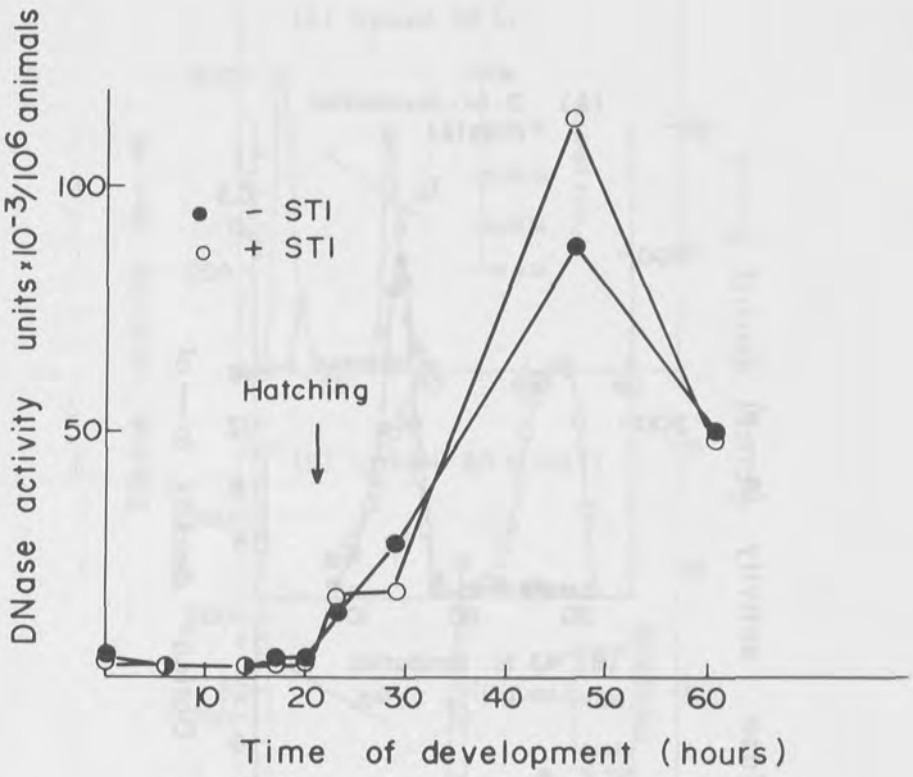

FIG. 1. DNase activity during early development in *Artemia*.

In order to further characterize the activity, its purification was attempted. It was found that the enzyme activity recovered in the cytosolic fraction (see below) at zero time of incubation, did not bind to many of the resins tested (DEAE-cellulose, CM-cellulose and PC-cellulose) but it did bind to hydroxyapatite (Fig. 2A). Therefore, purification by hydroxyapatite chromatography was carried out throughout development. It was found that after hatching, a new peak of activity appeared eluting with the void volume of the column (Fig. 2B). Nevertheless, no catalytic differences between the peaks could be demonstrated and significant variations in the ratio of both peaks were detected from experiment to experiment. Moreover, the recoveries of the hydroxyapatite columns were consistently low. Therefore, there was a possibility that, as for RNA polymerase I (Osuna *et al.*, 1977b), the new peak resulted from a proteolytic modification of the adsorbed peak produced by the increased proteolytic activities observed after hatching (Osuna *et al.*, 1977a). The experiments presented in Fig. 3 confirmed this idea. The inclusion of STI in the homogenization medium and during the elution of the column eliminated the presence of the first peak while improving the recovery of the DNase activity.

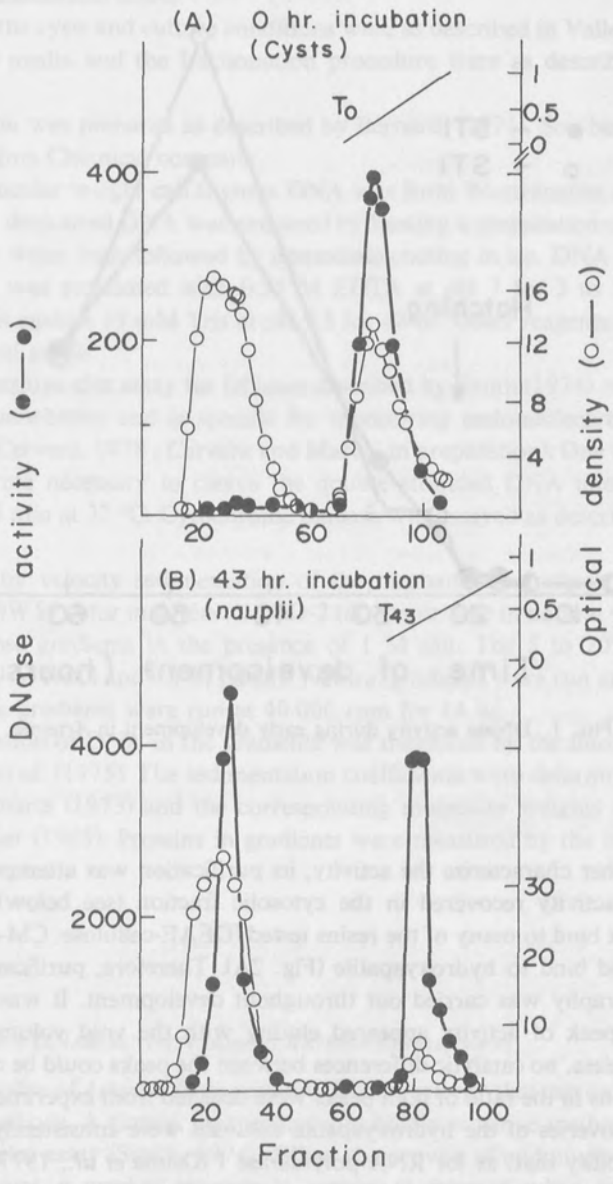

FIG. 2 Hydroxyapatite purification of DNase activity.

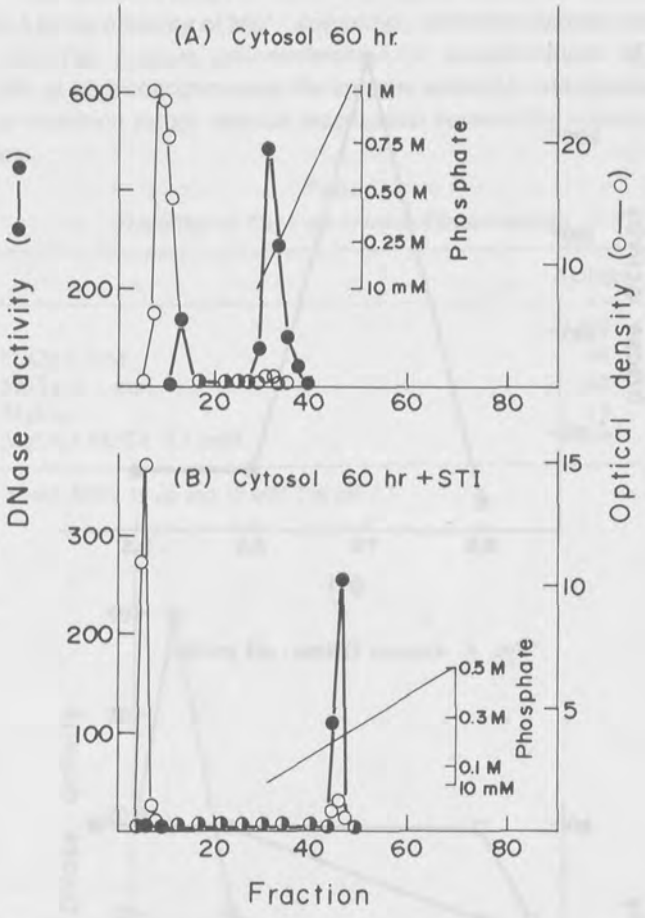

FIG. 3. Effect of STI on the hydroxyapatite purification of DNase activity in *Artemia* nauplii.

PROPERTIES OF THE DNASE ACTIVITY

The enzyme is a neutral DNase (Fig. 4). It acts specifically on DNA. The addition of concentrations of transfer RNA, 10 times in excess over the DNA in the assay, does not inhibit at all the activity on the DNA, indicating that it is a true DNase and not a nuclease. On the other hand, single stranded DNA competes with the activity on double stranded DNA, but a fourfold excess in single stranded molecules is necessary to decrease 50% the activity on double stranded DNA. This result suggests that the enzyme is active on both single and double stranded DNA but with lower affinity for the monocatenary substrate.

The *Artemia* DNase requires divalent cations to cleave the DNA, although it can utilize the endogenous metals present in variable amounts in commercial preparations of high molecular weight DNA from calf thymus (Fig. 5). In contrast with DNase I which requires Ca^{++} in

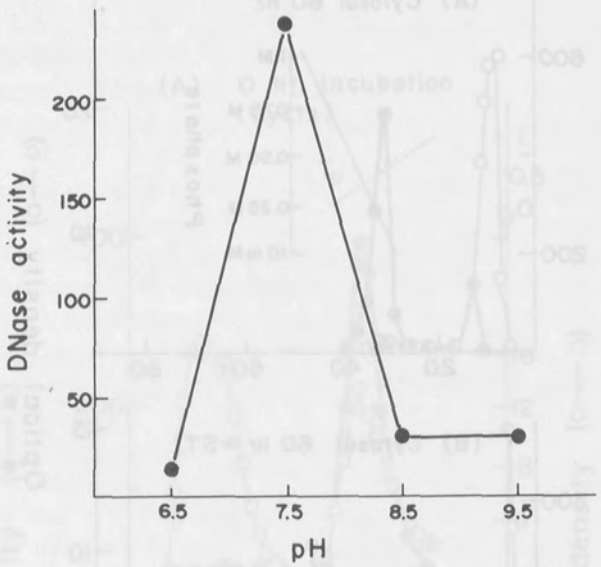

FIG. 4. *Artemia* DNase : pH profile.

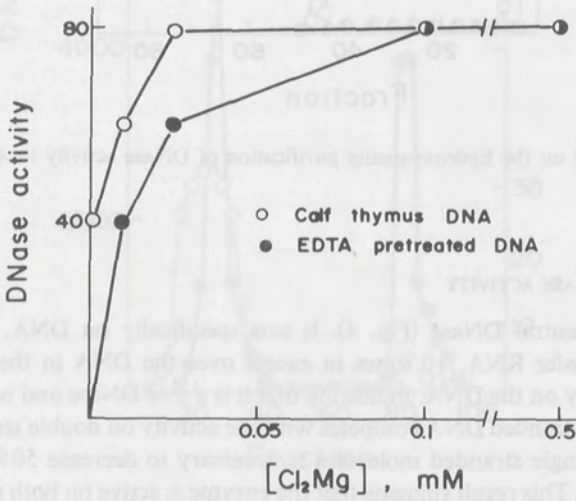

FIG. 5. Divalent cation requirement of *Artemia* DNase activity.

addition to Mg^{++} for activity (Price, 1975), Ca^{++} is slightly inhibitory (Table I). The addition of EGTA to DNase I in the presence of Mg^{++} completely inhibits its activity, while this treatment is stimulatory for the *Artemia* endonuclease. Low concentrations of salt are slightly stimulatory, while at high concentrations the enzyme activity is completely inhibited (Fig. 6). This effect is an inhibition rather than an inactivation because the activity was recovered in dyalized fractions.

TABLE I

The effect of Ca^{++} on *Artemia* DNase activity

	Activity
Normal assay*	100
Normal assay + $CaCl_2$ 5 mM	60
Normal assay + EGTA 0.1 mM	160
Normal assay – $MgCl_2$	10
Normal essay – $MgCl_2$ + EGTA 0.1 mM	30

* Includes $MgCl_2$ 5 mM, DNA 20 μg and 10 mM Tris pH 7.5.

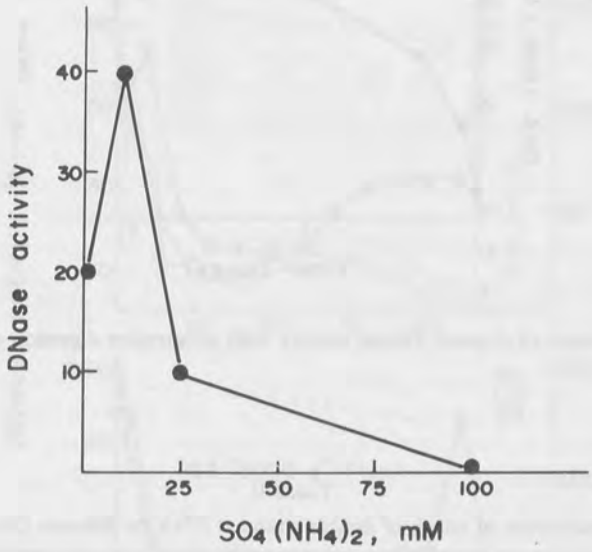

FIG. 6. Effect of the concentration of salt on *Artemia* DNase activity.

The mechanism of attack of the *Artemia* DNase was approached by comparing the behavior of the substrate after the action of the DNase in neutral and alkaline sucrose gradients as summarized in Table II. The native DNA from calf thymus has an average molecular weight of 10.3×10^6 daltons which should become 5×10^6 molecular weight, once denatured. In contrast, the results indicate that the molecular weight is only 1×10^6 daltons, showing that it has an average of 8 nicks per molecule. Thirty units of the enzyme degrade the

native DNA to 2.5×10^6 daltons in 20 min, suggesting that it diplotomically cuts each molecule three times in this period. On the other hand, the same amount of enzyme reduces the denatured molecular weight to 0.45×10^6 daltons suggesting that it nicks more than 10 times the DNA molecule in the same time. The number of nicks preexisting in the DNA molecule has also been checked with the single stranded specific S_1 nuclease and a comparison between the degradation mode of both enzymes indicate that the *Artemia* endonuclease is not specifically nicking the second DNA chain opposite to the preexisting nick in the substrate. In fact, the *Artemia* DNase can degrade the DNA into much smaller double stranded fragments upon very long incubation. A plot of the results of this experiment (Fig. 7) shows that there is a retardation in the velocity of hydrolysis upon further substrate degradation, as has been reported for other DNases (Laskowski, 1971).

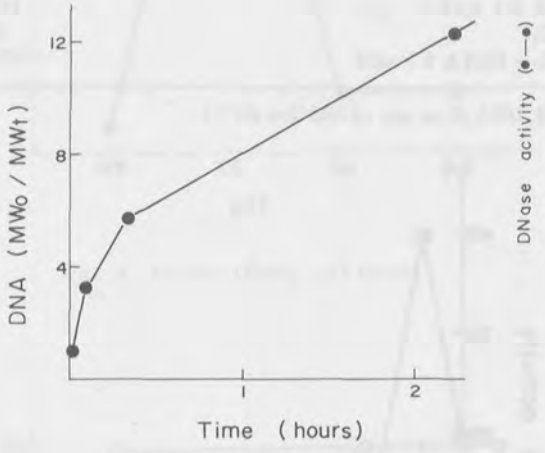

FIG. 7. Retardation of *Artemia* DNase activity with progressive digestion of the substrate.

TABLE II

Mechanism of attack of double stranded DNA by *Artemia* DNase

Enzyme units added	Neutral gradients		Alkaline gradients	
	Sedimentation coefficient	M.W	Sedimentation coefficient	M.W
30	14.5	2.55×10^6	9.6	0.45×10^6
15	17.2	4.1×10^6	10.5	0.56×10^6
7.5	n.t.	n.t.	11.7	0.73×10^6
3.7	18.7	5.3×10^6	n.t.	n.t.
1.8	20.9	7.3×10^6	n.t.	n.t.
None	23.5	10.3×10^6	13.3	1.0×10^6

THE SUBCELLULAR LOCALIZATION OF *ARTEMIA* DNASE

The total activity of *Artemia* DNase and its subcellular localization was found to be very dependent on the homogenization conditions. As can be seen in Table III at zero time of development, when the "ficoll" medium is used to stabilize the yolk platelets (Marco *et al.*, 1980), only 30% of the activity with the sucrose medium is found. Moreover, the activity in "ficoll" homogenates is recovered primarily in particulate fractions while that obtained with the disruptive sucrose is mostly cytosolic. When analyzed by 0.96 to 2 M sucrose gradients, the particulate activity was found to be associated with the heavy material that pellets through these gradients in parallel with cytochrome oxidase (Fig. 8). These pellets appeared under electron microscopic examination to be made up of yolk platelets (Marco *et al.*, 1980).

The effect of "ficoll" medium on the subcellular distribution of the enzyme continues briefly after hatching (Fig. 9) when the total activity begins to increase. The homogenization in "ficoll" medium still reduces the total activity detected and maintains it in particulate fractions.

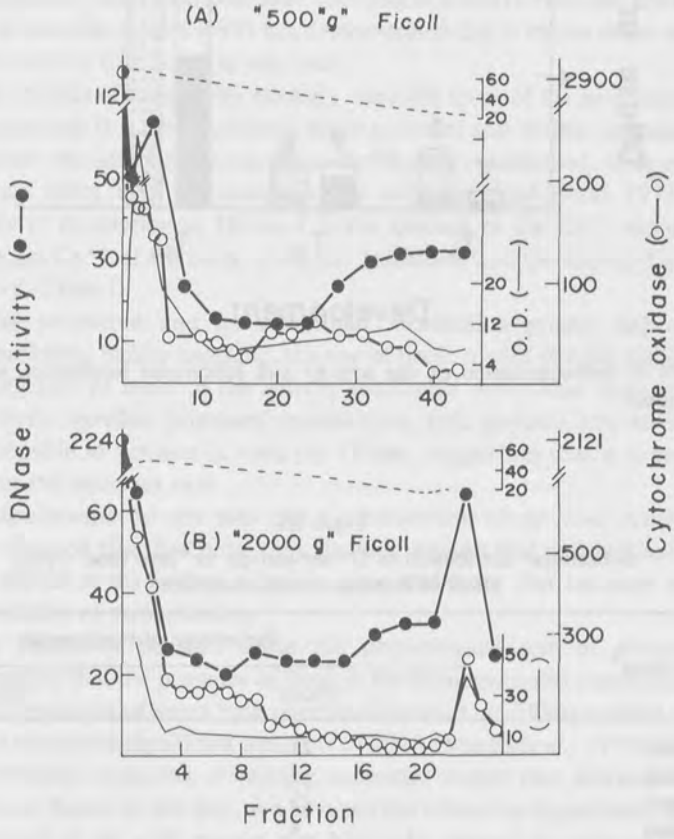

FIG. 8. Sucrose gradient purification of particulate DNase activity in *Artemia* cysts incubated for 12 hr.

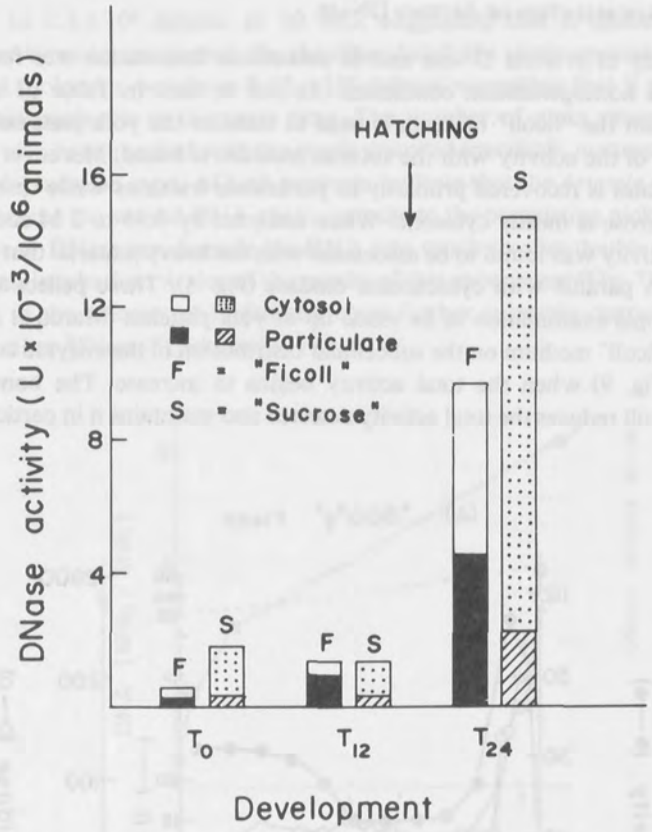

FIG. 9. Effect of homogenization on the activity and subcellular localization of *Artemia* DNase during development.

TABLE III

Subcellular distribution of DNase activity in "zero time" cysts, effect of homogenization conditions

Subcellular fractions*	Percentage of homogenate	
	"Ficoll"	"Sucrose"
500 g fraction	21	3
2 000 g fraction	13	1
27 000 g fraction	25	14
150 000 g fraction	4	4
Cytosol	53	93
Activities in homogenates (U/10⁶)	550	1500

* Recovery is more than 100%.

These results suggest that *Artemia* DNase might be located in the yolk platelets and become unmasked during the process of physiological degradation of these structures. In accordance with this idea, several treatments of the isolated fractions, including in *vitro* incubation, are capable of increasing the DNase activity. In particular, the solubilization procedure of Ballario *et al.* (1978) not only releases the majority of the activity from the particulate fractions but also results in an activation of more than 200% of the DNase. This can explain the difference in total activity observed in "ficoll" or sucrose media. The activity detected in the latter homogenates is higher probable because it has been solubilized during the extraction procedure.

Discussion and conclusions

A DNA-specific endonuclease has been detected in extracts of *Artemia* embryos, the activity of which markedly increases after hatching. Although initial hydroxyapatite chromatography suggested the appearance after hatching of a newly excluded activity (Fig. 2), the addition of the proteolytic inhibitor STI has demonstrated this to be the result of an *in vitro* modification of the activity that binds to the resin.

The enzymatic properties of the activity strongly resemble those of the pancreatic DNase. It has a neutral pH optimum. It is active on both single stranded and double stranded DNA but preferentially degrades the latter substrate by a haplotomic mechanism. This enzyme also shows the retardation effect upon degradation of the substrate (Laskowski, 1971). The only distinguishing property in relation to DNase I is the absence of the Ca^{++} requirement for optimal activity ; in fact Ca^{++}, if anything, is slightly inhibitory and the specific Ca^{++} chelator EGTA is stimulatory (Table I).

The total activity recovered and its subcellular localization greatly depends on the homogenization conditions. Before hatching, the use of ficoll, a yolk platelet stabilizing agent (Fig. 8), permits detection of most of the activity associated with these storage structures. The increase in activity parallels processes metabolizing yolk platelets and at this stage of development it is possible to activate *in vitro* the DNase, suggesting that it is present as an inactive precursor in the dormant cyst.

Although our experiments do not rule out a contribution of *de novo* synthesis to the increase in activity detected after hatching, they strongly suggest that a significant part of the enzyme activity is stored in the yolk platelets in a masked form that becomes active upon physiological degradation of yolk platelets.

At present very little can be said about the physiological role of *Artemia* DNase. Nevertheless, our finding that the presence of ficoll in the homogenization medium allows the isolation of a pure preparation of intact yolk platelets (Marco *et al.*, 1980) enabled us to report that these structures contained significant amounts of DNA (Marco *et al.*, 1977). This DNA is composed mainly of linear molecules of varying molecular weight that decrease during yolk platelet metabolization. Based on the data, we advance the following hypothesis : this DNA is a structural component of the yolk platelet that has to be cleaved in order to facilitate the physiological process metabolizing these storage structures. Therefore, the DNase activity is embedded in the yolk platelet and becomes active when massive mobilization of these structures is required. Later in development, it will dissapear or require additional controlling

mechanism(s). The decrease in activity found at later times of development (Fig. 1) may be related to this regulatory mechanism(s).

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Occurrence of poly(A) polymerase in particles rich in poly(A) RNA in the developing embryos of *Artemia*

Nirmala Jeyaraj[1], *Sohel Talib*[2], *John Louis*[1],
Christy Susheela[1], *and Kunthala Jayaraman*[1,3]

[1] *Department of Molecular Biology, School of Biological Sciences*
Madurai Kamaraj University
Madurai-625 021, India

[2] *Roche Institute of Molecular Biology*
Nutley, New Jersey 07110, USA

Abstract

It was revealed from ^{32}P-phosphate incorporation studies that there was an enrichment of poly(A) RNA in the pre-ribosomal particulate fraction during the early stages of development of *Artemia*. The association of an active poly(A) polymerase with this poly(A) rich particle had been demonstrated. In dormant cysts, the poly(A) polymerase activity of the particulate fraction was not detectable while the soluble extracts exhibited high levels of enzyme activity.

There was a decline of the soluble poly(A) polymerase activity with a concomitant increase in the activity of the particulate enzyme during development. The presence of two distinct poly(A) polymerase activities in the nucleus was demonstrated at all stages of development. The association of an active poly(A) polymerase with the particulate fraction rich in poly(A) RNA and the observed changes in the subcellular levels of this enzyme during development implicated its involvement in the processing of poly(A) RNA during development.

Introduction

Activation of the dormant cysts of *Artemia* has provided us with a model system for the study of the regulation of utilization of the stored messengers inasmuch as it has been well established that transcription and translation are the principal forces behind early developmental processes (Hentschel and Tata, 1976 ; Heip *et al.*, 1977). The occurrence of preformed and translatable poly(A) RNA in the dormant cysts has been unequivocally established and indicates a definitive role for the poly(A) RNA in the developmental process of this organism (Felicetti *et al.*, 1975, Nilsson and Hultin, 1975). In our earlier studies we have observed that the poly(A) RNA in the particulate fractions were rapidly turned over (Susheela and Jayaraman, 1976).

[3] To whom reprint requests should be addressed.

Post-transcriptional polyadenylation of mRNAs and their occurrence in the form of mRNP particles is well established in eukaryotic systems (Greenberg, 1975 ; Brawerman, 1976). Although the role of poly(A) is not yet clearly understood it has been speculated to be involved in regulating the mRNA structure and function. (Darnell, 1976). The enzyme poly(A) polymerase (EC.0.7.7.19) which is involved in the polyadenylation of RNA has been well characterised in a variety of systems (Rose and Jacob, 1976 ; Nevins and Joklik, 1977 ; Peters and Jeffery, 1978). Inorder to elucidate the distribution and turnover of poly(A) RNA during development in *Artemia*, the levels of poly(A) RNA and of the enzyme poly(A) polymerase in the subcellular fractions have been monitored. We are presenting here the first report on the occurrence of nuclear and membrane associated poly(A) polymerases of *Artemia*.

Materials and methods

Uniformly labeled (^{3}H) ATP (5 mCi/9 μmoles) and ^{32}P-orthophosphate (carrier free) were purchased from BARC, Bombay, India, Sephadex G-25 was obtained from Pharmacia, Sweden, DEAE-cellulose (0.85 meq/g-medium mesh), phosphocreatine, creatine phosphokinase, tRNA, unlabeled nucleotide triphosphates and poly(U) were from Sigma, USA. Source and method of activation of dormant *Artemia* cysts have been described previously (Susheela and Jayaraman, 1976). All experimental procedures were carried out under sterile conditions to minimize the action of RNAses.

Poly(A) RNA in subcellular fractions during development

Cysts were labeled during activation with ^{32}P-phosphate (500 mCi/50 mg cysts) and the subcellular fractions from the homogenates made in Buffer A (Tris HCl 0.01 M, pH 7.5, NaCl 0.01 M, $MgCl_2$ 3 mM, Triton X-100 1.0%, diethylpyrocarbonate 0.1%, polyvinylsulphate 0.002%) were obtained after pronase treatment and differential centrifugation as outlined by Susheela and Jayaraman (1976). After sedimentation of the particulate fractions at 16 000 rpm (30 000 $\times$ g) the supernatants were spun at 50 000 rpm ($2.10^6 \times$ g) for the pelletting of ribosomal fraction. RNA was extracted from these fractions and the proportion of radioactivity in the poly(A) RNA was determined by poly(U) filter binding assay as described by Sheldon *et al.* (1972).

Radioactive chase experiments were carried out by labeling the cysts with ^{32}P-phosphate for the first 4 hr of activation and after exhaustive washing with saline containing 0.1 M phosphate pH.7.0, they were suspended in fresh saline for further activation. After 1,2, and 4 hr, RNA from the subcellular fractions was extracted and its poly(U) binding capacity assayed.

Preparation of nuclear and cytoplasmic extracts for poly(A) polymerase (PAP)

Cysts were homogenized in 0.05 M Tris HCl, pH 8.0 and the subcellular fractions were obtained as described earlier. The nuclear extract was prepared by lysing the nuclei with 0.3 M NaCl in the same buffer and then desalted by passing through Sephadex G-25 column. The membrane extracts were obtained by suspending the particulate fraction in Buffer TME (20 mM Tris HCl, pH 8.0, 0-2 mM β-mercaptoethanol and 0.5 mM EDTA) and sonicating in a Vibronics (India) 30-KC Sonicator, for a total period of 2 min at 0 °C with cooling after each

30 sec sonication period. The debris were spun down at 5 000 rpm for 10 min and the supernatant which was turbid was used as the source of enzyme. The soluble proteins were concentrated by precipitating with 60% ammonium sulphate and the precipitate was dissolved in Buffer TME and desalted with Sephadex G-25.

PAP assay

The enzyme activity was determined by following the incorporation of (^{3}H) ATP into acid insoluble products. The assay mixture contained in a total volume of 0.2 ml ; 0.1 M Tris HCl, pH 8.0, 1 mM $MnCl_2$, 4 mM dithio-threitol, 5 mM NaF, 25 μg phosphocreatine, 2 μg creatine phosphokinase, 100 μg tRNA, 3.6 nmoles ATP (150 cpm/pmole), 10% glycerol (V/V) and the enzyme. The assay mixture was incubated at 37 °C for 30 min and the reaction was terminated by the addition of 1 ml of ice cold 10% TCA and the precipitate was filtered on millipore filters and processed for counting. The enzyme specificity was checked with labeled ADP as substrate or by the addition of unlabeled nucleotide triphosphates as described by Hadidi and Sethi (1976).

Fractionation of the enzyme on DEAE-cellulose

The crude extracts were applied to a column of DEAE-cellulose (1.5 × 20 cm), eluted with a linear gradient of 0.0-0.5 M KCl in Buffer TME and the fractions monitored for OD_{280} and PAP activity.

Results and discussion

The cystshells of *Artemia* are made up of chitinous materials that are impermeable to the usual precursors or inhibitors of macromolecules. Hence we have been using ^{32}P-phosphate to label the RNA in order to monitor their changes particularly during early periods of development (Susheela and Jayaraman, 1976). The distribution of labeled RNA and their poly(A) content in the different sub-cellular fractions during development is shown in Table I.

Table I

Distribution of labeled poly(A) RNA in the subcellular fractions from developing *Artemia* embryos

Developmental stages	Percentage of poly(A) RNA[1]			
	Nuclear	Particulate	Ribosomal	Soluble
4 hr	2.53	16.55	7.14	9.5
Emergence	3.77	16.39	9.31	---
Nauplius	4.70	20.34	3.94	15.98

[1] The labeled RNA from each fraction was extracted as described in Materials and methods and its poly(A) content expressed as percent acid precipitable CPM that was bound to poly(U) filters.

--- not tested.

It is clearly seen that the particulate fraction was enriched in poly(A) RNA throughout development and was the major site of storage for poly(A) RNA. However, there was a doubling of poly(A) RNA in the nucleus and in the cytoplasm by the naupliar stage of development.

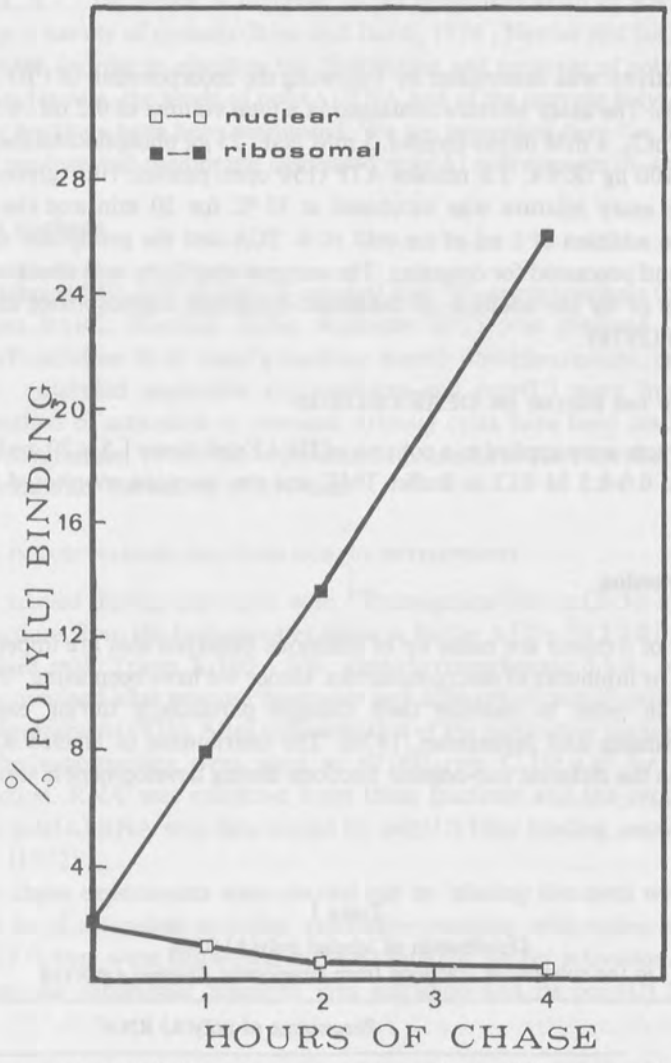

FIG. 1. Changes in the poly(U) binding capacity of pre-labeled RNA from nuclear and ribosomal fractions during development. RNA was extracted from the nuclear and ribosomal fractions at the time intervals indicated after the chase of ^{32}P-phosphate in the activation medium, as described in Materials and methods. The poly(U) binding capacity of the RNA samples was assayed by using poly(U) filters.

The results of the experiments aimed at chasing the label from the poly(A) RNA populations in the subcellular fractions during the early hours of development (4-8 hr) are shown in Fig. 1. The steep rise of the labeled poly(A) RNA in the ribosomal fractions indicates

the active mobilization of the poly(A) RNA species to the site of translation. The decrease of the prelabeled poly(A) RNA in the nuclear fractions suggests either an active turnover of poly(A) RNA in the nucleus and/or their transport to the cytoplasm during development. The significant mobilization of pre-labeled poly(A) RNA in the ribosomes is in agreement with the observation of Amaldi *et al.* (1977) who had shown by using another approach that the ribosomal associated poly(A) RNA increased during early hours of development in *Artemia*.

Since the processing of the poly(A) tail of the RNA populations can be mediated by an enzymatic cleavage or addition of poly(A), we looked for the presence of the enzyme poly(A) polymerase in the subcellular fractions of the embryos. The crude extracts of all the fractions showed very little dependence on the added substrate (Table II). When the nuclear extracts were fractionated on DEAE-cellulose columns, the presence of two distinct PAP activities were detected (Fig. 2). One was eluted in the void volume of the column and the other eluted by 0.2 M KCl. The particulate fraction, however, had only one type of PAP activity which was eluted in the void volume and there was a considerable amount of purification (25 fold) achieved in this step. Further, using ^{32}P-labeled cysts we have demonstrated the separation of poly(A) RNA of this fraction from PAP on DEAE-column. Thus the removal of endogenous RNA apparently is necessary prior to the demonstration of the enzyme activity. The soluble fraction also showed activity in the wash fraction of the column.

TABLE II

Subcellular distribution of poly(A) polymerase activity during development in *Artemia*

Developmental stages	Poly(A) polymerase activity[1]						
	Nuclear extract			Particulate extract		Soluble extract	
	Crude	DEAE-I	DEAE-II	Crude	DEAE-I	Crude	DEAE-I
Dormant	n.d.	398	103	n.d.	n.d.	30	1260
6 hr	26	636	354	84	1737	n.d.	598
Pre-nauplius	3	335	217	164	2149	n.d.	63
Nauplius	n.d.	562	317	n.d.	4688	n.d.	n.d.

[1] Expressed as pmoles of (^{3}H) ATP incorporated/mg protein/hr.

n.d. – not detectable (the limit of detection in the assay was : 3 pmoles/mg protein/hr).

DEAE I and II represent the enzyme activity in the wash fraction and in 0.2 M KCl eluate of the DEAE-cellulose column respectively.

The subcellular distribution of PAP during development is presented in Table II. In the dormant cysts PAP activity was detectable only in the nuclear and soluble fractions. However, upon resumption of development, the activity appeared in the particulate fraction as well and this activity increased towards the later stages of development. On the contrary, the soluble PAP activity showed a definite decline during development. A similar decrease of soluble PAP activity had been observed during post-fertilization development of sea urchin embryos by Slater *et al.* (1978). The observed properties of the PAP not retained by DEAE column

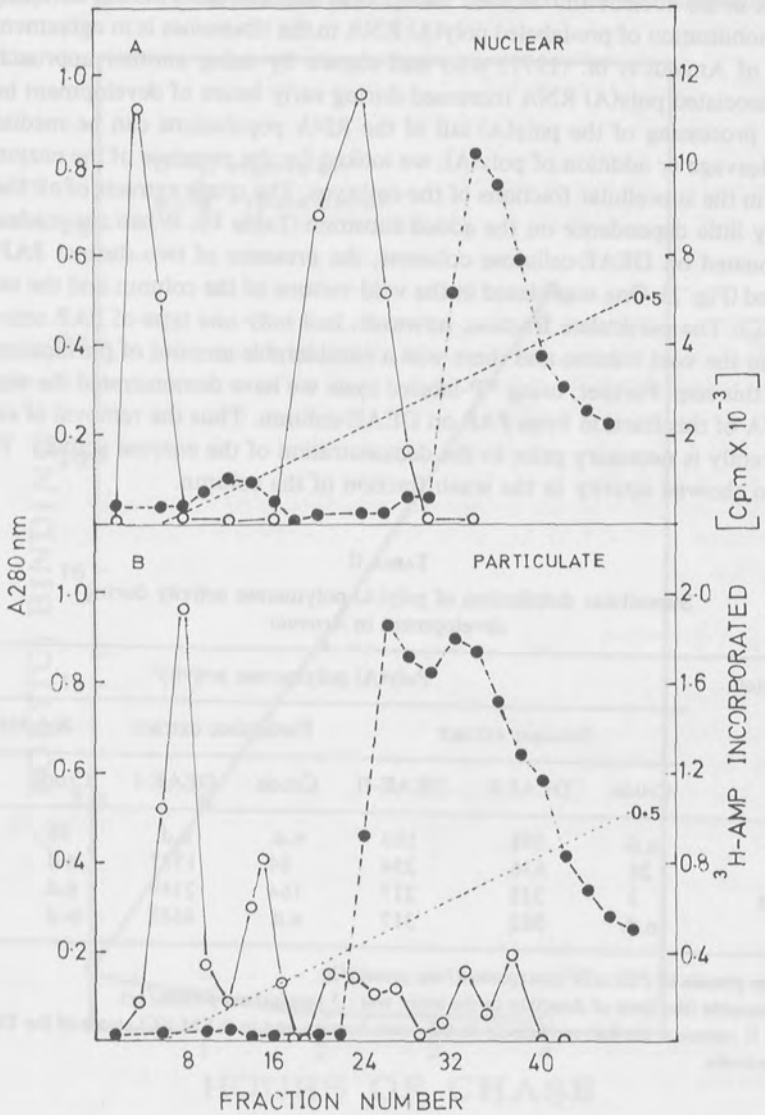

FIG. 2. DEAE-cellulose column profiles of poly(A) polymerase : nuclear and particulate extracts from cysts activated for 4 hr, were obtained as outlined in Materials and methods and fractionated on DEAE-cellulose column (1.5 × 20 cm) with a linear gradient of 0-0.5 M KCl in Buffer TME. (Fractions were monitored for OD 280 nm (● – – – ●) and PAP activity (○ —— ○).

indicated that they are of different types. The nuclear enzyme (DEAE I) exhibited higher activity with tRNA and nuclear RNA as primers, whereas the particulate enzyme showed an enhanced activity with particulate RNA, the soluble RNA and polynucleotides such as poly(U) and poly(A) (Table III). In addition, the kinetics of ATP incorporation by nuclear and particulate PAP was also found to be different (Fig. 3A and B). The observed rate of reaction of the nuclear enzyme was slower than that of the particulate enzyme. There was a rapid degradation of the product in the incubation mixture containig the particulate PAP suggesting

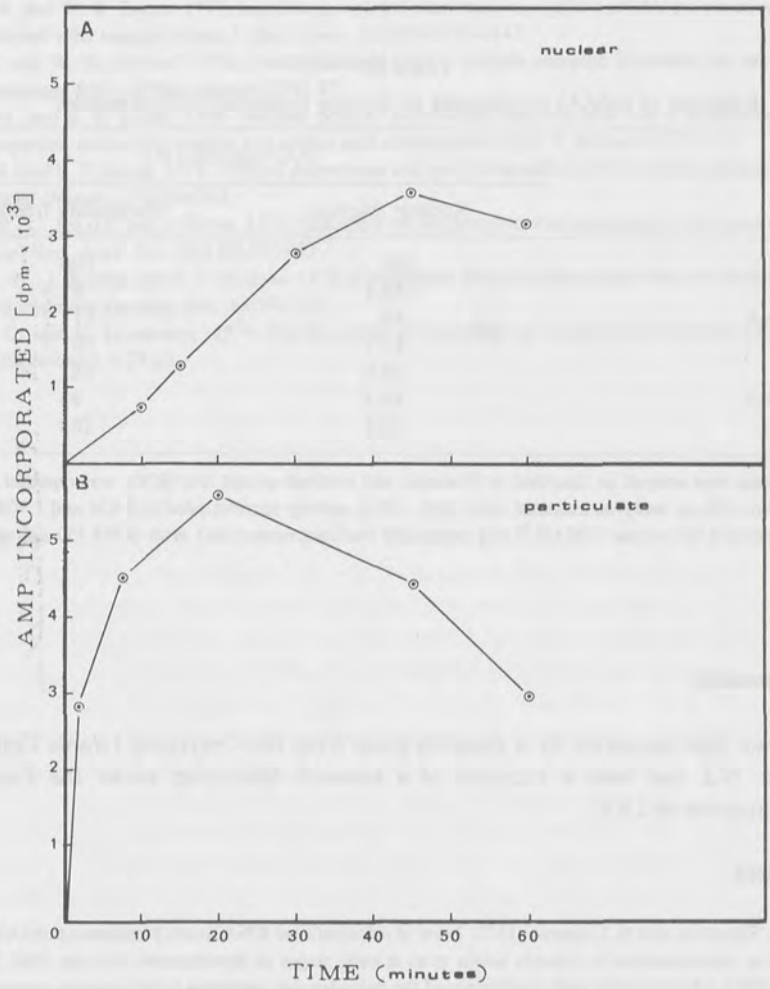

FIG. 3. Kinetics of nuclear and particulate poly(A) polymerase activities. Partially purified PAP from nuclear (1.4 μg/assay) and particulate (1.3μg/assay) fractions (eluted in the void volume of DEAE-columns) were incubated with (^{3}H) ATP in the standard PAP assay mixture for various time intervals as indicated and the activity levels were determined.

that this fraction may also harbor poly(A) degradative activity. The appearance of particulate PAP activity in the activated cysts and its increase during further development suggest a positive role for this enzyme in the turnover and processing of poly(A) RNA present in this fraction. We have observed that the particulate fraction obtained after Triton treatment, a procedure used in our poly(A) RNA studies, also exhibited activity indicating a very firm association of this enzyme with the poly(A) RNA rich particles. Recently, it has been reported by Rose and Jacob (1979) that PAP is one of the proteins of the poly(A) ribonucleoprotein particles in Morris hepatoma cells. Further characterization of nuclear and particulate RNP fractions for their poly(A) content and PAP activity is under progress.

TABLE III

Response of poly(A) polymerases of *Artemia* to primer polynucleotides

Primer	PAP activity (%)	
	Nuclear fraction	Particulate fraction
tRNA	100	100
Nuclear RNA	148.4	82.9
Particulate RNA	88	117.2
Poly(A)	43.7	107.7
Poly(U)	60.0	182
Ribosomal RNA	96.3	93
Soluble RNA	69.8	203

Poly(A) polymerase was assayed as described in Materials and methods except that tRNA was replaced by various other primer RNAs (50 μg/assay) as indicated inthe table. 100% activity corresponded to 0.636 and 1.900 nmoles of (^{3}H) ATP incorporated for nuclear (DEAE-I) and particulate fractions respectively with tRNA (50 μg) as primer.

Acknowledgements

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A hypothesis on the activation process of proteolytic activities during *Artemia* early development

R. Marco, C. G. Vallejo, R. Garesse, and R. Perona

Instituto de Enzimología y Patología Molecular del CSIC
Facultad de Medicina de la Universidad Autónoma
Arzobispo Morcillo s/n, Madrid-34, Spain

Abstract

The activation of development of the dormant cysts of *Artemia* probably requires, as has been suggested in other systems, the action of proteolytic activities. However, no proteolytic activity in the physiological pH range has been reported until late after hatching (Osuna *et al.*, 1977) when a considerable morphogenesis has already occurred in the absence of cell division. The availability of a very sensitive fluorescamine-based method for the assay of proteases that uses their natural substrates (Garesse *et al.*, 1979) has helped us to reexamine the problem. In this paper, we advance a hypothesis suggesting that the proteolytic activities previously described to appear late after hatching might be the result of the unmasking of a precursor stored in the cyst.

Introduction

Important events occurring in the early stages of development appear to be under the control of cytoplasmic determinants. It is clear that the maternal message, which is masked in ribonucleoprotein particles, has to be activated. It has been suggested, for example in sea urchin (Mano and Nagano, 1970), that proteolytic activities play an important role in this activation. When the dormant cysts of *Artemia* are rehydrated, they undergo, up to the nauplius stage, a dramatic morphogenetic change in the absence of significant cell division (Nakanishi *et al.*, 1962 ; Olson and Clegg, 1978). Moreover, about 50% of the yolk platelets of the cyst are metabolized by the hatching time (Warner *et al.*, 1972 ; Olalla *et al.*, 1977). However, no significant proteolytic activity has been detected in the physiological pH range up to late in the nauplius stage (Osuna *et al.*, 1977). The availability of a very sensitive fluorescamine-based method for the assay of proteinases (Garesse *et al.*, 1979) has permitted us to reexamine the problem. The method uses the natural substrates, *i.e.* proteins, and allows the determination of the activity in the presence of chaotropic agents, like potassium iodide, that recently have been shown to dissociate proteases from their inhibitors (Hernández-Jodra and Gancedo, 1979). The results obtained suggest that the proteolytic activities described previously (Osuna *et al.*, 1977 ; Olalla *et al.*, 1978) are the result of the unmasking of a precursor stored in the cyst that is physiologically activated as development proceeds. The properties of this precursor have been studied at different stages of development and the

possible relationship with the acid protease described by Nagainis and Warner (1979) in cysts is discussed. A hypothesis on the whole process of activation is presented.

Materials and methods

Cysts from San Francisco Bay (Metaframe, Menlo Park, California 94025) were used.

Culture of embryos, counting, extraction, and fractionation of cysts and nauplii were carried out as described by Vallejo *et al.* (1979). The precursor in the unmasking of proteolytic activities found after hatching was detected using the medium of Nagainis and Warner (1979) for homogenization, the medium of Osuna *et al.* (1977) and the media described by Marco *et al.* (1980). The data on the acid protease have been obtained using only the medium for homogenization reported by Nagainis and Warner (1979) unless otherwise stated.

Gel filtrations were carried out on a 62 × 1.3 cm column equilibrated with 20 mM sodium borate buffer (pH 8.5) in the absence and the presence of 1.2 M KI. The DEAE-cellulose chromatography of the cyst extracts was carried out as described by Nagainis and Warner (1979). Hydroxyapatite chromatography was carried out in a (5 × 1.7 cm) column equilibrated with 0.01 M sodium phosphate buffer (pH 6.8) and eluted with a gradient of phosphate from 0.01 M to 0.5 M (pH 6.8).

The determination of proteolytic activity at alkaline pH was carried out with the fluorescamine method described by Garesse *et al.* (1979). The determination of proteolytic activity at acid pH was made in the presence of acetate buffer as described by Nagainis and Warner (1979) although routinely, protamine sulfate was used as substrate. The posterior reaction with fluorescamine was made as described by Garesse *et al.* (1979). In every case, appropriate blanks were carried out in parallel. The reactions were always followed at different times and only the linear part of the assay was considered. The acid pretreatment of fractions was carried out as follows : 100 μl of the fraction were mixed with 100 μl of 0.2 M acetate buffer, pH 2.5 (the final pH of the mixture was 3.0) and preincubated for 6 min in ice. The velocity of the reaction was determined with 100 μl of the mixture in the presence of 0.1 M universal buffer (phosphate-citrate) from pH 2.5 to 7.3. The reaction at pH 8.0-8.5 was in the presence of 0.2 M borate buffer. The control reactions were made diluting the sample with the same volume of distilled water and assaying at the different pHs with the indicated buffers.

Results

Identification of a precursor in the unmasking of proteolytic activities after hatching

Using the sensitive fluorescamine method, we have been able to detect alkaline proteolytic activity immediately after hatching. The activity observed differs in several aspects from the one found later in the development. Data from early (E) nauplii (5 hr after hatching) and late (L) nauplii (45 hr after hatching) will be compared. The activity of E nauplii is activated about a 6-fold by potassium iodide (KI). As development proceeds, the directly assayable activity increases as reported by Osuna *et al.* (1977) while the activation by this chaotropic agent decreases. When the activity has reached the maximum (L nauplii), KI is still able to activate by a factor of about 2. These results indicate that the proleolytic activity of *Artemia* was already present immediately after hatching in an inhibited form.

The molecular weight of the proteolytic activity in the cytosol depending on the time of development (as well as the effect of KI on it) has been investigated. The data are summarized in Table I. The molecular weight in the absence of KI varies little, from about 100 000 to about 80 000. Nevertheless, while this relatively high molecular weight complex in the E nauplius is almost insensitive to dissociation by the chaotropic agent, the complex of L nauplius is completely dissociated down to the 20 000 molecular weight range. In conclusion it can be said that KI is able to activate the inhibited proteolytic activity present immediately after hatching but is not able to dissociate the corresponding complex. As development proceeds, the amount of proteolytic activity assayed directly increases and the complex becomes sensitive to dissociation by KI.

TABLE I

Effect of potassium iodide on the molecular weight of the protease complex, depending on time of development

Age of nauplii	Proteolytic activity	
	High molecular weight	Low molecular weight
5 hr-(E)		
− KI	125 000-66 000 (100%)	
in the column		
+ KI	125 000-91 000 (88%)	25 000 (12%)
45 hr-(L)		
− KI	76 000 (100%)	
in the column		
+ KI	71 000 (5%)	21 000 (95%)

The corresponding percentage of the total activity of the extracts is given in parenthesis. Activity was assayed in the fractions of a Sephacryl S-200 gel filtration column.

A marked change in the chromatographic behavior of the proteolytic activity at both times of development is also observed (Table II). The detailed work on which the tables are based has been published elsewhere (Garesse *et al.*, 1980).

Is the acid protease detected in the cysts a true acid protease ?

Nagainis and Warner (1979) have only found proteolytic activity in the cyst when the determination was carried out at an acid pH. In addition to this finding we have also detected a lower activity at alkaline pH (Fig. 1A, closed circles). The alkaline activity of zero time cysts behaves – by chromatographic criteria – similarly to the complex detected later. The KI treatment of the subcellular fractions of the cyst was found completely inhibitory at an acid pH while, as said above, it activated the alkaline activity complex after hatching. For these reasons, we considered the possibility that the acid pH was acting as an *in vitro* unmasking factor of the complex before hatching. The following observations suggest that something of this sort may be actually occurring. Firstly, the pH optimum of the activity detected at an acid pH varied with purification (Fig. 1). In the cytosol, it showed a sharp peak at pH 3.5 ; after

DEAE-cellulose elution, the peak moved to pH 4 with a shoulder in the pH 5 region and, after subsequent hydroxyapatite chromatography, the eluted activity had a pH optimum of 4.5. In addition, if the fractions were pretreated for a few minutes at pH 3.0, a further shift in the pH optimum was observed, particularly with the purified fractions (Fig. 1, open circles). The pH optimum of the "acid" activity was then 6.5.

TABLE II

Chromatographic behavior of the alkaline proteolytic activity during the nauplius stage

	Distribution of the activity			
	Early nauplii		Late nauplii	
	Excluded activity	Adsorbed activity	Excluded activity	Adsorbed activity
[a] DEAE-cellulose				
− KI	30%	70%	< 1%	100%
in the assay				
+ KI	70%	30%		
Total recovery (− KI)	180%-300%		100%	
Hydroxyapatite	20%	80%	100%	< 1%
Total recovery	150%-300%		100%	

[a] The change in distribution observed in the DEAE-cellulose chromatography of the early nauplii cytosol between excluded and adsorbed activity when adding KI to the proteolytic assay is due to the activation of the complex found in the excluded fractions.

THE BEHAVIOR OF THE "ACID" PROTEASE UPON DEVELOPMENT

As shown in Fig. 2, the "acid" activity remains at the same level during development inside the cyst. Shortly after hatching, there is a burst of activity immediately followed by an exponential decrease. The decrease precedes the increase in the directly detected level of the alkaline activity (Osuna *et al.*, 1977). Interestingly, the lag between the decrease in "acid" protease and the increase in the alkaline one coincides with the time of appearance of the alkaline activity complex that is strongly activated by KI. This activity, as shown elsewhere (Garesse *et al.*, 1980), represents about 60% of the maximal activity found in the late nauplius stage.

THE SUBCELLULAR LOCATION OF THE "ACID" PROTEASE

When studied during development, a significant change in the subcellular localization of the "acid activity" was detected (Table III). Well before hatching, less than 10% of the activity detected in the homogenate was recovered in the cytosol. The cytosolic percentage begins to increase immediately before hatching reaching near 70% at 48 hr of development. In contrast, the alkaline activity is detected mainly in the soluble fraction, except immediately after hatching when it is partially associated with the particulate fractions.

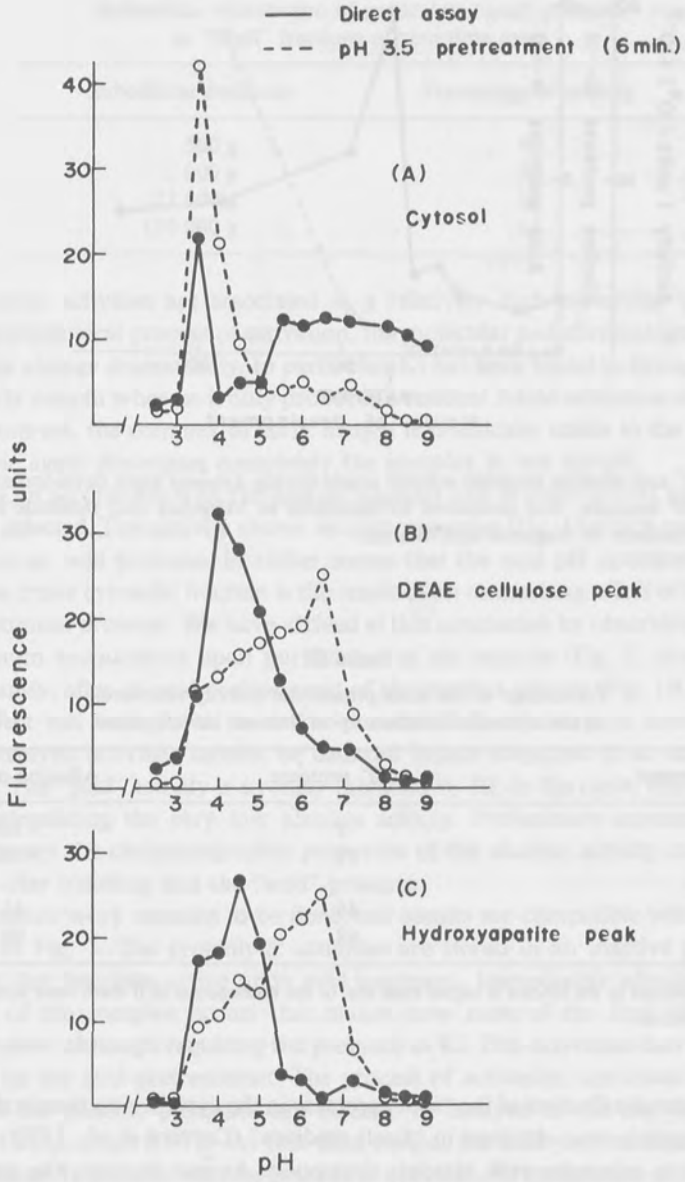

FIG. 1. Change in pH optimum of "acid" protease. Solid lines correspond to the control activity, directly assayed, and the broken lines to the same fractions pretreated with acid as described in Materials and methods. (A) The cytosol was obtained from homogenates in the Nagainis and Warner's medium of 14 hr incubated cysts by centrifugation at 150 000 × g for 150 min. (B) The DEAE-cellulose pool corresponds to the peak eluated from a DEAE-cellulose chromatography of the cytosol. (C) The hydroxyapatite pool corresponds to the peak activity eluted from a hydroxyapatite chromatography of the DEAE-cellulose pool.

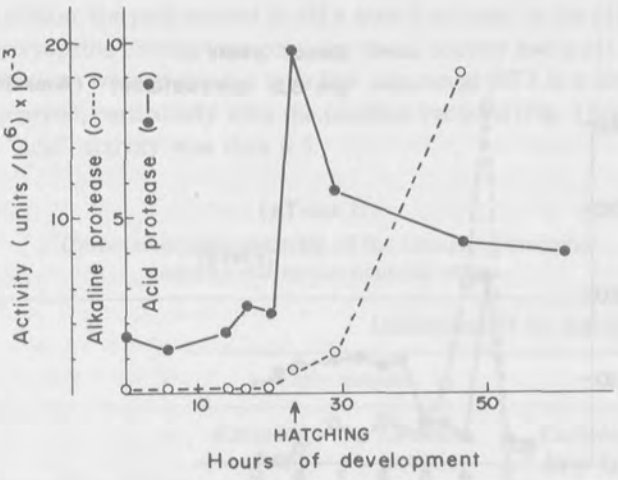

FIG. 2. "Acid" and alkaline protease activity levels during *Artemia* early development. The activity, expressed per 10^6 animals, was measured as described in Materials and methods in homogenates obtained in the medium of Nagainis and Warner.

TABLE III

Percentage of the total proteolytic activity recovered in the cytosolic fraction upon *Artemia* development

Time of development	"Acid" protease	Alkaline protease
14 hr	9	>100[a]
20 hr	19	>100[a]
HATCHING —		
23 hr	46	46
48 hr	68	90

[a] The activity detected in the cytosol is higher than that of the homogenate as if there were some inhibitos in the particulate fractions.

The subcellular distribution of the "acid" protease in the cysts at zero time is shown in Table IV. The homogenate was obtained in "ficoll medium" (Cervera *et al.*, 1980 ; Marco *et al.*, 1980) in order to minimize yolk platelets disruption. As can be seen, the activity appears mainly linked to the yolk platelets-rich fractions.

Discussion and conclusions

The data published elsewhere (Garesse *et al.*, 1980) and summarized in this paper suggest that the alkaline proteolytic activities reported by Osuna *et al.* (1977) to increase markedly in the late nauplius stage are also present, but in an inhibited state, immediately after hatching.

TABLE IV

Subcellular distribution of particulate "acid" protease in "ficoll" fractions of zero time cysts

Subcellular fractions	Percentage of activity
500 g	46
2 000 g	30
27 000 g	10
150 000 g	14

These proteolytic activities are associated in a relatively high molecular weight complex. During the physiological process of activation, the molecular and chromatographic properties of the complex change dramatically. In particular, KI has been found to strongly stimulate the activity of early nauplii whereas it only produces a residual 2-fold activation of the late nauplii activity. In contrast, the complex in early nauplii is practically stable to the KI action while this chaotropic agent dissociates completely the complex in late nauplii.

In the cysts, in accordance with the data of Nagainis and Warner (1979), an "acid" protease can be easily detected. This activity shows several properties (Fig. 1) which make us think that it is likely not an acid protease. In rather seems that the acid pH optimum shown by the enzyme of the crude cytosolic fraction is the result of an unmasking effect of the low pH on a higher pH optimum protease. We have arrived at this conclusion by observing the changes on the pH optimum encountered upon purification of the enzyme (Fig. 1, closed circles) and, more importantly, after an acid pretreatment of the purified activity (Fig. 1B,C, open circles). A similar effect has been found in crude extracts of *Saccharomyces cerevisiae* were the different proteolytic activities cannot be detected unless subjected to an acid pretreatment (Wolf, 1980). The "acid" activity is strongly inhibited by KI. In the cysts, this treatment is uneffective in stimulating the very low alkaline activity. Preliminary experiments indicate a similarity between the chromatographic properties of the alkaline activity complex observed immediately after hatching and the "acid" protease.

Although much work remains to be done, our results are compatible with the hypothesis summarized in Fig. 3. The proteolytic activities are stored in an inactive precursor in the dormant cyst that becomes active upon acid treatment. Immediately afte hatching, another modification of the complex occurs that makes now most of the final alkaline proteases activity detectable, although requiring the presence of KI. This activation can also be achieved at this stage by the acid pretreatment. The process of activation continues as development proceeds and the alkaline proteases can directly be assayed in the late nauplius stage as reported by Osuna *et al.* (1977). At this time period, the acid pretreatment inactivates the alkaline proteases. In parallel with the process of activation of the alkaline activity, the level of the "acid" protease decreases.

The actual nature of the high molecular weight complex is suggested by data indicating that the "acid" protease is mainly particulated in the cyst. The subcellular distribution of this activity is zero time cysts (Table IV) points to its association to the yolk platelets structures. The observed burst in activity of the "acid" protease corresponds to the moment of yolk platelets metabolization and the accompanying protein solubilization (Marco *et al.*, 1980). The solubilization of the yolk platelets-rich fraction of the cysts by the procedure of Ballario *et al.*

(1978) results in the solubilization and activation of the "acid" protease. This probably explains the above mentioned burst of this activity. The same effect is observed with the DNase (Cervera *et al.*, 1980) that is also recovered in the cytosol in a similar complex.

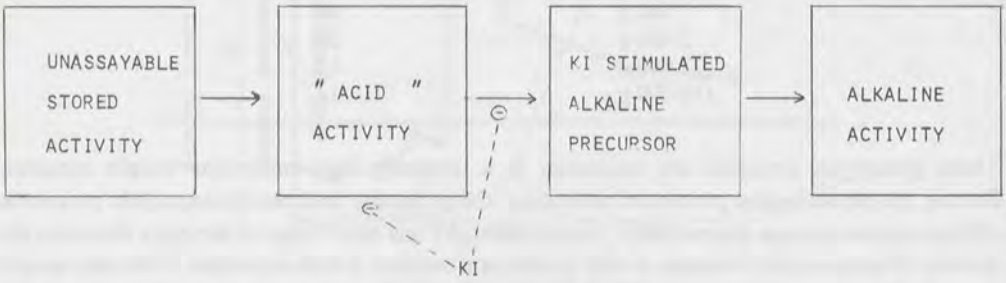

FIG. 3. Hypothetical evolution of the molecular state of the proteolytic activity during *Artemia* development. The activity of the dormant cyst, located in the particulate fraction, is highly inhibited and only detectable at acid pH. After hatching, an inhibited precursor of the alkaline activity is found, being now stimulated by caotropic agents. The activation process proceeds and the protease can be assayed directly in the late nauplius.

According to our data, at least some of the enzymes needed for the metabolism of the yolk platelets are stored in an inhibited form inside the latter. This is probably related to the regulation of the physiological degradation of the yolk platelets. For this regulation, we postulate an autocatalytic and feedback controlled activation of these enzymes. A potent trypsin inhibitor located in the yolk platelets of Amphibia has been described (Slaughter and Triplett, 1976) to slowly leach from the yolk in a soluble form less inhibitory for the active enzyme. The inhibitors of the "acid" protease found by Nagainis and Warner (1979) in the cytosol may be the result of a similar process. These inhibitors are not very potent ; they only produce a lag of the initial velocity of the reaction. Moreover, we have found that the "acid" protease is strongly inhibited while in the yolk platelets and can be activated by the solubilization of these organelles.

The reported complete inhibition by cycloheximide of alkaline proteolytic activities in the late nauplius stage (Cano *et al.*, 1979) raises the interesting possibility of an interaction between the protein synthesis level and the activation process. Further work will be required to reject or complete, in this and other aspects, the hypothesis postulated here.

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Poly(A) polymerase activity during the early development of *Artemia*

Leandro Sastre and Jesús Sebastian

Instituto de Enzimología y Patología Molecular del CSIC
Facultad de Medicina, Universidad Autónoma
Arzobispo Morcillo s/n, Madrid-34, Spain

Abstract

Extracts from dormant embryos of *Artemia* contain a poly(A) polymerase activity that has been partially purified. It catalyzes the synthesis of a polyadenylic acid chain of an average of 10 nucleotides covalently bound to the 3' end of an RNA or poly(A) molecule. The enzyme uses ATP as substrate and the reaction is dependent on the presence of manganese. More than 80% of the activity is located in the cytosol fraction of dormant embryos. During embryogenesis there is an increase of the nuclear poly(A) polymerase activity, representing 50% of the total at the time of hatching.

Introduction

The expression of genetic information in higher organisms involves different steps between the first event of chromatin activation to the final synthesis of the active gene products. The existence of this complex pathway in eukaryotic systems amplifies the levels of control of gene expression. Therefore, the enzymes implicated in genome modification, transcription, postranscriptional processing of the RNAs, translation and postranslational modification of proteins are candidates to play a role in the regulation of the information transfer. The role of these enzymes can be specially important in developing systems, where the expression of developmental program requires both, the differential transcription of the genome and the activation of previously synthesized and stored mRNAs.

Polyadenylation is one of the events described in several systems that occurs along the activation of stored mRNAs after fertilization or resumption of development (Wilt, 1973 ; Slater and Slater, 1974 ; Davis and Davis, 1978 ; Harris and Dure, 1978). Therefore, the investigation of the enzyme(s) involved in this mRNA modification is relevant to understand the regulation of the process.

The postgastrular embryonic development of *Artemia* offers an interesting model system to study the mechanisms underlying the transition from a dormant to a metabolically and genetically active state and, moreover, the activation of messenger RNAs present in dormant encysted embryos.

The ATP : RNA-terminal riboadenylate transferases (poly(A) polymerases) are enzymes that have been implicated in the polyadenylation of the mRNA molecules (Edmonds and Winters, 1976). The poly(A) polymerase activity was first described by Edmonds and Abrams

(1960) in animal tissues and found thereafter in many biological systems. The enzyme catalyzes the synthesis of a poly(A) chain covalently bound to the 3' end of a primer RNA.

In this paper we report the identification and properties of a poly(A) polymerase present in dormant and developing embryos (Sastre and Sebastian, 1978, 1979) as well as the levels of the enzyme during the early development of *Artemia*.

Material and methods

Chemical and buffers

Nucleotides, polyadenylic acid, and ribohomopolymers were obtained from Sigma Chemical Co. ^{3}H-ATP and other labeled nucleotides were from the Radiochemical Centre, Amersham, England. Calf thymus DNA from Worthington Biochemical Co., RNA from *Torula*, pancreatic ribonuclease and RNase T_1 were from Calbiochem., Sephadex G 150 and Poly(U) Sepharose from Pharmacia, DEAE-cellulose from Serva.

Buffer L contains : 50 mM Tris-HCl ; 0.4 mM EDTA, 5 mM mercaptoethanol, and 20% glycerol, pH 8.5.

Buffer F contains : 25 mM Hepes, pH 7.5 ; 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 M sucrose, and 15% Ficoll.

Buffer D contains : 50 mM Tris-HCl ; 0.4 mM EDTA, 5 mM mercaptoethanol, and 0.6 M ammonium sulfate, pH 8.5.

Organism and growth conditions

Artemia cysts were obtained from San Francisco Bay Brand Inc., Division of Metaframe Co., Menlo Park, California 94025. Treatment of dry embryos and growth conditions were as described elsewhere (Renart and Sebastian, 1976 ; Osuna *et al.*, 1977).

Preparation of subcellular fractions and extracts

The subcellular fractions of dormant and developing embryos were obtained according to Marco *et al.* (1977) using buffer F.

The extraction of the poly(A) polymerase activity from the particulate fractions was performed using buffer D. The homogenates were centrifuged at 105 000 × g for 2 hr to obtain the corresponding solubilized proteins.

The soluble extracts were prepared with buffer L. The embryos and nauplii were homogenized with two volumes of buffer L in a Kontes Duall grinder. The homogenate was centrifuged at 105 000 × g for 2 hr. The resulting supernatant was the soluble extract.

Poly(A) polymerase assay

The assay mixture contains in a final volume of 0.15 ml : 50 mM Tris-HCl, pH 8.5 ; 2 mM $MnCl_2$; 0.12 mM ATP ; 1 μCi (8 ^{3}H)ATP ; 0.2 mg of RNA primer and enzyme preparation. The specific activity of the ATP was 30 cpm/pmol. The assay mixture was incubated at 30 °C for 30 min and the reaction was stopped by addition of 5 ml ice-cold TCA containing 2% tetrasodium pyrophosphate. The TCA insoluble material was collected on Whatman glass fibre filters, washed and counted in a liquid Scintillation Counter. One unit of poly(A)

polymerase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nanomole of AMP into TCA insoluble material in 60 min under the assay conditions.

Protein determination

Proteins were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results

Poly(A) polymerase activity in dormant encysted embryos from *Artemia*

Soluble extracts from *Artemia* dormant embryos contain an enzymatic activity able to incorporate AMP into a TCA insoluble material. Table I shows the requirements of this activity. The enzyme requires a divalent cation and it is activated by RNA. The activity becomes fully dependent on the presence of RNA in the assay mixture after the chromatography of the extract on DEAE-cellulose. The requirements of this enzymatic activity are similar to those of the poly(A) polymerases from other biological systems (Edmonds and Winters, 1976).

Table I

Requirements of the poly(A) polymerase activity of extracts from *Artemia* dormant embryos

Assay components	pmol ATP incorporated	% activity
ATP, tRNA, Mn^{2+}, Mg^{2+}	648	100
Minus Mg^{2+}	776	120
Minus Mn^{2+}	97	15
Minus Mg^{2+} and Mn^{2+}	18	3
Minus tRNA	73	11

About 80% of the poly(A) polymerase activity was found in the cytosol and only 20% in the particulate fraction containing nuclei and yolk platelets in dormant embryos. The pattern is the same when using poly(A) as RNA primer and magnesium as divalent cation instead of manganese.

The cytosolic poly(A) polymerase was partially purified by DEAE-cellulose chromatography, ammonium sulfate precipitation and filtration on Sephadex G 150. Fig. 1 shows the chromatography on DEAE-cellulose of the extract. The enzyme has a very low affinity for DEAE-cellulose and it is eluted with 80 mM KCl. This step allows to eliminate most of the RNA present in the extract. About 90% of the poly(A) polymerase pool precipitates between 30 and 45% ammonium sulfate. The final step is a filtration on Sephadex G 150. The purification procedure allows to obtain a preparation of poly(A) polymerase purified 50 fold with a recovery of 40%.

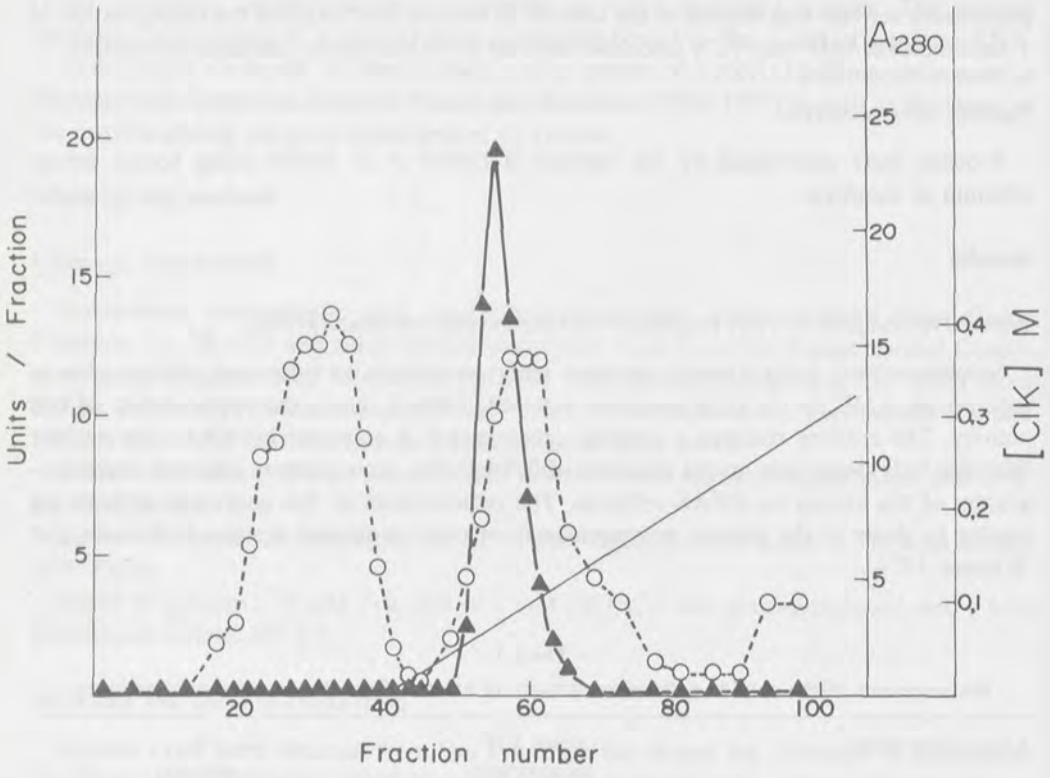

FIG. 1. DEAE-cellulose chromatography of the soluble extract from *Artemia* dormant embryos. 35 ml of soluble extract of dormant embryos prepared with buffer L were applied to a 50 ml DEAE-cellulose column equilibrated with buffer L containing 0.01 M KCl. After sample application the column was washed with 20 ml of buffer L plus 0.01 M KCl and eluted with a 75 ml × 2 linear gradient of 0.01 M to 0.5 M KCl in buffer L. Fractions of 2.5 ml were collected and aliquots of 50 μl were assayed for poly(A) polymerase activity. Absorbance at 280 nm (○- - -○). Poly(A) polymerase activity (▲——▲).

PROPERTIES OF THE POLY(A) POLYMERASE ACTIVITY FROM DORMANT EMBRYOS

We have studied the substrate specificity and the nature of the product of the partially purified poly(A) polymerase in order to characterize the enzyme.

Table II shows the substrate specificity of the enzyme for the nucleotides ; ATP (but not CTP, GTP or UTP) is the substrate for the enzyme. The lack of the activity with CTP alone or in combination with ATP when using labeled CTP allows to conclude that the enzyme is not a tRNA nucleotidyl transferse (Schofield and Williams, 1977). Moreover, the lack of enzymatic activity with other nucleotides indicates that the enzyme is not a ribohomopolymer polymerase (Niessing and Sekeris, 1974).

The poly(A) polymerase requires RNA for activity. Table III shows that the enzyme has the same activity with RNA from *Torula* and poly(A) and 10% of activity on poly(C) and poly(G).

The enzyme has indetectable activity on poly(U) and single or double stranded DNA. Fig. 2 shows the effect of the RNA concentration on the activity of the poly(A) polymerase. The saturation curves are similar for RNA from *Torula* and poly(A).

The poly(A) polymerase activity requires manganese for maximal activity with an optimal concentration of 2 mM. The optimum pH of the reaction is 8-8.5 and the activity is inhibited by low concentrations of salt. 70 mM KCl causes 50% inhibition of the enzymatic reaction.

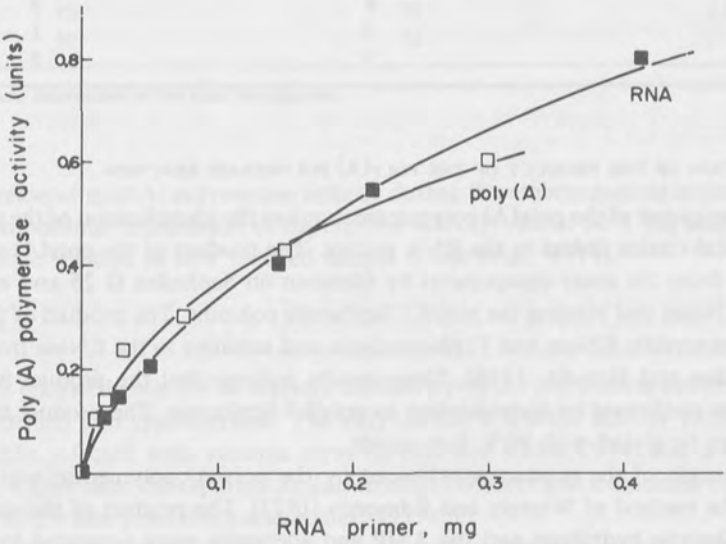

FIG. 2. Effect of RNA concentration on poly(A) polymerase activity. The reaction velocity was measured using the standard assay mixture and varying concentrations of poly(A) (□——□) and RNA from Torula (■——■).

TABLE II

Nucleotide specificity of poly(A) polymerase

Nucleotide	Labeled nucleotide	pmol nucleotide incorporated
ATP	ATP	245
CTP	CTP	6
GTP	GTP	7
UTP	UTP	6
ATP + CTP	CTP	5

TABLE III

RNA primer specificity of poly(A) polymerase

Polynucleotide primer	pmol ATP incorporated	% activity
Torula RNA	288	100
poly(A)	270	93
poly(G)	39	14
poly(C)	26	9
poly(U)	6	2
ss DNA	3	1
ds DNA	3	1

CHARACTERIZATION OF THE PRODUCT OF THE POLY(A) POLYMERASE REACTION

The characterization of the poly(A) polymerase requires the identification of the products as polyadenylic acid chains linked to the RNA primer. The product of the poly(A) polymerase was separated from the assay components by filtration on Sephadex G 25 and analyzed by sensitivity to RNases and binding the poly(U) Sepharose columns. The product of the reaction is resistant to pancreatic RNase and T_1 ribonuclease and sensitive to the RNase from *Artemia* nauplii (Sebastian and Heredia, 1978). These results indicate that the product is a poly(A) chain. This was confirmed by hybridization to poly(U) Sepharose. The product binds to the column and can be eluted with 90% formamide.

The chain length of the product synthesized by the poly(A) polymerase was calculated according to the method of Winters and Edmonds (1973). The product of the reaction was subjected to alkalyne hydrolysis and the AMP and adenosine were separated by thin-layer chromatography using poly(ethyleneimine)-cellulose (Reyes, 1972). The average length calculated for the poly(A) chains was 10 nucleotides.

The poly(A) chain was shown to be covalently bound to the RNA primer by using $\alpha^{32}P$ labeled ATP as substrate and looking for the interchange of radioactivity to the 3'-end nucleotide of the RNA after alkalyne hydrolysis.

LEVELS OF POLY(A) POLYMERASE ACTIVITY DURING EARLY DEVELOPMENT OF *ARTEMIA*

The levels of poly(A) polymerase activity were measured in dialyzed soluble extracts from embryos and nauplii at different stages of development. The extracts from nauplii were prepared in the presence of soybean trypsin inhibitor to prevent proteolytic attack by the proteases induced after hatching (Osuna *et al.*, 1977). The amount of soluble extracts used for the enzymatic assays contained low ribonuclease activity and do not interfere with the poly(A) polymerase assays.

Table IV shows the poly(A) polymerase levels expressed in terms of specific activities and activity per nauplius. There is an increase of activity during embryogenesis that is maximal at the time of hatching and decrease thereafter during the early larval development. The enzyme levels after hatching were measured in fed nauplii, since starvation of the cultures leads to a decrease in the levels of some enzymes (Sebastian *et al.*, 1979 ; Osuna, 1979).

TABLE IV

Poly(A) polymerase activity during the early development of *Artemia* embryos

Hours of development	Activity	
	U/10^5 embryos	U/mg proteins[1]
0	63	0.96
10	71	0.77
18	120	2.75
24	121	2.60
32	79	2.69
50	32	1.59

[1] Proteins were determined in the total homogenate.

The increase of poly(A) polymerase activity during the embryogenesis occurs along with a change in subcellular localization of the enzyme activity. About 50% the activity is found in the particulate fraction in new hatched nauplii (Cano *et al.*, 1979).

Discussion

Dormant encysted embryos of *Artemia* contain a poly(A) polymerase activity that has been partially purified and characterized. The enzyme has a specific activity comparable to that found in cells infected with vaccinia virus (Brakel and Kates, 1974) and it is one order of magnitude higher than the reported for calf thymus (Winters and Edmonds, 1973ab), rat liver (Niessing, 1975) and yeast (Half and Keller, 1975).

A purification procedure for the poly(A) polymerase has been worked out that allows to purify the enzyme 50-fold. We have no indication for the existance of more than one isoenzyme of the poly(A) polymerase in *Artemia* embryos. In several systems it has been reported two or three isoenzymes with different RNA primer specificities and metal dependence (Half and Keller, 1975 ; Niessing, 1975 ; Pellicer *et al.*, 1975 ; Edmonds and Winters, 1976 ; Nevins and Joklik, 1977).

The cytosolic poly(A) polymerase from *Artemia* embryos is dependent on manganese for maximal activity. It can use natural RNAs and poly(A) as primers with the same efficiency. These properties and the average size of the poly(A) chain synthesized make the enzyme comparable to other manganese-dependent poly(A) polymerases characterized in other eukaryotic systems (Edmonds and Winters, 1976).

The high specific activity and the cytosolic location of the enzyme in dormant embryos suggest a role for the poly(A) polymerase in the polyadenylation of cytoplasmic stored mRNAs. A cytoplasmic poly(A) polymerase has been identified in sea urchin embryos (Slater *et al.*, 1978) and it is supposed to be implicated in the postfertilization polyadenylation of latent messenger RNAs. In the case of *Artemia* it is tempting to postulate that the poly(A) polymerase present in encysted embryos is involved in the polyadenylation of poly(A)-minus mRNAs or in the extension of the poly(A) chain in the poly(A)-containing mRNAs stored in dormant embryos (Nilsson and Hultin, 1972, 1975 ; Felicetti *et al.*, 1975 ; Grosfeld and

Littauer, 1975 ; Sierra *et al.*, 1976 ; Susheela and Jayaraman, 1976 ; Amaldi *et al.*, 1977 ; Slegers and Kondo, 1977 ; Simons *et al.*, 1978). This could be a mechanism for their activation after resumption of development.

The increase of poly(A) polymerase activity in the nuclear fraction around hatching suggests a function for the enzyme in the polyadenylation of newly synthesized mRNAs. The poly(A) polymerase activity is maximal at the time of hatching and decrease thereafter. The physiological significance of these fluctuations is being investigated in relation to the *in vivo* polyadenylation during early larval development of *Artemia*.

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Role of the RNA polymerases in the regulation of transcription during the early development of *Artemia*

Jesús Sebastian, Jesús Cruces, Carmen Osuna, and Jaime Renart

Instituto de Enzimología y Patología Molecular del CSIC
Facultad de Medicina, Universidad Autónoma
Arzobispo Marcillo s/n, Madrid-34, Spain

Abstract

Artemia embryos and nauplii contain three isoenzymes of RNA polymerase with different enzymatic and molecular properties. The levels and ratios of the RNA polymerases I, II and III are different along the embryonic and larval development, indicating the existence of an independent regulation for these enzymes. An inhibitor of the *in vitro* transcription of native DNA has been characterized from encysted embryos. The inhibitor seems to act at the level of initiation and could have a role in the maintenance of the repression of transcription during the dormant stage.

Introduction

The elucidation of the mechanisms involved in the regulation of transcription is an important step in order to understand the molecular basis of cell differentiation and development. The control of transcription in eukaryotic organisms can be carried out at the level of DNA template and at the level of the enzymatic machinery responsible for the transcription process, the RNA polymerases and the factors involved in the modulation of their specificity and/or activity.

The existence of multiple forms of the RNA polymerase with different functions in eukaryotic organisms makes possible the control of transcription mediated by these enzymes (Chambon, 1975). RNA polymerase I is responsable for the transcription of ribosomal RNA, RNA polymerase II for the precursors of messenger RNAs and RNA polymerase III for transfer RNAs and 5s rRNA. Changes in the concentration and/or activity of the isoenzymes can produce a specific change in the rate of synthesis of rRNA, tRNA and mRNA precursors. Besides the role of the RNA polymerases in the quantitative control of the RNA synthesis, the enzymes can play an important role in the regulation of the expression of specific informational genes by changes in the specificity of RNA polymerase II. These changes can be obtained by different factors or enzyme modifications that alter the recognition of specific promotor regions on DNA by the RNA polymerase II. The role of the RNA polymerases in the control of transcription has been reported in bacterial and eukaryotic organisms (Rutter *et al.*, 1974 ; Doi, 1977 ; Zillig *et al.*, 1977).

We have approached the role of the RNA polymerases in the regulation of transcription during *Artemia* development by studying three different aspects : 1) the existence of factors controlling the transcription process, 2) the structural modifications of the RNA polymerases and 3) the determination of the levels of these enzymes. In this paper we report the identification of an inhibitor of the *in vitro* transcription isolated from dormant encysted embryos and the fluctuations of the levels of the RNA polymerases during early development. Several aspects of the role of the RNA polymerases during development of *Artemia* have been reported by different laboratories (Birndorf *et al.*, 1975 ; Renart and Sebastian, 1976 ; Sebastian *et al.*, 1976 ; D'Alessio and Bagshaw, 1977 ; Hentschel and Tata, 1977 ; Osuna *et al.*, 1977b ; Sebastian *et al.*, 1979).

Material and methods

Chemical and buffers

Nucleotides and soybean trypsin inhibitor type I-S were obtained from Sigma Chemical Co. ^{3}H-UTP from the Radiochemical Centre, Amersham, England. Calf thymus DNA from Worthington Biochemical Co., α-amanitin from Boehringer, Manheim and DEAE Sephadex from Pharmacia Fine Chemicals. All other chemicals were of analytical grade.

Buffer B contains : 50 mM Tris-HCl ; 0.2 mM EDTA ; 5 mM mercaptoethanol, and 20 % glycerol, pH 7.5.

Buffer C is buffer B containing 75 mM ammonium sulfate.

Buffer H contains : 50 mM Tris HCl ; 0.2 mM EDTA ; 5 mM mercaptoethanol, and 0.6 M ammonium sulfate, pH 7.5.

Organism and growth conditions

Artemia cysts were obtained from Longlife Fish Food Products, Division of Sternco Industries Inc., Harrison, New Jersey 07029 and from San Francisco Bay Brand Inc., Division of Metaframe Co., Menlo Park, California 94025. Treatment of dry embryos and nauplii were as described elsewhere (Renart and Sebastian, 1976 ; Osuna *et al.*, 1977b). The feeding of the nauplii population was carried out by addition to the culture of an homogenate of the blue green algae *Spirulina* (Sorgeloos and Persoone, 1975).

RNA polymerase assay

The RNA polymerase assay was carried out according to Renart and Sebastian (1976) using native calf thymus DNA as template. One unit of RNA polymerase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 pmole of UMP into TCA insoluble material in 10 minutes under the assay conditions.

Results and discussion

RNA polymerases from *Artemia* larvae

The solution of the eukaryotic RNA polymerases requires the use of high ionic strength in the homogenization buffer in order to remove the enzymes from the chromatin complex

(Chambon, 1974). The solution of the RNA polymerases from *Artemia* embryos and nauplii is obtained by preparing the extracts in the presence of 0.4 M ammonium sulfate. Lower salt concentrations lead to a partial solubilization of these enzymes, especially of RNA polymerases I and III (Renart and Sebastian, 1976 ; Osuna, 1979).

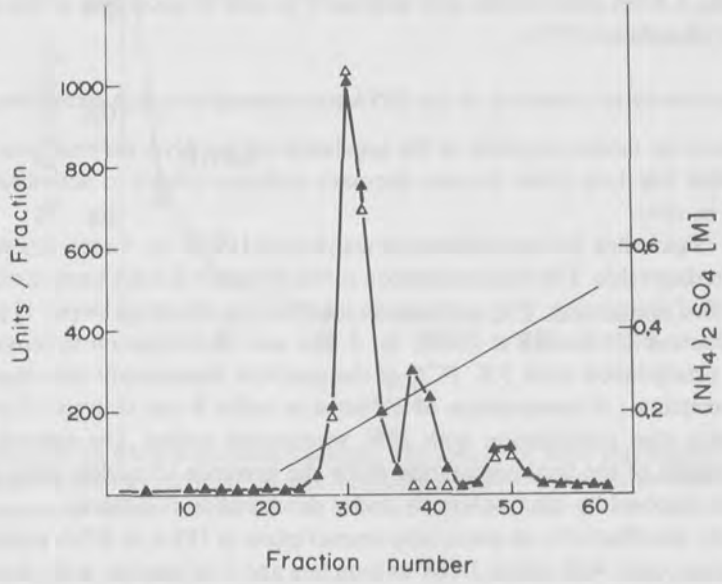

FIG. 1. Separation of the RNA polymerases from extracts of *Artemia* nauplii (T18) by chromatography on DEAE Sephadex. 5 g of new born nauplii (T18) were homogenized with two volumes of buffer H. The homogenate was centrifuged at 27 000 × g for 30 min and the supernatant was diluted with one volume of buffer B and centrifuged at 105 000 × g for 2 hr. The soluble extract was diluted with buffer B to get a final concentration of 0.075 M ammonium sulfate and was applied to a 30 ml column of DEAE Sephadex equilibrated with buffer C. After the sample application, the column was washed with 30 ml buffer C and eluted with a 60 ml × 2 linear gradient of 0.075 M to 0.6 M ammonium sulfate in buffer B. Fractions of 3 ml were collected and aliquots of 50 μl were assayed for RNA polymerase activity. The incubation time was 20 min (▲) activity in the absence of α-amanitin. (△) activity in presence of 10 μg α-amanitin.

The RNA polymerase activity present in the soluble extract is resolved into three isoenzymes by chromatography on DEAE Sephadex. Fig. 1 shows the separation of RNA polymerases I, II and III from a soluble extract prepared from new born nauplii. The peaks corresponding to these enzymes elute at 0.15 M, 0.25 M and 0.35 M ammonium sulfate. The elution pattern of the enzymes is the same for the embryos and nauplii RNA polymerases. However the levels and ratio of the isoenzymes are different along the embryonic and larval development. The nomenclature adopted for the *Artemia* RNA polymerases is the one proposed by Roeder and Rutter (1969) for the multiple enzymes from eukaryotic organisms.

The DNA template and metal specificities of the *Artemia* RNA polymerases are comparable to those of the RNA polymerases from other eukaryotic organisms (Chambon, 1974). RNA

polymerases I and III are insensitive to concentration of α-amanitin up to 500 μg/ml and RNA polymerase II is 50% inhibited by 0.005 μg α-amanitin per ml (Renart and Sebastian, 1976 ; Osuna, 1979). The insensitivity of enzyme III to α-amanitin makes this enzyme comparable to the RNA polymerase III from another arthropod, the *Bombix mori* (Sklar *et al.*, 1976) and different from enzyme III from most animal systems. Therefore, enzymes I and III belong to class A RNA polymerases and enzyme II to class B, according to the nomenclature proposed by Chambon (1975).

Characterization of an inhibitor of the RNA polymerases isolated from dormant embryos

In the search for factors involved in the regulation of the RNA polymerases it was found that the soluble fractions from *Artemia* dormant embryos inhibit to activities of the RNA polymerases *in vitro*.

The factor responsible for the inhibition is resistant at 100 °C for 5 min, it is precipitable by TCA and non dialyzable. The characterization of the inhibitor (I_1) has been carried out with a partially purified preparation. The purification involves the following steps : 1) preparation of the soluble fraction ; 2) heating at 100 °C for 5 min and centrifugation to remove denatured proteins ; 3) precipitation with 5% TCA of the previous supernatant and centrifugation to obtain the precipitate ; 4) resuspension of the pellet in buffer B and dialysis ; 5) preparation of the supernatant after precipitation with 30% ammonium sulfate. The spectral and electrophoretical analysis of the final preparation show the presence of nucleic acids and about 20 protein bands resolved by electrophoresis under denaturating conditions.

Fig. 2 shows the effect of I_1 on the *in vitro* transcription of DNA by RNA polymerase I. The inhibition occurs only with native DNA as template and it is inactive with denatured DNA. The inhibition by I_1 of the transcription of double strand DNA is independent of the source of DNA, including *Artemia* DNA, calf thymus DNA and *E. coli* DNA. On the other hand, the inhibitor is active with the three isoenzymes of *Artemia* RNA polymerase as well as with bacterial RNA polymerase.

The specificity of the inhibition with native DNA suggests the interaction of the inhibitor with the DNA template. To test this hypothesis, we studied the effect of different concentrations of DNA on the extent of inhibition. Fig. 3 shows that the percentage of inhibition of a certain amount of I_1 decreases with increasing concentrations of DNA template in the assay mixtures. Therefore, the inhibitor interacts with the DNA rather than with the RNA polymerase molecule. This conclusion is also supported by results showing that the percentage of inhibition for a given amount of I_1 is independent of the concentrations of RNA polymerases.

Initiation of the RNA synthesis seems to be the step of the transcription process that is inhibited by I_1. Fig. 4 shows the effect of I_1 on the RNA polymerase activity when the inhibitor is added to the incubation mixture after initiation of the reaction. I_1 has no effect in these conditions on the *in vitro* transcription. The inhibitor has to interact with DNA before the formation of the initiation complex in order to inhibit the reaction. This is shown in Table I. Preincubation of DNA with RNA polymerase and ATP or GTP prevents the inhibitory effect of I_1, while preincubation with CTP does not prevent the inhibition. These results are compatible with the hypothesis that I_1 acts at the level of initiation, since the interaction of I_1 with DNA has to take place before the formation of the initiation complex.

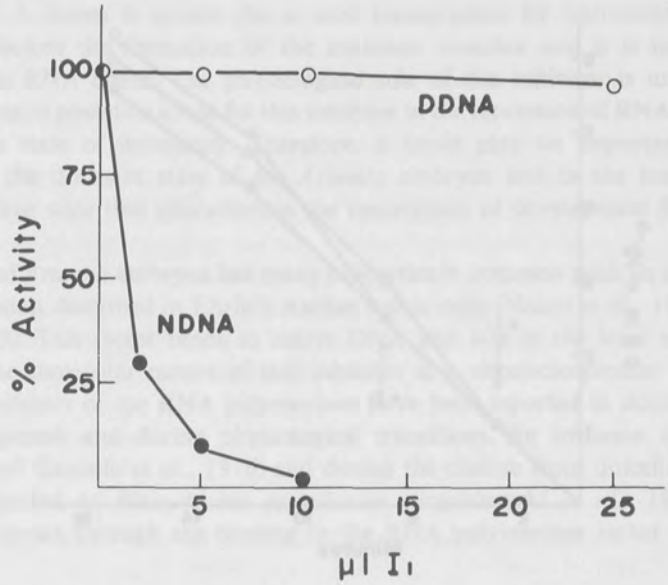

FIG. 2. Effect of I_1 on RNA polymerase I activity. The activity of RNA polymerase I from nauplii (DEAE Sephadex fraction) was determined with increasing amounts of partially purified I_1 using native (●) and denatured calf thymus DNA (○).

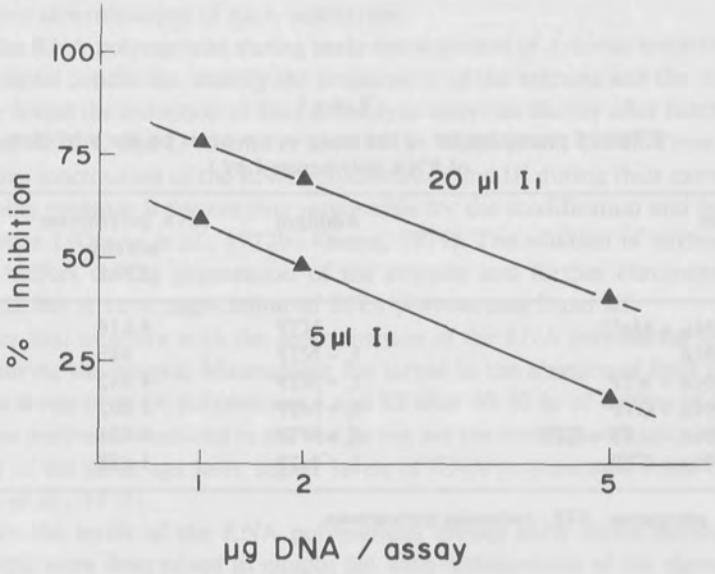

FIG. 3. Effect of DNA concentration in the inhibition of RNA polymerase I for I_1. The activity of RNA polymerase I (DEAE Sephadex fraction) was determined with three concentrations of native calf thymus DNA in the absence or presence of 5 μl and 20 μl of partially purified I_1.

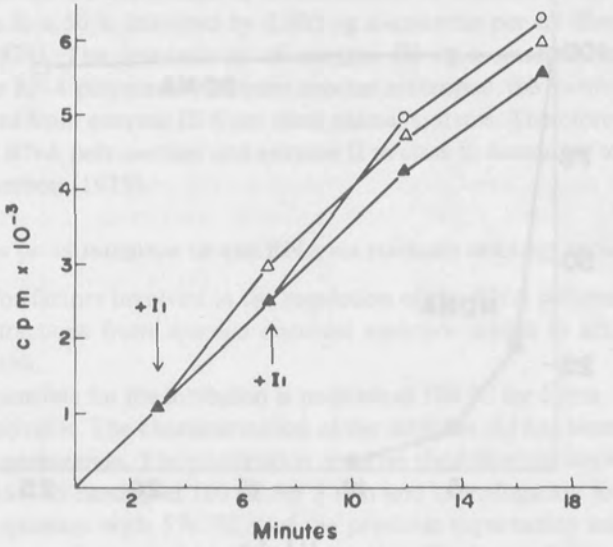

FIG. 4. Effect of addition of I_1 to the RNA polymerase reaction. RNA polymerase I (DEAE Sephadex fraction) was incubated in a serie of tubes and 5 μl of I_1 were added at 3 min (△) and 7 min (○) of the reaction. (▲) Control of activity without I_1.

TABLE I

Effect of preincubation of the assay components on the inhibition of RNA polymerase I by I_1

Preincubation	Addition	RNA polymerase activity CPM	% inhibition
RNAp I + DNA + Mn^{2+}	NTP	4 614	
RNAp I + DNA	I_1 + NTP	845	82
RNAp I + DNA + ATP	I_1 + NTP	4 042	12
RNAp I + DNA + GTP	I_1 + NTP	4 002	13
RNAp I + DNA + ATP + GTP	I_1 + NTP	4 621	0
RNAp I + DNA + CTP	I_1 + NTP	1 128	76

RNA_p : RNA polymerase ; NTP : nucleoside triphosphates.

In conclusion, I_1 seems to inhibit the *in vitro* transcription by interaction with double stranded DNA before the formation of the initiation complex and it is inactive on the elongation of the RNA chain. The physiological role of this inhibitor is unknown. It is, however, tempting to postulate a role for this inhibitor in the repression of RNA synthesis that characterizes the state of dormancy. Therefore, it could play an important role in the maintenance of the dormant state of the *Artemia* embryos and in the transition to the metabolically active state that characterizes the resumption of development (Hentschel and Tata, 1976).

The inhibitor of *Artemia* embryos has many properties in common with an inhibitor of the *in vitro* transcription described in Ehrlich Ascites tumor cells (Natori *et al.*, 1974 ; Kostraba and Wang, 1975). This factor binds to native DNA and acts at the level of initiation of transcription. The molecular nature of this inhibitor is a ribonucleoprotein (Natori *et al.*, 1975). Other inhibitors of the RNA polymerases have been reported in dormant spores of *Bacillus thuriengiensis* and during physiological transitions, for instance, during the T7 infection of *E. coli* (Iandolo *et al.*, 1976) and during the change from unicellular yeast to a multicellular mycelial in *Histoplasma capsulatum* (Boguslawski *et al.*, 1975). The last inhibitors seem to act through the binding to the RNA polymerases rather than to DNA template.

Levels of the RNA polymerases during the early larval development of *Artemia*

The levels of the RNA polymerases were determined in samples of nauplii at different stages of development obtained from synchronous cultures. The RNA polymerases were solubilized using buffer H and separated by DEAE Sephadex chromatography in order to have a quantitative determination of each isoenzyme.

The levels of the RNA polymerases during early development of *Artemia* are influenced by different experimental conditions, mainly the preparation of the extracts and the culturing of nauplii. We have found the induction or four proteolytic enzymes shortly after hatching of the nauplii : proteases A, B, C and D (Osuna *et al.*, 1977a ; Olalla *et al.*, 1978). These proteases produce the *in vitro* inactivation of the RNA polymerases I and III during their extraction and solubilization, being protease B the enzyme responsible for the modification and inactivation of RNA polymerase I (Osuna *et al.*, 1977b ; Osuna, 1979). The addition of soybean trypsin inhibitor to the buffers during preparation of the extracts and further chromatography is necessary to avoid the *in vitro* degradation of RNA polymerases I and III.

A second factor that interfere with the determination of the RNA polymerase levels is the condition of culturing the nauplii. Maintaining the larvae in the absence of food produces a sharp drop in the levels of RNA polymerases I and III after 40-50 hr of culture at 30 °C. The low levels of these enzymes measured in starved larvae are the consequence of the starvation, since fed nauplii of the same age have higher levels of RNA polymerases I and III (Osuna, 1979 ; Sebastian *et al.*, 1979).

Table II shows the levels of the RNA polymerases during early larval development of *Artemia*. The levels were determined in nauplii fed with homogenates of the algae *Spirulina* and the extracts were prepared in the presence of soybean trypsin inhibitor. The levels of RNA polymerase I remain in the order of 1 200 units/g nauplii during 15-20 hr after hatching and decrease to a value of 500 units/g nauplii during the following 50 hr. The levels of RNA

polymerase II and III remain almost constant during the first 100 hr of larval development. The change of RNA polymerase I produces a variation in the ratio of RNA polymerase I/ RNA polymerase II, which is 2,5 in new born nauplii at is about 1 in nauplii after 20 hr of larval development.

TABLE II

RNA polymarase levels during the early larval development of *Artemia*

Time of development (hr after hatching)	Units/g nauplii (ww)		
	RNA_p I	RNA_p II	RNA_p III
New-born nauplii (T18)	1 318	500	150
10 hours (T27)	1 270	680	120
20 hours (T37)	920	600	89
30 hours (T48)	534	646	73
43 hours (T60)	482	530	101
71 hours (T88)	420	675	129
79 hours (T96)	507	574	103

The investigation of the levels of the RNA polymerases has pointed out the existence of several methodological problems that can interfere with their determination. In the case of *Artemia*, the induction of multiple proteases and the effect of starvation on the nauplii population are the source of artefactual results. Shields and Tata (1976) have also shown the limitation of the RNA polymerase determination in other systems under different physiological transitions.

The results obtained during the early larval development together with the reported results during the postgastrular embryonic development of *Artemia* (Renart and Sebastian, 1976) indicate the existence of independent changes in the levels of activities of the RNA polymerases. Several mechanisms can be proposed to explain the changes in the levels of RNA polymerases, including mechanisms affecting the enzyme concentration by changes in the rate of synthesis or degradation as well as the enzyme activity. The independent regulation of the RNA polymerases has been found during the expression of the differentiation and development programs of many systems, as well as during different metabolic and physiological transitions (Rutter *et al.*, 1974).

The physiological meaning of the fluctuations of the RNA polymerases has to be evaluated after determining the relative rates of synthesis *in vivo* of the different classes of RNA. The increase of RNA polymerase I during the embryogenesis could be related with the activation of the RNA synthesis after resumption of development. The impermeability of the cysts to labelled precursors makes it difficult to study RNA synthesis during embryonic development. Clegg and Golub (1969) have reported the active synthesis of ribosomal RNA during this period using $^{14}CO_2$ as precursor. On the other hand, Susheela ad Jayarama (1976) did not measure any RNA synthesis during the embryonic development using ^{32}P as precursor. The last results are in contrast with the nuclear activation during the resumption of development that can be observed by electron microscopy and the existence of transcription complexes in dormant encysted embryos (Hentschel and Tata, 1977).

In contrast with embryos, nauplii are highly permeable to many molecules and RNA synthesis can be studied using different labelled precursors. McClean and Warner (1971) have reported that RNA synthesis is very active after hatching. However, after a period of several hours, the rate of RNA synthesis has a drop to almost undetectable values. This pattern of RNA synthesis is not correlated with the maintenance of the RNA polymerase levels during this stage. Therefore, the reported decrease in RNA synthesis could be a consequence of the fall of the RNA polymerases I and III found in starved nauplii. Hentschel and Tata (1976) had previously suggested that the fall in the rate of RNA synthesis during the larval development could be produced by the starvation of the cultures. In conclusion, it is necessary to investigate the synthesis of the different classes of RNA during the early development of *Artemia* under non starving conditions in order to understand the role of the fluctuations of the RNA polymerases in the regulation of transcription.

Acknowledgements

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Multiple proteolytic enzymes in *Artemia*

María A. Günther Sillero, Silvia L. Burillo, Elvira Domínguez, Asunción Olalla, Carmen Osuna, Jaime Renart, Jesús Sebastian, and Antonio Sillero

Instituto de Enzimología y Patología Molecular del CSIC and Departamento de Bioquímica Facultad de Medicina, Universidad Autónoma Arzobispo Morcillo s/n, Madrid-34, Spain

Abstract

Extracts from *Artemia* cysts contain very low levels of protease activity. This activity remains low in the first 24 hr of incubation and increases during development to larvae. From an 150 000 × g supernatant of larval extracts, four proteases termed A, B, C, and D have been characterized and partially purified. Some of their properties are presented here. The four proteases have lytic activity on yolk platelets *in vitro*, although their physiological function is not known at present. The low levels of proteases present in the cysts are theoretically enough to account for the degradation of platelets in the first 24 hr of development, a period in which half of the yolk platelets content of the cysts are degraded.

The four proteases have been found in two *Artemia* populations from Spain one parthenogenetic and other bisexual. The level of proteolytic activity in the parthenogenetic population was significantly higher than in the bisexual one. The investigation of the reason for this discrepancy led to the finding of proteases inhibitors in the bisexual population.

Introduction

It is now generally accepted that the proteases play an important role in the morphogenetic changes that occur during development (Reich *et al.*, 1975). In the case of *Artemia* cysts, the study of the proteases could have a special interest to understand, among others, the mechanism of the drastic decrease in the yolk platelet content during the first 2 days of development (Olalla *et al.*, 1977) and the possible changes, either physiological or artefactual in tissue extracts (Twardowski *et al.*, 1976 ; Osuna *et al.*, 1977a), in the structure and function of other proteins.

The proteolytic activity of the dormant embryos is very low (Osuna *et al.*, 1977b) and as happen with several other enzymatic activities studied (Cano *et al.*, 1979), increases steadly after hatching. Four proteolytic activities have been detected in larval extracts, all of them maximally active in the alkaline pH range. Their course of appearance during development has been already described (Osuna *et al.*, 1977b). In this report, we shall summarize the more relevant properties of the four proteases, termed A, B, C, and D, and discuss their possible role in the metabolism of yolk platelets. The presence of the four proteases in two populations of

Artemia adults, and the occurrence of inhibitors of proteases in one population will also be presented.

Other proteolytic ativities, optimally active at acid pH and unrelated to the ones presented here, have also been described in *Artemia* extracts (Nagainis and Warner, 1979).

Materials and methods

The following substrates and inhibitors were used to characterize the proteolytic activities reported here : α-N-benzoyl-DL-arginine p-nitroanilide (BAPNA) ; benzyloxycarbonyl-L-leucine p-nitrophenyl-ester (CLNE) ; (p-toluenesulfonyl)-L-arginine methyl ester (TAME) ; casein ; azocasein ; phenylmethylsulfonylfluoride (PMSF) ; N-α-p-tosyl-lysine chloromethyl ketone (TLCK) ; soybean trypsin inhibitor (STI) ; ovomucoid trypsin inhibitor (OTI).

Experimental details of the growth conditions of *Artemia* cysts and of the methods used to prepare the extracts and measure the proteolytic activities have been previously described (Osuna *et al.*, 1977b ; Olalla *et al.*, 1978).

Artemia adults, were obtained from salterns in Sigüenza (Guadalajara) and San Fernando (Cádiz), Spain. They were collected and throughly washed with deionized water. Immediately after, they were frozen and kept at -70 °C until the preparation of the extracts.

The source of yolk platelets was the precipitate of 700 × g obtained after stirring *Artemia* cysts in glass distilled water (1/10) for 3-4 hr. In some experiments the yolk platelets were further purified through chromatography in Sepharose 4B (unpublished results from this laboratory). The lysis of yolk platelets was measured with some of the methods described below and the results were very similar irrespective of the platelets used. A suspension of yolk platelets (20 A_{660nm} units) were incubated with *Artemia* extracts in a final volume of 0.5 ml of 50 mM Tris-HCl, pH 7.5 ; 20 mM $MgCl_2$; 40 mM sucrose, at 37 °C, and with vigorous agitation. At 5 min intervals, 25 μl aliquots were withdrawn and the decrease in turbidity followed at 660 nm. The reaction can also be followed counting the number of the remaining platelets or measuring the peptides liberated to the supernatant after the addition of 0.5 ml of 5 % trichloroacetic acid to the reaction mixture. In every case, controls without addition of enzyme were carried out (Olalla *et al.*, 1977).

Results and discussion

Chromatographic characterization of four proteases in *Artemia* larval extracts

When a 150 000 × g supernatant from larval extracts is applied to a DEAE-cellulose column and eluted with a lineal gradient of KCl, three peaks of proteolytic activities appear using casein as substrate. The peaks eluted at KCl concentration of around 0.4, 0.6, and 0.8 M, respectively. Two different enzymatic activities, A and B, can be uncovered under the first peak using CLNE and BAPNA as substrates, respectively. The activities under peaks II and III were termed proteases D and C, respectively. Protease C, but not D, was also active on BAPNA and TAME. In some chromatographic runs, peak II (protease D) appears as a shoulder of peak I. The chromatographic position of protease D can be easily localized with the help of the inhibitor STI and using (azo)casein as substrates. STI inhibits protease B, but not D (Table I).

TABLE I

Properties of the alkaline proteases induced during development of *Artemia* cysts

	Proteases			
	A	B	C	D
Substrates :				
CLNE	+	–	–	–
BAPNA	–	+	+	–
TAME	–	+	+	–
Casein	–	+	+	+
Azocasein	–	+	+	+
Inhibitors :				
STI	+	+	+	–
OTI	+	+	+	–
PMSF	+	–	–	–
TLCK	–	+	+	–
Heating for 5 min (half inactivation), °C	60	52	45	65
Elution from DEAE (KCl)	0.43 M	0.47 M	0.85 M	0.60 M
Molecular weight ($\times 10^3$)	38	33	34	36

Data taken from Osuna *et al.* (1977b), Olalla *et al.* (1978) and unpublished results from this laboratory.

The four proteases were purified following conventional methods and using as substrates : CLNE (for protease A), BAPNA (for proteases B and C) and casein (for protease D). Experimental details for the purification of proteases A, B and C have been presented (Olalla *et al.*, 1978) and those corresponding to protease D will be published elsewhere.

PROPERTIES OF PROTEASES A, B, C, AND D

The four proteases have maximal activities in the alkaline pH range and do not require the addition of metal to the reaction mixture for full activity. The following molecules were tested as substrates of the four proteases : CLNE, BAPNA, TAME, casein and azocasein. Those of them found to be substrates for each protease are consignated in Table I. It can be noted that, in our experimental conditions, protease D did not hydrolyze any of the synthetic substrates of proteases tested. The resistance to heating and the effect of several inhibitors are also presented in Table I. PMSF and STI are particularly strong inhibitors of protease A. This protease belongs probably to the chymotrypsinlike serine proteases as hydrolyzes the leucine ester bond of CLNE and is inhibited by PMSF. Although protease B is much less inhibited by PMSF than protease A, it is strongly inhibited by TLCK, a reagent of the histidyl residue present in the active site of certain proteases (Shaw *et al.*, 1965). As the arrangement Asp-His-Ser seems to be present in the active site of serine proteases, enzyme B could be included also among those belonging to this type. It is also a trypsin-like enzyme as hydrolyzes the amide group of BAPNA, in which the carboxyl group is apported by arginine.

The estimated molecular weights for proteases, A, B, C, and D were 38 000, 33 000, 34 000 and 36 000, respectively, as determined by, Sephadex G-75 column chromatography. The

possibility that the different proteases here described arise from a single protease by auto-digestion was investigated through the determination of the molecular weight of the proteases at different stages of purification (Olalla *et al.*, 1978). In all cases the values obtained for each protease were essentially equal to the molecular weights described here.

Possible physiological role of proteases A, B, C, and D on yolk platelets metabolism

As stated in the introduction, the study of proteases in *Artemia* was undertaken in order to get some insight into the mechanism of yolk platelets degradation during *Artemia* development. In parallel with the characterization of proteases, some methods were developed to measure the possible occurrence, in *Artemia* extracts, of a lytic activity on yolk platelets (see above, and Olalla *et al.*, 1977). With these methods this activity was detected and could be partially purified from *Artemia* larval extracts. Its chromatographic behavior was essentially alike to that of the proteases above described. As an example, the activity on yolk platelets and on casein present in the fractions collected after chromatography of an *Artemia* larval extract on a DEAE cellulose column is shown in Fig. 1. It can be easily appreciated that the lytic activities on yolk platelets are coincident with the three peaks of proteases obtained using casein as substrate (Fig. 1). Fractions corresponding to the three peaks were pooled and the relative activity of the four proteases on yolk platelets determined. PMSF (Table I) was utilized to discriminate between protease A and B, both present in the pooled fractions corresponding to the first peak. The relative activity on yolk platelets of the proteases A, B, C, and D, present in extracts of larvae obtained after 60 hr of incubation of the cysts, were 6, 56, 24, and 14%, respectively.

Although these proteases are able to degrade yolk platelets *in vitro*, we are still not certain on their physiological role *in vivo*. In relation to that the following comments could be made.

The rate of degradation of yolk platelets *in vivo* has been measured counting the number of platelets at different times after hydratation of the cysts. After 0, 1, 2, 3, 4, and 5 days of development, the total number of platelets, referred to 10^5 embryos/larvae was : 773, 361, 153, 38, 12, and 8×10^7, respectively (Olalla *et al.*, 1977). During the first 24 hr of development, the rate of disappearance of these particles is around $[(773 - 361) : (24 \times 60)] \times 10^7 = 28.6 \times 10^5$ yolk platelets lysed per min per 10^5 embryos, around 7×10^6/g of original cysts.

The lytic activity on yolk platelets measured in *Artemia* cysts extracts remains very low in the first 24 hr and increases steadily until 80-90 hr of development (Fig. 2), a very similar pattern of appearance to that of the proteases (Osuna *et al.*, 1977b). Actually we think that both activities, detected with help of different substrates, correspond to the same enzyme(s). The discrepancy between the timing of appearance of the proteolytic activities and the rate of yolk platelets degradation during development (*i.e.* half of the platelets content of the cysts has been metabolized before a significant increase in the proteolytic activities takes place) poses a question on the actual role of these proteases on the metabolism of yolk platelets *in vivo*. In this regard, several alternatives could be considered : the proteases present in the cysts are different to those present in the larvae ; they are of intestinal origin and digestive function ; they are actually implied in platelets degradation. Although further work is needed to clarify these points, it is worthy to note that, in the last case, the very low proteolytic activity detected in the cysts would be enough to account for the observed rate of degradation of yolk platelets

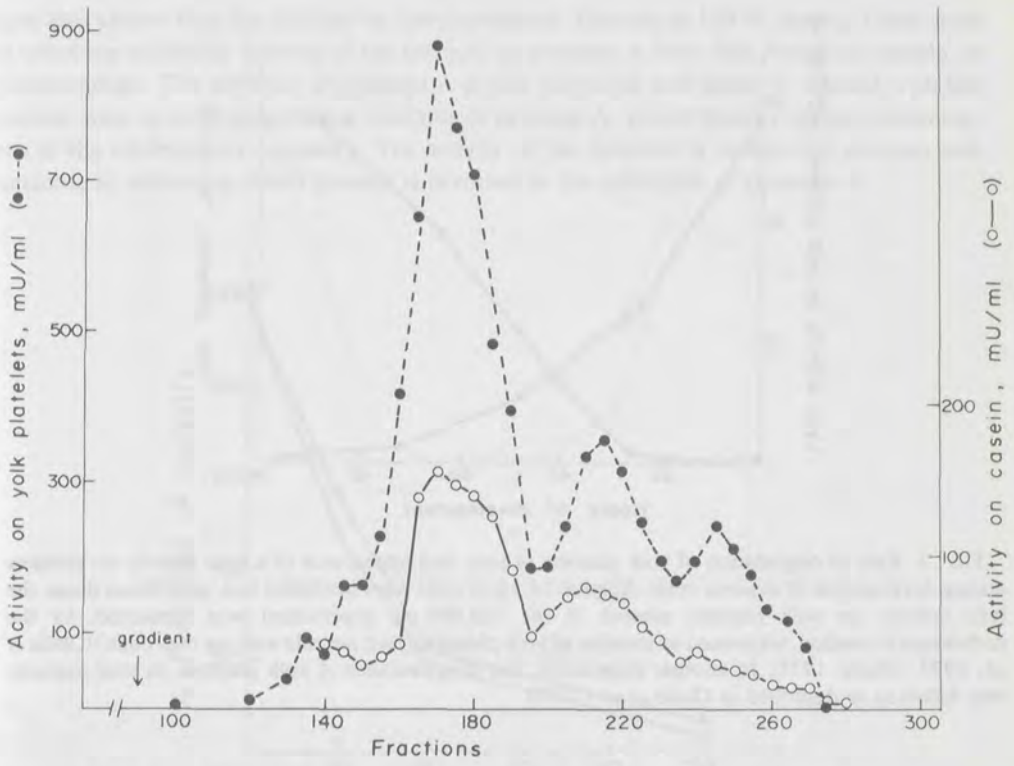

FIG. 1. Chromatography on DEAE-cellulose of proteolytic activities present in *Artemia* larvae. A Sephadex G-100 pooled fractions (50 mg protein) obtained from larvae of 60 hr (Olalla *et al.*, 1978) were applied to a DEAE-cellulose column (1 × 20 cm). The proteolytic activities were eluted with 200 ml of a lineal gradient of KCl (0.2-1 M) in 20 mM Tris-HCl, pH 7.5. Fractions of 1 ml were collected. The lytic activity on platelets was measured with the turbidimetric method, and the activity on casein, ás previously described (Olalla *et al.*, 1977 ; Olalla, 1978).

in vivo, according to the following calculations. The lytic activity detected in 26 hr larval extracts is enough to lyse 90×10^6 yolk platelets per min per gram of cysts. This figure is ten times higher than the theoretically needed to account for the rate of yolk platelets degradation in the first 24 hr (see above). If just this activity (enough to lyse 7×10^6 platelets/min/g) were present in the cysts it would be undetectable with our method to measure lysis of yolk platelets *in vitro*. In the same context, the hydrolytic activity on BAPNA, measured in the cysts, is around one order of magnitude lower than that present after 26 hr of incubation.

PROTEASES IN *ARTEMIA* ADULTS

Another approach that can give some information on the physiological role of larval proteases is the investigation of these enzymes at different developmental stages of *Artemia*. Therefore we have studied the proteolytic activities present in adults of two *Artemia*

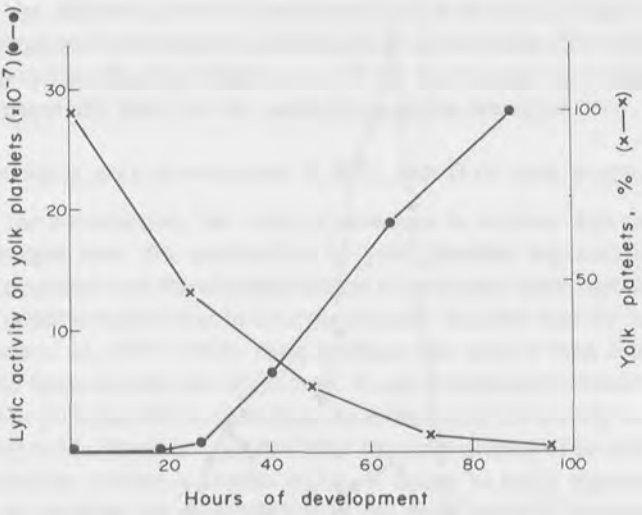

FIG. 2. Rate of degradation of yolk platelets *in vivo*, and appearance of a lytic activity on platelets during development of *Artemia* cysts. Aliquots of 5 g of cysts were incubated and, at different times, the lytic activity on yolk platelets present in the 105 000 × g supernatant was measured, by the turbidimetric method. (expressed as number of yolk platelets lysed per min and mg of protein) (Olalla *et al.*, 1977 ; Olalla, 1978). In another experiment, the disappearance of yolk platelets, in total extracts, was followed as described in Olalla *et al.* (1977).

populations obtained from salterns in Sigüenza (Guadalajara, Spain) and in San Fernando (Cádiz, Spain). The first one is a parthenogenetic population and the second one is bisexual.

Extracts prepared with *Artemia* adults from Sigüenza have proteolytic activities on CLNE, BAPNA and casein. DEAE-cellulose chromatography of the extracts shows a pattern of elution of proteolytic activities comparable to that obtained with nauplii from San Francisco (California) (Osuna *et al.*, 1977b). There is a peak of activity on CLNE, which corresponds to the position of elution of protease A from nauplii. There are two peaks of activities on BAPNA, one corresponding to protease B and the second eluting at slightly lower KCl concentration than protease C from larval extracts. The activity on casein is resolved in a peak inhibited by soybean trypsin inhibitor and a second broader peak insensitive to this inhibitor. The STI-sensitive peak coincides with the peak of activity on BAPNA of protease B. 25% of the activity on BAPNA of the extracts corresponds to enzyme B, and the remaining 75% to protease C. About 30% of the activity on casein of the extracts belongs to protease B and 70% to the STI-resistant protease D.

Extracts prepared with *Artemia* adults from San Fernando have low activities on CLNE and BAPNA in contrast to the results obtained with the population from Sigüenza. Table II shows the activities on CLNE, BAPNA and casein of both populations. Several hypotheses can be postulated to explain these results, including the existence of protease inhibitors in the populations from San Fernando. To test this hypothesis we have studied the effect of addition of extracts from San Fernando adults to partially purified proteases from nauplii. Fig. 3 shows the inhibition of protease A by different amount of extracts from San Fernando adults. The

figure also shows that the inhibitor is thermoresistant. Heating at 100 °C during 5 min does not affect the inhibitory activity of the extracts on protease A from San Francisco nauplii or Sigüenza adults. The inhibitor of protease A is non dialyzable and seems to interact with the protease, since to inhibit increasing amounts of protease A, proportionally higher concentrations of the inhibitor are necessary. The activity of the inhibitor is sensitive to pronase and proteinase K, indicating that a protein is involved in the inhibition of protease A.

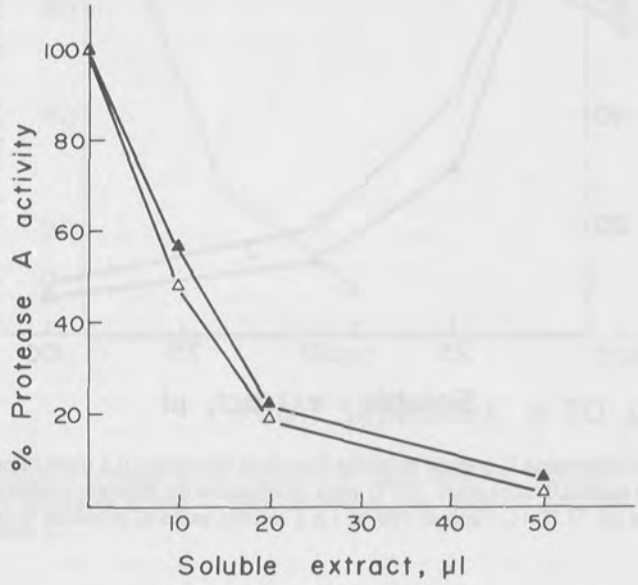

FIG. 3. Inhibitor of protease A present in adults from San Fernando. 2.5 units of protease A obtained from San Francisco nauplii (Osuna *et al.*, 1977) were incubated with different amounts of unheated (▲) and heated (△) (100 °C for 5 min) soluble extracts from adults from San Fernando.

TABLE II

Levels of proteolytic activities in *Artemia* adults

Population	Proteolytic substrate			
			Casein	
	CLNE	BAPNA	STI resistant	STI sensitive
Sigüenza	170	23	6.5	3
San Fernando	10	2	5.0	1

The date are given in units/g of animals (wet weight). Enzymatic units are as referred to in Osuna *et al.* (1977b).

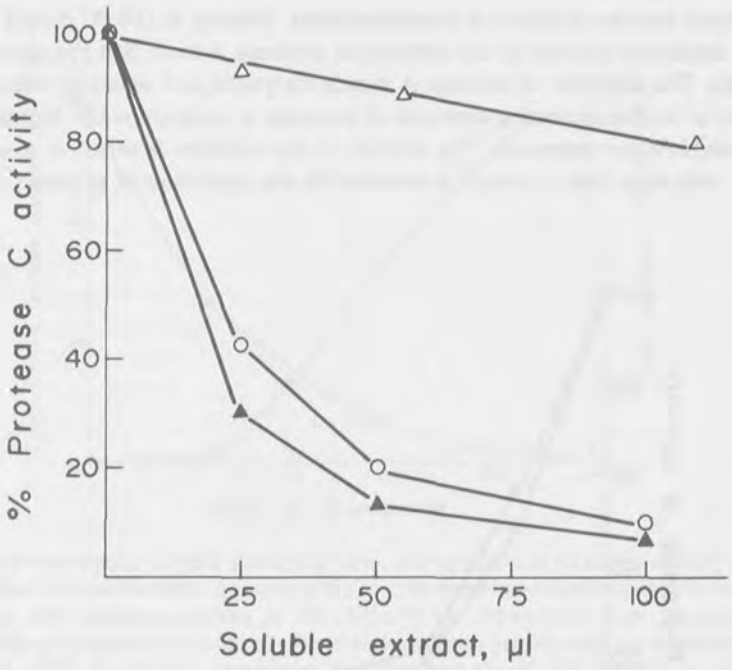

FIG. 4. Inhibitor of protease C present in adults from San Fernando. 0.5 units of protease C obtained from San Francisco nauplii (Osuna *et al.*, 1977) were incubated with different amounts of unheated (▲) or heated for 5 min [at 75 °C (○) and at 100 °C (△)] soluble extracts prepared with adults from San Fernando.

Fig. 4 shows the inhibition of protease C by extracts from San Fernando adults. The inhibitor is resistant to heating at 75 °C for 5 min but is sensitive to 100 °C. This inhibitor is non dialyzable and is found with the excluded volume of a Sephadex G-50 column. Preparations of this inhibitor, partially purified by heating the extracts at 75 °C for 5 min and by Sephadex G-50 gel filtration, also inhibits protease B, assayed with BAPNA or casein. Fig. 5 shows the inhibition of protease B and C by partially purified inhibitor of protease C. Whether there is one inhibitor active on both proteases or there are two different inhibitors specific for proteases B and C. with some common properties, remain to be elucidated.

The presence of inhibitors for proteases A, B, and C in San Fernando adults can explain the low levels of these proteases measured in the extracts. In relation to that we are trying to separate the proteases from the inhibitors using different fractionation methods and disassociating agents. It is difficult to explain the differences found between two populations of *Artemia* with respect to the presence of protease inhibitors. The existence of inhibitors of proteolytic enzymes is a biological control mechanism to prevent the deleterious activities on the own organism and it is commonly found in nature (Reich *et al.*, 1975). From other point of view, the biochemical differences found between the two *Artemia* populations raise the problem of the standardization of the biological materials for biochemical and physiological studies in order to make comparable the results obtained in different laboratories.

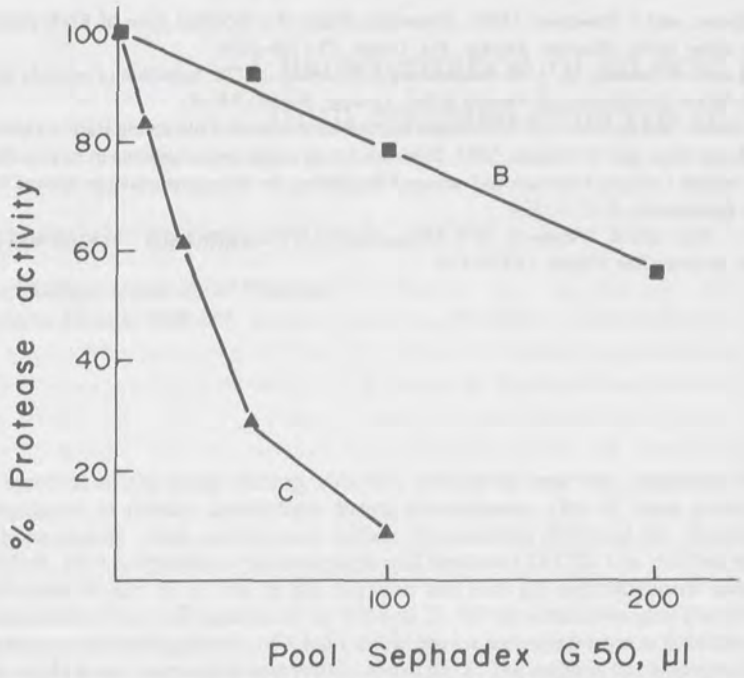

FIG. 5. Inhibitor(s) of protease B and C present in adults from San Fernando. 0.5 units (determined with BAPNA) of proteases B and C were incubated with different amounts of partially purified inhibitor of protease C.

Acknowledgements

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Characterization of an acid protease from encysted embryos of *Artemia*

A. H. Warner and V. Shridhar

Department of Biology, University of Windsor
Windsor, Ontario, Canada N9B 3P4

Abstract

Encysted embryos of the brine shrimp, *Artemia*, contain at least two proteases which may be important regulators of protein metabolism during development. One of these proteases has been purified to homogeneity using ammonium sulfate fractionation followed by chromatography on phosphocellulose, DEAE-Sphedex, hydroxyapatite and Sephadex G-150. The purified enzyme has an apparent molecular weight of 53 000 by gel filtration and SDS-gel electrophoresis indicates that the enzyme is composed of two polypeptides of 23 500 and 27 100 molecular weight. The purified enzyme is an acid protease with a pH optimum of 3.6-3.8 using bovine serum albumin as substrate. The enzyme is most stable at pH 6 and inactivated irreversibly above pH 8. The enzyme has a temperature optimum of 45 °C, but it is completely inactivated at 60 °C in the presence or absence of substrate. The acid protease is unaffected by phenylmethyl sulfonylfluoride, pepstatin, N-ethylmaleimide and chloromercuribenzoate ; however, the enzyme is strongly inhibited by Cu^{2+}, Hg^{2+}, iodoacetate and leupeptin. Neither Ca^{2+}, Mg^{2+} nor Co^{2+} has any significant effect on enzyme activity. Preliminary evidence indicates that the cyst acid protease is a cytosol enzyme with properties similar to cathepsin B type proteases.

Introduction

During the past few years we have been studying the possibility that proteases and protease inhibitors in *Artemia* embryos function in the regulation of developmental events that require proteolytic initiation. In our studies we have found that *Artemia* cysts contain significant quantities of at least two proteases which we believe to be important enzymes in protein metabolism during early development. Recently we presented evidence that one of these proteases has a pH optimum in the acid range (Nagainis and Warner, 1979), and we suggested that this enzyme may be responsible for the utilization of yolk during development. A second protease has a pH optimum near neutrality and it appears to have a high degree of specificity for the protein synthesis elongation factor, EF-2 (Yablonka-Reuveni and Warner, 1979). Both proteases are inhibited by an endogenous inhibitor(s) which renders the neutral protease completely inactive in crude homogenates. At this time it is not known whether the neutral protease observed by us in *Artemia* cysts preparations is the same as one of the proteases observed by Osuna *et al.* (1977).

In this paper we describe a procedure for the purification of the cyst acid protease to homogeneity and present evidence that this enzyme is a cathepsin B type protease found predominantly in the cytosol fraction of *Artemia* cysts.

Materials and methods

The *Artemia* cysts used for the enzyme purification studies were from the Utah salterns and they were obtained from Longlife Aquarium Products, St. Thomas, Ontario in 1970 and stored at -15 °C under vacuum. About 50 % of the cysts gave rise to nauplii after 24 hr incubation at 30 °C. The cysts were hydrated and washed as described by Warner *et al.* (1979) and ground in 25 g batches to a thick paste with a small volume of Buffer A (50 mM Tris-HCl, pH 7.3, 5 mM KCl, 1 mM DTT and 10 mM $MgCl_2$) using a Torsion grinder. The cyst paste was stirred with three and one-half volumes of additional Buffer A and the homogenate was centrifuged at 10 000 × g for 45 min. The supernatant fluid was collected avoiding the floating lipid layer and centrifuged at 150 000 × g for $2^1/_2$ hr. The resulting supernatant fluid (S-150 fraction) contained about 60 % of the total cyst acid protease activity and this fraction was used as the starting material in the purification scheme.

The assay method for the determination of protease activity during the purification procedure was similar to that reported previously with slight modification (Nagainis and Warner, 1979). Except where noted otherwise the incubation vessels contained the following in 0.2 ml final volume : 4 mg/ml N,N-dimethylated bovine serum albumin, 0.1 M acetate buffer, pH 3.8, 0.12 mM EDTA and 0.025 to 0.10 ml of the various column fractions. The reaction vessels were incubated at 40 °C and at 0 and 30 min 0.05 ml was removed from each vessel and assayed for the liberation of amino groups using trinitrobenzenesulfonic acid (TNBS) as described previously (Nagainis and Warner, 1979). One unit of protease activity is defined as that quantity of enzyme which gives rise to a ΔA_{420} of 0.01 in 1 min. Protein was measured by the method of Lowry *et al.* (1951) following delipidation according to Bligh and Dyer (1959). The SDS-polyacrylamide gel analyses were carried out on 10 % gels according to the method of Laemmli (1970).

The various subcellular fractions of *Artemia* cysts were prepared as described by Warner *et al.* (1972) using three different homogenization media. The composition of these media is described under Results. Prior to the assay for acid protease activity, each cyst fraction was equilibrated with a buffer containing 15 mM potassium phosphate, pH 6.8, and 25 mM KCl. In all cases the ratio of homogenization media to fully hydrated cysts was 10 to 1 (v/w), and each sediment was washed once with the homogenization buffer.

The N,N-dimethyl BSA was prepared according to Lin *et al.* (1969) and the yolk proteins according to Warner *et al.* (1972). Bovine hemoglobin, TNBS, phenylmethyl sulfonylfluoride (PMSF), ovomucoid and soybean trypsin inhibitor were from Sigma. Pepstatin and leupeptin were generous gifts from W. Troll (New York University) and the U.S.-Japan Cooperative Cancer Research Program. The Sephadex media was obtained from Pharmacia, phosphocellulose was from Whatman and hydroxyapatite was from Clarkson Chemical Co.

Results

Purification of *Artemia* cyst acid protease

In these experiments the postribosomal fraction (S-150 fraction), obtained as a by-product of our studies on the characterization of the ribosome-associated protein synthesis inhibitor (Warner *et al.*, 1977), was used as the source of soluble acid protease. The S-150 fraction was

adjusted to 30 A_{260}/ml by the addition of Buffer A, then solid $(NH_4)_2SO_4$ was added to a final concentration of 28 %. The preparation was stirred for 30 min at 4 °C then the precipitate was removed by centrifugation and discarded. The soluble fraction was adjusted to contain 40 % ammonium sulfate and treated as above for the 0-28 % fraction. The S-150 fraction which was soluble in 40 % ammonium sulfate was adjusted to contain 55 % ammonium sulfate and the resulting precipitate (40-55 %) was collected by centrifugation and stored at -20 °C. The 40-55 % ammonium sulfate fraction prepared in this way contains the bulk of the cytosol acid protease activity, and it is relatively free of the non-dialyzable acid protease inhibitor observed previously (Nagainis and Warner, 1979).

The 40-55 % ammonium sulfate fraction was suspended in a minimum volume of 25 mM KCl containing 15 mM potassium phosphate, pH 6.8, and desalted on a column of Sephadex G-25 (2.2 × 18 cm) using the same buffer. The protein-rich fraction from Sephadex G-25 was applied to a column of phosphocellulose (2 × 34 cm) equilibrated with the same KCl-phosphate buffer as above and the column was washed with the buffer until the effluent gave an absorbance of less than 0.1 unit at 280 nm. Under these conditions the acid protease activity washes through the phosphocellulose column immediately behind the bulk flow-through protein and the enzyme preparation is relatively free of orange pigment.

The phosphocellulose column fractions containing the acid protease activity were pooled, adjusted to pH 7 with N NaOH then applied to a column of DEAE-Sephadex (1.5 × 28 cm) equilibrated with a buffer containing 25 mM KCl and 15 mM potassium phosphate, pH 7.0. The protein was washed onto the column with 150 ml of the starting buffer and the bound proteins were eluted with a 500-ml linear gradient of KCl (25 mM to 750 mM) buffered with 15 mM potassium phosphate, pH 7.0. Using this procedure about 85 % of the soluble acid protease elutes as a symmetrical peak between 0.26 and 0.33 M KCl.

The contents of the DEAE-Sephadex column fractions which eluted between 0.26-0.33 M KCl were pooled and applied directly to a column of hydroxyapatite (1 × 23 cm) equilibrated with the same starting buffer used previously for the DEAE-Sephadex step. The column was washed with 50 ml of the starting buffer then developed with a 500-ml linear gradient of potassium phosphate, pH 7.0 (15 to 500 mM). The acid protease activity eluted from the column with the second UV-absorbing peak and the protein which eluted between 70 to 140 ml was pooled and concentrated to less than 1.5 ml by vacuum dialysis.

The concentrated acid protease from the hydroxyapatite step was applied to a column of Sephadex G-150 (1.5 × 86 cm, super fine) equilibrated with 25 mM KCl buffered with 15 mM potassium phosphate, pH 7.0, and eluted with the same buffer. The acid protease activity eluted as a symmetrical peak with a K_{av} identical to the third UV-absorbing peak to elute from the column. The column fractions containing the upper one-half of the activity peak were pooled, concentrated by vacuum dialysis and stored at -20 °C. The data shown in Table I summarize the purification procedure from two complete experiments using 50 g dry cysts as starting material for each experiment. This procedure results in a homogeneous protein representing about 1.4 % of the S-150 proteins.

Properties of the cyst acid protease

The apparent molecular weight of the pure cyst acid protease is about 53 000 as determined by chromatography on Sephadex G-150, and the protein is composed of two subunits.

Analysis of the subunits on a 10 % polyacrylamide gel slab in the presence of 0.1 % SDS gave values of 23 500 and 27 100 as the molecular weights of the subunits, the sum of which is in reasonable agreement with the molecular weight of the native enzyme (53 000). These data are shown in Fig. 1.

TABLE I

Summary of purification steps for cysts acid protease[1]

Step	Total protein (mg)	Specific activity (EU/mg)	Total enzyme units	Yield (%)
S-150	2 869	4.0	11 476	100
40-55 % $(NH_4)_2SO_4$	1 406	7.4	10 334	90
Phosphocellulose	4 80	16.2	7 785	68
DEAE-Sephadex	96.4	70.1	6 758	59
Hydroxyapatite	27.6	194	5 354	47
Sephadex G-150	7.1	289	2 052	18

[1] The values are based on 100 g dry cysts as starting material.

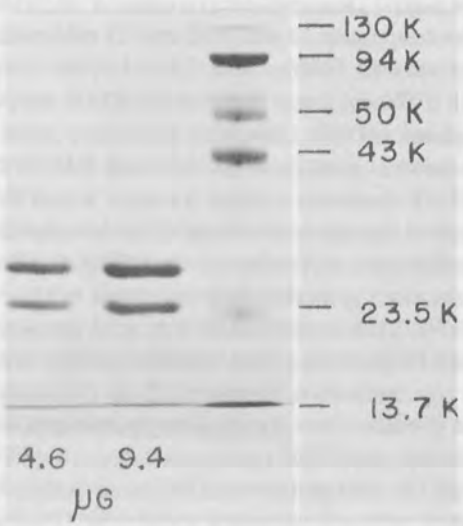

FIG. 1. Analysis of cyst acid protease by SDS-polyacrylamide gel electrophoresis. The Sephadex G-150 purified enzyme shown in Table I was subjected to electrophoresis on a 10 % polyacrylamide slab containing 0.1 % SDS as described by Laemmli (1970). Standard molecular weight markers are shown at the right and the amount of acid protease added to each gel slot is shown at the bottom of the figure.

Using either BSA or dimethyl BSA as substrate the pH optimum of the reaction is 3.6-3.8 for the pure enzyme. Moreover, the pure enzyme is catalytically inactive below pH 2 and above 6 and it undergoes autodegradation when stored in an ice bath in buffer alone at pH 3-5. The enzyme is inactivated when stored in ice at pH 8 or above and the inactivation at pH 8

is complete after 20 min at 40 °C. The pure acid protease is most stable when stored at pH 6.0-6.5 but under these conditions, and in the absence of substrate, it is inactivated completely after 20 min at 60 °C.

The effect of metals and various reagents on acid protease activity

In an attempt to characterize the type of acid protease in *Artemia* cysts, we tested the effect of several metal ions on the activity of the enzyme. The results of these experiments are shown in Table II. Although most divalent metal ions had very little effect on the acid protease activity at 5-10 mM concentrations in the incubation vessels, Cu^{2+} and Hg^{2+} were very inhibitory. In fact, Cu^{2+} and Hg^{2+} at 10^{-5} M produced 82 % and 30 % inhibition, respectively. The fact that Fe^{2+} is slightly stimulatory and Fe^{3+} inhibitory is curious but the reasons for these observations are unknown (see below). Collectively these data indicate that the cyst acid protease does not require exogenous metal ions for optimal activity and that the active site probably contains a carboxyl- or SH-group.

Table II

Effect of various metals on *Artemia* cysts acid protease

Metal	Concentration	% of control[1]
None	—	100
$MgCl_2$	10	93
$MnCl_2$	10	91
$CaCl_2$	10	107
$FeCl_2$	5	117
$FeCl_3$	10	39
$CdCl_2$	10	81
$CoCl_2$	10	100
$ZnCl_2$	10	69
$CuCl_2$	0.1	5
	0.01	18
$HgCl_2$	1.0	10
	0.01	70

[1] $MnCl_2$, $CoCl_2$ and $CuCl_2$ interfere with the TNBS reaction; thus their effect was corrected to controls containing alanine as a standard. All assays were conducted in the absence of EDTA using purified enzyme.

In another series of experiments we treated the purified acid protease with enzyme or protease reagents of known function. The results of these treatments are shown in Table III. Treatment of the acid protease with the serine protease inhibitor phenylmethyl sulfonylfluoride (PMSF) at neutral pH had no effect on the enzyme activity, whereas treatment of the enzyme with the alkylating reagent iodoacetate at pH 4 or 7 strongly inhibited the enzyme activity. Treatment of the enzyme with the SH-group blocking reagents p-chloromercuribenzoate (pCMB) and N-ethylmaleimide (NEM) were without significant effect on the

enzyme activity and the SH-reagent dithiothreitol (DTT) had no effect on the enzyme activity. Although these results suggest that the enzyme is not a thiol protease, the inhibitory effects of Cu^{2+}, Hg^{2+} and iodoacetate suggest that the cyst acid protease may be a thiol containing enzyme whose active site is conformationally restricted.

TABLE III

Effect of various treatments on *Artemia* cysts acid protease

Treatment	Concentration[1]	% of control
None	—	100
PMSF	0.13mM	100
Iodoacetate	1.0 mM	13
pCMB	1.0 mM	88
NEM	10.0 mM	96
Pepstatin	10.0 μg/ml	101
Leupeptin	1.0 μg/ml	2
Ovomucoid	1.0 mg/ml	97
Soybean trypsin inhibitor	1.0 mg/ml	98
DTT	10.0 mM	94
EDTA	1.0 mM	177
1,10-phenanthroline	1.0 mM	157

[1] Except for the first four treatments, these values represent the concentrations in the reaction vessels. The values for the first four treatments represent the concentration of these reagents incubated with the enzyme alone for 20 min at 23 °C prior to the enzyme assay.

The enzyme is also unaffected by pepstatin, a cathepsin D inhibitor, and chicken ovomucoid and soybean trypsin inhibitor, but it is sensitive to leupeptin, a well known inhibitor of cathepsin B-type proteases. In these experiments leupeptin was found to have an ID_{50} of 0.19 ug/ml which is equivalent to 4×10^{-7} M, and this amount of inhibitor is nearly equivalent to the molar amount of enzyme in the reaction. Since the B-type cathepsin is a thiol protease, our findings using leupeptin and other enzyme reagents support the view that the cyst acid protease may be a thiol protease.

The results in Table III, using the chelators EDTA and 1,10-phenanthroline suggest that the acid protease is not a metallo-protein. In fact, both chelating agents stimulate the acid protease markedly probably by removing trace amounts of heavy metals such as Cu^{2+} and Hg^{2+} from the enzyme preparations.

HYDROLYSIS OF DIFFERENT PROTEINS AS SUBSTRATES

Since hemoglobin is often the choice protein substrate for acid protease measurements, we compared hemoglobin as substrate to other proteins under identical conditions. The results of

this experiment are shown in Fig. 2. Of the four proteins tested (hemoglobin, bovine serum albumin, dimethyled bovine serum albumin and soluble cyst yolk proteins) hemoglobin was hydrolyzed the most efficiently by the acid protease under our experimental conditions. However, since dimethyl-BSA gives the lowest background values of the four proteins tested as substrate in the TNBS assay, the results obtained with this substrate have been the most reproducible. Using dimethyl-BSA as substrate the enzyme has an apparent K_m of 0.06 % or about 8.9 uM assuming a molecular weight of 67 000 for the substrate.

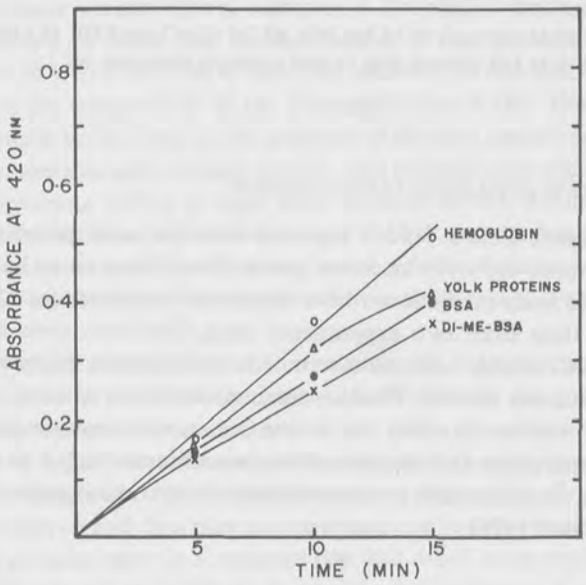

FIG. 2. Hydrolysis of different proteins by the purified acid protease. All protein substrates were tested at 4 mg/ml in a 0.4 ml reaction mixture containing 2.3 enzyme units and under conditions described in Material and methods.

DISTRIBUTION OF ACID PROTEASE IN *ARTEMIA* CYSTS

Acid proteases such as that described herein are usually considered to be lysosomal proteases but in *Artemia* cysts this does not appear to be the case. We have used three different homogenization media to prepare subcellular fractions and in all cases the bulk of the acid protease activity was found in the 15 000 × g supernatant or post-mitochondrial fraction. These results are shown in Table IV. In separate experiments we found only trace amounts of acid protease activity in crude ribosomal pellets and in yolk platelets purified using a previously established procedure (data not shown) (Warner *et al.*, 1972). Thus the bulk of the *Artemia* cyst acid protease appears to be in the cytosol fraction and not associated with lysosomes or other embryo organelles.

TABLE IV

Distribution of cyst acid protease among various embryo fractions

Cell fraction	Buffer A1[1]	Sucrose-Tris-EDTA[2]	Phosphate-KCl[3]
750 × g pellet	19.8	2.8	9.5
15 000 × g pellet	19.0	14.5	13.9
15 000 × g supernatant	61.2	82.7	76.6

[1] Buffer A1 is identical to buffer A described under "Methods and materials" except that it contains in addition 0.25 M sucrose.

[2] This buffer contains 0.25 M sucrose, 0.03 M Tris-HCl, pH 7.3 (22 °C) and 0.001 M EDTA.

[3] This buffer contains 0.10 M KCl buffered with 15 mM potassium phosphate, pH 7.0.

ACID PROTEASE LEVELS IN DEVELOPING *ARTEMIA* EMBRYOS

In an earlier report Bellini (1957) reported that the acid protease activity of H_2O homogenates of *Artemia* embryos increases nearly three fold prior to hatching. Using more refined isolation and assay methods we have found a different pattern of enzyme activity in *Artemia* embryos. Data from two experiments using California cysts show that the total amount of 0.1 M KCl soluble acid protease in *Artemia* embryos decreases by at least 50 % prior to hatching (data not shown). Whether these observations reflect the actual level of acid protease activity *in vivo* is unknown due to the presence of protease inhibitors in *Artemia* embryos, but the same pattern of enzyme activity was observed in 0.1 M KCl soluble extracts after removal of the non-dialyzable protease inhibitor by chromatography on DEAE-cellulose (Nagainis and Warner, 1979).

Discussion and conclusions

In a previous study from this laboratory the existence of an acid protease in *Artemia* embryos was confirmed (Nagainis and Warner, 1979). This observation extended an earlier study by Bellini (1957) and also demonstrated the presence of protease inhibitors which may be important in the regulation of protease activity during development.

In the study reported here we developed a five-step procedure for the purification of the cyst acid protease using conventional enzyme purification methods following several unsuccessful attempts by us to purify the enzyme by affinity chromatography on hemoglobin-agarose columns using the procedure of Smith and Turk (1974). In addition to the properties described above, it is noteworthy that the purified acid protease displays a light brown colour in solution at pH 7. This observation together with the fact that Fe^{2+} is slightly stimulatory whereas Fe^{3+} is inhibitory suggest that the enzyme contains Fe^{2+}. However, treatment of the enzyme with EDTA or 1,10-phenanthroline did not inhibit the enzyme activity. Thus, if the enzyme is an iron-containing protein the iron is either inaccessible to the chelators or not part of the binding or catalytic site of the molecule.

In an earlier communication we reported a molecular weight of 84 000 for the partially purified cyst acid protease (Nagainis and Warner, 1979). These results were obtained using

Ultrogel AcA34 which we have found to give higher molecular weight values than Sephadex (unpublished observations). In our view the results reported here for the molecular weight of the native acid protease (53 000) are more correct than previous data from this laboratory.

The results presented above are similar to those reported by Husain (1976) for calf liver cathepsin B2, but they appear to be different from the data of Ninjoor *et al.* (1974) who reported that rat liver cathepsin B2 is a carboxypeptidase of lysosomal origin. The pattern of substrate hydrolysis by the cyst acid protease as observed by SDS-polycrylamide gel electrophoresis suggests that the cyst protease is an endoprotease. Also, the bulk of the enzyme appears to be localized mainly in the cytosol and not in the lysosomes (see Table IV). Perhaps the *Artemia* acid protease is a new type of cathepsin B. Despite our findings on the localization of the enzyme it should be noted that the distribution of acid protease among the various subcellular fractions *in vivo* is difficult to ascertain because the distribution observed *in vitro* varies depending on the composition of the homogenization buffer. Homogenates prepared with low ionic strength buffers and in the presence of divalent cations such as Mg^{2+} (Buffer A1) have more sedimentable acid protease activity than homogenates prepared with low ionic strength buffers containing EDTA or high ionic strength buffers without EDTA. The true meaning of the cell fractionation experiments is still unclear, but the fact that at least 60 % of the total cyst acid protease activity is present in the soluble fraction of cyst homogenates prepared under different conditions supports the view that the cyst acid protease is primarily a cytosol protein.

The role of the cyst acid protease in protein metabolism is unknown at this time. However, in view of the fact that the acid protease appears to be a broad spectrum protease and the predominant active protease in the cyst (Osuna *et al.*, 1977 ; Nagainis and Warner, 1979), it appears reasonable to suggest that this enzyme is active in yolk utilization during prenaupliar development. It may also be involved in the activation or unmasking of other proteases in the cyst or developing embryo and thus play an important role in the initiation of developmental events. Clearly the protease story is a complex one and much more work will be needed in this area to elucidate the role of proteases in *Artemia* development.

Acknowledgement

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Regulation of histone acetylation in *Artemia*

A. Cano[1], *I. Estepa, and A. Pestaña*

Instituto de Enzimología y Patología Molecular del CSIC
Facultad de Medicina de la Universidad Autónoma
Arzobispo Moricillo s/n, Madrid-34, Spain

Abstract

Histone acetylation in *Artemia* has been studied as a tool to understand regulatory mechanisms in relationship to developmental processes. A critical reevaluation of previous studies on the acetylation of endogenous histones in nuclei, together with new data concerning the role of an endogenous inhibitor of the acetyltransferase, leads to the conclusion that developmental variation in endogenous acetylation is the result of changes in substrate availability, changes in enzyme specificity or both. Analytical and kinetic studies with the partially purified enzyme indicate that the specificity of the acetyltransferase is regulated by H4 histone, which is one of its substrates.

Introduction

Histone acetylation within the nucleosomes is currently viewed as one of the mechanisms involved in the regulation of the structure and function of chromatin (Yamamoto and Alberts, 1976). Little is known, however, about the enzymatic machinery involved in histone acetylation and the regulatory mechanisms involved in such an important process.

As a contribution to this promising field, we have been involved over the last 4 years in the study of the acetylation of histones in *Artemia*, using several *in vitro* approaches, such as the endogenous acetylation in nuclei, acetylation of histones by nuclear extracts (Cano and Pestaña, 1976) and the properties of the partially purified histone acetyltransferase (Cano and Pestaña, 1979). Some of the results relevant to the regulation of histone acetylation are critically examined in this communication.

Material and methods

Dormant gastrulae of *Artemia* (San Francisco Bay brand, from Metaframe, California USA) were rehydrated and grown as previously described (Cano and Pestaña, 1976). Embryos and early larvae were harvested at the indicated time (in hours) after the onset of development, washed in deionized water and kept frozen at -20 °C until used.

[1] Present adress : Department of Biology, Massachussets Institute of Technology, Cambridge, 02139 Massachussets, USA.

Homogenization and preparation of the nuclear fraction or soluble chromatin and incorporation of acetate in nuclei or histone acetyltransferase activity were assayed as described by Cano and Pestaña (1976) using (1-^{14}C) acetyl coenzyme A (58 mCi/mmol from Amersham) as acetyl donor. Histone acetyltransferase was purified from T40 nauplii up to the DEAE cellulose or hydroxylapatite steps (Cano and Pestaña, 1979) as indicated.

Histone extraction from *Artemia* nuclei and from acetyltransferase assay mixtures was carried out by procedures already described (Cano and Pestaña, 1976). Calf thymus histones were purified by conventional methods (Oliver *et al.*, 1972) and were shown to be more than 80% homogenous by electrophoresis. Electrophoretic analysis of histones was carried out by the Panyim and Chalkley method (Panyim and Chalkley, 1969) and the radioactivity in slices of electrophoretic gels was measured as described by Cano and Pestaña (1979). Endonuclease activity in nuclear extracts was ascertained by sucrose gradient analysis and quantified by the albumin clot assay (Smith, 1974). Protein concentration was estimated by the method of Lowry (Lowry *et al.*, 1951) while DNA in nuclear preparations was measured by the absorbance at 260 nm after perchloric acid hydrolisis (Cano and Pestaña, 1976).

Results and discussion

Endogenous acetylation in nuclei and chromatin

The *in vivo* approach for the study of histone acetylation during the early development of *Artemia* is hindered by the lack of permeability to radioactive precursors of the encysted gastrulae. In order to overcome this intrinsic difficulty, we developed an *in vitro* system for the acetylation of endogenous histones in nuclei and chromatin. In He La cells, it has been shown (Geraci *et al.*, 1974) that methylation of histones and DNA in nuclei corresponds with similar processes occurring *in vivo*.

In Fig. 1 summarizes the main characteristics of the endogenous acetylation in *Artemia* nuclei. The rate of the reaction was found to be linear within a wide range of nuclei concentration (Fig. 1A). Extraction of the acetylated nuclear preparation with 0.25 M HCl prior to trichloroacetic acid precipitation indicated that more than 80% of acetylated proteins are acid soluble (Fig. 1B). Thermal inactivation studies showed that the acetylation of nuclei is a thermolabile process that can be completely suppressed by preincubation for 10 min at 50 °C (Fig. 1D). The pH profile of endogenous acetylation had an optimum at pH 7.5 (Fig. 1E). Electrophoretic analysis of acid soluble proteins extracted from acetylated nuclei to T40 indicated that H3 and H4 histones account for most of the acetate incorporated (shaded bars in Fig. 3) under optimal assay conditions.

Marked changes in the endogenous acetylation in nuclei were observed during development (Fig. 2A) with a peak value at T18-20, and low incorporation at T0-T12 and T20-T40. This same pattern was obtained in studies carried out with soluble chromatin preparation (Fig. 2B), thus suggesting that both the acetyltransferase and endogenous acceptor are bound to DNA. These developmental changes in endogenous acetylation could in principle arise from varying levels of endogenous acceptor, changes in acetylating activity or through the action of an inhibitor which is detectable in nuclear extracts 24 hr after the onset of development (Cano and Pestaña, 1976).

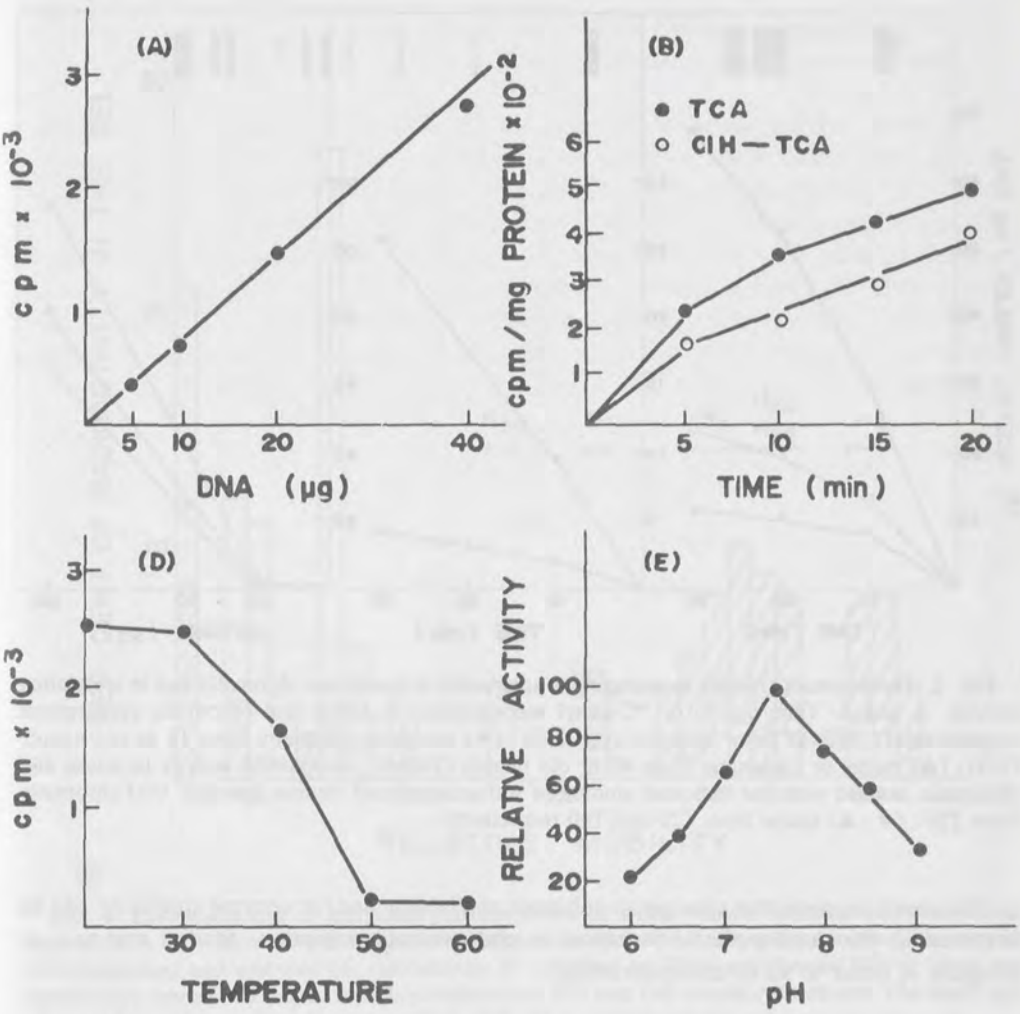

FIG. 1. Properties of acetylation in *Artemia* nuclei. A. Effect of nuclei concentration on ^{14}C-acetyl incorporation. B. Time course of ^{14}C-acetyl incorporation in (●) total ; (○) acid soluble proteins precipitable by TCA. D. Effect of temperature. E. pH dependence.

In order to explore these possibilities, we measured acetyltransferase activity in nuclei and chromatin with added calf thymus histones as substrate. As shown in Fig. 2C, no differences were found in acetylating activity, which was even higher at T40 than at T20 ; therefore we conclude that enzyme levels cannot account for the marked differences observed in endogenous acetylation.

Electrophoretic analysis of the reaction products of acetylated nuclei (Fig. 3), indicated that at T20, most of the acetate was found in non-histone proteins, while at T40, the acetate was preferentially bound to the four inner histones, H3, H4, H2A and H2B.

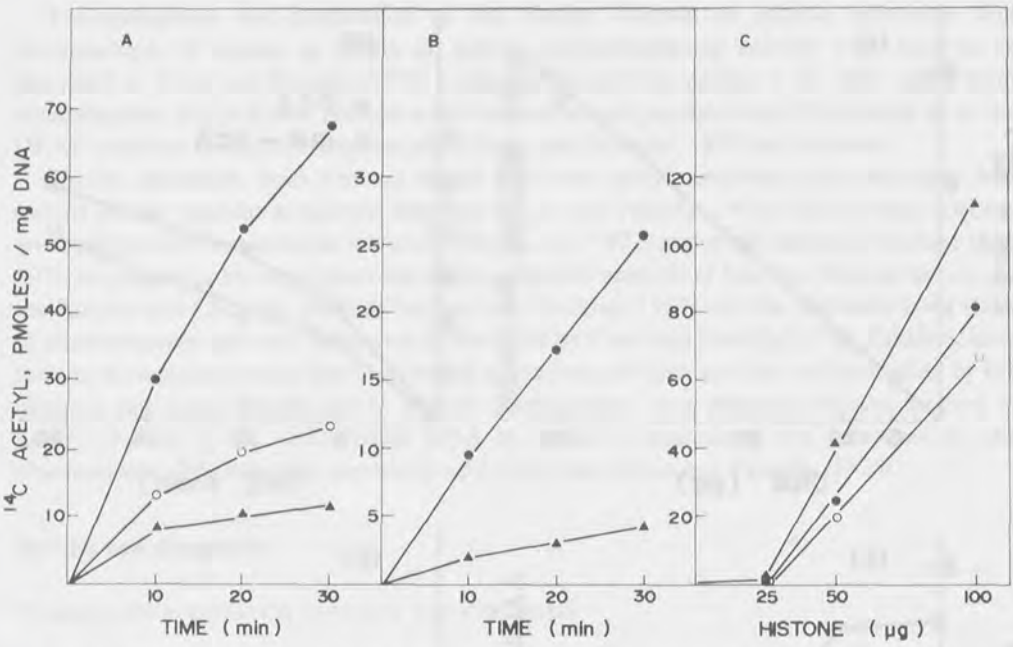

FIG. 2. Development changes in endogenous acetylation in nuclei and chromatin and in acetylating activity. A and B. Time course of ^{14}C-acetyl incorporation in nuclei and chromatin preparations respectively. (○) nuclei from hydrated cysts (TO) ; (●) nuclei or chromatin from 18 hr old nauplii (T18) ; (▲) nuclei or chromatin from 40 hr old nauplii (T40). C. Acetylating activity in nuclei and chromatin assayed with the indicated amount of unfractioned calf thymus histones. (○) chromatin from T20 ; (●, ▲) nuclei from T20 and T40 respectively.

This result suggests that changes in substrate availability and/or enzyme specificity can be involved in the developmental variation in endogenous acetylation. Studies are now in progress in order to solve this uncertainty.

CHARACTERIZATION OF THE ENDOGENOUS INHIBITOR OF HISTONE ACETYLTRANSFERASE

We have previously described an inhibitor of the acetylation of histones, which can be detected in nuclear extracts of *Artemia* nauplii from T24 onward. The inhibitor was characterized as a DNA fragment, which renders the histone inaccessible to the acetyltransferase through stoïchiometric interaction of the histone-DNA type (Cano and Pestaña, 1976). One critical question remaining to be answered was whether the presence of the inhibitor at defined time periods in the development of *Artemia* is an artifact arising from nuclease attack during the preparation of the nuclear extract, or whether it is endogenous as such to the undisrupted, living animal cells. Suspicions for an endonuclease involvement arose from studies in this laboratory (Cervera *et al.*, 1980) showing a Mg^{2+} dependent DNase activity in *Artemia* homogenates, which increased markedly between T12 to T24 after the onset of development and also from our thermal denaturation studies of the inhibitor, revealing a base composition identical to the bulk of the *Artemia* DNA.

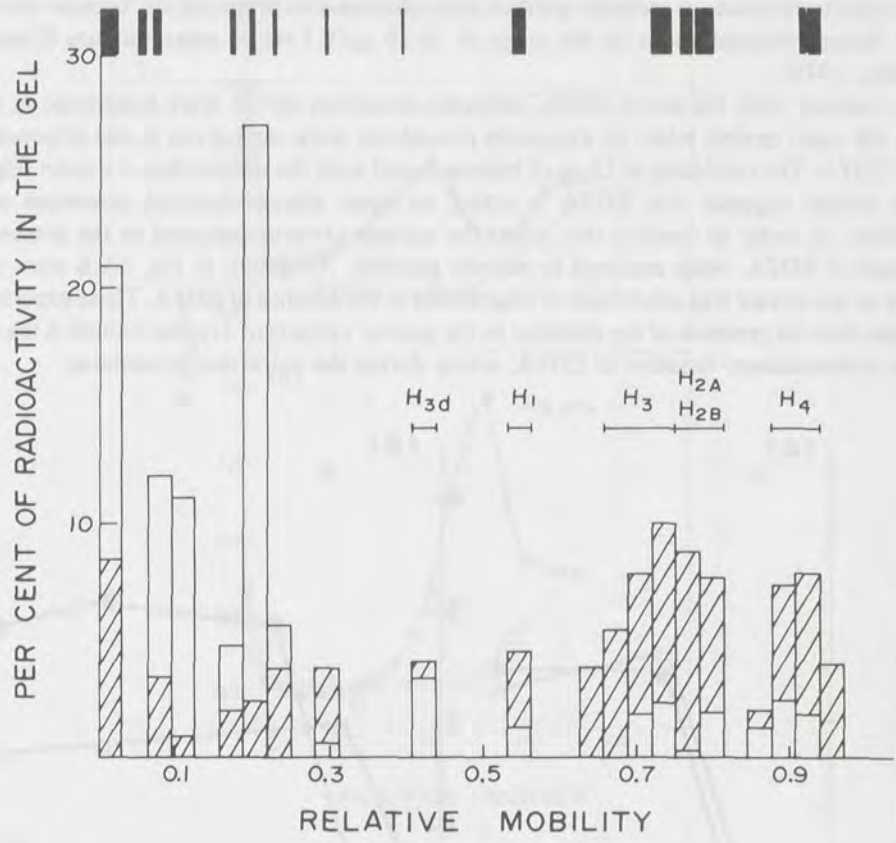

FIG. 3. Electrophoresis of acetylated proteins extracted from *Artemia* nuclei. Acetylated nuclei, derived from 1 g of animals (wet weight) were extracted with 0.4 M H_2SO_4 and the acid soluble material electrophoresed and analyzed for radioactivity as described by Cano and Pestaña (1979). Open and shaded bars correspond to acid soluble proteins from T20 and T40 nauplii respectively. The black bars on top of the figure are a schematic pattern of the main proteins in the gel.

The following experiments were undertaken to clarify the mechanism involved in the appearance of the inhibitor and its physiological role, if any.

The standard procedure for acetyltransferase isolation includes overnight dialysis to lower KCl concentration, followed by a centrifugation to clarify the extract. Histone saturation studies with these nuclear extracts from T24 onward, always gave sigmoidal curves of the type showed in Fig. 4A. We have previously shown (Cano and Pestaña, 1976) that this substrate saturation curve is the result of the interaction of increasing histone concentrations with a fixed amount of endogenous inhibitor. At low histone concentration (25 μg/0.5 ml of assay mixture), most of the histones are sequestered by the inhibitor, while at higher concentration of histones (50 μg), there is enough free substrate for the acetyltransferase to act.

The acetyltransferase, in partially purified and inhibitor-free preparations, became saturated with histone concentrations in the range of 10-20 μg/0.5 ml of assay mixture (Cano and Pestaña, 1979).

In contrast with the above results, substrate saturation curves were hyperbolic in shape (Fig. 4B, open circles) when all extraction procedures were carried out in the presence of 1 mM EDTA. The inhibition at 25 μg of histone found with the uncentrifuged extract (Fig. 4B, solid circles) suggests that EDTA is acting on some physicochemical properties of the inhibitor. In order to confirm this point, the nuclear extracts, prepared in the presence or absence of EDTA, were analyzed in sucrose gradient. As shown in Fig. 5A,B most of the DNA in the extract was solubilized or fragmented in the absence of EDTA. These experiments suggest that the presence of the inhibitor in the nuclear extracts of *Artemia* nauplii is the result of an endonuclease, sensitive to EDTA, acting during the extraction procedures.

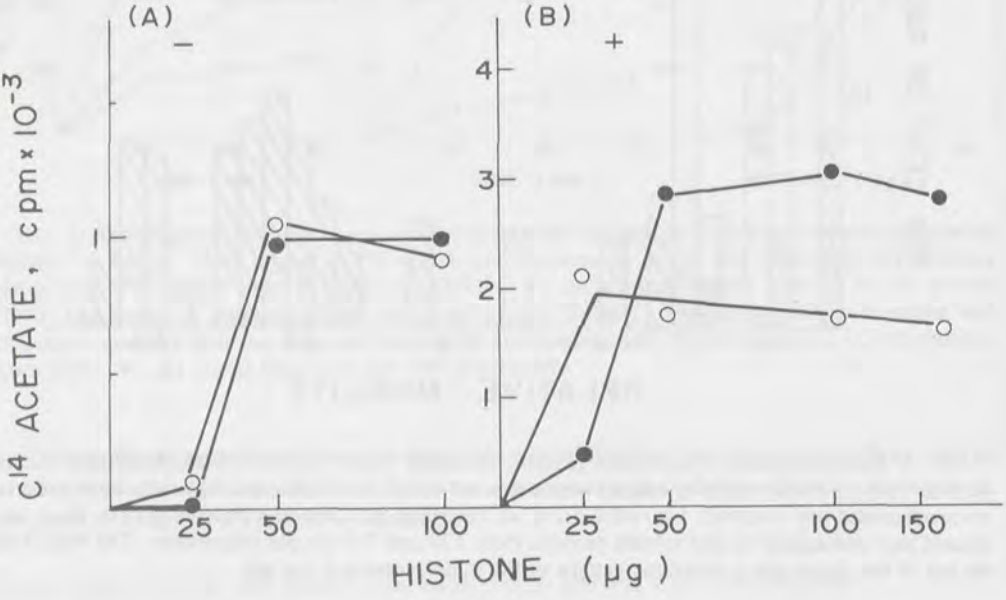

FIG. 4 Substrate saturation curves with nuclear extracts. Nuclear extracts from T24 nauplii were prepared in the absence (A) or presence (B) of 1 mM EDTA. Substrate saturation was studied with 100 μl aliquots of the extracts and the indicated amounts of unfractioned calf thymus histones. (●) Dialized nuclear extracts before centrifugation ; (○) dialized nuclear extracts after centrifugation.

This later inference was further explored in a serial study of the developmental changes in acetyltransferase inhibitor, soluble DNA and endonuclease activity. The nuclear extracts from *Artemia* embryos and nauplii were prepared in the absence of EDTA as described, including overnight dialysis and centrifugation. The inhibitor was titrated by the ratio of activities obtained with 25 and 50 μg of histone substrates ; the rationale for such an approach has been

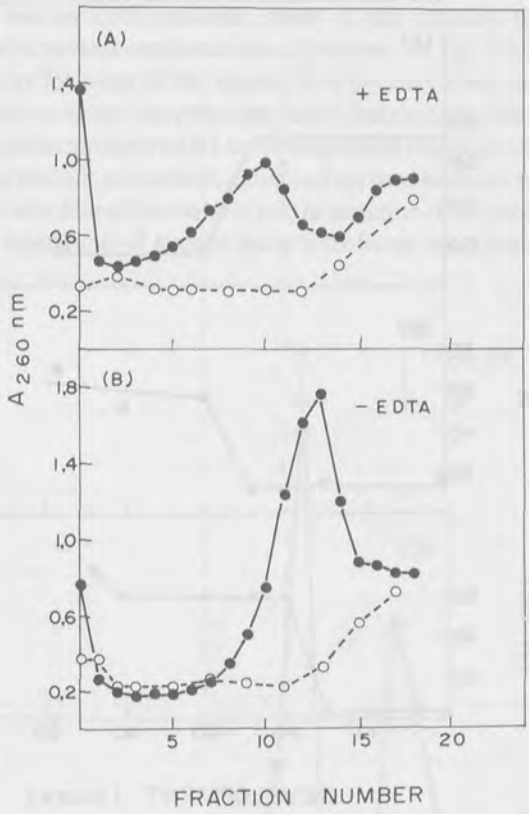

FIG. 5. Sucrose gradient analysis of nuclear extracts. Nuclear extracts from T24 nauplii were prepared as described in legend of Fig. 4. Aliquots of the dialized extracts were analysed in 5-20% sucrose gradients containing 2 M NaCl and 1 mM EDTA. The samples were centrifuged for 15 hr at 20 000 rpm. (A) extract prepared with addition of 1 mM EDTA ; (B) extract prepared without EDTA ; (●) total nucleic acids in the 1 ml fractions ; (○) RNA content of the 1 ml fractions (measured by absorbance at 260 nm after alkaline hydrolysis and precipitation of DNA with perchloric acid).

previously documented (Cano and Pestaña, 1976). Soluble DNA was measured by spectrophotometry of the perchloric acid hydrolyzates, and was represented as percent of the total DNA present in the extracts before centrifugation. Endonuclease was assayed by the clot method (Smith, 1974) and the activity given by the dilution titre. The results of this study are summarized in Fig. 6, whose main feature is the remarkable degree of synchrony between the appearance of the inhibitor, changes in soluble DNA and increase in endonuclease activity. Based on the experimental evidence in this section, it can be concluded with reasonable certainty that the inhibitor of acetyltransferase activity is an artifact arising from endonucleolytic attack of the DNA present in nuclear extracts.

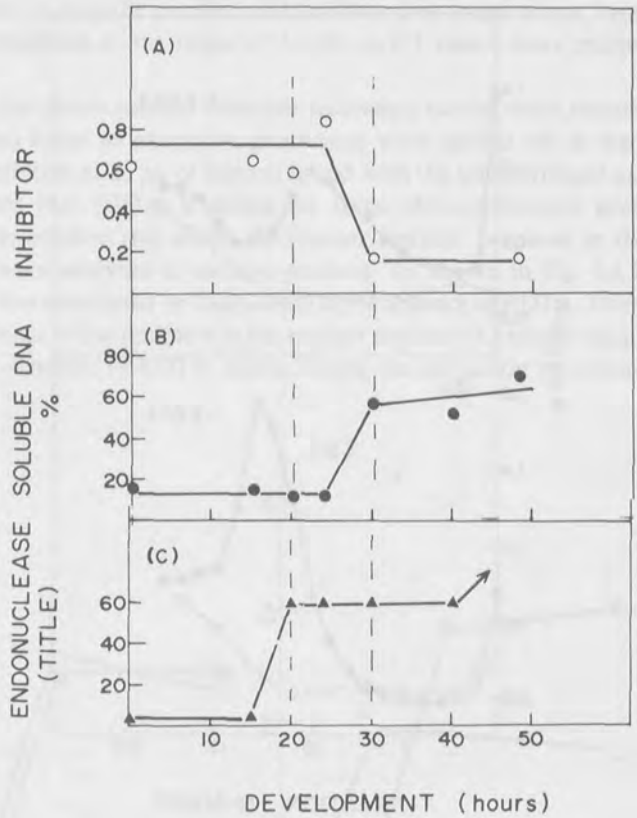

FIG. 6. Time course of developmental changes in endogenous inhibitor (A), soluble DNA (B) and endonuclease activity (C) in nuclear extracts. Nuclear extracts from animals of the indicated times of development were prepared as described in legend of Fig. 4. And analyzed for : A. Endogenous inhibitor. Histone saturation curves of each extract were obtained as described in Fig. 4. The presence of endogenous inhibitor is given as the ratio of ^{14}C-acetyl incorporation at 25/50 μg of histones. B. Soluble DNA. DNA content in the centrifuged extracts was measured by absorbance at 260 nm after hydrolysis in perchloric acid and is represented as the percentage of the DNA content in the corresponding uncentrifuged extracts, determined in the same way. C. Endonuclease activity. Aliquots of the dialized extracts were analyzed for endonuclease activity by the clot method (Smith, 1974). The title is expressed as the lowest dilution of the extracts needed to detect DNA digestion in the conditions of the assay.

SUBSTRATE REGULATION OF HISTONE ACETYLTRANSFERASE

We have previously observed a preferential acetylation of histone H1 by the *Artemia* acetyltransferase purified from 40 hr old nauplii (Cano and Pestaña, 1979). This behavior contrasts with the known specificity of the acetyltion *in vivo*, and also with our own observations of the acetylation of endogenous histones in nuclei (Fig. 3). In both cases, the acetylation was restricted to the arginine rich histones H3 and H4. In order to clarify this

question we carried out an electrophoretic study of the reaction products of the acetyltransferase, assayed with several combinations of histones. In Fig. 7 (circles) and Table I, it is shown that H1 accounts for most of the acetate incorporated when purified H1 histone was given as the only substrate to the enzyme. The small radioactivity found in other sections of the gel represents the contamination of H1 by other histone fractions or degradation products. When the five histones present in unfractionated calf thymus histones were given as substrate, the radioactive acetate was found (exclusively) in the arginine rich histones (Fig. 7, triangles), a result that suggests a regulation of histone acetyltransferase specificity by its substrates.

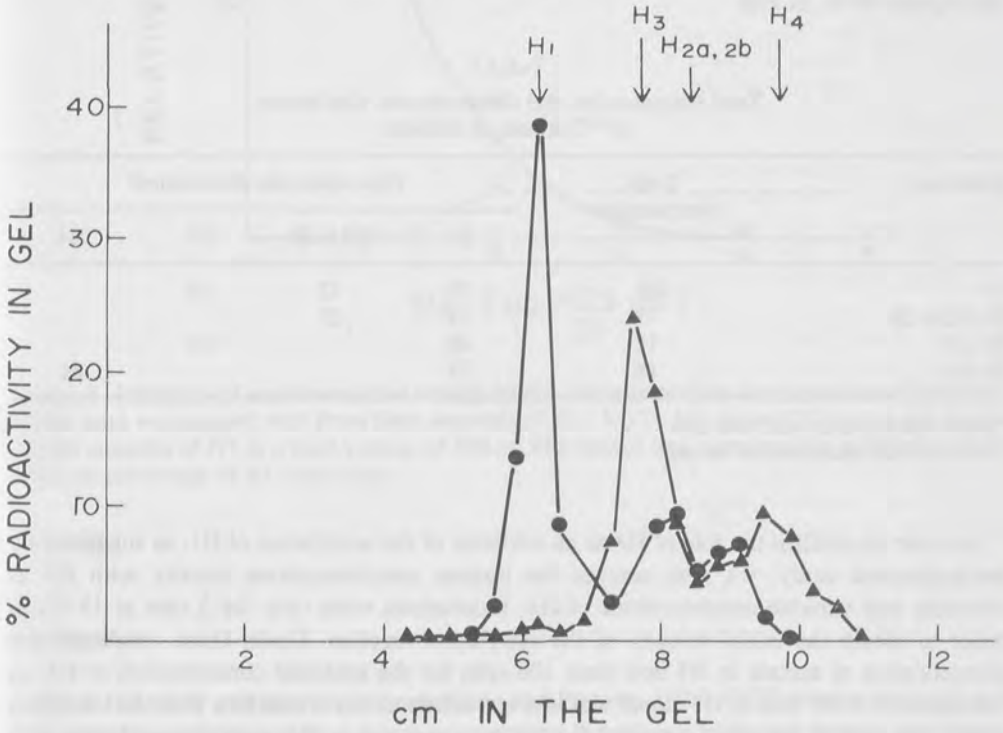

FIG. 7. Electrophoretic analysis of histones acetylated by the purified acetyltransferase. The acetyltransferase (DEAE-Cellulose step) from T40 nauplii was incubated in a final volume of 5 ml with saturating concentrations (20 μg/ml) of either fraction H1 purified (●) or unfractioned (▲) calf thymus histones. Proteins were precipitated with 20% TCA prior to electrophoresis and radioactive analysis of the gels was made as described in Fig. 3.

We next studied the influence of single histones upon the acetylation of H1. To this end, histones (100 μg of H1 alone or 50 μg of H1 and 50 μg of other histones as indicated) were incubated with 0.2 μCi of ^{14}C acetyl CoA and purified acetyltransferase (DEAE cellulose step) in a final volume of 5 ml. Total acetate incorporation after 30 min incubation was measured in a 0.5 ml aliquot. The remainder was precipitated with trichloroacetic acid and washed with

acetone, electrophoresed and assayed for radioactivity. The results of this experiment are summarized in Table I. With H1 as the only substrate, nearly 80% of the radioactive acetate in the electropherogram was found in the H1 region. When equal amounts of the H2A-H2B histones were present during the incubation, the acetylation reaction was inhibited for 30% with respect to H1 alone, but the electrophoretic distribution of acetyl radicals was not affected. The acetylation reaction was also 30% inhibited in the presence of H3, although the electrophoretic analysis indicated a redistribution of acetyl radicals between H1 and H3. Finally, the presence of H4 during the incubation markedly affects both the acetylating activity (80% inhibition) and the distribution of the radioactive acetate in the electropherogram (60% in H4).

TABLE I

Total incorporation and electrophoretic distribution of ^{14}C acetate in histones

Substrates	Total incorporation[1]	Electrophoretic distribution[2]			
		H1	H2A-2B	H3	H4
H1	100	78	12	10	
H1:H2A-2B	77	71	29		
H1:H3	72	48		52	
H1:H4	18	39			61

[1] 100% incorporation was 2 600 cpm.
[2] In % of total radioactivity of the gel.

In order to confirm the role of H4 as an inhibitor of the acetylation of H1, as suggested by electrophoretic study, we next assayed the histone acetyltransferase activity with H1 as substrate and variable concentrations of H4. Incubations were only for 5 min at 35 °C, in order to obtain the initial velocity of the acetylation reaction. Under these conditions the incorporation of acetate in H4 (less than 200 cpm for the maximal concentration of H4, as compared to 2 500 cpm in H1 alone) was low enough so as not to interfere with the inhibition study. The plot of the data, corrected for the incorporation in H4, gave a close family of S shaped curves of inhibition (Fig. 8) at any of the concentrations of H1 assayed. This result suggests that H4 acts as an allosteric, non-competitive inhibitor of the acetylation of H1, with an $I_{0.5}$ close to 0.2 μM. In other, as yet, unpublished studies, we found the substrate saturation curves with H1 and acetyl CoA to be sigmoidal in shape.

These two sets of experimental results point to the possible allosteric character of histone acetyltransferase from *Artemia*.

We conclude from these experiments that the substrate specificity of the histone acetyltransferase from *Artemia* is regulated by histone H4, which in turn is one of its substrates. Such a regulation by substrate can be seen as a regulatory mechanism by which the acetate transfer can be directed to the proper histone. In addition, this regulation allows to correlate the high activity with H1 shown by the acetyltransferase from *Artemia* with the known specificity of histone acetylation *in vivo*.

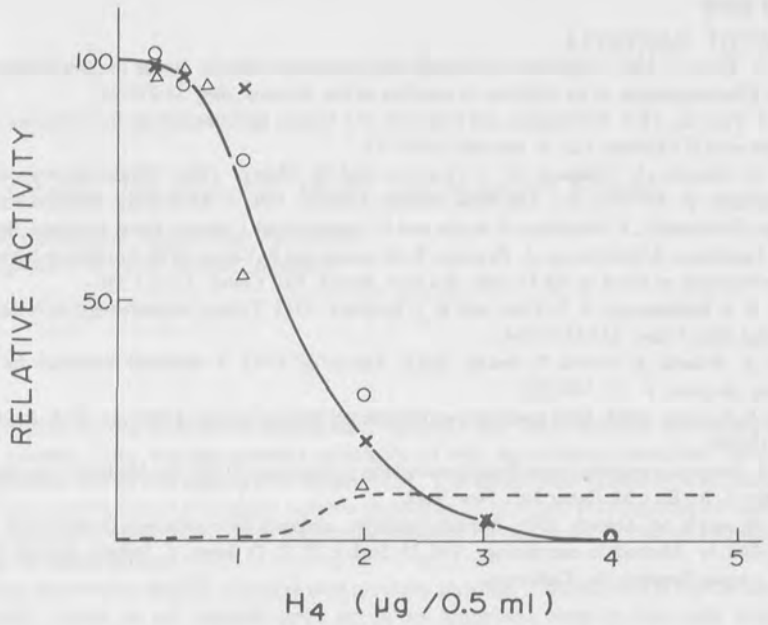

FIG. 8. Inhibition of acetyltransferase activity by H4. Partially purified acetyltransferase (hydroxylapatite step) was assayed with three fixed amounts of H1 : 5 (○), 10 (△) and 25 (×) µg and the indicated amounts of H4 in a final volume of 500 µl. The dashed line represents the acetylation of H4 alone, as percentage of H1 acetylation.

Conclusions

From this study and previous reports (Cano and Pestana, 1976, 1979) we can conclude that the acetylation of histones in *Artemia* is controlled by enzyme level and enzyme regulation.

The observed changes in endogenous acetylation in nuclei, which were supposed to arise from the developmental appearence of an endogenous inhibitor are now best explained by changes in substrate availability or enzyme specificty as discussed in section A.

The involvement of an endogenous inhibitor in the regulation of histone acetylation now seems very unlikely in view of the results presented in section B, which would indicate its artifactual origin.

The assumed allosteric character of the inhibition by H4, as shown in section C, together with our unpublished results showing that the substrate saturation curves with H1 and acetyl CoA follow sigmoidal kinetics, seem to indicate that histone acetyltransferase from *Artemia* is a regulatory enzyme. This conclusion, if proven to be true, would reinforce our view that histone acetyltransferase is a likely site for the regulation of the structure and function of chromatin.

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Artemia lipovitellin

Didier de Chaffoy, Jan Heip, Luc Moens, and Masatoshi Kondo[1]

Departement Celbiologie, Universiteit Antwerpen
Universiteitsplein 1, B-2610 Wilrijk, Belgium

Abstract

The lipovitellin of the anostracan crustacean, *Artemia* has been isolated and characterized. The lipovitellin isolated from oocytes consists primarily of two apoprotein molecules having estimated molecular weights of 190 000 and 68 000, respectively. The lipoprotein of embryos at the gastrula stage or nauplius larvae was found to contain various quantities of multiple polypeptides in addition to those detected in the oocyte lipovitellin ; their molecular weights ranged from 78 000 to 180 000, depending on the stage of development.

An alkaline lipovitellin-specific protease was partially purified ; this enzyme is tightly associated with the lipovitellin, either in an isolated state, or in an integrated state in the yolk granule. It was demonstrated that this lipvitellin-specific protease is fully responsible for the hydrolysis of the largest apoprotein molecule (Mr 190 000) during the embryonic development, producing multiple polypeptides of a defined size in addition to those of Mr 68 000 and Mr 78 000, respectively. The smallest apoprotein molecule (Mr 68 000) was found to be completely resistant to this proteolytic activity. However, the lipid globules accumulating in the nauplius larvae exhibit only trace amounts of lipovitellin proteins in addition to large quantities of low molecular weight polypeptides. This may indicate that the lipid globules represent the later stages of the yolk breakdown process and that proteases other than the alkaline lipovitellin-specific protease are involved in subsequent hydrolysis of the yolk apoproteins.

The native lipovitellin molecule has an estimated molecular weight of $0.6\text{-}0.7 \times 10^6$ (as determined by gel filtration and sucrose gradient analyses), and appears to represent a dimeric form of the polypeptide with the calculated molecular weight of 290 000. The integral components of the lipovitellin molecule, carbohydrates and lipids, were also characterized.

Introduction

Our understanding on the physical and biochemical properties of the major high density lipoproteins (vitellin or lipovitellin) isolated from the mature eggs of various vertebrates has expanded rapidly in recent years (Bergink and Wallace, 1974 ; Tata, 1976 ; Gordon *et al.*, 1977 ; Ohlendorf *et al.*, 1977).

In invertebrates, only the lipovitellin molecule in insects has been thoroughly investigated (Engelman, 1974 ; Hagedorn, 1974 ; Gellisen *et al.*, 1976 ; Chino *et al.*, 1977 ; Kambysellis, 1977). Information on lipovitellin in crustaceans (Wallace *et al.*, 1967 ; Zagalsky *et al.*, 1969 ; Zagalsky and Herring, 1972 ; Fyffe and O'Connor, 1974 ; Croisille *et al.*, 1974) and

[1] To whom correspondence should be addressed.

particularly in anostracan crustaceans (Warner *et al.*, 1972 ; Zagalski and Gilchrist, 1976) is relatively limited.

It has been determined that the lipovitellin of crustaceans is associated with various carotenoids, causing diversity of coloration in the eggs (Zagalsky, 1976). Even though the carotenoid components of anostracan crustaceans have already been examined (Hsu *et al.*, 1970 ; Zagalsky, 1976), no concrete analysis on the apoprotein structure of the lipovitellin has been presented yet. We have undertaken this study on *Artemia* to establish the subunit structure of the lipovitellin in this organism. Evidence is presented in this report that the *Artemia* lipovitellin contains primarily two apoprotein subunits having molecular weights of 68 000 and 190 000, respectively, and that an alkaline lipovitellin-specific protease hydrolyses preferentially the Mr 190 000-protein, but not the Mr 68 000-protein during embryonic development.

Materials and methods

Culture method of *Artemia*

Nauplius larvae and adult animals were obtained by culturing the encysted cryptobiotic gastrulae as previous described (Moens and Kondo, 1976 ; Heip *et al.*, 1978).

Preparation of yolk granules

In most of the experiments reported here, yolk granules were prepared in quartz bidistilled water (QBW) because this resulted in a minimal solubilization of the lipovitelin from yolk granules. The encysted gastrulae were hydrated in water at room temperature for 1 hr, or at 0 °C overnight. The hydrated embryos were homogenized in QBW at 0 °C using a Virtis homogenizer at 55 000 rpm for 3 min. The homogenate was filtered through a glass wool column and the filtrate was centrifuged in a Beckman JS 13 rotor at 10 000 rpm (10 200 × g) for 2-4 min at 4 °C. The pellet was resuspended in QBW and recentrifuged as described above. This operation was repeated until a clean supernatant was obtained. The final pellet was resuspended in an appropriate solution for extraction of the lipovitellin as below.

Solubilization of lipovitellin

The lipovitellin was solubilized at 0 °C by gentle stirring of the isolated yolk granules in 50 mM sodium citrate buffer (pH 5-6), 50 mM Tris-glycin buffer (pH 7-9) or 50 mM Tris-HCl buffer (pH 7-9), supplemented with different concentrations of NaCl as indicated in individual experiments. At different time intervals, an aliquot (1/15th of the original volume) was taken and the insoluble material was pelleted by centrifugation in a test tube in an Eppendorf centrifuge 3 200 at the maximal speed. The solubilized fraction was used for further analysis of the lipovitellin.

Female adults carrying oocytes in the oviduct were selected, washed several times in QBW and dissected carefully under a binocular microscope to recover the oocytes (de Chaffoy *et al.*, 1978). They were rapidly transferred into an Eppendorf test tube, homogenized in QBW with a glass fitted pestle, and centrifuged in the same test tube for 5 min as described above. This operation, was repeated until the supernatant became clear. The last pellet was used to solubilize the oocyte lipovitellin as described above.

Determination of protein concentration

Protein samples (2 μl) were spotted on a cellulose acetate sheet together with a control bovine serum albumin (BSA) of known concentration (1 mg/ml) and thoroughly dried. After staining with 0.25% amido black, each stained spot was cut out and dissolved by vigorous shaking in 2 ml of a solution containing 10% formic acid/1% trichloroacetic acid/80% acetic acid. The optical density at 630 nm was determined and the protein concentration was estimated on the basis of the reference protein, according to the method originally developed by Prof. W. Zillig.

Dodecylsulphate-polyacrylamide gel electrophoresis

Gel electrophoresis in the presence of 0.1% sodium dodecylsulphate (SDS) was performed as previously described (Moens and Kondo, 1978), except that 5% gels (0.3 × 10 cm) were used.

Isolation and analysis of carotenoid pigments

The carotenoid pigment was extracted from the lipovitellin with 67% ethanol by shaking at room temperature for *ca* 1 min, followed by centrifugation in an Eppendorf test tube as described above. The liquid phase was further extracted with an equal volume of pure petroleum ether. The petroleum ether phase was recovered, evaporated *in vacuo*, and the residue was dissolved in 90% ethanol. The reduction of carotenoids was obtained through the addition of a few crystals of sodium borohydride, added directly to the sample before spectral analysis in a Beckman Spectrophotometer Model 25.

The carotenoid pigments were saponified by incubation at room temperature overnight in 7.5% ethanolic potassium hydroxide. The reaction was stopped by the addition of a few drops of acetic acid as described by Arpin *et al.* (1969). Acetylation of the carotenoids was carried out in dehydrated pyridine, followed by addition of 10% (in volume) of acetic anhydride (Schimmer and Krinsky, 1966). The mixture was left to react for 24 hr at room temperature.

Thin layer chromatography on a Silicagel G plate was developed with a solvent containing benzene/diethyl-ether/methanol (17/2/1) using the authentic markers of astaxanthin, canthaxanthin and echinenone.

Electron microscopy

Electron microscopy of prenauplius or nauplius embryos was performed with a Siemens Elmiskop I.A. as described previously (de Chaffoy *et al.*, 1978). Animals were fixed in 2.5% glutaraldehyde, followed by 1% osmium tetraoxide in 0.2 M cacodylate buffer (pH 7.2), stained with uranyl acetate, and embedded in Epon. The ultra thin sections were first double stained with uranyl acetate and then with lead citrate.

Materials

The encysted gastrulae of *Artemia* were supplied by Metaframe (San Francisco, California, USA). Silicagel G plates came from E. Merck, Darmstadt, Fed. Rep. Germany. The authentic carotenoid samples were kindly supplied by Drs. U. Gloor and F. Weber (Hoffmann-La

Roche and Co., Basel, Switzerland). Other materials were indicated in our previous publications (de Chaffoy *et al.*, 1978, Heip *et al.*, 1978 ; Moens and Kondo, 1976, 1978).

Results

Solubilization of lipovitellin

Fig. 1a shows the solubilization kinetics of the lipovitellin from the yolk granules of gastrulae in either 50 mM Tris-HCl buffer (pH 7-9) or 50 mM sodium citrate buffer (pH 5-6) in the presence of 1 M NaCl. Below pH 6, the solubility of the *Artemia* lipovitellin was markedly reduced in comparison with that observed above pH 7. Under these conditions, the greater part of the lipovitellin solubilized in 3 hr was already extracted within 30 min (Fig. 1a). A better solubilization was achieved by using 50 mM Tris-HCl (pH 9) containing 1-2.5 M NaCl (Fig. 1b). The critical concentration of NaCl for the solubilization of lipovitellin was found to be about 0.4 M. From Fig. 1b it can be seen that the lipovitellin was very poorly solubilized at lower (12.5-50 mM), in contrast to higher (1-2.5 M), NaCl concentrations except in the case of a saturating concentration (6.11 M).

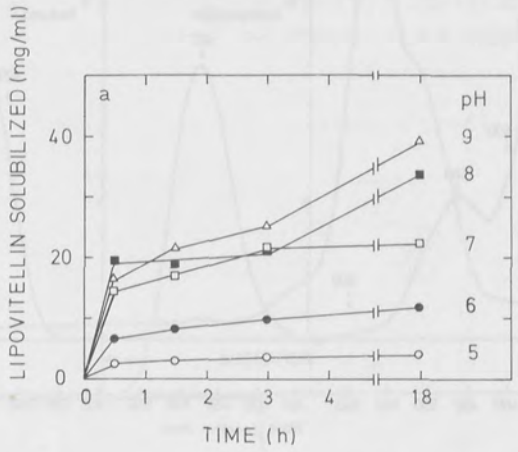
Carotenoid pigments of lipovitellin

The absorption spectrum of the *Artemia* lipovitellin in the solubilization buffer (50 mM Tris-HCl (pH 8-9)/1 M NaCl) exhibits two maxima at 368 and 460 nm with a shoulder at 400 nm (Fig. 2a), as would be expected from the red-orange coloration of the lipovitellin. The pigment, extracted from the lipovitellin with 67% ethanol, followed by re-extraction with pure petroleum ether, showed an absorption spectrum with a rather symmetrical peak at 473 nm (Fig. 2b). After reduction of this pigment with sodium borohydride, two maxima at 459 and 475 nm with a shoulder at 423 nm were observed (Fig. 2c). Spectral analysis of the authentic sample of canthaxanthin (4,4'-diketo-β-carotene) revealed that this pigment is reduced to isozeaxanthin (4,4'-dihydroxy-β-carotene). Furthermore, thin layer chromatography analysis of the carotenoid pigment (Fig. 2d) and gas-liquid chromatography (not shown) have demonstrated conclusively that about 97% of the total carotenoids in *Artemia* lipovitellin is canthaxanthin while the rest is essentially echinenone (4-keto-β-carotene).

Molecular size of lipovitellin

The native lipovitellin molecule was examined by three different techniques to estimate its apparent molecular weight. The electrophoresis of the lipovitellin in 4% gels at pH 9.3 exhibited one major band with a slowly migrating shoulder (Fig. 3a), indicating the existence of some heterogeneity in its molecular structure. The lipovitellin was obtained from the gastrulae in these studies. As will be shown below, the non-symmetrical peak may be caused by the presence of different hydrolytic polypeptides in the gastrula lipovitellin (LVg).

Gel filtration on Bio-Gel A1.5m was used to estimate the apparent molecular weight of the lipovitellin. As shown in Fig. 3b, the lipovitellin was eluted as a single peak with a trailing shoulder of varying size, depending on the experiment. The position of the major peak corresponds to a molecular weight of 0.6×10^6. Analysis on linear sucrose gradients containing 1 M NaCl indicated a sedimentation coefficient of about 15.5 S (Fig. 3c), which may be in



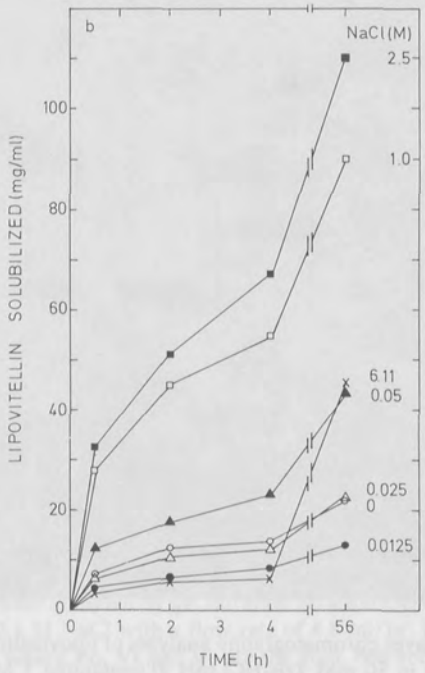
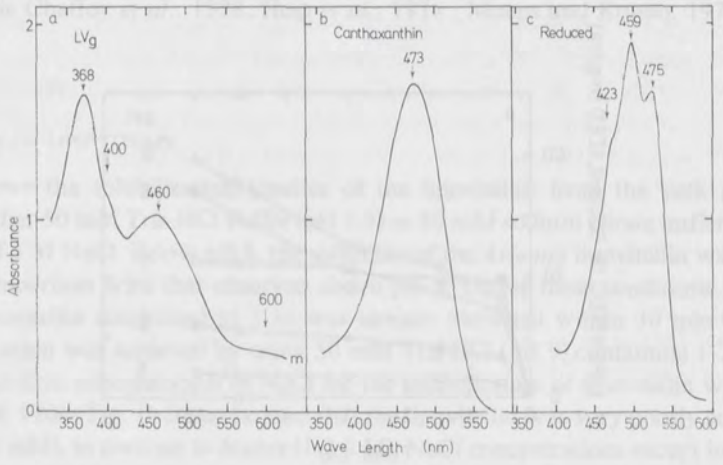


FIG. 1. Solubilization of *Artemia* lipovitellin from yolk granules. (a) The gastrula lipovitellin was solubilized in the presence of 1 M NaCl with either 50 mM Na citrate buffer (pH 5 and 6) or 50 mM Tris-HCl buffer (pH 7, 8 and 9) as described in Materials and methods. (b) The same lipovitellin preparation was solubilized with 50 mM Tris-HCl buffer (pH 9) in the absence or presence of different NaCl concentrations as indicated in the figure.



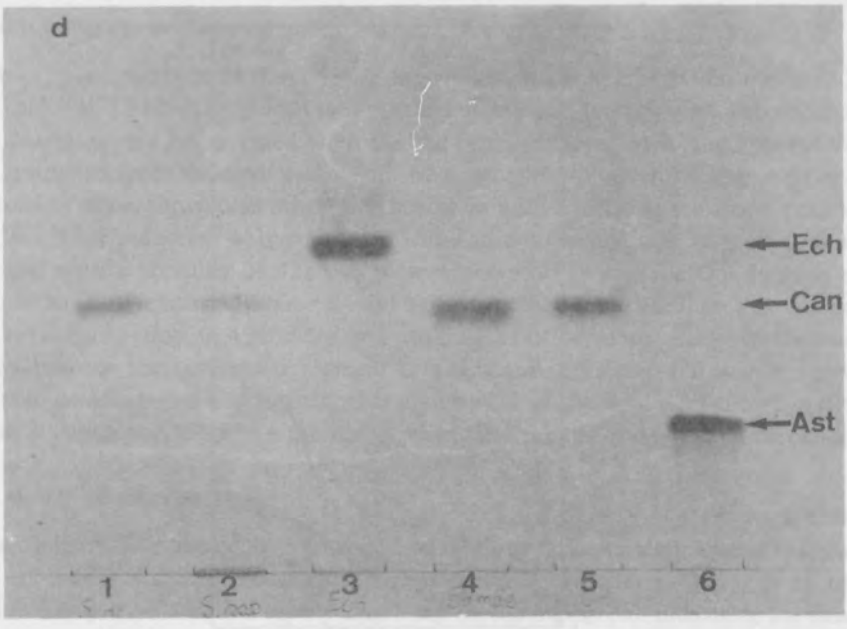


FIG. 2. Spectral and thin layer chromatography analyses of lipovitellin and carotenoids. (a) Spectral analysis of native lipovitellin in 50 mM Tris-HCl (pH 9) containing 1 M NaCl. (b) Carotenoids were extracted from the lipovitellin with petroleum ether whereafter the spectrum was determined in 90% ethanol. (c) The same sample as in (b) was reduced with sodium borohydride immediately before the spectral analysis. (d) The carotenoids of the lipovitellin were either acetylated or saponified as described in Materials and methods. Samples were analyzed on a thin layer plate with several authentic references. Slot 1, acetylated sample ; slot 2, saponified sample ; slot 3, echinenone (Ech) ; slot 4, untreated sample ; slot 5, canthaxanthin (Can) ; and slot 6, astaxanthin (Ast).

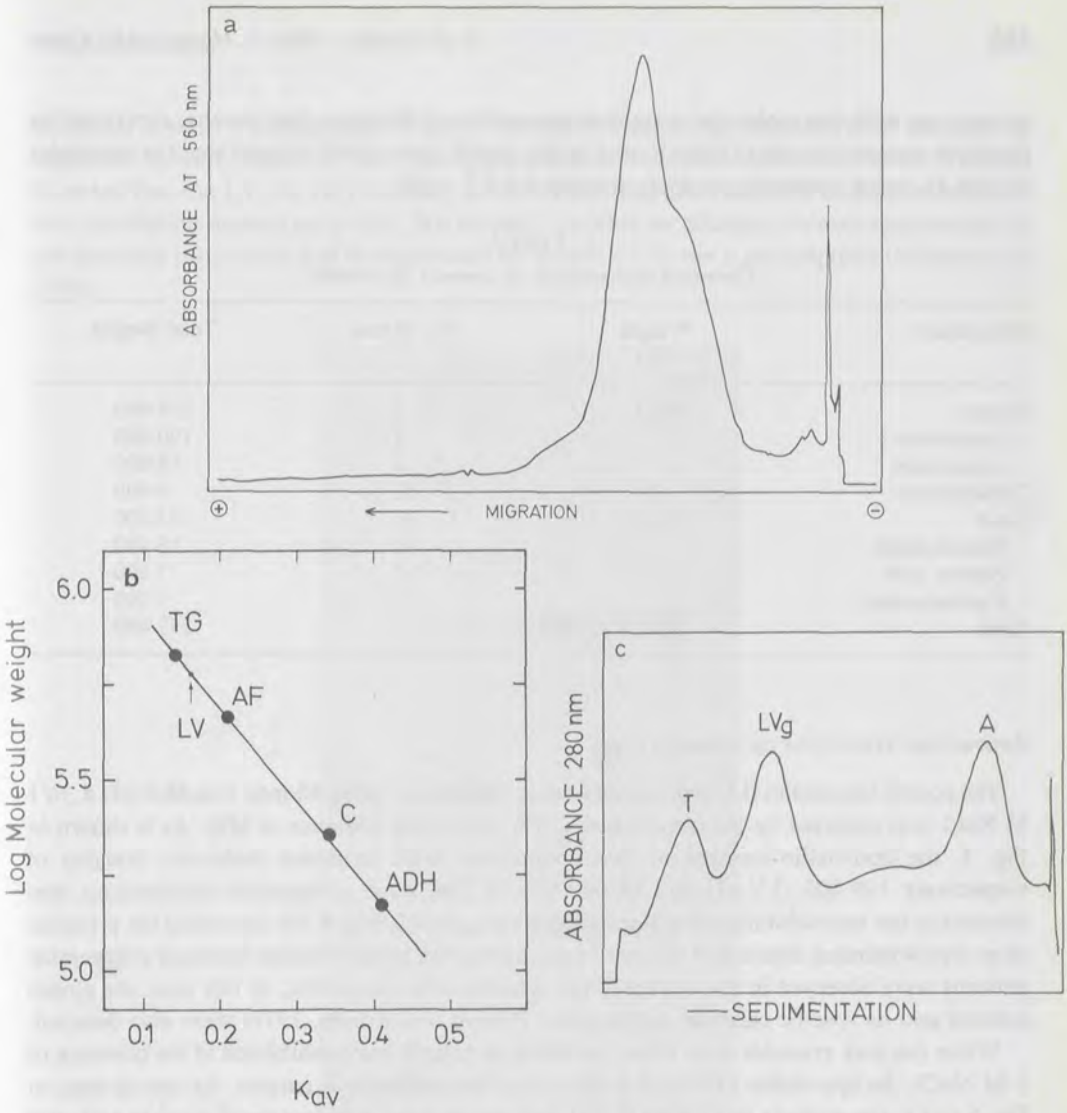


FIG. 3. *Gel electrophoresis, gel filtration and sedimentation analyses of the native lipovitellin.* (a) The gastrula lipovitellin (LVg) was extracted from the yolk granules with 50 mM Tris-HCl (pH 9)/1 M NaCl and electrophoresed in 4% polyacrylamide gels in 5 mM Tris-glycine buffer (pH 9.3) at room temperature for 5 hr with 1.25 mA per gel. The gels were stained with 0.1% coomassie brilliant blue and scanned at 560 nm with a Gilford type 240 spectrophotometer equipped with a linear transport. (b) similar samples as in (a) were analyzed by gel filtration on a Bio-Gels A 1.5m column using 50 mM Tris-HCl (pH 9) containing 0.1 M NaCl with a flow rate of 4.8 ml/hr. Kav is the partition coefficient, defined ad [Ve-Vo]/[Vt-Vo], where Vo, Vt and Ve are the void, bed and elution volumes,respectively. Data are means of three column runs. The molecular weight markers are horse liver alcohol dehydrogenase (ADH ; Mr 83 000), beef liver catalase (C, Mr 232 000), horse spleen apoferritin (A, Mr 440 000) and hog thyroid thyroglobulin (T, Mr 669 000). LV indicates the peak of the lipovitellin. (c) The LVg sample was analyzed by 5-30% sucrose density gradients in 50 mM Tris-glycine (pH 8.3)/1M NaCl using a Beckman SW 41 rotor at 41 000 rpm for 16 hr at 4 °C. The absorbance at 280 nm of each gradient was recorded by a ISCO UA5 UV detector. Different markers were used in different runs ; they are horse thyroid thyroglobulin (T, 19.3 S), beef liver catalase (11.2 S), *Artemia* hemoglobin (11.3 S), horse liver alcohol dehydrogenase (A, 5S), sheep hemoglobin (4.1 S) and horse heart cytochrome C (2.1 S).

accordance with the molecular weight estimated by gel filtration. Our results, supported by chemical composition data (Table I ; de Chaffoy and Kondo, 1980), suggest that the molecular weight of native lipovitellin is approximatey $0.6\text{-}0.7 \times 10^6$.

TABLE I

Chemical composition of *Artemia* lipovitellin

Component	Weight (%)	No. of mol	Total weight
Protein	88.1	2	258 000
Apoprotein 1		1	190 000
Apoprotein		1	68 000
Carbohydrate	3.3	52	9 400
Lipid	8.6	36	25 000
Phospholipid		21	16 400
Neutra lipid		13	7 500
Canthaxanthin		2	1 100
Total			292 400

APOPROTEIN STRUCTURE OF LIPOVITELLIN

The oocyte lipovitellin (LVo) prepared as described above using 50 mM Tris-HCl (pH 8.3)/1 M NaCl was analysed by electrophoresis in 5% gels in the presence of SDS. As is shown in Fig. 4, the lipovitellin consists of two apoproteins with estimated molecular weights of respectively 190 000 (LV-α1) and 68 000 (LV-ε). The same polypeptide composition was detected in the non-solubilized fraction of the oocyte as well (Fig. 4, P), indicating the presence of an overwhelming amount of lipovitellin in the oocytes in the oviduct. Identical polypeptide patterns were observed in the extract of the whole ovary except that, in this case, the globin subunit and its specific cleavage polypeptides (Moens and Kondo, 1978) were also detected.

When the yolk granules from either gastrulae or nauplii were solubilized in the presence of 1 M NaCl, the lipovitellin exhibited a more complex polypeptide pattern. As can be seen in Fig. 5 and 6, the gastrula lipovitellin (LVg) contains multiple polypeptides (LV α2 to α10, and LV-γ and LV-δ) in addition to those apoproteins (LV-α1 and LV-ε) detected in the oocyte lipovitellin (LVo). The nauplius lipovitellin (LVn), obtained from larvae cultured for 3-4 days without feeding, was found to have essentially two polypeptides having molecular weights of 120 000 (LV-α10) and 68 000 (LV-ε) (Fig. 5). However, lipovitellin preparations from young nauplii were observed to contain polypeptides in varied quantities quite similar to those found in the gastrulae (LVg). These results suggest that the LV-α1 (Mr 190 000) is preferentially hydrolyzed via multiple polypeptides of the defined size in the yolk granules during embryonic and larval development. On the other hand, the LV-ε (Mr 68 000) seems to be completely resistant to this proteolytic degradation.

An identical proteolysis of the LV-α1 can be demonstrated *in vitro* by incubating the solubilized lipovitellin in the solubilization medium. When the gastrula lipovitellin (LVg), which contains a large amount of the LV-α1, was incubated at 0 °C in the presence of different concentrations of NaCl, the LV-α1 was hydrolyzed progressively to lower

molecular weight α polypeptides (Fig. 6). The rate of hydrolysis was slower at zero and at saturation concentrations of NaCl than at intermediate NaCl concentrations (Fig. 6). It should be noted that the LV-ε is very resistant to this proteolysis and remained intact during the *in vitro* incubation as well as *in vivo*. We partially purified an alkaline protease responsible for the observed proteolysis and demonstrated its specificity for the α polypeptides (Vallejo *et al.* 1980).

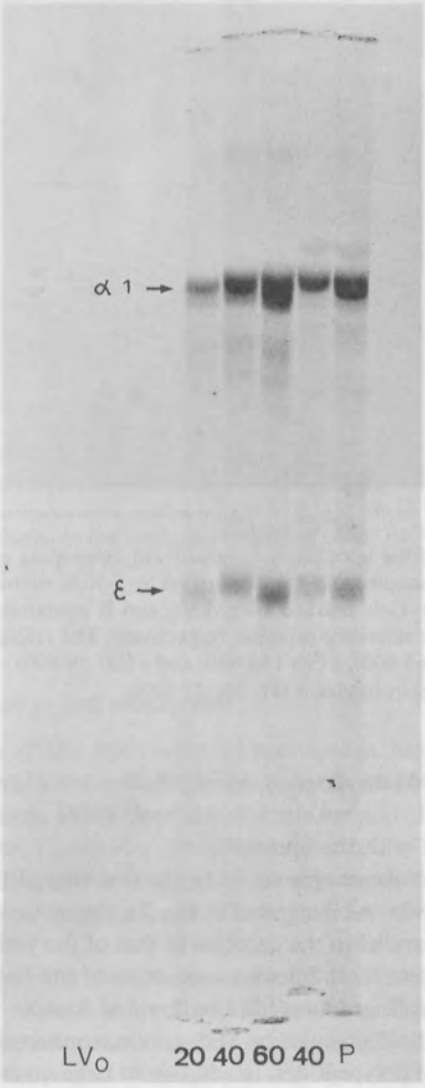


FIG. 4. SDS gel analysis of the oocyte lipovitellin. The lipovitellin (LVo) was solubilized from the oocytes with 50 mM Tris-HCl (pH 8)/1 M NaCl and analyzed in 5% gels as described in Materials and methods. The number marked under each gel indicates the amount of protein loaded in μg while P marks the insoluble fraction of the oocyte after solubilization at 0 °C for 1 hr.

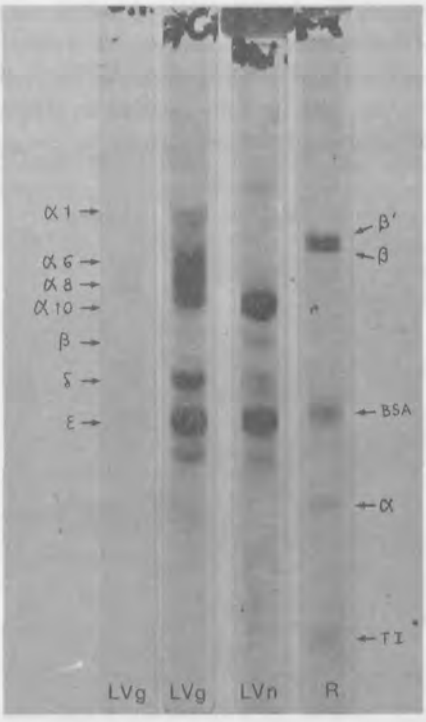


FIG. 5. SDS gel analysis of the lipovitellin isolated from developing embryos. The lipovitellin was solubilized from gastrulae or nauplii as in Fig. 4, purified by 5-30% sucrose density gradients as in Fig. 3c, and analyzed by 5% gels. Gels marked LVg, LVn and R contained the gastrula, nauplius lipovitellins and molecular weight reference proteins, respectively. The reference proteins are *E. coli* RNA polymerase subunits, β' (Mr 165 000), β (Mr 155 000) and α (Mr 39 000); bovine serum albumin (BSA, Mr 68 000) and soy bean trypsin inhibitor (TI, Mr 21 500).

We therefore conclude that the *Artemia* lipovitellin consists of two apoproteins (LV-α1 and LV-ε) of which only LV-α1, but not the LV-ε, is hydrolyzed stepwise to the LV-α10 by the specific protease associated with the lipovitellin.

The proteolysis described above appears to be the first step of the yolk degradation in the developing embryo of *Artemia*. As illustrated in Fig. 7a, the increase in the proportion of lipid globules was found to be parallel to the decrease in that of the yolk granules during the post-gastrula development. Moreover, the lipid composition of the lipid globules was practically identical with that of the yolk granules (de Chaffoy and Kondo, 1980). The analysis of the protein components of the lipid globules by SDS-gel electrophoresis indicated the presence of trace amounts of lipovitellin polypeptides, in addition to large quantities of heterogeneous low molecular weight polypeptides (Fig. 7b). As an interpretation of these observations we suggest that the lipid globules represent the later stages of yolk degradation, in which the LV-ε is also eventually hydrolyzed. As a consequence proteases other than the polypeptide α specific protease seem to be involved in the final phase of yolk utilization.

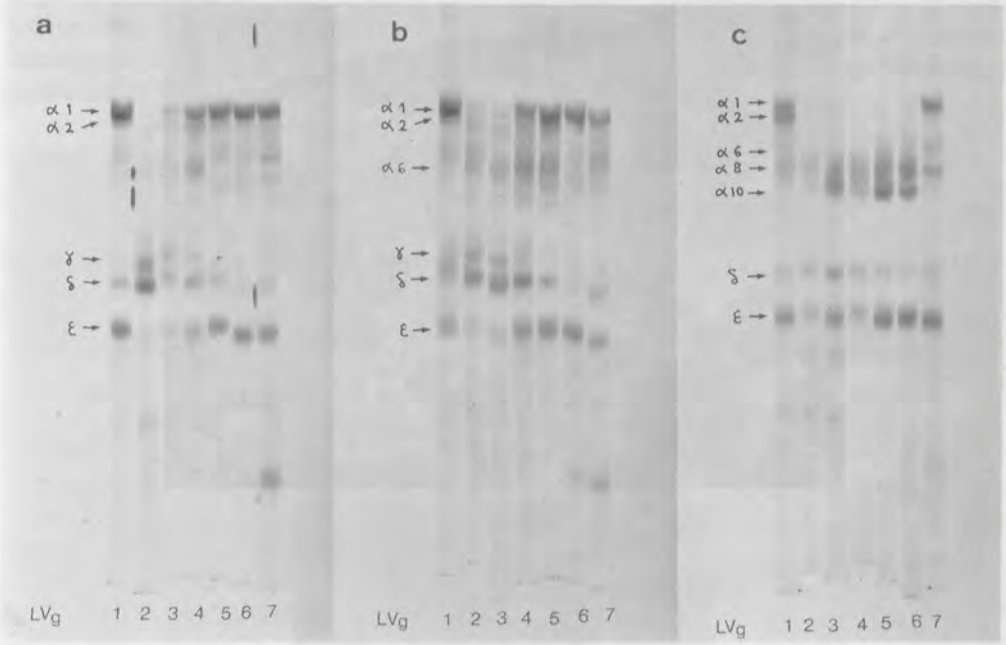
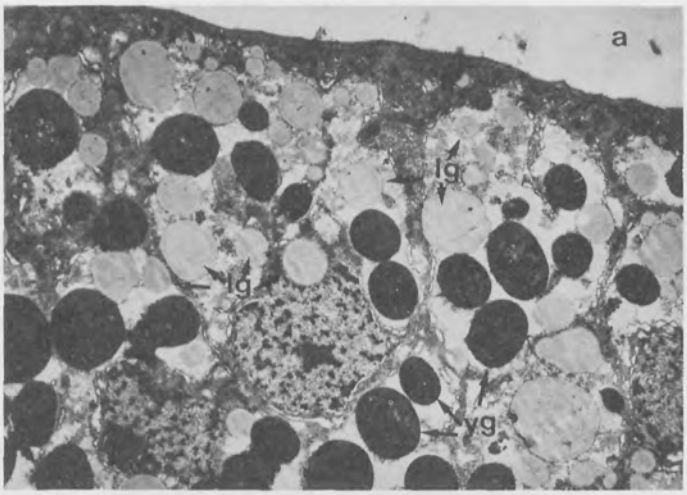


FIG. 6. SDS gel analysis of the gastrula lipovitellin during *in vitro* hydrolysis. The gastrula lipovitellin (LVg) was solubilized with 50 mM Tris-HCl (pH 9) containing different concentrations of NaCl and incubated at O °C in the corresponding medium for 0.5 (a), 4 (b) or 56 hr (c). In each panel, the lipovitellin was prepared and incubated in the medium containing either no NaCl (gel 1), or 12.5 mM (gel 2), 25 mM (gel 3), 50 mM (gel 4), 1 M (gel 5), 2.5 M (gel 6) or 6.1 M NaCl (gel 7).

THE PRESENCE OF LIPOVITELLIN IN THE HEMOLYMPH

The site of biosynthesis of the lipovitellin of crustaceans has not yet been definitively determined. An extraovarian site for vitellogenin synthesis has been suggested for the blue crab, *Callinectes sapidus* (Kerr, 1969), the fiddler crab, *Uca pugilator*, the spidercrab, *Libinia emarginata* and the crayfish, *Cambarus clarkii* (Wolin *et al.*, 1973). On the other hand, *in vitro* radiolabeling of the isolated ovary suggested that this organ is capable of synthesizing the vitellogenin in crayfish, *Orconectes immunis* (Ganion and Kessel, 1972) and *Procambarus* species (Lui *et al.*, 1974).

We therefore investigated the possible presence of lipovitellin in the hemolymph by immunological technique. A double immunodiffusion test of the hemolymph clearly demonstrated that immunoreactive material against rabbit anti-lipovitellin antiserum is pesent in the female-, but not in the male hemolymph (Fig. 8a). SDS-gel analysis of the total hemolymph indicated LV-α1, LV-α10 and LV-ε as minor components of female, but not of male hemolymphs (Fig. 8b). Furthermore, SDS-gel analysis of the immune complex confirmed the presence of the lipovitellin proteins in the hemolymph (not shown). Since the hemolymph contains not only the apoproteins (LV-α1 and LV-ε), but also its hydrolytic



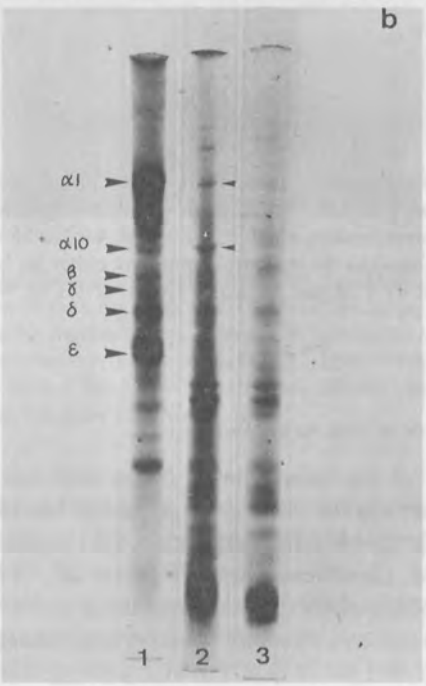


FIG. 7. Electron microscopy and SDS gel analyses of the lipid globules. (a) electron micrograph of the prenauplius, showing the presence of both yolk graules (yg) and lipid globules (lg). The magnification was 9 000 times. (b) The lipid globules were prepared in the same way as the yolk granules, except that centrifugation at 10 200 × g in a Beckman JS 13 rotor was done for a longer time (8-10 min). The upper yellow, loosely packed layer on top of the tightly packed yolk granules was removed by gently suspending the lipid globules in QBW. This fraction was further purified by repeated centrifugations as above. The last pellet was directly denatured with 1% SDS/1% 2-mercaptoethanol and analyzed in 5% gels as usual. Gel 1, LVg ; gels 2 and 3, the proteins recovered from the lipid globules of different preparations.

products (LV-α2 to LV-α10), it seems likely that these lipovitellin proteins are a result of leakage from the ovary into the hemolymph rather than transport to the ovary through the hemolymph route. However, other interpretations are equally possible and any conclusions on the site of lipovitellin biosynthesis must await further experimentation.

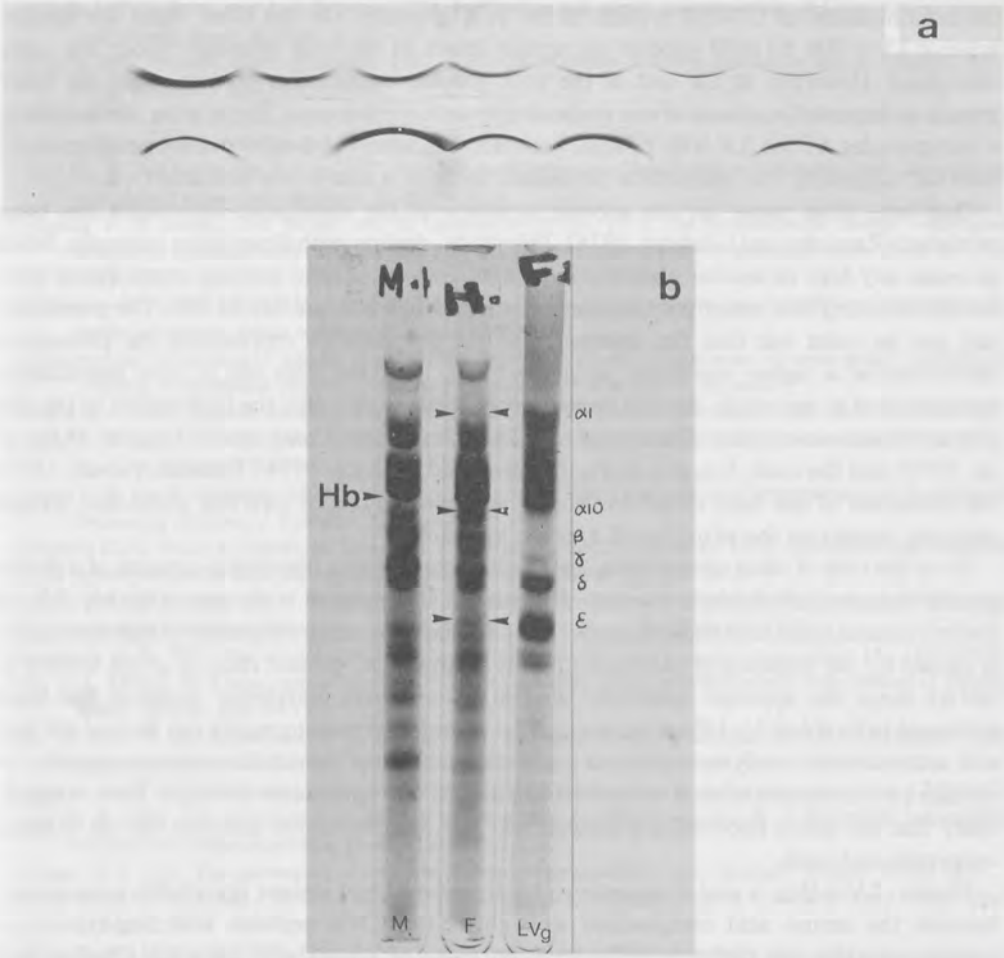


FIG. 8. Presence of the lipovitellin in the female hemolymph. (a) Double immunodiffusion test of the hemolymph proteins with rabbit anti-lipovitellin antiserum was performed as described in Heip *et al.* (1978). The antiserum was placed in the central canal. Wells in the upper panel contained from left to right 100, 40, 25, 20, 15, 12, and 10 μg lipovitellin, respectively. Wells in the lower panel contained from left to right 15 μg lipovitellin, male hemolymph, 100 μg lipovitellin, female hemolymph, 25 μg lipovitellin, male hemolymph and 20 μg lipovitellin, respectively. (b) SDS gel electrophoresis of both male and female hemolymph and carried out in 5% gels as above. Gels M, F and LVg indicate the male hemolymph, the female hemolymph and the gastrula lipovitellin, respectively. Hb indicates the globin subunits (Mr 125 000) of *Artemia* hemoglobins. Three small arrows placed on both sides of the gel marked «F» indicate the presence of small amounts of the female specific proteins, corresponding to LV-α1, LV-α10 and LV-ε, respectively.

Discussion

The present study intended a first elucidation of the apoprotein subunit structure of *Artemia* lipovitellin. The results indicate that the lipovitellin is composed of two apoprotein subunits. The larger subunit LV-α1 (Mr 190 000) undergoes a sequential proteolytic cleavage, leading to the accumulation of LV-α10 protein in the yolk granules. On the other hand the smaller subunit LV-ε (Mr 68 000) appears to remain intact in the yolk granules under the same conditions. However, at the end of the yolk granule degradation process, even the latter protein is degraded *in vivo* to lower molecular weight components. The *in vitro* proteolysis of α polypeptides to the LV-α10 protein was not inhibited by 1 mM phenylmethylsulfonyl fluoride, suggesting the responsible protease(s) to be of a non-serine protease type.

Only one other paper on the subunit structure of the anostracan lipovitellin has been published (Zagalsky and Gilchrist, 1976). This study, dealing with *Branchipus stagnalis*, failed to reveal any high molecular weight components. Instead, several multiple components were found, including five major polypeptides between Mr 45 000 and Mr 85 000. The possibility can not be ruled out that the detected multiple polypeptides represented the proleolytic derivatives of a higher molecular weight subunit, resulting from the *in vitro* degradation demonstrated in our study. Several recent reports have shown that the lipovitellins of the silk worm, *Philosamia cynthia*, (Chino *et al.*, 1977), the mosquito, *Culex pipiens fatigens*, (Atlas *et al.*, 1978) and the toad, *Xenopus laevis*, (Bergink and Wallace, 1974 ; Ohlendorf *et al.*, 1977) are composed of one high molecular weight subunit and one or two low molecular weight subunits, similar to the structure of *Artemia* lipovitellin.

As in the case of other invertebrate lipovitellins, the *Artemia* lipovitellin consists of a glycoprotein moiety (carbohydrate content of about 3%) complexed with approximately 9% of various neutral lipids and phospholipids (Table I). The red-orange coloration of this lipovitellin is caused by the presence of canthaxanthin at a carotenoid/protein ratio of about 0.3-0.4% (w/w). Since the apparent molecular weight of the native lipovitellin molecule has been estimated to be $0.6\text{-}0.7 \times 10^6$ on the basis of gel filtration chromatography (on Bio-gel A1.5m) and sedimentation analysis in sucrose gradients each native lipovitellin molecule appears to contain two molecules of each of the 190 000 and 68 000 apoprotein subunits. Thus, it seems likely that the native lipovitellin is dimeric and that each monomer contains one set of these apoprotein subunits.

Finally, LV-δ (Fig. 5 and 6) appears to be an independent, minor lipovitellin component, because the amino acid composition as well as the CNBr-peptides and *Staphylococcus* protease-peptides are distinctly different from those of LV-α1 and LV-ε (de Chaffoy and Kondo, 1980). On the other hand the minor components, LV-β and LV-γ may possibly be degradation products of α proteins.

Acknowledgements

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The 27 000-Mr protein of the 19 S cytoplasmic complex of *Artemia* is one of the major RNA-binding proteins

Erik De Herdt, Herman Slegers, and Masatoshi Kondo[1]

Departement Celbiologie, Universiteit Antwerpen
Universiteitsplein 1, B-2610 Wilrijk, Belgium

Abstract

Small free cytoplasmic particles of the cryptobiotic gastrulae of *Artemia* have been investigated. A unique 19 S protein complex with a diameter of *circa* 15 nm has been found to exist in a large quantity in the gastrulae cytoplasm. The 19 S protein complex has an estimated molecular weight of *circa* 0.6×10^6 and bands at a buoyant density of 1.25-1.26 (g/cm^3) in sucrose or 1.31 (g/cm^3) in CsCl, respectively. Protein analysis has indicated that a slightly acidic 27 000-Mr protein is an exclusive constituent of the 19 S protein through extensive intramolecular disulfide bridges by virtue of the presence of about five cystein residues per molecule.

By affinity chromatography on *E. coli* ribosomal RNA covalently linked to Sepharose 4B, at least four distinct protein complexes have been isolated from the cytoplasm of the cryptobiotic gastrulae. One of the most prominent protein complexes eluted at about 0.14 M KCl, sediments at 19 S in sucrose gradients and contains only a 27 000-Mr protein upon sodium dodecyl sulphate gel analysis. Immunological examination using rabbit anti-19 S complex IgG fraction has shown the identity of the 27 000-Mr RNA-binding protein with the previously characterised 27 000-Mr protein of the 19 S complex. Although this protein exhibits a binding affinity for RNA, none of the protein components associated with the repressed polyadenylated messenger ribonucleoprotein particles of the cryptobiotic gastrulae has been demonstrated to be identical to the 27 000-Mr protein by immunological as well as electrophoretical analyses.

Finally, it has been shown by immunological cross-reaction that the 27 000-Mr protein is closely related with *Artemia* elongation factor eEF-Ts, of protein synthesis.

Introduction

Encysted cryptobiotic gastrulae of *Artemia* offer a unique opportunity for biochemists to study various aspects of the organisation, function and regulation of cellular components. They are a suitable source for obtaining free polyadenylated ribonucleoproteins, which are transiently in a functionally repressed state due to their association with a small translational inhibitor RNA (Slegers and Kondo, 1977 ; Slegers *et al.*, 1977), free non-polyadenylated ribonucleoproteins, which are found in a template active state because of their insensitivity to this inhibitor RNA (De Herdt *et al.*, 1979cd), membrane-bound polyadenylated ribonucleoproteins (Simons *et al.*, 1978), or free 80 S ribosomes, which are readily active in protein biosynthesis (Sierra *et al.*, 1974).

[1] To whom correspondence should be addressed.

Besides these cellular components, it has long been recognized in this laboratory that a 19 S component exists in abundance in the cytoplasmic extract. We have now purified this 19 S component completely free from ribonucleoprotein particles as well as other small protein complexes and investigated in detail its biochemical, biophysical and immunological nature. This cytoplasmic component has been demonstrated to be a pure 19 S protein complex composed of a single protein species of Mr 27 000 (De Herdt *et al.*, 1979a). This paper presents further characterisation of the 27 000-Mr protein, and the results are discussed especially with respect to the elongation factor Ts and RNA-binding proteins of *Artemia*.

Materials and methods

Purification of the 19 S protein complex

From the crude cytoplasmic extract (postnuclear supernatant) of the cryptobiotic gastrulae, the postmitochondrial supernatant (PMS) and postribosomal supernatant (PRS) were prepared by differential centrifugation in an appropriate buffer (as indicated in the figure legends) essentially according to De Herdt *et al.* (1979a) and Slegers *et al.* (1979ab). The 19 S complex was purified in the same manner as described in De Herdt *et al.* (1979a). Concentration of cytoplasmic or sucrose gradient fractions was accomplished by precipitation with 10% (w/v) polyethylene glycol (PEG) in the presence of 10 mM $MgCl_2$.

Centrifugation

Sucrose isopycnic and density gradient centrifugations were previously described in detail (Slegers and Kondo, 1977 ; De Herdt *et al.*, 1979a). Convex sucrose density gradients from 5% to 50% in buffer A were constructed by preparing linear sucrose gradients of 5% to 45% and 45% to 50% in the upper-half and lower-half of the centrifuge tube, respectively. Centrifugation was carried out at 4 °C in a Beckman SW 27 rotor at 130 000 × g for 16 hr. The gradient fractions were collected after centrifugation by giving an appropriate air pressure on top of the gradient and the absorption at 254 nm was recorded by a LKB UVICORD II.

Analytical methods

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS) or of urea at pH 2.4 or pH 9.6 was performed as described in De Herdt *et al.* (1979ad). Alkylation of protein samples was done according to Weber *et al.* (1972). The presence of disulfide bridges was detected by the reaction with phenazine methosulfate after treatment with bisulfite. Amino acid analysis was carried out exactly as described by Moens and Kondo (1978). Double immunodiffusion test was performed according to De Herdt *et al.* (1979a).

Preparation of rabbit anti-19 S antibodies

The highly purified 19 S complex was the source of antigen and injected with complete Freud adjuvant into adult rabbit. A partially purified γ-immunoglobulin (IgG) was prepared from the crude antiserum as in De Herdt *et al.* (1979a). The IgG fraction was purified by affinity chromatography with the purified 19 S complex coupled to Sepharose 4B.

Rabbit antiserum raised against *Artemia* $EF\text{-}1_H$ and eEF-Ts were kindly provided by Drs. W. Möller, L. Slobin and K. Roobol.

RNA-Sepharose affinity chromatography

Ribosomal RNA was isolated from either *E. coli* ribosomes or *Artemia* ribosomes by SDS/phenol/chloroform as described in Slegers *et al.* (1977). Coupling of ribosomal RNA to cyanogen bromide-activated Sepharose 4B was conducted essentially according to Ovchinnikov *et al.* (1978a). The efficiency of RNA coupling to Sepharose was estimated to be about 85%.

After packing RNA-Sepharose resin into a column (0.8 cm × 7 cm), it was sufficiently washed first with buffer O and then with buffer N. The PRS previously dialyzed against buffer N at 4 °C was charged onto the RNA-Sepharose affinity column and the unbound proteins were completely eliminated by washing with buffer N. The column was then developed with a linear KCl gradient (10 mM-600 mM) in buffer N at 4 °C. Fractions were analysed by SDS-gels, sucrose gradients and double immunodiffusion as described above.

Electron microscopy

The purified samples of the 19 S complex were negatively stained with 2% uranyl acetate solution for 30 sec and used for electron microscopy performed with a JEOL-10B.

Buffers

Buffer A, 35 mM Tris-HCl (pH 7.5)/20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid)/70 mM KCl/9 mM $MgCl_2$; buffer C, 10 mM sodium phosphate (pH 6.8)/5mM $MgCl_2$/50 mM NaCl; buffer D, 1 mM sodium phosphate (pH 6.8)/0.1 mM $MgCl_2$; buffer N, 10 mM triethanolamine-HCl (pH 7.8)/1.5 mM $MgCl_2$/10 mM KCl; buffer O, 10 mM KCl in buffer N replaced by 1 M KCl.

Materials

Encysted cryptobiotic gastrulae of *Artemia* were purchased from Metaframe, San Francisco Bay Brand (Newark, California, USA). CNBr-activated Sepharose 4B and molecular weight reference proteins were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Polyethylene glycol was purchased from Merck (Darmstadt, Fed. Rep. Germany). Pancreatic RNAase A and calf thymus histones were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Triton X-100 was from R. Coles N.V. (Diegem, Belgium). Uranyl acetate was from Fluka, A.G. (Switzerland).

Results

Identification of a 19 S component in the cryptobiotic gastrulae cytoplasm

Triton X-100 (5%) treated cytoplasmic extract (PMS) of the cryptobiotic gastrulae of *Artemia* gives only one distinct ultraviolet absorbing peak (peak 2) between ribosomal (peak 1) and soluble fractions upon preparative sucrose isopycnic centrifugation (Fig. 1a). The

detergent-resistant peak fraction was found to band at a buoyant density of 1.25-1.26 (g/cm³) in sucrose by analytical isopycnic centrifugation under the condition, where ribosomes band at 1.35-1.36 (g/cm³) (Slegers and Kondo, 1977). It contains predominantly a single component sedimenting at about 19 S besides contaminating ribosomal particles (Fig. 1b). In the absence of Triton X-100 essentially the same result was obtained as above except that several other detergent-sensitive minor peaks were present at lower densities [1.20-1.22 (g/cm³)] (De Herdt *et al.*, 1979a).

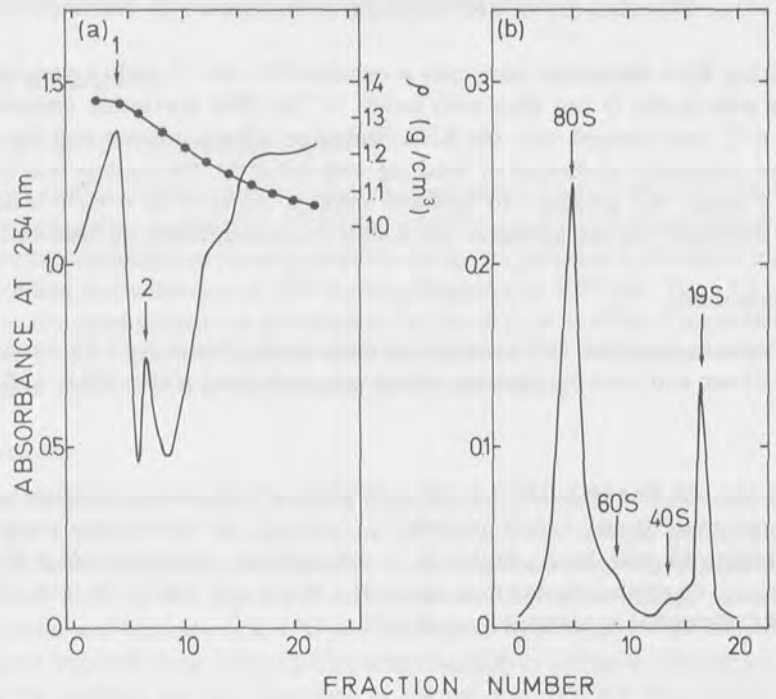

FIG. 1. Demonstration of a detergent-resistant 19 S component. (a). The PMS prepared in buffer A in the presence of 5% Triton X-100 was analysed by sucrose isopycnic centrifugation which was performed at 4 °C in a Beckman SW 41 rotor at 210 000 × g for 40 hr. Sucrose layers were made also in buffer A. Absorbance at 254 nm (——) and buoyant density (• —— •). (b). The pooled fraction of the peak 2 in (a) was first pelleted by centrifugation in a Beckman R 60 rotor at 210 000 × g for 14 hr., followed by suspending the pellet in buffer A. This suspension was then analysed by a linear 5-20% sucrose gradient in buffer A. Centrifugation was carried out in a Beckman SW 41 rotor at 290 000 × g for 100 min. Absorbance at 254 nm (——).

The abundant presence of the detergent-resistant 19 S component in the cytoplasm was also demonstrated by direct centrifugation of PMS in 5-50% convex sucrose density gradients. As shown in Fig. 2, the 19 S component was clearly seen in these gradients and a minor peak recognised at 10-11 S region (Fig. 2a) was completely sensitive to Triton X-100 (Fig. 2b), which was likely to correspond to those detergent-sensitive peaks of sucrose isopycnic centrifugation.

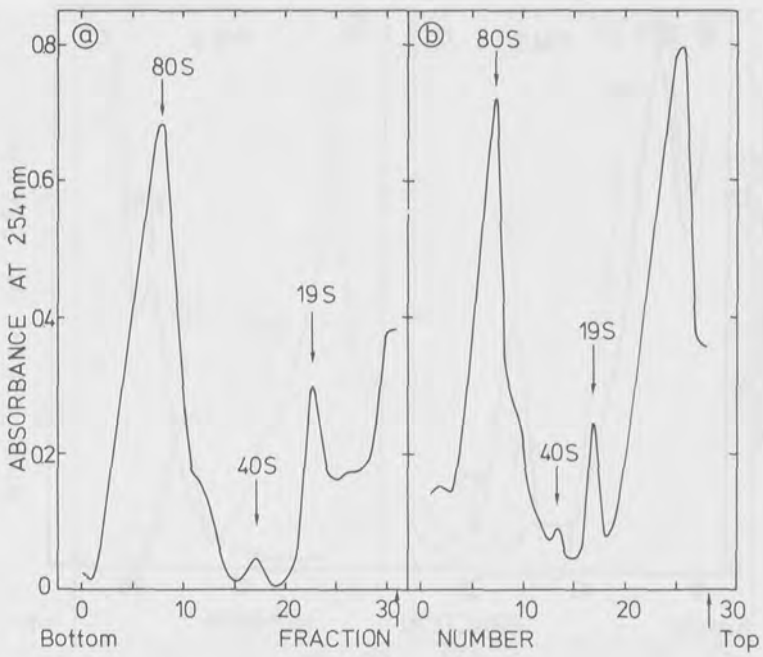

FIG. 2. Identification of the 19 S component by convex sucrose density gradients. The PMS prepared in buffer A in the absence (a) or presence (b) of 5% Triton X-100 was analysed by convex sucrose density gradients constructed as described in Materials an Methods. Absorbance at 254 nm (——).

Since the 19 S component was present in abundance, a technique of differential centrifugation (360 000 × g, 50 min) was tried to eliminate ribosomal particles from the PMS. The pellet of this centrifugation contained practically all the ribosomes with only a minor quantity of the 19 S component (Fig. 3a), whereas the ribosome-free supernatant (PRS) could recover most of the 19 S component (Fig. 3b). Therefore, the differential centrifugation was routinely employed to prepare this component in subsequent experiments. Further enrichment of the 19 S component was achieved by 10% (w/v) PEG precipitation of the PRS, by which a greater portion of 5-6 S material (Fig. 3b) was preferentially eliminated (De Herdt *et al.*, 1979a).

The detergent-resistant 19 S component was found to be also resistant to pancreatic RNAase A treatment (12 μg/ml at 37 °C for 10 min) as seen in Fig. 4b. Furthermore, the possibility of the 19 S component being a ribonucleoprotein complex was ruled out by the following evidence : (a) negative result by orcinol test, (b) a buoyant density of 1.31 (g/cm^3) in CsCl for the formaldehyde fixed 19 S component, and (c) no template activity in a cell-free translation system (De Herdt *et al.*, 1979a). Therefore, it has been concluded that the 19 S component represents a pure protein complex.

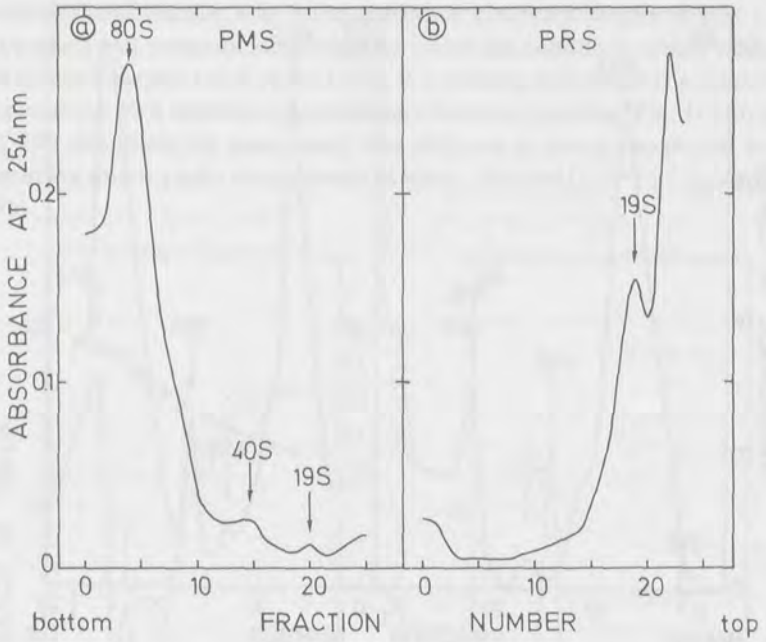

FIG. 3. Differential centrifugation of the 19 S component. (a). The PMS prepared in buffer A was centrifuged to pellet ribosomes in a Beckman R 60 rotor at 360 000 × g for 50 min. The ribosomal pellet was suspended in buffer D and analysed by a linear 5-20 % sucrose density gradient in buffer D. Centrifugation was performed in a Beckman SW 41 rotor at 260 000 × g for 179 min. (b). Small cytoplasmic particles in the PRS derived from (a) were pelleted by centrifugation in a Beckman R 60 rotor at 360 000 × g for 6 hr. The pellet was suspended in buffer D and analysed as in (a). Absorbance at 254 nm (——).

BIOCHEMICAL AND PHYSICO-CHEMICAL ANALYSES OF THE 19 S PROTEIN COMPLEX

The purified 19 S protein complex was examined under electron microscope. As shown in Fig. 5, the preparation consisted of a homogeneous population of spherical particles of a uniform size (15 ± 1.5 nm in diameter). Surprisingly, upon SDS-polyacrylamide gel analysis, a 27 000-Mr protein was found to be essentially a sole component of the 19 S complex (Fig. 6). Physico-chemical analyses of the 19 S protein complex indicated an approximate molecular weight of 0.6×10^6 (De Herdt *et al.*, 1979a). Thus, about twenty molecules of the 27 000-Mr protein were estimated to constitute the 19 S protein complex.

The amino acid composition of the 19 S complex is presented in Table I. The 27 000-Mr protein is a slightly acidic protein having about five cystein residues, which apparently form very extensive intramolecular disulfide bridges in the 19 S protein complex. The presence of disulfide bonds was confirmed by chemical analysis (De Herdt *et al.*, 1979a). This nature of the 19 S complex is well illustrated in Fig. 7, in which one part of the highly purified 19 S complex was reduced and alkylated, and both alkylated and non-alkylated samples were electrophoresed either in acid-urea gels (pH 2.4) or in basic-urea gels (pH 9.8). The reduced

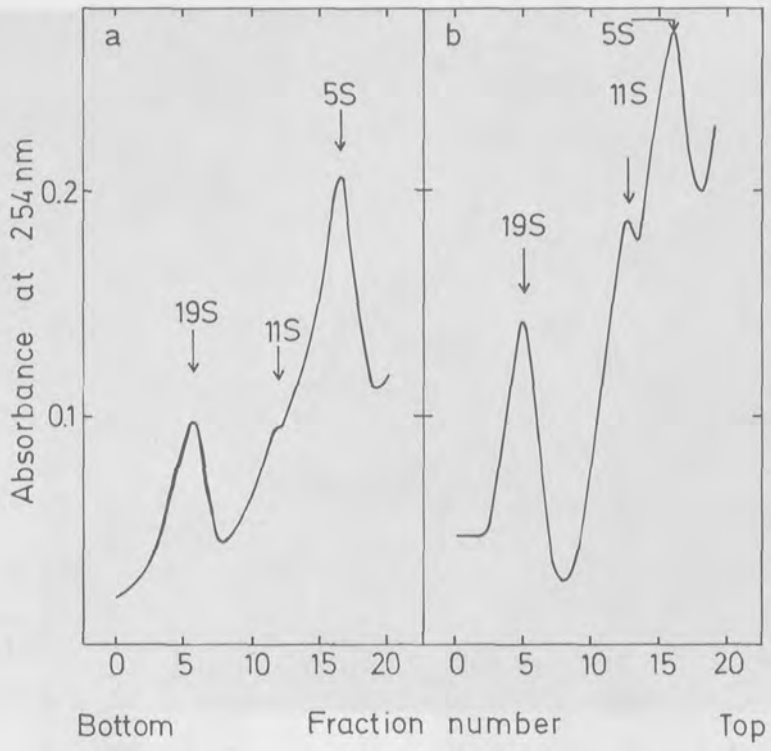

FIG. 4. Effect of RNAase A on the 19 S component. A preparation of the small cytoplasmic particles prepared as in Fig. 3b was divided into two parts. (a). One part was directly analysed by a linear 5-20% sucrose density gradient in buffer D. Centrifugation was done in a Beckman SW 41 rotor at 180 000 × g for 16 hr. (b). The second part was treated by pancreatic RNAase A (12 μg/ml) at 37 °C for 10 min before sucrose density gradient analysis. Absorbance at 254 nm (——).

19 S complex was able to enter only basic-urea gels and migrated as one major band with a few minor slowly migrating ones (gel 5, Fig. 7). The minor bands were possibly corresponding to the incompletely reduced 19 S complex. On the other hand, the non-reduced 19 S complex could not enter basic gels at all, indicating the failure of dissociation of this protein complex in the absence of a reducing agent. In acid-urea gels even the reduced sample of the 19 S complex (27 000-Mr protein) did not migrate into the gels (gel 2, Fig. 7).

THE 27 000-MR PROTEIN IS A RNA-BINDING PROTEIN

Certain cytoplasmic proteins have been shown to have the capacity to bind specifically to RNA *in vitro* in several eukaryotic systems (Blanchard *et al.*, 1974 ; Egly *et al.*, 1974 ; Liautard *et al.*, 1976 ; Sundquist and Persson, 1977 ; Ovchinnikov *et al.*, 1978a). In order to test the possibility that the *Artemia* 27 000-Mr protein be one of the cytoplasmic RNA-binding

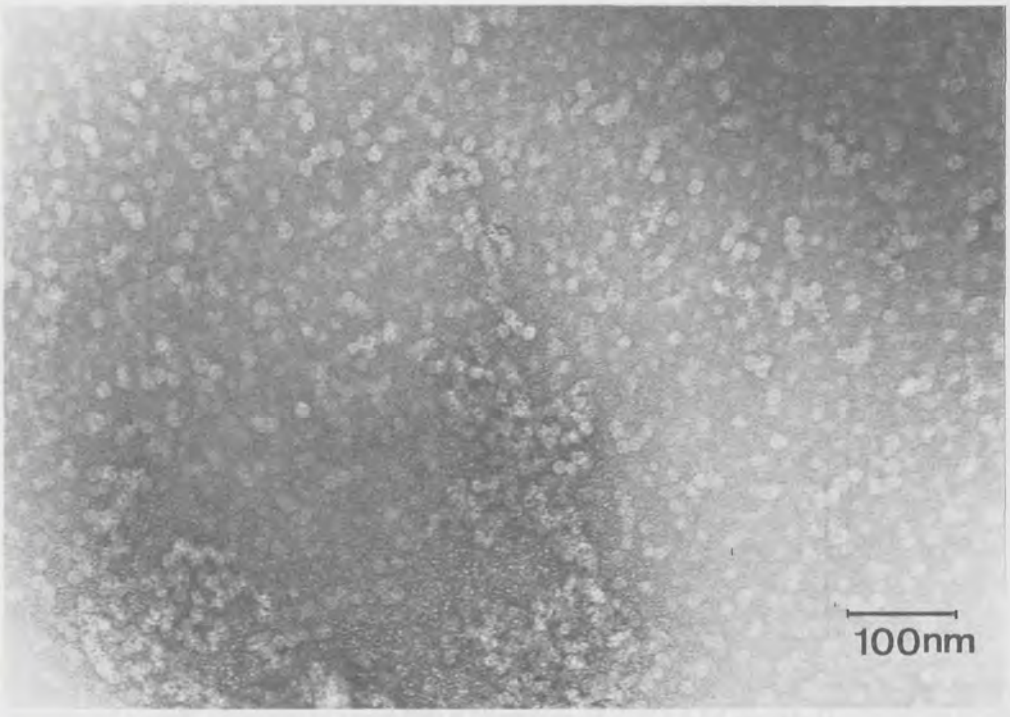

FIG. 5. Electronmicroscopic examination of the purified 19 S protein complex. Samples of the 19 S complex were prepared and examined by electron microscopy as described in Materials and Methods. A scale of 100 nm is indicated in the figure.

proteins, a technique of affinity chromatography on *E. coli* ribosomal RNA coupled to Sepharose 4B has been employed (De Herdt *et al.*, 1979b). After removing the unbound material of the ribosome-free cytoplasmic extract (PRS), the affinity column was developed by a KCl-gradient to elute the bound proteins as shown in Fig. 8. As in the case of rabbit reticulocyte RNA-binding proteins (Ovchinnikov *et al.*, 1978a), the majority of the bound proteins was eluted at 0.3 M KCl and the elution was practically completed at 0.5 M KCl (Fig. 8).

Each pool of three fractions of the affinity chromatogram was then analyzed by SDS-polyacrylamide gel electrophoresis. As illustrated in Fig. 9, a protein with an apparent molecular weight of 27 000 was largely eluted at fractions between 10 and 15. In order to ascertain the possible identity between the 27 000-Mr protein of the 19 S complex and thatof RNA-binding proteins, double immunodiffusion test was performed for several fractions of the affinity chromatogram against the purified rabbit anti-19 S complex IgG fraction (Fig. 10). It is quite evident that the intensity of the immune complex formed (Fig. 10) is proportional to the quantity of the 27 000-Mr protein present in the fractions of RNA-binding proteins (Fig. 9).

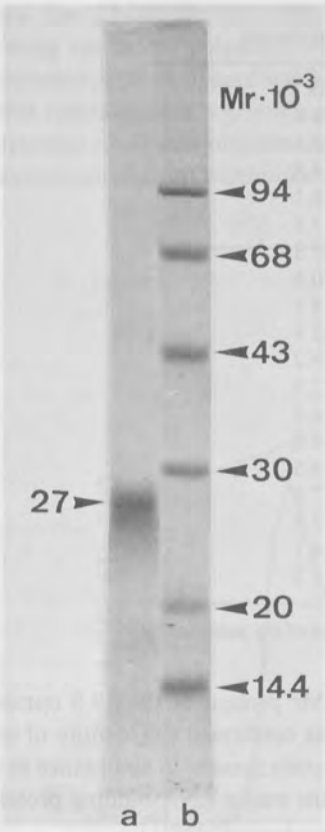

FIG. 6.

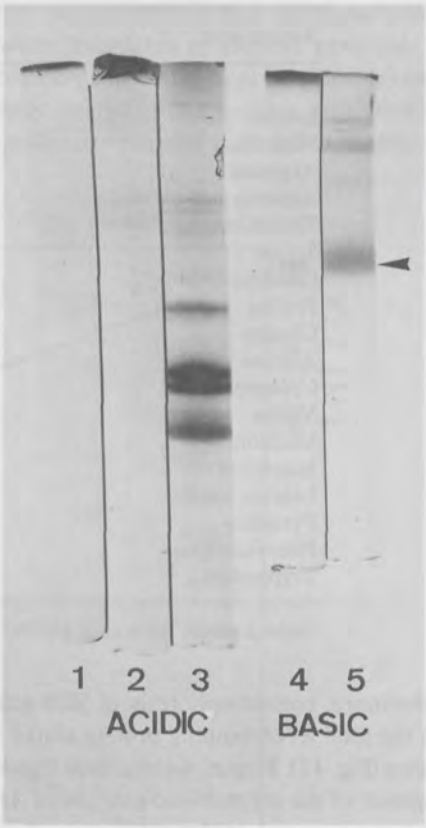

FIG. 7.

FIG. 6. SDS-polyacrylamide gel analysis of the purified 19 S complex. The acid-aceton precipitate (Barriault *et al.*, 1976) of the 19 S complex was dissolved in a buffer (80 mM Tris-HCl (pH 7.8)/ 100 mM dithiothreitol/4% SDS/10% glycerol) and electrophoresed in 10% gels (0.6 cm × 10 cm) at 2 mA/gel for 4 hr., essentially according to Studier (1973). (a). The 19 S complex protein component, (b). molecular weight reference proteins : rabbit muscle phosphorylase b (Mr 94 000), bovine serum albumin (Mr 68 000), egg white ovalbumin (Mr 43 000), bovine erythrocyte carbonic anhydrase (Mr 30 000), soybean trypsin inhibitor (Mr 20 000) and bovine milk α-lactalbumin (Mr 14 400).

FIG. 7. Analysis of the 19 S complex by acid or basic-urea gels. Non-alkylated samples (20 μg, gel 1, and gel 4) and alkylated ones (20 μg, gel 2, and gel 5) were analyzed by acid-urea gels (for gels 1-3) or basic-urea gels (for gels 4 and 5) as described in Materials and Methods. Gel 3 contained 30 μg of calf thymus histones. Arrow demonstrates the major protein band migrated into the basic-urea gel (gel 5). Taken from De Herdt *et al.* (1979a) with kind permission of the publisher.

TABLE I

Amino acid composition of *Artemia* 19 S complex

Amino acid	19-S complex
	mol %
Lysine	6.6
Histidine	3.5
Arginine	4.8
Aspartic acid	10.1
Threonine	3.8
Serine	7.8
Glutamic acid	10.4
Proline	4.1
Glycine	5.9
Alanine	9.2
Cysteine	2.3
Valine	6.2
Methionine	4.0
Isoleucine	5.5
Leucine	7.9
Tyrosine	2.8
Phenylalanine	4.1
Tryptophan	1.2

Taken from De Herdt *et al.* (1979a) with kind permission of the publisher.

Furthermore, coelectrophoresis in SDS-gels of the 27 000-Mr protein of the 19 S complex with the total RNA-binding protein eluted with 1 M KCl has confirmed the identity of both proteins (Fig. 11). Hence, we conclude that the 27 000-Mr protein present in abundance in the cytoplasm of the cryptobiotic gastrula of *Artemia* is one of the major RNA-binding proteins.

THE 27 000-MR PROTEIN IS NOT AN INFORMOSOMAL PROTEIN

It has been reported that RNA-binding proteins of frog, *Rana temporaria*, isolated by affinity chromatography on poly(U)-Sepharose can specifically be incorporated *in vivo* into newly formed informosomes in frog oocytes (Elizarov *et al.*, 1978). Since the cytoplasm of the cryptobiotic gastrulae of *Artemia* contains large amounts of the 27 000-Mr protein (Fig. 1, 2, and 3), and since this protein is a RNA-binding protein (Fig. 8 and 9), it has been conceivable that the 27 000-Mr protein may be involved in the formation of free cytoplasmic informosomes stored in a repressed state in these gastrulae (Slegers and Kondo, 1977 ; Slegers *et al.*, 1979ab).

A protein corresponding to a molecular weight of 27 000 has been observed by SDS-gel analysis of polyadenylated ribonucleoproteins stored in the cryptobiotic gastrulae, especially those sedimenting below 10 S (Slegers *et al.*, 1979ab).

However, the conclusive evidence that the 27 000-Mr protein associated with the ribonucleoprotein particles (informosomes) is not equivalent to the 27 000-Mr RNA-binding protein has been provided by immunological test. The total protein extracted from the polyadenylated ribonucleoproteins was examined by double immunodiffusion against the rabbit

anti-19 S complex IgG. As is evident from Fig. 12, no cross reaction can be detected under conditions, in which the 27 000-Mr protein of the 19 S complex forms a clear precipitin arc. Thus, the 27 000-Mr RNA-binding protein is not represented at least by those proteins forming the stored polyadènylated ribonucleoprotein complexes in *Artemia* gastrulae. This conclusion appears to conform with the finding that only one component (49 000-Mr) among rabbit reticulocyte RNA-binding proteins is possibly related to the proteins associated with polysomal ribonucleoproteins and that none of the former proteins is found in nuclear 30 S ribonucleoproteins (Ovchinnikov *et al.*, 1978b).

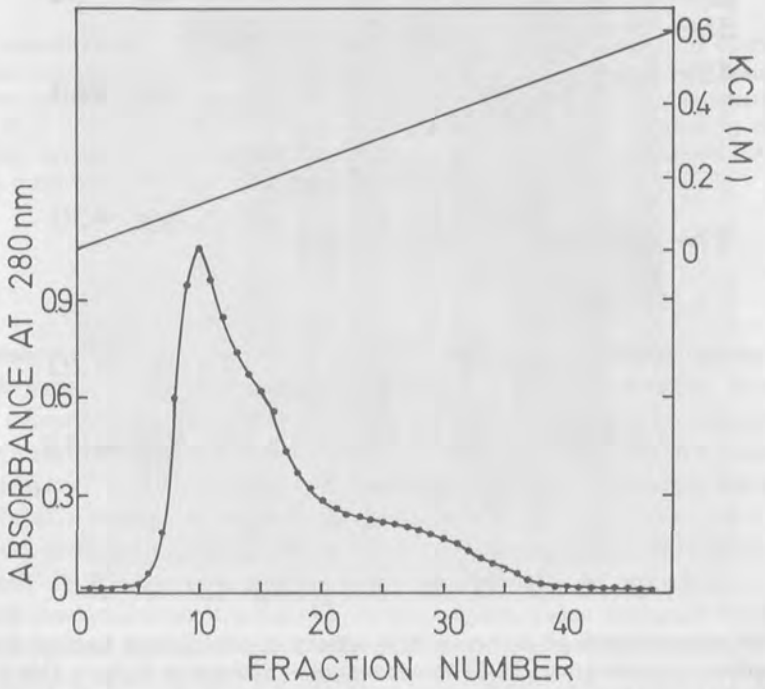

FIG. 8. Affinity chromatography of *Artemia* extract on *E. coli* ribosomal RNA-Sepharose. The PRS prepared in buffer C was dialysed at 4 °C overnight against buffer N before charging and was chromatographed as described in Materials and Methods. Absorbance at 280 nm (● —— ●).

THE 27 000-MR PROTEIN IS STRUCTURALLY RELATED TO *ARTEMIA* ELONGATION FACTOR TS

Artemia translation elongation factor 1 exists apparently as a high molecular weight complex (11 S EF-1_H) in the cryptobiotic gastrulae (Nombela *et al.*, 1976 ; Slobin and Möller, 1976) as in other eukaryotic organisms (Moon *et al.*, 1973 ; Bolla *et al.*, 1975 ; Grasmuk *et al.*, 1976 ; Lanzani *et al.*, 1976 ; Ejiri *et al.*, 1977 ; Nagata *et al.*, 1978). The 11 S EF-1_H seems to be an aggregated form of elongation factors, eEF-Tu, eEF-Ts and one extra polypeptide of unknown function (Mr 51 000) (Ejiri *et al.*, 1977 ; Slobin and Möller, 1978), whose amino acid composition is rather similar to that determined for the 27 000-Mr protein (Table I).

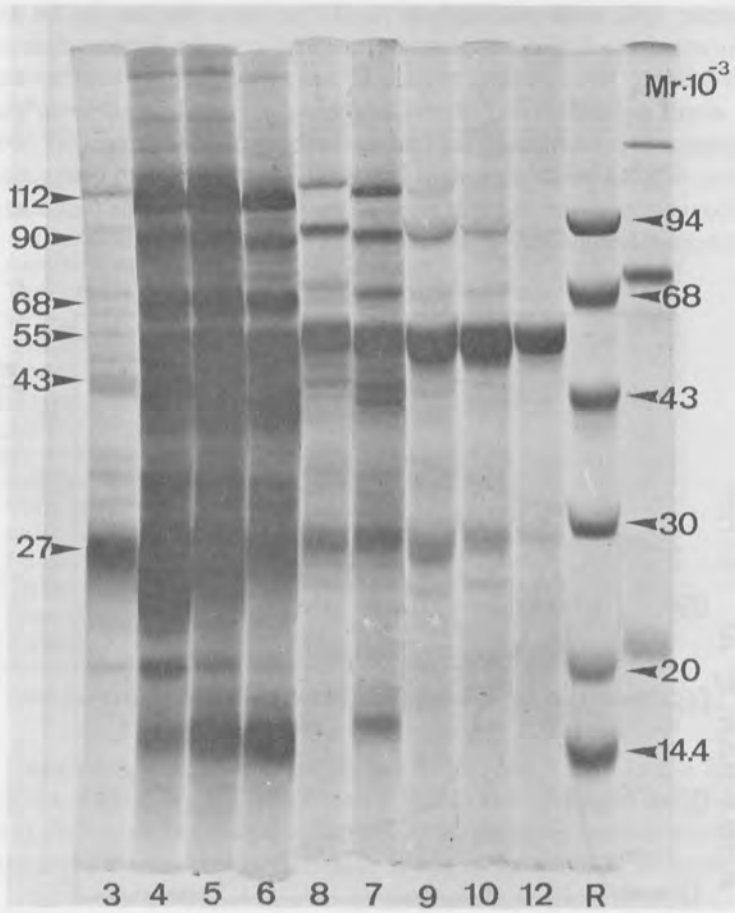

FIG. 9. SDS-polyacrylamide gel analysis of RNA affinity chromatography fractions. Pools of three fractions of affinity chromatogram (Fig. 8) were examined by SDS-gels as in Fig. 6. Gels 3 through 12 correspond to the pooled fractions of 7-9, 10-12, 13-15, 16-18, 22-24, 19-21, 25-27, 28-30, 31-33, and 34-36 (Fig. 8), respectively. The gel marked R contained reference proteins as in Fig. 6.

Therefore the possible relationship between eEF-Ts and the 27 000-Mr protein has been investigated by immunological cross-reactivity test. The result in Fig. 13a demonstrates the formation of a single continuous precipitin arc against rabbit anti-EF-1_H antiserum with the 27 000-Mr protein of the 19 S complex, partially purified eEF-Ts and the RNA-binding protein fraction containing the 27 000-Mr one. Furthermore, these same protein preparations have also produced a continuous precipitin arc against the rabbit anti-19 S complex IgG fraction (Fig. 13b). In the latter case, a few extra precipitin lines are also seen with the eEF-Ts and the RNA-binding protein preparations, but not with the purified 19 S complex. These data strongly suggest that these two polypeptides are structurally related to each other.

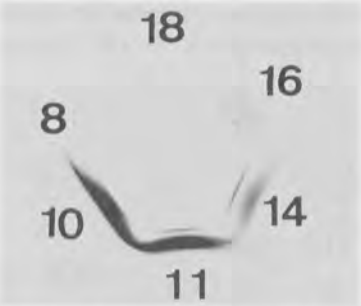

FIG. 10. Identification of the 27 000-Mr protein as a RNA-binding protein by double immunodiffusion test. Double immunodiffusion was performed at 4 °C for at least 24 hr against 60 μg of purified rabbit anti-19 S IgG fraction which was placed in the center well. The peripheral wells were filled with 20 μl of several affinity chromatography fractions (Fig. 8) as marked by their fraction number. After developing, the plate was washed with 50 mM sodium barbital (pH 8.6) containing 0.9% NaCl, dried with filter paper and stained with amido black.

Discussion

The existence of large quantities of the unique 19 S component in the cytoplasm of *Artemia* cryptobiotic gastrulae has been established since 1973 in this laboratory. However, the conclusive demonstration that the 19 S component constitutes a pure protein complex was delayed by a persistent residual contamination in the 19 S preparation of a template active non-polyadenylated 22 S ribonucleoprotein particle containing 9 S messenger RNA (De Herdt *et al.*, 1979acd). Evidence presented in this paper casts some insight into several interesting characteristics of the sole constituent of the 19 S protein complex, the 27 000-Mr protein, with respect to eEF-Ts, RNA-binding proteins as well as informosomal proteins.

The recent observation that *Artemia* eEF-Ts has a slightly higher molecular weight (30 000-31 000) (Roobol and Möller, personal communication ; our unpublished results) than the value initially published (Slobin and Möller, 1978) indicates the existence of the obvious difference in molecular size between the 27 000-Mr protein and eEF-Ts, *e.g.* the former protein being shorter by about 25-35 amino acid residues. This possibly suggests that the 27 000-Mr protein is produced from eEF-Ts by specific proteolytic cleavage. At this moment, we do not have direct data to verify this attractive hypothesis.

The fact that the 27 000-Mr protein is not involved in the formation of the repressed polyadenylated ribonucleoproteins stored in the cryptobiotic gastrulae (Fig. 12) might suggest other functions for this cytoplasmic protein. Considering the abundant presence in the cytoplasm, the 27 000-Mr protein forming a specific 19 S complex may possibly be involved somehow in cytoskeletal structures of dried encysted gastrula embryos. The study in this line is being conducted in this laboratory.

Although the 27 000-Mr protein is not an informosomal protein (*e.g.* non-messenger RNA associated protein), this protein has been found to be one of the major RNA-binding proteins isolated by *E. coli* ribosomal RNA-Sepharose affinity chromatography (Fig. 9). This capacity of the 27 000-Mr protein to interact with structural RNAs might possibly be connected with

the above-mentioned hypothesis, *e.g.* as components of the cytoskeletal structure. These aspects of this specific protein component of the 19 S complex remain for further studies.

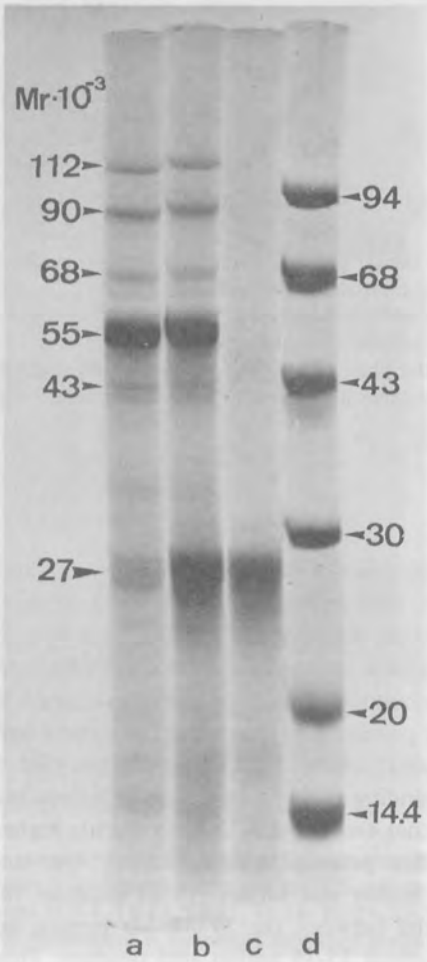

FIG. 11. Coelectrophoresis of the 27 000-Mr protein with RNA-binding proteins. The RNA-binding proteins obtained by elution with buffer O from the *E. coli* ribosomal RNA-Sepharose affinity column were compared with the 27 000-Mr protein of the 19 S complex by SDS-gels (as in Fig. 6). Gel a contained 30 μg of RNA-binding proteins ; gel c, 15 μg of the 19 S complex ; gel b, both proteins together ; and gel d, reference proteins. Taken from De Herdt *et al.* (1979a) with kind permission of the publisher.

Among RNA-binding proteins of *Artemia*, besides the 27 000-Mr protein, proteins with molecular weight of 43 000, 55 000, 68 000, 90 000, and 112 000 are the principal components, when the affinity column is developed by a linear gradient of KCl (Fig. 9). However, if the same column is eluted directly with 1 M KCl, the 55 000-Mr protein has been found to be the most prominent RNA-binding protein (Fig. 11). This observation parallels closely with the

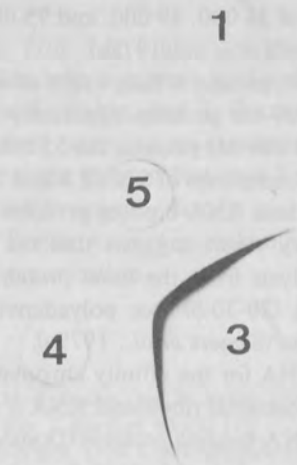

FIG. 12. Non-identity of the 27 000-Mr protein with polyadenylated ribonucleoprotein associated proteins. The proteins were prepared from the polyadenylated ribonucleoproteins purified by affinity chromatography on oligo(dT)-cellulose according to Slegers *et al.* (1979a). 10 μg of these proteins was placed in the wells 1 and 2, and 10 μg of the 19 S complex protein in well 3. The purified anti-19 S IgG (60 μg) was placed in the wells 4 and 5. Double immunodiffusion was performed as in Fig. 10. Taken from De Herdt *et al.* (1979a) with kind permission of the publisher.

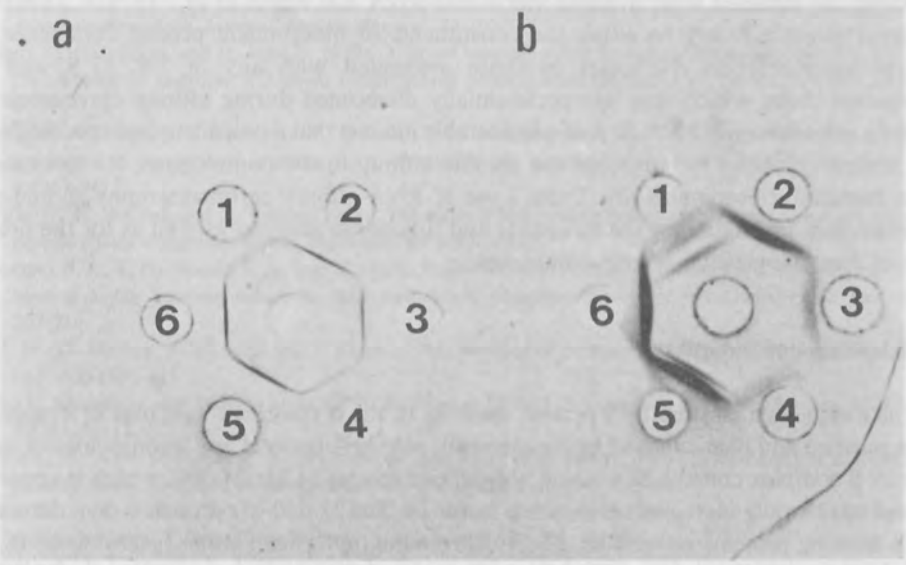

FIG. 13. Double immunodiffusion tests of the 27 000-Mr protein, eEF-Ts and RNA-binding proteins. The central wells of (a) and (b) contained 60 μg each of rabbit anti-EF-1_H antiserum and anti-19 S IgG, respectively. The wells 1 through 6 in both panels were placed with eEF-Ts (5μg), the 19 S complex (10 μg), (5 μg) and (5μg), RNA binding proteins (40 μg), and the 19 S complex (10 μg), respectivey. Double immunodiffusion was carried out as in Fig. 10.

report that the principal RNA-binding proteins of rabbit reticulocytes using the same method are those with molecular weights of 36 000, 49 000, and 95 000, the 49 000-Mr protein being the most prominent one (Ovchinnikov *et al.*, 1978a).

As in the case of the 27 000-Mr protein which exists as an aggregated form in the cytoplasm, the 68 000-Mr and 112 000-Mr proteins apparently form a single protein complex sedimenting at about 14 S. The 43 000-Mr protein, the 55 000-Mr protein and the 90 000-Mr protein similarly form the protein complexes of 5 S, 12 S and 7 S, respectively (De Herdt *et al.*, 1979e). The possible function of these RNA-binding proteins in cellular metabolisms has not yet been known. Our preliminary result suggests that the 68 000-Mr protein is possibly indistinguishable by SDS-gel analysis from the main protein component (Mr 68 000) associated with the fast sedimenting (20-30 S) free polyadenylate ribonucleoprotein particles stored in the cryptobiotic gastrulae (Slegers *et al.*, 1979a).

The use of *E. coli* ribosomal RNA for the affinity chromatography of eukaryotic proteins has been justified by the fact that bacterial ribosomal RNA is effectively able to compete with eukaryotic messenger RNA for RNA-binding proteins (Domogatsky *et al.*, 1978). We are able to support principally this conclusion by analysis of *Artemia* RNA-binding proteins isolated by affinity chromatography using homologous *Artemia* ribosomal RNA linked to Sepharose. The cytoplasmic complexes sedimenting at approximately 5 S, 7 S, 12 S, 14 S, and 19 S are recovered by their affinity to homologous ribosomal RNA as is found with *E. coli* ribosomal RNA. The protein components constituting the 7 S and the 19 S complexes are exactly those of Mr 55 000 and of Mr 27 000, respectively. However, in addition to those identified with *E. coli* ribosomal RNA (Fig. 9 and 11), extra polypeptides of Mr 48 000, Mr 76 000 and Mr 103 000 are retained with *Artemia* ribosomal RNA (De Herdt *et al.*, 1979e). Thus, these proteins might possibly be either the constituents of independent protein complexes with similar sedimentation coefficient or those associated with any of 5 S, 12 S, and 14 S complexes, from which they are preferentially dissociated during affinity chromatography with *E. coli* ribosomal RNA. It is of considerable interest that a polyadenylate-specific 76 000-Mr protein (Blobel, 1973) exhibits the specific affinity to the homologous, but not heterologous bacterial, ribosomal RNA. Thus, a use of RNA-affinity chromatography should prove an important technique for the structural and functional analyses as well as for the preparation of free cytoplasmic protein complexes.

Conclusions and summary

The unique cytoplasmic 19 S protein complex of the cryptobiotic gastrulae of *Artemia* has been purified and characterised by biochemical, physico-chemical and immunological means. The 19 S complex consists of a single polypeptide species of Mr 27 000, which is apparently related structurally to *Artemia* elongation factor Ts. The 27 000-Mr protein is one of the major RNA-binding proteins isolated by affinity chromatography with either heterologous (*E. coli*) or homologous (*Artemia*) ribosomal RNA. Despite its RNA binding capability, the 27 000-Mr protein has been found to be non-informosomal protein. The possible function of this unique protein in the cryptobiotic gastrulae is postulated.

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Protein and nucleic acid composition of free cytoplasmic messenger ribonucleoprotein particles isolated from *Artemia* cysts and nauplii

L. Felicetti, P. Pierandrei-Amaldi, and D. Griso

C.N.R. Laboratory of Cell Biology
Via Romagnosi 18/A. Rome, Italy

Abstract

Free cytoplasmic messenger ribonucleoprotein (mRNP) particles with sedimentation values lower than 40S were isolated from the postmicrosomal supernatant of *Artemia* cysts and nauplii by high speed centrifugation and separated according to their size by sucrose density gradient analysis. The particles appear to be a mixture of at least four discrete components with sedimentation values of 16S(P1), 9S(P2), 8S(P3) and 4S(P4) respectively. Each particle has a characteristic protein pattern when examined by acrylamide gel electrophoresis. The RNA extracted from the heavier particles (P1, P2 and P3) has an heterogeneous size distribution (18 to 4S) as determined by (^{3}H)-polyuridylic acid (polyU) hydridization and stimulates protein synthesis in a wheat germ cell-free system. The lighter particles (P4) contain RNA species devoid of template activity such as free polyadenylic acid (polyA) tails and tRNAs. During embryonic differentiation from gastrulae to nauplii, the pool of the heavier particles is depleted suggesting that they play a role in the activation of protein synthesis which follows rehydration of encysted embryos.

Introduction

In a previous paper we have shown that informational RNA is stored in the post-mitochondrial supernatant of *Artemia* cysts under the form of polyA-containing mRNP particles with sedimentation values ranging from 80S to 4S (Felicetti *et al.*, 1975). The polyA-containing mRNP particles can be found in the cytoplasm either free or bound to membrane and contain mRNA species of heterogeneous size which code for specific polypeptides in a wheat germ cell-free system (Nilsson and Hultin, 1974, 1975 ; Felicetti *et al.*, 1975 ; Grosfeld and Littauer, 1975 ; Sierra *et al.*, 1976 ; Grosfeld *et al.*, 1977 ; Simons *et al.*, 1978). Approximately 50% of the free cytoplasmic polyA-containing particles are complexed with ribosomes and ribosomal subunits : after EDTA treatment the heavier sedimenting particles (50 to 90S) can be converted to free 20-30S particles (Slegers and Kondo, 1977).

Ribosome-free mRNP particles with sedimentation values lower than 40S are very abundant in the cytoplasm of encysted embryos and can be isolated as discrete particles of various size by sucrose density gradient centrifugation without the use of high concentrations of chelating agents. This purification procedure has allowed us to study the protein and

nucleic acid content of these particles and to investigate their physiological role during embryonic development.

Materials and methods

Buffers

STNM : 150 mM sucrose, 30 mM Tris-HCl pH 7.4, 100 mM NaCl, and 10 mM $MgCl_2$; TNM : as above, with no sucrose ; TKE : 10 mM Tris-HCl pH 7.5, 500 mM KCl, and 2 mM EDTA ; NDS : 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, and 0.5% Na-dodecyl-sulfate.

Preparation of free cytoplasmic mRNP particles from cysts and nauplii

20 g *Artemia* cysts (San Francisco Bay brand, Newark, California) were hydrated for 20 min at 0 °C in artificial seawater (Hultin and Morris, 1968), washed several times in a Buchner funnel with seawater, rinsed with 200 ml of STNM buffer and finally ground with two volumes of the latter containing 500 μg/ml of heparin. The homogenate was filtered through cheesecloth, and centrifuged at 1 000 × g for 5 min and subsequently at 15 000 × g for 10 min. The postmitochondrial supernatant was spun at 133 000 × g for 2 hr in a 50 Ti rotor at 4 °C to separate the microsomes and ribosomal subunits. The upper 3/4 of the supernatant were carefully removed with a Pasteur pipette, the salt concentration brought to 0.5 M KCl with a 4 M KCl solution and the sample centrifuged at 225 000 × g for 5 hr in a SW 50.1 rotor at 4 °C. The mRNP pellets were used as the starting material for the following cycles of sucrose density gradient centrifugation.

To prepare mRNP particles from swimming nauplii, 40 g of cysts were distributed into four glass trays (40 × 60 × 5 cm) filled with 2 l of artificial seawater and incubated in a thermostatic chamber at 28 °C for 24 hr. At the end of the incubation, swimming larvae were concentrated in one section of the tray by their positive phototactic response to a light source (0.5 Watt electric bulb) and syphoned out. Nauplii were collected at low speed n conical centrifuge tubes, washed with STNM buffer and homogenized in a teflon-glass Thomas B with two volumes of the same buffer containing 500 μg/ml of heparin. The extracts were then submitted to differential centrifugation as described above.

Acrylamide gel electrophoresis of the protein moiety of mRNP particles

The fractions of the sucrose gradients containing mRNP particles of different sedimentation values (see diagrams in Fig. 3 and 6) were precipitated with two volumes of 95% ethanol, dried under vacuum, and finally dissolved in the electrophoresis buffer. Samples were loaded on 12% acrylamide slab gel according to Laemmli (1970).

RNA extraction

RNA was extracted from gradient fractions by the plenol-Na dodecylsulfate procedure (pH 9) as previously described (Felicetti *et al.*, 1975). Before ethanol precipitation the aqueous phase was shaken with two volumes of chloroform containing 1% isoamyl alcohol.

(^{3}H)-polyU hybridization assay

Intact mRNP particles or ethanol-precipitated RNA fractions from sucrose gradients were incubated with 20 000 cpm of (^{3}H)-polyU (New England Nuclear, sp. act. 5 Ci/mmol UMP) in a final volume of 0.5 ml as described by Rosbash and Ford (1974). When RNA fractions in NDS buffer were assayed directly, Na-dodecylsulfate was removed by adding K-acetate at a final concentration of 2%, the potassium salt centrifuged at 15 000 × g for 10 min and the clear supernatant incubated with (^{3}H)-polyU.

mRNA translation in a wheat germ lysate and electrophoretic analysis of translational products

Preparation of the wheat germ lysate and cell-free translation were carried out as previously described (Pierandrei-Amaldi *et al.*, 1977). 50 μl of incubation mixture were applied to 12% acrylamide slab gel as above. Fluorography of stained gels was carried out according to Laskey and Mills (1975).

Extraction of the protein moiety from the 16S mRNP particle

The P1 particle pelleted from two identical sucrose gradients (Fig. 6) was dissociated by the LiCl-urea method and dialyzed as described by Traub *et al.* (1971). The protein was further dailyzed against 10 mM Tris-HCl buffer pH 7.4, lyophilized and finally dissolved in sterile water.

Results

Free and ribosome-bound polyA-containing mRNP particles in the postmicrosomal supernatant of *Artemia* cysts

In preliminary experiments native subunits and mRNP particles were prepared from the postmicrosomal supernatant of encysted embryos by high speed centrifugation and analyzed by sucrose density gradients in low ionic strength buffer (TNM) as described in the legend to Fig. 1. In a typical experiment the 40S subunit sediments in the lower portion of the gradient, while the remaining UV adsorbing material shows sedimentation values lower than 40S with a prominent peak in the 20S region (Fig. 1). The gradient fractions were then tested for their ability to form ribonuclease-resistant hybrids with (^{3}H)-polyU, either directly or after RNA extraction. If the fractions are directly used for hybridization experiments, a peak of hybridizing activity is found in the region of the 40S subunit, with scarce activity left in the lighter regions of the gradient. After RNA extraction the hybridization pattern is completely reversed and lighter fractions have the highest capacity of hybridizing to (^{3}H)-polyU. Since the lighter particles do not contain significant ribonucleolytic activity (not shown) the most likely explanation of this result is that mRNP particles sedimenting in the 40S region have polyA tails which are accessible to labeled polyU, while lower sedimenting particles have masked polyA traits which are uncovered by RNA extraction.

In addition RNA was extracted from mRNP particles of different size and tested in a wheat germ cell-free system for its ability to stimulate (^{35}S)-methionine incorporation.

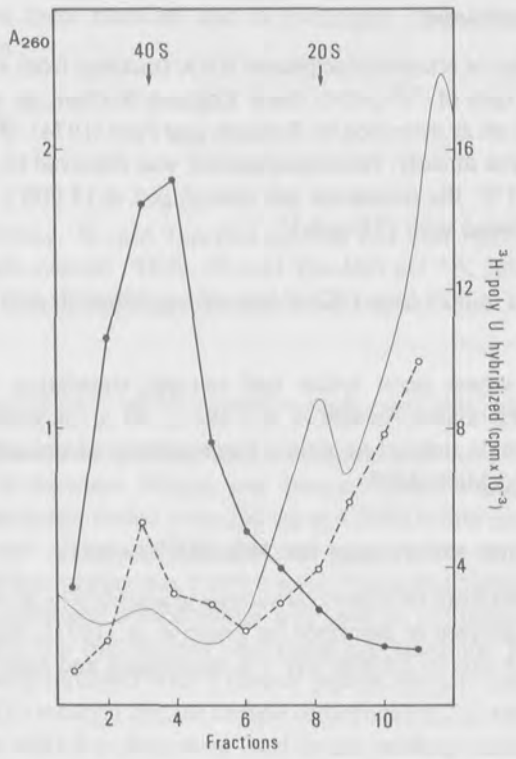

FIG. 1. Sedimentation pattern of polyA-containing mRNP particles from the postmicrosomal supernatant of dormant embryos. The postmitochondrial supernatant (see Materials and methods) was centrifuged at 133 000 × g for 60 min to remove to 80S monomers. The upper 3/4 of the supernatant were then spun at 225 000 × g for 5 hr. 20 A_{260}units of RNP pellets were applied to 16.5 ml 5-20% linear sucrose gradients in TNM buffer. Centrifugation was at 25 000 rpm for 16 hr in a SW 27.1 rotor at 4 °C. The A_{260} was automatically recorded by pumping the gradients from the bottom through a 0.2 cm flow cell of a Gilford spectrophotometer. Fractions of 1.5 ml were collected from two identical sucrose gradients. —— A_{260} ; ●——●——● 100 μl of each gradient fraction were directly used for the (^{3}H)-polyU hibridization assay ; ○ – – ○ – – ○ RNA was extracted from gradient fractions by the phenol-Na dodecylsulfate procedure (see Materials and methods), dissolved in 50 μl of sterile water and 10 μl aliquots tested for their ability to hybridize to (^{3}H)-polyU.

Fig. 2 shows the fluorography of translational products after slab gel electrophoresis : a characteristic set of proteins with a molecular weight ranging from 20 000 to 30 000 is synthesized in the cell-free system by RNA fractions 2 to 9 shown in Fig. 1. RNA extracted from the top fractions of the gradient (10 and 11) does not stimulate cell-free protein synthesis, although it contains polyadenylic acid.

In conclusion the mRNP particles isolated from the postmicrosomal supernatant have sedimentation values ranging from 40S to 4S and RNA extracted from the particles exhibit similar template activity in a wheat germ lysate (3 fold stimulation over the background level).

However the (^{3}H)-polyU binding capacity of native particles as compared to extracted RNAs is quite different for the heavier and the lighter mRNP particles. This observation suggests that free mRNP particles with a sedimentation value lower than 40S have a different structural conformation with polyA residues buried by peculiar protein-nucleic acid interactions.

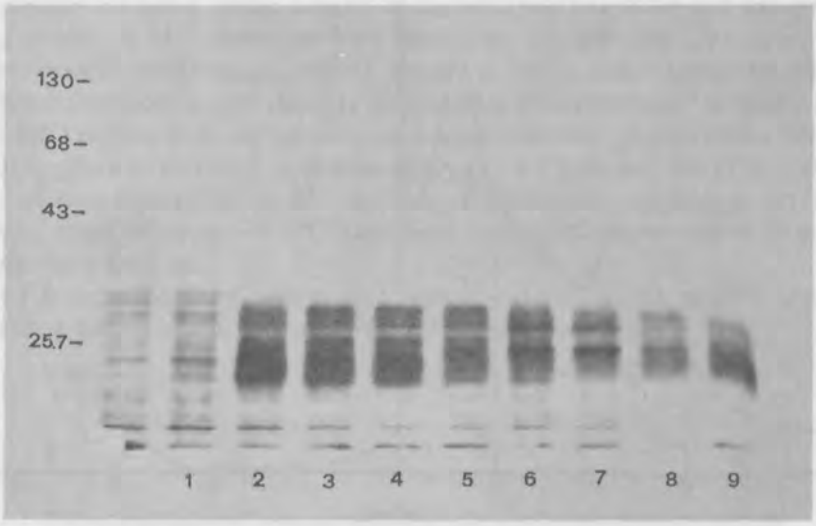

FIG. 2. Fluorography of (^{35}S)-methionine labeled polypeptides synthesized in a wheat germ lysate by RNA fractions corresponding to cyst mRNP particles of different size. RNA extracted from gradient fractions 1 to 9 (Fig. 1) was dissolved in 50 μl of sterile water and 10 μl aliquots tested for their template activity in a wheat germ cell-free system. Electrophoretic analysis and fluorography wer carried out as described in Materials and methods.

CHANGES IN THE SEDIMENTATION PATTERN OF FREE MRNP PARTICLES FROM THE POSTMICROSOMAL SUPERNATANT DURING EMBRYONIC DEVELOPMENT

Free mRNP particles were prepared from the postmicrosomal supernatant of cysts and nauplii as described in Methods. During high speed centrifugation the salt concentration was increased up to 0.5 M in order to remove supernatant proteins contaminating the mRNP particles. The mRNP pellets were then submitted to sucrose density gradient centrifugation in a high ionic strenght buffer (TKE) and separated according to their sedimentation values. The sedimentation patterns of mRNP particles from cysts and nauplii are shown in Fig. 3. The cyst supernatant contains mRNP particles with S values ranging from 30 to 4S, with a prominent peak at 20S and 4S. During embryonic development from gastrulae to nauplii a depletion of heavier mRNP particles is observed, while the lighter peak at the top of the gradient is preserved. This result suggests that heavier mRNP particles play a role in the activation of protein synthesis which follows rehydration of dehydrated embryos.

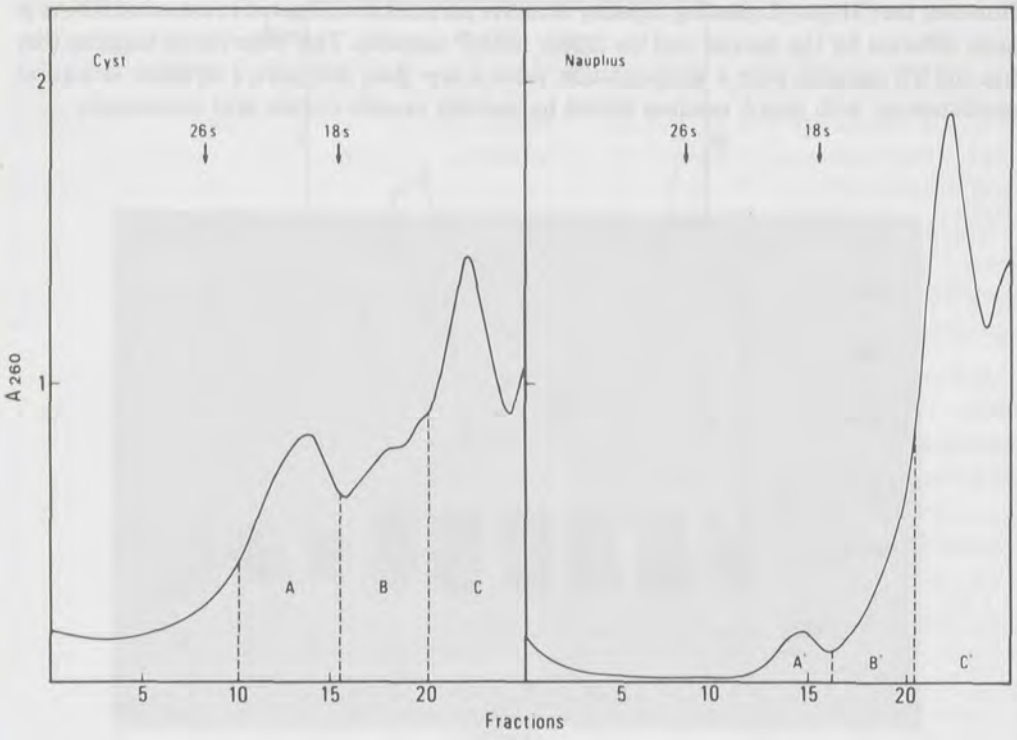

FIG. 3. Sedimentation patterns of free mRNP particles from the postmicrosomal supernatant of undeveloped and developed embryos. 20 to 30 A_{260} units of mRNP particles, prepared as described in Materials and methods, were applied to 16.5 ml 5-20% linear sucrose gradients in TKE buffer containing 100 μg/ml of heparin. Centrifugation was at 26 000 rpm for 16 hr in the SW 27.1 rotor at 4 °C. Fractions of 0.75 ml were collected from 3-4 sucrose gradients and pooled as outlined in the diagrams (Pool A, B and C).

PROTEIN AND NUCLEIC ACID CONTENT OF FREE MRNP PARTICLES OF DIFFERENT SEDIMENTATION VALUES FROM CYSTS AND NAUPLII

Gradient fractions corresponding to mRNP particles of different S values were pooled as shown in the diagrams of Fig. 3, precipitated with ethanol and the protein pattern analyzed by acrylamide gel electrophoresis (Fig. 4). It can be seen that the heavier particles from both cysts and nauplii (a,a') contain a characteristic protein band of 26 000 MW. The lighter particles show a polydisperse protein pattern ranging from 10 000 to 130 000 MW (b,b',c,c'). Although minor differences in the intensity of single bands are observed, the protein pattern of the mRNP particles does not undergo a significant change during embryonic development.

The RNA composition of cyst mRNP particles was investigated by extracting RNA from heavier (A + B) and lighter particles (C) and analyzing its size distribution by sucrose density gradient centrifugation as shown in Fig. 5. The A_{260} profile of RNA derived from heavier

particles (Fig. 5 A) is very broad, ranging from 18S to 4S. Single fractions from the gradient were assayed for their ability to hybridize to (^{3}H)-polyU and tested for template activity in a wheat germ lysate. Fractions with messenger activity are prominent in the 15S region with a smaller peak in the 8S region : they also contain polyadenylic acid as shown by the hybridization test. The highest hybridizing activity is confined to the lightest fractions of the gradient which are almost inactive in the cell-free system. These results indicate that heavier mRNP particles contain a certain amount of degraded polyA(+)RNA and free polyA tails; The A_{260} profile of RNA extracted from the lighter particles (Fig. 5B) shows a sharp, symmetric peak in the 4S region. mRNA activity is almost absent troughout the gradient while fractions hybridizing to (^{3}H)-polyU are present in the upper region of the gradient. The lack of mRNA activity in the lighter particles indicates that they mainly contain RNA species devoid of template activity such as degraded polyA (+) RNAs, and free polyA residues. In addition we have shown that 4S RNA has aminoacid acceptor activity and can be aminoacylated by using a mixture of (^{14}C)-aminoacids and a crude preparation of aminoacyl-synthetases from rat liver.

The RNA extracted from heavier and lighter particles at the nauplius stage has a sedimentation pattern similar to that shown for encysted embryos.

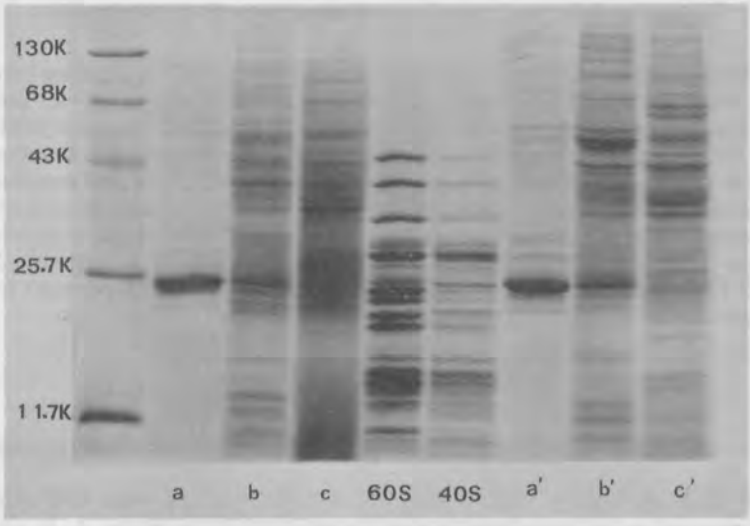

FIG. 4. Acrylamide gel electrophoresis of the protein moiety of mRNP particles derived from cysts and nauplii. mRNP particles corresponding to pools A, B, C as well as A', B', C' (see diagrams of Fig. 1) were analyzed for their protein content as described in Materials and methods. The reference markers were : beta-galactosidase (130 000 MW), bovine serum albumin (68 000 MW), ovalbumin (43 000 MW), chymotrypsinogen (25 700 MW) and cytochrome c (11 700 MW). The ribosomal proteins derived from 60S and 40S cyst ribosomal subunits were run as control. a, b, c : protein moiety of cyst A, B, C mRNP particles ; a', b', c' : protein moiety of nauplius A', B', C' mRNP particles.

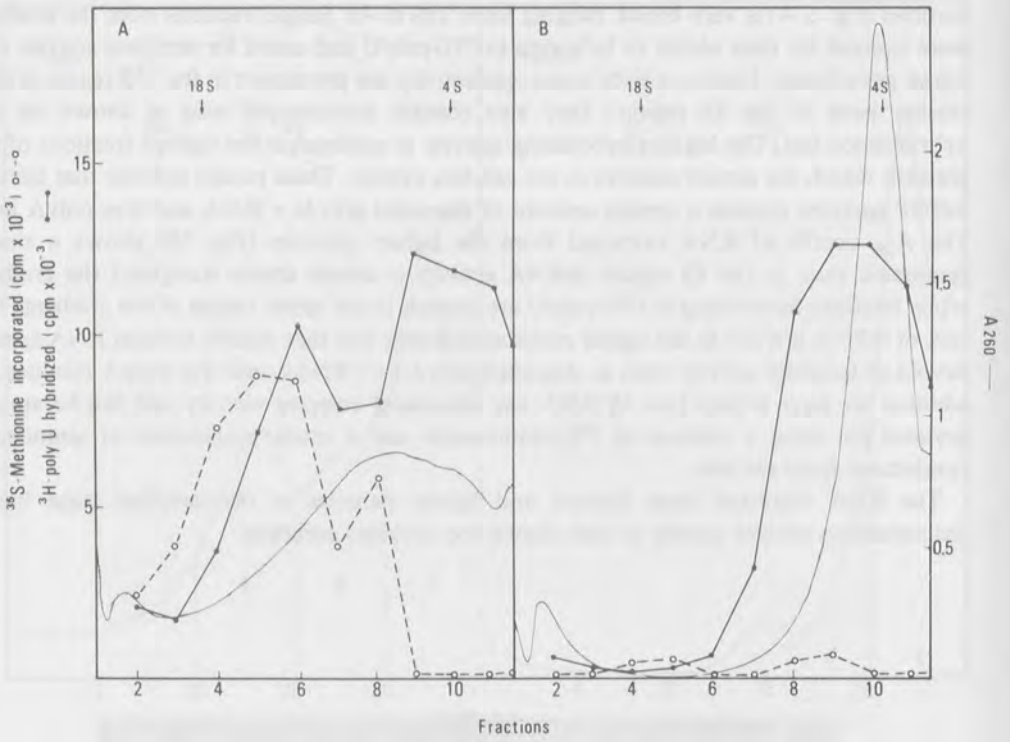

FIG. 5. Template activity and (^{3}H)-polyU hybridization capacity of RNA extracted from cyst heavier and lighter mRNP particles. Cyst mRNP particles corresponding to pool (A + B) and pool C (see diagrams of Fig. 1) were precipitated with two volumes of 95% ethanol, dissolved in NDS buffer and extracted by the phenol-Na dodecylsulfate procedure as described in Materials and methods. RNA was then applied to the top of 16.5 ml 5-20% sucrose gradients in NDS buffer and centrifuged at 26 000 rpm for 16 hr in the SW 27.1 rotor at 20 °C. Fractions at 1.5 ml were collected, ethanol precipitated and the RNA resuspended in 50 μl of sterile water. 25 μl aliquots were tested for their stimulatory activity in a wheat germ lysate and for their ability to hybridize to (^{3}H)-polyU as described in Materials and methods. (A) RNA extracted from the heavier mRNP particles ; (B) RNA extracted frm the lighter mRNP particles.

ISOLATION OF MRNP PARTICLES OF DIFFERENT SIZE FROM CYST POSTMICROSOMAL SUPERNATANT

A second cycle of sucrose density gradient centrifugation in high ionic strenght buffer (TKE) was required to isolate relatively homogeneous mRNP particles. A, B, and C pools as shown in the diagram of Fig. 3 were concentrated by ammonium sulfate precipitation and sedimented through linear sucrose gradients in TKE buffer as described in Fig. 6. At least four mRNP particles can be separated from each other : the heaviest P1 particle and the lightest P4 appear as symmetric peaks with sedimentation values of 16S and 4S respectively. P2 and P3 particles have very close sedimentation values (9S and 8S respectively) and are cross-contaminated.

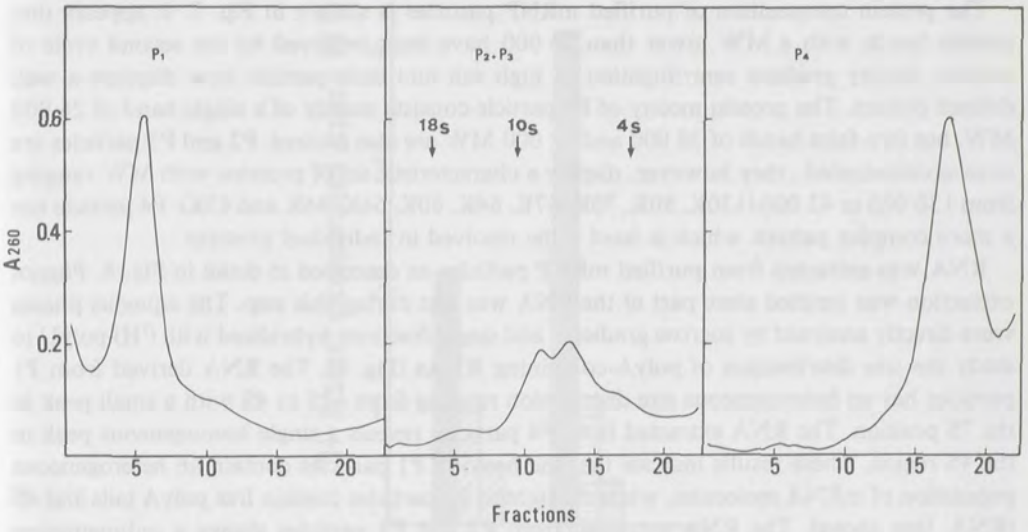

FIG. 6. Isolation of homogeneous cyst mRNP particles by a second cycle of sucrose density gradient centrifugation. Cyst mRNP particles corresponding to pool A, B, and C (see diagrams of Fig. 1) were concentrated by precipitation with ammonium sulfate (70% saturation), briefly dialyzed against TKE buffer and centrifuged through 5-20% linear sucrose gradients at 32 000 rpm for 16 hr in the SW 41 rotor at 4 °C. Average sedimentation values for purified mRNP particles were 16S (P1), 9S (P2), 8S (P3) and 4S (P4).

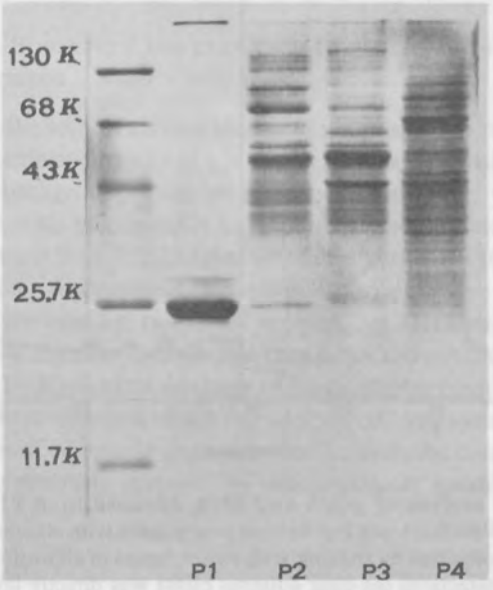

FIG. 7. Acrylamide gel electrophoresis of the protein moiety of cyst purified mRNP particles. Ethanol precipitation of purified particles and slab gel electrophoresis were carried ut as described in Materials and methods.

The protein composition of purified mRNP particles is shown in Fig. 7. It appears that protein bands with a MW lower than 25 000 have been removed by the second cycle of sucrose density gradient centrifugation in high salt and each particle now displays a well defined pattern. The protein moiety of P1 particle consists mainly of a single band of 26 000 MW, but two faint bands of 30 000 and 52 000 MW are also present. P2 and P3 particles are cross-contaminated ; they however, display a characteristic set of proteins with MW ranging from 130 000 to 43 000 (130K, 80K, 70K, 67K, 64K, 60K, 54K, 46K and 43K). P4 particle has a more complex pattern which is hard to be resolved in individual proteins.

RNA was extracted from purified mRNP particles as described in detail in Fig. 8. Phenol extraction was omitted since part of the RNA was lost during this step. The aqueous phases were directly analyzed by sucrose gradients and single fractions hybridized with (^{3}H)-polyU to study the size distributijon of polyA-containing RNAs (Fig. 8). The RNA derived from P1 particles has an heterogeneous size distribution ranging from 12S to 4S with a small peak in the 7S position. The RNA extracted from P4 particles reveals a single homogeneous peak in the 4S region. These results indicate that the heaviest P1 particles contain an heterogeneous population of mRNA molecules, while the lightest P4 particles contain free polyA tails and 4S tRNA. (see above). The RNA extracted from P2 and P3 particles shows a sedimentation pattern intermediate between that of P1 and P4 (data not shown).

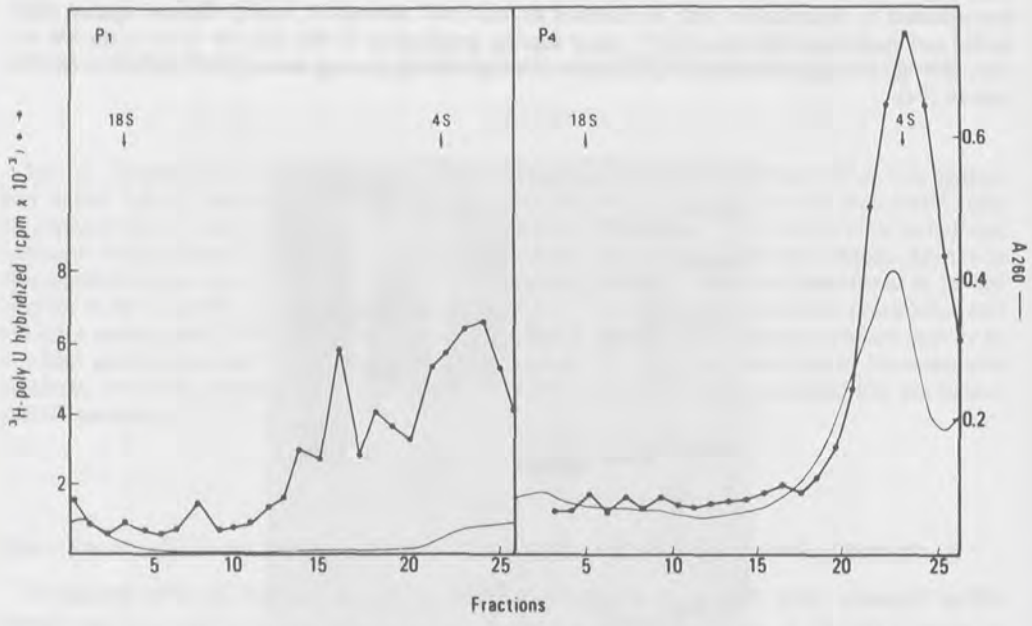

FIG. 8. Sedimentation analysis of polyA (+) RNA extracted from P1 and P4 mRNP particles. Highly purified P1 and P4 particles (see Fig. 6) were precipitated with ethanol, resuspended in 1 % NDS buffer and the RNA deproteinized by shaking with two volumes of chloroform containing 1 % isoamyl alcohol. After repeated extractions the clear aqueous phase was directly layered on the top of 5-20 % linear sucrose gradients in NDS buffer and centrifuged as described in Fig. 5. Fractions of 0.75 ml were collected and the RNA directly used in the (^{3}H)-polyU hybridization assay after removal of Na-dodecylsulfate as described in Materials and methods.

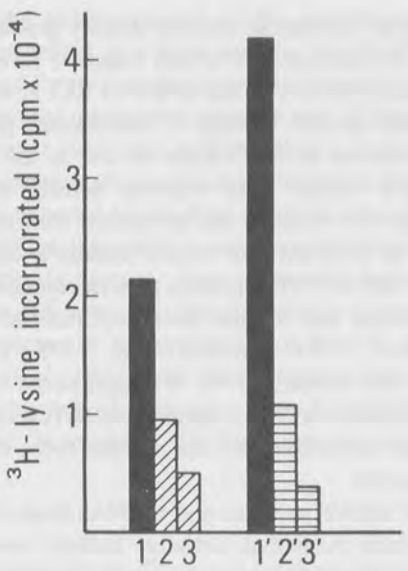

FIG. 9. Effect of the purified P1 particle and P1 protein on globin mRNA translation in a wheat germ cell-free system. A wheat germ lysate was programmed with globin mRNA (0.05 A_{260} units per 50 μl reaction mixture) and incubated at 25 °C for 60 min as described in Materials and methods. Incorporation of (^{3}H)-lysine into trichloroacetic acid insoluble material refers to 5 μl of incubation mixture. 1, 1' : control lysate ; 2, 3 : 0.02 and 0.04 A_{260} units of P1 particle added ; 2', 3' : 0.02 and 0.04 A_{280} units of P1 protein added.

EFFECT OF HOMOGENEOUS P1 PARTICLE AND EXTRACTED P1 PROTEIN ON MRNA TRANSLATION IN A WHEAT GERM CELL-FREE SYSTEM

The 16S particle from the second sucrose gradient in high salt was pelleted (100 000 × g for 24 hr) and tested for its template activity in a wheat germ lysate. No stimulation of aminoacid incorporation over the background level was observed. However, when the wheat germ lysate was programmed with globin mRNA, addition of the particle resulted in a marked inhibition of globin synthesis (Fig. 9, 2-3). In order to check whether the inhibitory effect was due to the RNA or protein components of the particle, proteins were dissociated from RNA by the LiCl-urea procedure used for ribosomal proteins (see Methods) and tested in the cell-free system after extensive dialysis. Even in this case a marked inhibition of globin synthesis was observed. (Fig. 9,2'-3'). Equivalent amounts of the purified protein did not have any effect on endogenous protein synthesis in a rabbit reticulocyte cell-free system (not shown). These results suggest that the purified protein does not interfere with the components of the protein synthetic machinery, but probably prevents the attachment of "naked" globin mRNA to the ribosomes.

Discussion

The procedure used to isolate mRNP particles from the postmitochondrial supernatant of *Artemia* embryos was derived from Bag and Sarkar (1975) with slight modifications. Al-

though more tedious and time consuming, sucrose density gradient centrifugation was preferred to oligodT-cellulose chromatography which required formamide elution and higher temperatures (Jain and Sarkar, 1979). The use of 0.5 M KCl in the centrifugation steps was believed to minimize the non specific binding of cytoplasmic proteins to mRNP particles (Blobel, 1972). KCl was preferred to NaCl since its use in the preparation of sea urchin mRNPs did not significantly change their buoyant density in both Mg^{++} and EDTA (Kaumeyer *et al.*, 1978). After two cycles of sucrose density centrifugation, we could isolate at least four mRNP particles of different size which possess a characteristic set of proteins ranging from 130 000 to 26 000 MW. The protein pattern resembles that reported for mRNP particles from other sources as sea urchin, duck erythroblast, chick embryonic muscle (Gander *et al.*, 1973 ; Baglioni, 1974 ; Barrieux *et al.*, 1975 ; Liautard *et al.*, 1976 ; Van Venrooij *et al.*, 1977 ; Jain and Sarkar, 1979). We must stress the fact that low molecular weight proteins in the range 15 000 to 25 000 daltons which resemble the typical ribosomal or cellular proteins in size, are removed after the second cycle of sucrose density gradient centrifugation in high salt buffer.

During the preparation of mRNP particles and mRNAs from *Artemia*, special precautions were taken in order to minimize nucleolytic activity : buffers, sucrose solutions and reagents were made with autoclaved distilled water ; sterilized pipettes and glassware were used throughout and heparin (100 to 500 μg/ml) was added to the homogenization medium and sucrose gradients. Nevertheless RNA fractions which had no template activity in cell-free systems were usually isolated from sucrose gradients. These fractions were rich in polyadenylic acid and had a characteristic sedimentation value of 4-5S. Although one can not exclude a certain degree of RNA degradation during the isolation procedure, this result indicates the presence of free polyA traits associated with proteins in the cytoplasm of *Artemia* cysts. Similar findings have been reported by Filimonov *et al.* (1977) in the cytoplasmic extracts of dry wheat embryos.

A characteristic feature of free mRNP particles from *Artemia* cysts is the relative abundance of a 20S particle which changes its sedimentation value into 16S after further purification through sucrose gradients (Felicetti *et al.*, 1975). This particle named P1 in this paper has a peculiar protein pattern which is mostly represented by a 26 000 MW protein and contains polyA (+) RNA with an heterogeneous size distribution from 12 to 4S. Recently De Herdt *et al.* (1979) have isolated, from *Artemia* cysts, a large quantity of a unique 19S complex which is a specific aggregate of a 27 000 MW protein and lacks polyA residues. The protein seems to be related to eEF Ts on the basis of aminoacid composition and immunological cross-reactivity. Although the 19S complex resembles the 16S particle purified by us, we are convinced that the P1 particle is not a simple aggregate of a 27 000 MW protein for at least two reasons : a) after two cycles of sucrose density centrifugation in high salt, the particle still contains polyA (+) RNA as detected by (^{3}H)-polyU hybridization ; and b) in addition to the main 26 000 MW band, the protein pattern of the particle contains two faint bands of 30 000 and 52 000 MW respectively. It is likely that the 16S mRNP particle and the 19S protein complex have a very close sedimentation constant after sucrose density analysis in high salt and are not easily separated from each other.

The last point we wish to comment on is the inhibition of mRNA translation produced by intact P1 particle in a wheat germ cell-free system. Similar results have been reported by Civelli *et al.* (1976) for the 20S mRNP particle from duck erythroblasts. In this paper we give

clear evidence that it is the protein moiety of the particle, and not the RNA, which is responsible for the inhibitory effect. It is likely that the 26 000 MW protein has a high affinity for informational RNAs and forms protein-nucleic acid complexes which are unable to interact with 40S ribosomal subunits during the initiation step of translation. This interpretation is in agreement with the peculiar structural conformation of free mRNP particles as shown by (^{3}H)-polyU hybridization experiments (see the first chapter in the Results). One may speculate that a sequence of events is required for the flow of information from mRNP particles into the polysome cycle. Free mRNP particles have a compact conformation with both 5' and 3' ends of the message hidden by specific proteins. They necessarily have to undergo a conformational change to interact with the 40S subunits. This hypothesis is substantiated by the existence of frozen 40S initiation complexes in the cytoplasm of *Artemia* cysts (Grosfeld and Littauer, 1975; Pierandrei-Amaldi *et al.*, 1977) with polyA residues accessible to labeled polyU. The final steps would be the activation of these complexes by initiation factors, the joining of the 60S ribosomal subunit and the entrance into the polysome cycle.

Acknowledgements

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Artemia extracellular hemoglobins : ontogeny, structure and *in vivo* radiolabeling

Jan Heip[1], *Luc Moens*[1], *Robert Hertsens*[1], *Edward J. Wood*[2], *Harie Heyligen*[1], *Annie Van Broekhoven*[3], *Raf Vrints*[1], *Didier de Chaffoy*[1], *and Masatoshi Kondo*[1,4]

[1] *Departement Celbiologie, Universiteit Antwerpen Universiteitsplein 1, B-2610 Wilrijk, Belgium*

[2] *Department of Biochemistry, University of Leeds 9 Hyde Terrace, Leeds LS2 9LS, England*

[3] *Department of Neurobiology, The Weizmann Institute of Science Rehovot, Israel*

Abstract

The brine shrimp (*Artemia*) is able to synthesize three distinct hemoglobin molecules (Hb-I, -II and -III) only after hatching into a nauplius larva. The point of induction (or deinduction) and the level of concentration in the hemolymph of a particular hemoglobin species during development have been found to be largely controlled ontogenetically under a wide range of salt and oxygen concentrations as long as the oxygen concentration in the culture medium is above 0.09 mM. Each of three hemoglobin exhibits different oxygen-binding characteristics ; Hb-I (P_{50} 5.34 mm Hg), Hb-II (P_{50} 3.7 mm Hg) and Hb-III (P_{50} 1.8 mm Hg), respectively (D'hondt *et al.*, 1978).

The *in vitro* hydrolysis with trypsin of the *Artemia* hemoglobins in either native or partially denatured state has confirmed our previous conclusion (Moens and Kondo, 1977) that the low molecular weight (< Mr 20 000) polypeptides obtained under certain denaturation conditions are the hydrolytic products of the high molecular weight globin subunits (Mr 125 000) by a contaminating protease. Furthermore, cross-linking of the native hemoglobin molecules with either a non-reversible reagent (dimethyl suberimidate) or a reversible reagent (methyl-4-mercapto-butyrimidate) has been able to render further support to our model on the subunit structure of *Artemia* hemoglobins (Moens and Kondo, 1978a).

The rapid and extensive synthesis of Hb-II and Hb-III in the freshly hatched nauplius can lead to the accumulation of the hemoglobin as much as 20% of the total cytoplasmic proteins within 24 hr. It is further suggested that both transscription and translation events are closely coupled for the observed biosynthesis of *Artemia* hemoglobins in young nauplii.

Introduction

The first detailed physiological studies on *Artemia* hemoglobin were described by Gilchrist (1954, 1956, 1958). However, the biochemical characterization of the extracellular hemo-

[4] To whom all correspondence should be addressed.

globins in the brine shrimp (*Artemia*) has considerably been delayed and reported only in the last decade mainly from two laboratories in San Francisco and Antwerp (Bowen *et al.*, 1969, 1976 ; Waring *et al.*, 1970 ; Moens and Kondo, 1976a, 1976c, 1977, 1978a).

Unlike the extracellular hemoglobins of molluscs and annelids which have been found to exist in a giant aggregated form having a molecular weight of $1\text{-}4 \times 10^6$ (Svedberg and Eriksson, 1933 ; Svedberg and Eriksson-Quensel, 1934 ; Rossi Fanelli *et al.*, 1970 ; Figueiredo *et al.*, 1973 ; Shlom and Vinogradov, 1973 ; Andonian and Vinogradov, 1975 ; Waxman, 1975 ; Wood and Mosby, 1975 ; Wood *et al.*, 1976), the corresponding hemoglobins of crustaceans have a considerably lower molecular weight of $2\text{-}5 \times 10^5$ (Ar and Schejter, 1970 ; Bowen *et al.*, 1976 ; Hoshi *et al.*, 1976 ; Moens and Kondo, 1976a ; David *et al.*, 1977 ; Moens and Kondo, 1977, 1978a ; Manwell, 1978). Hoshi *et al.* (1976) have reported that *Moina macrocopa* hemoglobin is made up of several low molecular weight subunits (Mr 8 900, 11 700 and 23 000). Similarly, David *et al.* (1977) have presented evidence that *Cyzicus cf. hierosolymitanus* hemoglobin consists of 20 small polypeptides of Mr 15 000.

In contrast to these crustacean hemoglobins, three extracellular hemoglobins of *Artemia* have been shown to exhibit a much simpler subunit structure of a dimeric form composed of two high molecular weight globin subunits (Moens and Kondo, 1976c, 1977, 1978a). Our evidence sharply contradicts the proposed subunit structure reported by Bowen *et al.* (1976), which predicts the similar structure to that of *Cyzicus* hemoglobin, namely the native *Artemia* hemoglobins are consisting of 12 low molecular weight subunits (Mr 19 000).

In this report, further studies on the molecular structure of *Artemia* hemoglobins are presented to gain more insight in this problem stated above. The results obtained from experiments utilizing techniques of *in vitro* trypsin hydrolysis and of cross-linking of the hemoglobin support strongly the model of a dimeric form. In order to pursue our investigation on the hemoglobin gene organization and gene expression at the molecular level, we have tried to isolate specific polysomes synthesizing the hemoglobin subunits by immunological technique. These results are discussed in the light of hemoglobin biosynthesis in *Artemia*.

Materials and methods

Purification of *Artemia* hemoglobins and preparation of globin subunits and their fragments

The hemoglobin was purified from the $105\,000 \times g$ supernatant (S-105) by $(NH_4)_2SO_4$ fractionation, three times repeated ion-exchange chromatography on DEAE-Sephadex A50 and gel filtration chromatography on Sepharose 6B as detailed in Moens and Kondo (1978a). The preparation of globin chain and their fragments for amino acid analysis was also described in Moens and Kondo (1978a).

Analytical methods

Cellulose acetate electrophoresis and isoelectric forcusing polyacrylamide gel electrophoresis of *Artemia* hemoglobins were described in Moens and Kondo (1976a). Spectral analysis and the determinatijon of iron and heme contents of the hemoglobin were already described in Moens and Kondo (1978a).

Double immunodiffusion was carried out at 4 °C for 24 hr in 1% agarose (5 ml) on glass plates (5 × 7.4 cm) in 50 mM sodium barbital/0.05% sodium acetate (pH 8.6). After incubation the gel plates were washed with the same buffer, dehydrated with filter paper and stained with benzidine-H_2O_2.

Crossed immunoelectrophoresis was performed essentially according to Laurell (1965). Electrophoresis in the first dimension using Tris-barbiturate buffer (pH 8.6) was at 10 V/cm for 1 hr, followed by electrophoresis in the second dimension at 1.5 V/cm for 18 hr. Before electrophoresis in the second dimension, 10 ml of 1% agarose containing the anti-*Artemia* Hb γ-globulin (IgG) was placed on the same glass plates (7 × 10 cm) having already 15 ml of 1% agarose used for the first dimension electrophoresis. Washing and staining of the electrophoregrams were carried out as double immunodiffusion plates.

Polyacrylamide gel electrophoresis

Hemoglobin samples were denatured by heating at 95 °C for at least 3 min in 5 mM Tris-glycine (pH 7.5)/1% sodium dodecylsulfate (SDS)/1% 2-mercaptoethanol/24% glycerol/0.01% bromphenol blue and electrophoresed in 6% gels either in perspex tubes (0.3 × 5 cm) with 2-4 mA per tube or in a slab (0.15 × 8 × 13 cm) with 8 mA per gel using electrode buffer 1 (0.01 M Tris base/0.34 M glycine/0.1% SDS, pH 7.5) at room temperature until the tracking dye reached the end of the gel.

In certain experiments for the analysis of trypsin treated hemoglobins, 7.5% gels or 5-30% gradient gels (Manwell, 1977) in a slab (0.15 × 7 × 10 cm) were electrophoresed using electrode buffer 2 (0.36 M Tris/0.043 M glycine/0.1% SDS, pH 9.8).

Preparation of anti-Hb IgG

Artemia hemoglobin preparation (11.5 mg/ml in 5 mM Tris-glycine buffer, pH 8.3) containing all three hemoglobins in different proportions was mixed with complete Freund adjuvant. 2 mg of the antigen (0.5 ml) was injected into adult rabbits, and similar injections were made during the first 2 weeks after the priming one. Subsequently the injection was carried out every 2 months. Antiserum was obtained by bleeding at different intervals and IgG was partially purified according to Kekwick (1970). Hb-II specific IgG was prepared by affinity chromatography on Sepharose 4B coupled with the purified Hb-II. Binding of IgG was done in 50 mM borate-NaOH (pH 8.3)/100 mM NaCl and after washing Hb-II specific IgG was eluted with 200 mM glycine buffer (pH 2.8)/500 mM NaCl from the column.

Hydrolysis of *Artemia* hemoglobin with trypsin

Hemoglobin samples were incubated at 37 °C in 22.5 mM Tris-glycine buffer (pH 8) with or without trypsin (205 U/mg, Worthington) in the presence or absence of 2.33 M urea/0.025% SDS/1.25% 2-mercaptoethanol. Trypsin was dissolved in 90 mM Tris-HCl (pH 8)/10 mM $CaCl_2$ and added to the reaction mixture in the weight ratio of enzyme/hemoglobin to 1/100 or 5/100 as indicated in the figure legends. Urea was deionized by ion-exchange chromatography on Biorad AG 501-X8(D) immediately before use. The hydrolysis was stopped by heating samples in 1% SDS/1% 2-mercaptoethanol at 95 °C for 5 min and the protein samples were analyzed by SDS-gel electrophoresis.

Preparation of polysomes and binding of [^{125}I]IgG

Polysomes were prepared essentially according to Moens and Kondo (1976b). The pelleted polysomes were suspended in a buffer (25 mM Tris-HCl (pH 7.6)/25 mM naCl/5 mM $MgCl_2$/140 mM sucrose/100 μg/ml sodium heparin) to a concentration of 31 A^{260}/ml. Anti-*Artemia* Hb IgG (RNAase-free) was iodinated with [^{125}I] by chloramine-T method (Hunter, 1973) and was dissolved in 10 mM sodium phosphate buffer (pH 7.2)/15 mM NaCl to a concentration of 0.36 mg/ml (specific activity, 0.2 μCi/μg IgG).

The reaction for antibody-binding was carries out at 0 °C for 1 hr by mixing 150 μl of polysomes with varied amountsof [^{125}I] antibodies (10-200 μl). After incubation, the reaction mixture was analyzed by 0.5-1.5 M sucrose density gradients in 35 mM Tris-HCl (pH 7.6)/70 mM KCl/9 mM $MgCl_2$, which were run in a Beckman SW41 rotor for 90 min at 41 000 rpm.

Cross-linking of *Artemia* hemoglobins

One volume of 0.5 mg/ml hemoglobin dialyzed against quartz distilled water was mixed with an equal volume of 12 mg/ml dimethyl suberimidate (DMS) in 0.2 M triethanolamine-HCl (pH 8.5) and the mixture was incubated at room temperature for desired period as indicated in the figure legend. The reaction was terminated by the addition of an equal volume of 30 mM sodium phosphate buffer (pH 7.2)/12% 2-mercaptoethanol/3% SDS/30% glycerol, followed by heating at 95 °C for 5 min. The treated hemoglobin samples (20-30 μg) were analyzed by SDS-gel electrophoresis.

When a reversible cross-linker, methyl-4-mercaptobutyrimidate (MMB), was employed, both the hemoglobin and MMB were dissolvedin 0.2 M treithanolamine-HCl (pH 7.2)/0.4 M 2-mercaptoethanol. The reaction mixture was made as for DMS, but was incubated at 0 °C for 20 min, followed by dialysis at 0 °C for 2 hr against 0.2 M triethanolamine-HCl (pH 7.2). The dialysate was oxydized with 5 mM H_2O_2 (final concentration) by incubating at room temperature for 30 min. The samples for gel electrophoresis were prepared as for DMS-treated hemoglobin except the omission of 2-mercaptoethanol from the buffer. SDS-gel electrophoresis was also performed in the absence of 2-mercaptoethanol. The reversal of the cross-linking disulfide bridges was done by dialysis against 10 mM sodium phosphate buffer (pH 7.2)/4% 2-mercaptoethanol/1% SDS/10% glycerol before SDS-gel electrophoresis.

Materials

Bovine pancreas trypsin was obtained from Worthington Biochemical Corp. (Freehold, NJ, USA). DMS and MMB were purchased from Pierce Chemical Co. (Rockford, Ill., USA). Radioactive iodine and [^{3}H] leucine were bought from Radiochemical Center (Amersham, UK). All other materials were indicated in our previous publications (Moens and Kondo, 1976a, 1976b, 1977, 1978a ; Heip *et al.*, 1978b).

Results

Ontogeny and number of species of *Artemia* hemoglobins

The 50% $(NH_4)_2SO_4$-precipitate of S-105 extract prepared from the adult brine shrimp (*Artemia*) contains three chromatographically distinguishable hemoglobin molecules. As shown in Fig. 1a, a gradient of NaCl (125-225 mM) developed on ionexchange chromato-

graphy using DEAE-Sephadex A50 has resulted in the separation of Hb-I, -II and -III, whose isoelectric points have been determined to be 5.6, 5.7, and 5.9, respectively (Moens and Kondo, 1976a). On the other hand, an analogous chromatography of the naupliar extract has demonstrated only two species of hemoglobins (Fig. 1b) ; a major peak (designated Hb-N) and a minor one corresponding to adult Hb-II and Hb-III, respectively. Although a definitive proof for the identity between Hb-N and Hb-II is still lacking, it seems highly likely at present that they are the same hemoglobin molecule. Therefore, no distinction will be made between Hb-N and Hb-II in the following discussion.

Hachted nauplius larvae contain already Hb-II and a small amount of Hb-III (Fig. 1b), but these hemoglobins can be detected by the spectral analysis only after the hatching event (Moens and Kondo, 1977). Furthermore, it has been shown that in the developing gastrula no protein molecules being able to cross-react with anti-Hb IgG are synthesized or previously stored (Heip *et al.*, 1978b). This was shown by double immunodiffusion experiments, in which S-105 extracts from various stages up to 36 hr after the resumption of embryonic development in the cryptobiotic gastrula were tested against anti-Hb IgG fraction. As shown in Fig. 2, the extract from 18 hr-incubated culture exhibited the earliest recognizable precipitin arc, when a reasonal portion of the gastrulae had already hatched into nauplii. Thus, it has been concluded that the capability of hemoglobin synthesis in *Artemia* is typically characteristic to the animals which require a large supply of oxygen for their energy production.

The appearance of a specific hemoglobin species in the hemolymph after the postlarval development in *Artemia* has been demonstrated to be essentially controlled ontogenetically (Heip *et al.*, 1977, 1978b). Hb-II is the first hemoglobin to be detected in the freshly hatched nauplius and is also the predominant species in the most of adult life-span. Hb-III follows immediately after Hb-II in its appearance in the nauplius, but its overall concentration in the hemolymph remains at all times significantly lower than that of Hb-II. Under the standard culture conditions (0.51 M NaCl, 0.17-0.21 mM oxygen, 28 °C), Hb-III disappears completely from the hemolymph in the young adult animal at around 30 days after hatching (Heip *et al.*, 1977, 1978b). Interestingly, Hb-III can be reinduced in those animals lacking this hemoglobin within 5-7 days after lowering the oxygen concentration below 0.07-0.09 mM and can be deinduced at will upon increasing again the oxygen concentration to 0.17-0.21 mM (Heip *et al.*, 1978a).

The induction of Hb-III by altering the oxygen concentration is accompanied by a substantial increase in the concentration of Hb-II in the hemolymph, which returns to the normal level upon the deinduction of Hb-III (Heyligen *et al.*, unpublished observations). This observations may suggest a close relationship between Hb-II and Hb-III in the regulation of hemoglobin biosynthesis. In fact, immunological analysis has demonstrated that Hb-II and Hb-III are structurally related to each other, but not to Hb-I. By double immunodiffusion test using the purified anti-Hb-II IgG fraction, Hb-II and Hb-III were able to form a continguous preciptin arc, whereas no arc was detected with Hb-I (Moens *et al.*, unpublished observations). Furthermore, as evident from crossed immunoelectrophoresis experiments in Fig. 3, the electrophoretical peaks of Hb-I and Hb-II produced the clear crossing-over lines, where two peaks joined to each other (Fig. 3a and 3b), whereas those of Hb-II and Hb-III exhibited a continuous peak line without crossing-over (Fig. 3a and 3c). These results have been interpreted to indicate that Hb-II and Hb-III contain a common (or at least

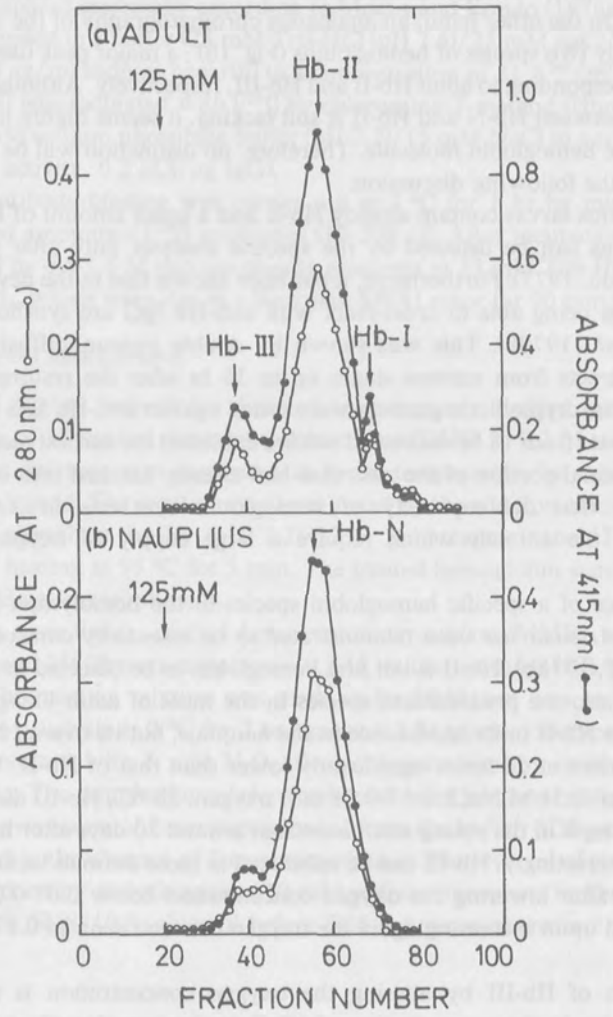

FIG. 1. Separation of *Artemia* hemoglobins. The step-wise elution fraction containing three hemoglobins of the first DEAE-Sephadex A50 column was then chromatographed on the second DEAE-Sephadex A50 column with a linear gradient of NaCl (125-225 mM). For further purification, separate pools of each hemoglobin fraction were individually chromatographed as above. (● —— ●), absorbance at 280 nm ; (○ —— ○) absorbance at 415 nm. Taken from Moens and Kondo (1978a) with kind permission of the publisher.

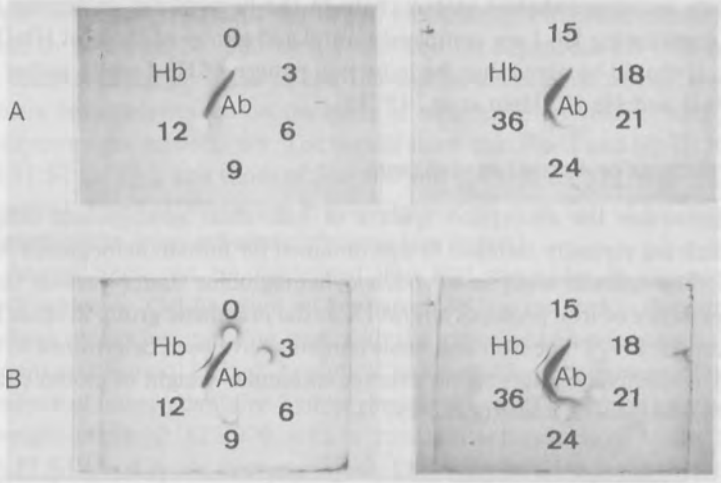

FIG. 2. Double immunodiffusion analysis of the developing gastrular extracts. The S-105 extract of gastrulae developing under the standard condition for the indicated hours was examined by double immunodiffusion test as described in Materials and methods. Hb, a mixture of *Artemia* hemoglobins ; Ab, the partially purified IgG raised against the total hemoglobin ; the number on the peripheral wells, the hour incubated. The extracts in (A) were prepared in the absence of 1% Triton X-100, whereas those in (B) were in its presence. The diffusion plates were stained with benzidine-H_2O_2.

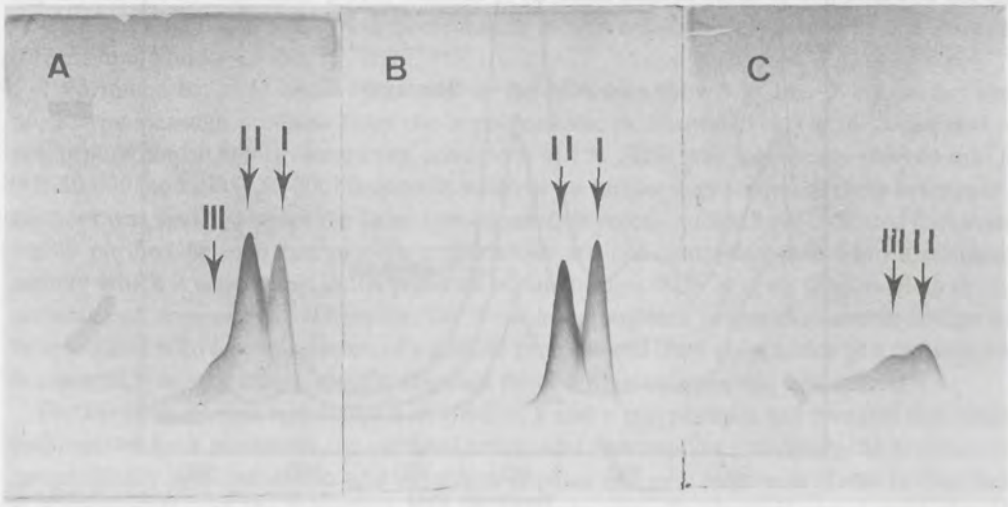

FIG. 3. Crossed immunoelectrophoresis analysis of hemoglobins. Crossed immunoelectrophoresis was performed as described in Materials and methods. (B), Hemolymph taken from old adult animal containing only Hb-I and Hb-II ; (C) S-105 extract of 20 hr nauplius culture containing Hb-II and Hb-III, and (A), a mixture of samples from (B) and (C). Electrophoresis plates were stained with benzidine-H_2O_2 as in Fig. 2.

immunologically indistinguishable) globin chain in the hemoglobin molecules and that the globin chains constituting Hb-I are completely unrelated to any of those of Hb-II and Hb-III. In this respect, it should be noted that the induction pattern of Hb-I seems rather independent of those of Hb-II and Hb-III (Heip *et al.*, 1978b).

Molecular structure of *Artemia* hemoglobins

Fig. 4 demonstrates the absorption spectra of individual hemoglobins (unpurified preparations), which are virtually identical to that obtained for human hemoglobin A (Moens and Kondo, 1977). The spectral analysis of *Artemia* hemoglobins under various conditions has indicated the presence of iron protoporphyrin IX as the prosthetic group in these hemoglobins (Moens and Kondo, 1977). The iron and heme contents have been determined to be 0.299 and 3.60% (w/w), respectively, implying an average molecular weight of globin chain per heme group to be 18 000 (Moens and Kondo, 1977).

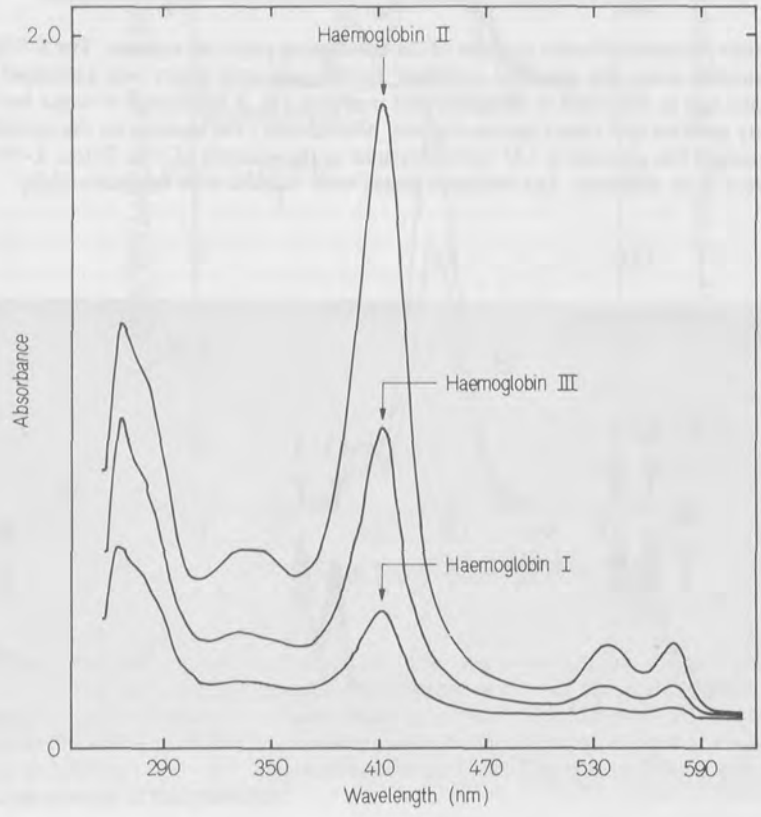

Fig. 4. Absorption spectra of *Artemia* hemoglobins. Individual hemoglobins were eluted from the cellulose acetate electrophoresis strip and their spectra were determined with a Brückl S. E. M. HRS 4001 C spectrophotometer. Taken from Moens and Kondo (1976a) with kind permission of the publisher.

An apparent molecular weight of the native molecule of three *Artemia* hemoglobins has been estimated to be about 240 000 by gel filtration on Sepharose 6B (Moens and Kondo, 1978a). The sedimentation coefficients and the weight-average molecular weights of Hb-II and Hb-III have been determined on the basis of sedimentation velocity and sedimentation equilibrium experiments, respectively. The results show that Hb-II and Hb-III have the values of 11.57 and 11.52 for $S^{\circ}_{20,w}$ and those of 240 000 and 217 000 for M°_{w}, respectively (Wood *et al.*, in preparation). These estimates are in good agreement with that of gel filtration and also to the calculated values from gel electrophoresis (see below).

All three hemoglobins can be dissociated into half molecules in the presence of 6 M guanidine hydrochloride. Gel filtration on Sepharose 4B has resulted in the estimate of about 126 000 for these molecules, agreeing well with the expected value for one-half hemoglobin molecule (Moens and Kondo, 1978a). Analytical sedimentation experimentsof dissociated Hb-III in the presence of either guanidine hydrochloride or acetate buffer (pH 4) have indicated a molecular weight of about 113 000 with a concomitant reduction of the sedimentation constant to 6.17 S (Wood *et al.*, in preparation). These results all indicate that *Artemia* hemoglobins are composed of two equal size submolecules which could simply be produced by treatment with denaturants, or acidic or alkaline pH. This conclusion has been strenghtened by polyacrylamide gel electrophoresis analysis of *Artemia* hemoglobins in the presence of SDS. As shown in Fig. 5, each hemoglobin species contains one major polypeptide (α-band) with an estimated molecular weight of 122 000, which can not be altered even by severe denaturation conditions (1 % SDS, 60 min at 95 °C). Thus, the half submolecule of *Artemia* hemoglobins demonstrated by gel filtration and sedimentation experiments is not a simple aggregated form of smaller polypeptides, but is rather a genuine single polypeptide chain. This implies inevitably that each globin subunit of *Artemia* hemoblogins is composed of six or seven heme-containing small basic protein units which are covalently joined to one another (Moens and Kondo, 1976c, 1977, 1978a).

Two minor bands (β and γ) observed in the SDS-gels shown in Fig. 5 are in fact the proteolytic cleavage products from the α polypeptide, as illustrated in Fig. 6. Reisolated α polypeptide under lightly denaturing conditions (0.1 % SDS) was specifically cleaved into β (Mr 80 000) and γ (Mr 50 000) fragments whereas no further degradation of these latter polypeptides was detected under the same conditions. Our recent studies have indicated that even highly purified *Artemia* hemoglobin preparations are not completely free from a protease activity which is active even in the presence of denaturants (SDS or urea) *in vitro* (Heip *et al.*, unpublished observations). Therefore, the *Artemia* hemoglobin preparation seems always to be associated with a small amount of a specific protease and the α polypeptide in a native state is susceptible at one highly specific cleavage point within its molecule (see below).

Furthermore, amino acid analysis of Hb-II α, β and γ polypeptides has revealed that these polypeptides have practically the identical amino acid composition, indicating the presence of no specifically localized amino acid sequences in either of β or γ fragments (Table I). This fact is in agreement with the hypothesis that six or seven virtually identical repeat units should constitute the globin subunit.

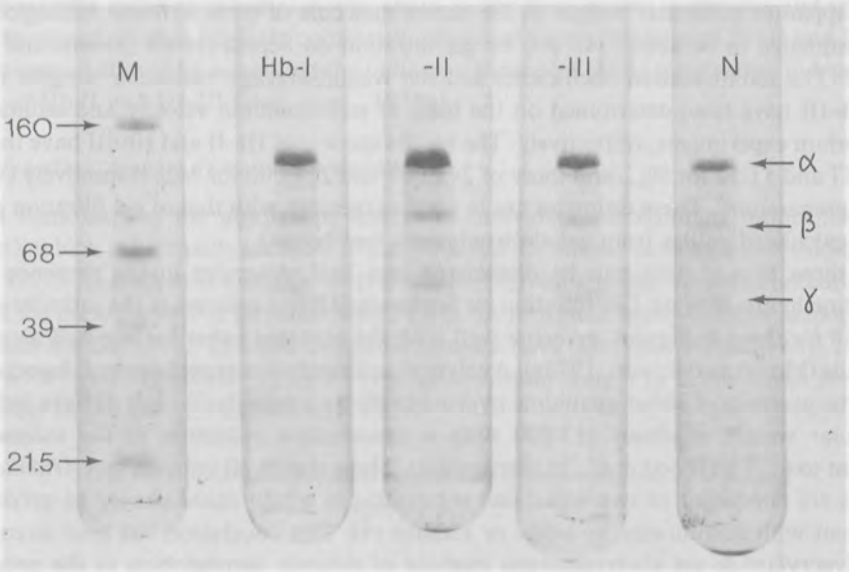

FIG. 5. SDS-polyacrylamide gel analysis of purified hemoglobins. The hemoglobin was purified as in Fig. 1 and Materials and methods. 20 μg of each hemoglobin was electrophoresedin 6% gels in the presence of 0.1% SDS as described in Materials and Methods. The gel M contained the molecular weight reference proteins : *E. coli* RNA polymerase β′, β subunits (Mr ~ 160 000), bovine serum albumin (Mr 68 000), *E. coli* RNA polymerase α subunit (Mr 39 000) and soybean trypsin inhibitor (Mr 21 500). (α), the globin subunit ; (β) and (γ), the hydrolytic products from the α polypeptide. Taken from Moens and Kondo (1978a) with kind permission of the publisher.

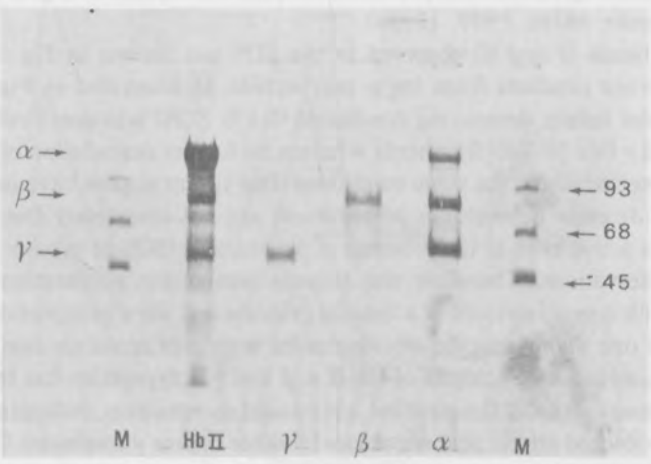

FIG. 6. SDS-polyacrylamide gel analysis of isolated α, β and γ polypeptides. Individual polypeptides were prepared by extracting the corresponding bands from a preparative slab gel and were electrophoresed n a 6% slab gel in the presence of 0.1% SDS as in Fig. 5. The gel slots marked M contained the molecular weight reference proteins : rabbit phosphorylase a (Mr 93 000), bovine serum albumin (Mr 68 000) and chicken ovalbumin (Mr 45 000). Taken from Moens and Kondo (1978a) with kind permission of the publisher.

TABLE I

Amino acid compositions of *Artemia* hemoglobins and their subunits.

Purified Hb-II and Hb-II α, β and γ polypeptides were prepared and amino acid analysis was conducted as described in Materials and methods. All amino acid determinations were the averaged values obtained after 24, 48, and 96-hr hydrolysis, except threonine and serine which were extrapolated to zero-time hydrolysis, and valine, isoleucine and histidine for which the 96-hr hydrolysis values were assigned. Half-cysteine was determined as cysteic acid and tryptophan was estimates spectrophotmetrically. n.d. = not determined. Taken from Moens and Kondo (1978a) with kind permission of the publisher

Amino acid	Hb-II		Hb-II α		Hb-II β		Hb-II γ	
	mol/ 18×10^3g	% (w/w)	mol/ 122×10^3g	% (w/w)	mol/ 80×10^3g	% (w/w)	mol/ 50×10^3g	% (w/w)
Aspartic acid	15.24	9.76	102.82	10.12	67.43	10.24	42.13	9.89
Threonine	7.10	4.07	52.62	4.64	31.49	4.28	21.22	4.46
Serine	8.17	4.13	54.04	4.20	37.03	4.43	24.79	4.59
Glutamic acid	17.98	12.73	120.96	13.16	78.45	13.15	49.95	12.97
Proline	5.46	3.03	35.62	3.03	25.54	3.35	16.53	3.36
Glycine	10.54	3.81	68.55	3.81	44.39	3.80	29.58	3.92
Alanine	11.86	5.09	75.84	5.00	49.22	5.00	31.16	4.90
Half-cysteine	1.19	0.69	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	12.30	6.94	82.85	7.18	54.75	7.31	34.30	7.09
Methionine	2.84	2.04	18.15	2.00	14.43	2.45	8.93	2.35
Isoleucine	8.19	5.17	53.97	5.24	36.55	5.46	23.41	5.41
Leucine	16.84	10.63	115.30	11.18	73.61	11.00	47.85	11.07
Tyrosine	3.46	3.02	21.66	2.90	14.29	2.95	9.60	3.08
Phenylalanine	9.90	7.87	64.90	7.93	44.13	8.31	29.92	8.72
Lysine	10.18	7.16	64.16	6.94	41.66	6.97	25.56	6.59
Histidine	6.11	4.56	41.22	4.73	23.08	4.08	14.88	4.07
Arginine	9.54	8.00	61.74	7.95	36.46	7.23	24.61	7.56
Tryptophan	1.31	1.29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

PROTEOLYTIC HYDROLYSIS *IN VITRO* OF *ARTEMIA* HEMOGLOBINS

Experiments described in the previous section have clearly demonstrated that *Artemia* hemoglobins are all in a dimeric form composed of two globun subunits (α) of the similar size and that during the isolation procedure a preferential proteolytic cleavage occurs *in vitro* at one specific site in the native globin chain, creating two fragments (β and γ) of the unequal size (Fig. 5 and 6). The latter conclusion has further been supported by the observation that when the native Hb-II (or others) was incubated with trypsin at 37 °C in the absence of denaturants, two groups of polypeptides with the molecular weights of around 80 000 and 50 000, respectively, were preferentially accumulated (Fig. 7), which were most likely equivalent to the β and γ polypeptides observed by the proteolysis *in vitro* without added protease (Fig. 5 and 6). Only very minor quantities of the low molecular weight polypeptides ($<$ Mr 30 000) were produced even after 60 min of incubation (Fig. 7). On the other hand, when the incubation was conducted under the identical conditions, but in the presence of denaturants (2.33 M urea and 0.025% SDS), almost complete breakdown of the α polypeptide into the low

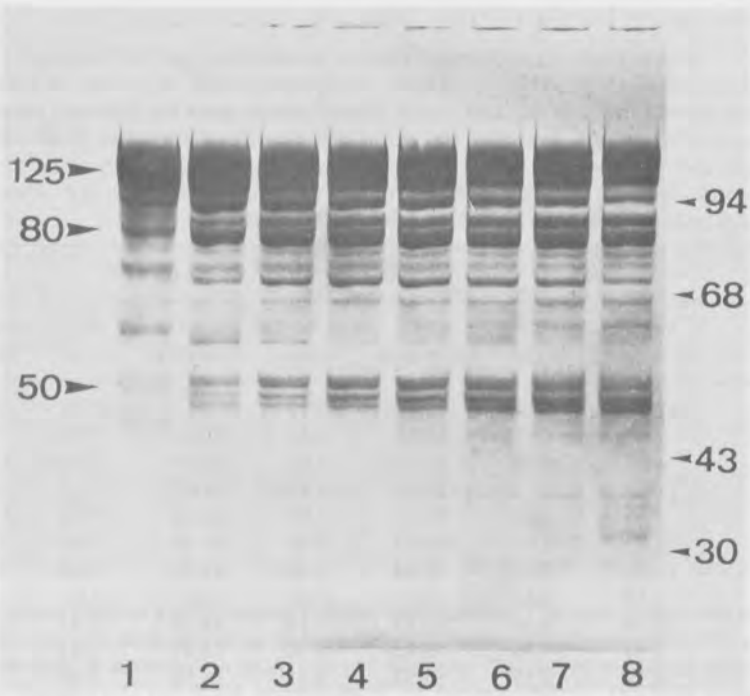

FIG. 7. SDS-polyacrylamide gel analysis of trypsin-treated hemoblogin-II in the absence of denaturants. Hb-II in 22.5 mM Tris-glycine buffer (pH 8) was incubated at 37 °C with trypsin (the weight equivalent to one-hundredth of the Hb-II) for 60 min. Samples were taken at different time intervals, processed and electrophoresed in a 7.5% slab gel in the presence of 0.1% SDS. The slots 1 through 8 were samples taken at 0, 5, 10, 15, 20, 30, 40 and 60 min, respectively. The number on both sides of the gel indicates the molecular weight ($Mr \times 10^{-3}$) : 125, 80 and 50 on the left correspond to the α, β and γ polypeptides ; 94, 68, 43, and 30 to rabbit phosphorylase a, bovine serum albumin, egg white ovalbumin and bovine erythrocyte carbonic anhydrase, respectively.

molecular weight fragments (< Mr 20 000) was observed within 10 min of incubation (not shown, but analogous experiments under altered conditions are presented in Fig. 8 and 9). These results strongly suggest that the native conformation of the globin subunits is able to determine the specific cleavage point within the α polypeptide for a possibly trypsin-like protease which would otherwise hydrolyze totally the globin subunits into small fragments.

Trypsin hydrolysis of *Artemia* hemoglobins II and III has further been studied under the denaturing conditions. Fig. 8 demonstrates SDS-gel analysis of Hb-II and Hb-III which are treated at 37 °C with trypsin in the presence of 2.33 M urea, 1.25% 2-mercaptoethanol and 0.025% SDS. Under these conditions, both Hb-II and Hb-III were almost completely degraded within 5 min of incubation to two groups of low molecular weight polypeptides (one at around Mr 30 000 and the other at about Mr 15 000) (Fig. 8, slots 3 and 5). Further hydrolysis of the hemoglobin molecules proceeded very slowly and even after 4hr-incubation the polypeptide pattern was not largely altered except the accumulation of the Mr 28 000

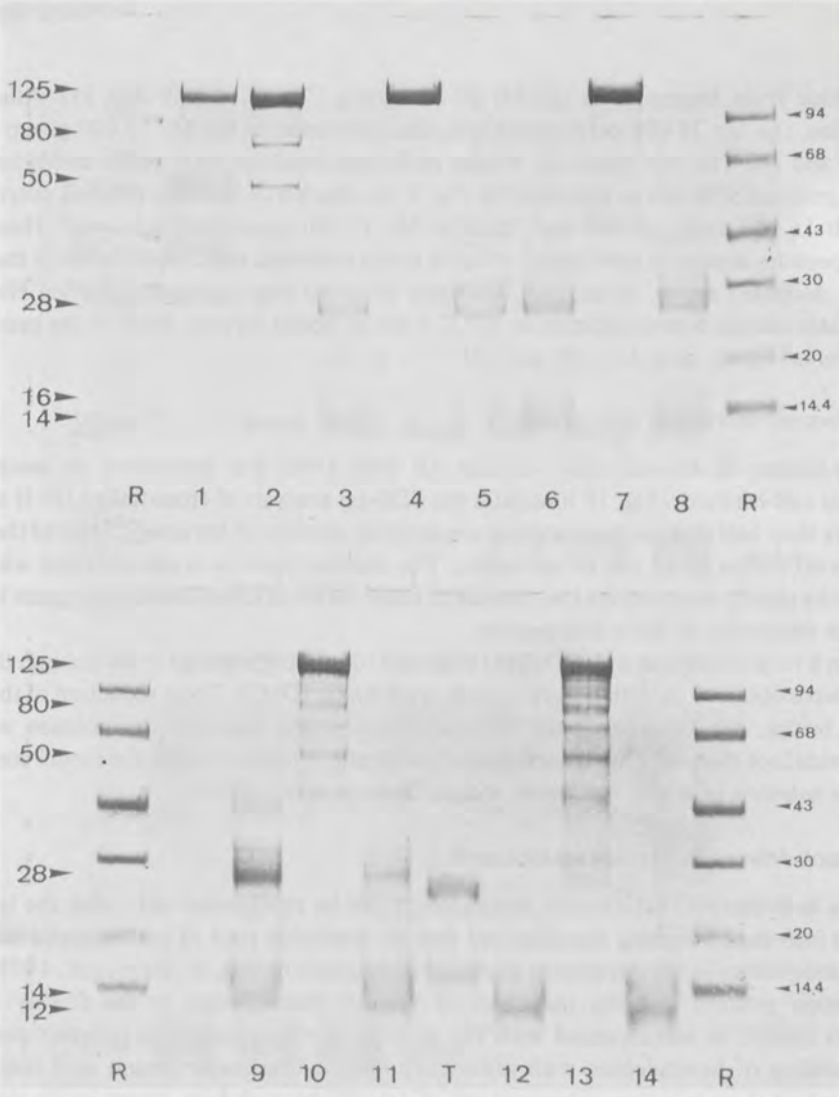

FIG. 8. SDS-polyacrylamide gel analysis of trypsin-treated hemoglobins in the presence of denaturants. Hb-II or Hb-III in 22.5 mM Tris-glycine buffer (pH 8) containing 2.33 M urea, 0.025% SDS and 1.25% 2-mercaptoethanol was incubated at 37 °C with or without trypsin (the weight equivalent to 1/20 of the hemoglobin) for 6 hr. Samples were taken at different time intervals for analysis in 7.5% slab gels as in Fig. 7. The sots 1, 4, 7, 10, and 13 contained Hb-II incubated for 0, 5, 20, 240, and 360 min in the absence of trypsin, respectively. The slot 2 contained Hb-III without incubation. The slots 3, 6, 9, and 12 contained Hb-II incubated for 5, 20, 240, and 360 min in the presence of trypsin, respectively. The slots 5, 8, 11 and 14 contained Hb-III treated with trypsin for 5, 20, 240, 360 min, recpectively. The slot marked T contained trypsin, while the slots R contained the molecular weight reference proteins : rabbit muscle phosphorylase b (Mr 94 000), bovine serum albumin (Mr 68 000), egg white ovalbumin (Mr 43 000), bovine erythrocyte carbonic anhydrase (Mr 30 000), soybean trypsin inhibitor (Mr 20 000) and bovine milk α-lactalbumin (Mr 14 000). The number on the left of the gels indicates the molecular weight ($Mr \times 10^{-3}$) : 125, 80, and 50 correspond to α, β and γ polypeptides ; 28, 16, 14, and 12 correspond to the major hydrolytic products during trypsin treatment.

polypeptide from fragments in the Mr 30 000-group (Fig. 8, slots 9 and 11). After 6 hr-incubation, the Mr 28 000 polypeptide was also converted to the Mr 15 000-group (Fig. 8, slots 12 and 14). The low molecular weight hydrolytic products were better distinguished by 5-30 % gradient SDS-gels as illustrated in Fig. 9, in which a conversion of small polypeptides from Mr 16 000 to Mr 14 000 and finally to Mr 12 000 could clearly be seen. These small tryptic peptides appear to correspond roughly to the predicted basic repeat units of the globin subunit discussed above. In addition, it should be noted that the highly purified Hb-II was quite stable during 6 hr-incubation at 37° C without added trypsin, even in the presence of denaturants (Fig. 8, slots 4, 7, 10 and 13).

Cross-linking of *Artemia* hemoglobins

Cross-linking of *Artemia* Hb-I, -II and -III with DMS was performed as described in Materials and Methods. Fig. 10 illustrates the SDS-gel analysis of cross-linked Hb-II and Hb-III. More than half of these hemoglobins are in either dimeric or tetrameric form of the globin subunits (α) within 20-40 min of incubation. The identical result was also obtained with Hb-I. The results clearly demonstrate the absence of other forms of cross-linked aggregates than the expected multimers of the α polypeptide.

When a reversible cross-linker (MMB) was used for the hemoglobin cross-linking, the same results were obtained as with non-reversible cross-linker (DMS). Upon reduction of the cross-linking bridge, all the cross-linked materials were found again at the position of the α polypeptide (not shown). This observation offers strong evidence against the model containing multiple subunits of a low molecular weight (Bowen *et al.*, 1976).

In vivo biosynthesis of *Artemia* hemoglobins

It has been shown that *Artemia* hemoglobins can be synthesized only after the larva has hatched into the swimming nauplius and that no detectable pool of yet unassembled globin polypeptides exists in the developing gastrula before hatching (Fig. 2 ; Heip *et al.*, 1978b). This observation predicts that the induction of *Artemia* hemoglobins in the freshly hatched nauplius should be accompanied with the *de novo* synthesis of globin polypeptides. Thus, pulse-labeling of hemoglobins with radioactive leucine (the major amino acid component) should reflect the actual rate of biosynthesis of *Artemia* hemoglobins, assuming no significant delay in the assembly process.

Table II summarizes the result obtained by pulse-labeling *in vivo* of freshly hatched nauplii for 24 hr with [^{3}H] leucine. After 1 day culture, the nauplius can synthesize hemoglobins as much as 20 % of the total cytoplasmic proteins and about 20 % of the total radioactivity taken up into the animal is incorporated into the newly synthesized hemoglobins (Table II). This fact indicates that the hemoglobin synthesis be the predominant fraction of the posthatching protein synthesis in these young nauplii. Our preliminary experments have suggested that the observed extensive synthesis of globin molecules is apparently not dependent on the messenger RNA (mRNA) stored in the developing gastrulae (Moens *et al.*, 1978b). Therefore, it seems highly likely that the active formation of hemoglobins is closely coupled with the *de novo* synthesis of hemoglobin mRNA in *Artemia* nauplii.

The rate of biosynthesis of three hemoglobins was examined by the 24 hr pulse-labeling with [^{3}H] leucine during the first 24 days after hatching. As illustrated in Fig. 11, a burst of Hb-

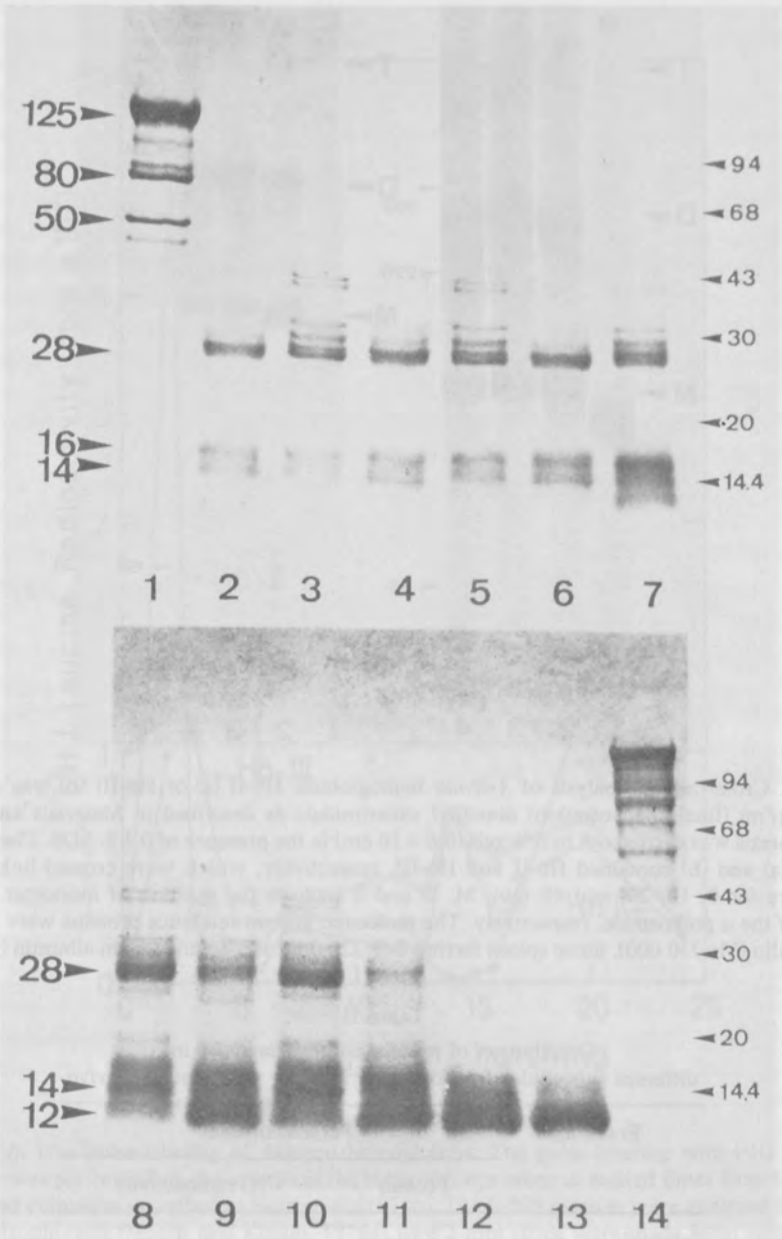

FIG. 9. Gradient SDS-polyacrylamide gel analysis of trypsin-treated hemoglobins in the presence of denaturants. Hb-II and Hb-III were treated with trypsin exactly as in Fig. 8. The product analysis was carried out in 5-30% gradient slab gels in the presence of 0.1% SDS. The slots 1, 3, 5, 7, 9, 11, and 13 contained Hb-III incubated for 0, 5, 20, 60, 120, 240, and 360 min, respectively. The slots 2, 4, 6, 8, 10, 12, and 14 contained Hb-II incubated for 5, 20, 60, 120, 240, and 360 min, and 360 min without trypsin, respectively. The number on the both sides of the gel indicates the molecular weight ($Mr \times 10^{-3}$) as in Fig. 8.

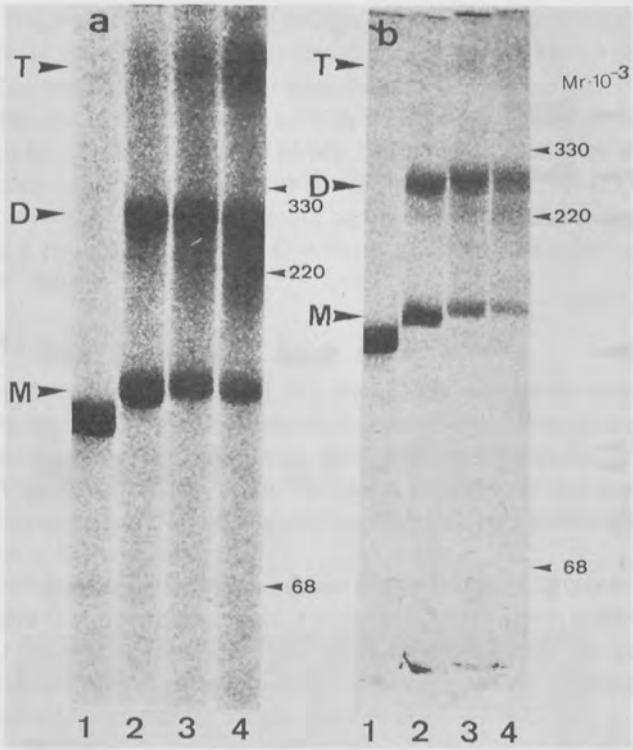

FIG. 10. Cross-linking analysis of *Artemia* hemoglobins. Hb-II (a) or Hb-III (b) was cross-linked with 6 mg/ml (final concentration) dimethyl suberimidate as described in Materials and Methods. Electrophoresis was carried out in 6 % gels (0.6 × 10 cm) in the presence of 0.1 % SDS. The gels 1, 2, 3, and 4 in (a) and (b) contained Hb-II and Hb-III, respectively, which were crossed-linked at room temperature for 0, 10, 20, and 40 min. M, D and T indicate the position of monomer, dimer and tetramer of the α polypeptide, respectively. The molecular weight reference proteins were hog thyroid thyroglobulin (Mr 330 000), horse spleen ferritin (Mr 220 000) and bovine serum albumin (Mr 68 000).

TABLE II

Distribution of protein and radioactivity in different subcellular fractions after labeling with leucine *in vivo*

Fraction	Percent of total	
	Protein	(^{3}H) radioactivity
Soluble*	73.1	51.3
Hemoglobin	21.0	20.2
Ribosome	0.5	15.3
Polysomes	5.4	13.2

(^{3}H) Leucine was added to the freshly hatched naupliar culture and maintained for the following 24 hr until the animals were harvested.

* The soluble fraction excluding the hemoglobin.

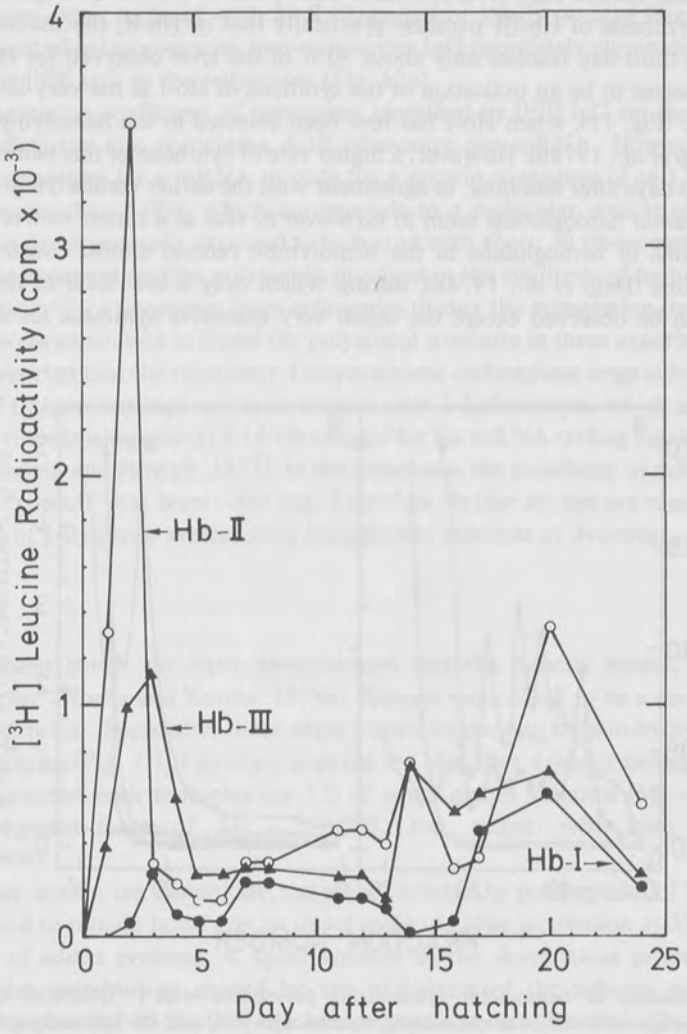

FIG. 11. *In vivo* pulse-labeling of *Artemia* hemoglobins. The pulse-labeling with (^{3}H) leucine for every 24 hr was performed on the sample of the brine shrimps taken at desired times from the partially synchronized culture as described in Heip *et al.* (1978b). The S-105 extracts were analyzed by cellulose acetate electrophoresis (Moens and Kondo, 1976a), and 2 mm strips were made from electrophoretic sheets, and the radioactivity was determined (Moens and Kondo, 1976b) after the dehydration of strips in methanol.

II synthesis occurs during the first 2 days but thereafter the rate of synthesis remains at the low level until the second high level synthesis takes place at around 20 days after hatching. Although the synthesis of Hb-III parallels practically that of Hb-II, the maximal rate of its synthesis at the third day reaches only about 30% of the level observed for Hb-II (Fig. 11). Further, there seems to be an indication of the synthesis of Hb-I at the very low level before the seventh day (Fig. 11), when Hb-I has first been detected in the hemolymph by staining techniques (Heip *et al.*, 1978b). However, a higher rate of synthesis of this hemoglobin occurs at around 17-20 days after hatching, in agreement with the earlier results (Heip *et al.*, 1978b). In addition, *Artemia* hemoglobins seem to turn-over *in vivo* at a rather slower rate, because the concentrations of hemoglobins in the hemolymph remain almost constant for a long period of life time (Heip *et al.*, 1978b), during which only a low level of the hemoglobin biosynthesis can be observed except the initial very extensive synthesis for a short period (Fig. 11).

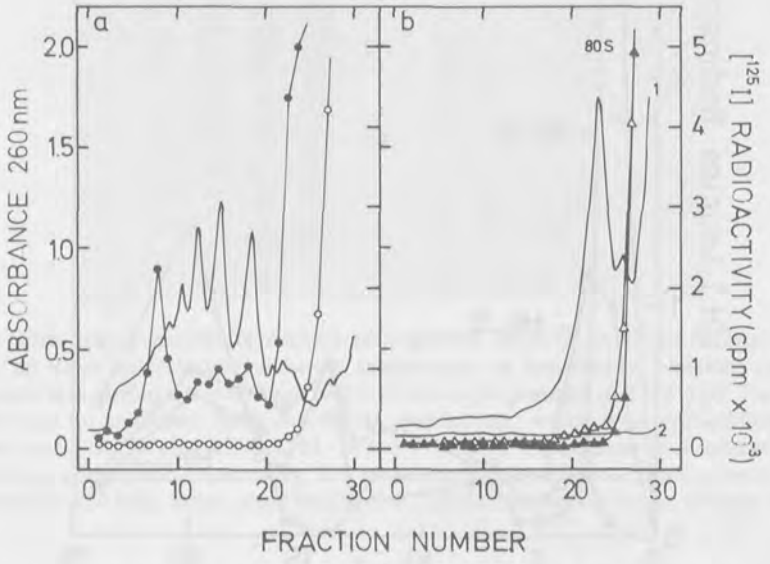

FIG. 12. Localization of hemoglobin-synthesizing polysomes with [125I] labeled antibodies. The polysomes were prepared from 24 hr old nauplii, reacted with [125I] anti-Hb IgG and analyzed on 0.5 M-1.5% sucrose gradients as described in Materials and methods. (a) (● —— ●), polysomes bound with [125I] IgG ; (○ —— ○), polysomes prebound with non-radiolabelled IgG and then challenged with [125I] IgG. (b) (▲ —— ▲), [125I] IgG alone treated as others ; (△ —— △) 80 S ribosomes from the cryptobiotic gastrulae reacted with [125I] IgG ; (——), absorbance at 260 nm. (1) and (2) in (b) indicate the absorbance of the gradients containing 80 S ribosomes and [125I] IgG alone, respectively.

In order to purify the mRNA specific for *Artemia* globin chains, polysomes have been prepared from 24hr old nauplii, when the high level of the hemoglobin synthesis should take place. The hemoglobin-synthesizing polysomes were identified by the formation of immunocomplexes on polysomes with [125I] anti-Hb IgG on sucrose density gradients. As shown in Fig. 12a, the [125I] labeled antibodies were found preferentially to bind to the polysomes

sedimenting at 250-300 S. This binding of the radioactivity has been prove to be the specific one, since no radioactivity is detected at the corresponding position of the gradient when polysomes were either replaced with 80 S ribosomes or totally omitted (Fig. 12b), and since the pretreatment of polysomes with non-radioactive IgG completely eliminates the subsequent binding of the [^{125}I] IgG to the polysomes (Fig. 12a).

The sedimentation coefficient of polysomes identified by [^{125}I] IgG on the sucrose density gradient predicts the size containing 8-10 ribosomes per mRNA. However, the minimal number of nucleotides for a mRNA to code for a protein consisting of ca 1 000 amino acids should be greater than 3 000, which corresponds to a molecular weight of about 10^6. The mRNA of this size is generally expected to be bound with about 30 ribosomes in its polysomal structure. The observed smaller polysomes involved in the synthesis of hemoglobin might be caused by "run-off" of ribosomes from polysomes during the preparation, because no special precautions were excercised to freeze the polysomal structure in these experiments. However, it has been reported that the ribulose-1,5-bisphosphate carboxylase large subunit-synthesizing polysomes of *Chlamydomonas reinhardi* contain only 2-5 ribosomes, which are far fewer than the expected ribosome number (14-16 ribosomes) for the mRNA coding for the protein having Mr 55 000 (Gelvin and Howell, 1977). In the latter case, the possibility of mRNA degradation or ribosome "run-off" has been ruled out. Therefore further studies are required to establish the structure of polysomes synthesizing hemoglobin subunits in *Artemia*.

Discussion

In the present study, we have demonstrated that the dimeric model, which we have proposed earlier (Moens and Kondo, 1978a), appears most likely to be a correct hemoglobin structure in *Artemia*. Especially, those experiments employing trypsin-hydrolysis and cross-linking techniques (Fig. 7-10) strongly support the idea that *Artemia* hemoglobin molecules are not constructed with multiples (*ca* 12) of small globin subunits (Mr ~ 20 000) into a native aggregated form of Mr ~ 250 000, but rather with two large subunits (Mr ~ 125 000).

Even under denaturing conditions, the globin subunit (α polypeptide) of Hb-II and Hb-III has been found to remain largely as an intact molecule after incubation at 37 °C for 4-6 hr in the absence of added protease. A small amount of the degradation products of relatively high molecular weight was caused by the hydrolysis of the subunit polypeptides by a contaminating protease in the hemoglobin preparation (see, Results). On the other hand, under the same conditions except the added trypsin, the globin subunit was completely degraded withint 5 min into chiefly two groups of polypeptides having the molecular weight of about 30 000 and 15 000, respectively (Fig. 8 and 9). These results and others (Hertsens and Heip, unpublished observations) appear to prove that the low molecular weight polypeptides observed under certain conditions (Moens and Kondo, 1977), which are assumed to constitute the basic subunits of *Artemia* hemoglobins (Bowen *et al.*, 1976), are the proteolytic degradation products.

Furthermore, upon denaturation and SDS-gel analysis, the cross-linked *Artemia* hemoglobin has not exhibited any other polypeptides (*e.g.* multiples of low molecular weight subunits) than those expected ones, namely the unreacted monomeric α polypeptide, the intramolecular cross-linked dimeric form and the intermolecular cross-linked tetrameric one

(Fig. 10). Moreover, after cleavage of the reversibly cross-linked hemoglobin, no polypeptides other than the original globin subunit (α) has been found. At present, further analysis in this line is being progressing in this laboratory. On the basis of foregoing evidence, we conclude that the *Artemia* hemoglobins are in a dimeric form in their native state. In support of these biochemical studies electromicroscopic observations have indicated that the *Artemia* Hb-II molecule consists of two stacked rings. The diameter of the ring and the height of the two stacked rings are estimated to be 120 Å ± 10 Å and 70 Å ± 10 Å, respectively (Jacob *et al.*, in preparation). Similar measurements on the clam shrimp (*Cyzicus fc. hierosolymitanus*) hemoglobin (*e.g.* 130 Å ± 10 Å, 85 Å ± 5 Å, respectively) have also been reported (David *et al.*, 1977).

It should also be noticed from the trypsin-hydrolysis experiment that the degradation pattern of the globin polypeptideis virtually identical in both Hb-II and Hb-III (Fig. 8 an 9). This indicates that not only the subunit structure but also the primary structure of the globin polypeptides of these hemoglobins are very similar. In fact, the amino acid compositions of three *Artemia* hemoglobins have been found to be almost identical (Moens and Kondo, 1978a).

However, immunological examinations have demonstrated that Hb-II is closely related to Hb-III, but not the Hb-I (Fig. 3 ; Moens *et al.*, unpublished results). Therefore, one or both of the globin subunits of Hb-II (or one being at least immunologically indistinguishable from Hb-II) should be constituting the Hb-III molecule. Our preliminary result using acid-urea gel electrophoresis has indicated the possibility that Hb-II consists of two electrophoretically separable chains whereas Hb-III contains only type of polypeptide (Heyligen *et al.*, unpublished observations). Thus, we tentatively suggest that Hb-II is a heterodimer molecule, while Hb-III is a homodimeric hemoglobin.

Bowen *et al.* (1977), and Sterling and Bowen (1977) have proposed that Hb-X (in their designation) is a homopolymer consisting solely of one of the two types of basic subunits of Hb-2 (Hb-II), which is a heteropolymer complex, and that the subunits of Hb-3 are not common to those of either Hb-II or Hb-X. From the proposed relationship of subunits of these hemoglobins and the relative electrophoretical mobility (Bowen *et al.*, 1977 ; Sterling and Bowen, 1977), it is most likely that our Hb-III be identical to their Hb-X, but not to Hb-3. In addition, Hb-1 has been proposed to be a homopolymer containing the other subunit type of Hb-2 (Hb-II) than the one involved in Hb-X (Hb-III) (Bowen *et al.*, 1977 ; Sterling and Bowen, 1977). However, this model contradicts our immunological evidence, if Hb-1 is assumed to be equivalent to our Hb-I. It should also be mentioned here that under a variety of culture conditions we have unable to detect a hemoglobin equivalent to Hb-3 of Sterling and Bowen (1977).

Finally, based on these studies it is suggested that the *Artemia* genome must contain at least three different genes to code for three hemoglobin molecules, assuming that Hb-I is a homodimer with the subunits unrelated to those of both Hb-II and Hb-III. The nature of hemoglobin gene organization in *Artemia* remains to be investigated.

Summary and conclusions

Artemia hemoglobins (Hb-I, -II and -III) consist of two high molecular weight globin subunits (Mr ~ 125 000), each containing multiple heme groups (6-7). Hb-II and Hb-III

contain one identical or at least immunologically indistinguishable globin subunit in common. Hb-I is completely unrelated to Hb-II (and also to Hb-III) on the immunological basis. The hemoglobin biosynthesis does not occur before hatching and the highest rate of synthesis has been observed for Hb-II and Hb-III in the nauplius within 2-3 days after hatching.

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Role of cytoplasmic membranes in the latency of protein synthesis in *Artemia* embryos

T. Hultin, M. Lake, M. O. Nilsson, and O. Nygård

Department of Cell Physiology, Wenner-Gren Institute, University of Stockholm
Norrtullsgatan 16, S-11345 Stockholm, Sweden

Abstract

Encysted embryos of *Artemia* contain latent mRNA, partially associated with a fraction of cytoplasmic membranes. The membranes, purified by banding in a sucrose gradient at 1.17 g/cm^3, contain endoplasmic vesicles and mitochondria. The origin of the membrane-associated poly(A)$^+$RNA was therefore investigated. In gel electrophoresis poly(A)$^+$RNA from the purified membranes of dormant cysts forms two bands of approximately 3×10^5 and 5×10^5 daltons. Later during development the lighter component decreases. Nuclei from dormant cysts are devoid of poly(A)$^+$RNA, while nuclei from embryos at emergence contain a predominant poly(A)$^+$RNA component of approximately 5×10^5 daltons. ^{125}I-labeled poly(A)$^+$RNA was used in excess DNA hybridization experiments. Poly(A)$^+$ membrane RNA from dormant cysts hybridized to nuclear DNA to the same extent as the nuclear RNA from developing embryos. The hybridization of labeled nuclear RNA to nuclear DNA was strongly inhibited by membrane RNA from either cysts or developing embryos. It is concluded that the stored, membrane-associated poly(A)$^+$RNA in dormant cysts is entirely or predominantly of nuclear origin. The 5×10^5-dalton component is probably homologous with the corresponding nuclear RNA component at later stages.

Poly(A)$^+$RNA-containing particles can be released from the membranes of dormant cysts by non-ionic detergents at low concentrations. Sucrose gradient centrifugation shows a major fraction of poly(A)$^+$RNA-containing particles around 40S. Poly(A)$^+$RNA extracted from these particles sediments as a broad band around 14S. The particles band in Cs_2SO_4 around 1.26 g/cm^3. Electron microscopy reveals fairly homogenous particles measuring 17-26 nm. Purified 40S particles are characterized by a protein pattern different from that of polyribosomal messenger ribonucleoportein particles from developing embryos.

The cytoplasm of dormant and developing embryos contain about equal amounts of initiation factor eIF-2. At both stages a minor part of this activity is bound to the membranes.

Introduction

The cytoplasm of encysted *Artemia* gastrulae contains latent mRNA, which is to a considerable extent associated with membranes (Nilsson and Hultin, 1972, 1974, 1975 ; Felicetti *et al.*, 1975 ; Sierra *et al.*, 1976 ; Grosfeld *et al.*, 1977 ; Hultin *et al.*, 1977 ; Simons *et al.*, 1978). After gentle homogenization and adequate dilution of the homogenate most mRNA-containing membranes sediment at 15 000 × g, and can be purified by banding in sucrose gradients at a density of 1.17 g/cm^3. The purified preparations contain endoplasmic

vesicles and mitochondria, which are difficult to separate effectively (Nilsson and Hultin, 1974). The mRNA is not dissociated from the membranes by EDTA.

The membrane-associated mRNA in *Artemia* cysts raises several questions of general interest, pertaining to its origin, relations to other mRNA classes, protein connections, and mode of interaction with the membrane structure. It would furthermore be of interest to know, whether other components of the disassembled protein synthesizing system of the cysts are similarly associated with membrane components. In the experiments described below purified membrane fractions were used to elucidate some of these questions.

Materials and methods

Materials

Artemia cysts were obtained from the Carolina Biological Supply Co., Burlington, NC, oligo(dT) cellulose T-2 from Collaborative Research Inc., Waltham, Mass., Sephadex G-10 from Pharmacia, Uppsala, Sweden, RNase A from Sigma Chemical Co., St. Louis, Mo., and *Escherichia coli* B tRNA from Calbiochem, Lucerne, Switzerland. The zwitterionic detergent, 3-(tetradecyldimethylammonio)-1-propanesulfonate was synthesized as described by Gonenne and Ernst (1978). [^{3}H]Methionine and [^{125}I]iodine were from the Radiochemical Centre, Amersham, England, and [^{3}H]poly(U) from the Miles Laboratories, Stoke Poges, England.

Cell fractionation

In most experiments cysts and nauplii were homogenized in a medium containing 0.15 M sucrose in TKM[1], and were fractionated as described previously (Nilsson and Hultin, 1974). For the preparation of RNA, nuclear fractions, obtained by 4 min centrifugation at 1 000 × g, were washed two to three times by recentrifugation in the same medium. When the nuclear fractions were to be used for DNA preparations, cysts and nauplii were homogenized in a buffer containing 0.1 % (v/v) Triton X-100, 0.32 M sucrose, 1 mM $MgCl_2$ and 1 mM potassium phosphate (pH 6.5), essentially as described by Lövtrup-Rein and McEwen (1966). The nuclear fractions were washed twice in detergent-free buffer before DNA extraction.

The membrane fractions, obtained by centrifuging the 1 000 × g supernatant for 10 min at 15 000 × g, were resuspended either in homogenization medium, or (for the preparation of poly(A)$^+$RNA) in Mg^{2+}-free medium containing 20 mM EDTA (Nilsson and Hultin, 1974). The membranes were purified by banding in 0.5-1.8 M sucrose gradients (buoyant density 1.17 g/cm^3). The ionic composition of the gradients was as in the TKM medium, when not otherwise indicated.

Polyribosomes were prepared from the post-mitochondrial fractions of developing embryos by the method of Wettstein *et al.* (1963), and were used for the preparation of messenger ribonucleoprotein particles essentially as described by Burny *et al.* (1969).

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecylsulfate; 1 × SSC, 150 mM NaCl and 15 mM sodium citrate (pH 7.0); TKM, 35 mM Tris-HCl (pH 7.7), 75 mM KCl and 5 mM $MgCl_2$; Tris, tris(hydroxymethyl)aminomethane.

Hybridization experiments

DNA was prepared from the purified nuclear fractions by the method of Marmur (1961), and was degraded to 300-500 nucleotide segments by alkali treatment as described by Tibbetts *et al.*, (1973). After neutralization, the DNA segments were precipitated with two volumes of ethanol (-20 °C), washed with 70 % (v/v) ethanol, and dissolved in 6 × SSC containing 50 % (v/v) purified formamide.

RNA was extracted from the nuclei and from the EDTA-treated and purified membrane fractions by the method of Holmes and Bonner (1973). Poly(A)$^+$RNA was prepared by repeated oligo(dT) cellulose chromatography as described previously (Nygård and Hultin, 1975). Samples of the RNA preparations were analyzed by polyacrylamide gel electrophoresis. Poly(A)$^+$RNA was labeled with ^{125}I by the method of Getz *et al.*, (1972). The labeled RNA was passed through a Sephadex G-10 column equilibrated with 2 × SSC, precipitated with two volumes of ethanol (-20 °C), and resuspended in 6 × SSC. Samples were taken for the determination of specific radioactivity.

Hybridization was performed in glass capillaries at two different DNA/RNA ratios as specified in the legends. The volumes were 10 µl. The sealed capillaries were heated for 2 min at 100 °C. Three capillaries were immediately plunged into an ice-bath, while the rest was transferred to a 45 °C water bath for different periods. Incubated samples, diluted with 1.0 ml 2 × SSC, were maintained at 4 °C. After RNase treatment (10 µg/ml) for 30 min at 35 °C the tubes were placed in an ice-bath. Undegraded material was precipitated with 0.5 ml 10 % (w/v) trichloroacetic acid. After 15 min (0 °C) the precipitates were collected on Millipore HAWP filters (0.45 µm pore size), washed and used for radioactivity determinations. At different periods of hybridization, control samples were processed in the same way, but without RNase digestion.

Preparation of poly(A)$^+$RNA-containing particles

Purified membranes from 4 g (dry weight) of cysts were suspended in 3 ml homogenization medium containing 0.1 % Triton X-100. After 10 min at 0 °C the suspension was centrifuged for 20 min at 12 000 × g and layered on sucrose gradients in TKM. After centrifuging for 18 hr at 41 000 × g (Beckmann SW 25 rotor) the gradients were monitored at 260 nm and 2.14 ml fractions were collected. Aliquots were used for poly(A) analyses.

The poly(A)-containing 40S fraction was diluted with TKM and pelleted by centrifugation for 3 hr at 166 500 × g (Beckmann 50 Ti rotor). The particles were suspended in TKM without or with the addition of 0.1 % sulfobetaine detergent, and were purified by recentrifugation in sucrose gradients.

Alternatively, the 40S fractions were used for the formation of linear Cs_2SO_4 gradients (1.10-1.80 g/cm^3). Centrifugation was for 18 hr (0 °C) at 150 000 × g (Beckmann SW 50.1 rotor). The gradients were monitored at 260 nm, and 0.26 ml fractions were collected.

RNA sedimentation analysis

Poly(A)-containing sucrose gradient fractions were precipitated with 2.5 volumes of ethanol at -20 °C. RNA was extracted according to Holmes and Bonner (1973). The RNA, precipitated in the presence of *E. coli* tRNA carrier, was dissolved in 250 µl buffer containing

20 mM sodium acetate, 5 mM EDTA, 0.1% SDS and 40 mM Tris-HCl (pH 7.7), layered on 0.15-0.46 M sucrose gradients in the same buffer, and centrifuged for 3.5 hr (0 °C) at 190 000 × g in a Spinco rotor SW 50.1. The gradients were monitored at 260 nm, and 0.43 ml fractions were collected for poly(A) analysis.

Polyacrylamide gel electrophoresis

RNA was analyzed by gel electrophoresis essentially as described by Loening (1967, 1969).

Protein samples were heated for 1 min with SDS and mercaptoethanol in a boiling water bath and analyzed in 7-15% polyacrylamide gradient gels usng the SDS system of Laemmli (1970).

Poly(A) analysis

Gradients were tested directly for poly(A)-containing material by a modification of the [^{3}H]poly(U) hybridization method of Lamb and Laird (1976). The 1.12 ml reaction mixtures contained 100 μl aliquots from gradient fractions, 0.5 μg of [^{3}H]poly(U) (9.5 Ci/mole phosphate) and 1 ml of 2 × SSC. After incubation for 2 hr at 45 °C the suspensions were allowed to cool for 10 min at room temperature, then placed in ice and treated for 20 min with 25 μg ribonuclease A. The reaction was stopped and the polynucleotides precipitated wiht 0.5 ml of ice-cold 10% TCA. The precipitates were collected on Millipore EGWP filters (0.2 μm pore size), dried and counted in a liquid scintillation counter.

eIF-2 determinations

The specific activity of initiation factor eIF-2 was determined in whole cytoplasmic extracts and in the extracts of purified membrane fractions.

Hydrated, dormant cysts and developing embryos at 50% emergence (50 g dry weight of each) were homogenized in a medium containing 0.2 M KCl, 5 mM magnesium acetate, 20 mM Tris-HCl (pH 7.6) and 10 mM mercaptoethanol. A cytoplasmic fraction was prepared as described previously (Nilsson and Hultin, 1974), and 4 M KCl was added to a final concentration of 0.5 M. The suspension was stirred for 10 min (0 °C) and centrifuged for 10 min at 15 000 × g. The supernatant was recentrifuged for 16 hr at 90. 000 × g and the extract fractionated with ammonium sulfate (Erni, 1976). The fraction at 40-50% saturation was collected.

Purified membranes were prepared as described previously (Nilsson and Hultin, 1974), but a buffer containing 35 mM KCl, 5 mM magnesium acetate, 35mM Tris-HCl (pH 7.6) and 5 mM mercaptoethanol was used throughout. The membranes were extracted with 0.5 M KCl as described by Schreier and Staehelin (1973), and centrifuged for 16 hr at 90 000 × g. The material was concentrated by ammonium sulfate precipitation at 70% saturation.

The activity of eIF-2 was measured as described previously (Nygård and Hultin, 1976).

Electron microscopy

Poly(A)-containing fractions from Cs_2SO_4 gradients were collected and dialyzed overnight against TKM. One drop of the suspension was placed on a formwar carbon film and negatively stained with 1% uranyl acetate (pH 4.6), or with 1% sodium phosphotungstate (pH 6). The preparations were examined in a Jeol 100S electron microscope.

Results

Sequence homology of membrane-associated poly(A)$^+$RNA with nuclear DNA and polyadenylated transcription products

Poly(A)$^+$RNA from the purified membrane fraction of dormant cysts was characterized by two distinct gel electrophoretic bands at approximately 3×10^5 and 5×10^5 daltons (Fig. 1). At the stage of 50 % emergence the proportion of the light band was markedly reduced (Grosfeld *et al.*, 1977). No poly(A)$^+$RNA was obtained from the nuclear fraction of dormant cysts. At 50 % emergence, the nuclear poly(A)$^+$RNA contained one dominant component, similar in electrophoretic mobility to the heavier poly(A)$^+$RNA from the membranes (Fig. 1). The appearance of this material during development indicates that it represents polyadenylated transcripts of nuclear genes.

^{125}I-labeled poly(A)$^+$RNA from the membranes of dormant cysts hybridized in solution with excess nuclear DNA to approximately the same saturation levels as the labeled nuclear poly(A)$^+$RNA from developing embryos (Fig. 2). The kinetics of hybridization were also essentially similar. In hybridization-competition experiments different concentrations of unlabeled poly(A)$^+$RNA (12 or 18 times the concentration of labeled RNA) were included in the hybridization mixtures. The inhibition was equally effective in both heterologous combinations (Fig. 2). Fig. 3 compares the competitive activities of unlabeled poly(A)$^+$RNA from the membranes of dormant and developing embryos in the hybridization of ^{125}I-labeled nuclear poly(A)$^+$RNA to DNA. The efficiency of the membrane RNA from developing embryos was not reduced despite its lower proportion of the light poly(A)$^+$RNA component (Fig. 1 B). Rather, it was somewhat increased. The data suggest a close similarity not only in size, but also in sequence distribution, between the 5×10^5 dalton component in the membrane-associated poly(A)$^+$RNA and the corresponding component in the polyadenylated nuclear RNA transcribed during early development.

The structural organization of membrane-associated poly(A)$^+$RNA

Poly(A)$^+$RNA-containing particles were easily detached from the purified cytoplasmic membranes. Fig. 4 shows that by gentle treatment with the non-ionic detergent, Triton X-100, 65 % of the poly(A)- containing material was solubilized already at a concentration of 0.1 %. Some poly(A)-containing structures remained sedimentable at 12 000 × g even at high Triton concentrations.

When analyzed by sucrose gradient centrifugation, a great part of the detached, poly(A)-containing material accumulated in a 40S fraction (Fig. 5). A fraction of slightly more rapidly sedimenting particles had much lower poly(A) content. The separation became more effective with increasing distance of sedimentation. Only a minor part of the poly(A)$^+$RNA was associated with heavier aggregates, *e.g.* ribosomes. Sedimentation analysis of the RNA extracted from the 40S fraction in the presence of tRNA carrier showed a poly(A) peak at 14S (Fig. 6).

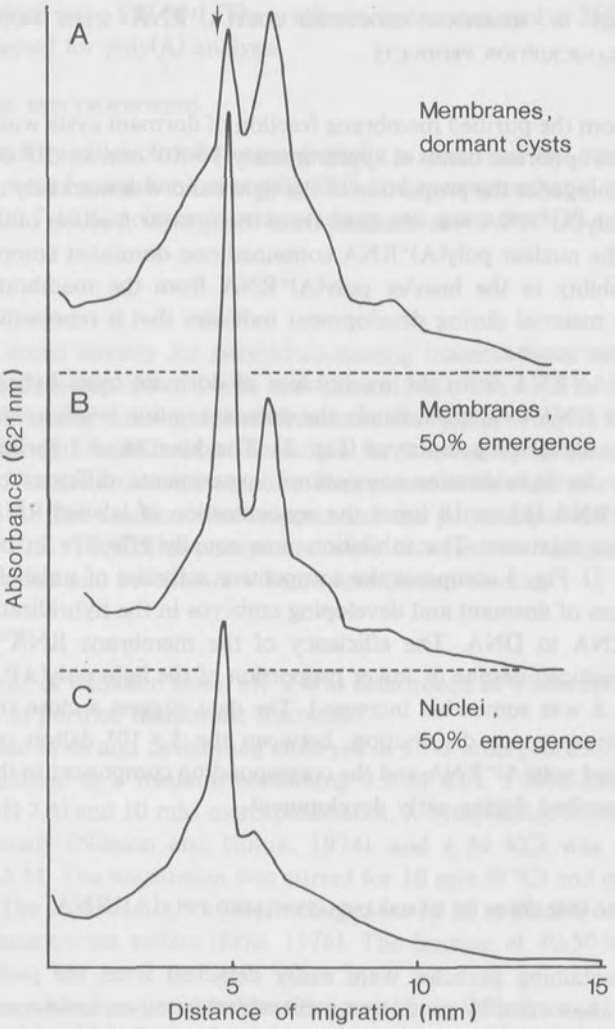

FIG. 1. Electrophoretic patterns of poly(A)⁺RNA from purified membrane and nuclear fractions of cysts and developing embryos at 50% emergence. Of the RNA preparations 1.3-1.5 A_{260} units were analyzed in 2.6% polyacrylamide gel columns provided with 2.1% spacer gels. Electrophoresis was for 150 min at 200 V. Gels were stained in 0.2% toluidine blue and recorded by use of a Joyce-Loebl microdensitometer.

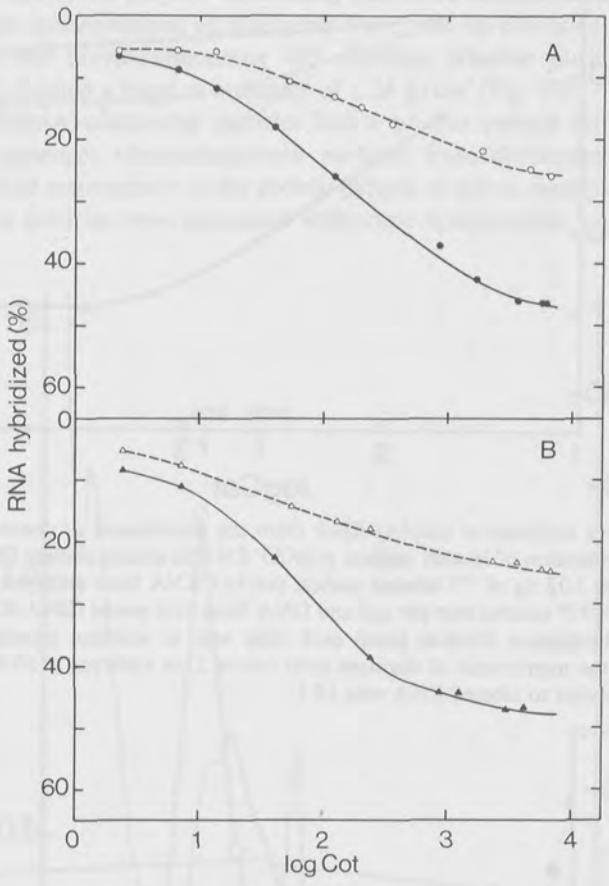

FIG. 2. Hybridization experiments indicating the nuclear origin of membrane-associated poly(A)$^+$-RNA from dormant cysts. Nuclear DNA was from nauplii. (A) ^{125}I-labeled poly(A)$^+$RNA from the purified membrane fraction of dormant cysts (specific activity 0.6×10^6 counts/min per μg). The hybridization curve (solid line) was constructed from two series of incubation in capillary tubes with DNA:RNA ratios of 1 400:1 and 36 000:1. The [^{125}I]RNA concentrations were 1.58 and 1.13 ng per 10 μl, respectively. For competitive hybridization (broken line) unlabeled nuclear poly(A)$^+$RNA from embryos at 50% emergence was included at a ratio of 12:1 to the labeled membrane RNA. (B) ^{125}I-labeled nuclear poly(A)$^+$RNA from embryos at 50% emergence (specific activity 1.3×10^6 counts/min per μg). The hybridization curve (solid line) was constructed as in A, but with DNA:RNA ratios of 1 100:1 and 29 000:1 ([^{125}I]RNA concentrations 2.13 and 1.42 ng per 10 μl, respectively). For competitive hybridization (broken line) unlabeled membrane RNA from dormant cysts was included at a ratio of 12:1 to the labeled nuclear RNA.

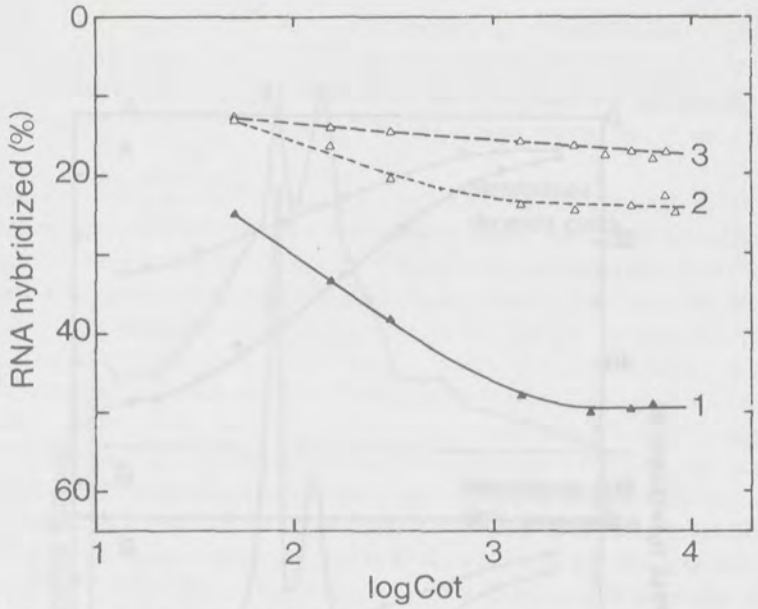

FIG. 3. Competitive activities of poly(A)⁺RNA from the membranes of dormant and developing embryos in the hybridization of labeled nuclear poly(A)⁺RNA to excess nuclear DNA. Each capillary tube (10 μl) contained 3.02 ng of ^{125}I-labeled nuclear poly(A)⁺RNA from embryos at 50% emergence (specific activity 0.5×10^6 counts/min per μg) and DNA from cyst nuclei (DNA:RNA ratio 17 000:1). For competitive hybridization (broken lines) each tube was in addition provided with unlabeled poly(A)⁺RNA from the membranes of dormant cysts (curve 2) or embryos at 50% emergence (curve 3). The ratio of unlabeled to labeled RNA was 18:1.

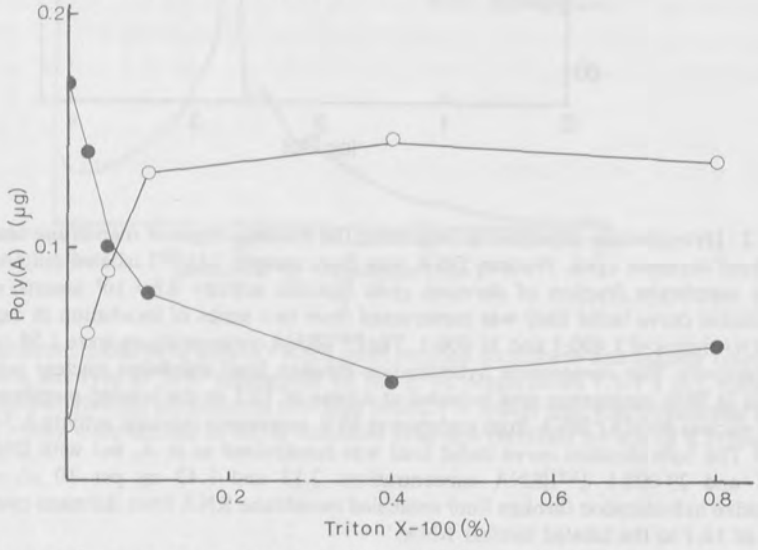

FIG. 4. Detachment of membrane-associated poly(A)-containing particles with non-ionic detergent. Purified membranes from dormant cysts were suspended in TKM, treated for 10 min (0 °C) with different concentrations of Triton X-100, and centrifuged for 20 min at 12 000 × g. RNA was extracted from supernatants (○) and sediments (●), and analyzed for poly(A) by hybridization to [^{3}H]poly(U).

The 40S particles could be purified by resedimentation in sucrose gradients. After treatment of the 40S fraction with sulfobetaine detergent (0.1 %) some light-absorbing material was solubilized, but most of the poly(A)$^+$-containing structures sedimented in the 35-40S region (Fig. 7A). At high concentrations of detergents there was no extensive loss of material. In Cs_2SO_4 gradients the poly(A)-containing 40S particles, whether purified by sulfobetaine treatment or not, formed a band at a density of 1.26 g/cm^3 (Fig. 7B).

The purified poly(A)-containing particles had a protein pattern different from that of polyribosomal messenger ribonucleoprotein particles from developing embryos (Fig. 8). Certain proteins had counterparts in the protein pattern of whole membranes. Lipid analyses suggested that the particles were associated with some lipoprotein(s).

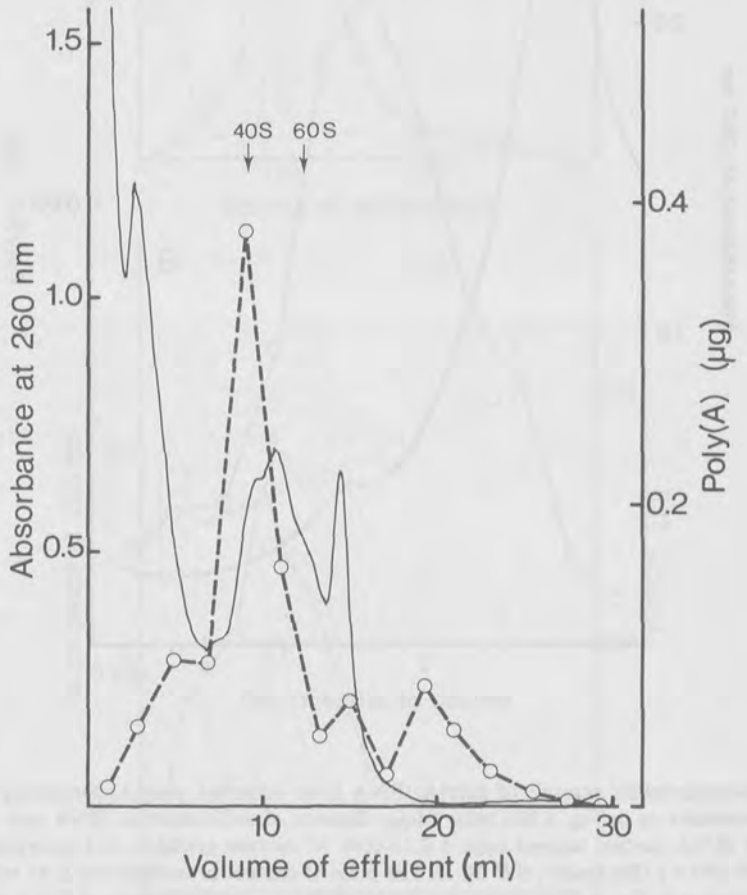

FIG. 5. Sedimentation analysis of detached, poly(A)$^+$-containing structures. Purified membranes were treated for 10 min (0 °C) with 0.1 % Triton X-100 in TKM, and the 12 000 × g supernatant was centrifuged for 16 hr (41 400 × g) in a 0.5-1.8 M sucrose gradient (Beckmann SW 25). Ribosomal subunits from rat liver were centrifuged in a parallel gradient for indicating 40S and 60S positions (arrows). The poly(A) content of consecutive 2.14 ml fractions (○) was determined by direct hybridization to [^{3}H]poly(U). Continuous line represents the A_{260} profile.

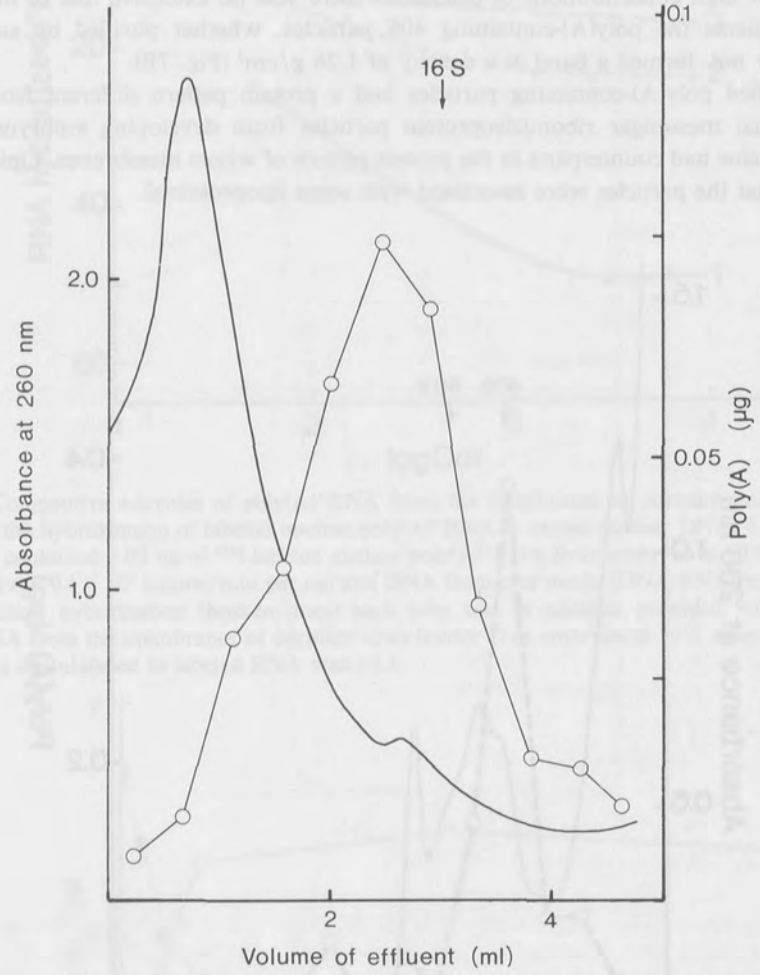

FIG. 6. Sedimentation analysis of poly(A)⁺RNA from detached, poly(A)-containing 40S particles, obtained essentially as in Fig. 5 but with longer distance of sedimentation. RNA was isolated in the presence of tRNA carrier, layered onto a 0.15-0.46 M sucrose gradient, and centrifuged for 3.5 hr (0 °C) at 190 000 × g (Beckmann SW 50.1). The poly(A) content of consecutive 0.43 ml fractions (○) was determined as in Fig. 4. Continuous line indicates the A_{260} profile (mainly of tRNA carrier). Arrow indicates the peak of *E. coli* 16S rRNA, centrifuged in a parallel gradient.

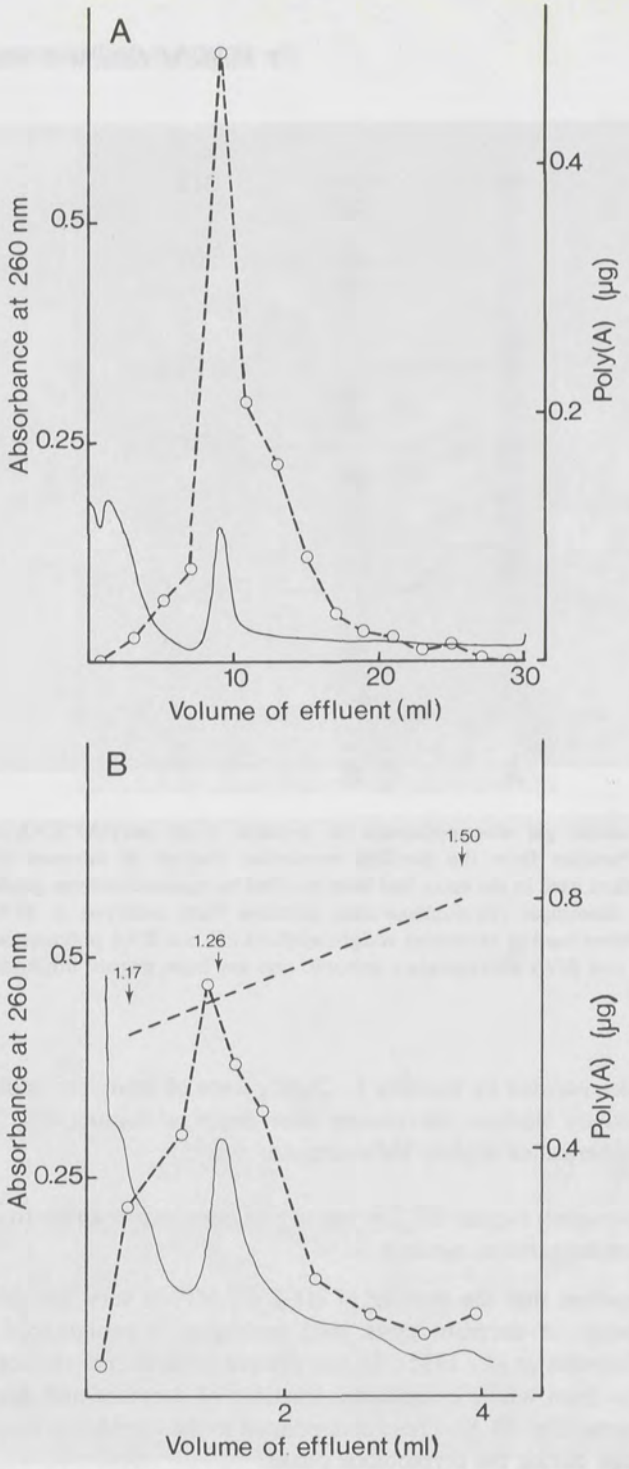

FIG. 7. Purification of detached, poly(A)-containing 40S particles. (A) The poly(A)-containing 40S fraction was suspended in TKM containing 0.1% zwitterionic sulfobetaine detergent, and was recentrifuged in a sucrose gradient as described in Fig. 5. (B) The particles were centrifuged for 18 hr (0 °C) without fixation in a preformed Cs_2SO_4 gradient. Consecutive fractions (○) were used for poly(A) determinations. Continuous lines represent A_{260} profiles, oblique line in B the density profile.

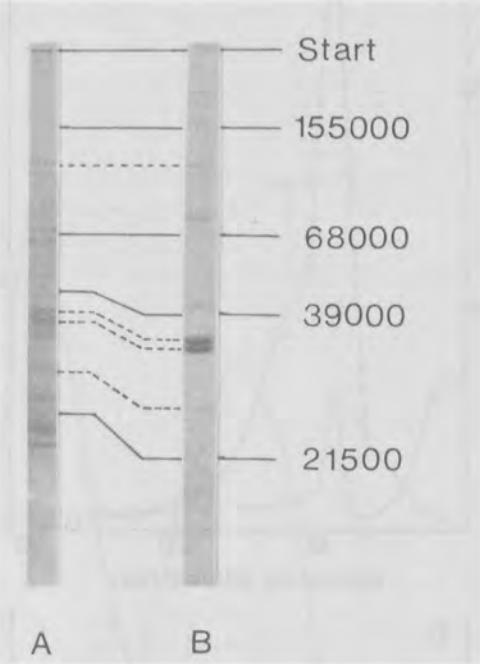

FIG. 8. SDS gradient gel electrophoresis of proteins from poly(A)$^+$RNA-containing particle preparations. (A) Particles from the purified membrane fraction of dormant cysts. The poly(A)-containing 40S fraction used in the assay had been purified by repeated sucrose gradient centrifugation. (B) Polyribosomal messenger ribonucleoprotein particles from embryos at 50% emergence. The following proteins were used as molecular weight markers : *E. coli* RNA polymerase β-subunit, bovine serum albumin, *E. coli* RNA polymerase α-subunit, and soy bean trypsin inhibitor.

The 40S particles purified by banding in Cs_2SO_4 were of relatively uniform size (26 × 17 nm) as determined by electron microscopy after negative staining (Fig. 9). Their typical appearance was spherical or slightly bullet-shaped.

THE ACTIVITY OF INITIATION FACTOR eIF-2 IN THE CYTOPLASMIC AND PURIFIED MEMBRANE FRACTIONS FROM DORMANT AND DEVELOPING EMBRYOS

It has been reported that the activity of eIF-2 (IF-MP) is very low in the cytosol and ribosomal KCl wash of dormant cysts, and undergoes a pronounced increase during development (Filipowicz *et al.*, 1975). In the present experiments the total yield of eIF-2 activity in extracts from whole cytoplasmic fractions of dormant and developing embryos were nearly the same (Fig. 10 A). These data pointed to the membrane fraction as a possible site of eIF-2 storage during the cryptobiotic phase.

The eIF-2 activity was determined in 0.5 M KCl extracts of purified membrane fractions from dormant and developing embryos (Fig. 10 B). Although there was some difference in the specific activities, the total activities were closely similar. The specific activities of the

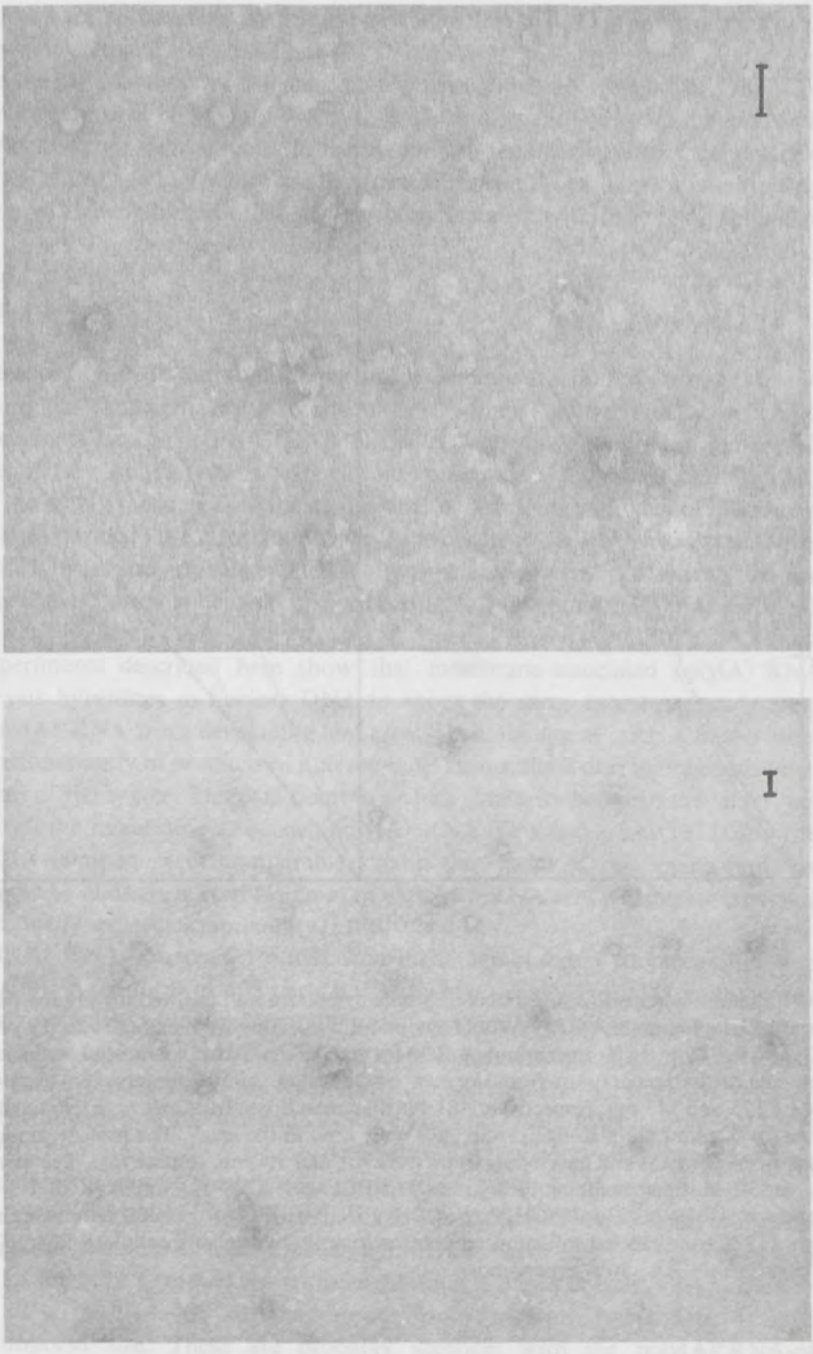

FIG. 9. Electron micrographs of 40S particles purified by banding in Cs_2SO_4. Poly(A)-containing Cs_2SO_4 fraction as shown in Fig. 7 B was negatively stained with 1% uranyl acetate (pH 4.6). Scales indicate 50 nm.

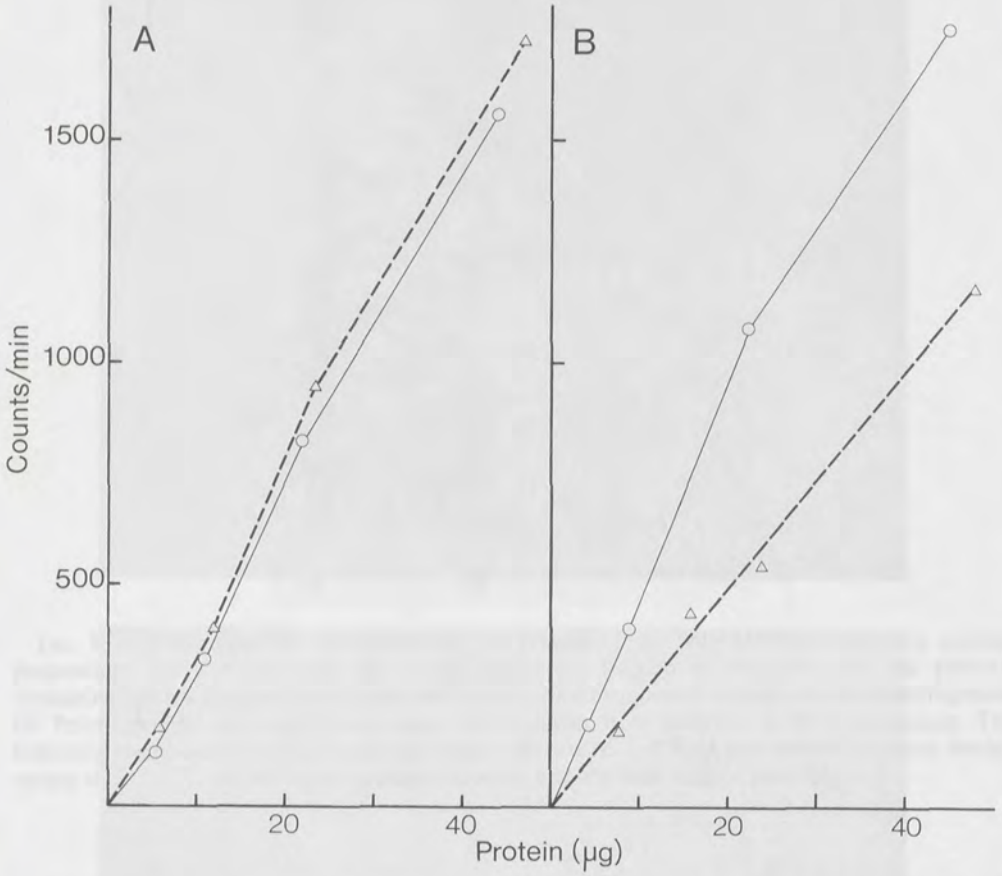

FIG. 10. Initiation factor eIF-2 in extracts of whole cytoplasm and purified membrane fractions of dormant and developing embryos. (A) Whole cytoplasmic fractions were extracted in the presence of 0.5 M KCl, and the 40 to 50% ammonium sulfate fraction of the extracts was used in the assay. The protein contents of the preparations from dormant and developing (50% emergence) cysts (50 g dry weights) were 125 and 143 mg, respectively. (B) Purified membrane fractions were extracted in 0.5 M KCl, and the 70% ammonium sulfate precipitates were used in the assay. The protein contents of the preparations from dormant and developing cysts were 6.1 and 9.4 mg, respectively. The assay system contained, in 50 μl final volumes, 1.0 μmol Tris-HCl (pH 7.5), 5.0 μmols KCl, 0.5 μmol 2-mercaptoethanol, 10 pmols GuoPP(CH_2)P, and 2.4μg [^{3}H]Met-tRNA$_f^{Met}$ (7 300 counts/min per μg). After 5 min (37 °C) the labeled initiation complex was collected on nitrocellulose filter. ○——○, Dormant cysts; △– – –△, 50% emergence.

preparations were comparable with those of the whole cytoplasmic extracts, but the total activities were relatively low (less than 10%).

Most of the eIF-2 activity of the membranes was solubilized with 0.1% Triton X-100. In view of the recent data of Spirin (1978) and Ovchinnikov *et al.* (1978), in support of factor-carrying ribonucleoprotein particles in the cytoplasm, the distribution of the detached eIF-2 activity was determined by sucrose gradient centrifugation. In gradients similar to that in Fig. 5 a fraction of the solubilized eIF-2 activity cosedimented with the poly(A)-containing 40S particles.

Discussion

The presence of mitochondria in the purified membrane fraction of dormant *Artemia* cysts made the origin of the membrane-associated mRNA somewhat uncertain (Nilsson and Hultin, 1974). In mammalian cells poly(A)$^+$RNA of nuclear and mitochondrial origin may differ in the lengths of their poly(A) chains (Hirsch and Penman, 1973 ; Molloy and Darnell, 1973). However, the poly(A) sequences in the membranes of dormant cysts were of intermediate size, and therefore provided little information about the origin of the RNA molecules (Nilsson and Hultin, 1975). It has recently been stated that only a low percentageof membrane-associated mRNA in *Artemia* cysts is capable of hybridizing to mitochondrial DNA (Grosfeld *et al.*, 1977).

The experiments described here show that membrane-associated poly(A)$^+$RNA from dormant cysts hybridizes to nuclear DNA to about the same extent as newly transcribed nuclear poly(A)$^+$RNA from developing embryos. Thus, the latent mRNA in the membranes consists predominantly of products of nuclear genes, transcribed during oogenesis or the early development of the zygote. The data point to a close similarity between the larger, persistent component of the membrane-associated poly(A)$^+$RNA (Grosfeld *et al.*, 1977) and the major poly(A)$^+$RNA component of comparable size in the nuclei of developing embryos. This similarity may be of interest from the point of view of mRNA renewal during embryogenesis, and would justify a closer examination.

The poly(A)$^+$RNA, detached from the purified membranes with non-ionic detergents, appears mainly in the form of 40S particles. Their low density in Cs_2SO_4 is compatible with the relatively complex protein pattern, different from that of polyribosomal mRNP particles, and the presence of some lipid. The lipid content is furthermore suggestive of hydrophobic sites, which may participate in the membrane interaction. The particles can be purified by repeated centrifugation in sucrose and Cs_2SO_4 gradients. Like polyribosomal mRNP particles (Olsnes, 1970) they are sensitive to ionic detergents, but low concentrations of zwitterionic or non-ionic detergents can be used in their purification. The poly(A) sequences of the particles are available to hybridization with poly(U) under standard assay conditions, and the particles bind to oligo(dT) cellulose at high ionic strength (Hultin *et al.*, 1977). This suggests that the interaction of the poly(A) sequences with neighboring proteins is easily reversed under these conditions. The purified 40S particles appear in the electron microscope as spheres of relatively uniform size. These are probably identical with the poly(A)$^+$RNA-carrying structures, but conclusive evidence is still lacking.

Like whole cytoplasmic fractions, purified membranes from dormant and developing embryos contain about the same amounts of initiation factor eIF-2. Only a minor part of the

overall activity is membrane-bound. Our results do not confirm the earlier data of Filipowicz *et al.*, (1975), and disfavour the possibility of eIF-2 sequestration during the cryptobiotic phase. Some of the membrane-bound eIF-2 activity seems to be associated with mRNA-containing particles. This possibility is currently investigated.

Acknowledgements

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Ribosomes from *Artemia* cysts in cell-free translation of eukaryotic mRNA

Gisela Kramer and Boyd Hardesty

Department of Chemistry, Clayton Foundation Biochemical Institute
University of Texas at Austin
Austin, Texas 78712, USA

Abstract

Ribosomes from dry cysts of *Artemia* were isolated and analyzed for their ability to support translation of exogenous mRNA and their usefulness in the studies of eukaryotic peptide initiation. KCl-washed *Artemia* ribosomes did not support protein synthesis in the absence of exogenous mRNA and enzymes. They appear to be devoid of endogenous mRNA and mRNA fragments. The intact ribosomes or ribosomal subunits translated exogenous eukaryotic mRNAs efficiently, when supplemented with initiation factors and elongation enzymes from rabbit reticulocytes. Ribosomal subunits derived from *Artemia* ribosomes were found to be at least as active as monomeric ribosomes from the same organism or ribosomal subunits from reticulocytes for translation of exogenous mRNA. 40S ribosomal subunits from *Artemia* cysts proved useful in the study of individual steps of peptide initiation. Some requirements for the binding of ^{125}I-labeled globin mRNA to these particles are presented. Ribosomes from dry cysts of *Artemia* were found to be low in polyamines. Thus requirements for spermine and spermidine may be studied with these ribosomes.

Introduction

Dry cysts of *Artemia* represent a dormant form that is metabolically inactive. Thus ribosomes should be present as 80S monomeric particles that should be devoid of peptide initiation factors. It has been demonstrated that radiolabeled initiation factors eIF-2 and eIF-3 form stable complexes with 40S ribosomal subunits and that these factors are not present in 80S complexes (Safer *et al.*, 1976 ; Benne and Hershey, 1978).

Eukaryotic peptide initiation involves at least seven factors, probably more, and requires the hydrolysis of GTP and ATP. The initiation factor eIF-2 forms a ternary complex with Met-tRNA$_f$ and GTP. This ternary complex can bind to 40S ribosomal subunits in the presence of other initiation factors. Evidence has been presented (Odom *et al.*, 1978) and recently confirmed (Merrick, 1979) that GTP is associated with the proper binding of the ternary complex to 40S ribosomal subunits. It is generally accepted that binding of Met-tRNA$_f$ precedes binding of mRNA in eukaryotic systems. The mechanism and role of initiation factors in the latter reaction is less clear than for the binding of Met-tRNA$_f$. 40S ribosomal subunits from the dry cysts of *Artemia* may provide a useful tool to study eukaryotic peptide initiation in that they may lack fortuitously adherent non-ribosomal proteins.

Materials and methods

Materials

Dry cysts of *Artemia* were purchased from Longlife Aquarium Products, Harrison, New Jersey. ATP, GTP, creatine phosphate and creatine phosphokinase were from Sigma Chemical Company, St. Louis, Missouri. [^{14}C]-leucine (312 Ci/mol) and [^{14}C]arginine (312 Ci/mol) were from Schwarz/Mann, Orangeburg, New Jersey, sodium [^{125}I]iodide (carrier-free) from Amersham/Searle, Arlington Heights, Illinois, and carrier-free [^{32}P]phosphate from ICN Life Science Group, Irvine, California. [γ-^{32}P]ATP was prepared by minor modification (Keenan *et al.*, 1972) of the procedure described by Glynn and Chappell (1964).

Methods

Preparation of 80S ribosomes from Artemia *cysts*

Ribosomes were prepared from viable cysts of *Artemia* by minor modifications (Kramer *et al.*, 1975) of the procedure described by Zasloff and Ochoa (1971). This preparation includes centrifugation of the ribosomes through a solution containing 0.4 M KCl. The ribosomal pellet was resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM KCl, 0.1 mM $MgCl_2$, and 1 mM dithioerythritol at a concentration of 20 mg/ml. The ribosomes were stored frozen at -80 °C in small aliquots.

Preparation of ribosomal subunits

1. From *Artemia*

Subunits were prepared from the ribosomes obtained as described above by a procedure reported in detail previously (Kramer *et al.*, 1979). The ribosomes were dissociated in a solution containing 50 mM Tris-HCl (pH 7.5), 0.7 M KCl, 11 mM $MgCl_2$, and 20 mM β-mercaptoethanol. Ribosomal subunits were separated by sucrose gradient centrifugation.

2. From rabbit reticulocytes

These ribosomal subunits were prepared as described by Kramer *et al.* (1977). The main difference to the procedure under a) is that ribosomes from rabbit reticulocytes were first washed with 0.5 M KCl to obtain the ribosomal wash fraction described below. They were then dissociated in a solution containing 20 mM Tris-HCl (pH 7.5), 0.5 M KCl, 3 mM $MgCl_2$, 1 mM β-mercaptoethanol at 4 °C for 30 min before the subunits were separated.

Preparation of mRNA

Globin mRNA was prepared from the 0.5 M KCl-washed rabbit reticulocyte ribosomes as reported earlier (Konecki *et al.*, 1975a). Briefly, total RNA was extracted and chromatographed first on cellulose (Sigma cell type 50), then on Sepharose 4B (Pharmacia). By this procedure, 9S RNA was obtained essentially free of contaminating ribosomal RNA. Protamine mRNA was a generous gift from Dr. G. H. Dixon, Calgary, Canada.

^{125}I-labeling of globin mRNA

The procedure of iodination of mRNA has been published earlier (Henderson *et al.*, 1979). It is a modification of that described by Getz *et al.* (1972).

Preparation of enzyme fractions from rabbit reticulocytes necessary for in vitro *mRNA translation*

The preparation of 40-70% ammonium sulfate fraction from the postribosomal supernatant was carried out as described earlier (Hardesty *et al.*, 1971). The salt wash fraction from reticulocyte ribosomes (Morrisey and Hardesty, 1972) was either used directly as a source of initiation factors or was fractionated by chromatography on DEAE-cellulose (Cimadevilla *et al.*, 1975). The initiation factors eIF-2 and eIF-3 were further purified and separated from each other by chromatography on phosphocellulose (Odom *et al.*, 1978).

In vitro *protein-synthesizing system*

The standard assay reaction mixture (Kramer *et al.*, 1975) contained in a final volume of 0.1 ml : 20 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 75 mM KCl, 5 mM reduced glutathione, 50 μM each of 19 amino acids excluding the labeled one (generally leucine), 1 mM ATP (adjusted to pH 6.5), 0.4 mM GTP, 15 mM creatine phosphate, 13 μg creatine phosphokinase, 5 μg rabbit liver tRNA, 20 μM [^{14}C]leucine (40 Ci/mol), 100 μg protein of the postribosomal 40-70% ammonium sulfate fraction, about 90 μg of the ribosomal salt wash fraction, 80S *Artemia* ribosomes or ribosomal subunits as indicated, generally about 10 pmol, and mRNA as given in the legends. Reaction mixtures were incubated for 30 min at 37 °C. Reactions were terminated by the addition of 100 μl 1N NaOH, incubation was continued for another 5 min at 37 °C, then about 2 ml of 5% trichloroacetic acid were added. Precipitated protein was collected by filtration through glass fiber filters (Schleicher and Schüll, # 29). The filters were washed with 5% trichloroacetic acid, dried and counted in 10 ml of a counting fluid containing 5 g 2,5-diphenyloxazole per liter of toluene.

Binding of mRNA to 40S ribosomal subunits

^{125}I-labeled globin mRNA was bound to *Artemia* 40S ribosomal subunits as reported previously (Henderson *et al.*, 1979). The reaction mixture contained in a final volume of 0.15 ml : 20 mM Tris-HCl (pH 7.5), 80 mM KCl, 2.5 mM $MgCl_2$, 5 mM reduced glutathione, 1 mM ATP, 0.4 mM GTP, 5 mM creatine phosphate, 5 μg of creatine phosphokinase, 10 μg partially purified Met-tRNA$_f$, *Artemia* 40S ribosomal subunits, ^{125}I-labeled mRNA plus initiation factors and other components as indicated in the legends. The reaction mixture was incubated for 8 min et 37 °C, then loaded on a linear 15-30% (w/v) sucrose density gradient in 20 mM Tris-HCl (pH 7.5), 80 mM KCl, 2.5 mM $MgCl_2$ and 5 mM β-mercaptoethanol. The gradients were centrifuged at 59 000 rpm for the time indicated at 4 °C in a Spinco SW60 rotor (Beckman Instruments, Palo Alto, California).

Phosphorylation of ribosomal subunits and analysis by polyacrylamide gel electrophoresis

Ribosomal subunits were phosphorylated by the purified catalytic subunit of cAMP-dependent protein kinase from reticulocytes (Grankowski *et al.*, 1979), in a final volume of 30 μl, containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM dithioerythritol, 0.15 mM [γ-^{32}P]ATP (150 Ci/mol) and substrate and enzyme in the amounts indicated. Reaction mixtures were incubated for 10 min at 37°. Then the samples were processed for slab gel electrophoresis on 15% polyacrylamide gels with sodium dodecylsulfate as described previously (Grankowski *et al.*, 1979). Autoradiograms were prepared from dried gels by putting them under X-ray film ("No Screen", Eastman Kodak) for about 16 hr.

Results

Translation of exogenous mRNA on *Artemia* ribosomes

Ribosomes were isolated from the dry cysts of *Artemia* as described in the section "Methods". These ribosomes do not promote incorporation of amino acids into protein without added mRNA. However, they support translation of exogenous mRNA in the presence of enzyme fractions from rabbit reticulocytes. In the experiment shown in Fig. 1, crude fractions of elongation and initiation factors were used. The 40-70% ammonium sulfate fraction of the postribosmal supernatant provides aminoacyl-tRNA synthetases and peptide elongation enzymes. Peptide initiation factors are added as an enzyme fraction that was removed from reticulocytes ribosomes with 0.5 M KCl. This fraction has been called the ribosomal salt wash. We have studied the translation of two different mRNAs. The results with globin mRNA from rabbit reticulocytes are shown in Fig. 1 A ; those obtained with protamine mRNA from trout testis are given in Fig. 1 B. Globin mRNA translation was measured by incorporation of [^{14}C]leucine. Protamine is a small basic nuclear protein that does not contain leucine (Dayhoff, 1972), but is rich in arginine. Therefore incorporation of radioactive arginine was used to follow the synthesis of protamine.

Artemia ribosomes may be dissociated into their subunits by elevating the KCl concentration to about 0.7 M. These subunits are particularly useful in studies of peptide initiation as shown below. In contrast to the subunits derived from reticulocyte ribosomes, the subunits from *Artemia* ribosomes support only a very low [^{14}C]leucine incorporation in the absence of exogenous mRNA (Table I, experiment 1). The endogenous leucine incorporation seen with *Artemia* ribosomal subunits is caused to a large extent by mRNA that is present as a contaminant in the ribosomal salt wash fraction from reticulocytes (Kramer *et al.*, 1975). Addition of globin mRNA causes a large increase in [^{14}C]leucine incorporation with *Artemia* subunits.

Reticulocyte ribosomal subunits still contain an appreciable amount of mRNA so that [^{14}C]leucine incorporation is increased less than twofold by addition of exogenous mRNA. The data presented in Table I, experiment 2, compare translational activity of *Artemia* ribosomal subunits with 80S ribosomes. Combined 40S plus 60S subunits are at least as active as 80S particles in translation of added globin mRNA. Appreciable incorporation in the presence of mRNA is observed with only the 60S ribosomal subunit fraction. This is due to contaminating 40S subunits in the 60S subunit preparation. Some 40S ribosomal subunits sediment as dimers that cosediment with the 60S subunits during sucrose gradient fractionation.

The somewhat higher activity for globin mRNA translation observed with subunits derived from *Artemia* appears to be related to the higher activity of the 40S subunits of the *Artemia* ribosomes, as shown in Fig. 2. For these experiments, 60S subunits from reticulocyte ribosomes were added to equimolar amounts of 40S subunits from either reticulocyte or *Artemia*. Similar results were observed with the reciprocal protocol in which 60S ribosomal subunits from *Artemia* were used (data not shown). The lower activity of the reticulocyte 40S ribosomal subunits may be related to fragments of mRNA that lack a nucleotide sequence required for peptide termination. Peptides formed with these fragments may not be released from the ribosomes thus blocking subsequent peptide synthesis.

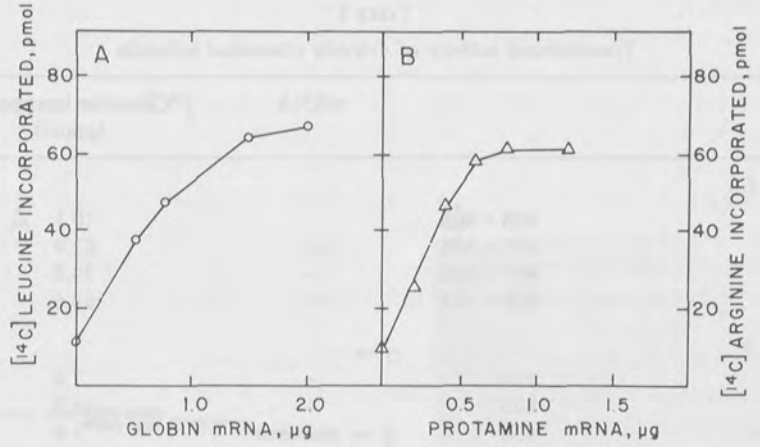

FIG. 1. Translation of exogenous mRNA in *Artemia* 80S ribosomes. A. Globin mRNA in the amounts indicated was translated on 10 pmol *Artemia* 80S ribosomes under conditions described under Materials and Methods. B. Protamine mRNA in the amounts indicated was translated on 12 pmol *Artemia* ribosomes as described except that ^{14}C-arginine (30 Ci/mol) was the radioactive amino acid used.

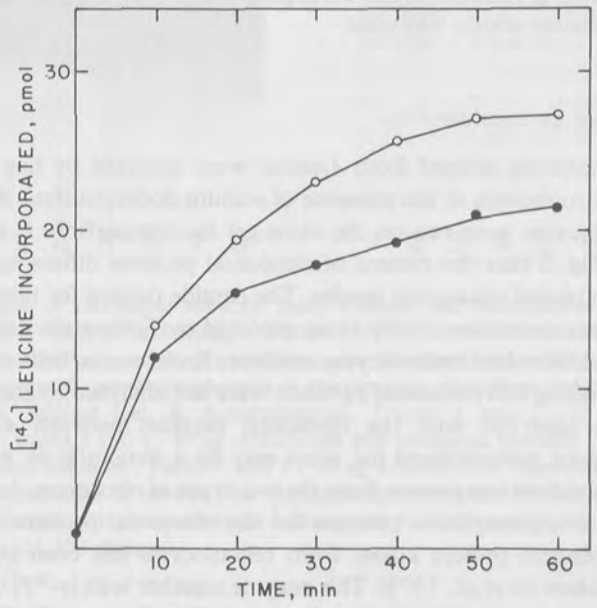

FIG. 2. Translational activity of *Artemia verus* reticulocyte 40S ribosomal subunits. The reaction mixtures, enlarged to 600 µl, contained 3.75 µg globin mRNA, 45 pmol reticulocyte 60S ribosomal subunits and either 45 pmol *Artemia* (○——○), or reticulocyte (●——●) 40S ribosomal subunits. From the incubation mixtures 75 µl were withdrawn at the times indicated and processed as described under Materials and methods.

TABLE I

Translational activity of *Artemia* ribosomal subunits

Ribosomes		mRNA	[^{14}C]leucine incorporated (pmol)
Experiment 1			
Artemia	40S + 60S	–	10.1
	40S + 60S	+	82.4
reticulocyte	40S + 60S	–	34.5
	40S + 60S	+	64.6
Experiment 2			
Artemia	80S	–	7.4
	80S	+	46.0
	40S	–	1.4
	40S	+	1.8
	60S	–	5.6
	60S	+	22.7
	40S + 60S	–	4.25
	40S + 60S	+	51.7

Ribosomes or ribosomal subunits as indicated were tested for their ability to translate globin mRNA. In these experiments, 5 μg of partially purified globin mRNA as obtained after cellulose chromatography was used. In experiment 1, the amount of all ribosomal subunits was 20 pmol. In experiment 2, either 12 pmol 80S ribosomes or 8 pmol of each of the ribosomal subunits were added.

CHARACTERIZATION OF *ARTEMIA* SUBUNITS

The ribosomal subunits isolated from *Artemia* were analyzed by one dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Ribosomal subunits from rabbit reticulocytes were run on the same gel for comparison. It is evident from the results shown in Fig. 3 that the pattern of ribosomal proteins differs appreciably between these two distantly related eukaryotic species. The peptide pattern for rabbit reticulocyte 40S subunits shown here resembles closely those reported by Freienstein and Blobel (1975) for 40S subunits from either duck reticulocytes, rat liver, Krebs ascites cells or mouse MOPC 41 cells. The corresponding 60S ribosomal subunits were not analyzed by these authors. In spite of the differences observed with the ribosomal peptides between *Artemia* and rabbit reticulocytes, evidence was obtained for what may be a structural as well as a functional homology between at least one protein from the two types of ribosomes. Included in Fig. 3 are the corresponding phosphorylation patterns for the ribosomal proteins. The catalytic subunit of cAMP-dependent protein kinase from reticulocytes has been purified to apparent homogeneity (Grankowski *et al.*, 1979). This enzyme together with [γ-^{32}P]ATP phosphorylates a protein of the reticulocyte 40S ribosomal subunit (Fig. 3, track 2). This protein has an apparent molecular weight of 32 000 and may be S6 by the nomenclature suggested for mammalian ribosomal subunits (McConkey *et al.*, 1979). It appears to be the same ribosomal protein reported by Traugh and Porter (1976) to be phosphorylated by fractions from reticulocytes that contained cAMP-dependent protein kinases or their catalytic subunit. One

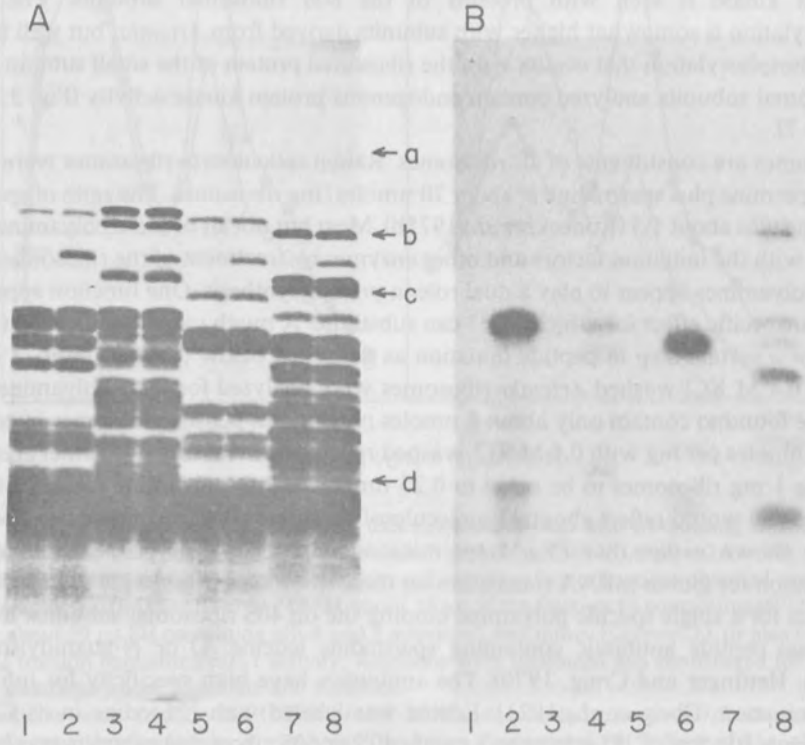

FIG. 3. Comparison of ribosomal subunits from *Artemia* and reticulocytes. Ribosomal subunits were incubated with [γ-^{32}P]ATP and without (tracks 1, 3, 5, 7) or with (tracks 2, 4, 6, 8) 1 μg of the catalytic subunit of cAMP-dependent protein kinase as described under Methods. They were then analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate followed by autoradiography. A. Stained Gel. B. Autoradiogram. Tracks 1 and 2 : 20 μg reticulocyte 40S ribosomal subunits ; tracks 3 and 4 : 40 μg reticulocyte 60S ribosomal subunits ; tracks 5 and 6 : 20 μg *Artemia* 40S ribosomal subunits ; tracks 7 and 8 : 40 μg *Artemia* 60S ribosomal subunits. Molecular weight markers are indicated by arrows : (a) bovine serum albumin (68 000 daltons) ; (b) fumarase (subunit = 48 500 daltons) ; (c) glyceraldehyde-3-phosphate dehydrogenase (subunit = 37 000 daltons) ; (d) trypsin inhibitor (21 300 daltons).

protein of the *Artemia* 40S ribosomal subunits also is phosphorylated by the reticulocyte cAMP-dependent kinase, although to a somewhat lesser extent than the reticulocyte ribosomal protein (Fig. 3, track 6). The *Artemia* ribosomal protein has a molecular weight of about 30 000 *versus* 32 000 for the phosphorylated reticulocyte ribosomal protein. However, the similarity in phosphorylation probably reflects a structural homology and possibly a functional homology between the two proteins. Only slight phosphorylation by the cAMP-dependent kinase is seen with proteins of the 60S ribosomal subunits. The level of phosphorylation is somewhat higher with subunits derived from *Artemia*, but well below the level of phosphorylation that occurs with the ribosomal protein of the small subunit. None of the ribosomal subunits analyzed contain endogenous protein kinase activity (Fig. 3, tracks 1, 3, 5, and 7).

Polyamines are constituents of all ribosomes. Rabbit reticulocyte ribosomes were found to contain spermine plus spermidine at about 70 nmoles/mg ribosomes. The ratio of spermine to spermidine was about 1:3 (Konecki *et al.*, 1975b). Most but not all of these polyamines may be removed with the initiation factors and other enzymes by treatment of the ribosomes with 0.5 M KCl. Polyamines appear to play a dual role in protein synthesis. One function appears to be a rather unspecific effect for which Mg^{2+} can substitute. A much more specific effect has been shown for a certain step in peptide initiation as described below (Kramer *et al.*, 1979).

When 0.4 M KCl-washed *Artemia* ribosomes were analyzed for their polyamine content, they were found to contain only about 8 nmoles polyamines per mg ribosomes in contrast to about 18 nmoles per mg with 0.5 M KCl-washed reticulocyte ribosomes (Kramer *et al.*, 1979). Assuming 1 mg ribosomes to be equal to 0.25 nmol, the low polyamine content of *Artemia* ribosomes still would reflect about 32 moleculesof spermine plus spermidine per ribosome. It has been shown earlier that 75 μM spermine added exogenously constitutes the optimal concentration for globin mRNA translation on these ribosomes (Konecki *et al.*, 1975b). There is evidence for a single specific polyamine binding site on 40S ribosomal subunits. Edeine is a small basic peptide antibiotic containing spermidine (edeine A) or N-guanidylspermidine (edeine B, Hettinger and Craig, 1970). The antibiotics have high specificity for inhibition of peptide initiation (Obrig *et al.*, 1971). Edeine was labeled with ^{125}I-iodine in its C-terminal tyrosine ring. Binding of ^{125}I-edeine to *Artemia* 40S or 60S ribosomal subunits was studied by a Millipore filter assay that has been described in detail previously (Odom *et al.*, 1978). It was found that ^{125}I-edeine will bind only to 40S, not to 60S ribosomal subunits (Odom *et al.*, 1978). The ratio of edeine bound to 40S ribosomal subunits at saturation concentrations approached 1. This result plus the experiments showing that edeine inhibits specifically eukaryotic peptide initiation (Obrig *et al.*, 1971 ; Konecki *et al.*, 1975b ; Odom *et al.*, 1978), may indicate that one polyamine binding site on the 40S ribosomal subunit plays a crucial role in peptide initiation.

Binding of globin mRNA to *Artemia* 40S ribosomal subunits

Artemia 40S ribosomal subunits offer several advantages over reticulocyte ribosomal subunits for the study of partial reactions of peptide initiation. These ribosomal subunits appear to be nearly devoid of contaminating mRNA or mRNA fragments as described above. Also, their polyamine content generally is much lower. Spermine has been shown to stimulate binding of ^{125}I-globin mRNA to *Artemia* 40S ribosomal subunits (Fig. 4 by Kramer *et al.*, 1979).

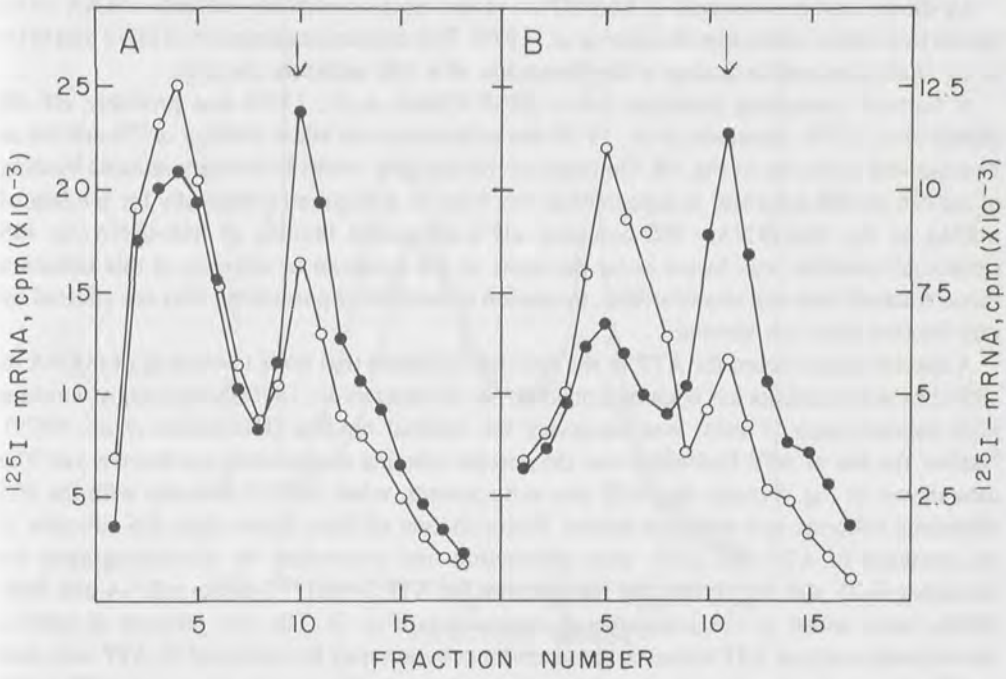

FIG. 4. Different initiation factor fractions that stimulate globin mRNA binding to *Artemia* 40S ribosomal subunits. A. Effect of eIF-1. ^{125}I-labeled globin mRNA (0.6 μg = 2.8×10^5 cpm) was incubated with 12 pmol of 40S ribosomal subunits and initiation factor fractions obtained from DEAE-chromatography (*cf* Cimadevilla *et al.*, 1975), about 39 μg of the fraction FI containing eIF-2, 3 and 4B activities ; about 59 μg FII containing eIF-4 and 5 activities ; and minus (○——○), or plus (●——●) 2 μg DE_{100} fraction containing eIF-1 activity. Reactions were incubated and centrifuged for 90 min at 59 000 as described under Materials and methods.

The gradients were fractionated (ISCO Model 273) and collected into small plastic tubes. Their radioactivity was determined in a γ-counter (Beckman Biogamma). B. Effect of the eIF-(3 + 4B) fraction. ^{125}I-labeled globin mRNA (0.6 μg = 1.2×10^5 cpm) was incubated with 40S subunits and fractions containing eIF-(4 + 5) and eIF-1 activities as described for A ; about 3 μg eIF-2 (approximately 85% pure) ; and minus (○——○), or plus (●——●) about 16 μg of the eIF-(3 + 4B) fraction. Centrifugation was for 100 min at 59 000 rpm. The gradients were processed as under A. The arrows indicate the position of 40S.

Requirements for different initiation factor fractions were studied. Using *Artemia* 40S ribosomal subunits and ^{125}I-labeled globin mRNA, the data in Fig. 4A present evidence that eIF-1 is necessary for the formation of a 40S initiation complex. This initiation factor has been shown to stimulate Met-$tRNA_f$ binding to 40S ribosomol subunits (Odom *et al.*, 1978 ; Kramer *et al.*, 1979 ; Staehelin *et al.*, 1979) however, the stimulatory effect for this reaction could be almost abolished, when AUG was included in the reaction mixture (Odom *et al.*, 1978). eIF-1 renders the Met-$tRNA_f$ · GTP · 40S complex insensitive to edeine (Odom *et al.*, 1978).

As shown earlier, omission of Met-tRNA$_f$ in the reaction mixture prevents mRNA to be bound to *Artemia* ribosomes (Kramer *et al.*, 1976). This implies requirement of eIF-2 and GTP or its non-hydrolyzable analog in the formation of a 40S initiation complex.

A fraction containing initiation factor eIF-3 (Odom *et al.*, 1978) and probably eIF-4B (Benne *et al.*, 1979 ; Staehelin *et al.*, 1979) has to be present to allow binding of ^{125}I-mRNA to *Artemia* 40S as shown in Fig. 4B. Omission of this fraction results in strongly reduced binding of mRNA to 40S subunits. It appears that this fraction is required specifically for binding of mRNA to the Met-tRNA$_f$ · 40S complex. eIF-2-dependent binding of Met-tRNA$_f$ to 40S ribosomal subunits was found to be the same in the presence or absence of this initiation factor fraction (data not shown). Also, formation of methionyl-puromycin was not affected by this fraction (data not shown).

A specific requirement for ATP in the reaction sequence that leads to binding of mRNA to 40S ribosomal subunits has been described earlier (Kramer *et al.*, 1976). Surprisingly, a rather high concentration, 1 mM, was necessary for optimal binding (Henderson *et al.*, 1979). Neither the site of ATP hydrolysis nor the specific reacting components are known yet. The data shown in Fig. 5 imply that ATP has to be present, when mRNA interacts with the 40S ribosomal subunits and initiation factors. Preincubation of these factors plus 40S subunits in the presence of ATP and GTP, then eliminating free nucleotides by chromatography on Sephadex G-25, did not abolish the requirement for ATP, when ^{125}I-globin mRNA and Met-tRNA$_f$ were added to the preincubated components (Fig. 5). The low amount of mRNA binding seen without ATP added in the second incubation may be explained by ATP adsorbed to 40S ribosomal subunits and carried over from the preincubation. In any event, ATP added together with mRNA causes a big increase in the amount of mRNA bound to 40S ribosomal subunits. This fact seems to rule out the possibility that ATP is needed in a reaction to modify initiation factors and/or the ribosomal surface prior to mRNA binding.

Discussion

Ribosomes and ribosomal subunits from dry cysts of *Artemia* have been shown to be particularly useful in the study of partial reactions of peptide initiation. These ribosomes are monomeric and are not actively engaged in protein synthesis. As a consequence they appear to be relatively devoid of mRNA, initiation factors and elongation enzymes that are required for protein synthesis. Therefore experiments with these ribosomes generally should provide a relatively clear answer to the requirements of compounds necessary for peptide initiation.

Controversy still exists about the exact role of most of the initiation factors and the mechanism of the individual steps in peptide initiation. Merrick and coworkers were unable to demonstrate that factor eIF-1 is involved in peptide initiation (Staehelin *et al.*, 1979). Trachsel and Staehelin (1978) did not require any other initiation factor to bind the ternary complex between eIF-2, GTP and Met-tRNA$_f$ to 40S ribosomal subunits. It is likely that some of these controversial issues in peptide initiation arise from the preparation of 40S ribosomal subunits used.

We demonstrated earlier (Konecki *et al.*, 1975 ; Odom *et al.*, 1978 ; Kramer *et al.*, 1979) the importance of polyamines in peptide initiation. Also, data were presented showing that salt-washed *Artemia* ribosomes contain less than half the amount of polyamines per mg ribosomes compared to reticulocyte salt-washed ribosomes. However, we have been unable to isolate

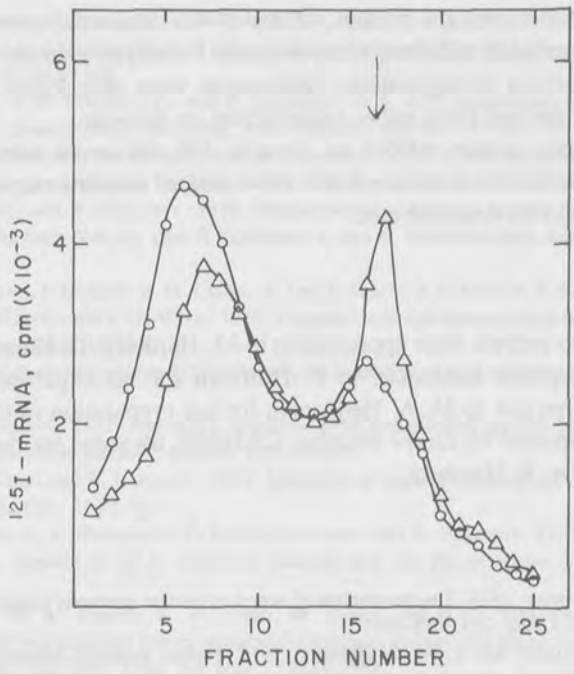

FIG. 5. Requirement for ATP during mRNA binding to 40S ribosomal subunits. About 80 pmol of *Artemia* 40S ribosomal subunits were incubated in a total volume of 0.2 ml under conditions described in Methods with about 6 μg of the eIF-1 fraction, 180 μg of the FII fraction (eIF-4 plus 5 activities), 9 μg of the eIF-2 fraction and 50 μg of the fraction containing eIF-3 plus eIF-4B. After incubation, the reaction mixture was chromatographed on a calibrated Sephadex G-25 column (0.5×15 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 80 mM KCl, 2.5 mM $MgCl_2$ and 5 mM reduced glutathione. Material eluting at v_o was collected, combined, and 125 μl each were used in the subsequent incubation. Met-tRNA$_f$, ^{125}I-globin mRNA (0.53 μg = 4.7×10^4 cpm), GTP minus (○——○), or plus (△——△) ATP were added to give a total volume of 150 μl. Incubation and gradient centrifugation (100 min at 59 000 rpm) were carried out as described under Methods. Fractions were collected and their radioactivity determined as described above in the legend to Fig. 4.

either reticulocyte or *Artemia* ribosomes that are free of polyamines, but retain activity for peptide synthesis. We believe that polyamines may play a critically important role in the function of eukaryotic ribosomes and that the lack of success to date in reconstituting eukaryotic ribosomal subunits may in part reflect this requirement.

Summary

Translation of added globin mRNA or portamine mRNA proceeds efficiently on ribosomes from dry cysts of *Artemia*. Subunits derived from these ribosomes appear to be devoid of endogenous mRNA, thus prove superior to those from reticulocytes in the translation of exogenous mRNA.

Despite functional similarity, the protein pattern of 40S ribosomal subunits from *Artemia* and reticulocytes appear quite different, when analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Differences were also found in the polyamine content of ribosomes derived from either reticulocytes or *Artemia*.

Binding of ^{125}I-labeled globin mRNA to *Artemia* 40S ribosomal subunits was studied. Stimulation of this reaction by initiation factor eIF-1 and an absolute requirement for factors eIF-3 plus eIF-4B could be demonstrated.

Acknowledgements

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Mitochondrial unmasking and yolk platelets metabolization during early development in *Artemia*

R. Marco, R. Garesse, and C. G. Vallejo

Instituto de Enzimología y Patología Molecular del CSIC
Facultad de Medicina de la Universidad Autónoma
Arzobispo Morcillo s/n Madrid-34, Spain

Abstract

The majority of the mitochondrial enzymes detected in the extracts of *Artemia* dormant gastrulae are present in the subcellular fractions rich in yolk platelets. This has been observed in conditions in which yolk platelets are stabilized. We have studied how the mitochondria stored in these structures are released during development. A few hours before hatching, most-of the mitochondrial activity still co-purifies with the yolk platelets down to the bottom of the sucrose isopycnic gradients. Immediately after hatching, when about 50% of the yolk platelets are already metabolized, an important increase in the total mitochondrial activity is detected (about 7-fold) that bands as a heavy peak associated with the partially metabolized yolk. At 24 hr after hatching, few yolk platelets still remain undigested. At this time, the differences depending on the isolation conditions are minimal. Most of the activity now bands at the proper buoyant density of free mitochondria.

In accordance with the data obtained using a biochemical approach, abundant mitochondria-like structures emerging from partially metabolized yolk platelets are visualized in thin sections of the partially developed cyst.

Introduction

Several old lines of evidence point to the important role of mitochondrial function during embryonic development (Giudice, 1973 ; Boell and Greenfield, 1975). They extend from the Warburg experiments (Warburg, 1908) indicating the existence of a sharp increase in oxygen consumption following sea urchin egg fertilization to the studies on mitochondrial localization in certain areas of the embryo and the observation that dislocation of these organelles leads to abnormal development (Wilson, 1928 ; Novikoff, 1961). In spite of this promising experimental background, more recent work using modern technology has failed to provide a definite picture at the molecular and biochemical levels. Oxygen consumption appears to be one of the determining mechanisms through which the environment can control the process of the resumption of development of *Artemia* (Clegg, 1976 ; Vallejo *et al.*, 1980). The consideration of all these data made it interesting to obtain more information on the properties of mitochondria during this process. Schmitt *et al.* (1973) had already indicated that important changes were occurring in the mitochondrial population of *Artemia* cysts. In this paper, we

summarize our work that indicates that, in parallel with a maturation process, the increase in mitochondrial activity observed both *in vivo* and *in vitro* around hatching is related to the unmasking of a mitochondrial population stored in the yolk platelets. Part of this work has been already published (Marco and Vallejo, 1976 ; Vallejo and Marco, 1976 ; Vallejo *et al.*, 1979).

Material and methods

Cysts

Were obtained from Metaframe, San Francisco.

Homogenization media

Two media were used; The "sucrose medium" is composed of 0.68 M sucrose, 25 mM Hepes buffer, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1 mM sodium borate, adjusted to pH 7.5. The "ficoll medium" is composed of 0.3 M sucrose, 15% Ficoll 400 (Pharmacia), 25 mM Hepes buffer, 5 mM $MgCl_2$ and 0.5 mM $CaCl_2$, adjusted to pH 7.5.

Data on chemicals, enzyme assays and extraction and fractionation of cysts as described elsewhere (Vallejo *et al.*, 1979).

Gradients

The subcellular fractions were run through sucrose isopycnic gradients made up of sucrose solutions from 2.0 M to 0.96 M in a final volume of 13.6 ml plus the volume sample. Once the sample was added, the gradients were centrifuged at 26 000 rpm for 12 hr in a SW27 rotor.

Electron microscopy

The details of the procedure to prepare the isolated frations for electron microscopy have been described previously (Vallejo *et al.*, 1979). After different times of incubation the sections of the cysts were prepared as follows. The cysts deposited in a drop of melted gelatin were frozen in liquid nitrogen and cut with a razor blade to allow the fixative to penetrate the thick shell. Fixation was carried out by immersion in a solution of equal amounts of 4% glutaraldehyde in 0.2 M cacodylate pH 7.2 and 2% osmium tetroxide in double distilled water during 1 hr at 0 °C. After thorough washing in 0.2 M cacodylate pH 7.2, the cysts were post-fixated in 2% osmium tetroxide for 2 hr at 0 °C. After washing, the cysts were dehydrated and embedded in Araldit and the thin sections stained as described for the isolated fractions (Vallejo *et al.*, 1979).

Results

The subcellular localization of the mitochondrial activity detected in the cysts immediately after hydration

The stability of *Artemia* yolk platelets has been found to depend on several factors (Marco *et al.*, 1977) : a) the homogenization medium ; b) the dilution at which the homogenization is

carried out and c) the origin of the cysts. With cysts obtained from Utah, the "500 g and 2 000 g" fractions were mainly composed of intact yolk platelets and nuclei (Vallejo *et al.*, 1979). However, in the same conditions with the cysts of San Francisco most of the yolk platelets appeared disrupted and the presence of Ficoll was required in the homogenization medium to improve the isolation of these organelles. This Ficoll effect can be seen in Fig. 1 where the "2 000 g fraction" obtained from San Francisco cysts with and without Ficoll are compared.

TABLE I

Effect of the homogenization conditions on the subcellular distribution of protein and mitochondrial enzyme activities in *Artemia* dormant cysts

Subcellular fractions	Ficoll			Sucrose		
	Protein (%)[2]	Cytochrome oxidase (%)[2]	ICDH[1] (%)[2]	Protein (%)[2]	Cytochrome oxidase (%)[2]	ICDH (%)[2]
500 g × 10 min	44	29	32	55	11	12
2 000 g × 10 min	33	45	40	7	14	24
7 500 g × 20 min	8	23	22	5	66	50
27 000 g × 30 min	1	3	5	2	9	11
150 000 g × 90 min	1	< 1	1	2	1	3
Recovery	95	75	87	83	92	100
Total activities (U or mg/10^6 animals)	270	3	5	225	2	7

[1] NADP-dependent isocitrate dehydrogenase.

[2] Percentages are obtained taking as 100% the sum of the figures found in the different particulate fractions. Recovery is the percentage of this sum in relation to the figure found in the total homogenate.

As shown in Table I, the presence of Ficoll in the homogenates from zero time cysts of San Francisco results in a marked increase in the protein recovered in the yolk platelet-rich fractions ("500 g and 2 000 g"), without affecting the amount of total protein recovered. Furthermore, in Table I, the subcellular distribution of cytochrome oxidase and particulate NADP dependent isocitrate dehydrogenase in "sucrose" and "ficoll" media are also compared. The total activities being similar, it is striking that in "ficoll medium" which has a higher viscosity than the "sucrose" one, both cytochrome oxidase and isocitrate dehydrogenase appear to sediment in parallel at lower centrifugal forces. In order to further investigate the properties of the mitochondrial activities of these fractions, they were analyzed by running through a continuous sucrose gradient (2.0 M to 0.96 M). In Fig. 2, the distribution of cytochrome oxidase in sucrose gradients of the combined particulate fractions obtained in "ficoll" and "sucrose" media are compared. In "ficoll" (Fig. 2A), the majority of the detected activity is associated with the pellet of the gradient. In contrast, a mitochondrial band is visible in the gradient from the "sucrose" particulate fraction. The electron microscopic examination of the pellets demostrated that they were composed of yolk platelets. Mitochondria sticking to these structures could not be observed.

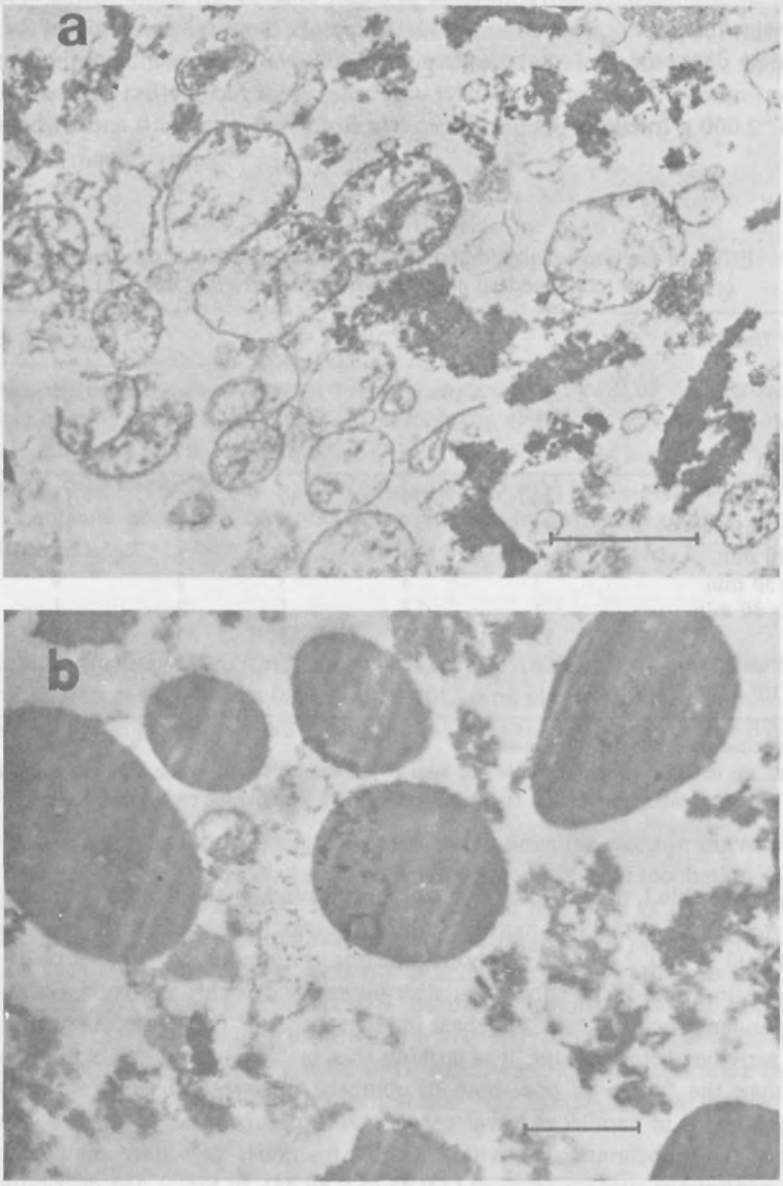

FIG. 1. Electronmicrographs of San Francisco cysts "2 000 g" subcellular fractions obtained either in "sucrose medium" (a) or "ficoll medium" (b). The "2 000 g" subcellular fraction is obtained after centrifugation of the homogenate at 500 × g for 10 min, resuspension and centrifugation at the same speed. The supernatant is centrifuged at 2 000 × g for 10 min and the pellet thus obtained is the "2 000 g" subcellular fraction.

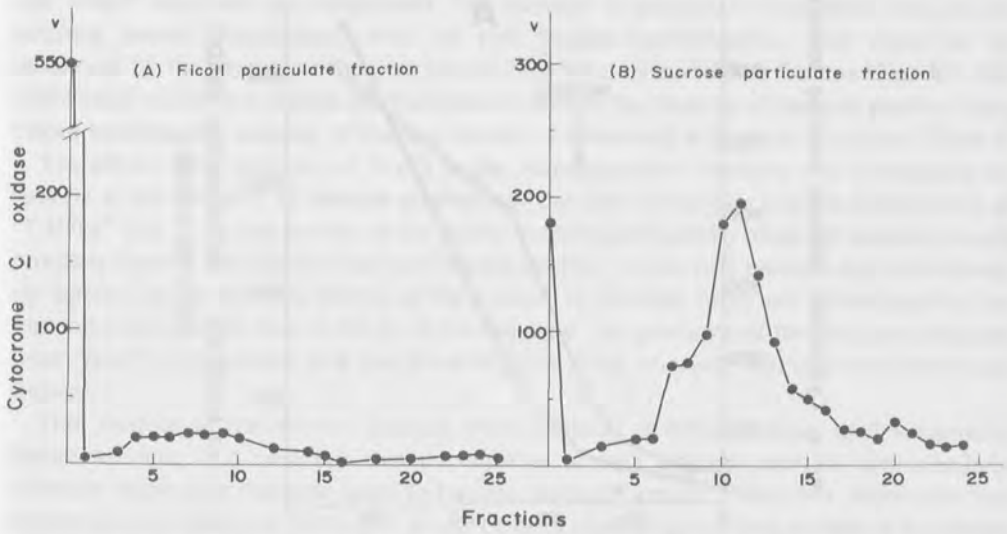

FIG. 2. Sucrose gradient analysis of mitochondrial enzyme activity of time zero cysts. The homogenates obtained either in "ficoll medium" (A) or "sucrose medium" (B) were directly centrifuged at 150 000 × g for 90 min and the pellets obtained run through isopycnic sucrose gradients.

Also biochemical controls were carried out. A "sucrose" free mitochondria-rich fraction was mixed with a "ficoll" yolk platelets fraction and the mixture run through the sucrose gradient in parallel with the two component controls. The mixture gave a band with the same activity and buoyant density as the free mitochondria-rich fraction alone, suggesting again that mitochondria had not stuck to the yolk platelets pellet of the gradient. The possibility that Ficoll itself were causing the abnormal sedimentation profile of mitochondrial activities was ruled out by resuspending a "sucrose" free mitochondria-rich fraction in "ficoll medium" and running it in parallel with the control (without resuspension in "ficoll"). Both samples produced the same band in the gradient (Marco, R., R. Garesse, and C. G. Vallejo, in preparation).

From these experiments, it can be concluded that a significant fraction of the mitochondrial activity detected immediately after hydration of the cysts appears associated with a population of mitochondria stored in the yolk platelets.

THE BEHAVIOR OF MITOCHONDRIAL ACTIVITY DURING THE EARLY *ARTEMIA* DEVELOPMENT

In Fig. 3A, two mitochondrial enzyme markers, cytochrome oxidase and particulate NADP-dependent isocitrate dehydrogenase, show a marked and coordinate increase in specific activity after the hatching period. In previous published work (Vallejo *et al.*, 1979), we have presented data indicating that this increase is detected in a similar way when the data are computed in total activity per million of animals. Moreover, this coordinated pattern is followed by all the mitochondrial enzymes examined (cytochrome oxidase, succinate cytochrome *c* redutase, malate dehydrogenase, glutamic oxalacetic transaminase, fumarase

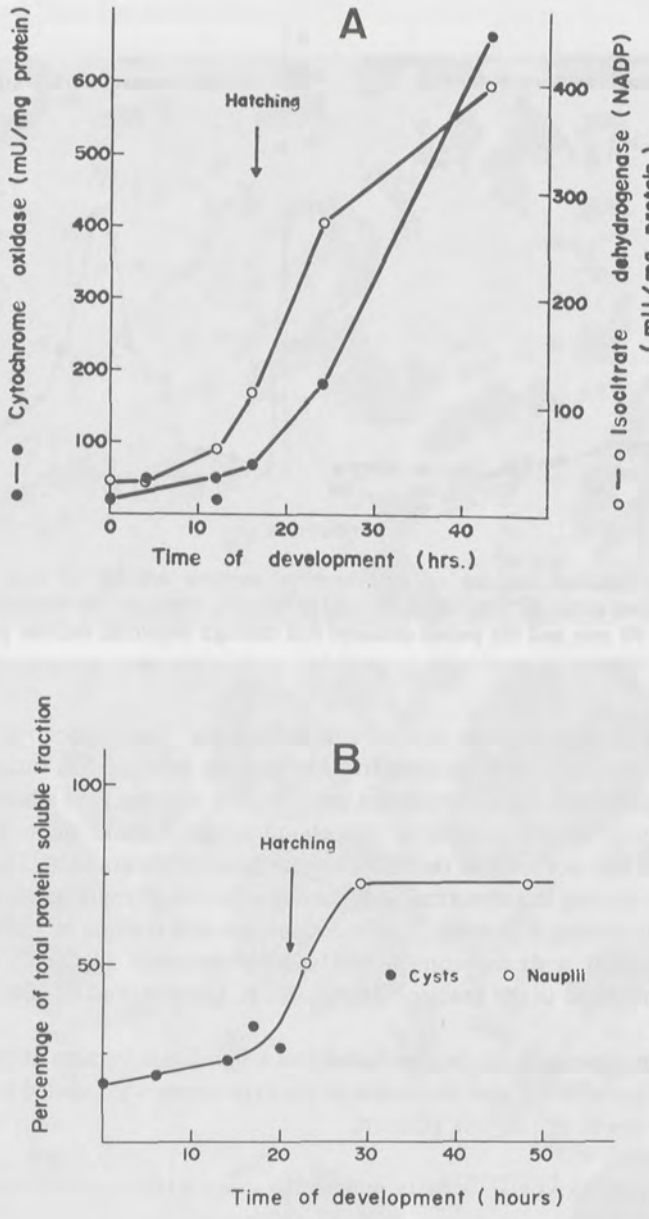

FIG. 3. Mitochondrial enzyme activity during *Artemia* early development (A) and effect of the time of development in the subcellular distribution of protein (B). A. Total homogenates of animals of different stages of development were centrifuged directly at 150 000 × g for 90 min and the mitochondrial enzyme activities assayed in the pellets. B. Total "ficoll" homogenates of animals of different stages of development wee centrifuged as in A and the subcellular distribution of protein determined. The percentage of the protein of the soluble fraction in relation to the total homogenate observed during development is shown.

and NADP isocitrate dehydrogenase). The increase is particularly important around the hatching period, coincidentaly with the yolk platelet metabolization. This event can be monitored by the amount of the total protein recovered in the soluble fraction (Fig. 3B). The distribution of the total protein is a parameter to follow the integrity of the yolk platelets since before hatching the majority of the cyst protein is associated with these structures (Table I).

The effect of the presence of Ficolll in the homogenization medium was investigated by looking at the behavior in sucrose gradients of the total particulate fraction sedimenting at "7 500 g" (Fig. 4). As can be seen in the figure, the increased activity observed as development proceeds appears associated to the yolk platelet fraction. As the yolk platelets are metabolized, the activity moves from the bottom of the gradient to fractions more and more typical of the free mitochondria. In fact, at 48 hr of development, the gradients of the fractions obtained from "ficoll" homogenates look like those obtained from "sucrose" homogenates (results not shown).

Thin sections of the *Artemia* gastrula were prepared at different times of development. Before hatching it is observed that the number of yolk platelets remains approximately constant, while their diameter tends to become gradually smaller. Moreover, before the first morphogenetic cleavages (that occur arund 12 hr of incubation) become evident, it is possible to see a large number of mitochondria-like structures under the electron dense coat of the yolk platelets (Fig. 5). These images are analogous to structures observed in isolated fractions (Vallejo *et al.*, 1979).

Schmitt *et al.* (1973) have also presented data showing mitochondrial maturation during *Artemia* early development but as they only investigated one particular subcellular fraction and one enzyme activity during the first 3 hr of development, they could not observe the process studied here.

Discussion and conclusions

In *Artemia*, similarly as it has been found in other well-studied developmental systems (Pikó *et al.*, 1967 ; Chase and Dawid, 1972), the majority of the mitochondria that will be needed in further development appears to be stored for its future utilization. The storage form of these mitochondria in *Artemia* is in the interior of the yolk platelet structures. The apparent increase in mitochondrial activity detected by us (Fig. 3 and Vallejo *et al.*, 1979) around hatching is related to the yolk platelet metabolization and the release of the stored population of mitochondria. To establish this conclusion, it was important to realize that there are several factors that affect the state and recovery of the yolk platelets in extracts from *Artemia* cysts (Marco, R., R. Garesse, and C. G. Vallejo, in preparation). In particular, the effect of the inclusion of Ficoll in the homogenation medium was most important when *Artemia* cysts from San Francisco were used for these experiments (Fig. 1), while the Utah cysts were less dependent on its presence (Vallejo *et al.*, 1979).

As development proceeds, the increase in mitochondrial activity detected in homogenates appears linked to the partial metabolized yolk platelet fraction. At 48 hr of development, when most of the yolk platelets have been already metabolized, mitochondrial activity begins to behave as associated to free organelles in sucrose gradients (Fig. 4).

The observation of the same unmasking process in this sections of the cysts strengthen the conclusion obtained by the biochemical approach. Similar structures have been also visualized

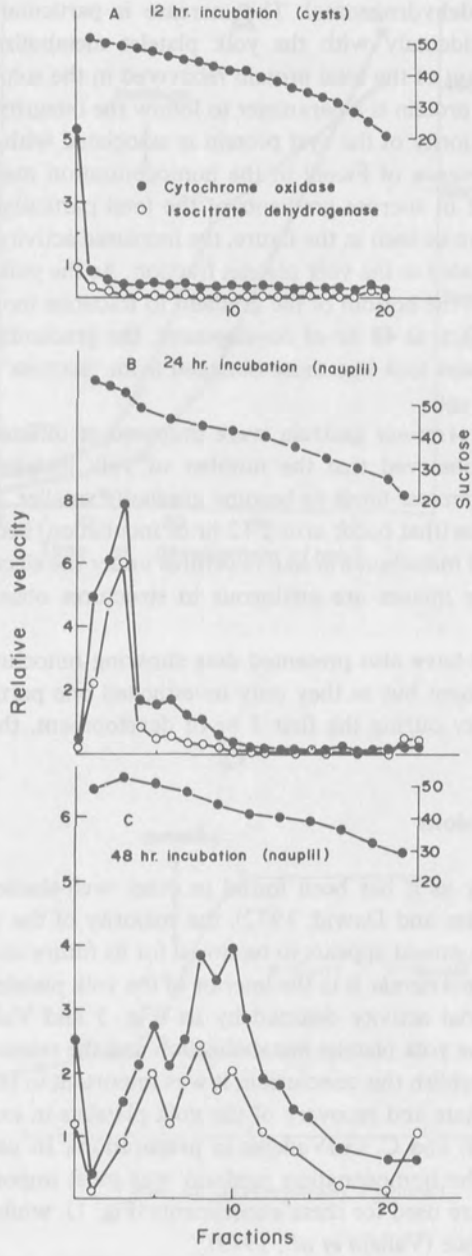

FIG. 4. Effect of development on the sedimentation of the Ficoll fractions. Homogenates of animals at different stages of development were obtained in "ficoll medium" and centrifuged as described in legend for Fig. 3A. The pellets were run through isopycnic sucrose gradients. The activities profiles are represented together with the buoyant density of the fractions of the gradients.

in *in vitro* isolated fractions later in development (Vallejo *et al.*, 1979). It has to be pointed out that the zero time cysts also has a population of free mitochondria that can be visualized in thin sections of the cysts and probably corresponds to the ones observed banding in the gradients of "ficoll" fractions.

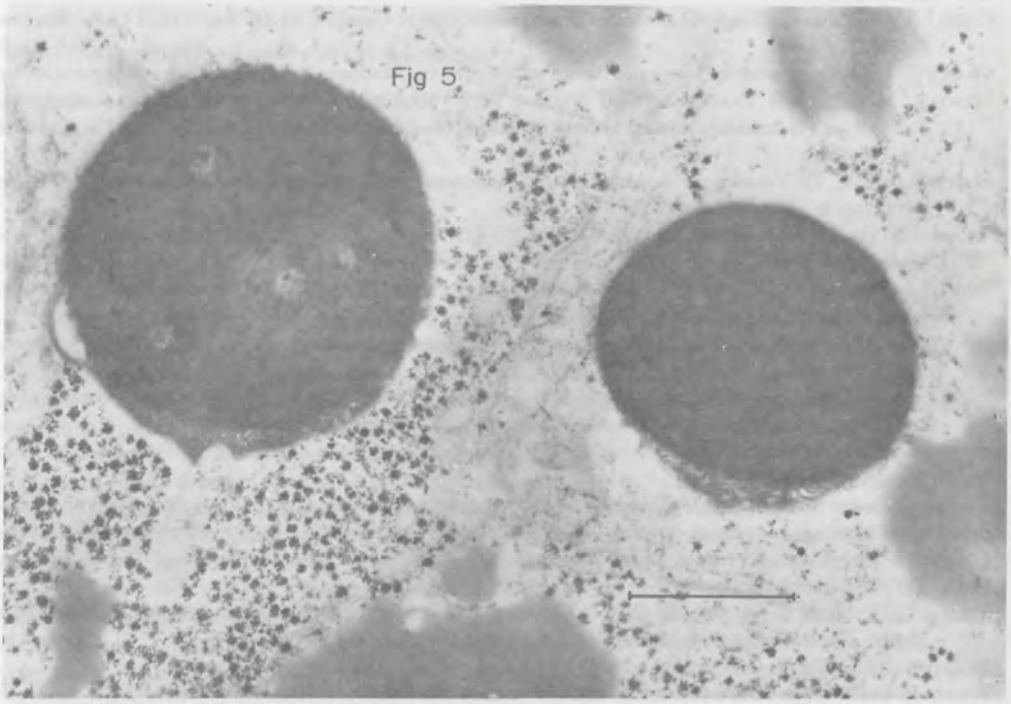

FIG. 5. Thin section of a cyst incubated for 12 hr. Yolk platelets with mitochondria-like membranes are shown.

In conclusion, the increase of mitochondrial activity detected during *Artemia* early development is associatedto the physiological metabolization of the yolk platelets, resulting in the unmasking and release of a population of mitochondria stored in these organelles. These events occur in parallel with a maturation process (Vallejo *et al.*, 1979) of both the free and stored mitochondria, the molecular basis of which remains to be established.

Acknowledgements

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Physical-chemical characterization of cytoplasmic ribosomal particles isolated from *Artemia*

Paul Nieuwenhuysen and Julius Clauwaert

Department of Cell Biology, University of Antwerp
Universiteitsplein 1, B-2610 Wilrijk, Belgium

Abstract

Cytoplasmic, complete ribosomes were isolated from the postmitochondrial supernatant of the cryptobiotic (and the developing) embryos of the brine shrimp (*Artemia*). They were further purified, or dissociated and separated into their large and small subunits by zonal centrifugation in a sucrose gradient containing a high concentration of KCl. Different ionic concentrations were tested to minimize aggregation of purified ribosomal particles. The ratio of their ribonucleic acid to protein content, and their absorbance at 260 nm has been determined ; RNA concentration was measured with the phosphorus method and protein concentration with the original and a modified Lowry method and also with the biuret method. Furthermore the buoyant densities in a CsCl gradient of the complete ribosomes and their large subunits have been determined ; these values are also related to their RNA/protein ratio. The density has been measured of solutions containing different concentrations of ribosomal particles ; using our values for their absorbance at 260 nm, this yields their density increment and partial specific volume $\bar{v}^o$; these are also related to their RNA/protein ratio. The standard sedimentation coefficient $s^o_{20,w}$ of the unfixed and formadehyde-fixed complete ribosomes has been determined by analytical boundary centrifugation, and their standard diffusion coefficient $D^o_{20,w}$ has been determined by photoncount autocorrelation spectroscopy of scattered laser light ; some analogous results have been obtained for the formaldehyde-fixed large subunits also. Combining $s^o_{20,w}$, $D^o_{20,w}$ and $\bar{v}^o$ finally yields the molecular weight ; furthermore it gives information about the hydrodynamic and dry-particle radius and volume, and about the hydrodynamic solvation.

Abbreviations used

A_{260} unit : amount of material which would give an absorbance of 1 at 260 nm in a light path of 1 cm and a volume of 1 ml ;
BSA : bovine serum albumin ;
DTT : dithiothreitol ;
Hepes : N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid ;
mRNA : messenger ribonucleic acid ;
PEG : poly(ethylene glycol) ;
PMS : postmitochondrial supernatant ;
RNA : ribonucleic acid ;
rpm : revolutions per minute ;
rRNA : ribosomal ribonucleic acid.

Introduction

Ribosomes

The biosynthesis of proteins is a process in which many complex molecules take part. It occurs on ribosomes ; these subcellular organelles, present in all organisms, appear to be essential for the correct recognition of the genetic information encoded in mRNA, and for the translation of this message into proteins. The ribosomes of prokaryotic and eukaryotic cells are structurally and functionally similar ; ribosomes from eukaryotic cells are larger and more complicated. Therefore, most structural and functional studies have been performed on the ribosomes from prokaryotes, particularly from the bacterial species *Escherichia coli*. All ribosomes consist of two dissimilar subunits ; these must associate with mRNA before protein synthesis can take place. Each ribosome can only be engaged in the synthesis of one protein at a time, but a messenger may be translated simultaneously by several ribosomes : the complex including mRNA and attached ribosomes is termed a polyribosome or polysome.

Elucidating the structure of the ribosome is necessary to understand in detail its involvement in protein synthesis. However, to perform its complex functions, the ribosome has also a very complex structure. Its two subunits consist of RNA and many different protein molecules. So any single experimental approach to a structural determination (such as by X-ray crystallography) would be inadequate. Research into ribosome structure can be divided very roughly into three categories. The first consists of general characterizations of ribosomes and their subparticles ; these studies include the determination of the RNA and protein content, and of physical properties such as molecular (or particle) weight, size, shape, and solvation. The second category consists of structure determinations of the isolated ribosomal components ; this includes the analysis of their primary structure (*i.e.*, the amino acid and nucleotide sequences of the protein and RNA molecules respectively), the determination of their secondary structure, and of their shape in solution. The third category is that of ribosomal "topography" : how are the various individual ribosomal components arranged relative to each other in space (and how do they interact) ?

A book on ribosomes had been edited by Nomura *et al.* (1974) and reviews of ribosome structure and function have been written more recently by Cox and Godwin (1975), Wittmann (1976), Brimacombe (1976), Brimacombe *et al.* (1976, 1978), Cox (1977), Stöffler and Wittmann (1977), and Kurland (1977ab).

Artemia

Information on the brine shrimp (*Artemia*) can be found in other papers of this volume, in earlier reviews of the biochemical, molecular aspects of its differentiation and morphogenesis (Finamore and Clegg, 1969 ; Hentschel and Tata, 1976), and in a report of a workshop (edited by Jaspers and Persoone, 1977) ; a bibliography on *Artemia* covers more than 1100 references (Sorgeloos, 1976).

Motivations for our choice of *Artemia* as a source of ribosomes are :

– the interest in *Artemia* to study the role of protein synthesis in executing the developmental program ;

– the availability of the seed-like, desiccated *Artemia* cryptobiotic embryos (cysts) which are rich in free, single ribosomes, with little or no polysomes being detectable ;

– the possibility to isolate ribosomes and polysomes from *Artemia* in a further stage of development (such as the free-swimming nauplii), which allows to compare the physical-chemical properties of ribosomes isolated during different developmental stages ; and

– the widespread use of the unprogrammed ribosomes isolated from *Artemia* cysts for cell-free protein synthesis.

Aim of our work

Detailed physical-chemical studies of the ribosomal particles isolated from the prokaryote *E. coli* have already been reported ; however, much less information is available about the general physical-chemical properties of ribosomal particles from eukaryotes (Van Holde and Hill, 1974). Therefore it is our aim to make accurate measurements of the ribonucleic acid and protein content, the partial specific volume, sedimentation and diffusion coefficient, of ribosomal particles isolated from *Artemia* ; their molecular weight, size and solvation can be derived from these data. All the results can then be compared with the corresponding published data on the smaller *E. coli* ribosomes.

In cooperation with Dr. K. Heremans (Katholieke Universiteit Leuven) we have also made a study of the pressure-dependent association-dissociation equilibrium between the free, single ribosomes and their subunits (from the cryptobiotic embryos), and of the pressure-sensitivity of polysomes (from nauplii), using light scattering under high hydrostatic pressures (Nieuwenhuysen *et al.*, 1975, 1978b). By cooperating with Dr. M. Koch and Prof.H. Stuhrmann (EMBL, DESY, Hamburg) a neutron scattering experiment with contrast variation could also be performed on the complete *Artemia* ribosomes ; this has given information on the spatial distribution of their RNA and protein. The results of both studies have been described in detail elsewhere (Nieuwenhuysen, 1978b). This report will only focus on the data obtained up to mid 1979 about the general physical-chemical properties of ribosomal particles isolated from *Artemia*.

Materials and methods

UV-absorbance measurements

UV-absorbance measurements were performed with a Zeiss PMQ3 spectrophotometer.

Preparation of the postmitochondrial supernatant

Cryptobiotic embryos of *Artemia* from San Francisco Bay, California, were stirred in a 5 % NaClO solution at room temperature for 7 to 10 min, extensively washed with distilled water and dried by suction. The cysts were used as such or suspended and incubated at 30 °C in a medium containing artificial sea salt until the hatching of free-swimming embryos (nauplii) ; these were collected for the preparation of polysomes and ribosomes. Cysts or nauplii were homogenized in a cooled mortar in the presence of Hepes buffer (pH 7.5), containing 20 mM Hepes, 1 mM DTT, 70 mM KCl, 9 mM Mg acetate, and 150 mM sucrose ; the homogenate was filtered through cheese cloth and centrifuged twice in a Beckman JA 20 rotor at 18 000 rpm and 4 °C for 20 min to obtain the PMS. More recently, a motorized grinder is used and

the filtration is replaced by a centrifugation in a JA 10 rotor at 10 000 rpm and 4 °C for 10 min.

The activity of carp liver polysomes in a homologous cell free system was higher in Hepes than in Tris/HCl buffer (Amthauer and Krauskopf, 1979). The K^+ and Mg^{2+} concentrations in our buffer are close to the optimal concentrations for *in vitro* translation of poly-U with ribosomes from dry cysts or developing embryos (Hultin and Morris, 1968), and for the *in vitro* translation of the polysomes from nauplii (Moens and Kondo, 1976). 1 mM DTT was included mainly because it has been reported that it suppresses dimerization of *E. coli* ribosomal particles, more efficiently than 2-mercapto-ethanol (Moore *et al.*, 1977).

Preparation of complete ribosomes from the cryptobiotic embryos

To obtain very pure ribosome solutions, a procedure was followed, which has been developed in our laboratory (Nieuwenhuysen and Slegers, 1978ab ; Nieuwenhuysen, 1978b). Briefly, this involved an overnight centrifugation of PMS in a high-density sucrose gradient in a Beckman R 60 fixed-angle rotor, precipitation of the ribosomes with PEG 6000, and sucrose density gradient centrifugation of the resuspended ribosomes in a SW 27 swinging-bucket rotor or in a Ti 14 zonal rotor. The ribosome solutions obtained contain no more regular, rodlike macromolecular particles, which were observed in the development of this isolation procedure and which have been partly characterized (Nieuwenhuysen *et al.*, 1979b). In all the steps of the isolation procedure, the ribosome solutions contained the same Hepes buffer as the one used for the preparation of the PMS.

Occasionally, the centrifugation of PMS in a high-density sucrose gradient and the subsequent PEG precipitation were replaced by a direct pelleting of the ribosomes. Simply centrifuging PMS in the R 60 rotor at 4 °C and 50 000 rpm for 130 min yielded a ribosome pellet covered by an orange tinted layer of membranous material which could be loosened at least partly from the surface (Zasloff and Ochoa, 1974). We have found that this contamination can be avoided by an overnight centrifugation (18 hr) in the same rotor at the same temperature and speed, through a 14 to 22 ml pad of 50 % sucrose ; this yiels a clear colorless ribosome-pellet (Nieuwenhuysen, 1978b). More recently, the KCl concentration has been increased from 70 mM to 100 mM in the complete isolation procedure, to make the aggregation of the ribosomes completely negligible. Complete ribosomes purified by following this slightly modified procedure, were used in two photon correlation spectroscopy experiments (indicated in Table V).

Preparation of ribosomes from nauplii

The PMS from nauplii was prepared as described higher in the Hepes buffer which was used throughout in the following preparation procedure. The ribosomal particles were pelleted from the PMS in a R 60 rotor. Ribosomes and polysomes containing a different number of ribosomes were separated by sucrose density gradient centrifugation of the resuspended particles in a Ti 14 zonal rotor. The ribosomes from the fractions in the 80S region were precipitated with PEG 6 000, resuspended, and subjected to another sucrose density gradient centrifugation. This yielded what we call complete ribosomes from nauplii.

To obtain only ribosomes all attached to mRNA, polysomes containing four or more ribosomes were incubated at 25 °C for 10 min with a minimal concentration of insolubilized

pancreatic ribonuclease. (This enzyme has a great affinity for the exposed single-stranded mRNA.) Subsequently, the fiber-bound RNAse was removed by a short centrifugation. The optimal fiber concentration, *i.e.* that which is needed for nearly complete conversion of polysomes to ribosomes (to perform analytical boundary sedimentation), was determined earlier in preliminary experiments by analyzing polysomes, incubated with different fiber concentrations, by zonal sucrose density gradient sedimentation in a SW 27 or SW 41 swinging-bucket rotor.

Preparation of ribosomes subunits

The preparation of pure and intact ribosome subunits is a prerequisit for the study of their general physical-chemical properties in solution. *E. coli* ribosomes are readily dissociated by decreasing to Mg^{2+} concentration in the suspending medium, and the subunits can be re-associated at higher Mg^{2+} concentrations to biologically active ribosomes. On the other hand, the isolation of intact subunits of ribosomes from eukaryotes has been impossible for many years ; this is due to the fact that the subunits obtained by the removal of Mg^{2+} ions, using chelating agents such as EDTA or sodium pyrophosphate, have a modified structure as reflected by their low sedimentation coefficient ; these particles do not combine to form active ribosomes. However, Martin and Wool (1968) gave a new impulse to the structural and functional studies by describing the isolation of intact subunits from rat muscle by treatment of these ribosomes with 1 M KCl (followed by zonal centrifugation) in the presence of Mg^{2+} ions and an antioxidant. Since then, some modifications of this method have made it possible to obtain pure preparations of intact ribosomal subunits from a large number of eukaryotes.

We have followed two procedures reported earlier for the dissociation of *Artemia* cyst ribosomes into intact subunits. Both involve treatment of the ribosomes with a high concentration of KCl, either in the presence of low (0.75 mM) (Snoeks *et al.*, 1975 ; Moens, 1976-77) or high (11 mM) (Zasloff and Ochoa, 1971, 1974) Mg acetate concentration. A modification of the last procedure seemed applied successful.

Pellets of the complete ribosomes from cryptobiotic embryos were obtained by centrifugation of PMS through a pad of 50 % (w/w) of sucrose as described above. To dissociate the ribosomes, such pellets were dissolved in a buffer containing 20 mM Hepes (pH 7.5) and 1 mM DTT, a high KCl concentration, 750 mM, and a low Mg acetate concentration, 0.75 mM (Snoeks *et al.*, 1975 ; Moens, 1976-77). Subsequently, the ribosomal particles were separated by an overnight centrifugation in a 15-50 % (w/w) sucrose gradient containing the same buffer, in a Ti 14 zonal rotor at 4 °C and 32 000 rpm. However, the continuous monitoring of the UV-absorbance during the fractionation of the gradient revealed not only the two expected absorbance peaks of the separated large and small subparticles, but two additional smaller peaks were also observed (Fig. 1A). The same results were obtained when the experiment was repeated with other pellets and when the ribosomal particles were separated by an overnight zonal centrifugation in a 10-30 % (w/w) sucrose gradient in a SW 27 swinging-bucket rotor at 20 000 rpm. One time fractions with a high absorbance were dialyzed separately against buffer containing 70 mM KCl and 2 mM Mg acetate, and concentrated by ultrafiltration to 1 ml ; another time the fractions were directly concentrated in pellet by low speed centrifugation after PEG precipitation and resuspended in buffer containing 70 mM KCl and 9 mM Mg acetate. The suspensions could then be analyzed by zonal

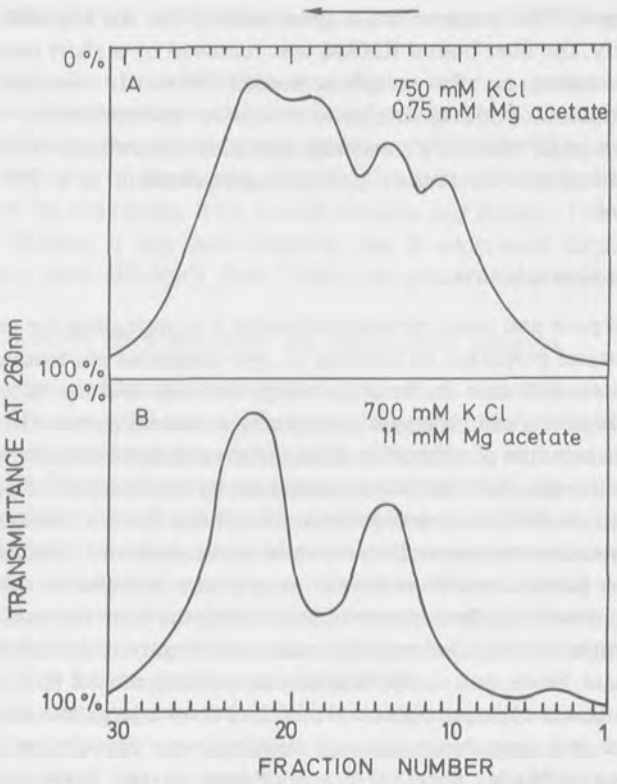

FIG. 1. Separation by zonal centrifugation of ribosomal particles in 20 mM Hepes buffer (pH 7.5), 1 mM DTT and the indicated concentrations of KCl and Mg acetate. About 500 A_{260} units were layered on a 15-50% (w/w) sucrose gradient in a Beckman Ti 14 zonal rotor and centrifuged at 4 °C and 32 000 rpm for 18 hr. (The arrow indicates the direction of sedimentation.)

centrifugation in gradients of 10-30% (w/w) sucrose in the same buffer, using swinging-bucket rotors. This showed that the two additional absorbance peaks (obtained after preparative centrifugation at high ribosome concentration), were due to physically altered large and small subunits, which had both a 20% lower sedimentation coefficient. The conversion to more slowly sedimenting forms in buffers with a high ratio of K^+ to Mg^{2+} concentration has been observed earlier with ribosome subunits isolated from eukaryotic cells such as rat liver (Clegg and Arnstein, 1970), Krebs II ascitic carcinoma in mice (Fais *et al.*, 1970), plants (Cammarano *et al.*, 1972a), sea urchin eggs (Gambino *et al.*, 1973) and human placenta (Vanduffel *et al.*, 1975). Differences between the sedimentation coefficients of the intact and altered subunits have not been measured accurately, because this is hardly possible by sucrose gradient centrifugation, due largely to the uncertainty in the buoyancy factor (Nieuwenhuysen, 1979a), and because the sedimentation coefficients of the changed subparticles depend on the Mg^{2+} and K^+ concentration in the buffer (Fais *et al.*, 1970; Cammarano *et al.*, 1972a). A higher Mg^{2+} concentration protects the subunits from the

transition. Separations by zonal sucrose gradient centrifugation of ribosomal particles in dissociation buffer, containing 20 mM Hepes (pH 7.5), 1 mM DTT, 700 mM KCl and 11 mM Mg acetate (Zasloff and Ochoa, 1971, 1974) using a SW 27 swinging-bucket rotor or a Ti 14 zonal rotor, yielded apparently intact ribosomal subunits (Fig. 1B). By zonal centrifugation of ribosome subunits in this buffer, in 10-30% sucrose gradients, using an SW 27 rotor, it was checked that decreasing the Mg acetate concentration to 5 mM or increasing the KCl concentration to 1 000 mM, caused the appearance of the slower sedimenting particles.

The ribosome subunits, obtained after separation in the zonal rotor, could be concentrated in pellet by a short centrifugation at low speed, after precipitation with PEG 6 000 ; however, pellets of the small subunits could not always be dissolved easily, like it has been mentioned later in the literature (Rodriguez-Pousada and Hayes, 1978). Therefore, the subunits were collected in pellets by an overnight centrifugation in a R 60 rotor at 40 000 rpm and 4 °C (Zasloff and Ochoa, 1974). The large subunits dissolved much faster in Hepes buffer than the small ones. The particles were then purified again by zonal sucrose gradient centrifugation. Also in these last steps of the purification procedure, the ionic composition of the buffer had to be chosen carefully, to avoid aggregation as outlined further.

Prevention of aggregation of the ribosome subunits

Ribosome subunits tend to form dimers and larger aggregates. This is a well-known property of the ribosomal particles isolated from *E. coli* (*e.g.* Zamir *et al.*, 1974 ; Laughrea *et al.*, 1978 ; Koch and Stuhrmann, 1979) and from eukaryotes (*e.g.* Hamilton *et al.*, 1971 ; Martin *et al.*, 1971 ; Van der Zeijst *et al.*, 1972 ; Wettenhall *et al.*, 1973 ; Vanduffel *et al.*, 1975 ; Shimizu *et al.*, 1977 ; Thompson *et al.*, 1977 ; Reboud *et al.*, 1978 ; Buisson *et al.*, 1979 ; Henshaw, 1979 ; Moldave *et al.*, 1979 ; Moldave and Sadnik, 1979), including *Artemia* (see further, and S. Ochoa, 1978, personal communication). The tendency to aggregate is greater for the eukaryotic than for the *E. coli* particles (Stuhrmann, 1978). Monodisperse solutions of macromolecules are required to study them by scattering techniques such as light scattering (including intensity fluctuation spectroscopy and measurements of the time-averaged scattering intensity), X-ray and neutron scattering; investigations with these techniques can yield a wealth of information (as reviewed by Damaschun *et al.*, 1979). Therefore we have tried to eliminate aggregation of ribosomal subunits as much as possible.

Pellets of subunits were obtained as described higher and were dissolved gently in 20 mM Hepes buffer (pH 7.5), containing 1 mM DTT and different concentrations of KCl and Mg acetate. The solutions were loaded on linear 10-30% sucrose gradients, containing the same buffer and salt concentrations, for a zonal centrifugation at 4 °C for 17 hr in a SW 27 swinging-bucket rotor, at 23 000 rpm for the large subunits, and at 27 000 rpm for the small subunits. During the fractionation of the gradients after centrifugation, the profile of the UV-transmittance at 260 nm was recorded, to detect the amount of aggregation. The fractions with lowest transmittance were dialyzed for 20 hr against the same buffer. Then the homogeneity of the particles was investigated by boundary sedimentation in the analytical ultracentrifuge at room temperature and at rotor speeds between 35 000 and 50 000 rpm. Fractions containing the subunits, obtained after the separation in the zonal rotor as described above, were also directly dialyzed against buffers with different salt concentrations, for investigation in the analytical ultracentrifuge. The amount of aggregation varied between

different preparations, as mentioned by Vanduffel *et al.* (1975) for human placenta ribosomes, and also by S. Ochoa (1978, personal communication) for *Artemia* ribosomes. Therefore we always compared the effect of different salt concentrations on the same preparation of subunits.

Using the same buffer as in the isolation and characterization of the complete ribosomes, *i.e.* Hepes buffer containing 1 mM DTT, 70 mM KCl and 9 mM Mg acetate, yielded sometimes more aggregated than single subunits. Increasing the KCl concentration yielded a drastic decrease of aggregation, as found also for other eukaryotic ribosomes by zonal sucrose gradient centrifugation (*e.g.*, Wettenhall *et al.*, 1973 ; Vanduffel *et al.*, 1975 ; Shimizu *et al.*, 1977). However, our additional sensitive analytical boundary sedimentations revealed than the prevention of aggregation was hindered by the appearance of slowly sedimenting particles at KCl concentrations higher than 400 mM. Alternatively, the Mg^{2+} concentration was decreased at a constant 70 mM of KCl. Using only 0.5 mM Mg salt would prevent the aggregation of *E. coli* ribosome subunits (Laughrea *et al.*, 1978) ; however, we found that *Artemia* particles remain intact at 2 mM Mg acetate, but that slowly sedimenting forms appear at 0.5 mM, while aggregation was worse than in 400 mM KCl and 9 mM Mg acetate. Cox and coworkers (1978, personal communication) have found minimal aggregation of eukaryotic ribosome subunits in a 10 mM Tris/HCl buffer, containing only 2 mM $MgCl_2$ and no KCl. By zonal centrifugation, we found that the large subunits aggregated more in this buffer than in Hepes buffer containing 400 mM KCl and 9 mM Mg acetate. Solutions of subunits obtained by zonal sucrose gradient centrifugation, were also directly investigated in the analytical ultracentrifuge, quickly, without prior dialysis to remove the sucrose ; nevertheless, aggregation was present. In conclusion, in 20 mM Hepes buffer (pH 7.5) containing 1 mM DTT, intact subunits with minimal aggregation were obtained with about 300 mM KCl and 10 mM Mg acetate ; however, less than 20% aggregation could not be achieved routinely.

In another series of tests, ribosome subunits were first fixed with formaldehyde, because this might reduce their tendency to aggregate, and because this prevents their conversion to slower sedimenting particles at high K^+ to Mg^{2+} ratio. Unfortunately, the fixation procedure itself causes the partial irreversible aggregation of subunits ; this effect increases with the concentration of ribosomal particles and aldehyde (*e.g.* Garcia-Patrone and Algranati, 1976). When 5% of freshly neutralized foraldehyde was added (*e.g.*, Spirin *et al.*, 1965 ; Perry and Kelley, 1966) to the sucrose gradient fractions containing the subunits (which were obtained by separation in the zonal rotor), the solutions became turbid ; this indicated complete aggregation. Diluting the solutions of subunits with dissociation buffer to 1 A_{260}/ml and decreasing the formaldehyde concentration to 1% allowed to reduce the fixation-induced aggregation to an acceptable degree. Sucrose and formaldehyde were then removed by exhaustive dialysis against Hepes buffer, containing different concentrations of KCl and Mg acetate in different tests. The solutions obtained in this way could be concentrated by ultrafiltration to 10 ml using a 200 ml Amicon stirred cell with anisotropic DIAFLO XM50A, XM100A or XM300A filters ; large aggregates were then removed by a short centrifugation, because they would reduce the flow rate in the second ultrafiltration in a 10 ml cell, to a volume of 1 ml. After both ultrafiltrations, gentle stirring of the concentrated solutions for 30 min without pressurization was necessary, to recover the particles which would otherwise be lost, due to adsorption to the filters. The concentrated solution, finally obtained, was then loaded on a linear 10-30% sucrose gradient in the same buffer for a zonal centrifugation as

described higher for unfixed ribosomal subunits. Fractions corresponding to the monomeric subunits were dialyzed against the same buffer, and their homogeneity was checked by analytical boundary sedimentation. The fixed particles were successfully found to remain intact at high K^+ to Mg^+ ratio : no slower sedimenting material was observed when the buffers contained 500 mM KCl and 5 mM Mg acetate, or even 700 mM KCl and no Mg acetate. However, even with such high K^+ to Mg^{2+} ratios, a complete elimination of the aggregation of ribosome subunits could not be achieved routinely. Occasionally, a monodisperse sample was obtained, as judged by analytical centrifugation ; this was then immediately used for photon correlation spectroscopy of scattered laser light.

Protein determination

Protein assays have been applied to solutions of ribosomal particles (containing their RNA) and of RNA-free protein obtained from complete ribosomes after extraction with LiCl/urea or acetic acid (Kaltschmidt and Wittmann, 1972 ; Sherton and Wool, 1974). The total protein content has been determined using the Folin-Lowry method (Stauffer, 1975), a modification of this method (Hartree, 1972), and the biuret method (Sacchi *et al.*, 1977). Bovine serum albumin (BSA) was used as a standard. The concentration of BSA in solution was determined by measuring the absorbance, accepting an absorbance of 6.6 at 280 nm and 1 cm path length for a 1% solution (Tanford and Roberts, 1952 ; Eisenberg *et al.*, 1977).

Pellets of purified large and small ribosome subunits obtained as described higher, were dissolved easily in phosphate buffer (pH 7.5) containing 20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 250 mM KCl, and 11 mM Mg acetate. The solutions were dialyzed in the cold against the same buffer before protein determination.

Ribonucleic acid determination

RNA concentration was calculated from the phosphorus content. Accepting an equimolar concentration of the four bases and taking no cations into account in the molecular weight of the nucleotides, we assume that the phosphorus content of RNA is 9.67%. Even a considerable deviaton of this molecular distribution of the nucleotides, as has been reported for the rRNA of rabbit, *Tetrahymena pyriformis* or *Xenopus laevis* (Cox *et al.*, 1976a), would not change the percentage that phosphorus contributes to the molecular weight of the nucleotides to a higher extent than the experimental inaccuracy of the phosphorus measurements. Solutions of Na_2HPO_4. 12 H_2O were used as standards for the phosphorus determinations ; their concentration was measured accurately by titration before each experiment.

Pellets of ribosome subunits were obtained as described higher and dissolved in 20 mM Hepes buffer (pH 7.5) containing 1 mM DTT, 250 mM KCl and 11 mM acetate, respectively. The solutions were dialyzed before the assay against the same buffer.

Ribosome digestion for phosphorus determination was performed as described by Van Aphen-Jager (1974) and by Ames (1966). Special care was taken to clean all glassware carefully by immersion into fresh sulfochromic acid for 24 hr, followed by extensive washing with twice-distilled water and steam. Amounts of phosphorus between 0.5 and 2.5 μg can be determined accurately.

Equilibrium centrifugation in a CsCl density gradient

To avoid the release of proteins due to the high ionic strength in the gradient (Meselson *et al.*, 1964), ribosomal particles have to be fixed by formaldehyde (Spirin *et al.*, 1965 ; Perry and Kelley, 1966) or glutaraldehyde (Baltimore and Huang, 1968). Formaldehyde solutions were neutralized with KOH before use. Fixation of concentrated solutions of complete ribosomes by dialysis against 5% formaldehyde in buffer or by adding formaldehyde to concentrated ribosome solutions before the zonal sucrose gradient centrifugation (the last step in the isolation procedure), yielded ribosomal aggregates. It has been reported that glutaraldehyde-fixation of *E. coli* ribosomal subunits also causes aggregation (Noll and Noll, 1974, 1976 ; Garcia-Patrone and Algranati, 1976). Fixation of dilute solutions of complete ribosomes (concentrations of 1 A_{260}/ml) by dialyzing them in the cold for 24 hr against buffer containing 5% formaldehyde gave satisfying results. The excess of formaldehyde was removed by further dialysis against buffer solutions. Complete ribosomes fixed in this way were used to determine their buoyant density in a CsCl gradient formed in the cell of an analytal ultracentrifuge. The initial densities of CsCl solutions containing the ribosomes were measured with a digital precision density meter DMA 02C (Anton Paar, Graz, Austria) or by weighing filled, calibrated micropipets. Centrifugation was performed in a MSE analytical ultracentrifuge equipped with a photoelectric scanner. A centrifuge run at 45 000 rpm for 16 hr resulted in equilibrium conditions.

The ratio of RNA to protein content can then be calculated from an empirical relation (Hamilton and Ruth, 1969 ; Hamilton *et al.*, 1971 ; Hamilton, 1971) : (buoyant density in a CsCl gradient)$^{-1} = (0.53 \times \%_{RNA} + 0.74 \times \%_{protein})/100$, where % represents percentage by weight. The validity of this method has been criticized (McConkey, 1974), but has been confirmed in detail more recently (Sacchi *et al.*, 1977).

To compare the buoyant densities and thus the RNA/protein ratios of ribosomes isolated from the cryptobiotic embryos and from the further-developed, free-swimming nauplii, a general procedure has been developed to determine a small difference or a maximal value for the possible difference in buoyant density of different species of macromolecules by analytical density gradient equilibrium centrifugation, as described elsewhere (Nieuwenhuysen, 1978b, 1979b). The procedure involves the measurements at sedimentation equilibrium of the bandwidths of the concentration distribution of the separate macromolecules and of a mixture of the different species. The eventual difference between the bandwidths can then be related to the eventual difference in buoyant density.

The buoyant density of the complete ribosomes and of their large subunits has also been determined by using swinging-bucket rotors in a preparative ultracentrifuge. Solutions of ribosomal particles, obtained after preparative sucrose density gradient centrifugation in the zonal rotor, were diluted to 1 A_{260}/ml with 20 mM Hepes buffer (pH 7.5) containing 1 mM DTT, 70 mM KCl and 9 mM acetate, or 700 mM KCl and 11 mM Mg acetate, respectively ; then they were dialyzed against the same buffers containg 1% freshly neutralized formaldehyde, to fix the particles and to remove the sucrose ; the CsCl was added to a mixture of the solutions of fixed ribosome particles ; this CsCl solution was centrifuged in the three tubes of a SW 65 rotor at 40 000 rpm and 4 °C for 26 hr. The density gradients obtained were fractionated and the transmittance at 260 nm continuously recorded. The densities of the fractions were measured gravimetrically using a calibrated 100 μl constriction pipette.

Measurements of the density increment

The density increment of the ribosomal solutions at zero concentration and constant chemical potential μ of all diffusible components $(\delta\rho/\delta c)^0_\mu$ (Eisenberg, 1976) was obtained from density *versus* concentration plots.

Pellets of complete ribosomes and ribosomal subparticles were dissolved in 20 mM Hepes buffer (pH 7.5), containing 1 mM DTT, 70 mM KCl, 9 mM Mg acetate, or 300 mM KCl and 11 mM Mg acetate respectively. The solutions were brought to thermodynamic equilibrium of diffusible components by exhaustive dialysis against buffer, using Visking dialysis membranes. Before the measurements, solutions were centrifuged in an Eppendorf centrifuge.

The densities ρ were determined with a digital Precision Density Meter DMA 02C (Anton Paar, Graz, Austria) (Kratky *et al.*, 1973). In this instrument the resonant frequency f of an oscillating U-shaped hollow glass tube depends on the density of the fluid contained in the tube : $f = (m + \rho V)^{-1/2}$, where m is the effective mass of the empty vibrator, and V is the volume of the sample taking part in the motion. The time τ, elapsed during a preselected number of periods, was measured. Thus if the indices i and j indicate different fluids, one has the simple relation

$$\rho_i - \rho_j = a(\tau_i^2 - \tau_j^2)$$

The cell constant a was determined by calibration with dry air and twice distilled water which had been made dustfree by filtration through a 0.1 μm Millipore filter and by centrifugation. The period is very sensitive to temperature, due to the thermal expansion of the fluid and to the mechanical properties of the cell itself ; so the temperature of the cell was kept constant within 0.005 °C at 25.0 °C using a Tronac Precision Temperature Controller Model 40 and two Haake thermostats in a temperature-stabilized room.

After dialysis the density of buffer inside and outside a dialysis bag was equal within experimental error, so that no corrections had to be made for membrane-asymmetry.

The precision of the $(\delta\rho/\delta c)^0_\mu$ determination is limited by the precision of the concentration measurements. We determined the concentration of the ribosomes by measuring the absorbance of the solution at 260 nm. To allow an accurate conversion here of A_{260}/ml to mg/ml, we have determined values for the absorbances $A_{260}^{1\,mg/ml}$.

Analytical boundary sedimentation

The sedimentation coefficients were measured by boundary sedimentation in a MSE analytical ultracentrifuge. This instrument has been described in a review (Coates, 1970), and in an introductory book about centrifugation (Bowen and Rowe, 1970). Double sector cells and absorption optics at 260 nm were used, in combination with an automatic photoelectric scanning device (as reviewed by Schachman and Edelstein, 1973). Temperatures were controlled within 0.1 °C between 15 and 25 °C. The use of a four- or six- hole rotor allowed simultaneous measurements on different samples. The concentration of ribosomal particles ranged from 40 to 80 μg/ml ; the concentration dependence of the sedimentation coefficient could thus be neglected, as it has been reported to be < -3 S per mg/ml for hepatic ribosomes (Sherman and Petermann, 1961 ; Tashiro and Siekevitz, 1975) and -2 S per mg/ml for dog pancreas ribosomes (Beeley *et al.*, 1968). Reduction of the sedimentation coefficient

to standard conditions of 20 °C and water yielded $s^0_{20,w}$ (Van Holde, 1971 ; Rowe and Mustafa Khan, 1972 ; Phelps, 1974).

These measurements also allow at the same time to check the absence of aggregation and dissociation, the purity and integrity of the ribosomes. So they were performed prior to photon correlation spectroscopy. The integral absorption patterns were recorded, because these would show the presence of heterogeneity more vividly than the derivate absorption pattern (or the schlieren pattern) (Schachman and Edelstein, 1973).

Photon-count autocorrelation spectroscopy of scattered laser-light

The diffusion coefficients of different ribosomal particles have been determined by single-clipped photoncount autocorrelation spectroscopy (Foord *et al.*, 1970 ; Koppel, 1974 ; Pusey *et al.*, 1974). This method is based on the efficient analysis of the intensity fluctuations of laser light scattered by a solution of the macromolecules in Brownian motion. Since the pioneering theoretical (Pecora, 1964) and experimental work (Cummins *et al.*, 1964) on dynamical light scattering, there have been published numerous reviews of the subject (*e.g.* Carlson, 1975 ; Schurr, 1977 ; Bloomfield and Lim, 1978) and some books (Chu, 1974 ; Cummins and Pike, 1974, 1977 ; Berne and Pecora, 1975). Fig. 2 shows a general block-diagram of an intensity-fluctuation spectrometer.

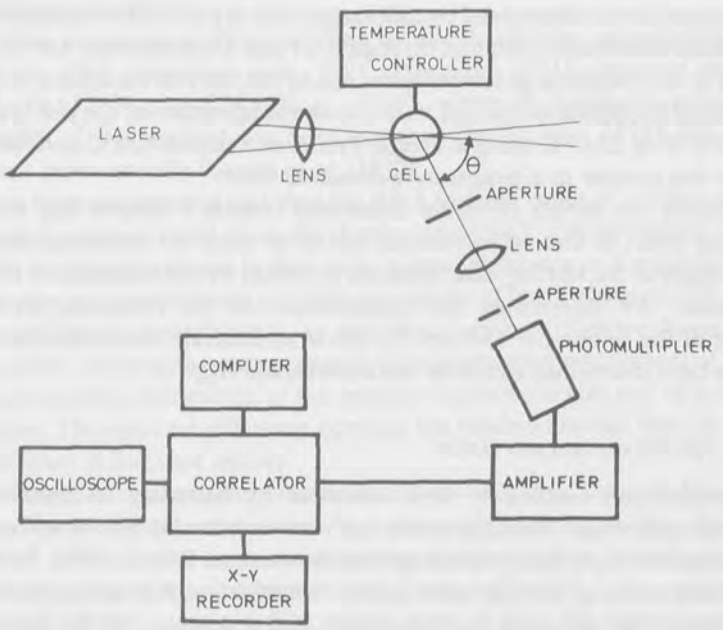

Fig. 2. General block-diagram of equipment for intensity fluctuation spectroscopy.

Most spectra were obtained with a setup from Malvern Instruments (Worcs, England) in a thermostatted room. In the preparative stage of the measurements, several steps were taken to avoid dust particles in the ligt scattering volume. Cylindrical, glass Malvern scattering cells

were immersed for at least a few days in a mixture of ethanol and HCl, and washed with twice distilled water, which had been made dustfree by filtration through a 0.1 μm Millipore filter ; they were further cleaned by flushing the surface with condensing acetone vapor, using an apparatus which had been constructed as described by Tabor (1972). Pasteur pipettes were cleaned with freshly distilled acetone and/or with buffer, which was made dust-free by centrifugation together with the ribosomes in a Beckman JA 20 or JA 21 fixed-angle rotor at speeds higher than 12 000 rpm (at 4 °C). One to 2 ml of the ribosomal solution was carefully pipetted out of the middle of the centrifuge tube into a cleaned scattering cell. A specially constructed adapter allowed a subsequent centrifugation of the filled cell up to 6 000 rpm in a JA 20 rotor. Afterwards, the cell was immersed in a refractive index matching water bath, carefully avoiding any impact so that the eventually present dust particles remained outside the scattering volume due to the final centrifugation. The temperature was maintained at 25.0 ± 0.1 °C with a Malvern Temperature Controller RR 56. A beam of light with wavelength $\lambda_0 = 4\,880$ Å of 40 to 150 mW from an intensity-stabilized Coherent Radiation argon ion laser (Model 55) was focused in the cell. The scattered light was detected with an ITT FW 130 photomultiplier, which is excellent for this application (Foord *et al.*, 1969). The single-clipped autocorrelation function of the photon counts was built up in a Malvern Type K 7023 24-channel high speed digital correlator (Foord *et al.*, 1970), and was continuously displayed on an oscilloscope. The sample times T and clipping levels k were chosen to yield optimal accuracy (Hughes *et al.*, 1973). Autocorrelation spectra were taken at scattering angles θ between 30° and 145° and were recorded on paper tape for subsequent analysis with a PDP 11/45 computer.

Theoretical considerations have yielded the following relation for the normalized single-clipped photon-count autocorrelation function $g_k^{(2)}(iT)$, with $i = 1, 2, \ldots 24$ for our setup, of the light scattered by a dilute mono-disperse solution of small Brownian particles with diffusion coefficient D (Foord *et al.*, 1970 ; Pusey *et al.*, 1974)

$$g_k^{(2)}(iT) - 1 = A \exp(-2DK^2iT).$$

In this expression, A is an experimental constant depending on the mean number of counts per sample time, the decay time, the clipping level k, the sample time T, the modulus of the scattering vector K, and on the geometrical arrangement of the optical components (Nieuwenhuysen and Clauwaert, 1977) ; the modulus of the scattering vector, K, is given by

$$K = (4\pi/\lambda_0)\, n \sin(\theta/2),$$

with n being the refractive index of the solution. In our data analysis method we combined unweighted and iteratively weighted least squares fitting procedures of the logarithm of $g_k^{(2)}(iT) - 1$ to straight lines and quadratic curves ; accurate values for D can be obtained, even when spectra are slightly distorted due to the scattering by dust (Nieuwenhuysen, 1978ab).

The reliability of our apparatus and data analysis procedure was checked by measurements on solutions of "monodisperse" 91 nm polystyrene latex spheres (Nieuwenhuysen and Clauwaert, 1977 ; Nieuwenhuysen, 1978ab) and of the small spherical bacteriophage MS2 (Nieuwenhuysen and Clauwaert, 1978a). After the $\sin\theta$ scattering-volume correction, the intensity of the laser light scattered by filtered water, benzene, and ribosomal solutions, was independent of the scattering angle, as can be expected with dust-free solvents and solutions of macromolecules which are much smaller than the wavelength of light (*e.g.*, Tanford, 1961).

Moreover, the diffusion coefficients of complete ribosomes have also been measured with a modified Malvern setup (Jolly and Eisenberg, 1976). There, the light source was an intensity-stabilized Spectra Physics argon ion laser, emitting light with $\lambda_o = 5145$ Å. Large, cylindrical high-quality glass scattering cells for a Sofica photo-diffuso-goniometer were used ; they were painstakingly cleaned with a flow of filtered, twice distilled water, following the complete procedure of Dubin *et al.* (1971). The index matching bath was filled with toluene, which was kept constant within 0.1 °C close to 20 °C. In these experiments, spectra were analyzed directly with a desk-top computer, mainly by weighted least square fits of $\ln[g_k^{(2)}(iT) - 1]$ to quadratic curves.

The diffusion coefficient of the large subunits has also been measured by photon correlation spectroscopy of the laser light scattered by the particles in a transparent centrifugation tube after being separated from their aggregates. This required special techniques and apparatus as outlined in the section "Results".

Reduction of the measured diffusion coefficient D to standard conditions of water at 20 °C yielded $D_{20,w}$ (Tanford, 1961 ; Koppel, 1974 ; Pusey *et al.*, 1974).

$$D_{20,w} = D \cdot (\eta/\eta_{20,w}) \cdot [293/(293 + t)]$$

with η the solvent viscosity at the temperature t (in °C) during the measurements.

Results

Protein content

The Folin-Lowry method (Stauffer, 1975) and the modified Folin-Lowry method (Hartree, 1972) were tried on solutions of ribosomes (containing their RNA) and of ribosomal protein (Nieuwenhuysen *et al.*, 1978b). The presence of LiCl and urea, which resulted from the LiCl/urea extraction procedure, did not influence the protein determinations as checked on blank and BSA solutions. After extraction, the supernatant which contained the proteins was dialyzed against 0.1 % formic acid or 1 N acetic acid. Lyophilization of these dialyzates yielded some insoluble material, so that the protein measurements of the samples gave doubtful results in that case. However, applying the protein determination methods directly on the protein solutions containing 0.1 % formic acid resulted in reliable results as could be concluded from control measurements of BSA solutions containing the same amount of formic acid. The efficiency of the protein extraction procedures was checked by measuring the uv-absorbance spectrum of the solutions. The removal of RNA results in a drastic lowering of the absorbance at 260 nm. Only the acetic acid extraction procedure gave reliable results from that point of view. The failure of the LiCl/urea method can be related to the low concentration of the starting solution (400 μg of protein/ml). It has been claimed that using BSA as the protein standard in the Folin-Lowry methods causes an underestimation of the real value of the protein content by 10 % ; the presence of ribosomal RNA would cause an overestimation of the protein content by the same amount (Sherton and Wool, 1974). On the other hand, it has been reported that eukaryotic ribosomal proteins yield more color per unit weight than BSA (Beeley *et al.*, 1968 ; Sacchi *et al.*, 1977). Anyway, the measured values in the first two rows of Table I give us 42 μg as a first estimate of the protein content of a solution containing 1 A_{260} unit of ribosomes. When the apparent protein content of the ribosomal

protein solutions after acetic acid extraction (obtained from BSA standard solutions) (third row of Table I) has to be increased by 10% to correct for the color yield of BSA as compared to ribosomal proteins, then this correction would give a protein content of 38.6 μg of ribosomal proteins. This indicates a loss of about 10% of the proteins during the extraction, a value which has been mentioned also in the literature (Sherton and Wool, 1974).

TABLE I

Determination of the protein content of solutions containing ribosomal particles isolated from the cryptobiotic embryos of *Artemia*

	Method used for the determination of protein	Measured equivalent micrograms of BSA per A_{260} unit of ribosomal particles		
		Complete ribosomes	Large subunits	Small subunits
Without extraction	Lowry	42.4 ± 1.6	–	–
Without extraction	Hartree	41.9 ± 1.6	27	35
After acetic acid extraction	Lowry	35.1 ± 1.8	–	–
After LiCl/urea extraction	Lowry	38.8 ± 1.3	–	–
Without extraction	biuret	41 ± 2.5	30	37

We have also used the biuret method (Sacchi *et al.*, 1977) for which the color yield is in general less dependent of the proteins involved ; in particular, eukaryotic ribosomal proteins would give the same color yield as BSA (Sacchi *et al.*, 1977). By adding ribosomal RNA in equimolar amounts to the standard BSA solutions, it was checked that its presence does not interfere with the color yield. From three series of measurements on three different preparations of complete ribosomes, we conclude (last row of Table I) that 1 A_{260} unit contains 41 ± 2.5 μg of protein (Nieuwenhuysen and Clauwaert, 1978b).

Analogous series of measurements have also yielded values for the large and small ribosome subunits as shown in Table I.

Ribonucleic acid content

Phosphorus determinations yielded that 1 A_{260} unit of large or small subunits contains 40.8 ± 1 μg RNA. The fact that the same value has been found for the different particles with different ratios of RNA to protein content is satisfying, because the contribution of the proteins to the absorbance at 260 nm can be neglected.

Buoyant densities in CsCl gradients

Analytical equilibrium centrifugation has yielded a value of 1.570 ± 0.005 g/cm³ for the buoyant density of the complete ribosomes in a CsCl gradient (Nieuwenhuysen *et al.*, 1978b). The symmetry of the UV-absorbance peaks at sedimentation equilibrium and the absence of any detectable material at the meniscus confirmed the good quality of the ribosomes.

Fig. 3 shows a typical UV-absorbance and density profile of a CsCl density gradient containing a mixture of the complete ribosomes and their large subunits, after centrifugation in a SW 65 swinging-bucket rotor (Nieuwenhuysen, 1978b). The initial density of the solution was made high enough to sediment the ribosomal particles into bands close to the meniscus ; the possible effects of the generated pressure on the buoyant density (see Discussion) were made minimal in this way. This experiment has yielded the same value for the buoyant density of the complete ribosomes as obtained with the analytical ultracentrifuge, and 1.60 ± 0.01 g/cm^3 for the large subunits.

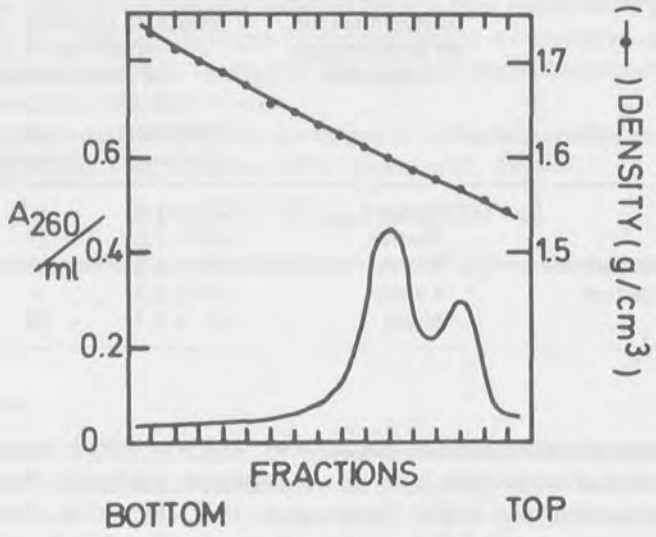

FIG. 3. Absorbance and density profile after centrifugation of a CsCl gradient containing a mixture of the large ribosome subunits (large peak) and the complete ribosomes (small peak) from the cryptobiotic embryos of *Artemia*. Centrifugation was in a Beckman SW 65 swinging-bucket rotor at 4 °C and 40 000 rpm for 26 hr.

With the empirical relation (given in Materials and methods) between the buoyant density and the RNA/protein ratio, values have been calculated for the RNA content of the ribosomal particles ; these are presented in Table II, together with the values derived from the chemical determinations. A survey of reports that give values as well for the RNA/protein ratio of ribosomal particles (chemically determined) as for their buoyant density in CsCl, has resulted in Fig. 4. Inspection of this figure indicates that the plotted published data should not all be used without criticism, and that our results are quite reasonable.

Application of our analytical ultracentrifugation method as described in Materials and methods, has yielded a maximal value for the possible difference $\Delta\rho$ between the buoyant densities of ribosomes isolated from the cryptobiotic embryos and the nauplii. Taking into account the experimental errors in determining the bandwidths of the concentration distributions at sedimentation equilibrium, it is estimated that $\Delta\rho \leqslant 0.005$ g/cm^3 ; a sharper

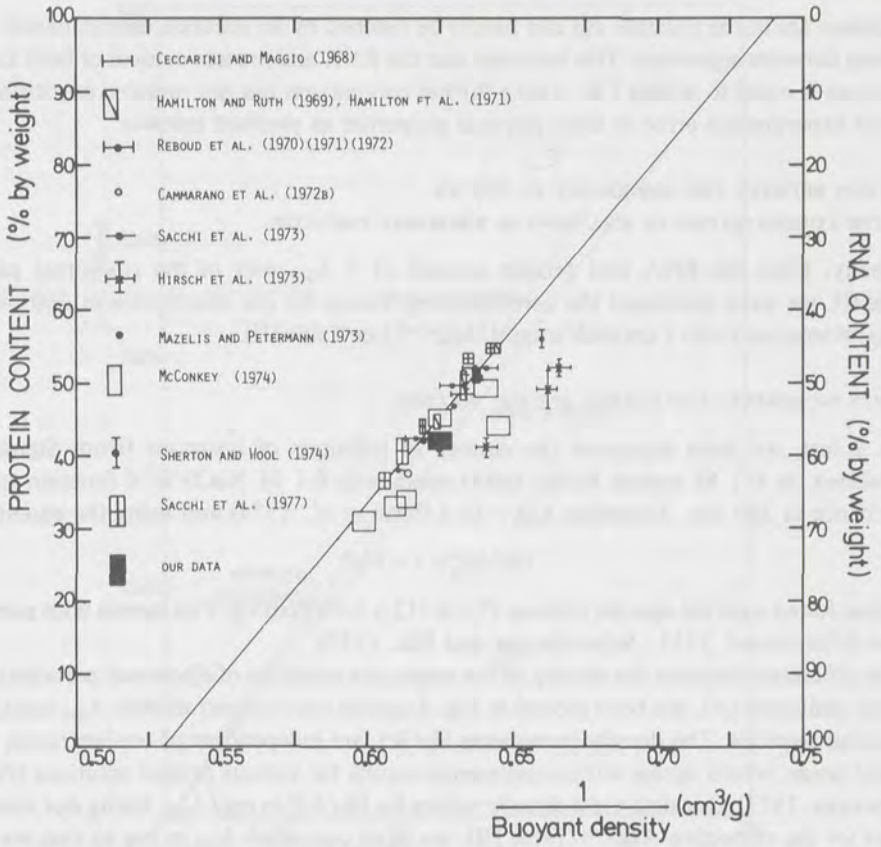

FIG. 4. Relation between published values for the chemically determined ratio of RNA protein content, and for the buoyant density in a CsCl gradient, of complete ribosomes and ribosomal subunits from *E. coli* and from the cytoplasm of different eukaryotes. Our own data on *Artemia* ribosomes particles have also been included. The straight line represents the empirical relation (buoyant density in a CsCl gradient)$^{-1} = (0.53 \times \%_{RNA} + 0.74 \times \%_{protein})/100$ (Hamilton, 1971 ; Sacchi *et al.*, 1977).

TABLE II

Protein and ribonucleic acid content of ribosomal particles isolated from the cryptobiotic embryos of *Artemia*

		Complete ribosome	Large subunit	Small subunit
From chemical determinations	Protein	42 $\pm$ 2 μg/A_{260}	29 $\pm$ 2 μg/A_{260}	36 $\pm$ 3 mg/A_{260}
	RNA	40.8 $\pm$ 1 μg/A_{260}	40.8 $\pm$ 1 μg/A_{260}	40.8 $\pm$ 1 μg/A_{260}
	Percentage by weight of RNA	49 $\pm$ 2%	58 $\pm$ 2%	52 $\pm$ 3%
From their buoyant density in a CsCl gradient	Percentage by weight of RNA	49 $\pm$ 1%	55 $\pm$ 2%	–

conclusion about the possible $\Delta\rho$ can hardly be reached by an accurate determination of the buoyant densities separately. This indicates that the RNA and protein content of both kinds of ribosomes is equal to within 1 %. Also a further comparison has not revealed any differences beyond experimental error in their physical properties as outlined below.

Relation between the absorbance at 260 nm and the concentration of solutions of ribosomal particles

Finally, from the RNA and protein content of 1 A_{260} unit of the ribosomal particles (Table II), we have calculated the corresponding values for the absorbance at 260 nm of a 1 mg/ml solution (with 1 cm path length) ($A_{260}^{1\,mg/ml}$) (see Table III).

Density increments and partial specific volumes

As a test, we have measured the density of solutions of lysozyme (from Sigma, 3× crystallized, in 0.1 M acetate buffer (pH4) containing 0.1 M NaCl) as a function of their absorbance at 280 nm. Accepting $A_{280}^{1\%} = 26.4$ (Span *et al.*, 1974) and using the equation

$$(\delta\rho/\delta c)_{\mu}^{o} = 1 - \bar{v}^{o}\rho^{o},$$

we have found a partial specific volume $\bar{v}^{o} = 0.712 \pm 0.003$ cm³/g. This agrees with published values (Charlwood, 1957 ; Schausberger and Pilz, 1977).

The difference between the density of the respective solutions of ribosomal particles (ρ) and of their dialyzates (ρ^{o}), has been plotted in Fig. 5 against the concentration in A_{260} units/ml of ribosomal particles. The density increments $(\delta\rho/\delta c)_{\mu}$ are independent of concentration in the studied range, which agrees with experimental results for various protein solutions (Pilz and Czerwenka, 1973). Our data yield directly values for $(\delta\rho/\delta c)_{\mu}^{o}$ in mg/A_{260}. Using our measured values for the respective $A_{260}^{1\,mg/ml}$ (Table III), we have converted A_{260} to mg so that we could calculate $\bar{v}^{o}$ of the respective ribosomal particles. All these results are shown in Table IV.

Table III

Experimentally determined absorbance at 260 nm of ribosomal particles isolated from *Artemia*

	$A_{260}^{1\,mg/ml}$
Complete ribosomes	12.1 ± 0.4
Large subunits	14.3 ± 0.6
Small subunits	12.9 ± 0.6

Fig. 6 clearly shows the correlation between our measured values for $\bar{v}^{o}$ and for the RNA content of the different ribosomal particles from *Artemia*. Published $\bar{v}^{o}$ values for RNA and ribosomal proteins have also been indicated on this figure, to make clear that our results are very reasonable. The corresponding published data on the complete ribosomes and ribosomal subunits from *E. coli* (Hill *et al.*, 1969a ; Igarashi *et al.*, 1973) lack such good correlation.

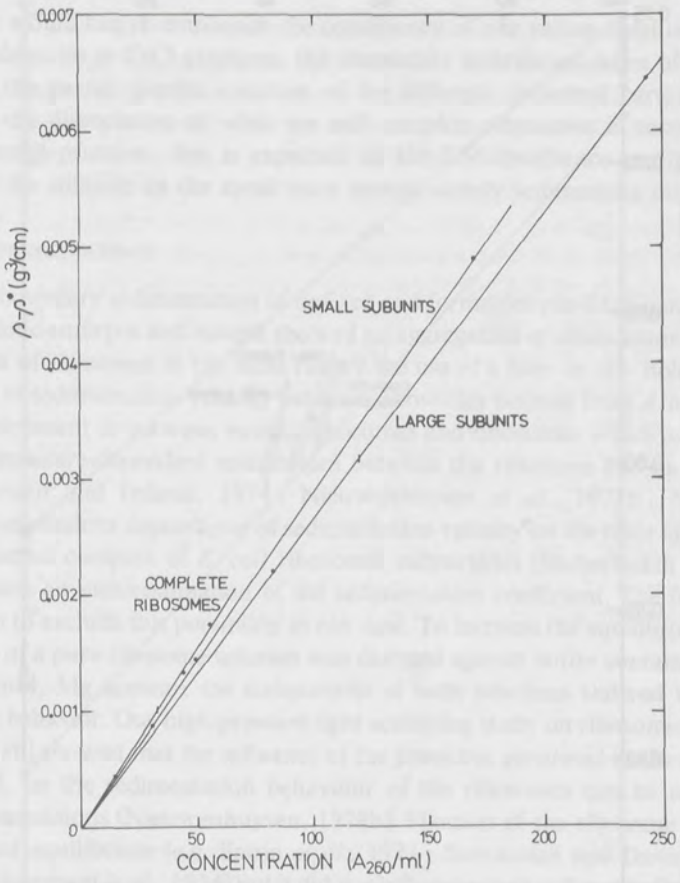

FIG. 5. Difference measured between the density ρ of the solutions of ribosomal particles, and the density ρ^o of their dialysate, plotted as a function of the concentration of ribosomal particles. Different symbols indicate different preparations of ribosomes ; data at low concentrations have been omitted for clarity.

TABLE IV

Measured density increments $(\delta\rho/\delta c)^o_\mu$ in buffers at 25 °C and partial specific volumes $\bar{v}^o$ of ribosomal particles isolated from *Artemia*

	$(\delta\rho/\delta c)^o_\mu$ (mg/A_{260})	$(\delta\rho/\delta c)^o_\mu$	$\bar{v}^o$ (cm^3/g)
Complete ribosomes	0.0312 ± 0.0003 (Nieuwenhuysen and Clauwaert, 1978b)	0.378 ± 0.01	0.621 ± 0.01
Large subunits	0.0266 ± 0.0003	0.380 ± 0.02	0.612 ± 0.02
Small subunits	0.0291 ± 0.0003	0.376 ± 0.02	0.615 ± 0.02

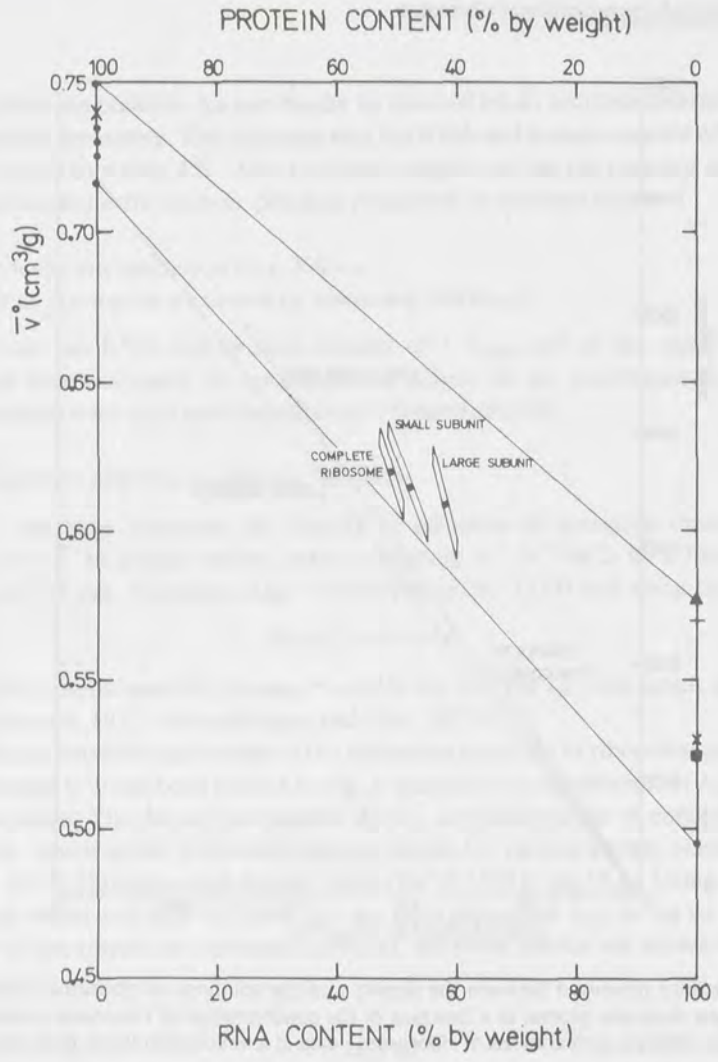

FIG. 6. Correlation between our measured values for the partial specific volume $\bar{v}^{o}$ and the RNA content of complete ribosomes and their subunits from the cryptobiotic embryos of *Artemia*. The symbols at 100% RNA indicate published values for the partial specific volume of different ribonucleic acids ; they were determined with density and concentration measurements for rRNA from *E. coli* (– Kurland, 1960) and from rat liver (X Petermann and Pavlovec, 1966), and for the RNA isolated from bacteriophage MS2 (● Slegers *et al.*, 1973) and from *E. coli* small ribosomal subunits (▲ Ortega and Hill, 1973), or they were derived from neutron scattering experiments for rRNA incorporated in the large subunits of *E. coli* ribosomes (■ Crichton *et al.*, 1977). At 100% protein, the points (●) indicate published values, measured with a digital density meter, for the partial specific volume of different ribosomal proteins from *E. coli* (Osterberg *et al.* 1976, 1977, 1978 ; Giri and Subramian, 1977 ; Giri *et al.*, 1977, 1978 ; Giri and Dijk, 1979) ; X indicates the value measured for the total preparation of *E. coli* ribosomal proteins denatured in 8 M urea (Möller and Crambach, 1967), while the partial specific volume of proteins changes only little on denaturation (Skerjanc *et al.*, 1970) ; the same value has also been derived from neutron scattering experiments for the proteins incorporated in the large subunits of *E. coli* ribosomes (Crichton *et al.*, 1977). The straight lines connect the maximal and minimal published values for $\bar{v}^{o}$ of *E. coli* ribosomal proteins with the extreme values for $\bar{v}^{o}$ of ribonucleic acids.

Finally we would like to emphasize the consistency of our values obtained up to now for the buoyant densities in CsCl gradients, the chemically determined ratios of RNA to protein content, and the partial specific volumes, of the different ribosomal particles. All the data indicate that the dissociation of what we call complete ribosomes is accompanied by the removal of some proteins ; this is expected, as the UV-absorbance profile obtained after separation of the subunits in the zonal rotor reveals slowly sedimenting material.

Sedimentation coefficients

Analytical boundary sedimentation of unfixed and formaldehyde-fixed complete ribosomes from cryptobiotic embryos and nauplii showed no aggregation or dissociation. Comparison of different kinds of ribosomes in the same run by the use of a four- or six- hole rotor indicated no difference in sedimentation velocity between ribosomes isolated from *Artemia* in different stages of development or between nauplii ribosomes and ribosomes which are all attached to mRNA. The pressure-dependent equilibrium between the ribosome and its large and small subunit (Baierlein and Infante, 1974 ; Nieuwenhuysen *et al.*, 1978b ; Nieuwenhuysen, 1978b), or an anomalous dependence of sedimentation velocity on the rotor speed as observed for the association complex of *E. coli* ribosomal subparticles (Shcherbukin and Guermant, 1975), can cause an underestimation of the sedimentation coefficient. The following results however seem to exclude this possibility in our case. To increase the equilibrium dissociation-constant, part of a pure ribosome solution was dialyzed against buffer containing only 2 mM, instead of 9 mM, Mg acetate ; the comparison of both solutions showed no difference in sedimentation behavior. Our high-pressure light-scattering study on ribosomes (Nieuwenhuysen *et al.*, 1978b) showed that the influence of the pressures generated in the analytical ultracentrifuge cell, on the sedimentation behaviour of the ribosomes can be neglected in our experimental conditions (Nieuwenhuysen, 1978b). Fixation of the ribosome particles blocks their dynamical equilibrium (*e.g.* Spirin *et al.*, 1971 ; Subramian and Davis, 1971 ; Subramian, 1972 ; Guermant *et al.*, 1974) but it did not influence their sedimentation behaviour. No rotor-speed dependence was observed between 12 000 and 38 000 rpm. So finally we feel confident assigning a value of 81 ± 1 S to the standard sedimentation coefficient $s^{0}_{20,w}$ of unfixed and fixed ribosomes from cryptobiotic embryos and nauplii of *Artemia* (Nieuwenhuysen and Clauwaert, 1978b).

To examine the formaldehyde-fixed large subunits, rotor speeds were chosen between 37 000 and 39 000 rpm. The rotor temperature was constant within 0.1 °C at 20.0 °C. Occasionally, complete ribosomes were sedimented simultaneously in the same rotor ; the same sedimentation coefficient was obtained as previously. Three independent preparations yielded for the standard sedimentation coefficient at infinite dilution, $s^{0}_{20,w} = 58 \pm 1$ S, (Nieuwenhuysen, 1978b ; Nieuwenhuysen *et al.*, 1979a).

Diffusion coefficients

Single-clipped photon-count autocorrelation spectroscopy of the light scattered from essentially monodisperse solutions of different kinds of complete ribosomes, has yielded their diffusion coefficient (Nieuwenhuysen and Clauwaert, 1978b). Before the measurements, solutions were dialyzed against 20 mM Hepes buffer (pH 7.5), containing 1 mM DTT, 70 (or 100) MM KCl and 9 mM Mg acetate. The Q-factors (Pusey *et al.*, 1974 ; Nieuwenhuysen,

1978ab), which reflect eventual deviations from mono-exponential decay of the measured correlation functions, were equal to zero to within experimental error ($|Q| < 0.02$).

Variation of the sample time T from its optimal value, resulting in spectra spanning from 0.5 to more than 5 decay times, did not change the obtained diffusion coefficient D. Correlation spectra of the light scattered at different angles yielded the same D. The diffusion coefficient of fixed ribosomes from cryptobiotic embryos of *Artemia* was also measured at higher temperatures. Reduced to 20 °C, the value remained constant up to 50 °C ; further heating up of the solution caused denaturation and aggregation, which was reflected by a decreasing D_{20} and an increase of the intensity of the scattered light. All these results justify the confidence in our measured values for the standard diffusion coefficient $D_{20,w}$ for different samples in function of the concentration (Fig. 7). No concentration dependence is evident below 1 mg/ml in standard buffer ; this is in agreement with theoretical expectations (Pusey, 1974 ; Rowe, 1977) and with experimental results for *E. coli* ribosomal particles (Koppel, 1974). Fig. 7 also shows that the same value for the standard diffusion coefficient was obtained with different experimental setups in different laboratories. Finally we conclude from the data that the complete ribosomes isolated from cryptobiotic embryos and nauplii of *Artemia* have the same standard diffusion coefficient at infinite dilution, which is not altered by fixation : $D^{\circ}_{20,w} = (1.41 \pm 0.02) \times 10^{-7}$ cm²/sec. Note that we have also found the same standard sedimentation coefficient for the different kinds of ribosomes.

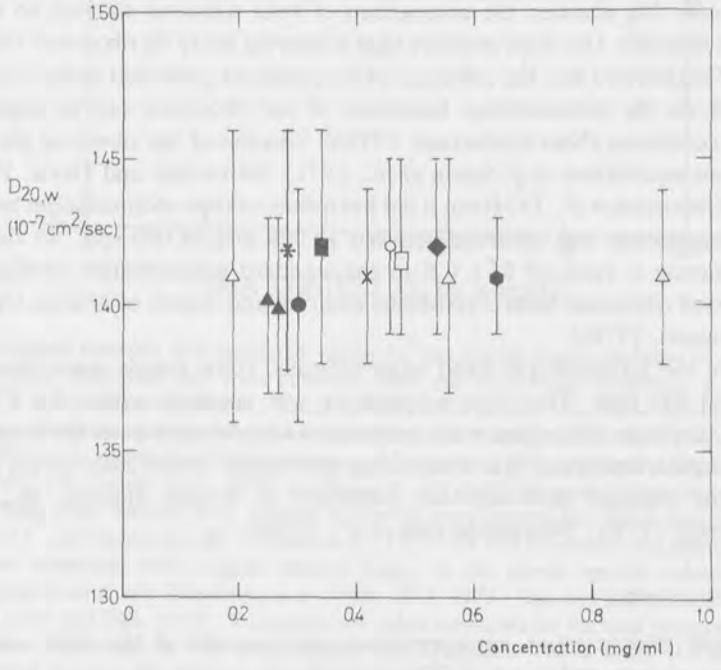

FIG. 7. Measured values for the standard diffusion coefficient $D_{20,w}$ of complete ribosomes as a function of their concentration. The different symbols indicate different preparations ; their meaning is explained in Table V.

TABLE V

Photon correlation spectroscopy of complete ribosomes from *Artemia*. Experimental conditions. (Meaning of the symbols in Fig. 7)

Symbol	Source of ribosomes	Method of preparation	Type of spectrometer
● ■ ▲ ♦	Cryptobiotic embryos	Centrifugation of PMS in a high-density sucrose gradient, PEG precipitation, and centrifugation in a zonal rotor	Standard
○ □	Cryptobiotic embryos	Idem + formaldehyde fixation	Standard
△	Nauplii	Pelleting of ribosomes from PMS, centrifugation in a zonal rotor, and formaldehyde fixation	Standard
▼	Cryptobiotic embryos	Pelleting of ribosomes from the PMS through a pad containing 50 % (by weight) of sucrose and centrifugation in a zonal rotor	Standard
*	Cryptobiotic embros	Idem	A transparent centrifuge tube was used instead of a glass scattering cell
⬢	Cryptobiotic embryos	Centrifugation of PMS in a high-density sucrose gradient, PEG precipitation, centrifugation in a zonal rotor, and PEG precipitation	Modified (Jolly and Eisenberg, 1976)

The presence of dimers and larger aggregates in solutions of ribosome subunits hinders the determination of the diffusion coefficient of the single particles. In general, photon correlation spectroscopy of a polydisperse solution can yield the normalized photon-count autocorrelation function $g^{(2)}(iT)$, where T is the sample time and i is the channel-number ; this "spectrum" can then be analyzed by curve fitting as follows (*e.g.*, Brown *et al.*, 1975) :

$$\ln[g^{(2)}(iT) - 1] = \ln A - \bar{\Gamma} \cdot (iT) + X \cdot (iT)^2 + Y \cdot (iT)^3 + \ldots$$

where A, $\bar{\Gamma}$, X, Y, *etc* . are to be determined. From $\bar{\Gamma}$ one obtains the so-called z-average diffusion coefficient $\bar{D}_z$ of the macromolecules, when these are all smaller than the wavelength of light, and when they have equal refractive index increments :

$$\frac{\bar{\Gamma}}{2\,K^2} = \frac{\Sigma_j N_j M_j^2 D_j}{\Sigma_j N_j M_j^2} = \bar{D}_z,$$

where K^2, the square of the scattering vector, is known for each spectrum ; N_j is the number of macromolecules in the solution of species j having molecular weight M_j and diffusion coefficient D_j. We can consider now a solution of ribosome subunits, that does not only contain single particles with molecular weight M and diffusion coefficient D, but also a certain weight-percentage of dimers,

$$\%_{dimers} = 100\ N_{dimer}M_{dimer}/(NM + N_{dimer}M_{dimer})$$

In that case we would have

$$\bar{D}_z = \frac{NM^2D + N_{dimer}M^2_{dimer}D_{dimer}}{NM^2 + N_{dimer}M^2_{dimer}} = \frac{(100-\%_{dimers})\,MD + \%_{dimers}M_{dimer}D_{dimer}}{(100-\%_{dimers})\,M + \%_{dimers}M_{dimer}} \quad (1)$$

In principle it would be possible to estimate $\%_{dimers}$ by analytical boundary centrifugation. D_{dimer} can be related to D, using the following equation (Eisenberg, 1976) which is valid as well for the dimers as for the single particles:

$$M = \frac{s^o\,R\,(273+t)}{D^o\,(\delta\rho/\delta c)^o_\mu}, \quad (2)$$

where R is the gas constant, and t the temperature in °C; the superscript ° indicates infinite dilution. The density increment $(\delta\rho/\delta c)^o_\mu$ can be taken equal for the subunits and their aggregates. Therefore $D^o_{dimer} = (s^o_{dimer}\ M/s^o\ M_{dimer})\ D^o$. Centrifugation experiments yield $s^o_{dimer} = 1.5\ s^o$ for the ribosome subunits, and obviously $M_{dimer} = 2M$. So we obtain $D^o_{dimer} = 0.75\ D^o$. Eliminating then D_{dimer} from expression (1) for $\bar{D}_z$ yields

$$\bar{D}^o_z = D^o\left[1 - \frac{\%_{dimers}0.5}{100 + \%_{dimers}}\right]. \quad (3)$$

This estimate of the relation between the searched value of D^o and the experimentally accessible value $\bar{D}^o_z$, when dimers are present in the solution, has been plotted in Fig. 8. In

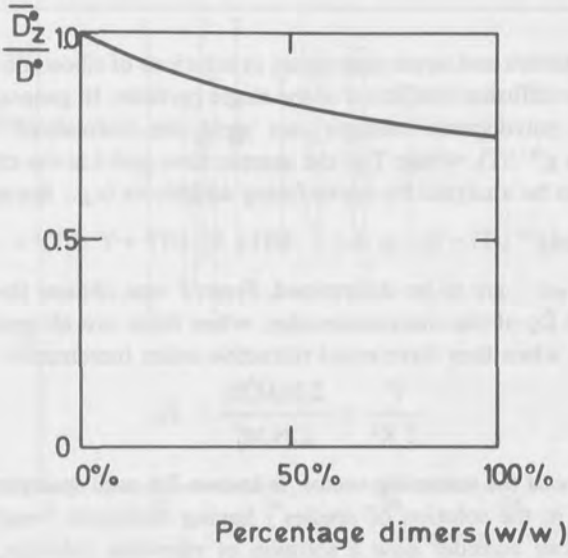

FIG. 8. Plot of the relation between the z-average diffusion coefficient of a mixture of monomers and dimers of ribosome subunits, $\bar{D}^o_z$, and the diffusion coefficient of the monomers, D^o, as a function of the percentage (w/w) of dimers in the mixture.

reality also larger aggregates are present ; so D^0 cannot be determined simply and accurately from measured values for $\%_{dimers}$ and for $\bar{D}^0_z$. The plot shows the need for monodispersity to determine D^0 with an experimental error of only a few percent. So the numerous attempts to eliminate aggregation, which were described in Materials and Methods were clearly necessary to allow the measurements, described hereafter.

A solution of formaldehyde-fixed large ribosome subunits in 20 mM Hepes buffer (pH 7.5) containing 1 mM DTT, 500 mM KCl and 5 mM Mg acetate was obtained by a final zonal sucrose gradient centrifugation as described in Materials and Methods. After dialysis against the same buffer, it was apparently monodisperse as judged by analytical boundary sedimentation. The solution was directly used for photon correlation spectroscopy. Series of spectra were recorded at 45°, 90° and 135°. The values for $D_{20,w}$ obtained at different scattering angles have been plotted in Fig. 9 ; they are independent of the scattering angle to within experimental error. The concentration of the ribosome subunits was as low as 0.3 mg/ml.

The obtained values for the diffusion coefficient of the complete ribosomes showed no concentration dependence up to at least 0.9 mg/ml, which is in agreement with the results for *E. coli* ribosomal particles and with theoretical expectations, as mentioned higher. So we conclude from the data in Fig. 9 that the standard diffusion coefficient at infinite dilution $D^0_{20,w}$ of the large ribosome subunits is equal to or slightly higher than 1.54 ± 0.03 cm²/s (Nieuwenhuysen, 1978b ; Nieuwenhuysen *et al.*, 1979a).

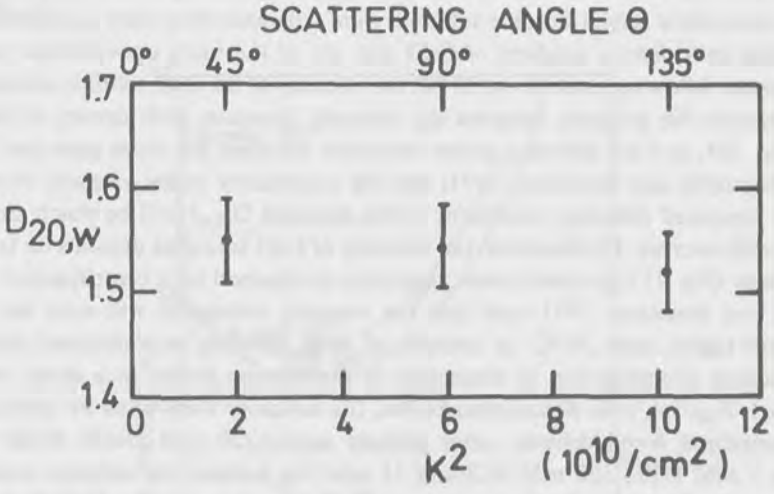

FIG. 9. Values for the standard diffusion coefficient, $D_{20,w}$, of the formaldehyde-fixed large ribosome subunits from *Artemia* determined by photon correlation spectroscopy at different scattering angles. (K^2 is the square of the corresponding scattering vector). Each value has been derived from a series of spectra, following the data analysis procedure described elsewhere (Nieuwenhuysen, 1978ab).

In the following, a method is described which has been developed to eliminate the uncertainty in the measured diffusion coefficient, caused by the aggregation of the subunits. The procedure includes the separation of eventual aggregates form the monomers (by zonal

centrifugation in a CsCl density gradient) and photon correlation spectroscopy of the laser light scattered by the monomers in the transparent centrifugation tube. Such plastic centrifuge tubes have a much lower optical quality than the glass scattering cells which are normally used. However, they allow a centrifugation of a macromolecular solution at high speed to remove dust particles from the scattering volume ; therefore they were used for intensity fluctuation spectroscopy of bacteriophage R17 and phage DNA (Newman *et al.*, 1974). Also, photon correlation spectroscopy has been directly performed of laser light scattered from *E. coli* ribosomal particles separated by zonal centrifugation in a 5-20 % sucrose density gradient (Koppel, 1974). We have constructed a simple holder to bring a centrifugation tube in the right position in the laser beam. It has been made out of aluminum and has been painted black completely. It allows the manual vertical displacement of a cellulose nitrate centrifugation tube (for the Beckman SW 40 swinging-bucket rotor) to scan its contents with the laser beam which passes through a pinhole and is focused in the tube. This tube is in close contact and in thermal equilibrium with the holder whose temperature is measured inside (near the scattering volume) with a calibrated mercury thermometer ; the whole setup is placed in a thermostatted room. Scattered light can be detected at all angles between 25° and 140°. Other components of the spectrometer have been described in Materials and Methods. As a test, a 0.3 mg/ml solution of complete ribosomes was centrifuged in a SW 40 tube at 20 000 rpm for 1 hr to remove dust ; this experiment has been included in Fig. 7 and Table V. The measured value for the diffusion coefficient was independent of scattering angle and in excellent agreement with the values determined earlier using glass scattering cells immersed in a water bath. The monomeric large ribosome subunits were separated from their aggregates by zonal centrifugation in a density gradient of CsCl and not of the more conventional sucrose (or glycerol) for the following reasons. At 20 °C, the viscosity of the CsCl solution almost does not vary throughout the gradient, whereas the viscosity increases with density in the case of sucrose (Fig. 10) ; so CsCl affords a better resolution between the zones separated by centrifugation (Kaempfer and Meselson, 1971) and the uncertainty in the viscosity correction (to reduce the measured diffusion coefficient to the standard $D_{20,w}$) will be much smaller with CsCl than with sucrose. Furthermore, the viscosity of CsCl solutions depends on temperature in such a way (Fig. 11) that even better resolution is obtained by a centrifugation near 0 °C (Kaempfer and Meselson, 1971) and that the viscosity correction will even be smaller at temperatures higher than 20 °C. A solution of large subunits was obtained after sucrose density gradient centrifugation of ribosomes in dissociation buffer in a zonal rotor ; after dilution to 1 A_{260}/ml with dissociation buffer, the subunits were fixed by addition of 1 % freshly neutralized formaldehyde ; after dialysis against 20 mM Hepes buffer (pH 7.5), containing 1 mM DTT, 300 mM KCl and 11 mM Mg acetate, the solution could be concentrated by ultrafiltration ; finally it was loaded on a density gradient of 3-11 % (w/w) CsCl in the same buffer without KCl, in a SW 40 tube. This type of tubes was chosen because they are the longest available, thus offering the best resolution. The density in the CsCl gradient is comparable (somewhat higher) to that in a 5-20 % sucrose gradient. Centrifugation was near 0 °C at 39 000 rpm for 140 min. Near the end of the run the cooling was switched off to warm up the gradient to room temperature. The zone of the monomeric subunits was located by measuring the laser light scattering intensity throughout the gradient. After photon correlation spectroscopy, the UV-absorbance profile of the gradient was measured (Fig. 12), which confirmed the light scattering intensity profile. No slowly sedimenting material was

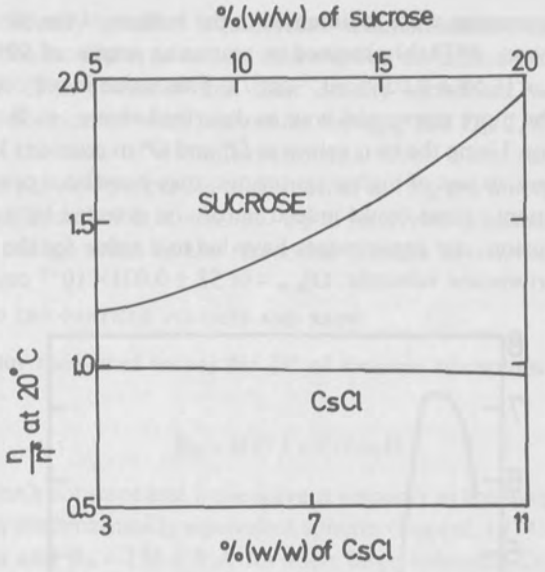

FIG. 10. Viscosity η in linear concentration gradients of CsCl and sucrose (in water) relative to the viscosity η^0 of water, at 20 °C. The 3-11 % CsCl gradient has about the same density as the standard 5-20 % sucrose gradient ; CsCl : from 1.023 to 1.091 g/cm³ ; sucrose : from 1.020 to 1.083 g/cm³. (Data from Weast, 1973.)

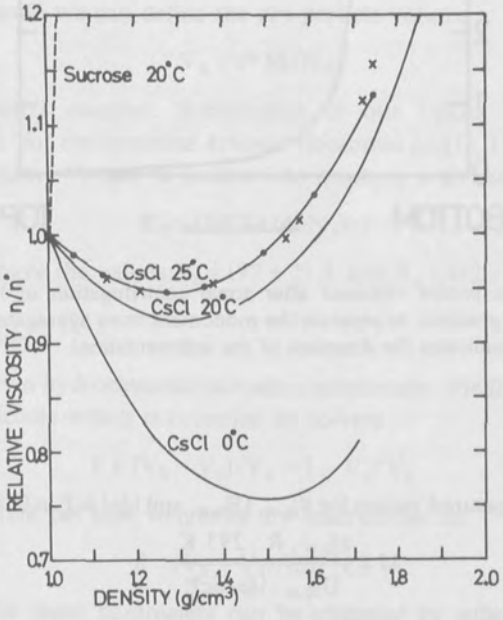

FIG. 11. The viscosity η of aqueous CsCl solutions relative to the viscosity η^0 of water, at 0 °C (Kaempfer and Meselson, 1971), 20 °C (Weast, 1973), and 25 °C (● Lyons and Riley, 1954 ; × Bruner and Vinograd, 1965).

detected and eventual aggregates were sedimented to the bottom of the tube. Analysing series of spectra (Nieuwenhuysen, 1978ab) obtained at scattering angles of 90° and 135° yielded consistent results : $D_{20,w} = (1.58 \pm 0.03) \times 10^{-7}$ cm²/s. This value is only slightly higher than the one determined in the more convential way as described above ; in fact both are equal to within experimental error. Using the two values as $\bar{D}_z^o$ and D^o in relation (3), we calculate that 5 (± 5)% (w/w) of dimers, or less of higher aggregates, may have been present in the solution used in the first experiment ; these could indeed hardly be detected by analytical boundary sedimentation. In conclusion, our experiments have led to a value for the standard diffusion coefficient of the large ribosome subunits, $D^o_{20,w} = (1.58 \pm 0.03) \times 10^{-7}$ cm²/s.

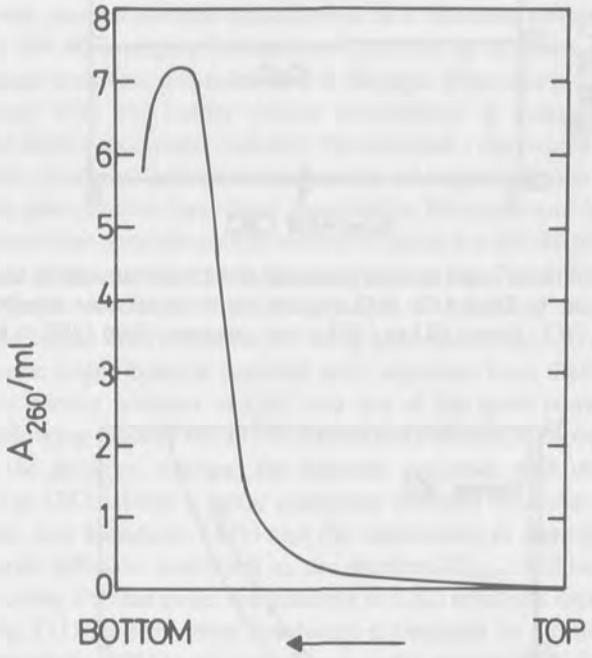

FIG. 12. UV-absorbance profile obtained after zonal centrifugation of large *Artemia* ribosome subunits in a CsCl density gradient, to separate the monomers from aggregates for photon correlation spectroscopy. (The arrow indicates the direction of the sedimentation).

MOLECULAR WEIGHTS

Substitution of our measured values for $s^o_{20,w}$, $D^o_{20,w}$, and $(\delta\rho/\delta c)^o_\mu$ in (Eisenberg, 1976)

$$M = \frac{s^o_{20,w} \cdot R \cdot 293\ K}{D^o_{20,w} \cdot (\delta\rho/\delta c)^o_\mu},$$

where R is the gas constant and 293 K the absolute temperature which corresponds to the standard 20 °C, yields the molecular weights $M = (3.7 \pm 0.1) \times 10^6$ for the complete ribosomes from the cryptobiotic embryos and $M = (2.3 \pm 0.1) \times 10^6$ for their large subunits.

Analytical CsCl density gradient equilibrium sedimentation experiments of ribosomes isolated from cryptobiotic embryos and nauplii showed no difference between their buoyant density (see higher). This indicates that their density increment will be equal to within experimental error. So together with our results for $s^0_{20,w}$ and $D^0_{20,w}$, this yields that also their molecular weight is the same to within experimental error. Thus, none of our data indicate large changes in the physical properties of ribosomes during pre-emergence development and concommitant cell differentiation in *Artemia*. Up to now, two-dimensional gel electrophoresis did not reveal any change either in the ribosomal proteins of *Artemia* (Möller *et al.*, 1975).

Hydrodynamic and dry-particle volumes and radii

Substitution of our measured values for D^0 of *Artemia* ribosomes in the Stokes-Einstein equation

$$R_h = k(273 + t)/6\pi\eta D,$$

where k is Boltzmann's constant and η the solvent viscosity at the temperature t (in °C), gives us the radii of the hydrodynamically equivalent spheres (Koppel, 1974) $R_h = 152 \pm 2$ Å for the complete ribosomes and $R_h = 136 \pm 3$ Å for their large subunits. Due to deviations of the ribosome shape from a sphere, their real hydrodynamic volume V_h will be smaller than that of the hydrodynamically equivalent sphere

$$V_{h,max} = (4\pi/3)\ R_h^3,$$

which has the value of $(14.7 \pm 0.6) \times 10^6$ Å^3 and $(10.4 \pm 0.6) \times 10^6$ Å^3 respectively.

For any macromolecule we can define the dry-particle volume

$$V_d = \bar{v}^o\, M/N_A$$

where N_A is Avogadro's number. Substitution of our values for $\bar{v}^o$ and M yields $V_d = (3.8 \pm 0.2) \times 10^6$ Å^3 for the complete *Artemia* ribosomes and $(2.3 \pm 0.2) \times 10^6$ Å^3 for their large subunits. The volume V_d can be packed into a sphere with radius

$$R_d = (3\bar{v}^o\, M/4\pi N_A)^{1/3}$$

The dry-particle radii have the values $R_d = (97 \pm 2)$ Å and $R_d = (82 \pm 2)$ Å respectively.

Hydrodynamic solvation

We can define here two hydrodynamic solvation parameters : the fraction of the volume of the solvated macromolecule which is occupied by solvent

$$F = (V_h - V_d)/V_h = 1 - V_d/V_h$$

and the volume of solvent per unit weight of dry macromolecule

$$\delta = (V_h - V_d)/(M/N_A)$$

The maximal values for these parameters can be obtained by substituting our values for $V_{h,max}$ and V_d in the equations. This yields $F_{max} = (0.74 \pm 0.01)$ and $\delta_{max} = (1.8 \pm 0.1)$ cm^3/g for the complete ribosomes and $F_{max} = (0.78 \pm 0.02)$ and $\delta_{max} = (2.1 \pm 0.2)$ cm^3/g for their large subunits.

Discussion

Buoyant density in CsCl gradients

The possible effects of the pressures, generated by centrifugation, have never been mentioned in reports of density gradient equilibrium sedimentation of ribosomal particles, as far as we know. However, the combined effects of compressing the solution and the macromolecular species may be significant ; they may affect band shape, band position, and buoyant density (Hearst *et al.*, 1961). Values of the standard pressure coefficient, ψ, which describes the variation of the buoyant density with pressure, have been reported for T-4 bacteriophage DNA and tobacco mosaic virus (Hearst *et al.*, 1961), for several additional nucleic acids (Bauer *et al.*, 1971), and for three proteins (Sharp *et al.*, 1978), all in CsCl solutions. The pressures generated in preparative ultracentrifuges are generally higher than in analytical ultracentrifuges. We have performed experiments using both types of centrifuges and two types of swinging-bucket rotors, which have all yielded consistent results.

Our value for the buoyant density of *Artemia* ribosomal particles in a CsCl density gradient, and thus for their RNA/protein ratio, is close to the values reported for intact cytoplasmic ribosomal particles isolated from different other eukaryotes (Table VI). Vournakis and Rich (1971) explain that the high density which they found for complete chick embryo ribosomes, 1.60-1.61 g/cm^3, probably reflects the fact that their preparation buffer contained 250 mM KCl, which may have released some ribosomal proteins. On the other hand, Kloppsteck and Schweiger (1973) think that the low density which they found for complete ribosoms from *Acetabularia*, 1.51 g/cm^3, may have been caused by some adherent non-ribosomal proteins.

Ribonucleic acid and protein content ; absorbance at 260 nm

Our values for the RNA content of ribosomal particles from *Artemia* agree with those obtained from chemical determinations on other eukaryotic ribosomal particles, as can be seen in Table VII.

Infante and Nemer (1968) have found no difference between the buoyant densities of ribosomes extracted from unfertilized eggs and from developing embryos of the sea urchin ; however, the results of Unsworth and Kaulenas (1975) indicate that ribosomes from the unfertilized eggs may have quantitatively more proteins than those from the blastulas. Two-dimensional gel electrophoresis has not revealed any differences between the proteins of the ribosomal subunits from *Artemia* cysts and nauplii (Möller *et al.*, 1975).

From the RNA and protein content and the absorbance at 260 nm of ribosomal particles, the absorbance at 260 nm of ribosomal particles, the absorbance at 260 nm of the RNA in the intact particles can be calculated. The contribution of the ribosomal proteins to A_{260} of ribosomes is estimated to be very small and is usually neglected (Zubay and Wilkins, 1960 ; Araco *et al.*, 1975). Kurland (1960) has determined the absorbance of the RNA from *E. coli* ribosomes : $A_{260}^{1\,mg/ml} = 22.7 \pm 0.5$. Cox (1970) has reported that the molar absorbance coefficients ε_{260} (P) of the free RNAs from rabbit reticulocyte large and small ribosomal subunits are $7\,750 \pm 100$ and $7\,600 \pm 100$ respectively ; accepting an equimolar base composition and thus a mean molecular weight of 321 for the nucleotides incorporated in RNA, we can calculate the respective values for $A_{260}^{1\,mg/ml}$ of 24.1 ± 0.4 and 23.6 ± 0.4. Further, the RNA chains incorporated in ribosomes have a very similar absorbance because their

TABLE VI

Literature values for the buoyant density in a CsCl gradient of cytoplasmic, ribosomal particles isolated from eukaryotes.
(Published values for subunits probably associated with non-ribosomal proteins, nucleic acids, or nucleoprotein-complexes have not been listed here)

Reference	Ribosome source	Centrifugation technique	Buoyant density (g/cm^3)		
			Complete ribosomes	Large subunits	Small subunits
Spirin *et al.* (1965)	Fish embryos	Swinging-bucket rotor	1.52	–	–
Perry and Kelley (1966)	Cultured L cells	Swinging-bucket rotor	1.55	1.57	1.49
Baltimore and Huang (1968)	HeLa cells	Swinging-bucket rotor	1.55	–	–
Maggio *et al.* (1968)	Sea urchin unfertilized eggs and embryos	Swinging-bucket rotor	1.55-1.56	–	–
Svetailo and Filippovich (1967)	*Dicotyledoneae*	Swinging-bucket rotor	1.53-1.55	–	–
Ceccarini and Maggio (1968)	Sea urchin	Swinging-bucket rotor	1.57	–	–
Ceccarini and Maggio (1968)	Slime molds	Swinging-bucket rotor	1.58	–	–
Infante and Nemer (1968)	Sea urchin unfertilized eggs and embryos	Swinging-bucket rotor	1.57-1.58	1.61	1.55
Parsons and McCarthy (1968)	Rat liver	Swinging-bucket rotor	1.60	1.62	1.55
Henshaw (1968)	Rat liver	Swinging-bucket rotor	1.54-1.55	1.59-1.60	1.53
Clegg and Arnstein (1970)	Rat liver	Swinging-bucket rotor	–	1.61	1.56
Fais *et al.* (1970)	Krebs ascitic carcinoma in mice	Swinging-bucket rotor	1.57	1.61	1.52
Henshaw and Loebenstein (1970)	Rat liver	Swinging-bucket rotor	1.55-1.57	–	1.53
Chi and Suyama (1970)	*Tetrahymena pyriformis*	Swinging-bucket rotor	1.56-1.58	1.57	1.53
Leick *et al.* (1970)	*Tetrahymena pyriformis*	Fixed-angle rotor	1.56	1.53	1.49
Olsnes (1970)	Rat liver (polysomes)	Swinging-bucket rotor	1.55	–	–
Ceccarini *et al.* (1970)	Slime mould	Swinging-bucket rotor	–	1.55	1.50
Reboud *et al.* (1970, 1971)	Rat liver	Swinging-bucket rotor	1.57	–	–
Grivell *et al.* (1971)	*Saccharomyces carlsbergensis*	Swinging-bucket rotor	1.55	–	–
Rosbach and Penman (1971)	HeLa cells	Swinging-bucket rotor	1.55	–	–

TABLE VI (continued)

Reference	Ribosome source	Centrifugation technique	Buoyant density (g/cm^3)		
			Complete ribosomes	Large subunits	Small subunits
Vournakis and Rich (1971)	Chick embryos (free, single ribosomes)	Fixed-angle rotor	1.61	–	–
Vournakis and Rich (1971)	Chick embryos (polysomal ribosomes, attached to a fragment of mRNA)	Fixed-angle rotor	1.60	–	–
Olsnes (1971)	Rat liver (polysomes)	Swinging-bucket rotor	1.57	–	–
Warner (1971)	Yeast	Swinging-bucket rotor	–	1.56-1.57	1.56-1.57
Hamilton *et al.* (1971)	Rat liver	Analytical ultracentrifuge	–	1.61	1.55
Reboud *et al.* (1972)	Rat liver	Swinging-bucket rotor	1.59	1.62	1.56
Ajtkhozin *et al.* (1972)	Pea seedlings	Swinging-bucket rotor	1.54-1.55	1.56-1.57	1.52-1.53
Vignais *et al.* (1972)	*Candida utilis*	Swinging-bucket rotor	1.53	–	–
Cammarano *et al.* (1972b)	Pea	Swinging-bucket rotor	1.57	1.59	1.55
Cammarano *et al.* (1972b)	Sea urchin	Swinging-bucket rotor	1.57-1.58	1.60	1.55
Cammarano *et al.* (1972b)	Chick liver	Swinging-bucket rotor	1.58-1.59	1.61	1.55
Cammarano *et al.* (1972b)	Rat liver	Swinging-bucket rotor	1.57-1.59	1.61	1.56
Sacchi *et al.* (1973)	Rat liver	Swinging-bucket rotor	–	1.61	1.55
Gambino *et al.* (1973)	Sea urchin eggs	Swinging-bucket rotor	1.57-1.59	–	–
Cammarano *et al.* (1973)	*Neurospora crassa*	Swinging-bucket rotor	1.58	1.59	1.55
Mazelis and Petermann (1973)	Yeast	Analytical ultracentrifuge	1.59	–	–
Zehavi-Willner and Danon (1973)	Rabbit reticulocytes	Swinging-bucket rotor	1.55	1.57	1.55
Hirsch *et al.* (1973)	Ehrlich-ascites-tumor cells	Swinging-bucket rotor	1.54	1.56	1.50
Kloppsteck and Schweiger (1973)	*Acetabularia*	Swinging-bucket rotor	1.51	–	–
Wettenhall *et al.* (1973)	Ehrlich-ascites-tumor cells	Swinging-bucket rotor	–	–	1.51-1.52

TABLE VI (continued)

Reference	Ribosome source	Centrifugation technique	Buoyant density (g/cm³)		
			Complete ribosomes	Large subunits	Small subunits
Leister and David (1974)	Ovaries of the frog *Xenopus laevis*	Analytical ultracentrifuge	–	1.63	1.55
Harris and Wool (unpublished observations cited by Sherton and Wool, 1974)	Rat liver	–	1.58	1.60	1.52
Yurina and Odintsova (1974)	*Chlorella pyrenoidosa*		1.56	–	–
McConkey (1974)	Mouse liver, HeLa cells	Swinging-bucket rotor	–	1.64	1.55
Sameshima and Izawa (1975)	Mouse ascites tumor cells	Swinging-bucket rotor	–	–	1.49
Smith and Henshaw (1975)	Ehrlich-ascites-tumor cells	Swinging-bucket rotor	–	–	1.52
Bag and Sarkar (1975)	Chick embryonic muscles	Swinging-bucket rotor	1.57	–	–
Bag and Sarkar (1976)	Chick embryonic muscles	Swinging-bucket rotor	–	1.58	1.52-1.53
Van Venrooij *et al.* (1976)	Ehrlich-ascites-tumor cells	Swinging-bucket rotor	–	1.59	1.54
Van Venrooij and Janssen (1976)	Ehrlich-ascites-tumor cells	Swinging-bucket rotor	–	1.59	1.54
Serdyuk and Grenader (1977)	Wheat (*Triticulum vulgare*)	Swinging-bucket rotor	1.57	–	–
Thompson *et al.* (1977)	Rat liver	Swinging-bucket rotor	1.57	1.61	1.51
Sacchi *et al.* (1977)	Rodents, rabbit reticulocytes ('Native')	Swinging-bucket rotor	–	1.62	1.56
	('Run-of')		–	1.64	1.56
	('Puromycin')		–	1.64	1.58
	('Highly purified')		–	1.65	–
Nieuwenhuysen *et al.* (1978b, 1979a), Nieuwenhuysen (1978b), Nieuwenhuysen and Clauwaert (1980)	*Artemia*	Analytical ultracentrifuge and swinging-bucket rotor	1.57	1.60	–
Longuet *et al.* (1979)	Mouse leukemia cells	Swinging-bucket rotor	–	1.59-1.60	1.56-1.57

TABLE VII

Values for the chemically determined RNA content (% by weight) and for the absorbance at 260 nm, $A_{260}^{1\,mg/ml}$, of ribosomal particles isolated from eukaryotes (derived from literature data).
From these values, the absorbance of the RNA incorporated in the ribosomal particles has been calculated; this can be compared with the expected value (see text)

Reference	Ribosome source	RNA content (% by weight) (chemical determinations)			$A_{260}^{1\,mg/ml}$			Calculated $A_{260}^{1\,mg/ml}$ of RNA incorporated in		
		Complete ribosomes	Large subunits	Small subunits	Complete ribosomes	Large subunits	Small subunits	Complete ribosomes	Large subunits	Small subunits
	(Prokaryotic)									
Tissières *et al.* (1959)	*Escherichia coli*	57, 61	62, 63, 64	61, 62	14.7, 15.7	18.2	16.2, 16.7	25.7, 25.9	28.7	26.4, 26.9
Kurland (1966)	*Escherichia coli*	67	–	–	–	–	–	–	–	–
Hess *et al.* (1967)	*Streptococcus pyogenes*	–	62-64	–	–	13.8	–	–	21.9	–
Miall and Walker (1967)	*Escherichia coli*	60-63	60-63	60-63	–	–	–	–	–	–
Spitnik-Elson and Atsmon (1968)	*Escherichia coli*	60	–	–	–	–	–	–	–	
Hill *et al.* (1969a)	*Escherichia coli*	–	–	–	14.5	14.5	14.8	–	–	–
Scafati *et al.* (1971)	*Escherichia coli*	59	–	–	13.2	–	–	22.4	–	–
Cammarano *et al.* (1972b)	*Escherichia coli*	62	–	–	–	–	–	–	–	–
Sacchi *et al.* (1973)	*Escherichia coli*	62	–	–	–	–	–	–	–	–
Igarashi *et al.* (1973)	*Escherichia coli*	68	65	64	14.8	15.3	14.0	21.7	23.5	21.9
McConkey (1974)	*Escherichia coli*	67	69	66	–	–	–	–	–	–
Giri *et al.* (1976)	*Escherichia coli* (Eukaryotic)	–	65	–	–	–	–	–	–	–
Chao and Schachman (1956)	Yeast	40-44	–	–	–	–	–	–	–	–
Ts'o *et al.* (1958)	Pea seedlings	40	–	–	–	–	–	–	–	–
Yin (1961), cited in Cotter *et al.* (1967)	Yeast	43	–	–	11.3	–	–	26.3	–	–

TABLE VII (continued)

Reference	Ribosome source	RNA content (% by weight) (chemical determinations)			$A_{260}^{1\ mg/ml}$			Calculated $A_{260}^{1\ mg/ml}$ of RNA incorporated in		
		Complete ribosomes	Large subunits	Small subunits	Complete ribosomes	Large subunits	Small subunits	Complete ribosomes	Large subunits	Small subunits
Ts'o and Vinograd (1961)	Rabbit reticulocytes	50	–	–	11.2, 11.3	–	–	22.5	–	–
Hamilton *et al.* (1962)	Rat liver	45	–	–	12.4	–	–	27.6	–	–
Lederberg and Mitchison (1962)	Yeast	41	–	–	11.1	–	–	27.1	–	–
Pogo *et al.* (1962)	Calf thymus nucleus	60	–	–	–	–	–	–	–	–
Madison and Dickman (1963)	Beef pancreas	–	–	–	8.9	–	–	–	–	–
Storck (1963)	*Neurospora crassa*	47	–	–	–	–	–	–	–	–
Moyer and Storck (1964)	*Aspergillus niger*	53	–	–	–	–	–	–	–	–
Mathias *et al.* (1964)	Rabbit reticulocytes	50	–	–	–	–	–	–	–	–
Clark *et al.* (1964)	Plant leaves	42	–	–	–	–	–	–	–	–
Tashiro and Siekevitz (1965)	Guinea pig liver	56	–	–	13.5	–	–	24.1	–	–
Breillatt and Dickman (1966)	Dog pancreas (ribosomes and polysomes)	45	–	–	11.2	–	–	24.7	–	–
Wolfe and Kay (1967)	Wheat embryo	55	–	–	–	–	–	–	–	–
Sussman (1967)	Slime mold	51, 53	–	–	–	–	–	–	–	–
Reisner *et al.* (1968)	*Paramecium aurelia*	57	–	–	–	–	–	–	–	–

TABLE VII (continued)

Reference	Ribosome source	RNA content (% by weight) (chemical determinations)			$A_{260}^{1\,mg/ml}$			Calculated $A_{260}^{1\,mg/ml}$ of RNA incorporated in		
		Complete ribosomes	Large subunits	Small subunits	Complete ribosomes	Large subunits	Small subunits	Complete ribosomes	Large subunits	Small subunits
Ceccarini and Maggio (1968)	Slime mold	52	–	–	–	–	–	–	–	–
Bielka *et al.* (1968)	Rat liver	51	–	–	–	–	–	–	–	–
Beeley *et al.* (1968)	Dog pancreas	60 (56, 64)	–	–	13.2	–	–	21.9	–	–
Odintsova and Yurina (1969)	Pea and bean seedlings	43-47	–	–	–	–	–	–	–	–
Rawson and Stutz (1969	Unicellular flagellate *Euglena gracilis*	44	32	64	–	–	–	–	–	–
Philips and Hersch (1970)	*Pisum sativum*	46	–	–	–	–	–	–	–	–
Reboud *et al.* (1970, 1971)	Rat liver	48	–	–	–	–	–	–	–	–
Hamilton *et al.* (1971)	Rat liver	–	55	45	–	–	–	–	–	–
King *et al.* (1971)	Rabbit reticulocytes	–	61	52	–	–	–	–	–	–
Cammarano *et al.* (1972b)	Plant	50	–	–	–	–	–	–	–	–
Cammarano *et al.* (1972b)	Liver	49	–	–	–	–	–	–	–	–
Mazelis and Petermann (1973)	Yeast	53	–	–	–	–	–	–	–	–

TABLE VII (continued)

Reference	Ribosome source	RNA content (% by weight) (chemical determinations)			$A_{260}^{1\,mg/ml}$			Calculated $A_{260}^{1\,mg/ml}$ of RNA incorporated in		
		Complete ribosomes	Large subunits	Small subunits	Complete ribosomes	Large subunits	Small subunits	Complete ribosomes	Large subunits	Small subunits
Hirsch *et al.* (1973)	Ehrlich-ascites-tumor cells	51	59	48	–	–	–	–	–	–
McConkey (1974)	Mouse liver, HeLa cells	–	67	56	–	–	–	–	–	–
Sherton and Wool (1974)	Rat liver	50	59	44	9.6	11.3	9.7	19.1	19.2	21.9
Leister and David (1974)	Ovaries of the frog *Xenopus laevis*	–	58	50	–	–	–	–	–	–
Vanduffel *et al.* (1975)	Human placenta	–	64	57	–	–	–	–	–	–
Rao *et al.* (1975)	Rat liver	49	–	–	10.2	–	–	20.8	–	–
Roman Palacios and Sánchez de Jiménez (1975)	Red cells of chick embryos	46	–	–	–	–	–	–	–	–
Unsworth and Kaulenas (1975)	Sea urchin (unfertilized egg) (blastula)	23	–	–	–	–	–	–	–	–
		50	–	–	–	–	–	–	–	–
Sacchi *et al.* (1977)	Rodents	–	55, 61, 63	45, 47	–	–	–	–	–	–
Sacchi *et al.* (1977)	Rabbit	–	57, 59, 64	46, 47	–	–	–	–	–	–
Van Agthoven *et al.* (1978)	*Artemia*	50, 52	–	–	–	–	–	–	–	–
Nieuwenhuysen and Clauwaert (1980)	*Artemia*	49	58	53	12.1	14.3	12.9	24.5	24.5	24.5

secondary structure is almost the same : Araco *et al.* (1975) have concluded from hypochromicity measurements that the absorbance of rRNA embedded in ribosomes is only 5% higher than that of free rRNA ; Elson *et al.* (1979) have given values ε_{260} (P) for *E. coli* small ribosomal subunits, which are only 1% higher than for their free RNA. So from all these data we expect an $A_{260}^{1\,mg/ml}$ of RNA in ribosomes between 22.9 ± 0.5 and 25.3 ± 0.4. Miall and Walker (1967) have measured values for the complete ribosomes from *E. coli* and for their large and small subunits of respectively 7 810, 7 650 and 7 710 ; Elson *et al.* (1979) have obtained ε_{260} (P) $= 7\,634 \pm 23$ for *E. coli* ribosomal subunits ; assuming again an equimolar base composition, we can calculate from these data values between 23.8 and 24.3 for $A_{260}^{1\,mg/ml}$ of RNA in *E. coli* ribosomes. In conclusion, different values for $A_{260}^{1\,mg/ml}$ of rRNA, incorporated in ribosomal particles, have been derived above from the literature, which range from 22.9 ± 0.5 to 25.3 ± 0.4. These can now be compared with the values in Table VII which have been calculated from the published RNA and protein content and $A_{260}^{1\,mg/ml}$ of different ribosomal particles and from our own data. The results for the complete ribosomes isolated from guinea pig liver (Tashiro and Siekevitz, 1965), for the mixture of ribosomes and polysomes from dog pancreas (Breillat and Dickman, 1966), and for the ribosomal particles from *Artemia* seem to be the most consistent. However, analytical centrifugation of the ribosomes from guinea pig liver showed faster and slower sedimenting particles (Tashiro and Siekevitz, 1965), and the polysomes from dog pancreas were not highly purified, which may explain partly the low value for their A_{260}/A_{280} (= 1.80) and for their chemically determined RNA content. The low values for $A_{260}^{1\,mg/ml}$ of rRNA calculated from data published for complete rat liver ribosomes (Sherton and Wool, 1974 ; Rao *et al.*, 1975) indicate that the RNA and protein content of 1 A_{260} unit of these ribosomes has been overestimated. The same criticism seems justified when we consider the values for $A_{260}^{1\,mg/ml}$ of rat liver ribosomal subunits (Sherton and Wool, 1974), which are the only quantitative published data, as far as we know, on the absorbance of eukaryotic ribosomal subparticles.

Partial specific volumes

The value that we have determined experimentally for the partial specific volume $\bar{v}^o$ of the complete ribosomes from *Artemia* can only be compared with a few published values for ribosomes from other eukaryotes, listed in Table VIII. Not all have been determined accurately with homogeneous ribosome solutions ; contamination with proteins may explain why the early values are so high.

Sedimentation coefficients

Published values for the standard sedimentation coefficients at infinite dilution, $s^o_{20,w}$, of ribosomal particles isolated from eukaryotes have been listed in Table IX. They are spread around the values we have measured for the corresponding particles from *Artemia*.

Glutaraldehyde fixation of the complete ribosomes from *E. coli* would change their $s^o_{20,w}$ from about 70 to 73 S (Subramian, 1972). Formaldehyde fixation however does not change the sedimentation coefficient of ribosomal particles isolated from eukaryotes (Spirin *et al.*, 1965 ; Perry and Kelley, 1966 ; Infante and Nemer, 1968 ; Chi and Suyama, 1970 ; Haga *et al.*, 1970) ; this agrees with our findings for the complete *Artemia* ribosomes.

TABLE VIII

Published values for the partial specific volume $\bar{v}^o$ (obtained by density and concentration measurements) of ribosomal particles, isolated from eukaryotes

Reference	Ribosome source	Methods of density measurements	$\bar{v}^o$ (cm^3/g)		
			Complete ribosomes	Large subunits	Small subunits
Chao and Schachman (1956)	Yeast	5 ml pycnometer	0.63-0.68	–	–
Hamilton *et al.* (1962)	Rat liver	3 ml pycnometer	0.66	–	–
Lederberg and Mitchison (1962)	Yeast	50 ml pycnometer	0.65	–	–
Wolfe and Kay (1967)	Wheat embryo	10 ml pycnometer	0.60	–	–
Mazelis and Petermann (1973)	Yeast	Pycnometer	0.63	–	–
Nieuwenhuysen and Clauwaert (1980)	*Artemia*	Digital precision density meter	0.62	0.61	0.62

TABLE IX

Published values for the standard sedimentation coefficient at infinite dilution, $s^o_{20,w}$, (determined using an analytical ultracentrifuge) of cytoplasmic ribosomal particles isolated from eukaryotes

Reference	Source of ribosomes	$s^o_{20,w}$		
		Complete ribosome	Large subunit	Small subunit
Chao and Schachman (1956)	Yeast	80	–	–
Chao (1957)	Yeast	80	60	40
Ts'o *et al.* (1958)	Pea seedlings	80	60	40
Hall and Doty (1959)	Calf liver	81	–	–
Takanami (1960)	Rat liver	79	58	39
Ts'o and Vinograd (1961)	Rabbit reticulocytes	78	58	40
Sherman and Petermann (1961)	Calf liver	81	–	–
Pogo *et al.* (1962)	Calf thymus nucleus	78	–	–
Lederberg and Mitchison (1962)	Yeast	83	60	38
Storck (1963)	*Neurospora crassa*	81	–	–
Hess and Lagg (1963)	Calf thymus lymphocytes	74	–	–
Mayer and Storck (1964)	*Aspergillus niger*	80	–	–
Taylor and Storck (1964)	Yeasts and molds	79-85	–	–
Clark *et al.* (1964)	Plant leaves	83	–	–
Mathias *et al.* (1964)	Rabbit reticulocytes	80	–	–
Dass and Bayley (1965)	Rat liver	83	–	–
Tashiro and Siekevitz (1965)	Guinea pig liver	77	–	–
Sissakian *et al.* (1965)	Pea seedlings	76	–	–
Pfuderer *et al.* (1965)	Rat liver	83	–	–
Boardman *et al.* (1966)	Tobacco leaves	82	–	–

TABLE IX (continued)

Reference	Source of ribosomes	$S^{o}_{20,w}$		
		Complete ribosome	Large subunit	Small subunit
Petermann and Pavlovec (1966)	Rat tumor	79	–	–
Sager and Hamilton (1967)	*Chlamydomonas*	83	–	–
Petermann and Pavlovec (1967)	Rat liver	83	–	–
Wolfe and Kay (1967)	Wheat embryo	80	–	–
Infante and Nemer (1968)	Sea urchin embryos	74	56	36, 37
Beeley *et al.* (1968)	Dog pancreas	80	–	–
Reisner *et al.* (1968)	*Paramecium aurelia*	85	–	–
Bielka *et al.* (1968)	Rat liver	80	–	–
Scott *et al.* (1970)	Unicellular flagellate *Euglena gracilis*	88	67	–
Fais *et al.* (1970)	Krebs ascites carcinoma in mice	77	60	40
Iwabuchi *et al.* (1970)	Slime mould *Dictyostelium discoidum*	83	61	42
Philips and Hersch (1970)	*Pisum sativum*	80	58	41
Haga *et al.* (1970)	Rat liver	82	59	–
Petermann and Pavlovec (1971)	Rat liver	81	59	40
Hamilton *et al.* (1971)	Rat liver	–	59	41
Bont *et al.* (1971)	Rat liver	77	–	–
Vournakis and Rich (1971)	Chick embryo (free)	78	–	–
Vournakis and Rich (1971)	Chick embryo (polysomal, attached to a fragment of mRNA)	85	–	–
Delihas *et al.* (1972)	*Euglena gracilis*	85	62	46
Petermann *et al.* (1972)	Rat liver	82	60	40
Mazelis and Petermann (1973)	Yeast	82	61	38
Meyer *et al.* (1974)	Rat liver	77	57	–
Damaschun *et al.* (1974)	Rat liver	77	–	–
Leister and David (1974)	Ovaries of the frog *Xenopus laevis*	–	62	37
Yurina and Odintsova (1974)	Plants	80	–	–
Cox *et al.* (1976b)	Rabbit reticulocytes	–	60, 58	–
Shimizu *et al.* (1977)	*Geotrichum candidum*	–	59	38
Nieuwenhuysen and Clauwaert (1978b, 1980)	*Artemia*	81	58	–

It has been reported that the polysomal ribosomes from chick-embryo muscle tissue sediment 9% more rapidly than the free, single ribosomes (Vournakis and Rich, 1971). Such a difference would be well beyond our experimental error, but we did not find it for *Artemia* ribosomes. Thus we believe that the more compact form of ribosomes attached to messenger RNA (Vournakis and Rich, 1971) should not be generalized. It should be noted that the chick-embryo ribosomes had a high buoyant density in CsCl (1.60-1.61 g/cm^3), which probably reflects the high salt content of their solvent (250 mM KCl) and the release of some proteins ; their molecular weight, however, was calculated to be the largest that has been reported for

ribosomes as far as we know (5.2×10^6), which is more than two times that of *E. coli* ribosomes (Koppel, 1974 ; Van Holde and Hill, 1974) ; furthermore, we have calculated that their solvation would be twice as large as for *Artemia* ribosomes. It may be relevant here to mention that the attachment of the ribosomes to mRNA-fragments influences their pressure dependence, as revealed by light scattering under high pressure (Nieuwenhuysen *et al.*, 1978b ; Nieuwenhuysen, 1978b).

Diffusion coefficients ; hydrodynamic size

The value that we have determined for the standard diffusion coefficient of the complete ribosomes from *Artemia* is considerably higher than those reported without experimental details for ribosomes isolated from chick-embryo muscle tissue ($1.04\text{-}1.14 \times 10^{-7}$ cm^2/sec) (Vournakis and Rich, 1971) ; however our comments on that study in the previous paragraph should be borne in mind.

Our values for the diffusion coefficient and the hydrodynamic size of the complete *Artemia* ribosomes can be considered in the light of results from X-ray scattering experiments, which have yielded 370 and 270 Å for the extreme dimensions of rat-liver ribosomes (Damaschun *et al.*, 1974). We have calculated the value of 15.2×10^6 Å^3 for the maximal rat-liver ribosome volume, *i.e.* the volume of a prolate ellipsoid of revolution with axes of 370 and 270 Å (and thus with an elongation $p = 1.32$). The prolate ellipsoid of revolution with the same elongation, which is consistent with our value for the diffusion coefficient of *Artemia* ribosomes, has a volume V_h which can be calculated from $V_h = V_{h,max} \cdot t^3(p)$, where $t^3(p) \leqslant 1$ is a tabulated function of the elongation p (Sadron, 1953) ; this yields $V_h = (14.4 \pm 0.6) \times 10^6$ Å^3. A closer agreement between the two volumes could not be expected.

Molecular weights

Our values for the molecular weight of ribosomal particles from *Artemia* can be compared with published values for cytoplasmic ribosomal particles from other eukaryotes in Table X. These have all been obtained with impure solutions (containing contaminations or aggregates), with physically altered particles, or with older methods which are not free from criticism (*cf.* the review by Van Holde and Hill, 1974.)

Comparison with ribosomal particles from *Escherichia coli*

Our results for the general physical properties of the ribosomal particles from the eukaryote *Artemia* are summarized in Table XI, together with literature data for the corresponding particles from the prokaryote *E. coli*. A comparison of both series of data shows primarily how much the size of the ribosome has increased during evolution. A comparison of *Artemia* and *E. coli* ribosomes by electron microscopy has shown that they have substantial similarity in overall shape and in mutual orientation of the subunits on the ribosomes (Boublik and Hellmann, 1978).

The ratio of the hydrodynamic radius of a macromolecule to its dry-particle radius, R_h/R_d, is larger than unity, due to deviations of the macromolecular shape from a sphere, and to the solvation of the macromolecule in solution. More detailed we have (Koppel, 1974) :

$$R_h/R_d = (V_{h,max}/V_d)^{1/3} = (V_{h,max}/V_h)^{1/3} \cdot (V_h/V_d)^{1/3}$$
$$= (f/f_o) \cdot (1\text{-}F)^{-1/3}$$

TABLE X

Published values for the molecular weight of cytoplasmic, ribosomal particles isolated from eukaryotes

Reference	Ribosome source	Method	Molecular weight (10^6)		
			Complete ribosome	Large subunit	Small subunit
Ts'o *et al.* (1958)	Pea seedlings	Sedimentation coefficient and intrinsic viscosity	4.0-4.5	–	–
Dibble and Dintzis (1960)	Rabbit reticulocytes	X-ray scattering intensity	4.0	–	–
Lederberg and Mitchison (1962)	Yeast	Sedimentation coefficient and intrinsic viscosity	3.8	–	–
Tashiro and Yphantis (1965)	Guinea pig liver	Sedimentation equilibrium	5.0	–	–
Reisner *et al.* (1968)	*Paramecium*	Sedimentation and diffusion coefficient, and partial specific volume	3.3	–	–
Hamilton *et al.* (1971)	Rat liver	Sedimentation equilibrium	–	3.0	1.5
Vournakis and Rich (1971)	Chick embryo	Sedimentation and diffusion coefficient	5.2	–	–
Cammarano *et al.* (1972b)	Plant	Calculated by combining estimated molecular weights of ribosomal	3.9	2.4	1.5
	Sea urchin	RNA with RNA/protein ratio	4.1	2.6	1.5
	Chick liver	estimated from buoyant density	4.3	2.8	1.5
	Rat liver	in CsCl gradient.	4.6	3.0	1.5
Cammarano *et al.* (1973)	*Neurospora crassa*	Idem	4.0	2.5	1.5
Mazelis and Petermann (1973)	Yeast	Sedimentation equilibrium	3.6 (3.4)	2.6	1.0
Leister and David (1974)	Ovaries of the frog *Xenopus laevis*	Calculated by combining published estimated molecular weights of ribosomal RNA with measured ratio of RNA to protein content	–	2.6	1.4
Sacchi *et al.* (1978)	Rodents,	Idem	–	2.7	1.3, 1.4
Nieuwenhuysen and Clauwaert (1980)	*Artemia*	Sedimentation and diffusion coefficient, and partial specific volume	3.7	2.3	(1.4)

TABLE XI

Comparison between the physical properties of complete ribosomes and their large subunits, isolated from the eukaryote *Artemia* and the prokaryote *Escherichia coli*

	Artemia		*Escherichia coli*		Reference	Method
	Complete ribosomes	Large subunits	Complete ribosomes	Large subunits		
$\bar{v}^o$ (cm^3/g)	0.62	0.61	0.64	0.64	Tissières *et al.* (1959)	Pycnometry
			0.60	0.59	Hill *et al.* (1969a, 1970)	Pycnometry
			0.60	0.59	Igarashi *et al.* (1973)	Pycnometry
$S^o_{20,w}$ (S)	81	58	69	50	Tissières *et al.* (1959)	Analytical ultracentrifugation
			69	–	Taylor and Storck (1964)	Analytical ultracentrifugation
			70	50	Hill *et al.* (1969b, 1970)	Analytical ultracentrifugation
			–	49-50	Imamura *et al.* (1976)	Analytical ultracentrifugation
			67-69	49-50	Allen and Wong (1979)	Analytical ultracentrifugation
$D^o_{20,w}$ (10^{-7} cm^2/sec)	1.41	1.58	1.83	1.91	Tissières *et al.* (1959)	Boundary spreading
			–	1.87	Serdyuk *et al.* (1970)	
			1.7-1.8	2.1	Hocker *et al.* (1973)	Intensity fluctuation spectroscopy
			1.71	1.90	Koppel (1974)	Intensity fluctuation spectroscopy
			1.7	1.9	Gabler *et al.* (1974)	Intensity fluctuation spectroscopy
			–	1.91	Han *et al.* (1979)	Quasi-elastic light scattering
M (10^6)	3.7	2.3	2.6	1.8	Tissières *et al.* (1959)	From their value for s, D and $\bar{v}$.
			2.7	1.6	Hill *et al.* (1969a, 1970)	Equilibrium centrifugation
			2.9	1.7	Scafati *et al.* (1971)	Light scattering
			–	1.5	Igarashi *et al.* (1973)	Light scattering
			2.5	1.55	Koppel (1974)	From his value for D and the value for s of Hill *et al.* (1969b) and for $\bar{v}$ of Hill *et al.* (1969a)
R_h (Å)	152	136	126	113	Koppel (1974)	From his value for D
$V_{h,max}$ (10^6 $Å^3$)	14.7	10.4	8.4	6.0		From the value for D of Koppel (1974)
V_d (10^6 $Å^3$)	3.8	2.3	2.5	1.5		
R_d (Å)	97	82	84	71		
R_h/R_d	1.57	1.66	1.50	1.59		
F_{max}	0.74	0.78	0.70	0.75	Koppel (1974)	
δ_{max} (cm^3/g)	1.8	2.1	1.4	1.8	Koppel (1974)	

Here $(V_{h,max}/V_h)^{1/3} = (f/f_o) > 1$ takes into account the deviation of the shape from a sphere ; it is the ratio of the real friction of the macromolecule, $f = (kT/D) = 6\ \pi\eta\ R_h$, to the lower friction f_o of a sphere with a volume equal to the hydrodynamic volume of the macromolecule, V_h. The factor $(V_h/V_d)^{1/3} > 1$ takes into account the hydrodynamic solvation of the macromolecule. The values determined for the ratios R_h/R_d of the large ribosome subunits from *E. coli* and *Artemia* are both slightly larger than the ratios for the complete ribosomes (see Table XI). So the solvation and/or the ratio f/f_o is slightly larger for the subunits than for the complete ribosomes. Electron microscopy after negative staining has revealed protuberances on the large ribosome subunits from *E. coli* and *Artemia* (Boublik and Hellmann, 1978) ; these will contribute to f/f_o in solution.

From the published values for the molecular weight of *E. coli* ribosomes and of their large subunit (see Table XI) we can derive the ratio

$$(M_{\text{large subunit}}/M_{\text{complete ribosome}}) = (1.55 \times 10^6/2.5 \times 10^6) = 0.62.$$

Our experimental data for the corresponding *Artemia* ribosomal particles yield for this ratio also a value of 0.62 ± 0.04.

Note

Experiments described in this paper were performed with subunits dissociated and separated in 20 mM Hepes/KOH buffer (pH 7.5), containing 1 mM DTT, 700 mM KCl, and 11 mM Mg acetate, because these ionic concentrations are used in the case of *Artemia* ribosomes in other groups (*e.g.*, Zasloff and Ochoa, 1971, 1974 ; Möller *et al.*, 1975). However, our measurements of their protein content and of their density increment have indicated the loss of proteins during the isolation. After the completion of the manuscript more experimental results have yielded evidence that the preparation procedure is indeed not suited to obtain intact subunits.

The absorbance profile after zonal centrifugation revealed that the peak of small subunits was sometimes too broad or even split-up. The ratio A_{260}/A_{280} of the obtained solutions of subunits is too high. The small subunits have a high buoyant density in CsCl, and a low sedimentation and diffusion coefficient. When the KCl concentration in the isolation is lowered to 400 mM, the absorbance profile after zonal centrifugation showed also complete dissociation, with a narrow zone of small subunits closer to that of the large subunits than in 700 mM KCl. Thus more intact subunits have been obtained and characterized.

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Proton magnetic resonance studies on the physical state of water in *Artemia* cysts

Patricia K. Seitz[1], *Carlton F. Hazlewood*[2], *and James S. Clegg*[3]

[1] *Department of Biochemistry, University of Texas Medical Branch Hendrix Building, Galveston, Texas 77550, USA*

[2] *Physiology Department, Baylor College of Medicine Houston, Texas 77030, USA*

[3] *Laboratory for Quantitative Biology, University of Miami Coral Gables, Florida 33124, USA*

Abstract

The cysts of *Artemia* provide an excellent system for studying the structure of water in a viable organism. Pulsed nuclear magnetic resonance (NMR) spectroscopy has been used to study the motional freedom of water as a function of hydration level in the cysts. The spin-lattice, T_1, and spin-spin, T_2, relaxation times of water protons were measured over a wide range of hydrations. T_1 and T_2 are not simple monotonic functions of water content : there are minima in the relaxation times at approximately 20 H_2O/100 g dried cysts. This is just below the hydration level where significant metabolic reactions begin to occur. The self-diffusion coefficient (D) of water in the cysts was also measured over a wide range of hydrations. Values of D for even the most hydrated cysts are almost an order of magnitude lower than that of pure water. Data were examined with respect to the structure of intracellular water. There is the suggestion of a large fraction of essentially immobile hydration water and the possibility of a reorganization of water structure at or near the onset of metabolism.

Introduction

While *Artemia* is an interesting and important organism in its own right, the dormant embryonic cysts provide a unique and useful tool for studying fundamental biochemical and biophysical phenomena (Clegg, 1967 ; Crowe and Clegg, 1973). The ability of the cysts to undergo repeated cycles of hydration and dehydration and to essentially arrest their metabolism while dehydrated (Clegg, 1974) make them ideal for studying the initiation of metabolism and other hydration dependent processes. One aspect that we have been concerned with is the role of water and water-macromolecule interactions in metabolism (Clegg, 1974, 1976, 1978 ; Seitz *et al.*, 1977 ; Seitz and Hazlewood, 1978).

The data presented here consist of pulsed nuclear magnetic resonance (NMR) relaxation times and diffusion coefficients of water as functions of hydration in *Artemia* cysts. These studies are part of an ongoing effort to describe changes in the physical properties of water inside the cysts. Other parameters that have been, or are being, measured include sorption

isotherms and derived thermodynamic parameters (Clegg, 1978), heat capacities by differential scanning calorimetry and dieletric constants over a wide frequency range.

The utility of pulsed NMR lies in the fact that it is a non-destructive technique, able to detect resonance absorption by only a few species of protons, specifically in this case, the water hydrogen protons (Abragam, 1961 ; Farrar and Becker, 1971). Brief pulses of radiofrequency energy to a sample placed in the proper magnetic field result in a nuclear precession (or rotation) about the direction of the field which relaxes with characteristic time constants describing the different directional components of decay (Bloch, 1946 ; Bloch *et al.*, 1946ab). These NMR relaxation times are related to the molecular correlation time (Bloembergen *et al.*, 1948) and thus reflect the motional freedom of the water molecules. The spin-lattice relaxation time (T_1) varies with limitations on mobility due to constraints by the surrounding environment. The spin-spin relaxation time (T_2) reflects spin transfer interactions with other nuclei. Long relaxation times reflect greater mobility. For instance, liquid water has a T_1 of approximately 3 sec and a T_2 of approximately 1.7 sec. Relaxation times of solids can be several orders of magnitude less.

NMR techniques can also be used to determine the water self diffusion coefficient, that is, the rate at which water molecules move through the sample, by varying the field gradient. Movement of the water protons through different parts of the magnetic field results in a decrease of one component of the sample magnetization and the rate of this decrease can be quantified (Torrey, 1956).

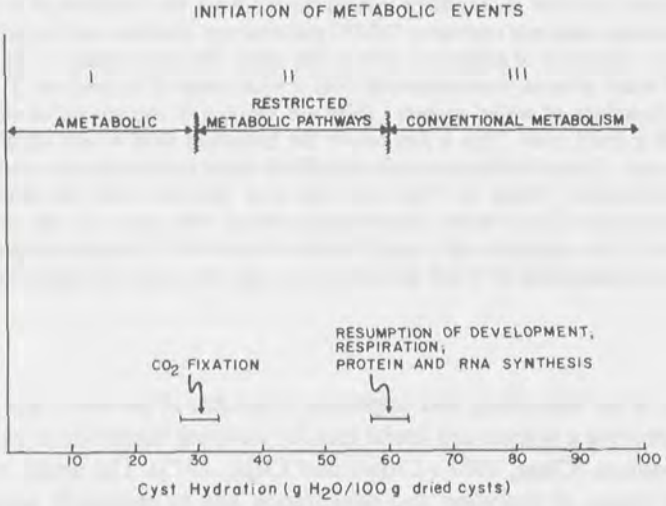

FIG. 1. Metabolic domains. Hydration is given in g H_2O/100 g dry cysts (g/100 g).

The detectable metabolic activity of the cysts increases with hydration as diagrammed in Fig. 1, after Clegg (1976). Levels of metabolic activity are summarized as a function of increasing hydration. Hydration is measured in g H_2O/100 g dry solids, abbreviated g/100 g for brevity. Note that there are two hydration levels of particular metabolic importance. Below 30 g/100 g, the cysts are essentially ametabolic. The only biochemical activity detected so far

is a gradual decrease in ATP concentration in the latter part of this region (Clegg and Cavagnaro, 1976). At about 30 g/100 g, however, short sequences of reactions begin to occur. This is not full metabolism and the cysts do not develop into larvae, but significant parts of known metabolic pathways can be detected. Above 60 g/100 g, conventional metabolism does resume (though it should be noted that this is less than half of the maximal hydration).

Materials and methods

In order to obtain cysts with hydration levels over the entire range, it was necessary to use two different methods for hydrating them. For the lower hydrations, (2 g/100 g to 30 g/100 g) cysts were allowed to sorb water from the vapor phase in sealed hydration chambers (Clegg, 1974). Different relative humidities were achieved by placing saturated solutions of various salts in the bottoms of the chambers (Winston and Bates, 1960). For higher hydrations (25 g/100 g to 140 g/100 g), cysts were immersed overnight at 0 °C in solutions of different sodium chloride concentrations (Clegg, 1974). Since the embryonic cuticle is impermeable to the solute, the osmolality of the solution determines the amount of water taken up.

T_1's were measured using $180°$-τ-$90°$ pulse sequences and T_2's by the conventional Carr-Purcell method (1954). The diffusion coefficients were determined by measuring the decrease in echo height after a $90°$-τ-$180°$ pulse sequence as a function of increasing field gradient (Torrey, 1956).

Results and discussion

Relaxation times

Fig. 2 shows the variation of T_1 as a function of hydration. It is clear that this is not a monotonic function. As the hydration increases from zero to 20 g/100 g, T_1 decreases. At that point, the T_1 values begin a smooth, steady increase and above 90 g/100 g, the T_1 is higher than that of dry cysts. The hydration where the T_1 minimum occurs slightly precedes the hydration level for the onset of partial metabolism (see Fig. 1) and thus occurs when the cysts are still essentially dormant.

In Fig. 3, the variation of T_2 with respect to hydration is shown. The pattern is similar, though not identical, to that of the T_2's. The relaxation time is relatively high at low hydrations and there is a minimum slightly preceding the point where metabolic activity begins. Above 20 g/100 g, there is a gradual increase in the T_1's. However, the early values drop much more abruptly than the T_1's do and the values at the highest hydrations never reach those of the lowest hydrations.

Three aspects of these data are important. First, T_1 and T_2 have very low values when compared to those of pure water. Second, there exist minima in both relaxation times, as functions of hydration. Third, the occurrence of these minima just precedes the onset of partial metabolism.

In simple physical systems, low values for T_1 and T_2 would indicate a decrease in the motional freedom of water molecule and perhaps an increase in structure (Bloembergen *et al.*, 1948). This is not necessarily the case in more complex biological systems, since the relaxation

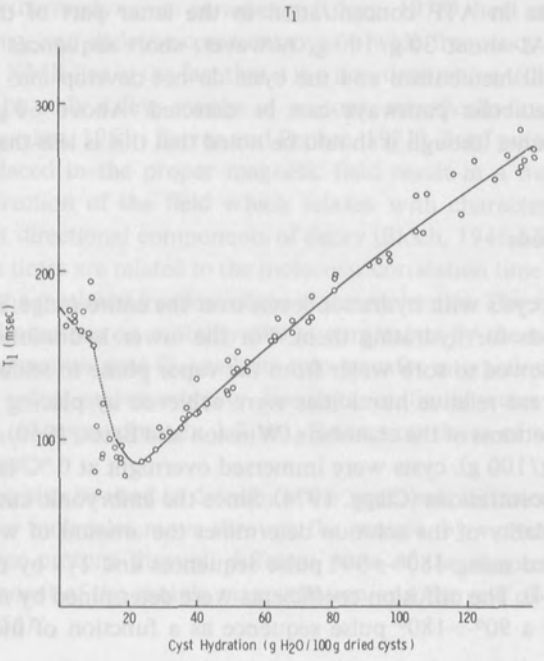

FIG. 2. Spin-lattice relaxation time, T_1 (msec), versus hydration (g/100 g).

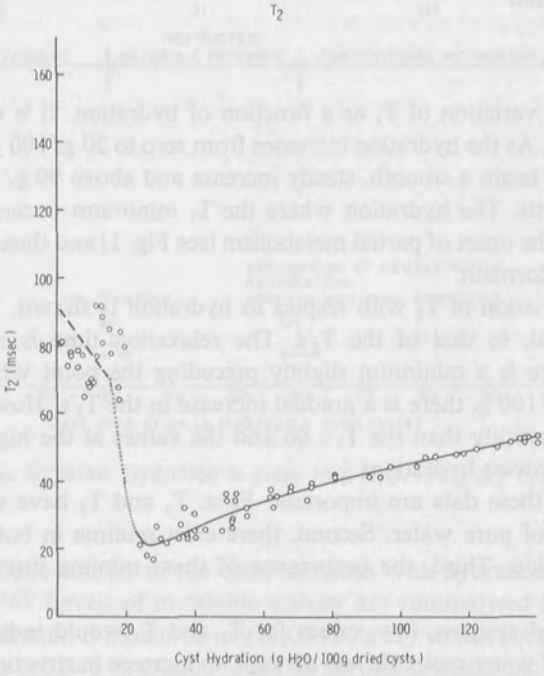

FIG. 3. Spin-spin relaxation time, T_2 (msec), versus hydration (g/100 g).

times are more heavily weighted by populations of faster relaxing protons (Zimmerman and Brittin, 1957). A popular two fraction model, first proposed by Bratton *et al.* (1965) and Clifford *et al.* (1968), pictures a small, tightly bound layer of fast relaxing macromolecular hydration water in rapid exchange with the vast majority of cell water which has the slower relaxing properties of bulk water. The simplest verson of this model predicts a smooth increase in relaxation times with increasing hydration, which, as Fig. 2 and 3 demonstrate, is certainly not the case.

Another prediction of the two fraction model is that a plot of 1/relaxation time *versus* 1/hydration should be linear and should extrapolate to 1/relaxation time of pure water, that is, the case of infinite dilution. Because of the non-monotonic nature of the T_1 and T_2 vs. H curves (Fig. 2 and 3), it is clear that this cannot result. But even if those points above 30 g/100 g are plotted inversely (as in Fig. 4), the points deviate from linearity. The intercept in Fig. 4 represents $1/T_1$ of water. This kind of analysis assumes that after a certain minimum amount of water is added, the hydration water is constant and that additional water goes into the bulk fraction. The nonlinearity of the results imply that this assumption must be modified.

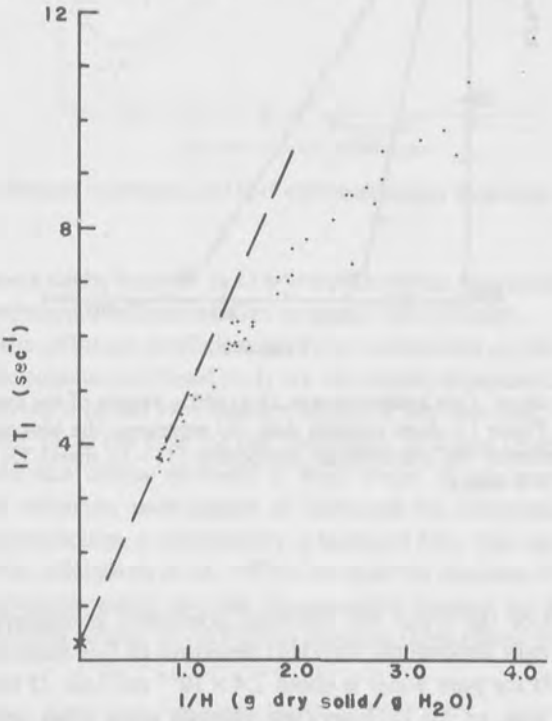

FIG. 4. Inverse plot of T_1 values for hydrations greater than 30 g/100 g. $1/T_1$ (sec^{-1}) *versus* 1/H (g dry solid/g H_2O). (X) represents $1/T_1$ for pure water.

Yet another prediction of this model is that the individual relaxation decays (from which T_1 and T_2 are determined) are single component (Zimmerman and Brittin, 1957) and would appear as straight lines in logarithmic plots. This would indicate that the exchange between

fractions is fast when compared to the time required for the NMR measurements, which was between 100 and 1000 msec. However, this was not the case for any of the observed relaxation decays. An example of a three component decay curve is given in Fig. 5. Relaxation times for the individual components were determined by curve stripping. The values for T_1 and T_2 reported in this paper are all initial (that is, weight averaged) relaxation rates.

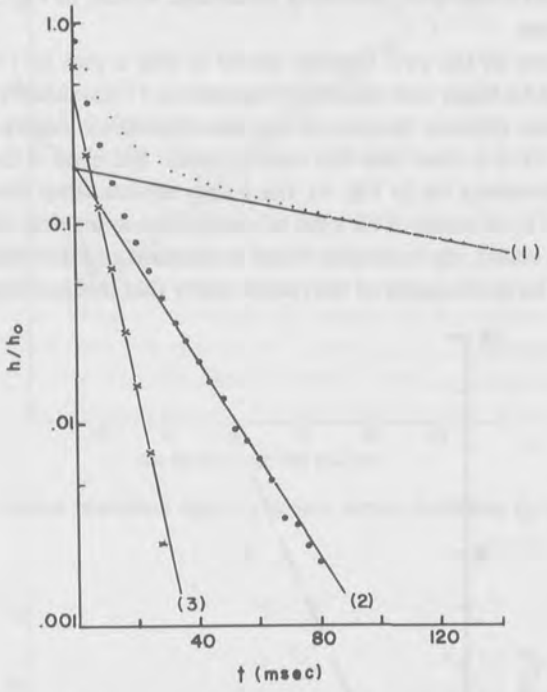

FIG. 5. T_2 relaxation decay. Cyst hydration was 28 g/100 g. Height of the magnetization vector (h/h_0) *versus* time (msec). Points (·) show original data. (1) represents the longest relaxing component ($T_2 = 143$ msec). (2) represents the intermediate component ($T_2 = 18$ msec). (3) represents the fastest relaxing component ($T_2 = 6$ msec).

DIFFUSION COEFFICIENT

In Fig. 6, variation of the water self diffusion coefficient is displayed as a function of hydration. The error bars indicate the standard deviation of five measurements on a single sample. The value of D for pure water is about 2.4×10^{-5} cm²/sec. D for the most hydrated cysts is about 1/8 of that, or 3×10^{-6} cm²/sec. Though some other cell types (such as red blood cells and liver cells) have similar values, the diffusion coefficient for most biological systems is reduced to only about one half that of pure water (Abetsedarskaya *et al.*, 1968 ; Hansen, 1971 ; Finch *et al.*, 1971 ; Cooper *et al.*, 1974). D for the cysts decreases from its maximum value to a minimum of about 3×10^{-7} cm²/sec. Experimental difficulties associated with measuring such low values (Farrar and Becker, 1971 ; Burnett and Harmon, 1972) suggest that this is an upper limit. It is only 1/100 the self diffusion coefficient for pure water and is lower that that reported for any viable system.

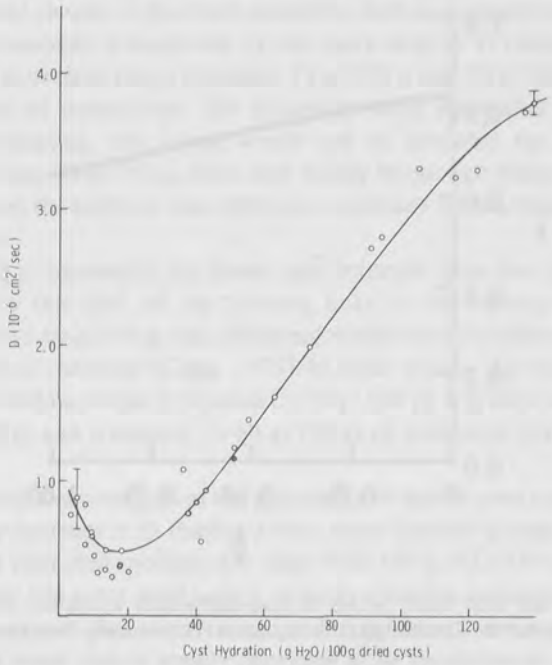

FIG. 6. Diffusion coefficient, D (10^{-6} cm^2/sec), *versus* hydration (g/100 g).

There appears to be a slight increase in D toward the lower hydrations, but because of the aforementioned experimental difficulties, this is somewhat tentative.

The low value of the diffusion coefficient also puts constraints on the interpretation of the relaxation data. While conclusions based on D are also model dependent, D is not weighted by one fraction. Considering only the two fraction model, if the observed diffusion coefficient is 1/6 that of pure water, (as is the case for the maximally hydrated cysts), then no more than 1/6 of the total water can diffuse as freely as bulk water. If one considers an obstruction effect, in which the diffusion path length is increased by obstructions such as cellular organelles and macromolecules, a decrease by a factor of two, but not much more, can be expected (Wang, 1954 ; Cleveland *et al.*, 1976). In order to estimate the combined effect of obstructions and hydration water, we use the equation derived by Rorschach [quoted in Cleveland *et al.* (1976) and Seitz *et al.*, (1977)] to describe these effects for spin echo measurements.

$$D'/D = (1-f)/1+\Phi)$$

where

D' = the observed diffusion coefficient
D = the diffusion coefficient of pure water (or, in this case, 3 % glycerol, to account for the small amount of glycerol in the cysts)
Φ = the volume fraction of macromolecules
f = the fraction of the total water which is macromolecular hydration water.

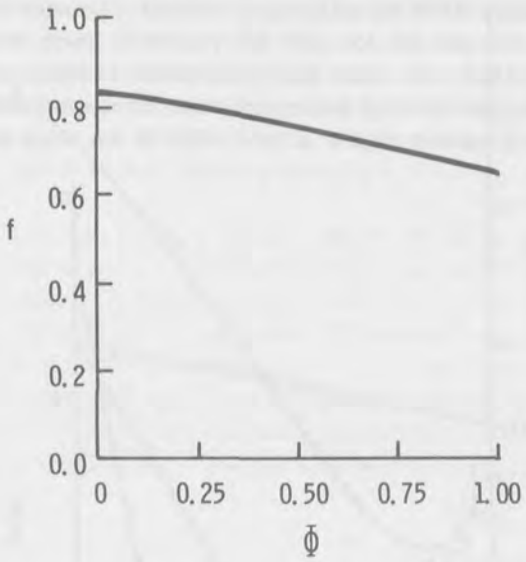

FIG. 7. Fraction of the total water which is macromolecular hydration water (f) versus volume fraction of macromolecules (Φ). Calculated from equation (1), assuming both obstruction and hydration effects.

Fig. 7 shows the solution for f as a function of Φ. Φ for fully hydrated *Artemia* cysts is about 0.35, if it is calculated using the average density of protein given by Fasman (1976). In other words, the fraction of the total water which comprises the macromolecular hydration shell would have to be very large (about 70-80%) and is not strongly dependent on Φ.

Conclusions

This leaves several possibilities, all of which imply some sort of restriction in the mobility of cyst water. There could be more than two fractions of water, as suggested by some of the relaxation decay curves, each with a different molecular correlation time (Outhred and George, 1973 ; Knispel *et al.*, 1974). In such a case, it would be reasonable to suggest that those water molecules closes to molecular surfaces would have the least mobility and those further away would have greater mobility (though not necessarily that of bulk water). Another possibility is slow exchange between fractions separated by differences in phase (Garlid, 1979) or by localization into compartments (Robertson, 1966 ; Tanner, 1978).

Returning to the role of water structure in metabolism (Fig. 1), recall that a significant change in the relaxation times of water protons occurs just before the onset of restricted metabolic pathways. The occurrence of the T_1 and T_2 minima suggests a reorganization of water molecules that precedes, and may be a prerequisite for, metabolic activity. This reorganization may represent a substitution of water for protective glycerol molecules closely associated with macromolecules in the dehydrated state [as suggested by Webb (1965) for

inositol] or a physical change in the water structure, such as a phase transition (Drost-Hansen, 1969). The latter possibility is supported by the sharp drop in T_2 values (from 65 to 20 msec) over a very short hydration range (between 19 g/100 g and 22 g/100 g ; see Fig. 3).

Before the onset of metabolism, the incoming water molecules could be required for hydration and orientation and hence would not be available for direct participation in metabolic events. Near 30 g/100 g, there may finally be enough water molelcules to properly solvate and transport the enzymes and substrates necessary for the observed reactions to occur to a limited extent.

Above 60 g/100 g, increasing the water concentration does not increase the number of active pathways. By this time, all the "missing links" in the various sequences of reactions must be filled. Above 60 g/100 g, the additional water seems to affect only the rates and not the kinds of metabolic reactions (Clegg, 1979). In other words, the functions of the incoming water molecules could be proper hydration (< 30 g/100 g), solvation and participation (30 g/100 g to 60 g/100 g) and transport (> 60 g/100 g) of molecules involved in the metabolic pathways.

The diffusion coefficient analysis of the maximally hydrated cysts raises an interesting point in this regard. The decrease in D implies a very large fraction (greater than 2/3) of the total water in a state of restricted mobility for cysts with 140 g H_2O/100 g dry solid. Since cysts with only 60 g H_2O/100 g dry solid have a virtually complete metabolism (and an even lower D^*/D ratio), this suggests that metabolism can be carried out almost solely with "hydration water", or at least water that is greatly restricted in its translational mobility.

One could speculate that this may even be the case for organisms which require greater amounts of water to survive and that the excess water is needed for long-range processes, such as transport, waste removal and temperature regulation.

These suggestions correlate very well with the vicinal-water network model of Clegg (1979).

Summary

In summary, we have found that there are large decreases in the values of the NMR relaxation times and diffusion coefficients and that T_1 and T_2, and possibly D, pass through minimal values at a hydration just preceding the onset of metabolism. The magnitude of the decreases suggests restriction in H_2O mobility and the location of the minima suggests a reorganization of water molecules prior to the beginning of metabolism. The overall outcome of these studies also strongly suggests that a large fraction of the intracellular water in fully hydrated cysts does not behave like ordinary bulk water.

Acknowledgements

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Eukaryotic elongation factor T and artemin : two antigenically related proteins which reflect the dormant state of *Artemia* cysts

Lawrence I. Slobin

Department of Biochemistry, University of Mississippi School of Medicine
2500 North State Street, Jackson, Mississippi 39216, USA

Abstract

The form of elongation factors eEF-Tu and eEF-Ts in *Artemia* embryos was investigated, taking into consideration the marked lability of eEF-Tu in the absence of glycerol. It was found that the bulk (about 65%) of the eEF-Tu and all the eEF-Ts was present as a high molecular weight aggregate (eEF-T) in *Artemia* cysts. During the course of *Artemia* development all the eEF-T is apparently dissociated to free eEF-Tu and free eEF-Ts, *i.e.* little, if any, eEF-T was detected in nauplii. Mixing experiments using supernatant proteins from cysts and nauplii demonstrated that the dissociation of eEF-T is not likely to occur *in vitro* during the course of factor analysis. This result agrees with our earlier suggestion (Slobin and Möller, 1976a) that the association of eEF-Tu with eEF-Ts in cysts may be a specific macromolecular adaptation to cryptobiosis. This hypothesis is supported by the marked differences in stability *in vitro* of eEF-Tu and eEF-T, with the former factor being very unstale in the absence of aminoacyl-tRNA.

Encysted embryos (gastrulae) of the brine shrimp *Artemia*, contain a major protein, designated as artemin, which is antigenically related to the eucaryotic elongation factor Ts (eEF-Ts). Artemin was purified from cyst extracts by immunoaffinity chromatography using anti-eEF-T antibodies coupled to Sepharose (eEF-T is a complex which contains eEF-Tu and eEF-Ts). Artemin was found to constitute approximately 13% of the postribosomal supernatant proteins of *Artemia* cysts or at least 4% of the total cyst proteins. In addition to immunological cross-reactivity, purified artemin was found to be related to eEF-Ts by the following criteria : 1) both proteins consist of a single polypeptide chain of similar MW (26 000-30 000) under denaturing conditions ; 2) artemin, like eEF-Ts, behaves as a high molecular weight aggregate under non-denaturing conditions ; 3) artemin and eEF-Ts have similar, albeit distinct, amino acid compositions. Despite these similarities, artemin was inactive in eEF-Ts mediated reactions. Analysis of 24 hr nauplii supernatants with anti-artemin antibodies revealed a 2.5-fold decrease in the amount of artemin. These data are consistent with the hypothesis that artemin plays an important role in either establishing or maintaining the state of biological dormancy in *Artemia* embryos.

Introduction

The protein synthetic apparatus of dormant brine shrimp embryos has now been extensively examined. What is striking is not that the components of protein synthesis – mRNA, initiation, elongation and termination factors and ribosomes – have been found. After all, a system lacking any of these components would be eternally dormant. Rather, it is orderly fashion in which the protein synthesis machinary in cysts has ground to a halt. Thus,

few, if any, polysomes are present (Golub and Clegg, 1968), mRNA is tucked away in informosome-like particles (Grosfeld and Littauer, 1975), some of which are membrane bound (Grosfeld *et al.*, 1977) and 80S ribosomes are quite clean with only small amounts of factors associated with them.

Several years ago we began an examination of the properties of eucaryotic elongation factor T (eEF-T)[1] in embryos of *Artemia*. This enzyme is responsible for binding aminoacyl-tRNA to polysomes in the presence of GTP. For a recent review of the factors involved in protein synthesis see Weissbach and Ochoa, 1976). Our initial observations (Slobin and Möller, 1975, subsequently confirmed by Twardowski *et al.* (1976), were that *Artemia* cysts contain abundant amounts of eEF-T and little, if any, free eEF-Tu. During the course of embryonic development eEF-T appears to be completely dissociated to eEF-Tu and its other component polypeptides. This conversion was shown to occur at or near the time when the organism emerges from the cyst in the form of a free-swimming nauplius.

At the time of these observations we were unaware that the γ-polypeptide of eEF-T is, in fact, eucaryotic elongation factor Ts (Slobin and Möller, 1978). Furthermore, we also did not know that eEF-Tu is markedly unstable in the absence of high concentrations of glycerol. In this report I have reinvestigated the state of these elongation factors during *Artemia* development. I have found, as originally reported, that most of the eEF-Tu activity in cysts is present in the eEF-T complex, although there is some present as eEF-Tu. All of the eEF-Ts in cysts in part of the eEF-T complex. During embryonic development, with the concomitant resumption of protein synthesis, all of the eEF-T is dissociated into eEF-Tu and eEF-Ts so that the complex eEF-T does not exist as such in nauplii. The possible importance of these findings in our understanding of the mechanism(s) whereby protein synthesis is regulated in *Artemia* development will be discussed.

During the course of our work on *Artemia* eEF-T and its component polypeptides, we made the serendipitous observation that cysts contain a major polypeptide, which is antigenically cross-reactive with eEF-T and may be structurally related to eEF-TS. Although a function for this protein, which we call artemin, has not yet been found, it may play a major role in either maintaining or helping to break the cryptobiotic state. Certainly, the finding that artemin comprise as much as 4% of the total protein of *Artemia* cysts and that the amount of the protein diminishes significantly during early embryonic growth suggests an important role for artemin in *Artemia* development.

Materials and methods

Materials

Prior to homogenization, desiccated cysts of *Artemia* (San Francisco Bay Brand, Newark, California) were washed with ice-cold 2% NaClO, followed by distilled water, as described by

[1] Eukaryotic elongation factor T (eEF-T) previously referred to as EF-1, is a complex consisting of eEF-Tu (α-chain), eEF-Ts (γ-chain) and a third polypeptide of Mr $\simeq$ 50 000 termed the β-chain. eEF-Tu has been referred to by other investigators as EF-1_α or EF-1_L : eEF-Ts has been called EF-1_β by Kaziro and coworkers : Iwasaki *et al.* (1976). A discussion of this nomenclature is given elsewhere (Slobin and Möller, 1979).

Slobin and Möller (1975). To prepare nauplii, cysts were developed in four percent artificial sea salt for 24 hr at 29 °C with vigorous aeration. All chemicals used were reagent grade.

Preparation of postribosomal supernatant proteins

Postribosomal supernatant proteins from both cysts and nauplii were prepared, as described previously by Slobin and Möller (1976a).

Assays

The eEF-Tu dependent binding of aminoacyl-tRNA to *Artemia* 80S ribosomes, the binding of GTP by eEF-Tu, the eEF-Ts catalyzed exchange of eEF-Tu.guagine nucleotide complexes and the synthesis of polyphenylalanine were performed as described elsewhere (Slobin and Möller, 1976ab, 1977).

Preparation of antibodies

Anti-eEF-T antibodies were prepared in rabbits. The peak of enzyme activity which eluted first upon Ultrogel AcA-34 chromatography (LKB, Stockholm, Sweden) was used as the antigen (Slobin and Möller, 1976a). The immunization protocol was identical to the one described previously for eEF-Tu (Slobin and Möller, 1976a). Immunoglobulins were prepared from serum by precipitation with 40% (w/v) saturated ammonium sulfate followed by chromatography on DEAE Bio Gel A (BioRad, Richmond, California) at Ph 7.5 using 0.02 M sodium phosphate as eluant. Anti-artemin antibodies were prepared in rabbits using artemin purified from cyst postribosomal supernatant proteins by immunoaffinity chromatography using anti-eEF-T antibodies followed by chromatography on Ultrogel AcA-44 (see below). A total of 3 mg of protein per rabbit in complete Freunds adjuvant was administered in 3 week intervals, as described by Slobin and Möller (1976a). Immunoglobulins were purified, as described for anti-eEF-T. Artemin was also purified by a more conventional procedure (to be published elsewhere) involving chromatography on Affi-Gel Blue (BioRad) and gel filtration on Sepharose-6B (Pharmacia). Artemin purifed without resort to eEF-T antibodies will be referred to as native artemin.

Chromatographic procedures

Immunoaffinity chromatography was performed in a manner essentially described by Livingston (1974). Immunoglobulins were coupled to CNBr activated Sepharose purchased from Pharmacia Fine Chemicals. The activated Sepharose was suspended in 0.1 N $NaHCo_3$ and 3 mg of IgC was added per gm of starting Sepharose. The mixture, having a final protein concentration of about 1 mg/ml, was shaken et 2 °C for 20 hr. The blocking of any remaining active groups on CNBr Sepharose, as well as the washing of the coupled product to remove noncovalently bound protein, was carried out according to the brochure on CNBr-activated Sepharose available from Pharmacia. Antibody-Sepharose columns were stored at 2 °C in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl containing 0.1% NaN_3.

Chromatography of postribosomal supernatant proteins on heparin-Sepharose was performed as described by Slobin (1976).

Protein mixtures were chromatographed on antibody-Sepharose columns at room temperature. Generally, the protein solution in a buffer containing 0.02 M Tris-HCl, pH 7.5 and 0.2 M KCl was passed through a small column (0.9 × 7 cm) of antibody-Sepharose, which was then washed with the following solutions: 50 ml of 0.02 M Tris-HCl, pH 7.5, 0.15 M NaCl, 10 ml of 0.5 M NH_4HCO_3, and 10 ml of 0.1 M NH_4OH. Fractions (1 ml) were collected after the application of the 0.1 M NH_4OH solution. The eluate, which contained the immunospecifically bound polypeptide(s), was neutralized to pH 8 with 1 M Tris-HCl, pH 7.0 and dialyzed *versus* 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM 2-mercaptoethanol. Purified proteins were stored at -70 °C prior to use. The immunoadsorbents were washed with starting buffer (0.02 M Tris-HCl, pH 7.5, 0.15 M NaCl) immediately after use and could be reused at least 10 times without any detectable loss of capacity. The capacity of anti-eEF-T Sepharose was at least 200 μg of artemin/ml of packed resin.

Miscellaneous procedures

Double diffusion analysis in agar gels using anti-eEF-T antibodies was performed as previously described by Slobin and Möller (1975). Electrophoresis in the presence of sodium dodecyl sulfate was performed according to Weber and Osborn (1969). Protein was determined by the procedure of Lowry *et al.* (1951).

Results

Form of eEF-Tu during *Artemia* development

As mentioned in the Introduction, at the time we reported that cysts contain exclusively an eEF-T complex we were unaware of the instability of eEF-Tu in buffers lacking high concentrations of glycerol. Therefore, we have repeated our work on the form of eEF-Tu in cysts and nauplii, ensuring that free eEF-Tu is not inactivated during the course of the analysis. Postribosomal supernatant proteins from both cysts and 48 hr nauplii were made 25% (v/v) in glycerol immediately after preparation and chromatographed separately on Ultrogel AcA-34 in the presence of 25% glycerol. The fractionated supernatants were assayed for their ability to catalyze the binding of aminoacyl-tRNA to ribosomes (eEF-Tu and eEF-T activity) support polyphenylalanine synthesis (EF-2 activity), and ability to bind [^{3}H]GDP (predominantly eEF-Tu activity). The results, shown in Fig. 1, illustrate that approximately 65% of the eEF-Tu activity of cysts exists in the form of the eEF-T complex, which we have previously characterized by Slobin and Möller (1976a). In 48 hr nauplii, however, only small amounts of the eEF-T complex persits, while more than 90% of the eEF-Tu activity elutes in the same position as purified eEF-Tu (Slobin and Möller, 1976a). Another indication of the large increase in free eEF-Tu activity is the marked increase in GDP binding activity of the nauplii supernatant proteins. This activity comigrates with the capacity to support aminoacyl-tRNA binding to ribosomes and presumably reflects the presence of eEF-Tu. We have shown previously that eEF-Tu has a much higher affinity for guanine nucleotides than does eEF-T. Figure 1 also show that there has been little, if any, alteration in the size distribution or activity of EF-2 during *Artemia* development.

Twardowski *et al.* (1976) have shown that extracts of 40 hr *Artemia* nauplii can convert rabbit reticulocyte eEF-T to a light form of the enzyme, presumably eEF-Tu. Their data

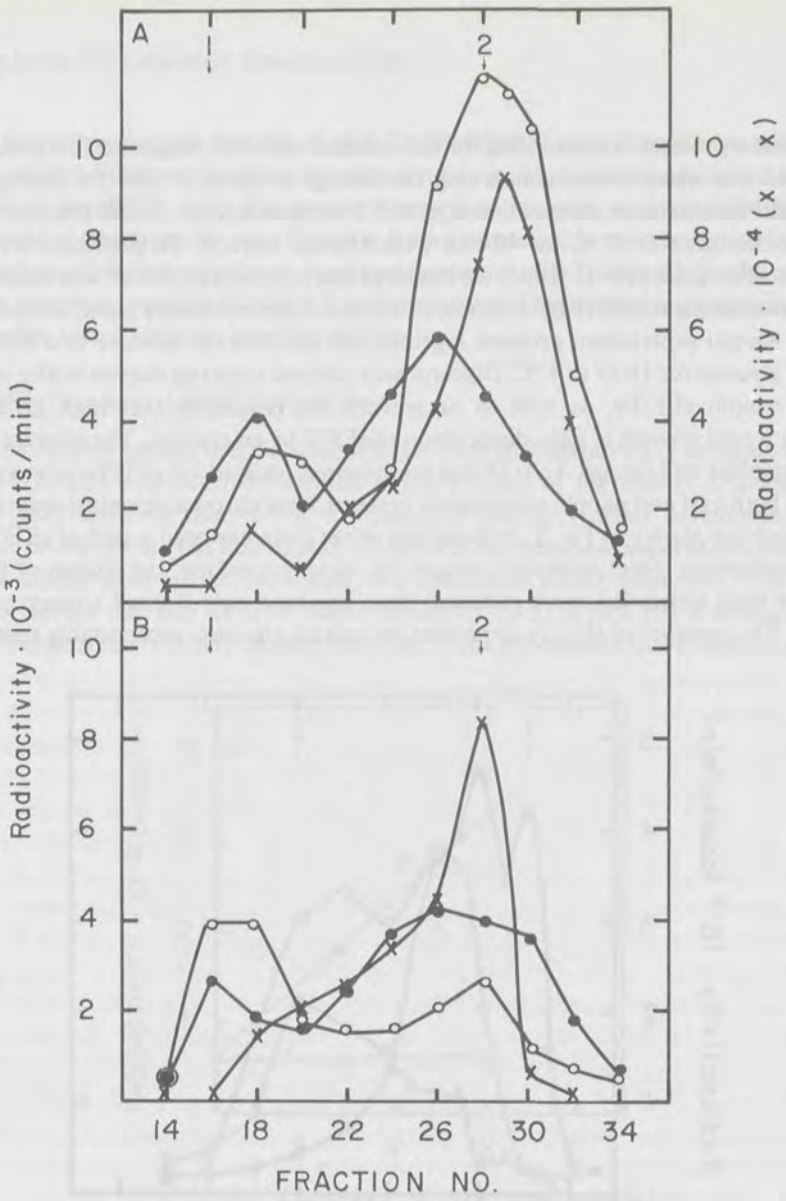

FIG. 1. Analysis of eEF-Tu activity of *Artemia* nauplii (A) and cyst (B) postribosomal supernatant proteins on Ultrogel AcA-34. A column (1 × 22 cm) of Ultrogel AcA-34 was equilibrated and developed with a buffer containing 20 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM dithiothreitol, and 25% (v/v) glycerol. Cyst and 48 hr nauplii supernatant proteins were each mixed with an equal volume of 50% v/v glycerol and applied separately to the column. A total of 4.1 mg cyst proteins (0.5 ml) and 5.2 mg (0.5 ml) of nauplii proteins were analyzed. Fractions (13 drops, approximately 0.5 ml) were collected and 2 μl aliquots of every other fraction were taken for aminoacyl-tRNA binding to ribosomes (○) as described in Materials and methods. The specific activity of (^{3}H) phenylalanyl-tRNA was approximately 10^3 cpm/pmol. For analysis of (^{3}H) GDP binding (X) 50 μl of every other fraction were mixed with 2.5 μl of (^{3}H) GDP (specific activity $\simeq$ to 8.4×10^3 cpm (pmol); final (GDP) = 4 μM) and after incubating for 5 min at 37 °C the tubes were processed for counting as previously described (Slobin and Möller, 1976b). Aliquots (10 μl) of every other tube were analyzed for polyphenylalanine synthesis (●) after the addition of 0.2 μg eEF-T as described (Slobin and Möller, 1976b). The arrows at positions 1 and 2 refer to the elution position of dextran blue and bovine serum albumin, respectively.

indicated that a protease is responsible for this reaction and they suggested that their findings may explain our observations concerning the change in form of eEF-Tu during *Artemia* development. To obtain the dissociation of eEF-T Twardowski *et al.* (1976) found it necessary to incubate the factor at 37 °C for 40 min with *Artemia* extracts. To determine whether the dissociation of *Artemia* eEF-T, which we observed had occurred *in vivo* or was simply a result of *in vitro* processing of eEF-T by a protease, we mixed approximately equal amounts of cyst and 48 hr nauplii supernatant proteins together and dialyzed the mixture in a buffer in the absence of glycerol for 16 hr at 4 °C. Glycerol was omitted from the dialysis buffer in order to inactivate nauplii eEF-Tu, as well as to prevent the possibility that high glycerol concentrations would prevent *in vitro* dissociation of eEF-T by an enzyme. The mixture was then chromatographed on Ultrogel AcA-34 and the fractions assayed for eEF-Tu activity. Control samples of both cyst and nauplii supernatant proteins were chromatographed separately. The results, which are shown in Fig. 2, indicate that while there has been a shift of eEF-T activity toward a somewhat lower molecular weight (or, strictly speaking, the elution of the eEF-T activity has been somewhat more retarded) there has been only a small amount of eEF-Tu generated. The amount of eEF-Tu generated by mixing the two supernatants together was

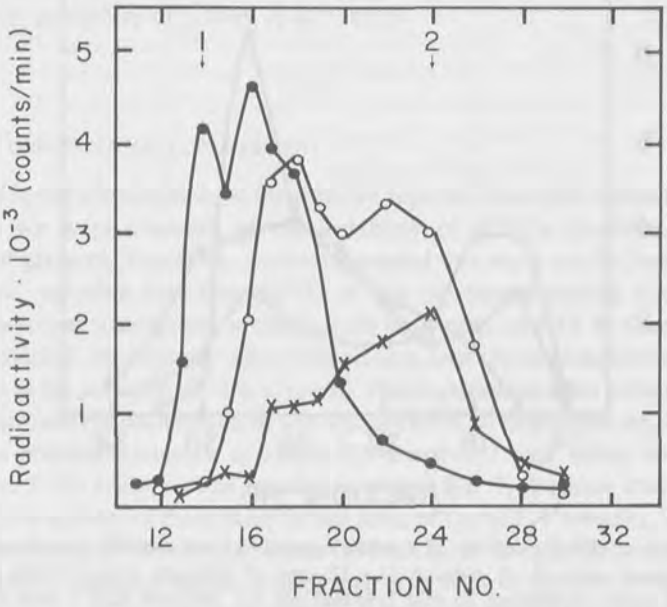

FIG. 2. Analysis of eEF-Tu activity of *Artemia* cyst (●), nauplii (X) and mixtures of cyst and 48 hr nauplii postribosomal supernatant proteins (○) on Ultrogel AcA-34. A column (1 × 22 cm) of Ultrogel AcA-34 was equilibrated and developed with a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM ditheothreitol (Buffer A). Cyst supernatant proteins (0.5 ml contain 6.5 mg protein) was mixed with an equal volume of Buffer A or 0.5 ml of nauplius supernatant proteins (containing 4.7 mg protein); the nauplius proteins (0.5 ml) were also mixed with 0.5 ml Buffer A. Each mixture was dialyzed separately against 100 volumes of Buffer A for 16 hr at 4 °C. The dialyzed mixtures were applied separetely to the Ultrogel column and 5 μl of every other fraction was analyzed for eEF-Tu activity as described in the Legend to Fig. 1. In the case of the cyst supernatant proteins (●) 3.5 μl aliquots were taken for analyses. For additional details see the legend to Fig. 1.

obtained by subtracting the amount of eEF-Tu activity still present in the nauplius proteins from the activity displayed by the supernatant mixture. This activity amounted to no more than 30% of the eEF-T. Since the nauplii supernatant proteins were analyzed immediately after removal of ribosomes by centrifugation (approximately 2 hr after homogenization of the embryos), I conclude that significant depolymerization of eEF-T into eEF-Tu did not occur *in vitro*. The possibility remains that eEF-T is depolymerized by a protease or proteases *in vivo* ; however, this seems unlikely (see Discussion).

Form of eEF-Ts during *Artemia* development

We have recently shown that elongation factor Ts is part of the eEF-T complex (Slobin and Möller, 1978) and is structurally identicl to the γ-chain of the factor. A question which remained after showing that eEF-T is dissociated during *Artemia* development was the disposition of eEF-Ts in cysts and nauplii. To answer this question, supernatant proteins from cysts and nauplii were applied separately to a column of DEAE-Sephadex A-50. We have previously shown that eEF-Ts is an acidic protein (pI ca 5), which has a strong affinity for anion-exchange resins. As may be seen from Fig. 3, nauplii extracts had abundant amounts of

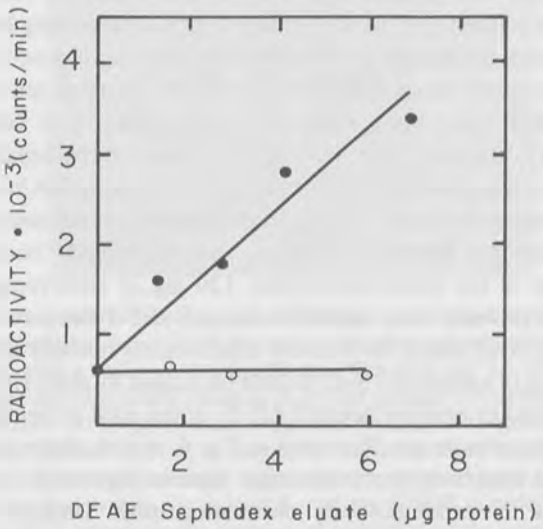

Fig. 3. Analysis of cyst (○) and nauplius (●) postribosomal supernatant proteins after chromatography on DEAE-Sephadex A-50. Nauplii postribosomal supernatant proteins (207 mg in 15 ml) were dialyzed against a buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM KCl, 1 mM ditheothreitol (Buffer B) and applied to a column (1.0 × 15 cm) previously equilibrated with the same buffer. The column was washed with Buffer B until no further protein was recovered and then eluted with Buffer B containing 500 mM KCl instead of 200 mM KCl. Varying amounts of protein were taken for determination of eEF-Ts activity using the ability of the factor to stimulate eEF-Tu-dependent binding of aminoacyl-tRNA to 80 S ribosomes (Slobin and Möller, 1978). For this purpose each assay mixture contained 40 mg (0.8 pmol) of purified eEF-Tu. For additional details see the legend to Fig. 1. Cyst postribosomal supernatant proteins (150 mg protein in 15 ml) were dialyzed against Buffer B and chromatographed on DEAE-Sephadex and the 0.5 M KCl eluate was tested for eEF-Ts activity as described above.

eEF-Ts, whereas no demonstrable eEF-Ts coud be observed in cyst extracts. We have tried several other methods of fractionation ; in each case extracts of nauplii contain a protein chromatographically indistinguishable from eEF-Ts. No such activity was found in cyst proteins. We conclude that all the eEF-Ts in *Artemia* cysts is present in the eEF-T complex and that the factor is liberated during the dissociation of the enzyme as embryonic protein synthesis is resumed.

Discovery of artemin

During the course of studies on the biosynthesis of eEF-T in developing *Artemia* embryos, I observed that the γ-polypeptide (eEF-Ts) of the factor appeared to be synthesized in large excess over the sum of the α and β chains. These findings suggested that either eEF-Ts is required in excess over eEF-Tu during early development, or that a polypeptide antigenically related to eEF-Ts is synthesized by the embryos. Since the polypeptides of eEF-T are present in the factor in essentially stoichiometric (1:1:1) ratios (Slobin and Möller, 1976a), this result indicated the presence of a pool of excess eEF-Ts, or an antigenically related protein in *Artemia* embryos.

To test this hypothesis the postribosomal supernatant proteins from *Artemia* cysts were chromatographed on a column of heparin-Sepharose. It has previously been shown that eEF-T is retained by heparin-Sepharose under conditions where the bulk of the supernatant proteins (90-95%) are eluted directly (Slobin, 1976). The proteins not retained by heparin-Sepharose (25 mM KCl elute) were then chromatographed on a column of anti-eEF-T Sepharose. Surprisingly, approximately 15% of the protein in the heparin-Sepharose eluate, based on the Lowry protein procedure (Lowry *et al.*, 1951) using bovine serum albumin as a standard, was immunospecifically retained by the antibody column and could only be eluted using conditions which are known to rupture antigen-antibody complexes. In order to estimate the specificity of the immunoabsorbent 120 mg of embryonic chicken liver post-ribosomal supernatant proteins were applied to the anti-eEF-T-Sepharose column. Using the elution procedure described above for *Artemia* supernatants resulted in the immunospecific retention of about 30 μg of embryonic liver protein (as judged by analysis of the NH_4OH eluate by the Lowry procedure) or approximately 0.025% of the total proteins applied. The results obtained using *Artemia* extracts are illustrated in Fig. 4, which shows dodecylsulfate gels of the cyst postribosomal supernatant proteins after heparin-Sepharose chromatography, both prior and subsequent to immunoaffinity chromatography. Dodecyl sulfate gels of the immunospecifically purified protein from cysts and nauplii (free-swimming larvae) supernatants are shown in the same figure.

The immunospecifically purified protein was assayed for eEF-Ts activity by measuring its ability to catalyze the guanine nucleotide exchange reaction using eEF-Tu.GDP complexes and free GTP. As can be seen from the results presented in Table I, the purified protein lacked measurable eEF-Ts activity. Since the effect of a little as 0.1 μg of eEF-Ts can readily be detected in the assay, it is apparent from the data that the purified protein contained less than 1% active eEF-Ts. Furthermore, the presence of artemin did not interfere with the enzymatic activity of eEF-Ts (Table I, experiment 8). In separate experiments it was established that eEF-Ts activity was not affected by the conditions required to elute immunospecifically bound protein from the antibody column. Evidence to the presented below, demonstrates that the

immunospecifically purified protein is structurally distinct from eEF-Ts. I have called the protein which is immunologically cross-reactive with eEF-Ts, artemin. Under denaturing conditions it consists of a single polypeptide chain with a molecular weight (26 000) identical to that of eEF-Ts[2].

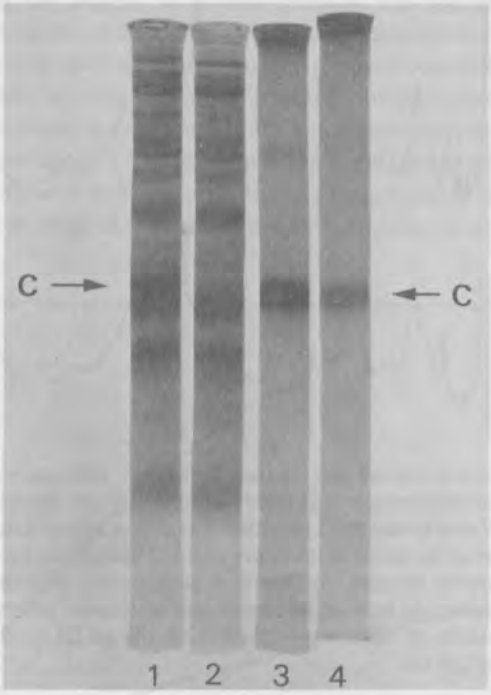

FIG. 4. Dodecylsulfate gels analysis of cyst postribosomal supernatant proteins and purified artemin. (1) 100 μg of cyst postribosomal supernatant protein, eluted after passage through a column of heparin-Sepharose equilibrated with a buffer containing 20 mM Tris-HCl, pH 7.5, 25 mM KCl ; (2) 100 μg of cyst postribosomal supernatant proteins (same as in 1) after passage through anti-eEF-T-Sepharose (see Experimental Procedures) ; (3) 10 μg of artemin purified from cysts by immunoaffinity chromatography ; (4) 8 μg of artemin purified from 48 hr nauplii by immunoaffinity chromatography. The bands at the top of the purified artemin gels represent aggregates of artemin. I have recently observed that it is necessary to boil the purified protein for at least 5 min in 1% SDS in order to reduce the amount of aggregates. The arrow at C represents the position of migration of the γ-polypeptide chain of eEF-T (eEF-Ts) and corresponds to 26 000 daltons.

Artemin was found to comprise 13% of the postribosomal supernatant proteins, as judged by the amount of the protein which was immunospecifically purified using the anti-eEF-T-Sepharose column. To check this estimate a densiometric scan was made of the dodecyl

[2] Recently it has been observed that purified eEF-Ts from *Artemia* embryos migrates somewhat less rapidly than artemin on dodecyl sulfate gels and has a molecular weight of about 30 000 compared with standard marker proteins (W. Möller, personal communication). If this observation is correct, it provides further evidence for the contention that artemin and eEF-Ts are different proteins.

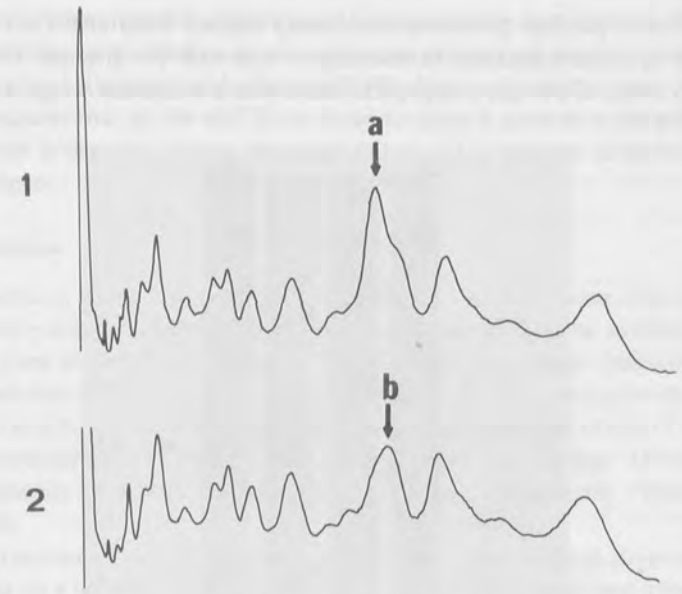

FIG. 5. Densimetric scans of stained gels (1) and (2) in Fig. 1. Gels were scanned at 550 nm using a Gilford model 240 spectrophotometer equipped with a Gilford model 2400-S linear transport attachment and Sargent Welch model SRG recorder. The percentage of artemin protein in gel (1) was estimted by weighing traces of the scans of the entire gels and in like manner the area under the artemin peak, indicated by the arrow beneath (a). For this purpose the contribution of a 24 000 dalton polypeptide, which also appears to be a major constituent of *Artemia* embryos (Grosfeld and Littauer, 1976) (indicated by the position of the arrow beneath (b) in the gel (2) scan), was substracted from the artemin peak by the use of gel (2).

TABLE I

Effect of eEF-Ts and artemin on the exchange of [^{3}H] GDP bound to eEF-Tu with GTP

Experiment no.	eEF-Ts added (μg)	Artemin added (μg)	[^{3}H] GDP bound[1] (pmol)
1	–	–	4.2
2	0.15	–	2.4
3	0.30	–	1.4
4	1.50	–	1.0
5[2]	–	–	1.0
6	–	6	4.3
7	–	12	4.1
8	1.50	6	1.1

[1] The assay for the guanine nucleotide exchange reaction was performed exactly as previously described by Slobin and Möller (1977), except that purified eEF-Ts (Slobin and Möller, 1978) was used instead of heat-treated eEF-T. Each reaction mixture (500 μl) contained 15 μg of eEF-Tu which previously had been incubated with [^{3}H]GDP (660 CPM/Pmol), 134 μM GTP and the indicated amount of artemin and eEF-Ts. After incubation for 5 mmin at 0 °C, aliquots (100 μl) were processed for filtration through a nitrocellulose filter. The results represent the average of three determinations.

[2] In experiment number 5 eEF-Tu, in addition to other factors, was omitted from the reaction mixture.

sulfate gels of the postribosomal supernatant proteins. From the scan (Fig. 5) it was estimated that 12% of the coomassie blue stain on the gel is bound to artemin, a value in good agreement with the result of 13% obtained by the Lowry procedure (Lowry *et al.*, 1951).

Although artemin could be purified from nauplii, it was of some interest to establish whether the amount of the protein is nauplii extracts was comparable to that of cysts. Quantitative precipitin analysis of cyst and 24 hr nauplii postribosomal supernatant proteins with anti-artemin antibodies showed that the nauplii proteins contained no more than half the amount of artemin present in cysts (Fig. 6). When artemin from equivalent amounts of cyst and nauplii supernatant proteins was purified by anti-artemin-Sepharose, it was found that the cyst material contained about 2.5 times as much artemin. Based on these results, I estimate that artemin comprises about 5% of the postribosomal supernatant proteins of 24 hr nauplii. Preliminary investigations suggest further reduction in amounts of artemin as embryonic development proceeds.

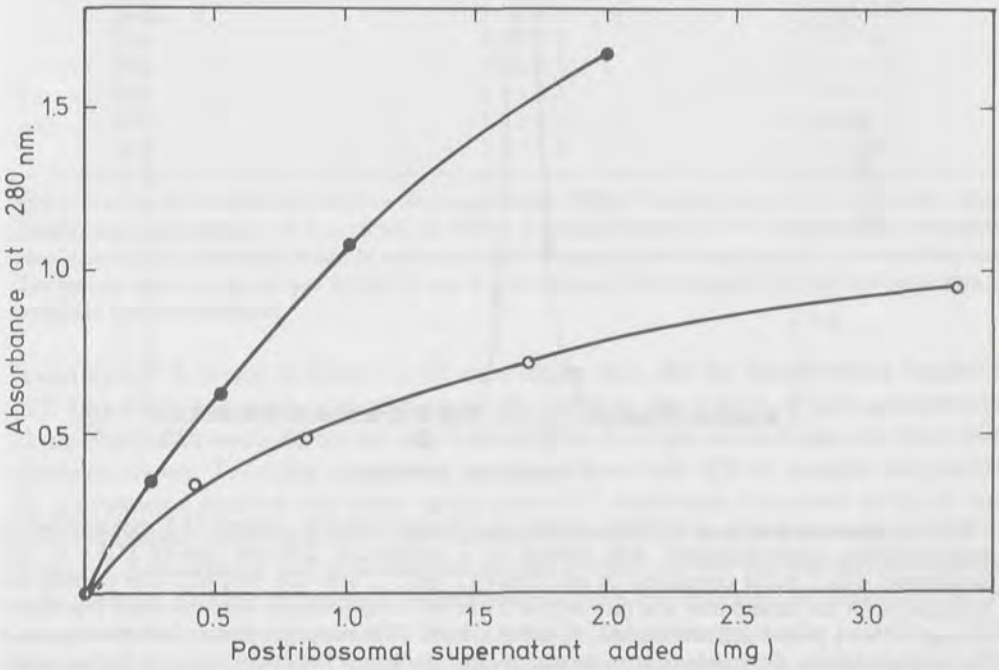

FIG. 6. Precipitation tests of cyst and nauplii supernatant proteins with anti-artemin antibodies. Freshly prepared cyst (●) and nauplii (○) supernatant proteins were incubated with 2.3 mg of anti-artemin IgG in buffer containing 20 mM Tris-HCl, pH 7.5, and 100 mM KCl (final volume 0.55 ml) for 13 hr at 4 °C. Incubation was terminated by layering the contents of each tube over 0.2 ml of a solution containing 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 M sucrose and 1% (v/v) triton X-100 in a 1.5 ml plastic centrifuge tube and centrifuging at room temperature for 6 min. in an Eppendorf Model 3200 centrifuge. The supernatants were carefully removed by aspiratijon and the immune precipitates washed once with 1 ml of a buffer containing 20 mM Tris-HCl, pH 7.5 and 0.15 M NaCl. After removal of the wash buffer the precipitates were dissolved in 0.5 M NaOH and the absorbance at 280 nm was measured in a Zeiss spectrophotometer. Incubation of 3.0 mg of non-immune IgG with 3 mg of either cyst or nauplii supernatant proteins resulted in absorbances at 280 nm of less than 0.02 by the procedure described above.

After purification of cyst artemin by immunoaffinity chromatography, a sample was subjected to gel filtration on Ultrogel AcA-44. The result shown in Fig. 7 indicates that in aqueous solution, under non-denaturing conditions, artemin behaves as a high molecular weight aggregate. Based on its elution position just after dextran blue artemin would appear to have a molecular weight $\geq 200\,000$. More precise hydrodynamic studies have confirmed this estimate. In fact, preliminary data indicate that artemin has a molecular weight $\geq 500\,000$. The single symmetrical peak of absorbancy observed after gel filtration further attests to the purity of artemin after immunoaffinity chromatography. Purified eEF-Ts eluted on AcA-44 columns at the same position as artemin although smaller amounts of the latter protein were found in less aggregated forms (Slobin and Möller, 1978).

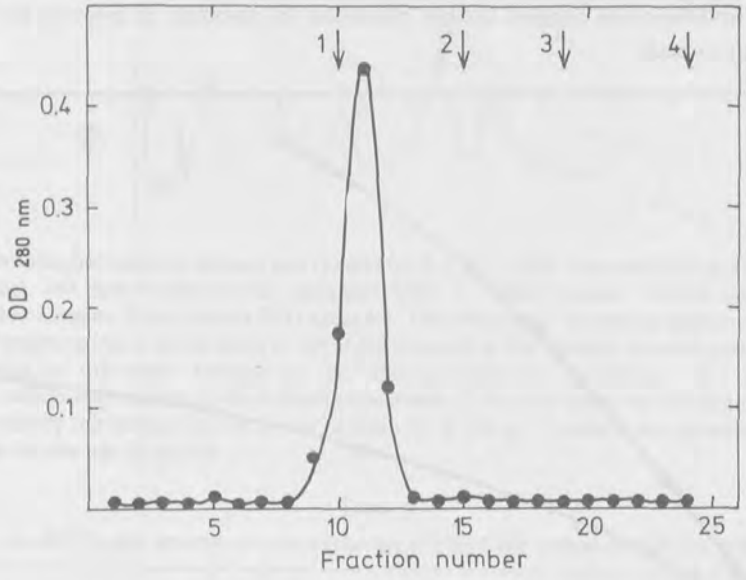

FIG. 7. Chromatography of purified artemin on Ultrogel AcA-44. Artemin (3.5 mg) purified by immunoaffinity chromatography was applied to a column of Ultrogel Aca-44 (1.5 × 70 cm) equilibrated with a buffer containing 20 mM Tris-HCl, pH 7.5, and -100 mM KCl. The column was developed with the same buffer at a flow rate of 23 ml/hr. 5 ml fractions were collected. Fractions 9 through 12 were pooled and concentrated by ultrafiltration. 95% of the protein applied was recovered. The arrows indicate the positions of molecular weight markers : (1) dextran blue ; (2) bovine serum albumin (66 000 daltons) ; (3) β-lactoglobulin (35 000 daltons) ; and (4) cytochrome C (13 000 daltons).

The amino acid composition of artemin is presented in Table II, together with the composition of purified eEF-Ts. As expected, the two proteins have rather similar compositions, although significant differences do exist, particularly among the basic amino acids. The only noteworthy feature of the composition of artemin is the high histidine content (about 5 mol %). Carbohydrate analysis gave less than 0.5% neutral sugar and artemin did not contain inorganic phosphate. The UV spectrum was typical for a protein and did not indicate the presence of any unusual prosethetic groups. The isoelectric point of artemin was

TABLE II

Amino-acid composition of artemin and eEF-Ts from *Artemia* cysts

Amino-acid	eEF-TS	Artemin
Asp	11.0 ± 0.8	11.5 ± 0.4
Thr	5.3 ± 0.2	3.5 ± 0.1
Ser	6.4 ± 0.2	8.8 ± 0.3
Glu	13.5 ± 0.3	10.6 ± 0.7
Pro	4.4 ± 0.3	3.5 ± 0.6
Gly	6.0 ± 0.3	5.4 ± 0.4
Ala	9.0 ± 0.3	10.3 ± 0.4
Cys		
Val	6.5 ± 0.1	5.3 ± 0.4
Met	2.0 ± 0.4	2.9 ± 0.2
Ile	5.9 ± 0.2	4.6 ± 0.3
Leu	9.1 ± 0.3	7.9 ± 0.5
Tyr	1.2 ± 0.4	1.9 ± 0.3
Phe	4.8 ± 0.1	5.3 ± 0.5
Lys	8.9 ± 0.2	6.6 ± 0.5
His	2.0 ± 0.1	4.7 ± 0.3
Arg	3.4 ± 0.1	4.9 ± 0.3

eEF-Ts was purified as described elsewhere (Slobin and Möller, 1978). The proteins were hydrolyzed under reduced pressure and under nitrogen for 22 hr in 6NHCl, 0.02% 2-mercaptoethanol at 110°. The amino acid compositions were determined in a Beckman Model M amino acid analyzer and are given in mole percent ± standard deviation. The data are based on four analysis for eEF-Ts and five for artemin. Cysteine was not detected in either protein and trytophan was not determined.

found to be 6.6, which is about 1.5 pH units higher than eEF-Ts. Besides being inactive in eEF-Ts mediated guanine nucleotide exchange reactions, the protein at high concentration (2 mg protein/ml reaction volume) was without effect in a reticulocyte lysate cell-free protein synthesis system. To further discriminte between artemin and eEF-Ts, artemin was purified by conventional methods and tested agaist anti-eEF-T antibodies. As shown in Fig. 8, anti-eEF-T antibodies cross-react with native artemin, although the precipitin line extending in the direction of the artemin well indicates that the two proteins are not, as expected, immunologically identical. The partial fusion of the immunoprecipitin line indicates the presence of related antigens in the two peripheral wells. Native artemin had properties similar to those of them protein purified by anti-eEF-T antibodies and did not have any detectable eEF-Ts activity. The immunological cross-reactivity between eEF-Ts and artemin has recently been confirmed (W. Möller, personal communication).

Discussion

In this report we have confirmed our initial observation that most of the eEF-Tu in *Artemia* cysts is in the form of a high molecular weight complex, eEF-T. We also have found that all the detectable eEF-Ts activity in cysts is part of the eEF-T complex. Upon resumption of metabolism, the eEF-T is dissociated into its constituent factors, so that in 48 hr nauplii these factors are completely separate from one another in the postribosomal supernatant proteins.

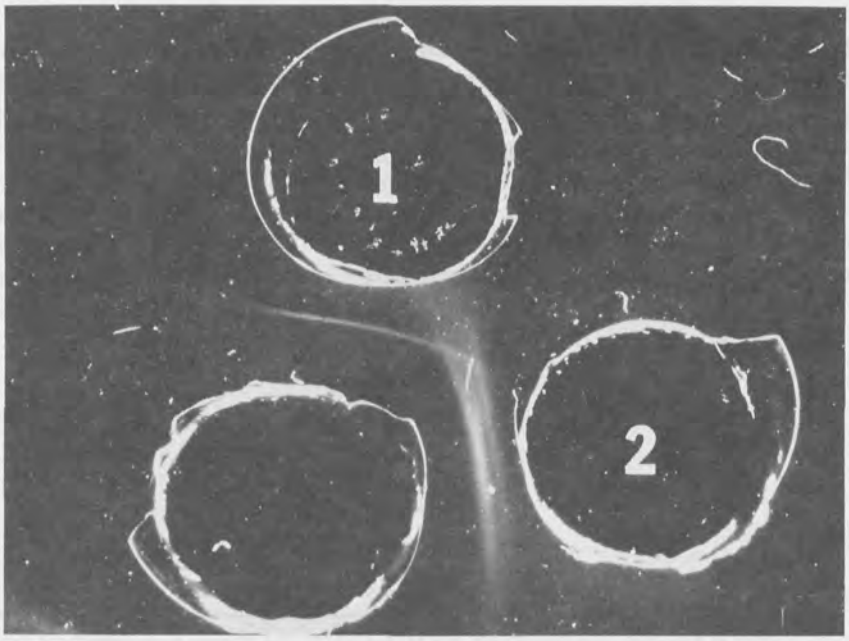

FIG. 8. Immunodiffusion of eEF-T and artemin. The center well contained 1.2 mg of rabbit anti-*Artemia* eEF-T antibodies. The peripheral well 1 contained 2 μg of native artemin and well 2 containd 8 μg of purified eEF-T.

Mixing of cyst postribosomal supernatant proteins with those from nauplii and incubation of the mixture for 16 hr at 4 °C causes the release of only a small amount of eEF-Tu from eEF-T (Fig. 2). This result strongly suggests that the dissociation of eEF-T occurs *in vivo* during *Artemia* development and is not the result of *in vitro* proteolysis of the factor by a protease from nauplii during the analysis of the extract.

Work in a number of laboratories, including our own, indicates that eEF-T is dissociated during the course of protein synthesis. In fact, it has been possible to dissociate the factor at physiological temperature and ionic strength by incubationwith GTP alone (Lanzani *et al.* 1976 ; Nombela *et al.*, 1976). Work to be presented elsewhere indicates that in rabbit reticulocytes, the predominant form of eEF-Tu is not complexed with other polypeptides ; this observation has also been made by Nagata *et al.* (1976).

Why, then, do *Artemia* cysts contain such a large amount of eEF-Tu in the eEF-T complex ? I believe there is a relatively straightforward explanation for the situation. First, we know the eEF-Tu from a variety of sources is extremely unstable in the absence of high concentrations of glycerol, whereas eEF-Tu in the eEF-T complex is relatively stable (Slobin and Möller, 1976b ; Nagata *et al.*, 1977). If eEF-Tu is also unstable *in vivo* in the absence of protein synthesis – the factor is stabilized by the joint presence of aminoacyl-tRNA and GTP – than a dormant organism, such as the brine shrimp cyst, must have a mechanism for providing for the long-term survival of the factor. However, rather than acquiring a special adaptive factor to stabilize eEF-Tu, the cyst simply makes use of elongation factor Ts, which

binds strongly to eEF-Tu to form the stable factor eEF-T. The conversion of this dissociated eEF-Tu into eEF-T is, thus, a consequence of a lack of protein synthesis. When protein synthesis is resumed, the factor is again dissociated in a reaction that, at the least, requires GTP and aminoacyl-tRNA and may possibly require other factors as well. In systems which are actively synthesizing protein, such as nauplii or reticulocytes, most, if not all of eEF-Tu, would be expected to be in the free from. According to this view, eEF-T may represent a spare capacity of the cell to carry out protein synthesis, a suggestion that has been made by Slobin and Möller (1976a), as well as by Legocki *et al.* (1974). A number of additional facts need to be established before we can be at all confident of this hypothesis. From the viewpoint of brine shrimp development, however, it seems likely that the preponderance of eEF-T in cysts and eEF-Tu in nauplii is a macromolecular reflection of the infinite difference in metabolic activity of the two developmental forms. It is possible that other labile factors in dormant organisms may also become part of larger, more stable complexes as the dormant state is established and then revert to their monomeric active form once dormancy is broken.

Dormant embryos of *Artemia* contain a major protein of 26 000 daltons, which is immunologically related to eEF-Ts. However, despite the immunological cross-reaction between artemin and *Artemia* eEF-Ts, the similarities in molecular weight and gel filtration properties, artemin and eEF-Ts appear to be different proteins. This conclusion is based on significant differences in isolectric point, immunological properties and amino acid composition, as well as the lack of eEF-Ts activity in purified preparations of artemin. It is possible that artemin is a precursor or degradation product of eEF-Ts, which does not have enzymatic activity, but is immunologically cross-reactive. However, purified artemin is not retained by DEAE-Sephadex equilibrated at neutral pH with 50 mM KCl, whereas eEF-Ts is eluted at 300 mM KCl (Slobin and Möller, 1978). It is unlikely, but not impossible that such a marked difference in properties could be conferred by the presence of a small polypeptide not shared by the two proteins.

A number of observations suggest that artemin may play an important role in the eary embryonic development of *Artemia*. Artemin accounts of 12 to 13% of *Artemia* cyst soluble proteins. It is also present in the microsomal fraction of the cells where it accounts for about 5% of the proteins released by washing the microsomes with 0.5 M potassium chloride (unpublished observations). Thus, artemin comprises about 4% of the proteins of *Artemia* cysts. The above estimate was made by determining that 30% of the total protein in cyst homogenates remained soluble after high speed centrifugation (100 000 × g for 3 hr). Further support for the suggestion that there is a 2.5-fold reduction in the amount of the protein in 24 hr nauplii compared with cysts. This reduction in the amount of artemin during development is reflected in the observation that artemin synthesis in nauplii accounts for less than 1% of soluble protein synthesis (unpublished conservation).

The relatively small of artemin synthesized by nauplii may indicate a continuing need for the protein during *Artemia* development. Alternatively, artemin synthesis may reflect the residual presence of artemin mRNA, which is in the process of decay. This latter suggestion is strengthened by recent observations by Grosfeld and Littauer (1976) and by Sierra *et al.* (1976). Both groups have observed on dodecyl sulfate fels a prominent protein band at 26 000 MW, which very likely corresponds to artemin. Translation of bulk cyst mRNA in a wheat germ cell-free system provided strong evidence that the mRNA for the 26 000 MW polypeptide is stored in considerably quantity in the cyst. The quantity of this mRNA remains

essentially unaltered during cyst development ; however, in nauplii the putative artemin mRNA disappears (Grosfeld and Littauer, 1976). Direct identification of the major translation product of cyst mRNA with artemin would provide additional opportunities for studying the role of artemin during *Artemia* development.

I have examined other biological specimens for the presence of artemin and found such an abundant protein to be lacking in sea urchin embryos, rat liver, embryonic chicken liver and rabbit reticulocytes. The criteria employed were cross-reacted with anti-artemia artemin and the appearance of a prominent band at about 26 000 Mr in SDS gels of postribosomal supernatant proteins. While these negative results obviously do not eliminate the possibility that artemin is widespread in the biosphere, they suggest a special role for the protein in the rather peculiar life cycle of *Artemia*.

Acknowledgement

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Polypeptide chain initiation during embryogenesis of *Artemia*

Albert J. Wahba, Thomas H. MacRae, and Charles L. Woodley

Department of Biochemistry, University of Mississippi Medical Center
2500 North State Street
Jackson, Mississippi 39216, USA

Abstract

Dormant and developing embryos of *Artemia* contain equivalent amounts of eIF-2, the eukaryotic chain initiation factor which forms a ternary complex with GTP and Met-tRNA$_f$. After purification, preparations of eIF-2 from both types of embryos have similar specific activities and are immunologically identical, and each stimulates amino acid incorporation in a cell-free system of protein synthesis.

The binding of [^{35}S]Met-tRNA$_f$ to 40S ribosomal subunits is catalyzed by eIF-2 isolated from dormant or developing embryos, and is dependent upon GTP and AUG. Another protein, cofactor 1, present in the 0.5 M NH_4Cl ribosomal wash, stimulates 2-3-fold the binding of [^{35}S]Met-tRNA$_f$ to 40S ribosomal subunits, as well as the formation of the eIF-2 ternary complex. This factor does not exhibit GTP-dependent binding of [^{35}S]Met-tRNA$_f$.

The eukaryotic chain initiation factor eIF-3, consisting of numerous polypeptides, was isolated by chromatography on heparin-Sepharose 4B, DEAE-cellulose, hydroxylapatite and Ultrogel AcA-34, and by centrifugation on sucrose density gradients. As assayed by its ability to stimulate [^{35}S]Met-tRNA$_f$ and (^{125}I)-labeled mRNA binding to 40S ribosomal subunits in the presence of eIF-2, the concentration of this factor is at least 5-fold greater in developing than in dormant embryos. Experiments are in progress to determine the significance of this difference in eIF-3 activity.

Introduction

During embryogenesis of *Artemia*, gastrulae may develop to adults, or may encyst, enter a dormant state and be released into the environment where they may become dessicated (Finamore and Clegg, 1969 ; Hentschel and Tata, 1976 ; Olson and Clegg, 1978). Cell-free extracts from dormant embryos contain active mRNA (Nilsson and Hultin, 1975 ; Sierra *et al.*, 1976 ; Amaldi *et al.*, 1977 ; Grosfeld *et al.*, 1977) large quantities of 80S ribosomes (Golub and Clegg, 1968 ; Hultin and Morris, 1968) elongation factors (Slobin and Möller, 1976a,b), termination factors (Reddington *et al.*, 1978) and aminoacyl tRNA synthetases (Bagshaw *et al.*, 1970) ; however, they are unable to translate natural mRNA (Sierra *et al.*, 1974). The absence of protein synthesis in cell-free extracts has been attributed partially to a deficiency of chain initiation factors (Filipowicz *et al.*, 1975 ; Filipowicz *et al.*, 1976). The GTP-dependent Met-tRNA$_f$ binding protein, initiation factor eIF-2, was reported to increase over 20-fold following resumption of embryo development (Filipowicz *et al.*, 1975). According to those results, embryo development subsequent to encystment would depend upon the replacement

of depleted or inactive initiation factors. Furthermore, the absence or inactivation of chain initiation factors in cell-free extracts of dormant embryos, may reflect intracellular conditions prior to encystment under which initiation factor deficiencies may trigger cessation of protein synthesis and eventual cyst formation.

To test some of these ideas, we began to purify and characterize chain initiation factors from dormant and developing embryos of *Artemia*. We have shown that eIF-2 is present in equivalent amounts at all stages of embryo development. In addition, a protein which stimulates eIF-2 activity was found in the 0.5 M NH_4Cl ribosomal washes (MacRae *et al.*, 1979). Embryo extracts were assayed for eIF-3, the eukaryotic chain initiation factor which is required for translation of natural mRNA and facilitates the binding of the eIF-2 ternary complex to 40S ribosomes (Schreier and Staehelin, 1973 ; Majumdar *et al.*, 1977 ; Trachsel *et al.*, 1977 ; Benne and Hershey, 1978). The quantity of eIF-3 in dormant embryos was only 20% of the amount in developing embryos. The results presented here indicate that eIF-3 and not eIF-2, as previously reported (Filipowicz *et al.*, 1975 ; Filipowicz *et al.*, 1976), may limit protein synthesis and resumption of embryo development subsequent to cryptobiosis.

Materials and methods

Preparations

Aminoacyl-tRNA

[^{35}S]Met-$tRNA_f$ was prepared by charging unfractionated yeast tRNA with [^{35}S]methionine in the presence of *E. coli* synthetase (RajBhandary and Ghosh, 1969 ; Gupta *et al.*, 1970).

(^{125}I)-labeled mRNA

Iodinated poly A-rich hemoglobin mRNA was prepared by the procedure of Getz *et al.* (1972). The specific activity of the (^{125}I)-labeled mRNA was 12.3×10^6 cpm/A_{260} unit when measured in a toluene-based scintillation fluid in a Beckman LS-9 000 counter.

Antisera to eIF-2

Antisera to eIF-2 from developing embryos were obtained from 4 month old mice which had been injected intramuscularly with the phosphocellulose fraction of eIF-2 (MacRae *et al.*, 1979).

Embryo development

Dormant embryos of *Artemia* were sterilized in Clorox diluted with an equal volume of water. Sterilized embryos were washed six times with water and collected by suction. For development, 500 g wet weight of sterilized embryos were suspended in 5 l of artificial seawater, and incubated 11 hr at 30 °C with vigorous aeration (Warner *et al.*, 1979). For the preparation of chain initiation factors from dormant embryos, cysts were not sterilized, but hydrated for 3 hr at 4 °C in 0.5 M NaCl.

Preparation of ribosomes and 40S ribosomal subunits

Hydrated embryos were ground in a motorized mortar and pestle (Warner *et al.*, 1979). The ground embryos were suspended in buffer A (KCl, 5 mM ; magnesium acetate, 10 mM ; 2-

mercaptoethanol, 10 mM ; Tris·Cl, pH 7.4, 50 mM), stirred for 15 min and centrifuged at 20 000 × g for 20 min. The supernatant was filtered through cheese cloth and glass wool, centrifuged at 37 000 × g for 30 min and the resulting supernatant was centrifuged at 150 000 × g for 2 hr. The ribosomal pellets thus obtained were resuspended overnight in buffer B (NH_4Cl, 500 mM ; magnesium acetate, 5 mM ; dithiothreitol, 1 mM ; Tris·Cl, pH 7.4, 50 mM), and this suspension was then centrifuged at 150 000 × g for 2 hr. 40S ribosomal subunits were prepared from the pellets and the high salt wash was stored at -70 °C for the preparation of eIF-2, eIF-3 and cofactor 1 (MacRae *et al.*, 1979 ; Warner, *et al.*, 1979).

Assays

Ternary complex formation with eIF-2, GTP, and [^{35}S]Met-tRNA$_f$, 40S ribosomal binding of [^{35}S]Met-tRNA$_f$, and translation of natural mRNA were performed as previously described (MacRae *et al.*, 1979). eIF-3 was assayed by its ability to stimulate eIF-2-dependent binding of [^{35}S]Met-tRNA$_f$ to 40S ribosomal subunits in the presence of AUG or (^{125}I)-labeled mRNA.

Isolation of chain initiation factors

Purification of eIF-2 and cofactor 1

These proteins were purified from the 0.5 M NH_4Cl ribosomal wash of *Artemia*. eIF-2 and cofactor 1 activities were found in the 35-60 % $(NH_4)_2SO_4$ fraction of the ribosomal wash. After dialysis, this fraction was chromatographed on heparin-Sepharose 4B and DEAE-cellulose. Cofactor 1 was not retained on DEAE-cellulose, whereas eIF-2 was eluted with a linear gradient from 50-350 mM KCl. Further purification of eIF-2 was achieved by chromatography on hydroxylapatite and phosphocellulose (MacRae *et al.*, 1979).

Purification of eIF-3

This factor was found in the 0-40 % $(NH_4)_2SO_4$ fraction of the 0.5 NH_4Cl M wash of *Artemia* ribosomes. This fraction, after dialysis against buffer C (Tris·Cl, pH 7.4, 50 mM ; KCl, 50 mM ; magnesium acetate, 0.2 mM ; 2-mercaptoethanol, 10 mM ; and glycerol, 10 %) was adsorbed and eluted stepwise from heparin-Sepharose 4B with increasing concentrations of KCl. The 0.5 M KCl eluate containing eIF-3 activity was further purified on DEAE-cellulose in buffer C. Fractions eluting between 170 and 300 mM KCl (with a linear gradient from 50-400 mM KCl) were dialyzed, adsorbed to hydroxylapatite and eluted stepwise with increasing concentrations of phosphate. eIF-3, which eluted with 500 mM potassium phosphate, was concentrated by addition of ammonium sulfate, dialyzed, and purified further by filtration on Ultrogel AcA-34 and by sucrose density gradient centrifugation.

Results

The total [^{35}S]Met-tRNA$_f$ bound in a ternary complex by the ribosomal salt washes and the post-ribosomal supernatants from dormant and developing embryos was identical (Table I). Variations in the length of embryo hydration and development did not alter the specific activity of the eIF-2 as measured by ternary complex formation (Table II).

TABLE I

eIF-2 activity in *Artemia* embryos

Embryo description	[^{35}S]Met-tRNA$_f$ bound per 100 g wet weight of embryos		
	Ribosomal salt wash[1] (pmol)	Post-ribosomal supernatant[2] (pmol)	Total (pmol)
Dormant	61	55	116
Developing (11 hr)	71	39	110

[1] 35-60% $(NH_4)_2SO_4$ fractions of NH_4Cl washes of ribosomes were used.

[2] Post-ribosolal supernatants were chromatographed on heparin-Sepharose 4B.

TABLE II

eIF-2 activity in dormant and developing embryos of *Artemia*

Embryo description	[^{35}S]Met-tRNA bound[1] (pmol/mg protein)
Dormant (1 hr hydration)	3.0
Dormant (3 hr hydration)	4.0
Dormant (24 hr hydration)	4.2
Developing (11 hr)	3.5
Emerged (nauplii)	3.1

[1] The specific activity of the eIF-2 fractions in the formation of a ternary complex was determined with 35-60% $(NH_4)_2SO_4$ fractions of ribosomal washes.

As previously observed (Van der Mast *et al.*, 1977 ; Hradec and Dusek, 1978), eIF-2 activity in $(NH_4)_2SO_4$ fractions was found to adsorb to heparin-Sepharose 4B. The elution pattern of eIF-2 from DEAE-cellulose is shown in Fig. 1. The column was initially washed with buffer containing 50 mM KCl and the fraction not adsorbed to DEAE-cellulose contained a factor, termed here cofactor 1, which stimulates 2-3-fold ternary complex formation with eIF-2. Therefore, in this and subsequent purification steps, 40 μg of cofactor 1 (DEAE-cellulose step) were added in the assays for eIF-2 activity.

At each stage of purification, the specific activity of eIF-2 from dormant and developing embryos was similar (Tables III, IV). A 123-150-fold purification of eIF-2, with a final specific activity of approximately 300 units/mg of protein and an overall yield of 5-7% was obtained. As shown by SDS gel electrophoresis (Fig. 2), phosphocellulose-purified eIF-2 consisted of three polypeptides with molecular weights of 52 000, 38 000 and 36 000. Based on densitometer tracings of the gels, the eIF-2 from either dormant or developing embryos was about 70% pure with the polypeptides present in a ratio of 1.00:1.24:1.20.

Preparations of eIF-2 from *Artemia* embryos participate in the formation of a 40S initiation complex. Maximal binding of [^{35}S]Met-tRNA$_f$ to 40S ribosomal subunits required GTP and

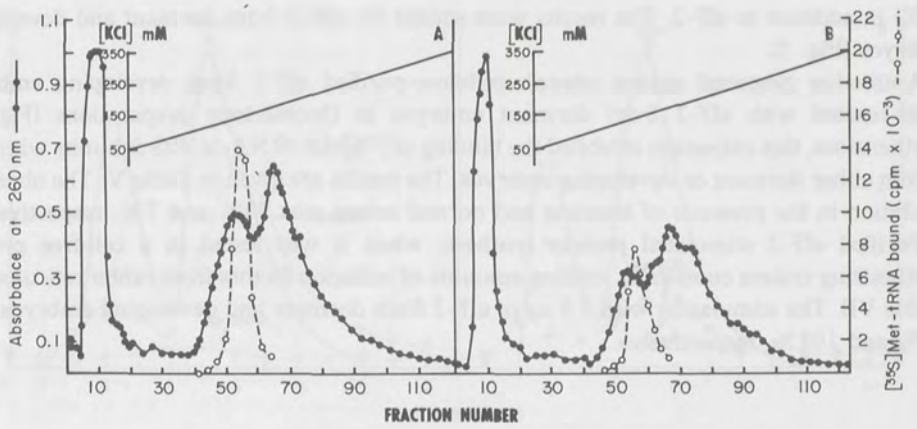

FIG. 1. Elution of eIF-2 from DEAE-cellulose. Chromatography of (A) 127 mg of step 3 eIF-2 from 5 064 g wet weight of dormant embryos, and (B) 112 mg of step 3 eIF-2 from 3 213 g wet weight of developing embryos was as described in Materials and Methods. Aliquots of each fraction (20 μl) were assayed for GTP-dependent [^{35}S]Met-tRNA$_f$ binding. [^{35}S]Met-tRNA$_f$ binding, ○ - - - - ○; absorbance at 280 nm, ● —— ●. From MacRae *et al.* (1979).

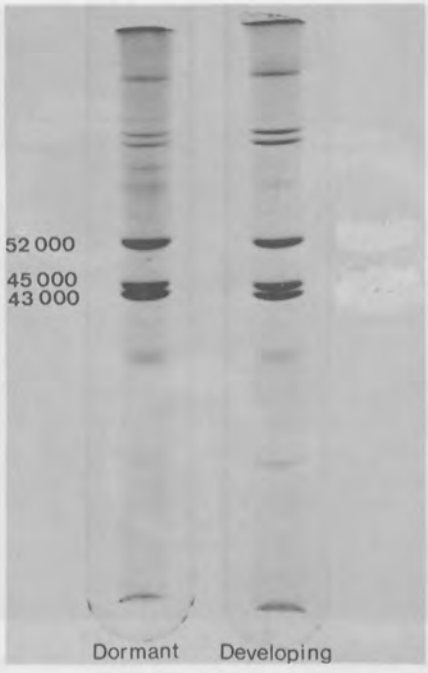

FIG. 2. SDS-polyacrylamide gel electrophoresis of the phosphocellulose fraction of eIF-2. From MacRae *et al.* (1979).

AUG in addition to eIF-2. The results were similar for eIF-2 from dormant and developing embryos (Fig. 3).

Antibodies prepared against phosphocellulose-purified eIF-2 from developing embryos cross-reacted with eIF-2 from dormant embryos in Ouchterlony preparations (Fig. 4). Furthermore, this antiserum inhibited the binding of [^{35}S]Met-tRNA$_f$ to 40S subunits with eIF-2 from either dormant or developing embryos. The results are given in Table V. The observed inhibition in the presence of immune and normal serum was 90% and 7%, respectively.

Purified eIF-2 stimulated protein synthesis when it was tested in a cell-free protein synthesizing system containing limiting amounts of initiation factors from rabbit reticulocytes (Table VI). The stimulation with 3.8 μg of eIF-2 from dormant and developing embryos was 72% and 108%, respectively.

TABLE III

Purification of eIF-2 from dormant embryos of *Artemia*

Step	Total protein (mg)	Total activity (units[1])	Specific activity (units/mg)	Yield (%)
1. 0.5 M NH_4Cl wash	1 649	3 298	2.0	100
2. 35-60% $(NH_4)_2SO_4$ fraction	671	3 489	5.2	106
3. Heparin-Sepharose 4B	127	2 286	18	69
4. DEAE-cellulose	15.7	1 036	66	31
5. Hydroxylapatite	4.9	495	101	15
6. Phosphocellulose	0.8	240	300	7

[1] One unit = 1 pmol of [^{35}S]Met-tRNA$_f$ bound in a ternary complex under standard assay conditions (10 min, 30 °C) (MacRae *et al.*, 1979).

TABLE IV

Purification of eIF-2 from developing embryos of *Artemia*

Step	Total protein (mg)	Total activity (units[1])	Specific activity (units/mg)	Yield (%)
1. 0.5 M NH_4Cl wash	969	2 229	2.3	100
2. 35-60% $(NH_4)_2SO_4$ fraction	459	2 295	5.0	103
3. Heparin-Sepharose 4B	112	1 568	14	70
4. DEAE-cellulose	10.2	459	45	21
5. Hydroxylapatite	1.9	266	140	12
6. Phosphocellulose	0.4	113	283	5

[1] One unit = 1 pmol of [^{35}S]Met-tRNA$_f$ bound in a ternary complex under standard assay conditions (10 min, 30 °C) (MacRae *et al.*, 1979).

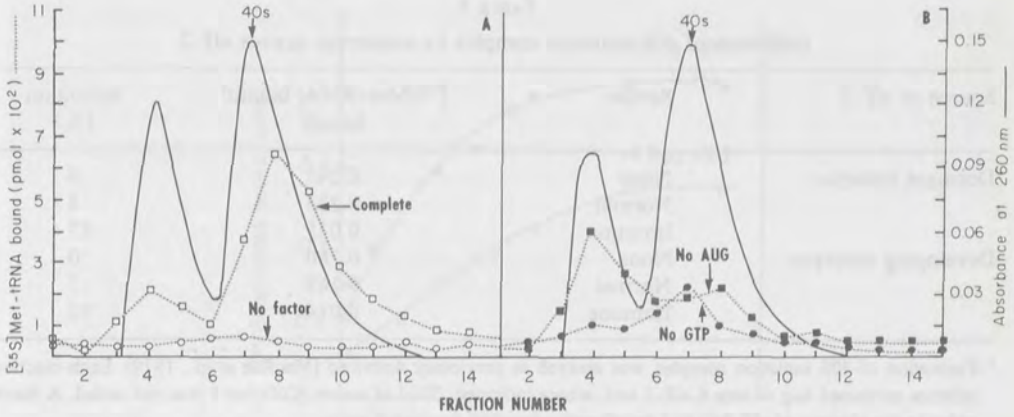

FIG. 3. 40S initiation complex formation with eIF-2 from dormant embryos. Binding of [^{35}S]Met-tRNA$_f$ to 40S ribosomal subunits with 5.7 μg of step 6 eIF-2 from dormant embryos. (A) Complete reaction mixture, □ ····· □ ; no factor added, ○ ····· ○ ; 260 nm absorbance, ——. (B) No AUG added, ■ ····· ■ ; no GTP added, ● ····· ● ; 260 nm absorbance, ——. Assay and centrifugation were as previously described (MacRae *et al.* 1979). Sedimentation was from left to right.

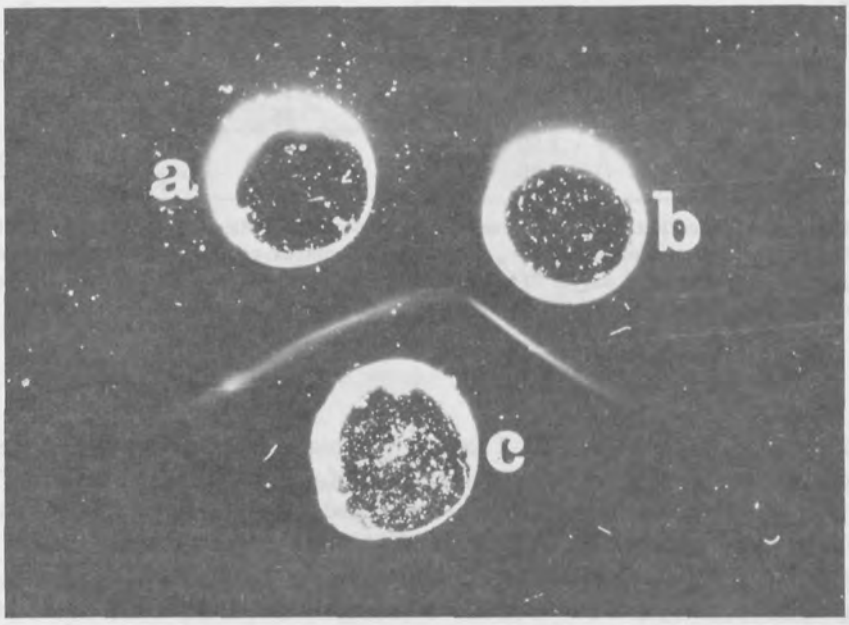

FIG. 4. Immunological cross-reactivity of eIF-2 from dormant and developing embryos. Mouse antibodies were prepared against step 6 eIF-2 from developing embryos. (a) contained 13 μg of step 6 eIF-2 from developing embryos ; (b) 6 μg of step 6 eIF-2 from dormant embryos ; (c) 25 μl of antiserum From MacRae *et al.* (1979).

TABLE V

Inhibition of 40S initiation complex by antiserum against eIF-2

Source of eIF-2	Serum	[^{35}S]Met-tRNA$_f$ bound[1] (pmol)	Inhibition (%)
Dormant embryos	None	0.245	0
	Normal	0.225	8
	Immune	0.031	87
Developing embryos	None	0.180	0
	Normal	0.169	7
	Immune	0.014	92

[1] Formation of 40S initiation complex was assayed as previously described (MacRae *et al.*, 1979). Each reaction mixture contained 6 μg of step 6 eIF-2 and, where indicated, 20 μl of serum. Cofactor 1 was not added. A blank value in the absence of eIF-2 (0.033 pmol) was subtracted from each value.

TABLE VI

Stimulation of polypeptide synthesis by eIF-2

Source of eIF-2	eIF-2 added (μg)	[^{14}C]Leucine incorporated[1] (pmol)	Stimulation (%)
—	–	70	0
Dormant embryos	0.76	89	27
	1.90	105	50
	3.80	121	72
Developing embryos	0.76	86	23
	1.90	116	66
	3.80	146	108

[1] Conditions for amino acid incorporation were as previously described (MacRae *et al.*, 1979). Each mixture contained 0.5 A_{260} unit of *Artemia* ribosomes, 0.15 A_{260} unit of poly(A)+ mRNA, and 300 μg of reticulocyte unfractionated ribosomal salt wash. Incubation was for 90 min at 30 °C. Blanks, in the absence of reticulocyte initiation factors or in the presence of eIF-2 from dormant or developing embryos, averaged 1.6 pmol, and were subtracted from each value.

Cofactor 1 may be involved in eIF-2 activity. This protein does not exhibit GTP-dependent binding of [^{35}S]Met-tRNA$_f$; however, with 4-6 μg of phosphocellulose-purified eIF-2 from either dormant or developing embryos, it stimulated 2-3-fold ternary complex formation (Fig. 5). Cofactor 1 isolated from DEAE-cellulose, from either dormant or developing embryo fractions, also stimulated 2-3-fold 40S ribosomal binding of [^{35}S]Met-tRNA$_f$ (Fig. 6).

The eukaryotic polypeptide chain initiation factor, eIF-3, was purified from 0.5 M NH_4Cl ribosomal washes of *Artemia* embryos. eIF-3 activity was found in the 0-40% $(NH_4)_2SO_4$ fraction of ribosomal washes and was adsorbed to heparin-Sepharose 4B. Chromatography on DEAE-cellulose partially separated eIF-3 from an inhibitor of cell-free protein synthesis which eluted at 50 mM KCl. Further purification of eIF-3 was achieved by chromatography

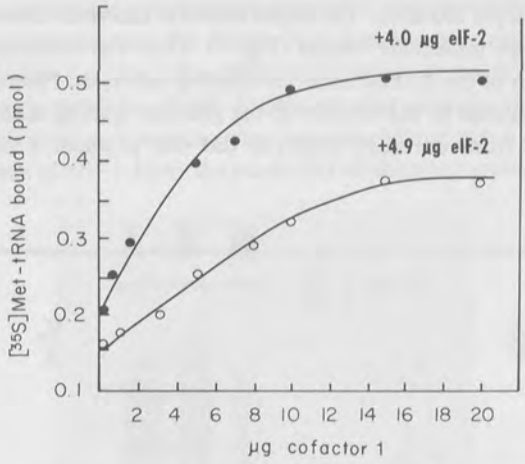

FIG. 5. *Effect of cofactor 1 on ternary complex formation.* Conditions were as described in MacRae *et al.* (1979). 4.9 μg of step 6 eIF-2 (stored in an ice bath) from dormant embryos, ○——○ ; 4.0 μg of step 6 eIF-2 (stored at -70°) from developing embryos, ●——●. Cofactor 1 (DEAE-cellulose step) was added as described.

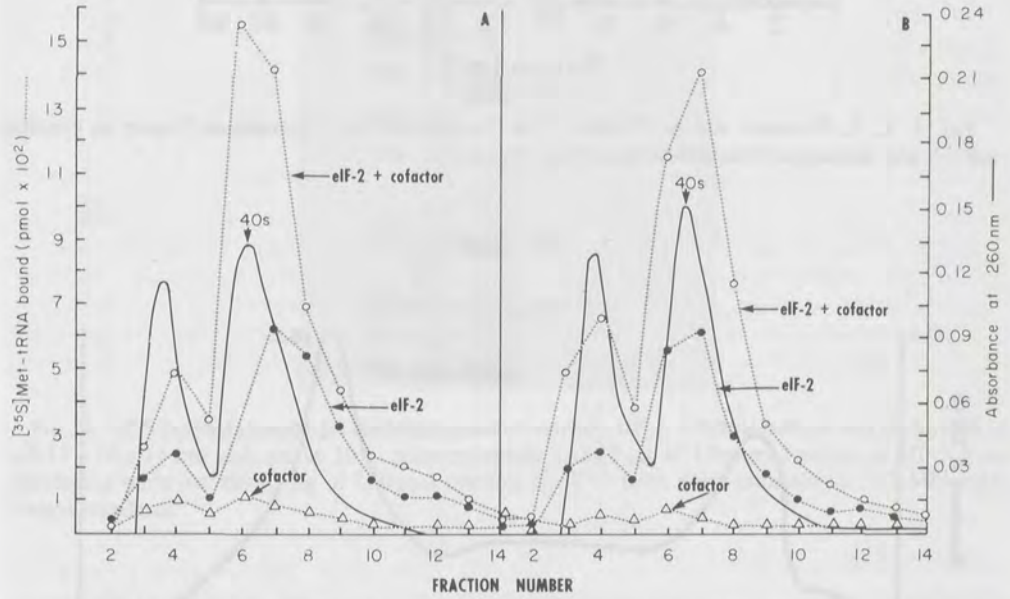

FIG. 6. Effect of cofactor 1 on eIF-2-dependent formation of 40S initiation complex. [35S]Met-tRNA$_f$ binding to 40S ribosomal subunits was assayed as in MacRae *et al.* (1979). (A) 40S initiation complex with 5.7 μg of step 6 eIF-2 from dormant embryos. (B) 40S initiation complex with 4.4 μg of step 6 eIF-2 from developing embryos. For panels A and B: 260 nm absorbance, ——; eIF-2 added, ●·····●; eIF-2 and cofactor 1 added, ○·····○; cofactor 1 added, but no eIF-2, △·····△. Where indicated, 38 μg of cofactor 1 (DEAE-cellulose step) were used per reaction mixture. Sedimentation was from left to right.

on hydroxylapatite and gel filtration. The factor eluted at the void volume of Ultrogel AcA-34, indicating it had a high molecular weight (Fig. 7). This was confirmed by sucrose density gradient centrifugation of the protein from developing embryos. Factor activity was found in the peak which sedimented to the bottom of the gradient during centrifugation (Fig. 8). The preparation of eIF-3 from dormant embryos did not produce a sharp peak on sucrose gradients.

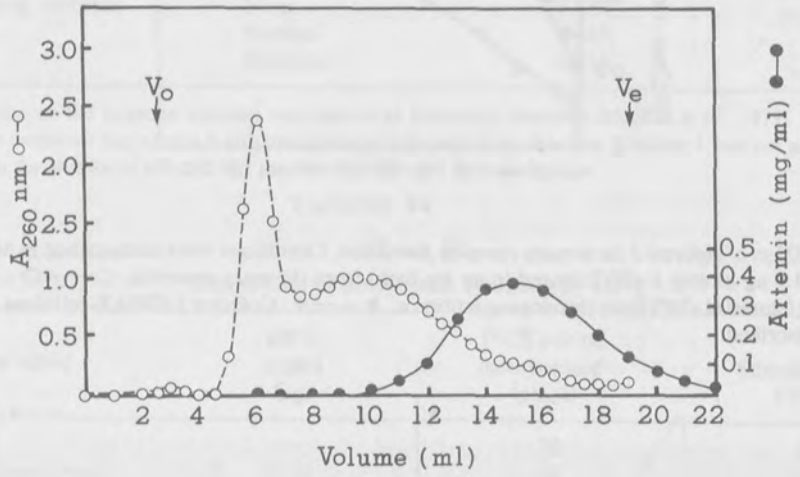

FIG. 7. C. L. Woodley and A. Wahba, "The Development of a Translation System to examine mRNA and messenger Ribonucloprotein from *Artemia*".

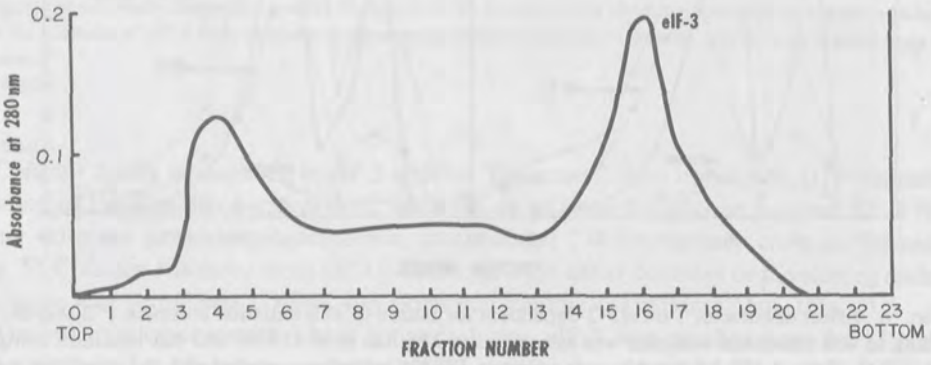

FIG. 8. Sucrose gradient purification of eIF-3. The Ultrogel fraction of eIF-3 from developing embryos was concentrated by ultrafiltration. A sample of 0.5 ml was layered onto a 11.6 ml, 5-15% linear sucrose gradient and centrifuged 20 hr at 40 000 rpm and 4 °C in a Beckman SW41 rotor.

Dodecylsulfate polyacrylamide gel electrophoresis of Ultrogel-purified eIF-3 from dormant and developing embryos shows that this factor from *Artemia* is a complex of many subunits (Fig. 9). At the present time, no detectable difference between these preparations of eIF-3 may be observed. Preliminary analysis of eIF-3 isolated after sucrose density gradient centrifugation indicates that several polypeptides may be removed and that a detailed comparison of the subunit composition of eIF-3 from dormant and developing embryos will be possible.

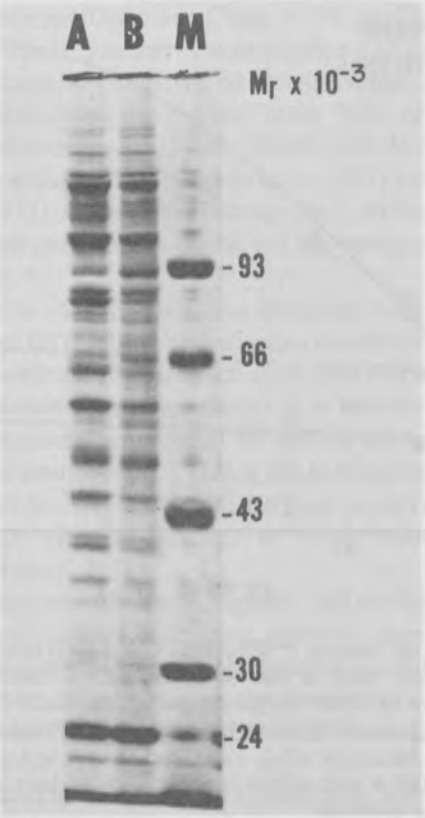

FIG. 9. SDS-polyacrylamide gel electrophoresis of *Artemia* eIF-3. Electrophoresis was performed in a $0.15 \times 10 \times 15$ cm slab gel in 10% polyacrylamide. (A) 20 μg of Ultrogel fraction of eIF-3 from developing embryos. (B) 25 μg of Ultrogel fraction of eIF-3 from dormant embryos. (C) Molecular weight standards.

The activity of eIF-3 was determined by its ability to stimulate AUG-dependent [^{35}S]Met-$tRNA_f$ binding to 40S ribosomal subunits in the presence of eIF-2. The specific activity of the heparin-Sepharose 4B fraction from dormant embryos was only 33% of that from developing embryos (Table VII). Similar results were obtained with the Ultrogel AcA-34 fractions of the factor from dormant and developing embryos. The amount of active eIF-3 in dormant embryos was determined to be only 20% as much as in developing embryos (Table VII).

Preparations of eIF-3 were also assayed for their ability to stimulate [^{35}S]Met-tRNA$_f$ and (^{125}I)-labeled hemoglobin mRNA binding to 40S ribosomal subunits in the presence of eIF-2 (Fig. 10). At all levels of factor tested, eIF-3 from developing embryos was at least 10-fold more active in stimulating the binding of [^{35}S]Met-tRNA$_f$ and (^{125}I)-labeled mRNA than eIF-3 from dormant embryos.

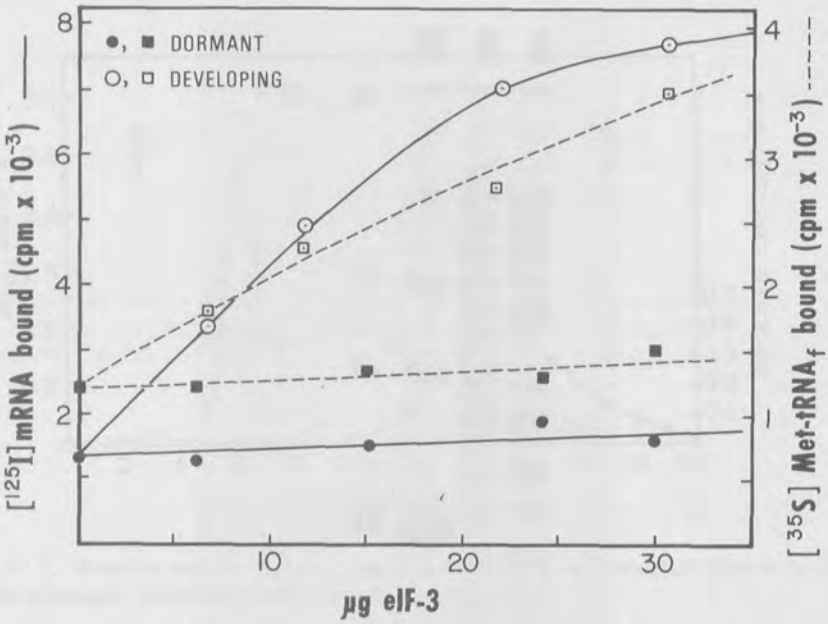

FIG. 10. Effect of eIF-3 on binding [^{35}S]Met-tRNA and (^{125}I)-labeled mRNA to 40S ribosomal subunits. Reaction conditions were as described for mRNA translation (Table VI), except no reticulocyte initiation factors or S100 fractions were added. Each 75 μl reaction contained: 40S ribosomal subunits, 0.45 A_{260} unit; [^{35}S]Met-tRNA$_f$, 3 pmols; (^{125}I)-labeled mRNA, 3 pmols (153 000 cpm); and phosphocellulose fraction of eIF-2, 3 μg. The Ultrogel AcA-34 fractions of eIF-3 were used. Incubation and analysis of tRNA and mRNA binding were as described in Fig. 3. ●, ■, dormant embryos; ○, □, developing embryos.

TABLE VII

eIF-3 activity in *Artemia* embryos

Embryo description	Specific activity (units/mg[1])	[^{35}S]Met-tRNA$_f$ bound per 100 g wet weight of embryos (pmol)
Developing	6	39
Dormant	2	8

[1] One unit = 1 pmol of [^{35}S]Met-tRNA$_f$ bound to 40S ribosomal subunits under standard assay conditions for AUG-directed binding. For each determination a blank, in the absence of initiation factors, was subtracted.

Discussion

Extracts from dormant and developing embryos were found to contain equivalent amounts of eIF-2. Factor activity in extracts of dormant embryos was comparable to that reported by others for developing embryos (Filipowicz *et al.*, 1975 ; Ochiai-Yanagi *et al.*, 1977) and much greater than what was reported for dormant embryos (Filipowicz *et al.*, 1975). Neither did the length of embryo hydration, nor the resumption of cell division which occurs upon emergence of developing embryos (Olson and Clegg, 1978), modify eIF-2 activity. The factor from either dormant or developing embryos was purified 125-fold, yielding a preparation with a specific activity of about 300 units/mg of protein. When analyzed by dodecylsulfate polyacrylamide gel electrophoresis, eIF-2 from either type of embryo exhibited three polypeptides with molecular weights of 52 000, 38 000, and 36 000. Preparations of eIF-2 from other sources, such as wheat germ (Spremulli *et al.*, 1979), rabbit reticulocytes (Benne *et al.*, 1976 ; Schreier *et al.*, 1977), ascites cells (Trachsel *et al.*, 1979) and pig liver (Harbitz and Hauge, 1976) are also composed of three subunits, but the middle polypeptide tends to have a molecular weight of 50 000.

Binding of [^{35}S]Met-tRNA$_f$ to 40S ribosomal subunits with eIF-2 from dormant or developing embryos required GTP and AUG, indicating the eIF-2 from either type of embryo functions similarly in 40S initiation complex formation. The GTP requirements are common to eIF-2 from all sources examined, whereas the AUG or mRNA requirements are less well defined. In some cases an absolute requirement for mRNA has been reported (Gupta *et al.*, 1975 ; Smith *et al.*, 1976 ; Chatterjee *et al.*, 1976), while in others mRNA exhibits a stabilizing effect (Harbitz and Hauge, 1976 ; Spremulli *et al.*, 1977), or is not required at all (Schreier and Staehelin, 1973 ; Adams *et al.*, 1975 ; Filipowicz *et al.*, 1975 ; Nombela *et al.*, 1976 ; Trachsel *et al.*, 1977 ; Benne and Hershey, 1978).

Immunological identity between eIF-2 from dormant and developing embryos was shown. Antibodies raised against eIF-2 from developing embryos cross-reacted with the protein prepared from dormant embryos in Ouchterlony preparations and 40S initiation complexes. Immunological cross-reactivity, in concert with the stimulation of polypeptide synthesis by eIF-2 from both types of embryos, provide further evidence that the factor is not modified during development of *Artemia*.

Cofactor 1, which stimulated ternary complex formation, as well as [^{35}S]Met-tRNA$_f$ binding to 40S ribosomal subunits, was found in dormant and developing embryos. Factors with similar activities, isolated from rabbit reticulocytes (Dasgupta *et al.*, 1976 ; Dasgupta *et al.*, 1978 ; Malathi and Mazumder, 1978) and developing *Artemia* embryos (de Haro *et al.*, 1978) have been reported. The relationship of cofactor 1 to the other eIF-2 stimulatory proteins and its role in protein synthesis remain to be determined.

The eukaryotic initiation factor eIF-3 was found in other laboratories to stimulate or stabilize Met-tRNA$_f$ and mRNA binding to 40S ribosomal subunits in the presence of eIF-2 (Majumdar *et al.*, 1977 ; Benne and Hershey, 1978). The factor from developing embryos of *Artemia* was also observed to possess these activities. The increase in [^{35}S]Met-tRNA$_f$ and (^{125}I)-labeled hemoglobin mRNA binding to 40S ribosomal subunits was 3-fold and 7-fold, respectively (Fig. 10).

Precipitation of eIF-3 in the 0-40 % $(NH_4)_2SO_4$ fraction of ribosomal washes, its large size as indicated by Ultrogel AcA-34 chromatography and sucrose density gradient centrifugation, as

well as its complex banding pattern on dodecylsulfate gel electrophoresis, strongly suggest that the *Artemia* factor which stimulates Met-tRNA$_f$ and mRNA binding to 40S subunits is similar to eIF-3 from other sources (Frienstein and Blobel, 1975 ; Safer *et al.*, 1976 ; Benne and Hershey, 1976 ; Ilan and Ilan, 1976 ; Schreier *et al.*, 1977 ; Thompson, *et al.*, 1977 ; Trachsel *et al.*, 1979).

With the AUG-directed binding assay (Table VII), the specific activity of eIF-3 was determined to be 3-fold greater and its concentration 5-fold more in developing than in dormant embryos. However, in the natural mRNA binding assay (Fig. 10), eIF-3 from developing embryos was at least 10-fold more active. In addition, the factor from each type of embryo behaves differently in its ability to stimulate binding of [^{35}S]Met-tRNA$_f$ to 40S subunits in the absence of message, a reported eIF-3 activity (Schreier and Staehelin, 1973 ; Trachsel *et al.*, 1977 ; Benne and Hershey, 1978). Only the factor from developing embryos was found to stimulate eIF-2-mediated binding of [^{35}S]Met-tRNA$_f$ to 40S subunits in the absence of message (data not presented).

Our results, in contrast to Filipowicz *et al.* (1975), demonstrate that dormant and developing embryos of *Artemia* contain equivalent amounts of eIF-2. Both types of embryos also contain a cofactor which stimulates eIF-2 activity. On the other hand, the quantity of eIF-3 is much less in dormant than in developing embryos. These data suggest that a modification of eIF-3 may occur during early embryo development, which could lead to cessation of protein synthesis and eventual encystment of embryos. Furthermore, eIF-3, and not eIF-2 as previously suggested, may limit the resumption of embryo development in *Artemia*. The modified eIF-3 may permit selective translation of various classes of mRNA, a function suggested for eIF-3 from other sources (Ilan and Ilan, 1976 ; Gette and Heywood, 1979). Such interesting possibilities are currently under investigation.

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The development of a translation system to examine mRNA and messenger ribonucleoproteins from *Artemia*

Charles L. Woodley and Albert J. Wahba

Department of Biochemistry, University of Mississippi Medical Center
2500 North State Street, Jackson, Mississippi 39216, USA

Abstract

A cell-free protein synthesis system was developed to determine which factors control the efficiency of mRNA translation. The reaction mixtures contained 0.5 M KCl-washed ribosomes from dormant *Artemia* embryos, yeast tRNA, rabbit reticulocyte initiation factors and a supernatant fraction. Two unusual low molecular weight components, as well as mRNP particles from dormant *Artemia* embryos, were examined for their involvement in protein synthesis. At 2.5 mM Mg^{2+} and 50 μM spermine, [^{14}C]leucine incorporation with poly(A)-rich mRNA (0.12 A_{260} unit), 220 pmol, and with polysomal RNA (2.3 A_{260} units), was 22 pmol. However, by the addition of 150-200 μM spermine, the translation of poly(A)-rich mRNA was greatly reduced (12 pmol [^{14}C]leucine incorporated) and polysomal RNA was 2-5-fold better as a template. With the high spermine concentration, the effficiency of poly(A)-rich mRNA translation was partially restored by the addition of an unusual low molecular weight component isolated from the post-mitochondrial supernatant of dormant *Artemia* embryos.

Free cytoplasmic mRNP particles were isolated from post-mitochondrial supernatants of dormant *Artemia* embryos. The major proteins associated with these particles have molecular weights of 32 000, 30 500, 27 000, 26 000, 24 000 and 20 500 as determined by SDS-polyacrylamide gel electrophoresis. Immunological cross-reactivity indicates that these particles contain significant amounts of artemin, a major protein found in *Artemia* cysts (Slobin, 1980).

Introduction

Encysted embryos of *Artemia* are characterized by the absence of protein synthesis (Sierra *et al.*, 1974), the accumulation of 80S ribosomes (Golub and Clegg, 1968 ; Hultin and Morris, 1968), along with mRNP particles (Nilsson and Hultin, 1974, 1975 ; Grosfeld and Littauer, 1975 ; Slegers and Kondo, 1977), artemin, which is a major protein of *Artemia* cysts (De Herdt *et al.*, 1979 ; Slobin, 1980), and large concentrations of unusual nucleotides (Warner and Finamore, 1967). With development, protein synthesis resumes, but at a slow rate, and most ribosomes remain as 80S monosomes (Golub and Clegg, 1968 ; Hultin and Morris, 1968). The fate of free cytoplasmic mRNP particles during development is as yet unknown. Grosfeld and Littauer (1975) suggested that these mRNP particles serve as a stored, cryptic form of mRNA in the dormant embryos which upon resumption of development shift into the ribosome pool.

We have examined various factors which may regulate the efficiency of polypeptide chain initiation during development. A cell-free system for protein synthesis analogous to that of Kramer *et al.* (1975) was established. It consists of salt washed 80S ribosmes from dormant *Artemia* embryos, and rabbit reticulocyte initiation and elongation factors. In this communication we will present preliminary evidence for the presence of a low molecular weight component in S15 extracts from dormant *Artemia* embryos which stimulates the translation of poly(A)-rich mRNA at high polyamine concentrations. The base in this compound is an unusual residue which has not been identified. We have also isolated two size classes of mRNPs from dormant *Artemia* embryos by rate-sedimentation and isopycnic sucrose gradient fractionation, and chromatography on CL-Sepharose 4B. The proteins associated with these mRNP particles consist primarily of six polypeptides having molecular weights of 32 000, 30 500, 27 000, 26 000, 24 000, and 20 500. One of these proteins, the 27 000 M_r polypeptide, corresponds to artemin which is a major protein in dormant *Artemia* embryos (De Herdt *et al.*, 1979 ; Slobin, 1980).

Materials and methods

Preparations

Initiation factors and aminoacyl-tRNA synthetases

Unfractionated initiation factors were obtained by washing rabbit reticulocyte ribosomes with buffer containing 0.5 M KCl and 3 mM Mg^{2+}. Crude aminoacyl-tRNA synthetases were prepared from the pH 4.8 precipitate of the rabbit reticulocyte S100 fraction (MacRae *et al.*, 1979).

Reticulocyte mRNA

Unfractionated reticulocyte polysomal RNA was prepared by phenol/SDS extraction of 0.5 M KCl washed rabbit reticulocyte ribosomes. Poly(A)-rich hemoglobin mRNA was prepared by chromatography of reticulocyte polysomal RNA on oligo dT-cellulose (Aviv and Leder, 1972).

Artemia *mRNP*

Washed and hydrated cysts (7.5 g) were ground according to the procedure of Grosfeld and Littauer (1975). Shells and unground cysts were removed by filtration through cheese cloth, and membranes, nuclei and mitochondria were pelleted by centrifiguation for 15 min at 15 000 × g. Two ml aliquots were layered onto 36 ml 15-30% linear sucrose gradients in buffer A (KCl, 200 mM ; $MgCl_2$, 5 mM ; Tris·Cl, pH 7.5, 20 mM) and centrifugated for 15 hr at 24 000 rpm and 4 °C in a Beckman SW 27 rotor. After fractionation, the 20S to 55S region was pooled, diluted 3 fold with buffer A, and centrifuged 6 hr at 43 000 rpm in a Beckman 45Ti rotor. The pellets were suspended in 2 ml of buffer A and layered onto a 10 ml isopycnic sucrose gradient formed from 2 ml layers of 28, 34, 41, 64, and 87% sucrose in buffer A. The gradients were centrifuged 65-75 hr at 35 000 rpm in a Beckman SW41 rotor. After fractionation, the A_{260} peak sedimenting between $\rho = 1.27$ and 1.32 g/cm^3 was diluted 4-fold with buffer A and pelleted by centrifugation at 60 000 rpm for 2 hr in a Beckman 65 rotor.

The mRNP particles were further characterized by chromatography on CL-Sepharose 4B in buffer containing 100 mM NH_4Cl, 2.5 mM $MgCl_2$, and Tris·Cl, pH 7.5, 10 mM.

Preparation of Artemia *ribosomes*

0.5 M NH_4Cl-washed 80S ribosomes were prepared as previously described (MacRae *et al.*, 1979).

Isolation of low molecular weight components from Artemia *cysts*

Dormant embryos of *Artemia* (100 g dry weight) were hydrated at 4 °C (Warner and Finamore, 1967) and ground in Tris·Cl, pH 7.5, 50 mM ; KCl, 200 mM ; EDTA, 10 mM ; sucrose, 0.44 M. Shells and unground embryos were removed by filtration through cheese cloth, and membranes were pelleted by centrifugation at 15 000 × g. The resulting S15 was made 75% in ethanol and allowed to stand 5 hr at -20 °C. The precipitate was collected by centrifugation for 15 min at -20 °C and 16 000 × g, dried under vacuum and dissolvd in Tris·Cl, pH 9.0, 10 mM ; SDS, 1% ; NaCl, 100 mM ; EDTA, 2 mM. This fraction was extracted with phenol:chloroform:isoamyl alcohol (1:1:0.02). The aqueous phase was re-extracted twice with chloroform, and made 75% in ethanol. After precipitation overnight at -20 °C, the RNA was collected by centrifugation, dissolved in 2 ml of Tris·Cl, pH 7.5, 10 mM, and EDTA, 1 mM, and chromatographed on a Bio-Gel P-150 column (2.5 × 500 cm) equilibrated with the same buffer. Fractions containing the low molecular weight components (900 A_{260} units) were pooled and applied to a 1 × 6 cm column of Norite A. The column was washed with Tris·Cl, pH 7.5, 10 mM ; EDTA, 1 mM to remove any unadsorbed material. The Norite A column was then thoroughly washed with water and the material which eluted (189 A_{260} units) was pooled, made 100 mM in ammonium acetate, pH 8.5 (at 1 M and 23 °C) and applied to a DEAE-cellulose column (1 × 30 cm) equilibrated with the same buffer. The column was eluted with a 250 ml linear gradient (100-300 mM) of ammonium acetate. The peak fractions eluting at 200 and 230 mM ammonium acetate were lyophilyzed separately. The fraction eluting at 200 mM ammonium acetate contained 7.4 A_{260} units and the 230 mM ammonium acetate eluate contained 8.5 A_{260} units.

ASSAYS

Protein synthesis

The translation of polysomal RNA or poly(A)-rich mRNA from rabbit reticulocytes was performed as previously described (MacRae *et al.*, 1979). Reaction mixtures contained, in a final volume of 75 μl : K-Hepes, pH 7.8, 20 mM ; KCl, 30 mM ; NH_4Cl, 60 mM ; $MgCl_2$, 2.5 ; dithiothreitol, 1 mM ; ATP, 1 mM ; GTP, 0.2 mM ; creatine phosphate, 8 mM ; creatine kinase (EC 2.7.3.2), 4 μg ; 0.5 M KCl washed *Artemia* ribosomes, 0.5 A_{260} units ; yeast tRNA, 2.7 A_{260} units ; [^{14}C]leucine (750 cpm/pmol), 18 μM ; each of the remaining unlabeled 19 amino acids, 50 μM ; spermine as indicated ; pH 4.8 fraction of reticulocyte S100, 80 μg ; 0.5 M KCl reticulocyte ribosomal salt wash, 0.25-0.30 mg ; and either poly(A)-rich hemoglobin mRNA, 0.15 A_{260} units or reticulocyte polysomal RNA, 1.5-2 A_{260} units. After incubation for 90 min at 30 °C, the amount of [^{14}C]leucine incorporated into hot trichloroacetic acid insoluble material was determined.

Purity of low molecular weight Artemia *component*

The purity of the low molecular weight components frm the *Artemia* S15 were determined by thin layer chromatography on mylar-backed cellulose sheets (EM Laboratories). Two solvent systems used for routine analysis were : Solvent A : 1 M sodium phosphate, pH 6.8 (10 ml):H_2O (90 ml):n-propanol (2 ml):ammonium sulfate (60 g) ; Solvent B : isobutyric acid (66 ml):NH_4OH (1 ml):H_2O (33 ml). Additional solvent systems used for characterization of the low molecular weight components included : Solvent C : NH_4OH (1.7 ml):isobutyric acid (66 ml):H_2O (33 ml) ; Solvent D : sat. $(NH_4)_2SO_4$ (80 ml):8.2% sodium acetate (18 ml):isopropanol (2 ml).

The purified components which eluted at 200 and 230 mM ammonium acetate from DEAE-cellulose were further characterized by their UV absorption spectra in acidic, neutral or alkaline buffers.

Results

A cell-free *in vitro* translation system was developed using ribosomes from dormant *Artemia* embryos. The effect of spermine and Mg^{2+} concentrations on the translation of rabbit reticulocyte poly(A)-rich mRNA and reticulocyte polysomal RNA was investigated. In the presence of spermine at 27-50 μM, poly(A)-rich mRNA translation was enhanced approximately 2-fold (Fig. 1). At concentrations greater than 50 μM, spermine reduced the level of translation of exogenous mRNA and the background incorporation was increased, probably due to the presence of mRNA in the reticulocyte initiation factor preparation (Kramer *et al.*, 1975). The Mg^{2+} optimum for translation of poly(A)-rich mRNA at various spermine concentrations was in the narrow range of 2.2-2.8 mM (Woodley *et al.*, unpublished observations).

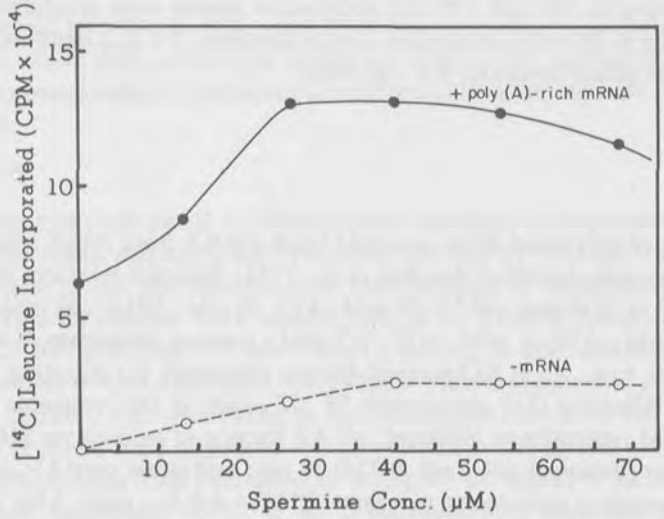

FIG. 1. Effect of spermine on protein synthesis. Translation with the *Artemia* ribosome system was as described in Materials and Methods. Poly(A)-rich mRNA, 0.125 A_{260} unit ; Mg^{2+}, 2.5 mM.

The stimulation of [^{14}C]leucine incorporation by reticulocyte polysomal RNA was only 50% at 27 μM spermine, whereas with poly(A)-rich mRNA amino acid incorporation was increased 8-fold over background (Table I). However, as the spermine level was raised to 200 μM, the background is reduced 7-fold and polysomal RNA translation was increased 40%. Therefore, the net stimulation of polysomal RNA translation at 200 μM was 14-fold. At this high level of spermine, poly(A)-rich mRNA translation was greatly inhibited, and amino acid incorporation was only 2-fold over background.

TABLE I

Effect of spermine on protein synthesis

Additions	[^{14}C]Leucine incorporated		
	Spermine added		
	27 μM	67 μM	200 μm
	pmol		
None	30.0	27.6	4.4
Polysomal RNA	45.2	47.9	62.5
Poly(A)-rich mRNA	176.7	97.1	8.3
Poly(A)-rich mRNA + low M.W. RNA[1]	152.8	156.8	32.8

Conditions for amino acid incorporation were as described in Materials and methods. Where indicated 2.3 A_{260} units polysomal RNA or 0.15 A_{260} unit poly(A)-rich mRNA and 0.14 A_{260} unit of the low molecular weight RNA.
[1] Isolated after chromatography on Bio-Gel P-150 and elution from charcoal.

A low molecular weight component which partially reversed the spermine inhibition of poly(A)-rich mRNA translation was isolated from the S15 fraction of dormant *Artemia* embryos. At low spermine concentrations (27-50 μM), this low molecular weight component had no effect or was slightly inhibitory (Table I). At 200 μM spermine, poly(A)-rich mRNA translation was only 2-fold over background, whereas with optimum concentrations of this low molecular weight compound translation was 7.5-fold greater than background.

The effect of 18S RNA on the translation of poly(A)-rich mRNA was studied (Table II). At 2.5 mM Mg^{2+} and 200 μM spermine, the incorporation of [^{14}C]leucine in response to poly(A)-rich mRNA was 2.4-fold over background. The addition of 18S RNA or the low molecular weight component resulted in the same degree of stimulation (11-fold over background).

This low molecular weight component was extensively purified according to the scheme shown in Fig. 2. A standard preparation from 100 g (dry weight) cysts produced approximately 4 000 A_{260} units of S15 RNA, from which 900-1 000 A_{260} units of low molecular weight nucleotides were recovered after chromatography on Bio-Gel P-150. The predominant nucleotide in this pool is diguanosine tetraphosphate (Warner and Finamore, 1967). Approximately 20% of the A_{260} material adsorbed to Norite A was eluted with water. This elution was dependent on the presence of EDTA in the sample buffer. The UV spectrum and thin layer chromatography showed that EDTA was not the unknown low molecular weight compound (Woodley, *et al.*, unpublished observations). This component was further purified by chromatography on DEAE-cellulose and three main peaks were resolved. The fractions

TABLE II

Effect of added low molecular weight component on translation of mRNA

Additions	[^{14}C]Leucine incorporation
	pmol
None	5.3
Polysomal RNA	91.3
Poly(A)-rich mRNA	12.9
Poly(A)-rich + 18S rRNA[1]	56.6
Poly(A)-rich + low M.W. RNA[2]	60.6

Conditions were as described in Materials and Methods. The standard reaction mixtures contained 200 μM spermine, 1.8 A_{260} units rabbit reticulocyte 18S rRNA, 0.125 A_{260} unit poly(A)-rich reticulocyte mRNA, 2.1 A_{260} units of unfractionated reticulocyte polysomal RNA, or 0.14 A_{260} unit of the low molecular weight *Artemia* component.

[1] Ribosomal RNA fraction from rabbit reticulocyte polysomal RNA which did not adsorb to oligo dT-cellulose was fractionated on a sucrose gradient containing formamide.

[2] Isolated after chromatography on Bio-Gel P-150 and elution from charcoal.

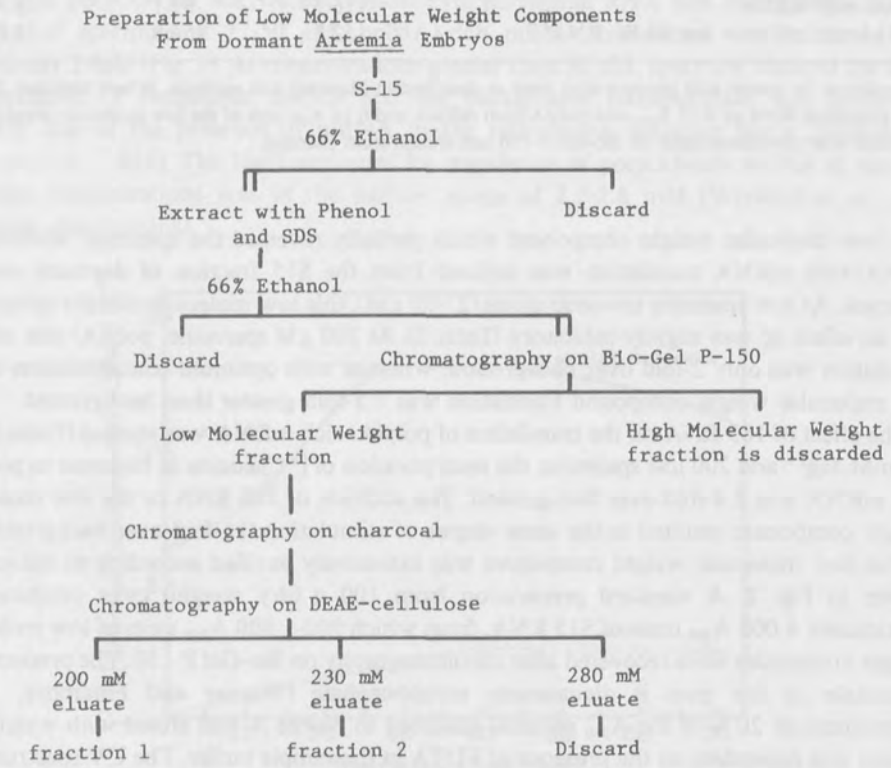

FIG. 2. Purification of the low molecular weight component from dormant *Artemia* embryos. Details of the preparation are given in Materials and methods.

eluting at 200 and 230 mM ammonium acetate, which had significant stimulatory activity, were extensively analyzed by thin layer chromatography (Fig. 3). Each fraction was greater than 90% pure, but could not be shown to correspond to any common nucleotide, including diguanosine or diadenosine polyphosphates.

The UV spectra of the low molecular weight compounds were essentially identical under acidic, neutral and basic conditions. The distinct UV spectrum was very similar under acid and neutral conditions (Fig. 4), having isobestic points at 236 and 272 nm. The absorption maximum was 260-262 nm. Under alkaline conditions, the absorption peak was sharply reduced.

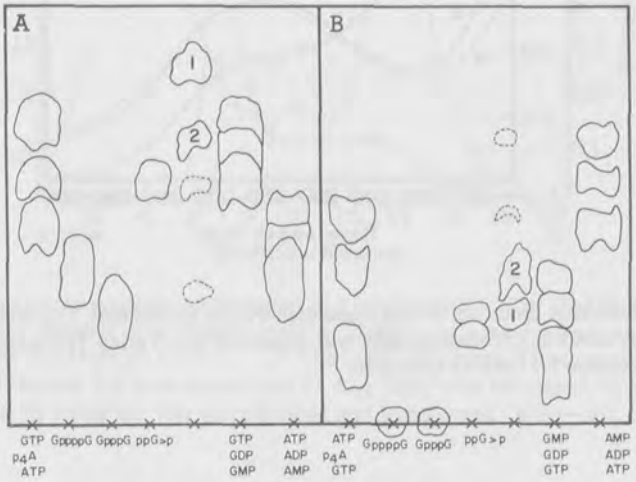

FIG. 3. Thin layer chromatography of *Artemia* low molecular weight component and marker nucleotides. The chromatographic medium was mylar-backed cellulose (EM Laboratories). Panel A was with solvent system D and panel B with solvent C as described in Materials and Methods. Spot 1 corresponds to the peak eluting at 200 mM ammonium acetate from DEAE-cellulose, and spot 2, the peak eluting at 230 mM ammonium acetate.

Cytoplasmic free mRNP particles were isolated from dormant *Artemia* embryos in buffer containing 20 mM Tris·Cl, pH 7.5, 200 mM NH_4Cl, and 5 mM $MgCl_2$, according to the scheme outlined in Fig. 5. Extraction of the mRNPs from the 15-30% rate sedimentation sucrose gradient yielded mRNA which had a pattern of activity very similar to that reported by Grosfeld and Littauer (1975). When the 20-55S region of the gradient was subjected to sucrose isopycnic gradient centrifugation, major peaks at $\rho = 1.27$-1.31 and $\rho = 1.1$ were obtained (Fig. 6). On a parallel gradient, a purified sample of artemin was found to band at $\rho \cong 1.24$. This mRNP pool was chromatographed on CL-Sepharose 4B equilibrated with buffer containing 100 mM NH_4Cl, 10 mM Tris·Cl, pH 7.5 and 2.5 mM Mg^{2+} (Fig. 7). Two distinct peaks and a trailing low molecular weight region were obtained. In a separate experiment, artemin was found to elute distinctly separated from the two high molecular weight mRNP peaks. Very similar banding patterns was obtained when each of the three peak regions from CL-Sepharose, as well as the mRNP fractions from the isopycnic gradient, were analyzed for protein content by SDS gel electrophoresis (Fig. 8).

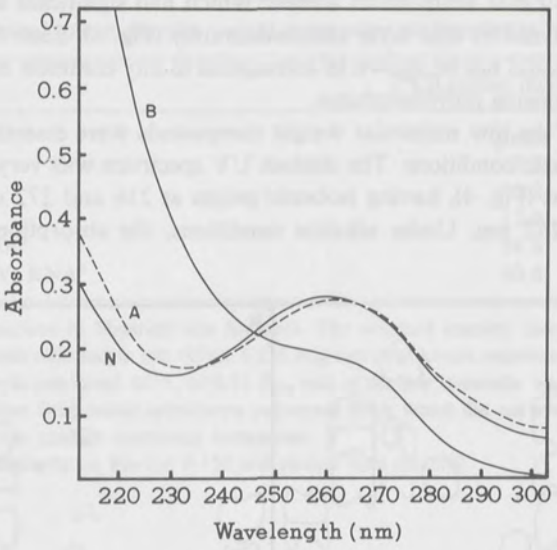

FIG. 4. UV absorption spectrum of low molecular weight nucleotides. The absorption spectra of the RNA after DEAE-cellulose chromatography was measured in a Varian 219 spectrophotometer : pH 1 (curve A) ; pH 7 (curve N) ; pH 13 (curve B).

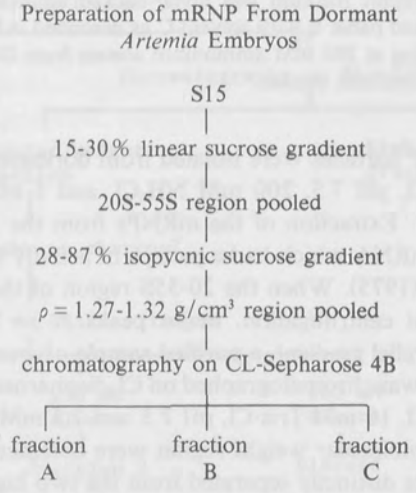

FIG. 5. Preparation of mRNP from dormant *Artemia* embryos. Details of this preparation are given in Materials and Methods.

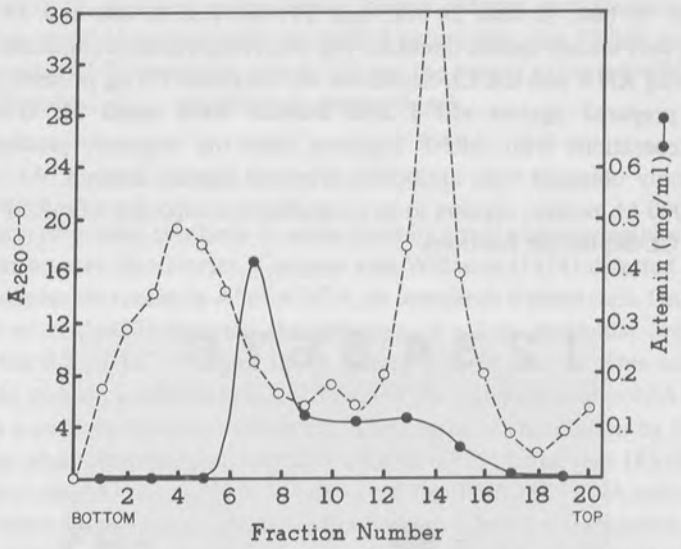

FIG. 6. Isopycnic sucrose gradient fractionation of *Artemia* mRNP. The 20-55S RNA from a rate-sedimentation sucrose gradient was layered onto a 28-87% sucrose gradient and centrifuged as described in Materials and Methods (○ — — ○). The tubes were pierced from the bottom and 0.5 ml fractions collected. Fractions 2-5 were pooled and 21 A_{260} units were recovered. In a parallel gradient 0.2 ml of artemin, at 13.2 mg/ml, was centrifugated and fractionated (● —— ●).

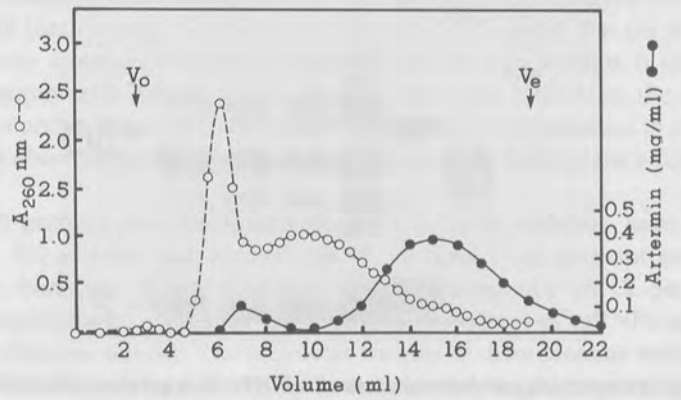

FIG. 7. CL-Separose 4B chromatography of mRNP. Isopycnic-purified mRNPs (7.8 A_{260} units in 0.25 ml) were applied to a 1 × 28 cm CL-Sepharose column as described in Materials and Methods, and 0.5 ml fractions were collected (○ — — ○). The first peak (5-6.5 ml) contained 2.2 A_{260} units, the second peak (7.5-11.5 ml) contained 3.2 A_{260} units, and the trailing edge 0.85 A_{260} unit. In a separate gel filtration, 2.6 mg of artemin in 0.2 ml was similarly chromatographed (● —— ●).

Six major bands were obtained, corresponding to proteins having molecular weights of 32 000, 30 500, 27 000, 26 000, 24 000, and 20 000. The 26 000 and 24 000 M_r bands appeared to be very closely spaced doublets. The isopycnic fractions contained approximately 1.7 μg protein/μg RNA and the CL-Sepharose 4B fractions, 1.3 μg protein/μg RNA.

Antibodies prepared against eEF-T and artemin were tested for cross-reactivity in Ochterlony preparations with mRNP fractions from the isopycnic gradients. A positive reaction was only obtained with antibodies prepared against artemin. As may be seen in Fig. 8, the 27 000 M_r protein appears to be a significant component of mRNP particles in the isopycnic and CL-Sepharose fractions.

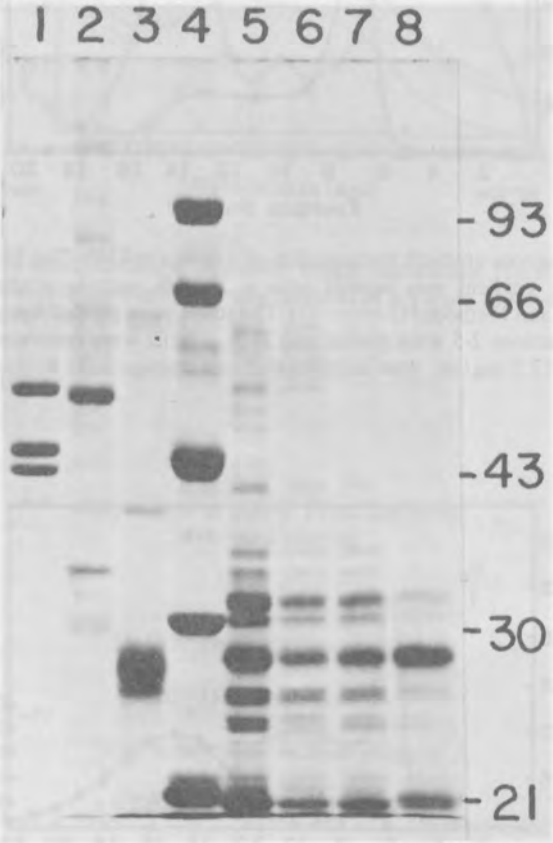

FIG. 8. SDS-polyacrylamide gel electrophoresis of mRNPs. Slab gel electrophoresis was carried out as previously described (MacRae *et al.*, 1979). Lane 4 contained BioRad molelcular weight standards, having molecular weights of 93 000, 66 000, 43 000, 30 000, and 21 000. The other lanes conained : Lane 1, *Artemia* eIF-2 ; lane 2, *Artemia* eEF-T ; lane 3, artemin ; lane 5, mRNP from isopycnic gradients ; lanes 6, 7, and 8 contained mRNP from the CL-Sepharose 4B chromatography step, in the order in which they eluted from the column.

The mRNP and mRNA fractions from the isopycnic sucrose gradient were examined for messenger activity in a wheat germ lysate system (Marcu and Dudock, 1974). Under conditions where poly(A)-rich hemoglobin mRNA translation was 30-fold greater than background, isopycnic mRNP translation was 2-fold and the phenol extracted mRNA 10-fold over background (Woodley *et al.*, unpublished observations).

Discussion

The regulation of protein synthesis in eukaryotes by small oligonucleotides has been under investigation in several laboratories. Clemens and Williams (1978) detected a highly potent inhibitor of polypeptide synthesis $A^{2'}p^{5'}A^{2'}p^{5'}A$, in interferon treated cells. On the other hand, Raymondjean *et al.* (1977) reported the presence of a low molecular weight, dialyzable nucleotide in the 0.5 M KCl wash of rabbit reticulocyte ribosomes. This nucleotide, which was reported to contain a unusual base, stimulated the translation of poly(A)-rich mRNA at 2mM Mg^{2+} in a partially fractionated system. The degree of stimulation by this RNA varied with messenger and polyamine concentrations. Kabat (1975) found that 18S rRNA stimulated the translation of poly(A)-rich mRNA. We observed that both 18S rRNA and a low molecular weight component from *Artemia* influenced poly(A)-rich mRNA translation at 200 μM, but not at 30 μM spermine. Both Raymondjean *et al.* (1977) and Kabat (1975) proposed that the low molecular weight RNA and 18S RNA formed a complex with the message, thereby facilitating its recognition by initiation factors and ribosomes.

The low molecular weight compound from *Artemia* cysts was resolved into two closely related species with apparently identical absorption spectra. The effect of these on translation was dependent on the spermine concentration. At low spermine levels, 0-70 μM, no stimulatory effect was seen. At the present time, the relationship of our low molecular weight component to that isolated by Raymondjean *et al.* (1977) is not known.

Protein synthesis may also be regulated by the availability of active mRNA or the inherent effectiveness of that message. Grosfeld and Littauer (1975) found that the stored mRNA in dormant *Artemia* embryos which is complexed with various proteins is not translated. In addition, Rudensey and Infante (1979) demonstrated that mRNA in the mRNP pool of unfertilized sea urchin eggs is an inherently poor message in comparison to polysome-bound mRNA. These observations suggest that several factors may regulate the efficiency of mRNA translation.

The mRNP proteins associated with dormant *Artemia* embryos have not been fully characterized. We isolated and purified mRNP particles from dormant embryos by rate-sedimentation, isopycnic sucrose gradients and chromatography on CL-Sepharose 4B. Six specific polypeptides were associated with the two size classes of mRNPs separated by CL-Sepharose 4B chromatography. The molecular weights of these proteins were in the range of 20 500 to 32 000. These are similar to the low molecular weight proteins that Huynh-Van-Tan and Schapira (1978) reported to be associated with rabbit reticulocyte mRNA. These included seven proteins with molecular weights from 21 000 to 31 000. Jeffery (1978) also reported a high molecular weight set of proteins associated with rabbit hemoglobin mRNA which we did not detect in the *Artemia* mRNP particles.

Upon careful examination of the SDS-polyacrylamide gels, the 24 000 and 26 000 M_r bands appear to be composed of very closely spaced doublets. Perhaps analogous to this,

Lestourgeon *et al.* (1977) reported the existenceof protein doublets associated with HeLa cell hnRNP particles. These doublets have molecular weights higher than the *Artemia* proteins. We have examined the mRNP proteins for any possible relationship to the subunits of eIF-2 (MacRae *et al.*, 1979), eEF-T (Slobin and Möller, 1976) and artemin (Slobin, 1980). Only one of these proteins, the 27 000 M_r polypeptide, corresponds to artemin.

The function of the proteins associated with mRNP particles in *Artemia* is not yet understood and no regulatory role in protein synthesis can be ascribed to them on the basis of the present evidence. Studies are currently in progress to purify extensively the particles, examine the different classes of mRNA associated with them and determine their products of translation.

Acknowledgements

We wish to thank W. Roth and D. Dinsmore for assistance in preparing ribosomal fractions, low molecular weight RNA and mRNP particles from *Artemia*. Dr. Lawrence Slobin is gratefully acknowledged for providing *Artemia* eEF-T, rabbit antisera to *Artemia* eEF-T and artemin, as well as for his aid in the immunological tests with the mRNP preparations. This work was supported by Grant GM 25451-01 from the National Institutes of Health, US Public Health Service.

Summary

1. A low molecular weight component was isolated from the S15 fraction of dormant *Artemia* embryos which stimulated translation of poly(A)-rich mRNA at high spermine concentrations.

2. This component was purified by gel-filtration and chromatography on charcoal. DEAE-cellulose chromatography resolved this component into two closely related compounds.

3. The low molelcular weight component contains an unusual base residue which was partially characterized by thin layer chromatography and UV absorption.

4. Two classes of mRNP particles isolated from dormant *Artemia* embryos contain the same set of six polypeptides.

5. One of these polypeptides has a molecular weight of 27 000 and is antigenically related to artemin, a major protein found in dormant embryos.

6. The major polypeptides associated with the mRNP from dormant embryos do not include any of the subunits of eIF-2 or eEF-T.

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Biochemical composition of *Artemia*

The Brine Shrimp Artemia. *1980. Vol. 2. Physiology, Biochemistry, Molecular Biology*
G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 664 p.

Carotenoid content of *Artemia* eggs and vitality of the young specimens of this crustacean

B. Czeczuga

Department of General Biology, Medical Academy
Kilinskiego 1, PL-Bialystok 15-230, Poland

Abstract

The cultivation of *Artemia* on poor foods, such as yeasts, and on rich foods, such as algae, is discussed with regard to the carotenoid content of the eggs.

Investigations were carried out on the following short food chains :

a) algae – brine shrimp : *Chlorella vulgaris – Artemia*
b) yeast – brine shrimp : *Saccharomyces cerevisiae – Artemia*.

Column and thin-layer chromatographic analysis showed that the carotenoid content in the eggs (main carotenoids : astaxanthin and β-carotene) depend chiefly on the carotenoid content of the food on which the *Artemia* have been fed.

In the case of *Artemia* fed on algae, large amounts of carotenoids were found in the eggs and conversely small amounts in eggs from *Artemia* fed with yeast.

Large amounts of carotenoids in *Artemia* eggs seems to be related with the vitality of the young specimens of brine shrimp.

The results are discussed in the light of the increase of carotenoids on the biology of *Artemia*.

The Brine Shrimp Artemia. *1980. Vol. 2. Physiology, Biochemistry, Molecular Biology*
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Carbon, nitrogen and phosphorous content in the developmental stages of the brine shrimp *Artemia*[1]

Carl H. Oppenheimer[2] *and Glória S. Moreira*[3]

[2] *Port Aransas Marine Laboratory, University of Texas Marine Science Institute Port Aransas, Texas 78373, USA*

[3] *Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo Caixa Postal 11.461, São Paulo 05421, Brazil*

Abstract

Total carbon, nitrogen, and phosphorus contents were measured for eggs, various developmental stages, and adults of *Artemia*, reared in the laboratory. Expressed as percentage of dry weight, the recorded ranges were : carbon, 27.5-55.6% ; nitrogen, 5.23-9.21% ; phosphorus, 1.15-1.59%. The recorded values are consistent with those given for animals of most planktonic groups.

Introduction

During research to develop an artificial food for use in shrimp mariculture (Subrahmanyam and Oppenheimer, 1970 ; 1971) experiments were conducted to determine the nutritive value of *Artemia* since these animals have been widely used as a food source for varous marine and freshwater vertebrates and invertebrates (Costlow and Bookhout, 1960 ; Wickins, 1972 ; Moller, 1978). The literature provides data on the rate of growth of various species fed on brine shrimp but no data have been found regarding the nutritiousness of the various stages of *Artemia*. This paper presents measurements of the total carbon, nitrogen, and phosphorus contents of eggs, larvae, and adults of this species.

Materials and methods

Dried eggs of *Artemia* from San Francisco Bay, California, were hatched in unfiltered seawater (20-25 °C, 35 ‰ salinity) contained in laboratory trays (40 × 25 × 5 cm). After 2-3 days the larvae were transferred to a small aerated aquarium (30 × 10 × 20 cm) and fed daily on fresh cultures of the alga *Phaeodactylum tricornutum* Bohlin.

For chemical analysis, egg cases, eggs and different larval stages (identified according to Heath, 1924) were pooled to make samples. Gravid females were not used in the determina-

[1] This investigation conducted in 1968 was supported by Armour and Co. and United Fruit Co. grant OPPE 83-872 Florida State Univesity.

tions. Males and females may be distinguished after the 8th stage, but since separate analyses gave almost identical results, selection according to sex was disregarded. Samples were briefly rinsed in distilled water, dried under light suction for 2 min, transferred to a previously tared piece of aluminium foil and weighed. They were subsequently dried for 1 hr at 105 °C, placed in a desiccator for 2 hr and reweighed.

Nitrogen content was determined using the micro-Kjehldahl technique (Strickland and Parsons, 1965) and total phosphorus following the method of Murphy and Riley (1962) after digestion of the samples in perchloric acid as recommended by Hansen and Robinson (1953). Total carbon was determined with a Beckman carbon analyser.

The maximum deviations from the mean nitrogen and phosphorus contents of 10 replicas of 1 000 to 1 500 animals for each element were 4.8 and 12.6% respectively. For carbon, the deviation values for 6 replicas of 2 000 animals each, ranged from 7.1-14.7%.

Results

Table I shows total carbon, nitrogen and phosphorus content, wet and dry weights and wet : dry weight ratios for some developmental stages of *Artemia*. Numbers of organisms to produce 1 g of carbon are included.

TABLE I

Total carbon, nitrogen, and phosphorus contents (% of dry body weight) and the wet : dry weight ratio for some developmental stages of *Artemia*

	% dry weight			Weights (μg)			
	Carbon	Nitrogen	Phosphorus	Wet	Dry	Ratio W/D	No. animals/gC
Egg case	51.3	5.91	0.17	–	0.80	–	2.4×10^9
Egg	50.2	7.22	0.97	–	3.72	–	5.3×10^8
1st instar	55.6	9.21	1.34	12.79	2.76	4.63	6.5×10^8
2nd instar	27.5	8.09	1.24	24.60	2.25	10.91	1.6×10^9
4th instar	9.7*	9.05	1.59	81.23	9.94	8.17	–
8th instar	45.1	8.76	1.42	232.94	38.39	8.44	5.8×10^7
11th instar	44.4	8.41	1.37	593.98	70.68	8.40	3.2×10^7
Adult	42.9	5.23	1.15	3952.67	391.56	10.09	6×10^6

* This value, considered abnormally low was disregarded. The approximated value, when calculated from the mean C:N:P ratio is about 50%.

Carbon content varied from 27.5% in the 2nd instar to 55.6% in the 1st instar, this maximum value coinciding with the minimum value for water content. Nitrogen content ranged from 5.23 to 8.09% for watery stages (2nd instar and adults), 8.41 to 9.05 in the intermediate stages, reaching a maximum value of 9.21% in the 1st instar, the least watery stage. Phosphorus content, not varying much in the different stages, ranged from 1.59% in the 4th instar to 1.15% in adults (excluding the eggs and egg cases).

Ratios of carbon-nitrogen-phosphorus for the various stages are given in Table II.

TABLE II

C:N:P ratios for some developmental stages of *Artemia*

	Carbon	Nitrogen	Phosphorus
Egg case	100	11.52	0.33
Egg	100	14.38	1.93
1st instar	100	16.56	2.41
2nd instar	100	29.42	4.51
8th instar	100	19.42	3.15
11th instar	100	18.94	2.09
Adult	100	12.19	2.68

Discussion

Different results for carbon, nitrogen and phosphorus contents in planktonic animals have been obtained by several authors working with related species, or with individuals of the same species from different areas. For example nitrogen levels for the copepod *Calanus finmarchicus* were 10.20% (Vinogradov, 1933), 7.03% (Krey, 1958), and 4.7 to 5.9% (Curl, 1962). The measured phosphorus content of Long Island Sound planktonic copepods was 0.82% (Harris and Riley, 1956) while Sargasso Sea copepods showed 0.79% (Beers, 1966). Although the measurements presented in this paper do not differ markedly from the data we obtained in preliminary experiments with eggs from Salt Lake, Utah, eggs of a different origin and batch might show variations in weight and composition.

According to Cowey and Corner (1963ab) and Raymont *et al.* (1964) non-proteinous nitrogen forms only a small part of the total body nitrogen. Using this assumption and the one that proteins contain an average of 16% nitrogen, the protein equivalent in the developmental stages of *Artemia* would vary from 32.69% in the adults to 57.56% in the 1st instar. These figures are lower than those presented by Krey (1950) for copepods and Raymont *et al.* (1964) for mysid *Neomysis integer, i.e.*, about 71-77% and 71% of dry weight respectively. Early studies (Brandt, 1898 ; Brandt and Raben, 1919 ; Vinogradov, 1953) suggest that marine zooplankton has a protein content of 64.5% of dry weight. The same authors gave values for total carbon varying from 43.0 to 47.7% dry weight. Raymont *et al.* (1964) calculated values around 30% for *Neomysis* total carbon. These values are comparable with our values of 27.5 to 55.6% for *Artemia* dry weight.

Redfield *et al.* (1963) proposed that an average atomic ratio for C:N:P of 103:16.5:1 represents the total zooplankton biomass. The data for *Artemia* in Table II show marked differences from this ratio, usually with greater values for nitrogen and phosphorus. The values presented in this paper are more similar to those given by Beers (1966) for separate planktonic groups.

Our results show that generalizations should not be made about the nutritiousness of individuals of a particular species used as food, even when they are obtained at the same time from the same area. Different developmental stages of *Artemia* show striking differences, the first

instar seeming to be a good source of protein, with the highest percentage of carbon and nitrogen, and the lowest wet : dry weight ratio.

The data in % carbon for instar I and II indicate a period of self absorption during the development of the rudamentary mandibles and a feeding mechanism.

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International Study on *Artemia*[1] XII. The carotenoid composition of eight geographical strains of *Artemia* and the effect of diet on the carotenoid composition of *Artemia*

Takumi Soejima[2], *Teruhisa Katayama*[3], *and Kenneth L. Simpson*[2]

[2] *Department of Food Science and Technology, Nutrition and Dietetics, University of Rhode Island Kingston, Rhode Island 02881, USA*

[3] *Department of Marine Biochemistry, University of Kagoshima Kagoshima 890, Japan*

Abstract

Echinenone and canthaxanthin were the only carotenoids isolated from the cysts and freshly-hatched nauplii of *Artemia* from nine samples representing eight geographical locations. The concentration of echinenone ranged from 0.5-5.5% of the total carotenoid composition. Canthaxanthin was the major pigment in all samples tested, ranging from 94.5%-99.5%. Three isolates obtained from San Francisco Bay and San Pablo Bay (California, USA) were very similar in their carotenoid concentrations.

Australian *Artemia* were fed on rice bran for 10 days. Racemic, synthetic astaxanthin and the pigments extracted from *Rhodotorula* and *Spirulina* were fed to the 10-day-old *Artemia*. The bio-accumulation of torularhodin, zeaxanthin and astaxanthin was observed. These pigments were observed to be lost more readily than canthaxanthin in *Artemia* returned to a carotenoid-free diet. The rapid turnover of β-carotene and echinenone from the diets is consistent with a biosynthetic pathway in *Artemia* : β-carotene → echinenone → canthaxanthin.

Introduction

Freshly hatched brine shrimp (*Artemia*) are the most commonly used source of food for the culture of fish and crustacean larvae (Wickins, 1972 ; Benijts *et al.*, 1975 ; Gallagher and Brown, 1975).

Unexpectedly heavy losses of fish have been reported when *Artemia* were used as a single diet source (Fujita, 1973). Various suggestions have been made to explain the poor nutritional value of some *Artemia* strains including possible differences in biochemical composition such

[1] International Interdisciplinary Study on *Artemia* Strains coordinated by the Artemia Reference Center, State University of Ghent, Belgium.

as mineral content, fatty acid composition and amino acid profiles (Hinchcliffe and Riley, 1972 ; Wickins, 1972 ; Benijts *et al.*, 1975 ; Gallagher and Brown, 1975).

Unexpectedly heavy losses of fish have been reported when *Artemia* were used as a single diet source (Fujita, 1973). Various suggestions have been made to explain the poor nutritional value of some *Artemia* strains including possible differences in biochemical composition such as mineral content, fatty acid composition and amino acid profiles (Hinchcliffe and Riley, 1972 ; Wickins, 1972 ; Benijts *et al.*, 1975 ; Gallagher and Brown, 1975 ; Claus *et al.*, 1977, 1979) and the level of pesticides (Bookhout and Costlow, 1970).

The color of crutaceans is mainly due to various carotenoids that are absorbed from the diet and deposited in the organism either as is, or in some transformed state.

A number of investigators have reported conflicting reports on the carotenoid composition of *Artemia*. Only echinenone and canthaxanthin were isolated by some authors (Krinsky, 1965 ; Hata and Hata, 1969 ; Hsu *et al.*, 1970), while others (Czeczuga, 1971 ; Czygan, 1968) reported a more complex carotenoid composition for *Artemia*. Davies *et al.* (1970) and Hsu *et al.* (1970) showed that β-carotene was converted to echinenone which was converted to canthaxanthin. A further conversion of isocryptoxanthin to 4-hydroxy-4'-keto-β-carotene was reported (Davies *et al.*, 1970). Czygan (1968) reported the isolation of astacene (not astaxanthin) and crustaxanthin from a Canadian strain that had been fed both a basal diet and an algae supplemented diet. These results were not obtained from the Californian *Artemia* strain. Zeaxanthin was reported to be absorbed but not further metabolized (Davies *et al.*, 1970).

The present study on the carotenoids of *Artemia* from various geographical areas is part of a larger study to determine the chemical and biochemical composition of various geographical strains of *Artemia* and to determine the suitability of these strains for specific predators. The present paper reports the carotenoid composition of the cysts and nauplii of nine *Artemia* samples taken from eight geographical locations. Extracts from *Rhodotorula* and *Spirulina* and racemic astaxanthin were fed to one strain to determine the response of *Artemia* to a variety of carotenoid-containing diets.

Methods and materials

Spirulina

Spirulina was provided in freeze-dried form by the Environmental Protection Agency (EPA), Environmental Research Laboratory, Narragansett, Rhode Island, USA.

Rhodotorula

Culture method

Rhodotorula was cultured on solid and liquid media (Table I). Ten liters of the medium were added to a Microferm Fermentor (New Brunswick Scientific Co., New Brunswick, New Jersey, USA). It was autoclaved at 121 °C for 1 hr and inoculated with 10 ml of yeast suspension. The culture medium was agitated by means of a mechanical stirrer, the temperature was held at 25 °C and filtered air was pumped through the fermentor.

TABLE I

The yeast (*Rhodotorula*) media (slants, starting and culturing media)

Ingredient	Slants	Starting	Culturing
Glucose	20 g	10 g	10 g
Peptone	5 g	3 g	
Yeast extract	1 g	1 g	0.1 g
Agar	18 g		
Ammonium nitrate			3 g
Potassium phosphate			1 g
Distilled water	1 000 ml	1 000 ml	800 ml
Seawater			200 ml

Harvesting of cells

Cells were harvested after 5 days of incubation by centrifuging at 4 500 rpm for 10 min. The centrifuged cells were washed twice with distilled water and recentrifuged as described above.

DISRUPTION OF *RHODOTORULA* AND *SPIRULINA* CELLS

Disruption of cells was accomplished using a French Pressure Cell (American Instrument Company, Inc., Silver Spring, Maryland, USA) in conjunction with a Wabash Hydraulic Press (Wabash Metal Product Company, Inc., Wabash, Indiana, USA) at a pressure of 1.4×10^8 psi (Simpson *et al.*, 1963).

PIGMENT EXTRACTION

The pigments were extracted from the disrupted *Rhodotorula* and *Spirulina* with acetone ; a slurry of the cells was first prepared with acetone and then the acetone-extracted pigments were separated from the cell mass by slow speed centrifugation. The procedure was repeated until the extract was colorless. The acetone solution of pigments was transferred to petroleum ether (PE) solution by the addition of distilled water (Simpson *et al.*, 1964).

SAPONIFICATION OF *SPIRULINA*

The PE solution of the pigments was transferred to an equal volume of 10% KOH in methanol. The solution was left overnight under nitrogen at room temperature in the dark. After saponification was completed, the alkali was removed by thorough washing with water.

ASTAXANTHIN

Crystalline, synthetic astaxanthin was provided by F. Hoffman-La Roche and Co., Basle, Switzerland (3R,3'R:3R,3'S:3S,3'S = 24.7:49.6:25.6).

Source of *Artemia*

Dehydrated cysts from the various geographical locations were either purchased commercially or were provided by the Artemia Reference Center, Ghent, Belgium. The origin of these cysts were : Shark Bay, Australia (World Ocean, lot no. 113) ; Macau, Brazil (Companhia Industrial do Rio Grande do Norte, CIRNE-Brand, harvested 1978) ; Margherita di Savoia, Italy (harvested 1977) ; Great Salt Lake, Utah, USA (harvested 1977) ; San Pablo Bay, California, USA (Living World, San Francisco Bay Brand, Inc., lot no. 1628) ; San Francisco Bay, California, USA (San Francisco Bay Brand, Inc., lot no. 313/3006 and 321995) ; Argentina (Buenos Aires) and Galera Zamba, Colombia.

Culture of *Artemia*

The cysts were hatched in sea water and after 48 hr the free-swimming nauplii were separated from the cyst residue. For the feeding study, approximately 14 000 newly-hatched nauplii (Australia : World Ocean, lot no. 113) were grown in 14 l of seawater. During the culture period, the seawater was kept in daylight at room temperature and aerated. Rice bran (1.4 g for 14 000 individuals) was dispensed into the seawater daily for 10 days.

Preparation of diets

Control diet

Rice bran was used as a control diet for the experiment.

Test diets

Three different pigmented diets were prepared as feed for *Artemia*. One diet contained a pigment from the yeast, *Rhodotorula*, a second diet contained synthetic astaxanthin and a third diet contained a pigment extract from the algae, *Spirulina*. The pigment content was absorbed on rice bran at a level of 1 mg/g diet.

Feeding experiments

All newly-hatched nauplii were fed rice bran for 10 days. They were then divided into seven groups (about 2 000 animals/group) and fed the following diets :

Group 1 : Rice bran (R.B.) only, 12 days.
Group 2 : *Rhodotorula*-extracted pigments, 6 days.
Group 2' : Same as Group 2 + R.B. only, 6 days.
Group 3 : Astaxanthin, 6 days.
Group 3' : Same as Group 3 + R.B. only, 6 days.
Group 4 : *Spirulina*-extracted pigments, 6 days.
Group 4' : Same as group 4 + R.B. only, 6 days.

Pigment analysis

The cysts and nauplii from the various geographical locations and the cultured adults were extracted with acetone. Pigments were separated and purified by column and thin layer

chromatography. Purified pigments were identified by absorption spectra in various solvents, chemical tests (*e.g.* acetylation, methylation, reduction and iodine test) and co-chromatography with authentic pigments (Hsu *et al.*, 1970 ; Davies, 1976).

Results

Feeding of the pigments from *Rhodotorula*

The carotenoid composition of *Rhodotorula* is shown in Table II. Torularhodin and torulene were found to be the major carotenoids present in this marine yeast. Table III shows the results of quantitative carotenoid analysis of *Artemia* fed a diet containing *Rhodotorula*-extracted pigments. Echinenone, canthaxanthin and torularhodin were identified in group 2 and 2' *Artemia* but torulene was not isolated. This result indicated that dietary torularhodin was absorbed from the diet and accumulated in *Artemia*. A small amount of a yellow pigment, less polar than echinenone, was observed in group 2. Its absorption spectrum showed only a general absorption in the blue region of the visible and near ultraviolet spectra. This pigment was not identified.

Table II

The carotenoid composition of the yeast (*Rhodotorula*)

Carotenoids	Relative abundances (%)
β-Carotene	8.51
γ-Carotene	0.92
Torulene	30.54
Torularhodin	57.87
Unknown	2.16

Total : 69.3 μg/g dry wt

Table III

The amounts of echinenone, canthaxanthin and torularhodin present in *Artemia* after feeding with pigments of the yeast (*Rhodotorula*)

	Concentration of carotenoids present (μg/g fresh wt)			
Feeding program	Echinenone	Canthaxanthin	Torularhodin	Unknown
Group 1	0.11	5.87		
Group 2	0.86	6.25	1.20	0.40
Group 2'	0.82	6.04	0.96	

Group 1 : rice bran only (12 days)
Group 2 : rice bran + pigments of *Rhodotorula* (6 days)
Group 2' : same as group 2 then rice bran only (6 days)

Feeding of astaxanthin

Table IV shows the results of a quantitative carotenoid analysis of *Artemia* fed a diet containing synthetic astaxanthin. Echinenone, canthaxanthin and astaxanthin were identified in both groups 3 and 3'. No other carotenoids were detected. This result indicated that dietary astaxanthin can also be absorbed from the diet by *Artemia*.

Feeding of pigments from *Spirulina*

The carotenoid composition of *Spirulina* is shown in Table V. β-Carotene and zeaxanthin were found to be the major carotenoids present. Table VI shows the result of the carotenoid analysis of *Artemia* fed a diet containing pigments extracted from *Spirulina*. β-Carotene, echinenone, canthaxanthin and zeaxanthin were identified in both groups 4 and 4' *Artemia*. A small amount of orange-colored pigment, more polar than zeaxanthin, was observed in group 4. It appears to be a mixture of degraded carotenoids. The amount of β-carotene in group 4' *Artemia* was much less than that of group 4 *Artemia*.

Analysis of geographical strains

An analysis of the cysts and nauplii from the nine geographical locations showed only echinenone and canthaxanthin. The amount of canthaxanthin ranged from 94 to 99% of the total carotenoid composition (Table VII).

Discussion

The pigments of a marine *Rhodotorula* yeast were determined and found to be typical of other analyses of these yeasts (Table II) (Simpson *et al.*, 1963). Of the major pigments, only β-carotene has been reported in *Artemia*. Although it was not detected, it is reasonable to assume that the increase in echinenone and canthaxanthin was due to a conversion of β-carotene to echinenone to canthaxanthin. Torularhodin is unique to red yeast, thus its presence shows the ability of *Artemia* to accumulate this carboxyl containing carotenoid.

The feeding of a mixture of stereoisomers of astaxanthin resulted in only the accumulation of astaxanthin over the pigments of the control. These results show that astaxanthin in the diet results in an accumulation of astaxanthin in the *Artemia*. These results compare with those of Czeczuga (1971) who found astaxanthin in the eggs of *Artemia* obtained from France. The finding of astacene in a Canadian strain and not the Californian strain by Czygan (1968) is difficult to explain apart from astacene being obtained from the diet. The results of the joint study (ISA) have shown considerable variation between the various geographical locations including the electrophoretic patterns (Seidel *et al.*, 1980). We had also shown the incorporation of pure astacene into rainbow trout, thus other biological systems incorporate this "artifact".

The greatest incorporation into *Artemia* came from the feeding of *Spirulina* pigments. β-Carotene and echinenone are major constituents and these two pigments were found together with canthaxanthin. Zeaxanthin had been reported in *Artemia* (Davies *et al.*, 1970) previously. The large turnover of β-carotene and echinenone in *Artemia* fed a carotenoid-free diet is consistent with the biosynthetic pathway proposed by Davies *et al.* (1970).

TABLE IV

The amounts of echinenone, canthaxanthin and astaxanthin present in *Artemia* after feeding with astaxanthin

Feeding program	Concentration of carotenoids present (μg/g fresh wt)		
	Echinenone	Canthaxanthin	Astaxanthin
Group 1	0.11	5.87	
Group 3	0.20	5.92	2.11
Group 3'	0.16	5.80	1.80

Group 1 : rice bran only (12 days)
Group 3 : rice bran + astaxanthin (6 days)
Group 3' : same as group 3 then rice bran only (6 days)

TABLE V

The caroteroid composition of the algae (*Spirulina*)

Carotenoids	Relative abundances (%)
β-Carotene	25.38
Echinenone	3.08
β-Cryptoxanthin	2.03
Zeaxanthin	65.30
Unknown	4.21

Total : 547.2 μg/g dry wt

TABLE VI

The amounts of β-Carotene, echinenone, canthaxanthin and zeaxanthin present in *Artemia* after feeding with pigments of *Spirulina*

Feeding program	Concentrations of carotenoids present (μg/g fresh wt)				
	β-Carotene	Echinenone	Canthaxanthin	Zeaxanthin	Unknown
Group 1		0.11	5.87		
Group 4	0.71	3.16	6.97	2.71	0.13
Group 4'	0.19	1.48	6.58	1.99	

Group 1 : rice bran only (12 days)
Group 4 : rice bran + pigments of *Spirulina* (6 days)
Group 4' : same as group 4 then rice bran only (6 days)

TABLE VII

The carotenoid composition of seven geographical strains of *Artemia*

	Fraction	λmax (in P.E.)	μg/g (dry wt)	%	Identification
Australia (Shark Bay)					
Nauplii	Fr.1	458	7.49	1.0	Echinenone
	Fr.2	463	741.50	99.0	Canthaxanthin
Cysts	Fr.1	458	5.19	1.1	Echinenone
	Fr.2	463	466.38	98.9	Canthaxanthin
Brazil (Macau)					
Nauplii	Fr.1	458	4.80	0.8	Echinenone
	Fr.2	463	595.75	99.2	Canthaxanthin
Cysts	Fr.1	458	4.90	1.2	Echinenone
	Fr.2	463	403.41	98.8	Canthaxanthin
Utah (Great Salt Lake)					
Nauplii	Fr.1	458	21.17	2.5	Echinenone
	Fr.2	463	865.60	97.5	Canthaxanthin
Cysts	Fr.1	458	8.90	1.9	Echinenone
	Fr.2	463	459.52	98.1	Canthaxanthin
California (San Francisco Bay, 1975) S.F.B. no. 321					
Nauplii	Fr.1	458	28.31	5.5	Echinenone
	Fr.2	463	486.42	94.5	Canthaxanthin
California (San Francisco Bay, 1975) S.F.B. no. 313					
Nauplii	Fr.1	458	17.43	3.9	Echinenone
	Fr.2	463	429.37	96.1	Canthaxanthin
Cysts	Fr.1	458	9.33	4.0	Echinenone
	Fr.2	463	223.94	96.0	Canthaxanthin
California (San Pablo Bay, 1978) S.P.B. no. 1 628					
Nauplii	Fr.1	458	8.83	2.0	Echinenone
	Fr.2	463	432.43	98.0	Canthaxanthin
Cysts	Fr.1	458	3.95	1.6	Echinenone
	Fr.2	463	242.75	98.4	Canthaxanthin
Italy (Margherita di Savoia)					
Nauplii	Fr.1	458	4.37	1.0	Echinenone
	Fr.2	463	432.96	99.0	Canthaxanthin
Cysts	Fr.1	458	3.46	1.8	Echinenone
	Fr.2	463	188.59	98.2	Canthaxanthin
Argentina (Buenos Aires)					
Cysts	Fr.1	458	1.92	0.5	Echinenone
	Fr.2	463	382.03	99.5	Canthaxanthin
Galera Zamba (Colombia)					
Cysts	Fr.1	458	0.90	0.6	Echinenone
	Fr.2	463	148.79	99.4	Canthaxanthin

Several generalizations can be made from this data.

1. *Artemia* accumulate a number of xanthophylls which they cannot biosynthesize. These pigments appear to be lost at a higher rate than canthaxanthin.

2. Nine samples of cysts and nauplii from eight geographical locations contained only echinenone and canthaxanthin. This is in contrast to the data of Czeczuga (1971) where these pigments represented only about 3 % of the total. This author reported a value for β-carotene of greater than 50 %, thus this strain would appear not to be closely related to the strains that we, as well as others, have worked with (Krinsky, 1965 ; Czygan, 1968 ; Gallagher and Brown, 1975 ; Hata and Hata, 1969 ; Hsu *et al.*, 1970 ; Davies *et al.*, 1970).

3. *Artemia* can be used as a carrier for a xanthophyll carotenoid pigment to a specific predator.

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