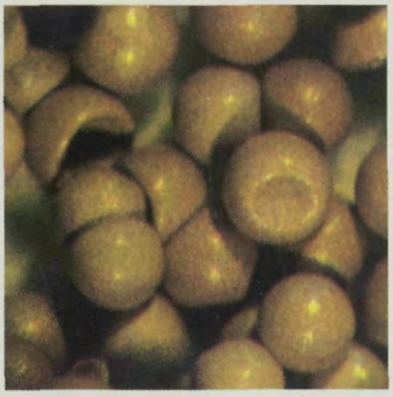
ARTEMIA

RESEARCH AND ITS APPLICATIONS



volume 2

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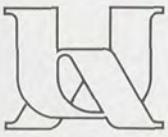
ARTEMIA

RESEARCH AND ITS APPLICATIONS

VOLUME 2

Walter DECLEIR, Luc MOENS,
Herman SLEGERS, Patrick SORGELOOS and Edmonde JASPERS
Editors

Proceedings of the
Second International Symposium on the
brine shrimp *Artemia*, organised under the
patronage of His Majesty the King of Belgium



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METABOLISM AND DEVELOPMENT

ENZYMES RELATED TO NUCLEOTIDE AND NUCLEIC ACID METABOLISM

GENOME STRUCTURE AND EXPRESSION

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Welcome address

Prof. Walter Decleir
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Mr. Chairman of the University of Antwerp, Representatives of the Ministries of Foreign Development and Cooperation, Agriculture, and Education, of the European Economic Community, and the Universities of Antwerp, Ghent and Brussels, Your Excellencies representing the Countries of Brazil, India, Israel, Malaysia, Panama, Peru, Spain, Thailand, and the United States of America, dear Colleagues, Ladies and Gentlemen,

It is both a great honour and a pleasure to welcome you to the University and City of Antwerp to participate in the Second International Symposium on the Brine Shrimp Artemia. This symposium has been honoured by the patronage of His Majesty the King of the Belgians. During a recent visit to Professor Persoone's Laboratory for Mariculture in Ghent and to the associated Artemia Reference Center, of which Dr. Sorgeloos is the Director, the King has already shown his keen interest in the research into and the applications of the brine shrimp.

As you may know, the first International Symposium on Artemia was held in 1979 in Corpus Christi, Texas, USA, and we are very happy to be able to organize this second symposium on the same subject here in Antwerp. This was made possible thanks to the generous help of many institutions. Therefore I want to thank the Antwerp State University Center (RUCA) and the Antwerp University Institution (UIA), the Belgian National Science Foundation, the Ministry of Education, the Belgian Administration for Development Cooperation, the Belgian Center for Oceanography, the Artemia Reference Center and the Institute for Marine Scientific Research (IZWO). The assistance of colleagues who kindly accepted to serve as Chairmen, Moderators, and Rapporteurs has been greatly appreciated, as has the help of all those members of the staff who have contributed to the organization of this symposium. Last but not least I wish to thank the Ambassadors and Consuls-General of Canada, France, Germany, Malaysia, Spain, and Thailand, who kindly contributed to the banquet by offering drinks typical of their country.

I am greatly indebted to the Antwerp State University Center (RUCA) for the use of the university's premises for this symposium and to the UIA, the second of the three bodies which together constitute the University of Antwerp, for making its facilities available in order to organize a workshop.

There were three reasons why we decided to organize this second symposium on the brine shrimp in Belgium. The first is the generous support of the Belgian National Science Foundation, which four years ago agreed to sponsor a research program in which various departments of the Universities of Ghent, Antwerp, and Leuven have participated. The organization of this symposium seemed to be the appropriate climax to this initiative. The second reason is that for many years the Artemia Reference Center of the State University of Ghent has played a central role in the coordination of research into *Artemia* and has created the 'International Study on Artemia' group, in which research laboratories from Italy, Spain, Great Britain, the USA, and Belgium are

participating. And thirdly, Belgium through its Administration for Development Cooperation is actively promoting Belgian know-how with regard to *Artemia* applications in third world countries. This is carried out by supporting research, sponsoring training courses, and setting up demonstration projects in various countries in Africa, Asia, and South America. It is not only Belgian governmental organizations which support *Artemia* research and development. The private sector too has recently shown an interest and has set up the Belgian joint venture company 'Artemia Systems'.

All this is sufficient proof of the key role which Belgium has played so far and is still continuing to fulfil in *Artemia* research. The organization of this symposium with 250 participants from 38 countries is an excellent illustration of this.

I should like to finish this short welcome address by expressing my sincere hope that you will enjoy your stay in our City of Antwerp and at our University. Furthermore, I hope that your participation in this symposium will be very fruitful and that this meeting will be a milestone on the way towards a growing knowledge about that tiny crustacean which we call *Artemia* and which seems to be predestined to play such an important role in future world food production.

Opening address

Mr. J. P. Goyens
Director-General of the Belgian Administration for Development Cooperation.

Mr. Chairman, Excellencies, Ladies and Gentlemen,

At a time when industrialized nations combine efforts to boost food productions in developing countries, when in 1985 alone an estimated amount of 3 billion US dollars will be spent on emergency food to only some 20 of the most effected countries, it is encouraging to realize that the scientific community contributes to these efforts by developing more pressing strategies and also inventing new resources.

Aquaculture is one of the fields where opportunities to increase food production in a relatively short time and at reasonable cost, seem very promising. Developing countries which are willing and able to exploit intensively their salt lakes and saltworks can obtain very profitable results by taking *Artemia* developing initiatives and introducing new techniques. In the first place, this food source creates a potential for improving local aquaculture production, and in most cases and at the same time, a better quality of the salt produced by solar evaporation. Secondly, in a number of third world countries, climatological and geological conditions prevail which favor mass production of *Artemia* cysts. Export of high quality cysts can become an important source of income. The promising results of past research initiated by the Belgian Artemia Reference Center, and implemented in collaboration with numerous national and international research and development organizations, have incited the Belgian Government to include in its national development cooperation programme a chapter on *Artemia* which aims at rendering the acquired know-how in the field of selection and reproduction of *Artemia* strains accessible to third world countries.

Each year, the Belgian Administration for Development Cooperation offers a number of fellowships to enable citizens of developing countries, interested in *Artemia* production, to participate in a special training course organized by Belgian universities. Since two years the same administration provides the necessary funds for a project of applied research in view of optimizing the use of *Artemia*, standardizing inoculation techniques, and selecting strains appropriate for inoculation in saltponds. More recently, two *Artemia* projects were started in the field. One aims at the inoculation of *Artemia* in saltponds in Malindi near Mombasa in Kenya, in view of intensive production in a region where *Artemia* are not present under natural conditions. A second project should help Thailand to master and improve the brine shrimp production techniques and coordinate its inoculation programmes. At the present moment, it is too early to evaluate these development cooperation activities in all their aspects. One conclusion we can draw already is that these *Artemia* projects, directed to practical applications in well-chosen environments, do not need important investments, as they are based for the major

part on results obtained by relatively simple techniques. We can also conclude that the exchange and most of all the pooling of numerous and different experiments on this micro-shrimp can only lead to new progress in the knowledge of *Artemia*.

Mr. Chairman, Excellencies, Ladies and Gentlemen, on behalf of the Belgian Administration for Development Cooperation, I wish the participants of this second international Symposium on *Artemia* fertile discussions and work, in view of improving this new resource for the benefit of the third world countries which ultimately is to the interest of the industrialized nations.

Speakers at the opening session (from left to right) : Dr. Patrick Sorgeloos, Prof. Dr. Walter Decleir (Symposium Organizers) and Mr. J.-P. Goyens (Director-General of the Belgian Administration for Development Cooperation).

Group picture of participants

Speakers at the opening session (from left to right) : Dr. Patrick Sorgeloos, Prof. Dr. Walter Declair (Symposium Organizers) and Mr. J.-P. Goyens (Director-General of the Belgian Administration for Development Cooperation).

Group picture of participants

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Notation of restriction endonucleases

The notation of restriction enzymes recently shows a high degree of variability. This is mainly caused by the daily discovery of new enzymes and subtypes. For uniformity in these Proceedings, the notation given in the "Directory of Restriction Endonucleases" Roberts (1980) is used.

ROBERTS R. J. 1980. Directory of restriction endonucleases. p. 1-15. In : *Methodes of enzymology.* Vol. 65. Nucleic acids, Part I. Grossman L. and K. Moldave (Eds). Academic Press, New York.

Metabolism and Development

Artemia *Research and its Applications. 1987. Vol. 2. Physiology, Biochemistry, Molecular biology*
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Comparison of the aerobic metabolism for four strains of *Artemia* [1]

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Abstract

The respiratory physiology in 35 ‰ artificial seawater has been studied for four strains of adult *Artemia*. 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. The experiments were carried out at temperatures ranging from 5 to 45 °C. The lower lethal value for male and female *Artemia* from the four strains (Great Salt Lake, Macau, San Francisco Bay, and Sfax) being 5 °C. The higher lethal value for male *Artemia* from Great Salt Lake, Macau, and Sfax being 45 °C, while male animals from San Francisco Bay only survive up to 35 °C. Female *Artemia* from the four strains show a temperature tolerance up to 40 °C. The measurements for the Macau strain illustrate the quick adaptability of *Artemia* to new environmental conditions.

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.

Although the distribution of *Artemia* is mainly controlled by the salinity, the temperature is most significantly influencing the survival of the animals in their local environment. From the literature data it is known that *Artemia*-biotopes show severe diurnal temperature fluctuations. Since these fluctuations are, however, not equal for different populations of *Artemia*, we might suspect that different *Artemia*-strains are equipped with different adaptational mechanisms to cope with the changing environment.

The influence of the ambient temperature upon the metabolic activity is one of the most intensively studied subjects related to respiratory physiology. Poikilotherms usually show an increasing oxygen consumption with increasing temperature until a maximum value is reached. Above this limit, the respiration decreases till the animal becomes nonmotile, while the animal will die by a slight temperature increase. The increased respiration due to an increase in temperature reflects a higher spontaneous activity of the organism.

The results obtained for the oxygen consumption give an indication of the scope for activity and the potential activity range (Fry, 1947) at a salinity of 35 ‰ for adult male and female *Artemia* from Great Salt Lake, Macau, San Francisco Bay, and Sfax. The measurements for the Macau strain illustrate the quick adaptability of *Artemia* to new environmental conditions.

[1] This research is part of the program 2.0012.82 of the Belgian National Science Foundation (NFWO).

Materials and methods

Animals

Only adult specimens of *Artemia* from the following strains were used :

- San Francisco Bayn, CA, USA = SFB ;
- Macau, Brazil = MAC ;
- Great Salt Lake, UT, USA = GSL ;
- Sfax, Tunisia = SFX.

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 automatically fed 24 times a day with a suspension of *Spirulina maxima.*

Seawater

For all experiments artificial seawater (HW-Wimex seasalt) with a salinity of 35 ‰, which was membrane filtered (0.2 µm) to remove all particles including bacteria, was used.

Respirometry

Respiration was measured in a Warburg constant-volume respirometer (Umbreit *et al.*, 1972). For each measurement a series of 15 ml flasks was used, each containing six individuals, incubated in 3 ml seawater. For CO_2 absorption, 0.3 ml KOH 20 % and a small filter paper were put in the center vial. The flasks were equilibrated for 30 min. Oxygen consumption was measured every 30 min over a period of 3 h. The flasks were continuously shaken. The total oxygen consumption was calculated by regression analysis of the oxygen decrease as a function of time. The oxygen consumption was expressed as QO_2 (µl O_2/mg dry weight/h). Under some circumstances mortality occurred, in which case the O_2-consumption was always corrected accordingly.

Anaesthesia was obtained by adding 0.25 ml of a 0.5 % chloroform suspension 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.

Results

Figs 1, 2, 3, and 4 show the rate of oxygen consumption (QO_2) at temperatures ranging from 5 °C to 45 °C of adult *Artemia* from the following strains :

- Great Salt Lake (GSL), UT, USA (Fig. 1) ;
- Macau (MAC), Brazil (Fig. 2) ;
- San Francisco Bay (SFB), CA, USA (Fig. 3) ;
- Sfax (SFX), Tunisia (Fig. 4).

In these graphs we have compared the oxygen uptake of actively swimming male and female animals with animals anaesthetized with chloroform and with total homogenates (only for male

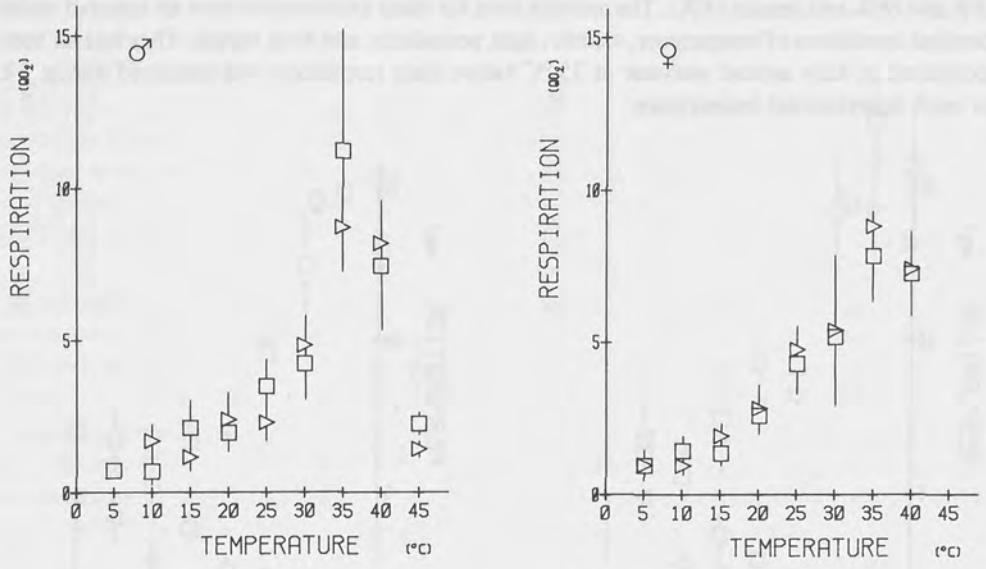

FIG. 1. The active and intermediate rate of respiration of *Artemia* (Great Salt Lake). The respiratory rate is plotted against temperature. (□) actively swimming animals ; (▷) anaesthetized animals.

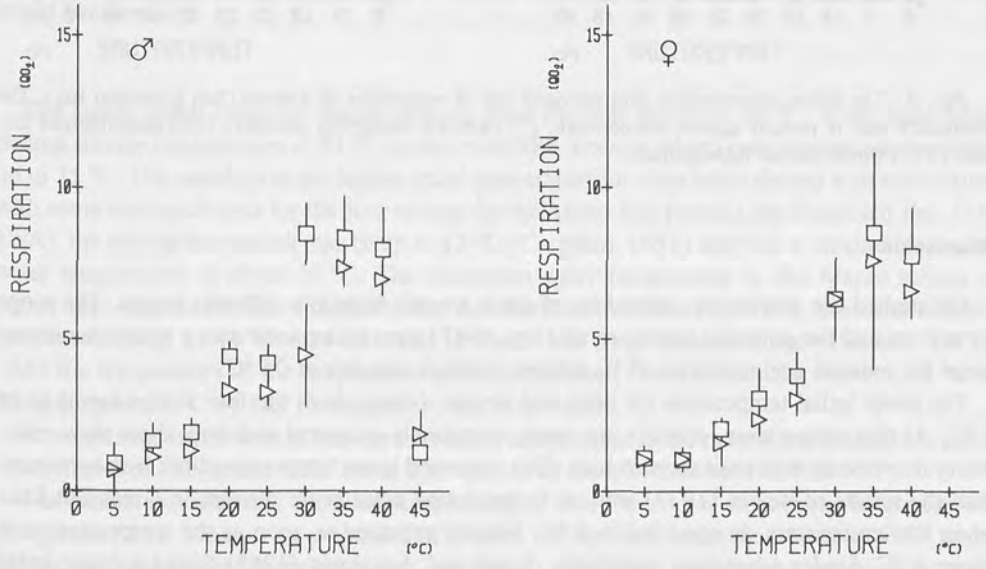

FIG. 2. The active and intermediate rate of respiration of *Artemia* (Macau). The respiratory rate is plotted against temperature. (□) actively swimming animals ; (▷) anaesthetized animals.

SFB and SFX and female SFX). The animals used for these experiments were all cultured under identical conditions of temperature, salinity, light periodicity, and food supply. They had all been acclimated in fully aerated seawater at 25 °C before their respiration was measured during 3 h for each experimental temperature.

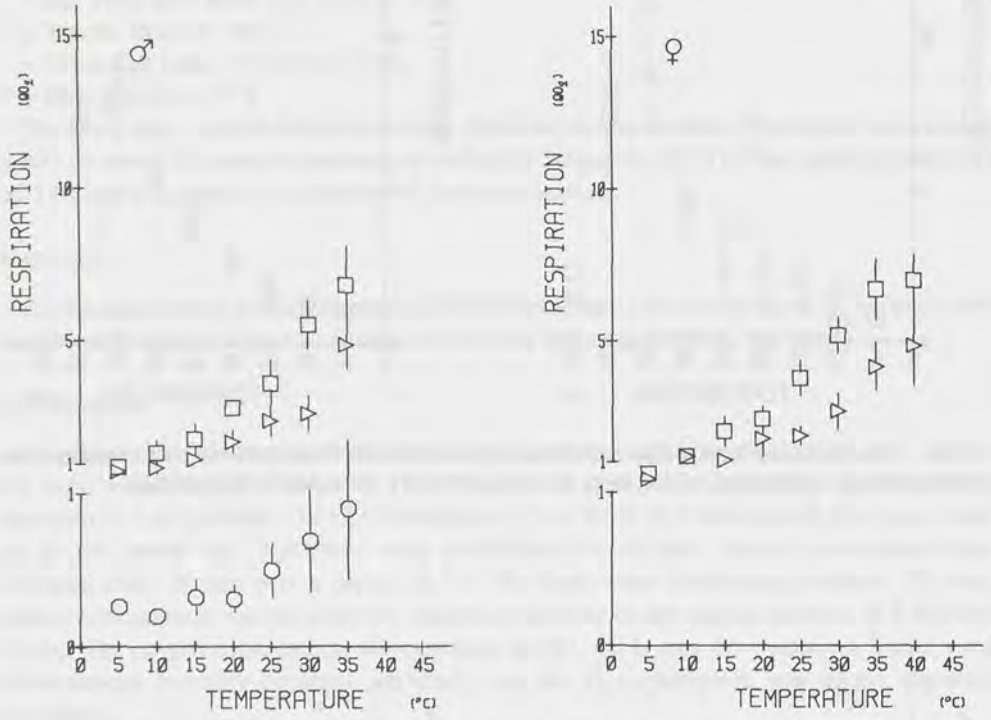

FIG. 3. The active, intermediate, and standard rate of respiration of *Artemia* (San Francisco Bay). The respiratory rate is plotted against temperature. (□) actively swimming animals ; (▷) anaesthetized animals ; (○) whole animal homogenates.

Discussion

We studied the respiratory physiology of adult *Artemia* from four different strains. 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 lower lethal temperature for male and female *Artemia* from the four strains seems to be 5 °C. At this temperature, animals are nearly completely quiescent and only show slow respiratory movements with their thoracopods. This measured lower temperature limit is in agreement with the results of Releya (1937) who no longer found adult brine shrimps in Great Salt Lake when the temperature dropped below 6 °C. Nauplii appeared as soon as the temperature rose above 9 °C. Under laboratory conditions, Engel and Angelovic (1968) found a lower lethal temperature between 5 °C and 8 °C for nauplii. An exception to these results are the findings of Lenz (1980) for Mono Lake *Artemia* (CA, USA), which are still abundant at 2 °C.

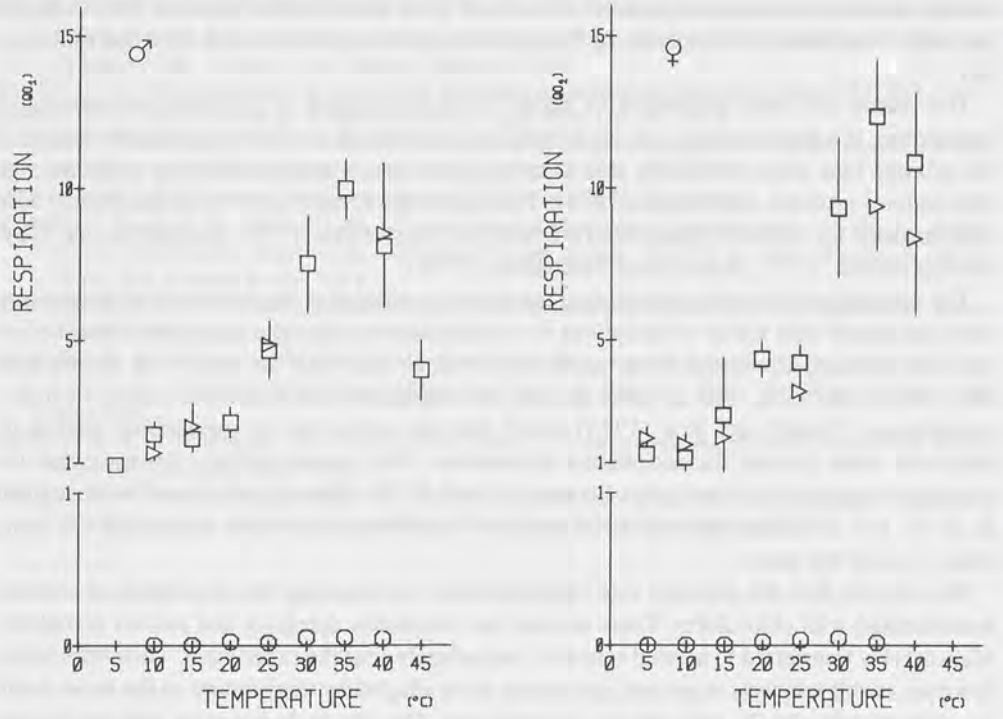

FIG. 4. The active, intermediate, and standard rate of respiration of *Artemia* (Sfax). The respiratory rate is plotted against temperature. (□) actively swimming animals ; (▷) anaesthetized animals ; (○) whole animal homogenates.

The higher lethal value for female *Artemia* from the four strains is 40 °C, while male brine shrimp survive temperatures of 45 °C except male SFB *Artemia*, which only tolerate temperatures up to 35 °C. The variation in the higher lethal temperature for male brine shrimp is in accordance with some ecological data for the four strains. In the Alviso Salt Ponds (San Francisco Bay, CA, USA), the diurnal fluctuation may be up to 12 °C (Carpelan, 1957) and with a maximal recorded water temperature of about 35 °C. The maximum water temperature in the Macau salinas is higher than that in the San Francisco Bay salt marshes (Vanhaecke, 1983). Seasonal changes between 0 °C up to 40 °C were recorded in Great Salt Lake (Stephens and Gillespie, 1972), while the temperature in the evaporation ponds near Sfax (Tunisia) fluctuate between 20 °C in January up to 39 °C in July (Vanhaecke, pers. commun.).

Summarizing, it can be said that the maximum temperature in the natural environment is about equal for MAC, SFX, and GSL while this value is at least 5 °C lower for SFB.

The results also illustrate the quick adaptability of *Artemia* to new environmental conditions. Indeed the MAC *Artemia* are originally SFB brine shrimps who were inoculated in 1977 in the Macau salt marshes by Sorgeloos (pers. commun.). The cysts used for our experiments were harvested in 1979, so within 2 years male *Artemia* show an adaptation to the higher environmental temperature in Macau compared to that in San Francisco Bay. This adaptation must

already be genetically encoded since we cultured all brine shrimp under the same conditions and yet males from Macau could survive 10 °C higher temperatures than the ones from San Francisco Bay.

The reason for these differences in higher lethal temperature is probably the presence of isoenzymes. If a single enzyme has no capability of supporting eurythermia, multiple variants of this enzyme may occur, which are able to catalyze the same reaction at different environmental temperatures (Addink and Zandee, 1978). The occurrence of such isoenzymes has already been demonstrated for different organisms (Baldwin and Hochachka, 1970 ; Hochachka and Clayton-Hochachka, 1973 ; Somero and Hochachka, 1976).

The respiration of actively swimming animals was considered as the active rate of metabolism, which increased with higher temperatures for the four strains. Near the upper lethal temperature we could measure a lowering of the active rate of metabolism only for male brine shrimp from SFX, MAC, and GSL. For all other groups, the respiratory rate reached a plateau at higher temperatures. Newell and Roy (1973) stated that the active rate of metabolism reaches its maximum value around the acclimation temperature. Our results indicate, however, that the maximum oxygen consumption lies between 30 and 35 °C, although all animals were cultured at 25 °C. For all strains the respiration seems to be affected to the same extent, the Q_{10} being about 2 in all the cases.

We tried to find the standard rate of metabolism 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 active animals, the Q_{10} being about 2 in both cases. Our results do not agree with the findings of Newell (1969) who showed that the anaesthetized metabolism of different intertidal animals is very low and remains unaffected by temperatures ranging from the lower lethal temperature up to their optimum. Therefore he considered his measurements as an indication of the standard rate of metabolism.

We also measured the respiration of total tissue homogenates of male and female SFX and male SFB *Artemia*. As can be seen on the appropriate figures, the respiration is much less affected by temperature fluctuations and may perhaps be a better way to express the standard metabolism. These findings are in agreement with the measurements of the respiration of total tissue homogenates from *Littorina littorea* (Newell, 1970).

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Developmental regulation of *Artemia* glycogen phosphorylase

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Abstract

Glycogen phosphorylase from cryptobiotic *Artemia* embryos was purified and K_m values relative to substrates and the allosteric effector AMP were determined.

The conversion reaction of form *b* into form *a* by an endogenous kinase was studied in an extract from dormant cysts.

The relative activity of form *a* and *b* was investigated throughout development up to the nauplius stage. Maximal *b* relative to *a* activity occurred at the pre-emergence stage.

Abbreviations

DTE : dithioerythritol ; PMSF : phenylmethyl-sulfonyl fluoride ; AMP : adenosine 5'-phosphate.

Introduction

The development of the embryos of the brine shrimp *Artemia* may be arrested at the gastrula stage ; the embryos enter a state of dormancy characterized by an almost complete cessation of metabolic activity. This period of dormancy may last several years but the cysts promptly resume development upon rehydration in a solute of adequate salinity (Finamore and Clegg, 1969).

Although some biochemical events associated with the resumption of development have been studied by many authors, the mechanisms by which metabolism can be turned off and on in this organism are largely unknown. Among the different problems to be elucidated, we focused our attention on the role played by the enzymes of carbohydrate metabolism.

Among the carbohydrate reserves present in *Artemia* cysts, glycogen is the storage polysaccharide whose degradation is known to be subjected to various metabolic controls. We have therefore investigated the molecular and kinetic properties of glycogen phosphorylase (E.C. 2.4.1.1.), the allosteric enzyme that occupies a key position in glycogenolysis.

The activity of glycogen phosphorylase is regulated through several different control mechanisms. One of these is the interconversion of a dephosphorylated form into a phosphorylated one by a specific kinase (ATP-phosphorylase phosphotransferase, E.C. 2.7.1.38). This enzyme, in turn, is activated through phosphorylation by cyclic AMP-dependent protein kinase (E.C. 2.7.1.37).

We have already described the biochemical properties of purified α,α-trehalase (α,α-trehalose 1-D-glycohydrolase, E.C. 3.2.1.28) from *Artemia* cysts (Ballario *et al.*, 1978). This enzyme hydrolyzes the other main storage disaccharide present in the dormant cysts, α,α-trehalose.

In this paper we present the characterization of the *Artemia* glycogen phosphorylase, extracted from dormant cysts. Its conversion from the dephosphorylated *b* form into the phosphorylated *a* form was also investigated.

Materials and methods

Artemia cysts were purchased from San Francisco Bay Brand Inc. They were first washed with a saturated sodium chloride solution to eliminate sand and debris and then treated with 1 % w/v sodium hypochlorite (expressed as available chlorine) solution in an ice-bath for 10 min. Non-viable embryos floating at the surface were removed by suction. The treated embryos were washed extensively with ice-cold distilled water and air dried over a nylon screen at room temperature. Embryos could be stored indefinitely over silica gel at room temperature.

Oyster glycogen, bovine serum albumin, dithioerythritol (DTE), adenosine 5′-phosphate (AMP) and sodium β-glycerophosphate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glucose-6-phosphate dehydrogenase, phosphoglucomutase and nicotinamide-adenine dinucleotide phosphate (NADP) were from Boehringer (Mannheim, Fed. Rep. Germany). All other chemicals used were reagent grade.

Assay of glycogen phosphorylase activity

Glycogen phosphorylase activity was assayed in the direction of glycogen breakdown by coupling the production of glucose-1-phosphate to the reduction of $NADP^+$ via phosphoglucomutase and glucose-6-phosphate dehydrogenase. The standard incubation mixture for the assay of phosphorylase activity contained the following in a total volume of 400 µl : 0.1 M phosphate buffer pH 6.8, 10 mM $MgCl_2$, 2 mM EDTA, 0.6 mM NADP, 5 mM AMP, 1.2 % glycogen, 4 µg/ml phosphoglucomutase, 2 µg/ml glucose-6-phosphate dehydrogenase. The reaction was started by the addition of the enzyme and the increase in absorbance at 340 nm was followed using a Gilford Mod. 2400-S recording spectrophotometer. The temperature of the assay mixture was maintained at 23 °C±1 °C. One unit of activity is defined as the amount of enzyme catalyzing the synthesis of 1 µmole of NADPH/min at 23 °C. A molar extinction coefficient of 6.3×10^3/M/cm was used to quantify NADPH.

Conversion of glycogen phosphorylase *b* into the *a* form by endogenous phosphorylase kinase

Crude extract was incubated at 37 °C in a medium consisting of : 3 mM ATP, 10 mM $MgCl_2$, 1 mM EDTA, 20 mM sodium β-glycerophosphate pH 6.8. At various times 2 µl aliquots were taken and assayed for glycogen phosphorylase activity both in the presence and in the absence of AMP. The dilution to the volume of the standard assay mixture (400 µl) was sufficient to arrest enzyme phosphorylation.

Enzyme determination during development

Embryos were allowed to develop by incubating the washed cysts in Millipore-filtered seawater at 18 °C. Nauplii free from shells and unhatched embryos were collected by taking advantage of their phototactic movements toward a light source.

Embryos were collected at various times during development by low speed centrifugation. One ml aliquots of packed embryos were then suspended in 3 ml of homogenization buffer containing 20 mM sodium β-glycerophosphate, pH 6.8, 0.1 M NaCl, 1 mM DTE, 1 mM EDTA, 0.1 mM

PMSF, 25 % (v/v) glycerol and homogenized in a Potter homogenizer fitted with a teflon pestle. The homogenate was centrifuged at 30 000×*g* for 30 min at 4 °C and the supernatant used for the determination of enzyme activity.

Protein determination

Proteins were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results

Enzyme characterization

Enzyme activity extracted from *Artemia* cysts was purified to homogeneity as described elsewhere (Sada *et al.*, submitted). The kinetic parameters of purified *Artemia* glycogen phosphorylase were calculated for glycogen, inorganic phosphate, and AMP. The K_m value for glycogen was 0.2 % (w/v), equivalent to 1.2×10^{-2} M (expressed as concentration of glycosidic monomeric units, Fig. 1). At high glycogen concentrations an inhibitory effect was observed. A glycogen concentration of 1.2 % (w/v) was selected to determine the kinetic parameters relative to AMP and P_i.

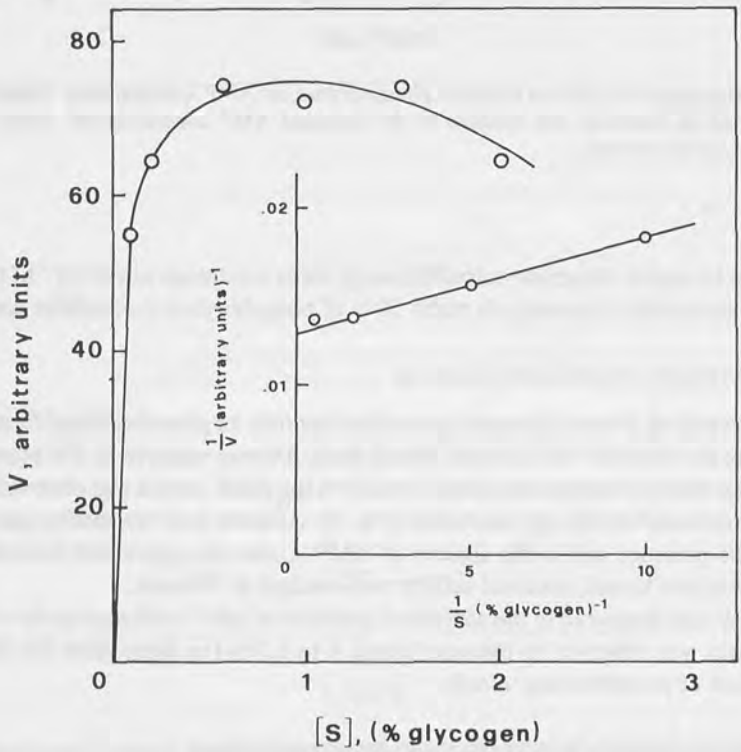

FIG. 1. Dependence of *Artemia* glycogen phosphorylase on glycogen concentration. Assays were carried out as described in Materials and methods, except for glycogen concentration which was as indicated. Insert : data plotted according to Lineweaver-Burk.

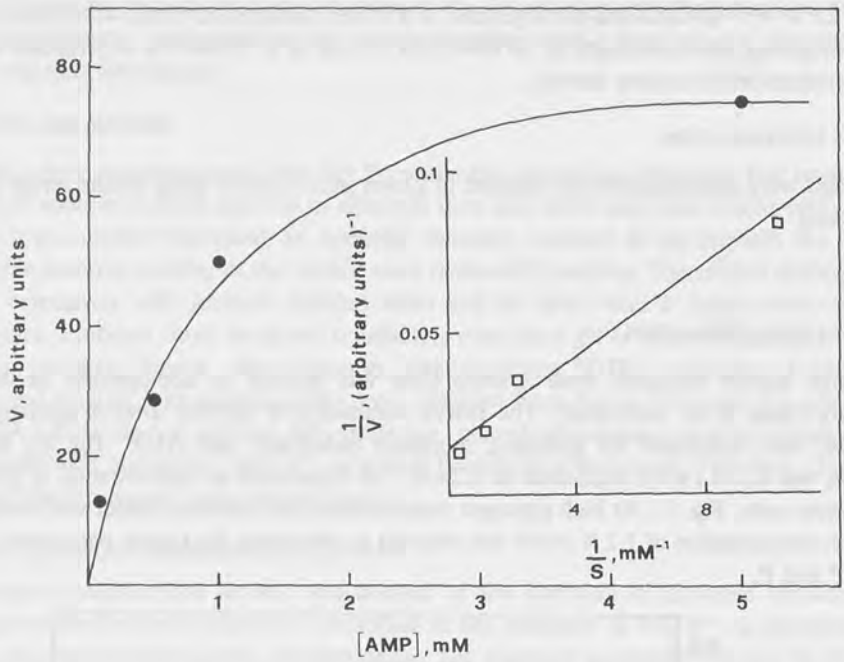

FIG. 2. Dependence of *Artemia* glycogen phosphorylase on AMP concentration. Assays were carried out as described in Materials and methods at the indicated AMP concentrations. Insert: data plotted according to Lineweaver-Burk.

The K_m for inorganic phosphate calculated under these conditions was 5×10^{-3} M (Fig. 2) while the AMP concentration necessary to reach 50 % of phosphorylase *b* activation was 6×10^{-4} M.

ENZYME CONVERSION BY ENDOGENOUS KINASE

The conversion of *Artemia* glycogen phosphorylase into its phosphorylated form was studied by incubating the enzyme with a crude extract from *Artemia* embryos in the presence of Mg^{++} and ATP. The effect of endogenous kinase present in the crude extract was observed. Phosphorylase activity increased during the incubation (Fig. 3). Aliquots from the incubation mixture were assayed in the presence and in the absence of AMP. Under the conditions used for incubation with phosphorylase kinase, maximal activity was reached in 100 min.

The activity ratio measured in the absence or presence of AMP, indicated as the -AMP/+AMP activation ratio, was observed to increase from 0.4 to 0.75. The latter value can be ascribed to the occurrence of phosphorylase *a* only.

DEVELOPMENTAL CHANGES IN GLYCOGEN PHOSPHORYLASE LEVELS

The presence of an active form of glycogen phosphorylase in *Artemia*, already at the stage of dormant gastrulae, indicates that this enzyme is stored (probably associated to glycogen

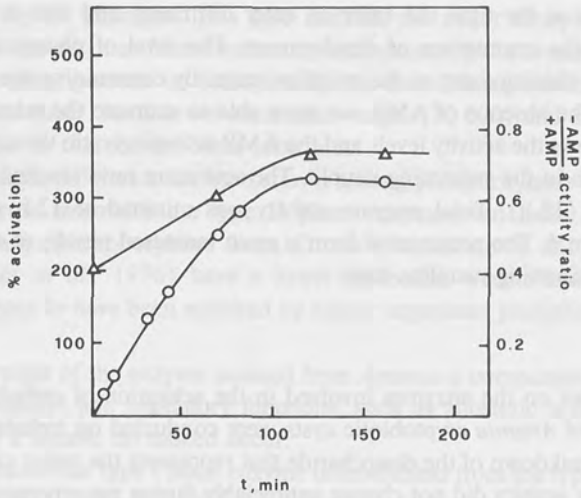

FIG. 3. Conversion of *Artemia* cyst glycogen phosphorylase by endogenous phosphorylase kinase. (○——○) % of activation (with respect to time 0 activity measured in the absence of AMP). (△——△) -AMP/+AMP activity ratio.

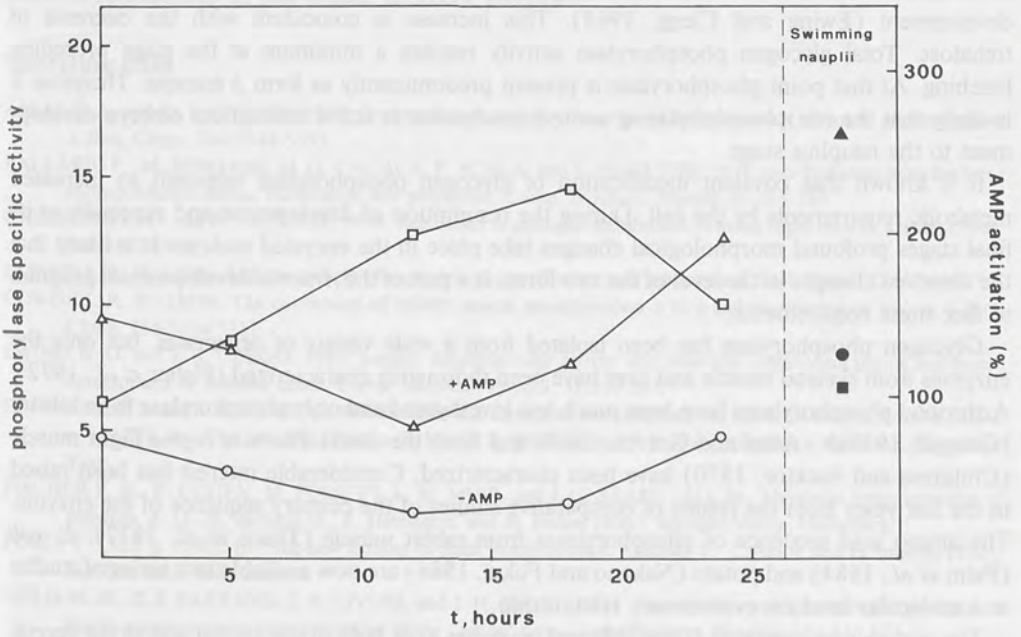

FIG. 4. Activity of glycogen phosphorylase during the development of *Artemia* embryos. Activity was measured in the absence (○——○) and in the presence (△——△) of AMP. (□——□) -AMP/+AMP activity ratio. Black symbols refer to the activity measured in swimming nauplii.

particles) in the cysts at the time the embryos enter dormancy and that it is ready to initiate glycogenolysis upon the resumption of development. The level of phosphorylase activity was measured throughout development to the nauplius stage. By determining the enzyme activity in the presence and in the absence of AMP, we were able to estimate the relative ratio of form *b* and form *a*. Fig. 4 shows the activity levels and the AMP activation ratio throughout development to emergence and also in the swimming nauplii. The activation ratio reached a maximum at the pre-emergence stage (18 h). Total enzyme activity was minimal at 12 h and it was present predominantly as form *b*. The presence of form *a* again increased rapidly at emergence to reach a maximum at the swimming nauplius stage.

Discussion

Our previous studies on the enzymes involved in the activation of carbohydrate metabolism during development of *Artemia* cryptobiotic cysts were conducted on trehalase, the glycosidase responsible for the breakdown of the disaccharide that represents the major carbohydrate reserve in the cysts. Trehalase activity did not change appreciably during pre-emergence development of the cyst. The 3-fold activation obtained after incubation with Mg^{++} and ATP indicates that *Artemia* glycogen phosphorylase exists in two interconvertible forms and that the interconversion is catalyzed by a kinase and a phosphatase. Such mechanism is effective in animal phosphorylases (Graves and Wang, 1972) and was also found to occur in *Neurospora crassa* (Gold *et al.*, 1974).

The activity of glycogen phosphorylase is also dependent on the developmental stage of *Artemia*. It is known that there are changes in the amount of glycogen of *Artemia* cysts during development (Ewing and Clegg, 1969). This increase is coincident with the decrease of trehalose. Total glycogen phosphorylase activity reaches a minimum at the stage preceding hatching. At that point phosphorylase is present predominantly as form *b* enzyme. Therefore it is likely that the (de)phosphorylating control mechanism is active throughout embryo development to the nauplius stage.

It is known that covalent modification of glycogen phosphorylase responds to increased metabolic requirements by the cell. During the resumption of development and especially at its final stages profound morphological changes take place in the encysted embryo. It is likely that the observed changes in the level of the two forms is a part of the *Artemia* developmental program to face these requirements.

Glycogen phosphorylase has been isolated from a wide variety of organisms, but only the enzymes from skeletal muscle and liver have been thoroughly characterized (Fisher *et al.*, 1972). Arthropod phosphorylases have been much less investigated and only phosphorylase from lobster (Cowgill, 1959ab ; Assaf and Graves, 1969) and from the insect *Phormia regina* flight muscle (Childress and Sacktor, 1970) have been characterized. Considerable interest has been raised in the last years from the results of comparative studies of the primary sequence of the enzyme. The amino acid sequence of phosphorylases from rabbit muscle (Titani *et al.*, 1977), *E. coli* (Palm *et al.*, 1984) and potato (Nakano and Fukui, 1986) are now available to a series of studies at a molecular level on evolutionary relationship.

The picture now emerging is that different isozymes exist both in the animal and in the vegetal kingdom. Higher plant phosphorylases have been classified into two types according to their molecular weight and glucan specificities (Fukui, 1983). Type I enzymes have a molecular weight of about 110 000 and show high affinities for amylopectin, amylose, and maltodextrin, but

extremely low affinity for glycogen. This type includes the major component of potato tuber phosphorylase and the enzymes from sweet corn and spinach leaf chloroplast.

The type II enzyme, present in the spinach leaf non-chloroplast fraction and a minor component in potato tuber, has a lower molecular weight (90 000) and exhibits an affinity for glycogen comparable to that for the other substrates.

No molecular weight difference has been shown among phosphorylases of animal origin. The rabbit muscle enzyme resembles the type II plant enzymes, but lacks the ability to utilize maltodextrins. Finally, phosphorylases from *E. coli* (Schächtele *et al.*, 1978) and from *K. pneumoniae* (Linder *et al.*, 1976) have a lower molecular weight and lack the regulatory properties which seem to have been acquired by higher organisms phosphorylases in the course of evolution.

The molecular weight of the enzyme isolated from *Artemia* is comparable to that of the rabbit enzyme (unpubl. results) and regulatory functions, such as allosteric activation by AMP and phosphorylation by a kinase, do indeed occur.

It has been suggested that type I plant enzyme differentiated from the type II isozyme through a large insertion in the middle of its polypeptide chain. Concurrently, in the animal kingdom, various changes have led to lower binding capabilities for glucans, accompanied by the acquisition of two different kinds of regulation : 1) the presence of the phosphorilatable and of AMP-binding sites ; and 2) the presence of the glycogen storage site.

Further studies on the biochemical properties of this arthropod enzyme and on the sequence homology with other enzymes may shed light on the evolutionary aspects of the structure-function relationship in this unique allosteric enzyme.

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Intracellular pH and anhydrobiosis in *Artemia* cysts

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Abstract

We have investigated the mechanism by which dormant cysts terminate dormancy. Our previous studies suggested that depressed rates of metabolism in anoxybiotic cysts are maintained by depressed pH_i. The available data support that premise. However, previous data also indirectly suggested that dormant cysts might be maintained in the dormant state by the same mechanism. That does not appear to be the case. Instead, the best available evidence suggests that pH_i may be elevated in the dormant cysts, although there is a suggestion in the data of the presence of a minor acidic compartment that may be important in terminating dormancy. Characterization of this compartment is a current goal. We have also studied the mechanism by which *Artemia* cysts survive dehydration. The ability of these cysts and other organisms to survive dehydration may be related directly to the interesting properties of trehalose discussed here. This molecule appears to be capable of replacing water around polar residues in membrane phospholipids and proteins and thereby to maintain the membrane in a fluid state in the absence of water. The increased fluidity in the dry membranes is, in turn, responsible for alleviating lateral phase separations of membrane lipids and proteins, and maintaining low permeability in the dry bilayer. The mechanism by which other biological molecules and structures are preserved in the dry cysts awaits study.

What controls termination of dormancy in *Artemia* cysts?

There is considerable confusion in the literature concerning use of the word "dormancy" as it applies to *Artemia* cysts, so we would like to describe carefully the way we think about the various states of arrested metabolism in which these cysts may exist and the relationship between those states and dormancy, as summarized in Fig. 1. Most of the terminology used here has been adapted from the classic review of Keilin (1959).

Cysts newly released from the ovisac can remain developmentally arrested for long periods, even under conditions that would seem to be favorable to hatching. Thus, these cysts possess endogenous controls over their own development. We call such cysts "dormant". Dormancy can be terminated by dehydration in the sense that before dehydration the cysts will not hatch, but after dehydration and rehydration they will. Since the rehydrated cysts will resume development under favorable conditions, they are no longer dormant, but instead exist in a quiescent state imposed by the environment, a state we call "anhydrobiosis." More recently, we have shown that dormancy can be terminated by storing the cysts at low temperatures (Fig. 2). Storing the cysts in the cold inhibits development, like dehydration, but cysts so treated are seen to develop readily when they are returned to favorable conditions. Thus, the cysts in the cold are no longer dormant,

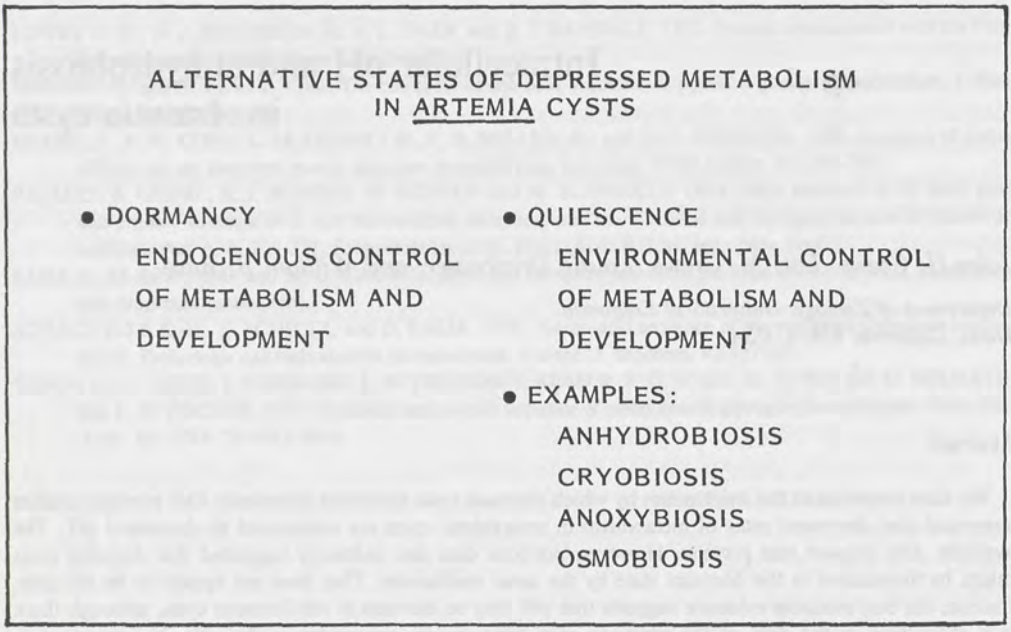

FIG. 1. Summary of the alternative states of depressed metabolism exhibited by *Artemia* cysts.

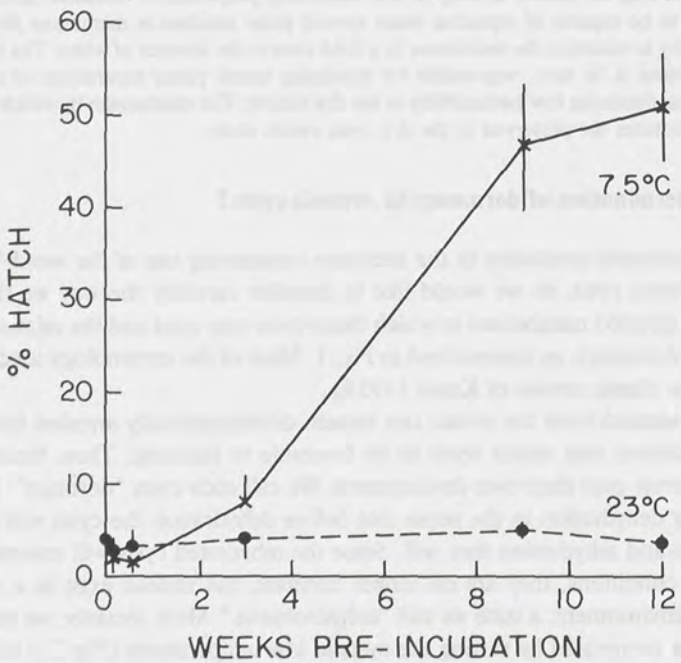

FIG. 2. Hatching of previously dormant cysts following pre-incubation at 7.5 °C for the indicated intervals, after which they were returned to room temperature and % hatching was recorded after 72 h.

but they are quiescent, a condition we call "cryobiosis." Finally, another form of quiescence can be induced by keeping the cysts under anoxic conditions (Dutrieu and Chrestia-Blanchine, 1966 ; Ewing and Clegg, 1969). Such cysts show greatly depressed metabolism, aside from metabolism of diguanidine tetraphosphate (Ewing and Clegg, 1969). We call this state "anoxybiosis." Dormancy and quiescence (of which there are several different types) thus represent two alternative states of the cysts, both of which result in developmental arrest. We have been studying for some time the basis for maintaining hypometabolic states in quiescent cysts and for terminating dormancy in dormant cysts, and we will review our progress in the following paragraphs.

Intracellular pH in anoxybiotic and dormant cysts

The last decade has witnessed an impressive advance in the ability of biologists to measure and manipulate intracellular pH (pH_i) in a wide variety of organisms (Busa *et al.*, 1982). A generality appears to be emerging from such studies that large excursions in pH_i may be responsible for maintaining cells in states of depressed metabolism. For example, sea urchin eggs show a depressed metabolism before fertilization, and they also appear to possess a depressed pH_i. When they are fertilized, pH_i rises by about 0.5 units (Shen and Steinhardt, 1978) and there is a coincident increase in metabolism (Shen and Steinhardt, 1980). Similarly, using ^{31}P-nuclear magnetic resonance to measure pH, we have shown that anoxybiotic *Artemia* cysts possess a depressed pH_i – as low as 6.2, in fact – and that when the cysts are supplied with oxygen pH_i rises by more than one pH unit (Busa and Crowe, 1983).

Effects of pH_i on metabolism

In order to assess the physiological significance of the pH_i decrease in anoxybiotic cysts, we have tested the effects of depressed pH_i on the ability of cysts to respire. These experiments were done in such a way that O_2 was maintained constant, while CO_2 was elevated. CO_2 diffuses into the cysts and ionizes as H^+ and HCO_3^-, thus depressing pH_i. Using combined ^{31}P-NMR to measure pH_i and polarography to record oxygen uptake, we have shown that treating non-dormant cysts with 60 % CO_2 depresses pH_i from >7.9 to 6.8. The depressed pH_i, in turn, decreases oxygen uptake by 2/3 (Busa and Nuccitelli, 1984) (Fig. 3). This inhibition of respiration is reversed by removal of CO_2. The basis for depression of metabolism in these cysts with low pH_i is almost certainly due to effects of pH on enzymatic activity. For example, phosphofructokinase, a key enzyme in regulating glycolysis, commonly has a pH optimum above pH 7.0, and below about pH 6.8 enzymatic activity is sharply inhibited (Busa *et al.*, 1982).

Effects of depressed pH_i on development

Development is also inhibited by acidification of the cytoplasm of the cysts by treating them with CO_2. By contrast with cysts hydrated under aerobic conditions in the absence of CO_2, cysts hydrated in the presence of either 11 % or 60 % CO_2 (which produces pH_i=7.4 or 6.8, respectively) do not hatch for at least 110 h in CO_2, despite the fact that oxygen was available at atmospheric concentrations. When CO_2 is removed, normal development proceeds, and, in fact, development appears to have an increased synchrony following the CO_2 incubation, a finding which should have practical applications (Busa and Nuccitelli, 1984) (Fig. 4).

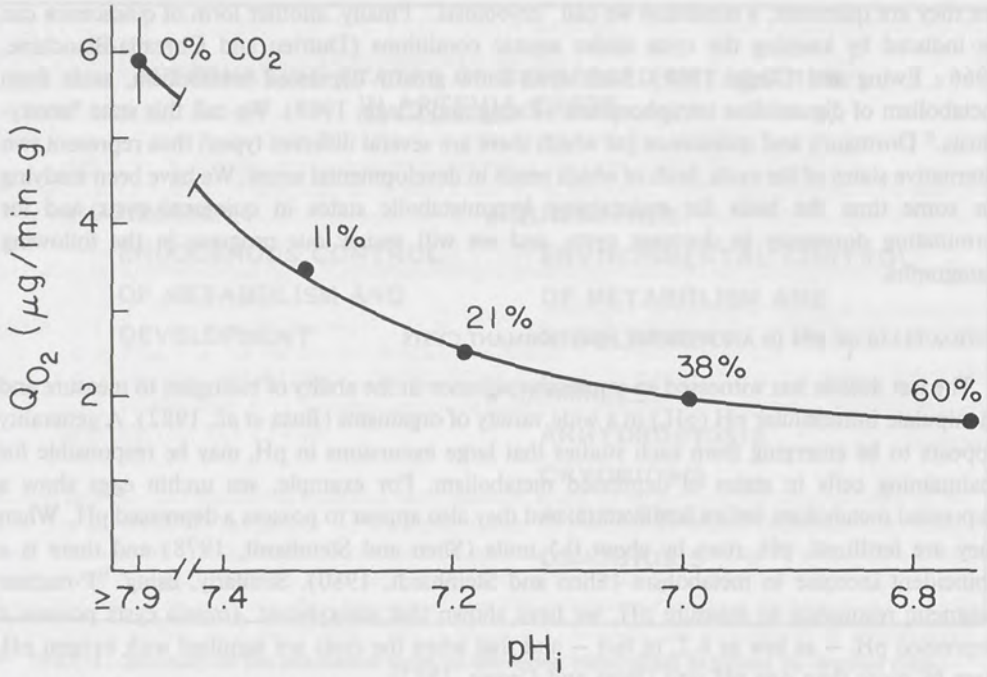

FIG. 3. Oxygen uptake by non-dormant cysts as a function of intracellular pH (from Busa and Nuccitelli, 1984).

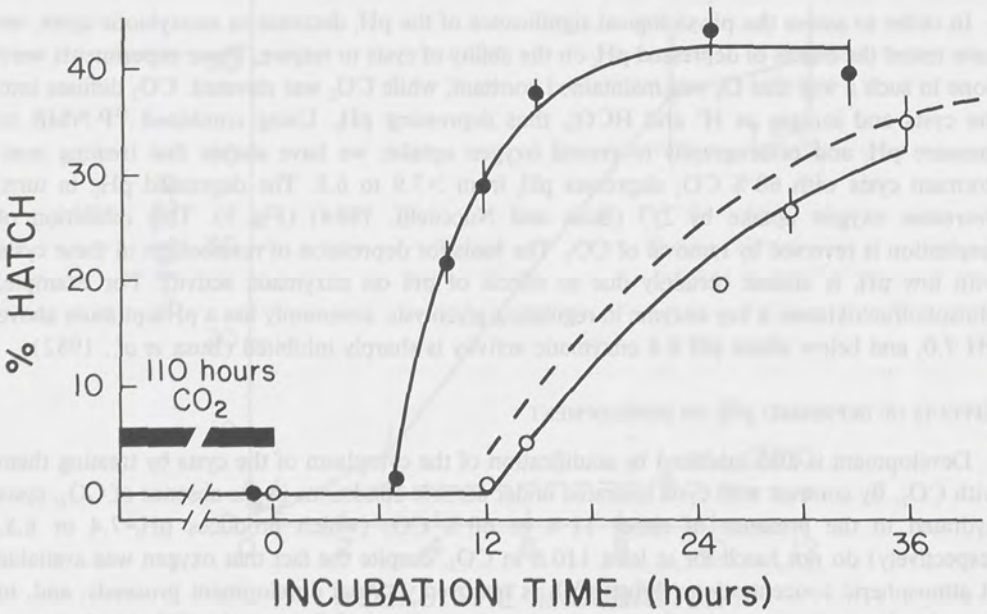

FIG. 4. Inhibition of hatching of non-dormant cysts by depression of intracellular pH in the presence of CO_2 (from Busa and Nuccitelli, 1984).

DOES pH_i MAINTAIN DORMANCY?

We have previously shown that elevation of pH_i by treating what we believed at the time to be dormant cysts with NH_3 will effect an increase in rate of development. Using NMR, we showed separately that NH_3 diffuses into anoxybiotic cysts and upon ionization increases pH_i. At the time these studies were done, we believed the cysts used in the hatching studies (Busa and Nuccitelli, 1984) to be dormant ; they were collected from females in the laboratory and never exposed to dehydration. We made the reasonable assumption that such cysts would remain dormant until they were dehydrated. What we did not realize at the time is that exposure to cold will also terminate dormancy, and consequently we stored the cysts at 4 °C before use. We now know that cysts so treated terminate dormancy, and we are forced to conclude that our previous data on effects of NH_3 on development (Busa and Nuccitelli, 1984) do not reflect responses of truly dormant cysts. Thus, the evidence for our previous inference that dormancy is maintained by depressed pH_i is not supported by these data, although the depressed pH_i is likely to play a major role in regulating metabolism in anoxybiotic cysts. We stress again that anoxybiotic cysts are not truly dormant. However, we still believe that pH_i may play a role in maintaining the cysts in a dormant state, even though the mechanism may be more complex than we previously thought. We have recently been studying pH_i in dormant cysts collected in the laboratory and carefully maintained in a dormant state. Such cysts can be kept at room temperature for extended periods without hatching. It is not an easy task to accumulate enough of such cysts for measurement of pH_i with ^{31}P-NMR, but we have been able to do so, nevertheless. When we attempted to measure pH_i in such cysts we obtained a surprising result : the major inorganic phosphate peak as seen with ^{31}P-NMR suggests that pH_i is not depressed in these cysts, instead it is elevated to some value greater than 7.9, not significantly different from pH_i in non-dormant cysts (Busa *et al.*, 1982 ; Busa and Crowe, 1983). Unfortunately, ^{31}P-NMR does not permit one to measure $pH > 7.9$, so at this point we do not know just how high pH_i might be in the dormant and non-dormant cysts. But we do know that pH_i is not depressed in dormant cysts, in contrast with our previous inference (Busa and Crowe, 1983). We are using other methods to measure pH_i that may provide the needed information, but have no data to report as yet that have direct bearing on this issue. However, we have done the following suggestive experiment. The dormant cysts were exposed to CO_2 at various concentrations and effects on pH_i were determined with ^{31}P-NMR. If an appropriate concentration of CO_2 is used, pH_i can be maintained in a range usually thought to be physiologically appropriate. For example, when dormant cysts are incubated in a stream of air containing 10 % CO_2, ^{31}P-NMR specta tell us that pH_i is depressed from >7.9 to 7.4. When dormant cysts so treated were kept in the CO_2 for a short time and then transferred to aerobic conditions at room temperature, they were seen to have broken dormancy and proceed to develop and ultimately hatch into nauplii (Fig. 5). It is tempting to conclude from these data that pH_i is elevated out of the physiological range in the dormant cysts and that breaking dormancy involves driving pH_i down into the physiological range. However, we are unwilling at this point to accept without further evidence this simple hypothesis for the following reasons. The ^{31}P-NMR spectra suggest that there may actually be two compartments in the dormant cysts that are separated in pH by about 0.5 pH units. We do not yet understand the role of these two compartments in dormancy, if any. Thus, breaking dormancy appears to be considerably more complex than acidification of a basic cytoplasm. The several possible mechanisms suggested by our data are under intense study at this time.

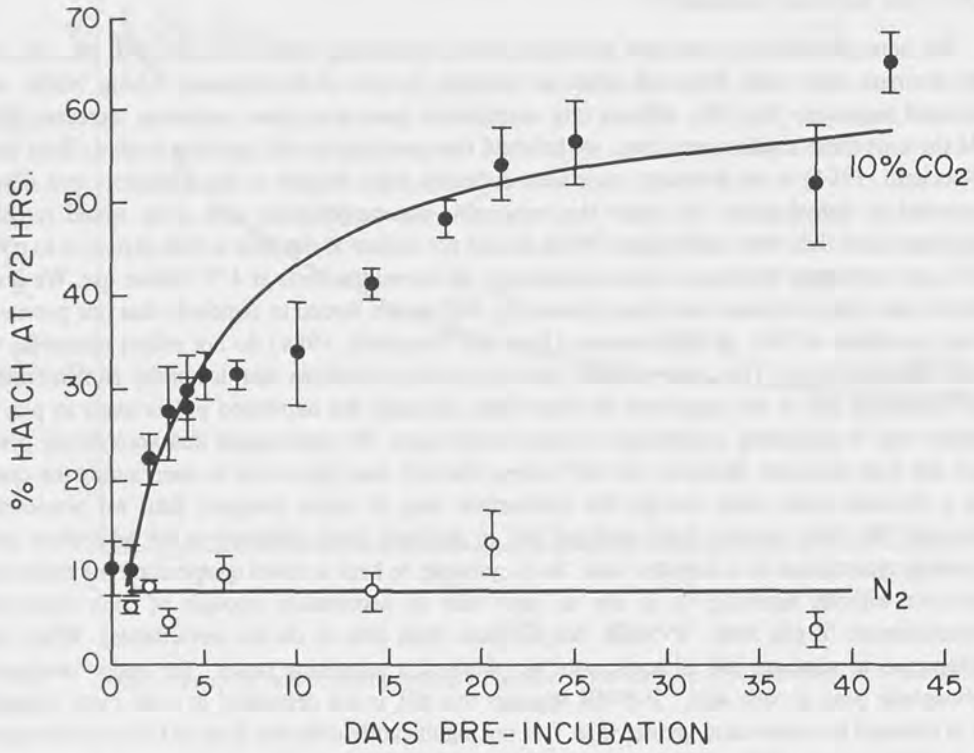

FIG. 5. Hatching of previously dormant cysts following pre-treatment with 10 % CO_2. Following the CO_2 treatment the cysts were aerated without CO_2, and % hatching was recorded after 72 h.

How do *Artemia* cysts survive dehydration?

Water is indispensable for maintenance of integrity of biological membranes and macromolecules (Keilin, 1959 ; Wieslander *et al.*, 1978 ; Tanford, 1980 ; Volke *et al.*, 1982 ; McDaniel *et al.*, 1983 ; Clegg, 1984ab ; Crowe and Crowe, 1984). Removal of the water in the vicinity of a biological membrane, for example, irreversibly violates its structural (Crowe and Crowe, 1982, 1983, 1984) and functional (Crowe *et al.*, 1983a) integrity. Nevertheless, *Artemia* cysts are capable of surviving removal of essentially all their intracellular water without being killed (*e.g.* Clegg, 1984a). The cysts, said to be in a state of anhydrobiosis (see Crowe, 1971 ; Crowe and Clegg, 1978 ; Bewley, 1979 ; Womersley, 1981 ; Crowe and Crowe, 1984 ; Crowe *et al.*, 1987 for reviews), can persist in the dehydrated state for long periods, and may resume active metabolism, sometimes within minutes, when they are rehydrated. We have been interested for some years in the mechanisms which permit these and other organisms to survive desiccation and most recently particularly in the means by which membranes in the dry organisms are stabilized in the dry state. We have made considerable progress towards achieving an understanding of the basic mechanisms and summarize this progress in the following paragraphs.

Biochemistry of anhydrobiotic organisms

Trehalose (Fig. 6) is a nonreducing disaccharide of glucose (Jeffrey and Nanni, 1985) commonly found at high concentrations (as much as 20 % of the dry weight) in anhydrobiotic organisms, including cysts of *Artemia* (Clegg, 1965). Some other anhydrobiotic organisms that contain high concentrations of this molecule include (reviewed in Crowe and Crowe, 1984 ; Crowe *et al.*, 1984b) : spores of certain fungi ; the desert resurrection plant ; macrocysts of the slime mold *Dictyostelium* ; dry active baker's yeast ; and the dry larvae and adults of many species of soil-dwelling rotifers, tardigrades, and nematodes. Many of these organisms can persist in the dry state for many years, but when they come in contact with water they rapidly swell and resume metabolic activities. Survival of dehydration by at least some of these organisms has been shown to be correlated with synthesis of trehalose (Madin and Crowe, 1975) or its degradation following rehydration (Crowe *et al.*, 1977). We have found that this molecule has some remarkable properties in that it is capable of stabilizing biological structure in the dry state.

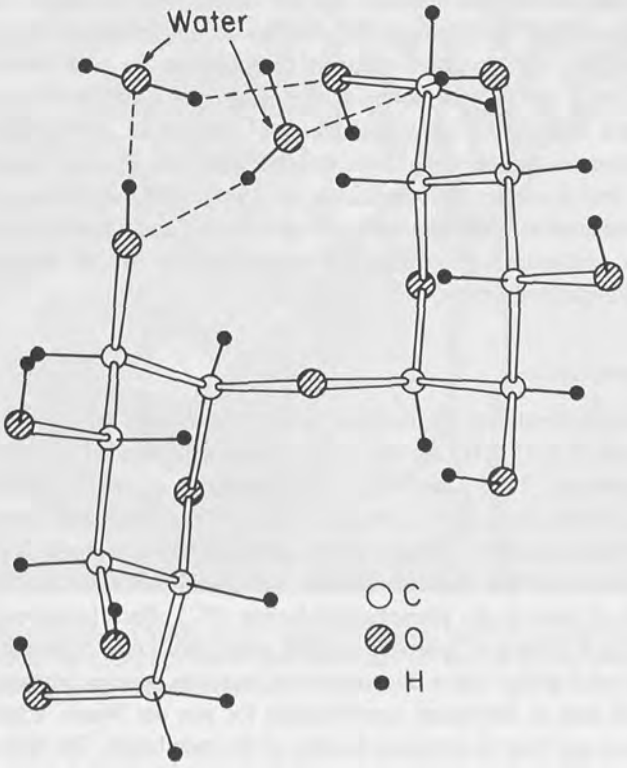

Fig. 6. Structure of trehalose.

Trehalose and stabilization of membranes

In order to study in detail the damaging events that occur during dehydration of a membrane, we have used Ca-transporting vesicles isolated from custacean muscle as a model system. We chose this particular membrane system because it has a characteristic morphology ; intramembrane particles, known to represent the Ca-ATPase in these membranes are predominantly on one half of the bilayer, as seen with freeze fracture (Fig. 7a). In addition, the membranes have a specialized biological function, they accumulate Ca from the surrounding medium. Thus, the consequences of dehydration can be studied for the morphological and functional integrity of the membrane. When the vesicles are dehydrated, the phospholipids form complex crystalline phases, some of which are nonbilayer structures (Crowe and Crowe, 1982), and intramembrane particles are excluded from these crystals (Crowe and Crowe, 1982 ; Crowe *et al.*, 1983ab). Upon rehydration the vesicles show evidence of extensive morphological damage – including fusion between vesicles and redistribution of intramembrane particles – and the ability to accumulate Ca is lost (Fig. 7d) (Crowe *et al.*, 1983b). By contrast, when the membranes are dried in the presence of trehalose at concentrations of at least 20 % of the dry weight (concentrations similar to those found in anhydrobiotic organisms) no evidence of formation of nonbilayer crystalline phases is seen during dehydration. In fact, in the dry state the vesicles appear as cup-shaped vesicles embedded in a matrix of trehalose (Fig. 7c), and upon rehydration of such preparations vesicles are obtained that are similar morphologically and functionally to freshly prepared ones (Fig. 7b) (Crowe *et al.*, 1983b). At physiological concentrations this effect is unique to trehalose ; the next most effective carbohydrate we have tested at stabilizing dry membranes is sucrose, and we have found that at least three times as much sucrose is required to achieve the same stabilization as with trehalose (Crowe *et al.*, 1984a). We have also shown that the dry membranes can be stored without significant loss of physiological function if they are kept dry and free of oxygen (Mouradian *et al.*, 1984, 1985) and that they can be frozen in the presence of trehalose without significant damage (Rudolf and Crowe, in press). We have most recently provided evidence concerning the mechanism by which trehalose stabilizes dry membranes, as summarized below.

Water and phospholipids

Membrane phospholipids are hydrated to some extent, with 10-12 water molecules (circa 20 % water content or 0.25 g H_2O/g dry weight) hydrogen bonded to each polar head group (Fringerli and Gunthard, 1976 ; Lee, 1977 ; Wieslander *et al.*, 1978 ; Schneider *et al.*, 1979 ; Chapman, 1982 ; Volke *et al.*, 1982 ; Smaby *et al.*, 1983 ; Crowe and Crowe, 1984). Little is known about the disposition of this water around the polar residues, but it is known that if affects the physical properties of the lipids profoundly (reviewed in Crowe and Crowe, 1984). For example, addition of water to dry phosphatidylcholine (PC) effects lateral expansion of bilayers, probably by forming a ribbon of hydrogen-bonded water molecules connecting polar residues in the phospholipid head group. Since this expansion increases spacing between head groups, one might expect it to lead to decreased opportunities for van der Waals' interactions among the hydrocarbon chains and thus to increased fluidity of the membrane. The first demonstration that this is the case came from Chapman *et al.* (cf. Chapman, 1982 ; Kodama *et al.*, 1982 ; Crowe and Crowe, 1984), who reported that the gel to liquid crystalline phase transition temperature

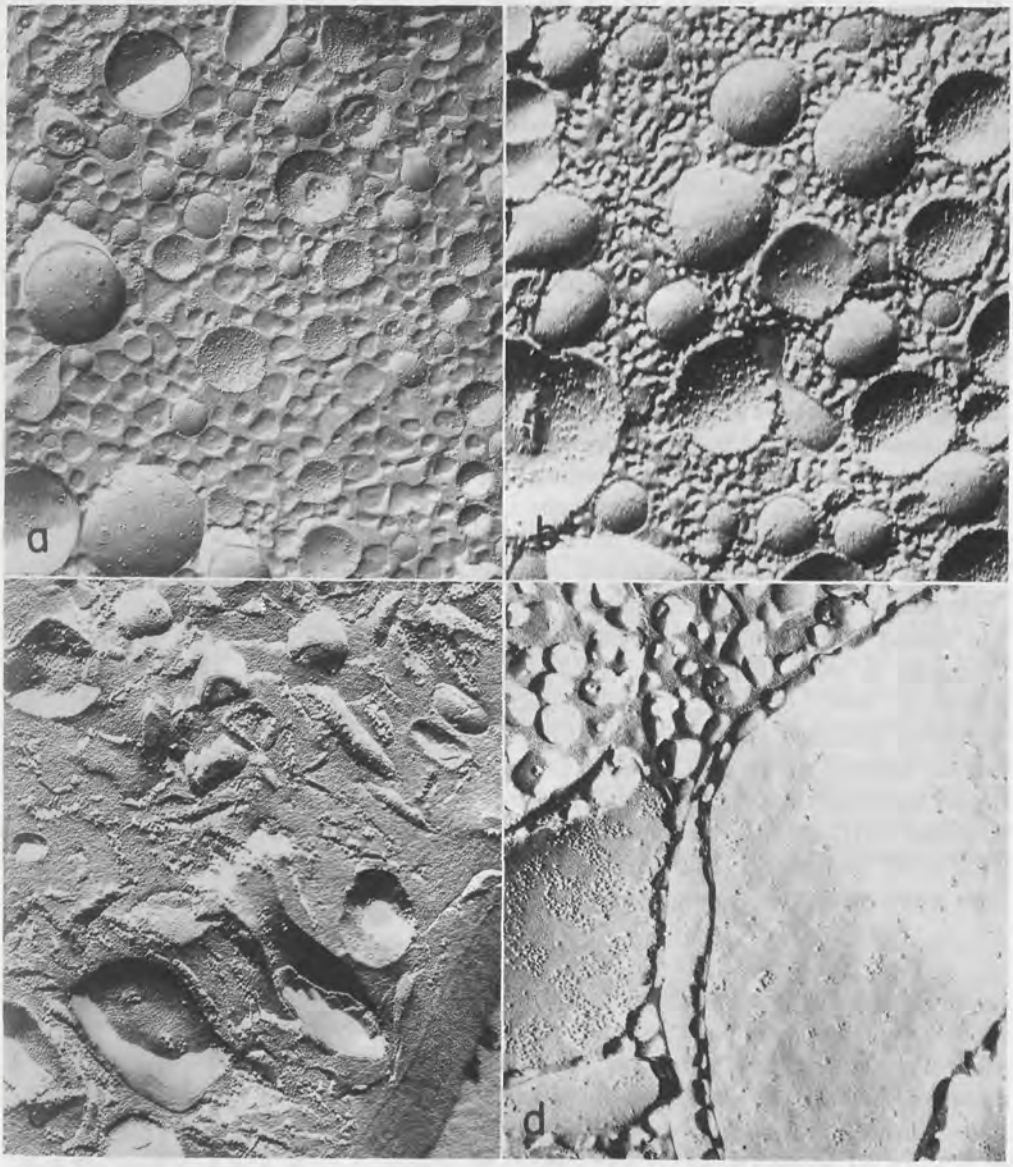

FIG. 7. Electron micrographs of freeze fracture preparations of : (a) fresh sarcoplasmic reticulum (SR) membranes ; (b) hydrated SR previously dried with trehalose ; (c) dry SR in the presence of trehalose ; (d) hydrated SR previously dried without trehalose.

of PC was elevated by about 30 °C in anhydrous dipalmitoyl phosphatidylcholine. When small amounts of water were added to the dry lipid the transition temperature steadily decreased until a hydration state of about 10-12 water molecules/phospholipid was achieved. In more recent years studies with physical techniques such as nuclear magnetic resonance (*e.g.* Griffin, 1976 ; Volke *et al.*, 1982) and infrared spectroscopy (*e.g.* Cameron *et al.*, 1980 ; Mantsch *et al.*, 1980, 1981 ; Crowe *et al.*, 1984a, 1985) have also suggested that fluidity of the membrane is altered by the hydration state.

Dehydration and membranes

Since dehydration of membrane phospholipids leads to increased packing density of the head groups, one might expect the increased opportunities for van der Waals' interactions among the hydrocarbon chains to lead to a phase transition of at least some membrane phospholipids from liquid crystalline phase to gel phase (Fig. 8). This phase transition is likely to be an important damaging event for the following reasons. It is well established that a liquid crystalline to gel phase transition induced by low temperature leads to lateral phase separation of phospholipid classes, cholesterol, and membrane proteins (Lee, 1977ab ; Wunderlich *et al.*, 1978 ; Crowe and Crowe, 1984 ; Quinn, 1985). This lateral phase separation can result in damage to the membrane since upon rehydration membrane proteins might be expected not to be redistributed in the bilayer in their original position, leading to loss of function. Furthermore, some phospholipids, most notably phosphatidyl ethanolamine (PE), in pure form prefer not to be found in bilayers, but instead form complex crystalline phases, the most common of which is the inverted hexagonal H_{II} phase (Reiss-Husson, 1967 ; Cullis and De Kruijff, 1978, 1979 ; Simon, 1978 ; Hui *et al.*, 1981 ; Boni and Hui, 1983 ; Seddon *et al.*, 1983 ; Boni *et al.*, 1984). We have previously published evidence based on freeze fracture (Crowe and Crowe, 1982) and ^{31}P-nuclear magnetic resonance (Crowe and Crowe, 1983b) that the non-bilayer crystals seen in dry model membranes discussed above are H_{II} phase PE. Although there is some evidence that short segments of H_{II} lipids may be involved in fusion between membranes in fully hydrated cells (Cullis and De Kruijff, 1978), formation of extensive non-bilayer structures is clearly incompatible with maintenance of integrity of the bilayer.

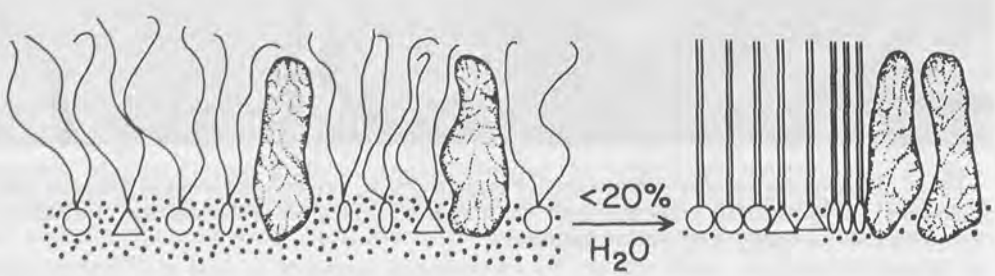

Fig. 8. Events accompanying dehydration of a membrane. Removal of water from around the polar head groups leads to decreased spacing between the head groups, which in turn leads to a transition from liquid crystalline to gel phase and to phase separations of lipid classes and proteins.

Hypothesized mechanism of inhibition of dehydration damage

In view of the above discussion on effects of dehydration on physical properties of lipids, the consequences seen on dehydration of a membrane in the absence of trehalose can be understood (Fig. 8). The damaging sequence would, by this line of reasoning, proceed by : 1) removal of water from the polar head groups, leading to a liquid crystalline to gel phase transition ; 2) lateral phase separation of phospholipids, other lipids, and proteins in the plane of the membrane ; and 3) formation of nonbilayer phases by lipids like PE. It follows, therefore, that if the destabilizing effects of dehydration are to be alleviated, the initial event in this sequence, transition from liquid crystalline to gel phase, must be inhibited. The most likely mechanism for an inhibition of the phase transition by trehalose is an interaction between trehalose and the phosphate head groups that increases the spacing between head groups. This "water replacement hypothesis" was proposed more than a decade ago (Crowe, 1971). One prediction from this hypothesis is that dry lipids in the presence of trehalose have physical properties similar to those of hydrated lipids, and we have provided some evidence that this is the case.

Effects of trehalose on phase transitions

We have measured effects of trehalose on the transition temperature (T_c) of one phospholipid, dipalmitoyl phosphatidylcholine (DPPC), using conventional differential scanning calorimetry (Crowe *et al.*, 1984b, 1985). The results (Fig. 9) show that the dry and hydrated DPPC show T_c's of 341 and 314 K, respectively. When even small amounts of trehalose were added to the DPPC before it was dried, the melting endotherm was broadened and displaced to a lower temperature. With increasing amounts of trehalose, T_c decreased steadily until, at the highest concentration, it was actually below that of the fully hydrated DPPC. Coincident with this decrease in T_c, as the mole ratio of trehalose to DPPC was increased, there was an increase in the enthalpy of the phase transition. Hydration of dry phospholipids similarly results in depression of T_c and increases in the enthalpy of that transition (Fig. 9). In other words, addition of trehalose to dry DPPC in some respects mimics additions of water. In fact, trehalose appears to be even more efficient than water in this regard.

Effects of trehalose on infrared spectra of membranes

The most likely mode of interaction between trehalose an membrane phospholipids involves formation of hydrogen bonds between OH groups on trehalose and the polar head group of the phospholipid. Modern Fourier transform infrared spectroscopy provides means for studying interactions between functional groups on trehalose and phospholipids, and thus provides means for clarifying the mechanism of interaction between the two compounds (*e.g.* Cameron *et al.*, 1980ab ; Mantsch *et al.*, 1980, 1981). As an example of the kind of information that can be obtained, we have studied effects on OH stretching vibrations in trehalose and on vibrations of the phosphate head group of phospolipids (Crowe *et al.*, 1984ab, 1985 ; Crowe *et al.*, in prep.). Some typical results are shown in Fig. 10. The most striking changes in the spectra were seen in bands assigned to the phosphate head group (Mantsch *et al.*, 1980, 1981) ; the band at 1 246/cm (which is a stretching vibration of the P=0 of the phosphate head group) was broadened, depressed, and shifted to 1 240/cm in the presence of trehalose. Concurrent with the changes in the phosphate vibration, alterations in bands assigned to trehalose were seen. For

example, the series of bands between 1 400 and 1 300/cm, assigned to OH deformations, were completely missing when DPPC was present. We interpret these data to mean that there is an interaction between the OH of trehalose and phosphate head group, probably by means of hydrogen bonding. More recently, we have shown similar interactions between trehalose and phospholipids in biological membranes and that analogous interactions occur between trehalose and membrane proteins. Infrared spectroscopy showed that when membranes were dried without trehalose the content of disordered proteins rose, but when they were dried with trehalose the proteins appeared to be maintained in their native state (Crowe *et al.*, 1984a).

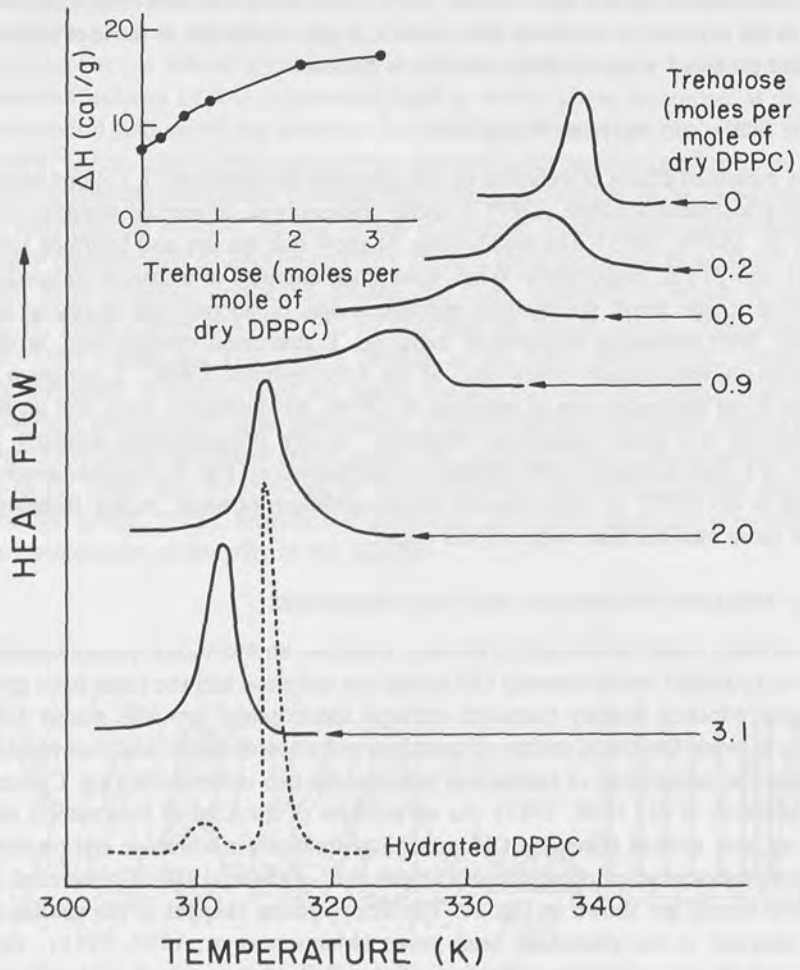

FIG. 9. Melting endotherms of dry and hydrated dipalmitoylphosphatidylcholine. The dry samples were also dried in the presence of the indicated concentrations of trehalose (from Crowe *et al.*, 1984b).

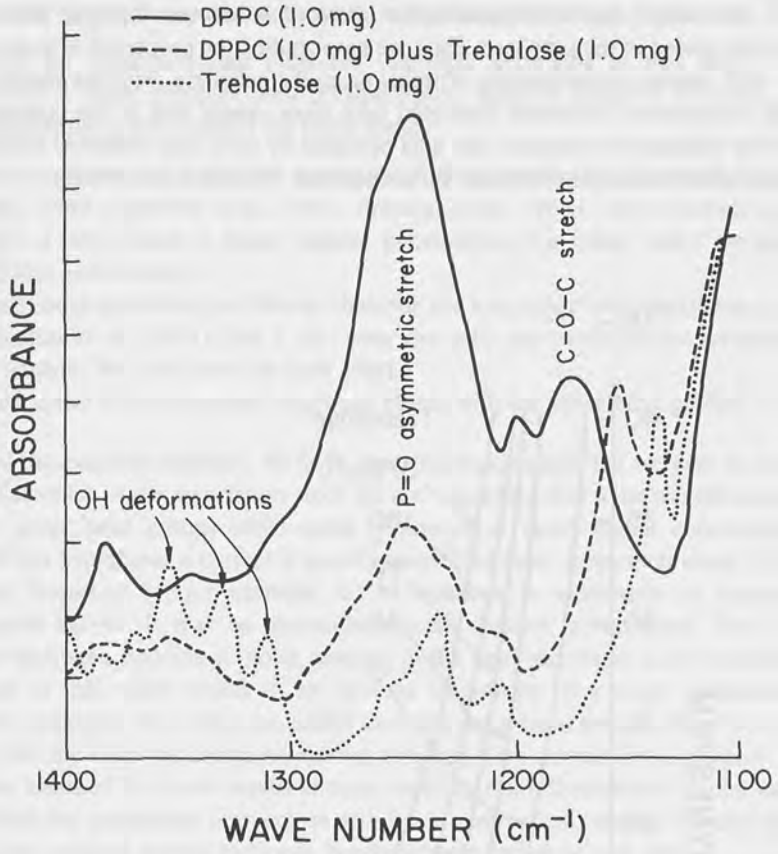

FIG. 10. Infrared spectra of dry dipalmitoylphosphatidylcholine (DPPC) in the presence and absence of trehalose and of trehalose alone. Note apparent interactions between OH groups of trehalose and P=0 of the phospholipid (from Crowe *et al.*, 1984b).

EFFECTS OF TREHALOSE ON MONOMOLECULAR FILMS OF PHOSPHOLIPIDS

One might expect trehalose to depress T_c by in some way decreasing the packing density of the head groups and thereby decreasing van der Waals' interactions between the hydrocarbon chains. What we are suggesting is that trehalose effects a lateral spreading of the dry lipids, much as hydrating them does. As yet we have been unable to test this hypothesis with dry lipids, but we have provided some information along these lines with monomolecular films of DPPC spread on an aqueous surface in a Langmuir trough. The surface pressure of such films can be measured, and from curves showing changes in surface pressure during isothermal compression of the film it is possible to evaluate the area occupied by a lipid molecule in the film (Gaines, 1966 ; Cadenhead and Demchak, 1969 ; Blume, 1979 ; Crowe *et al.*, 1984c ; Johnston *et al.*, 1984 ; Rudolph *et al.*, 1986). Previous work and work subsequent to our own report has shown that

considerable information can be obtained about interactions between the lipid film and extraneous molecules placed in the subphase. Thus, we have studied the properties of monomolecular films of PC with various concentrations of trehalose in the subphase, with the results shown in Fig. 11. The compression isotherms illustrated here show clearly that as the concentration of trehalose in the subphase is increased, the area occupied by each lipid molecule increases. This increase in area/lipid molecule depresses T_c, as expected (Crowe *et al.*, 1984c).

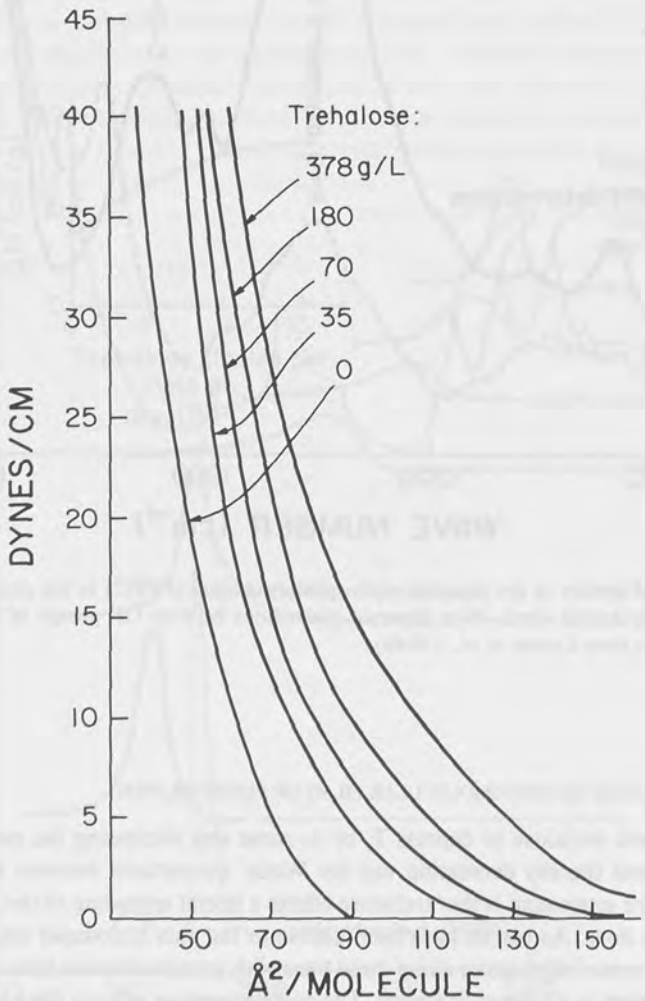

FIG. 11. Compression isotherms for monomolecular films of phosphatidylcholine spread on aqueous solutions of various concentrations of trehalose. Note that with increasing concentration of trehalose surface pressure develops at larger area/molecule, suggesting that interactions between trehalose and polar head groups of the phospholipid increases spacing between head groups (from Crowe *et al.*, 1984c).

A COMMENT ON THERMODYNAMICS OF PHOSPHOLIPID INTERACTIONS WITH TREHALOSE

The results for monomolecular films are somewhat surprising in that they suggest that trehalose forms a stable complex with phospholipids, event at relatively low concentrations. Several explanations might account for these results :

a) Trehalose could expand lipid films by mixing with the hydrocarbon chains (Cadenhead and Demchak, 1969 ; Herbette *et al.*, 1983 ; Brasseur *et al.*, 1985). This explanation is difficult to accept *a priori* since it would require penetration of a bulky, polar molecule into a hydrophobic environment.
b) Trehalose could affect the lipid film by changing the long range ordering of water in solution (cf. Johnston *et al.*, 1984). But if this were the case one would expect trehalose to alter surface tension. We have seen no such effect.
c) Trehalose could form coordinate hydrogen bonds with the polar head groups.

In view of the available evidence, we favor the latter explanation. But we wish to comment on the thermodynamics of the association since we are suggesting that trehalose replaces the water around the polar head groups when water is present at much higher concentrations than trehalose. If this hypothesis is correct it would require that water (present at about 55 M in bulk solution) be displaced by, for example, 0.5 M trehalose – seemingtly an unsurmountable thermodynamic barrier. It may be thermodynamically feasible, nevertheless. The ordering of water by its hydrogen bonding to polar residues in the lipid represents a low entropy state, so that removal of that water results in an increase in entropy. If a single trehalose molecule displaced, for example, four water molecules from the polar head groups, there would be a net increase in entropy since the decreased entropy resulting from association of trehalose with lipid is more than balanced by the increased entropy resulting from displacement of the water. Thus, we suggest that the association of trehalose with lipid is entropically driven. We also suggest that the association requires several hydrogen bonds between trehalose and lipid.

INTERACTIONS OF TREHALOSE WITH LIPOSOMES

We established in the work discussed above that trehalose is capable of preserving structure and function in dry biological membranes. Another source of damage to membranes due to desiccation is an increase in permeability of the bilayer during dehydration, which would result in leakage of cellular contents to the medium (Simon, 1978). We have explored this possibility using vesicles of pure phospholipids (liposomes). With trehalose both inside and outside the bilayer, almost 100 % of trapped solute is retained in rehydrated vesicles previously freeze-dried with 0.75 g trehalose/g lipid (Crowe *et al.*, 1986) (Fig. 12). By contrast, when the liposomes are dried without trehalose essentially all the trapped solute is released during rehydration. We have sought an explanation for this interesting effect, with the following results.

Freeze fracture studies

Freeze fracture studies showed us that extensive fusion between liposomes occurs during drying in the absence of trehalose, but that the fusion is completely inhibited in the presence of trehalose. Since it is known that membrane vesicles leak during fusion (Quinn, 1985), it seems likely that inhibition of fusion could be an important part of the preservation process. We have

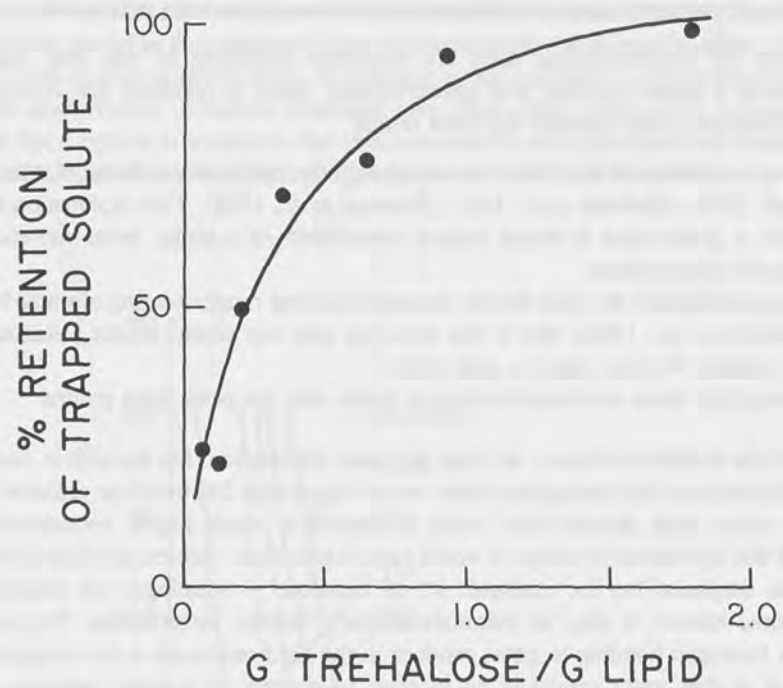

FIG. 12. Retention of trapped solute by phospholipid vesicles previously dried in the presence of various concentrations of trehalose and subsequently rehydrated (Crowe *et al.*, in press).

quantified the amount of trehalose required to inhibit fusion, using resonance energy transfer (MacDonald and MacDonald, 1981 ; Uster and Deamer, 1981 ; Crowe *et al.*, 1986). This technique involves preparing two populations of liposomes with a different hydrophobic fluorescent probe in the bilayer in each population. When the two probes, chosen so that their excitation and emission energies overlap, are in close proximity the donor probe will transfer energy to the acceptor, thus quenching the donor and exciting the acceptor. With a spectrofluorometer it is possible, therefore, to quantify the close approach of the probes (and thus to estimate fusion between liposomes). Results of these studies show that a surprisingly small amount of trehalose – much less than that required to prevent leakage – will completely inhibit fusion (Fig. 13). Thus, inhibition of fusion alone cannot account for preservation of the liposomes.

Phase transitions

It is also known that as hydrated liposomes are passed through their thermotropic phase transition they leak, possibly due to defects in the bilayer at the interface between gel and liquid crystalline domains (Lee, 1977ab). Therefore, it is possible that the dry liposomes are in gel phase and during rehydration pass through a transition to liquid crystalline phase, as a result of

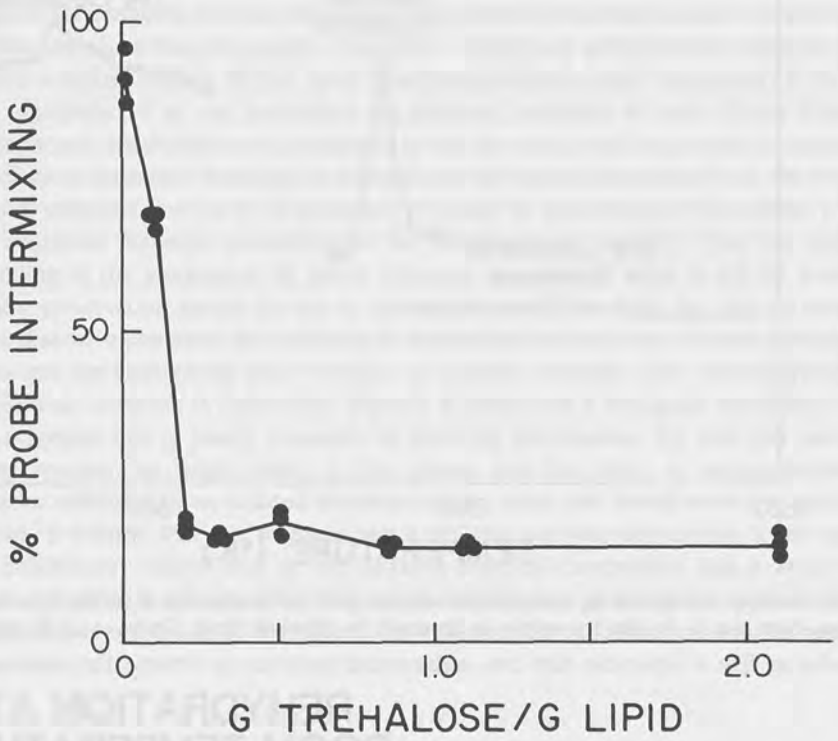

FIG. 13. Fusion between phospholipid vesicles (recorded by resonance energy transfer ; see text for details) during drying in the presence of various concentrations of trehalose. Note that fusion is completely inhibited by relatively low concentrations of trehalose (from Crowe *et al.*, in press).

which they leak. We tested that hypothesis using differential scanning calorimetry and found that the dry liposomes with minimal trehalose are indeed in gel phase at room temperature. When the liposomes are dried with trehalose, by contrast, T_c is depressed, and they are in liquid crystalline phase at room temperature (Fig. 14). Thus, when they are rehydrated they do not undergo a phase transition and they do not leak (Fig. 15). The ability to preserve dry liposomes is a property nearly unique to trehalose (Crowe *et al.*, 1986 ; Womersley *et al.*, 1986), a finding that is of considerable practical importance ; liposomes are used in the pharmaceutical industry for delivery of water soluble drugs to cells. The ability of trehalose to preserve the dry liposomes suggests means for preserving liposomes for clinical applications.

Summary

Results of our studies over the past several years have brought us closer to understanding on a molecular level how organisms like *Artemia* cysts are maintained in dormancy and how they are capable of surviving dehydration. This is a problem that has fascinated scientists and

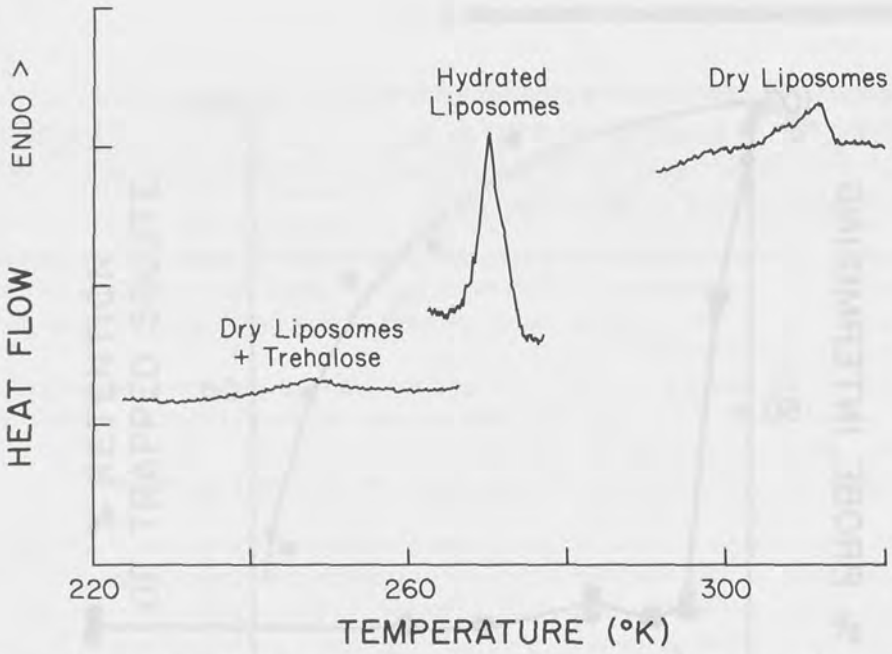

FIG. 14. Melting endotherms for phospholipid vesicles dried in the presence of various concentrations of trehalose. Note that T_c for the dry vesicles is depressed by trehalose (from Crowe *et al.*, in press).

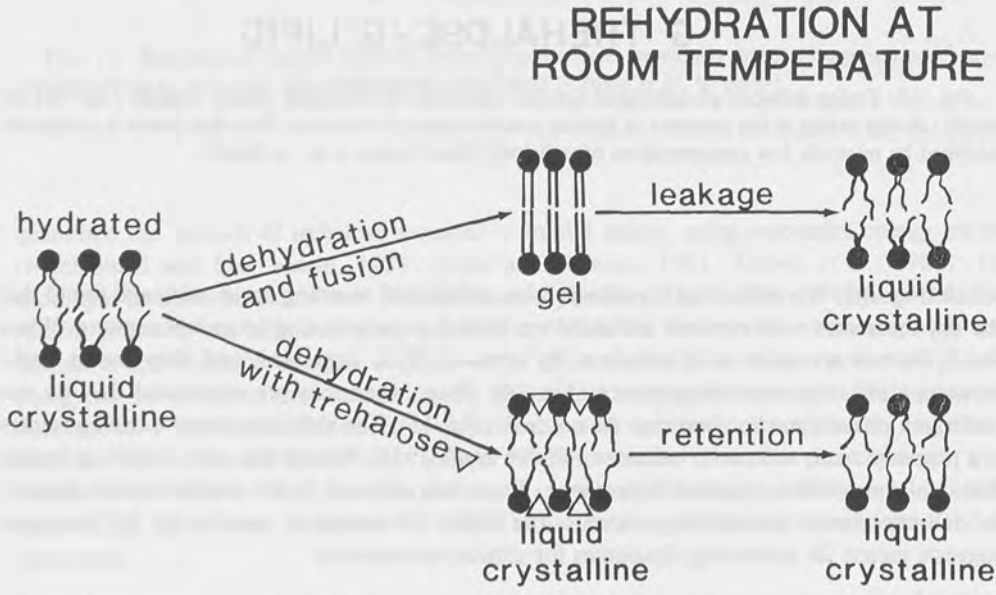

FIG. 15. Interpretive diagram of the cause of leakage from dry phospholipid vesicles during rehydration and its prevention by trehalose. Without trehalose T_c is elevated, so the vesicles are in gel phase at room temperature. When such vesicles are rehydrated they undergo a transition from gel to liquid crystalline phase, as a result of which they leak. When the vesicles are dried in the presence of trehalose, T_c is depressed, and they remain in liquid crystalline phase at room temperature. As a result, when they are rehydrated, they do not undergo a phase transition, and they do not leak.

philosophers for centuries not only because of its inherent intellectual interest but also because many of the organisms that are capable of entering dormant and anhydrobiotic states are of great importance in human welfare. In fact, some practical applications arise immediately from studies on these organisms : if we can understand the processes necessary to keep a living system in a state of depressed metabolism or to preserve it in the dry state, it will be possible to improve the viability of those organisms beneficial to humankind. On a more immediate level, the results we have already obtained have led to development of means for preservation of liposomes, a finding that has enormous potential applications in the pharmaceutical industry. If we can achieve an understanding of the mechanism by which trehalose imparts this effect it will be possible to design new preservation agents for use in specialized situations. With this end in mind, it is important that we understand the variability in interactions between the common carbohydrates and lipids and the interactions likely to occur in complex mixtures. Our previous results have also shown that trehalose is remarkably effective at preserving a biological membrane isolated from an organism that is clearly incapable of surviving dehydration. We find this particularly interesting because one might think, at first glance, that the ability to survive dehydration, whether it be exhibited by an isolated membrane or an intact cell, would involve a multitude of adaptations. It follows that one might expect a cell that survives dehydration to be equipped through evolutionary history with so specialized a chemical composition that it would not be possible to dehydrate a cell whose evolutionary history has not generated the same adaptations. We suspect this is not the case. We believe that relatively few modifications may be required to make an intact cell capable of surviving dehydration, and that ultimately it will be possible to do so.

Acknowledgements

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The structure of *Artemia* sp. haemoglobins II. A comparison of the structural units composing the *Artemia* sp. globin chains

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Abstract

Eight different types of structural units (E_1 to E_8) were isolated from the *Artemia* haemoglobins. Amino acid analysis and tryptic peptide mapping show that they are rather different from one another. The alignment of the partially amino acid sequence (86 residues) of E_7 with this of E_1 and the human β chain reveals 40 % homology (E_1/E_7). Essentially these residues are functionally identic, and are important in the formation of the tertiary structure.

Introduction

The *Artemia* globins (M_r 260 000) are dimers of two high M_r globin chains (M_r 130 000) (Moens and Kondo 1978 ; Wood *et al.*, 1981).

Each globin chain is built up by the covalent linkage of eight myoglobin-like structural units (M_r 16 000) (Moens *et al.*, 1984, 1985, 1987). Similar polymeric structures are found in mollusc and arthopod haemocyanin polypeptide chains (Gielens *et al.*, 1977, 1980 ; Wood, 1980).

Globin chains with multiple structural units may be produced by a gene originated by a series of tandem duplications of an ancestral globin gene. Originally the gene product should be a homopolymeric globin chain. Owing to genetic events within the multiplied genes the structural units and/or the linking regions within a globin chain may, however, show variations in the basic structure of that chain, resulting in a heteropolymeric globin chain.

In this communication we describe the comparison of the structural units composing the *Artemia* high M_r globin chains.

Materials and methods

A collection of structural units (fraction E) was isolated from *Artemia* haemoglobin as described (Moens *et al.*, 1984, 1985, in prep.)

Residual subtilisin was separated from the haemoglobin fragments by DE 52 chromatography according to Schroeder and Huisman (1980). The protease-free material was separated by chromatofocusing on a Mono P HR 5/20 column equilibrated in 25 mM Bis-Tris, iminodiacetic acid pH 7.1 buffer and developed with 9 % Polybuffer 7-4, iminodiacetic acid pH 4.0. The obtained fractions (E_1-E_8) were analyzed for purity by two-dimensional technique using a pH gradient from 3 to 10 and a 15 % SDS gel.

Peptides were obtained by cleavage with trypsin, and were separated by reversed phase chromatography on a C_{18} column (Mahoney and Hermodson, 1980).

Amino acid analysis and sequence determinations were performed according to the published methods (Spackman *et al.*, 1958 ; Chang *et al.*, 1978 ; Hewick *et al.*, 1981).

Results and discussion

The structural units (fraction E) composing the *Artemia* globin chains were separated by chromatofocusing in, at least, eight fractions (E_1 to E_8) with a pH of elution between 4.6 to 6.0 (Fig. 1, Table I). Two-dimensional analyses of these fractions reveal that, with exception of E_8 and E_4, they are fairly homogeneous. Their amino acid composition shows that they form a family of related polypeptides with discrete differences (E, R, T, A, V, L, Y) (Table II).

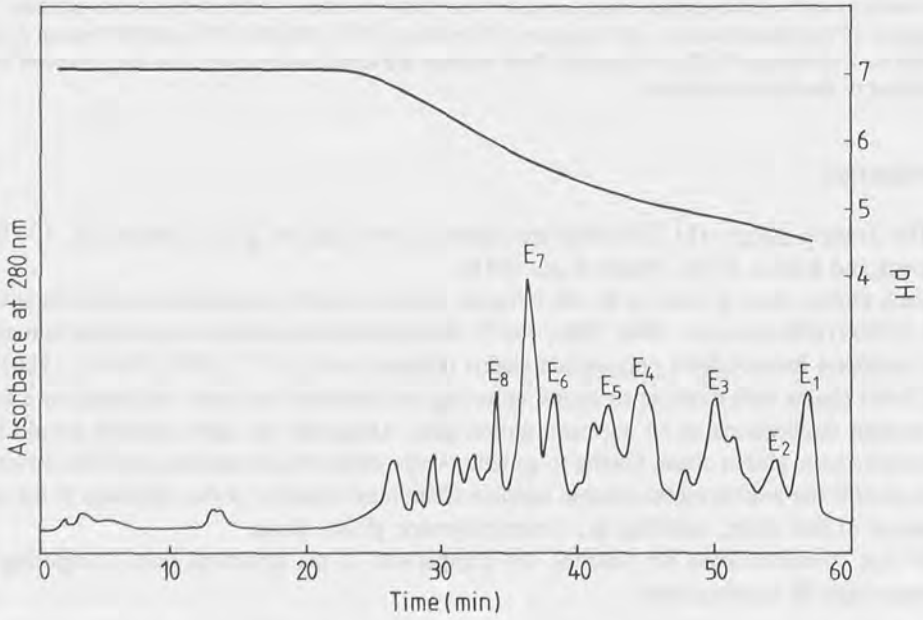


FIG. 1. Chromatofocusing of the structural units (fraction E). Composing the *Artemia* haemoglobins. Chromatofocusing was performed on a mono P HR 5/20 column equilibrated in 0.025 M Bis-Tris/ iminodiacetic acid pH 7.1. The proteins (15 mg) were eluted with 9 % Polybuffer (7-4) iminodiacetic acid pH 4.0 at a flow rate of 1 ml/min. Detection was done at 280 nm and the separated compounds were collected manually.

TABLE I

pH of elution of the structural units after chromatofocusing

	pH	sd	n	%
E_1	4.60	0.13	18	6.00
E_2	4.70	0.10	17	2.72
E_3	4.92	0.09	17	9.15
E_4	5.11	0.09	16	9.29
E_5	5.38	0.09	16	9.32
E_6	5.60	0.10	17	4.36
E_7	5.79	0.10	18	9.38
E_8	5.98	0.09	18	3.10

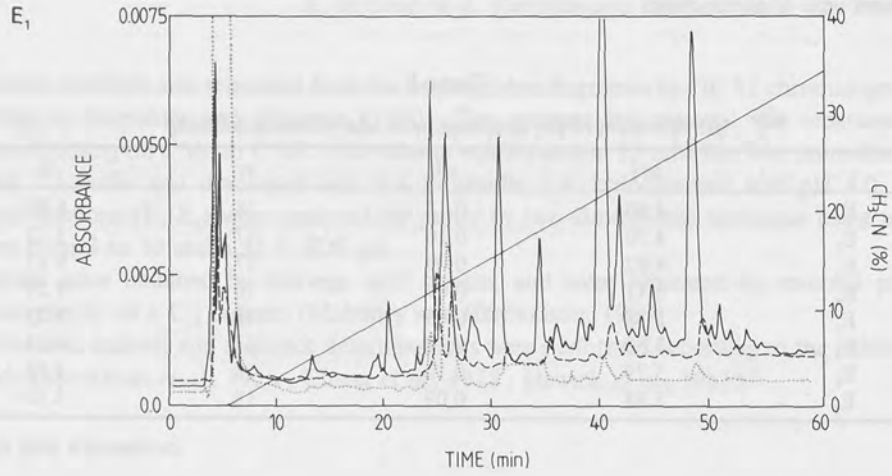
TABLE II

Amino acid composition of the structural units E_1-E_8.
Amino acid analysis was performed as described in Materials and methods.
The values (residues/molecule of M_r 17 000) were the data obtained after 24 h hydrolysis

	E_1		E_2		E_3		E_5		E_6		E_7	
	n	sd	n	sd	n	sd	n	sd	n	sd	n	sd
w	1.00	n.d.	1.00	n.d.	1.00	n.d.	1.00	n.d.	1.00	n.d.	1.00	n.d.
K	7.63	0.22	8.36	0.37	8.46	0.48	3.11	0.64	9.93	0.11	7.04	0.68
H	4.25	0.26	3.84	0.37	4.19	0.25	4.57	0.37	4.10	0.27	4.76	0.61
R	6.37	0.38	7.20	0.20	7.57	0.46	9.28	1.41	8.47	2.48	9.16	0.97
D	16.78	0.65	16.20	0.86	15.48	0.57	16.31	0.34	14.98	0.97	16.88	0.44
T	6.96	0.18	6.62	0.36	7.11	0.39	7.99	0.25	7.07	0.38	8.86	0.28
S	8.12	0.51	7.51	0.50	8.20	0.45	8.17	0.29	8.33	0.70	7.84	0.31
E	19.48	0.59	18.34	0.39	17.18	0.43	15.49	0.27	17.35	0.77	13.29	0.27
P	5.29	0.85	6.17	0.66	5.61	0.30	4.16	0.69	5.14	0.25	3.96	0.22
G	11.64	0.60	10.96	0.67	10.64	0.67	10.27	0.39	11.72	1.30	10.51	0.48
A	10.04	0.20	10.92	0.50	11.15	0.63	13.41	0.18	13.21	1.48	14.45	0.30
V	13.54	0.57	13.62	0.12	12.27	0.32	9.54	0.19	11.31	0.67	8.75	0.19
M	1.70	0.57	1.72	0.30	2.39	0.87	1.91	1.18	2.43	0.83	2.18	0.18
I	5.20	1.85	6.19	0.38	n.d.	n.d.	7.75	0.47	5.50	1.66	7.68	1.90
L	13.32	0.31	13.93	0.53	14.99	0.72	16.91	1.37	11.69	3.73	16.78	0.55
Y	3.77	0.53	3.30	0.45	2.88	0.34	2.32	0.45	4.06	0.43	1.77	0.10
F	12.68	3.93	10.53	0.79	10.41	0.58	11.33	0.53	12.29	0.54	12.30	0.30

The tryptic peptide maps obtained by C_{18} reversed phase chromatography of the isolated structural units were compared (Fig. 2). They clearly show that all units are rather different proteins with only a limited number of common peptides (*e.g.* the Trp-peptide), and that the peptide map of E_1, purified by chromatofocusing, is essentially similar to that of E_1, purified by isoelectric focusing (Moens *et al.*, 1985). This suggests that they are identic. The latter was proven by the determination of the amino acid sequence of some selected peptides.

E_7 was selected to compare its primary structure with that of E_1. The 41 amino-terminal residues were determined automatically with a gas-phase sequencer (Hewick *et al.*, 1981).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0.0025
0.0
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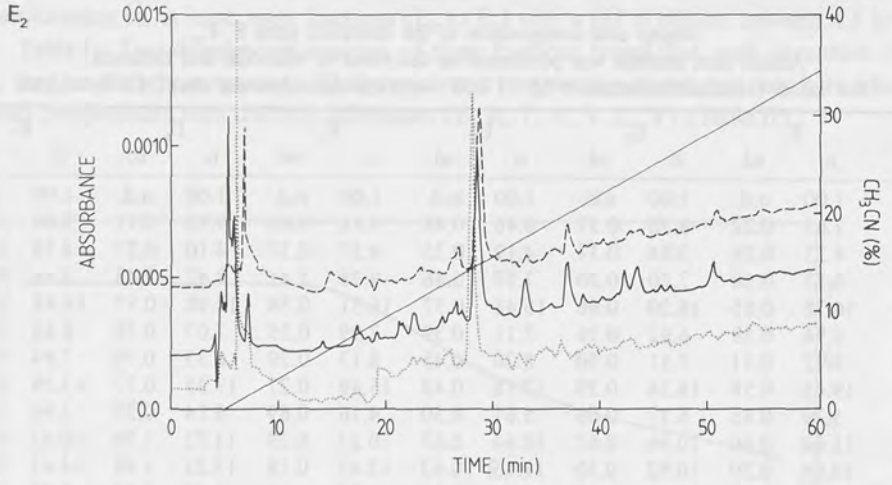
30
40
50
60
TIME (min)
40
30
20
10
0
CH3CN (%)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10
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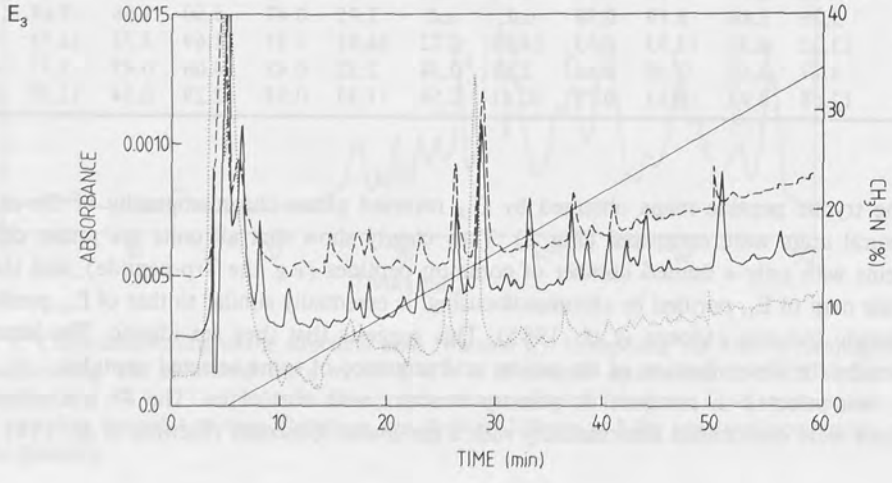
CH3CN (%)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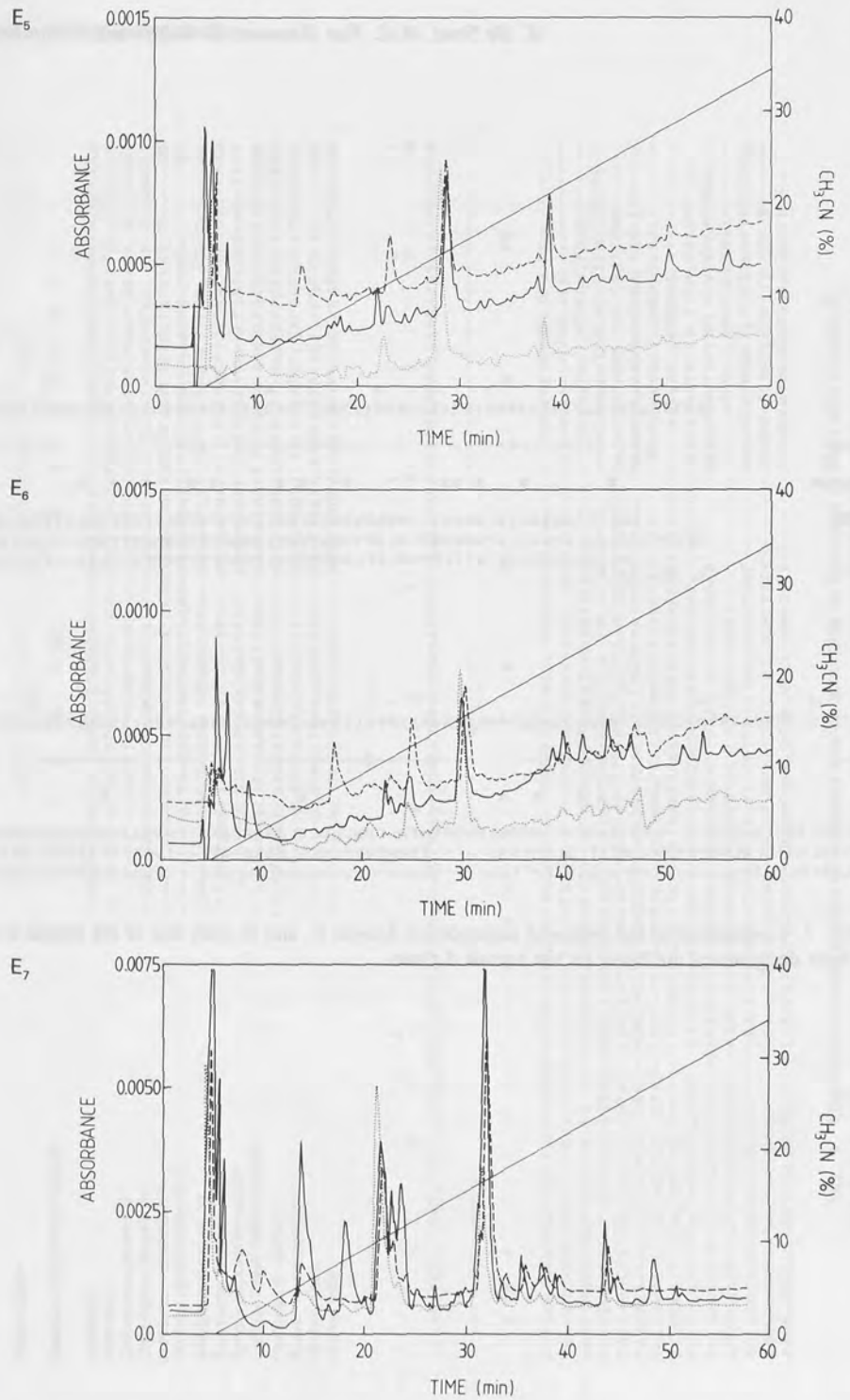

FIG. 2. Comparison of the tryptic peptide maps of the structural units E_1, E_2, E_3, E_5, E_6, and E_7. The peptide maps were obtained by C_{18} reversed-phase chromatography as described in Materials and methods. (———) A_{220} ; (---------) A_{280} ; (· · · ·) A_{254}.

FIG. 3. Comparison of the proposed sequence for *Artemia* E_1 and E_7 with that of the human β chain. The helix designations are those for the human β chain.

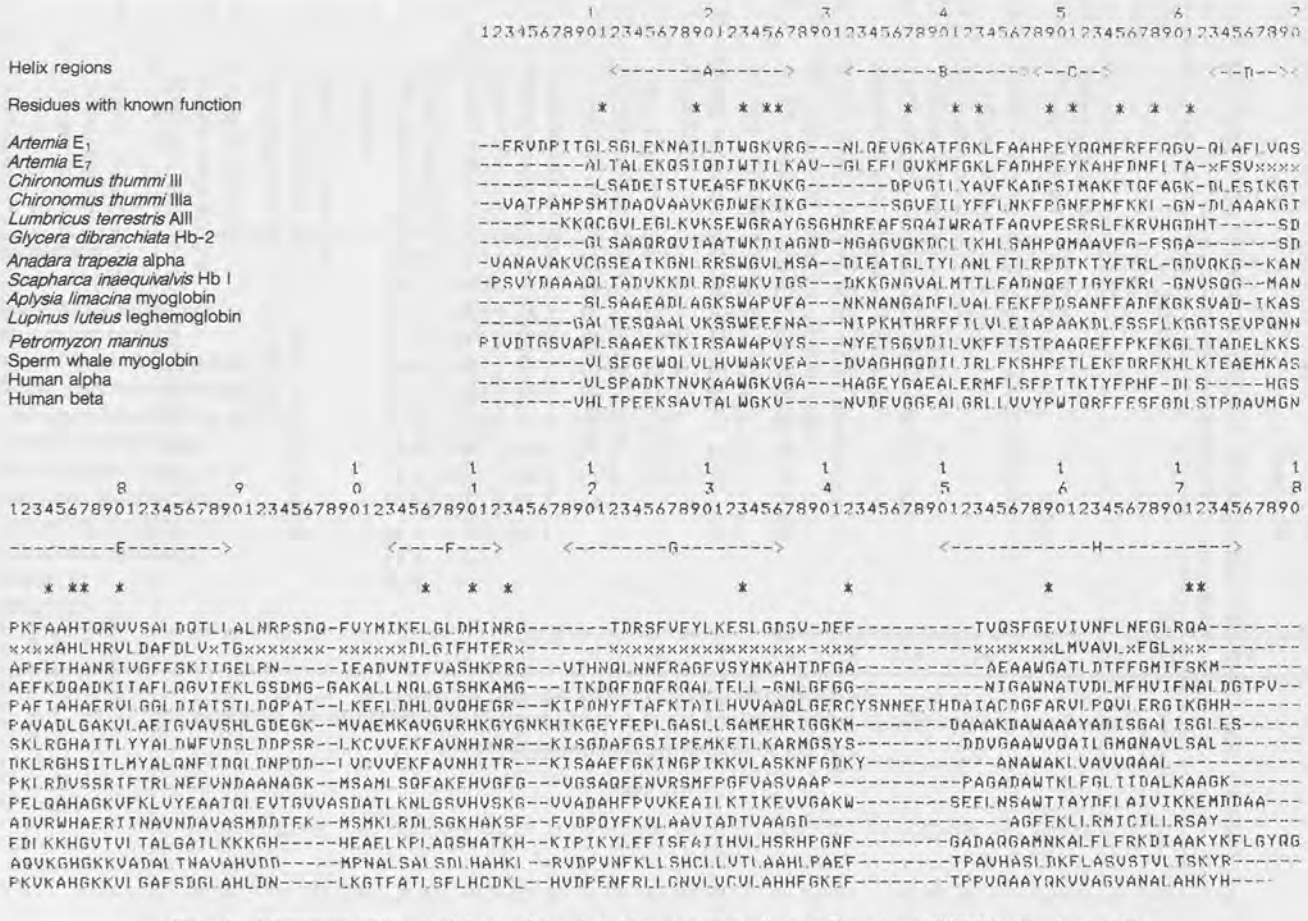

FIG. 4. Alignment of some globins of unrelated species according to Runnegar (1984) with *Artemia* E_1 and E_7. The helix designations are those for the human β chain. Asterisks refer to the residues of Table IV.

TABLE III

Homologies between the globins of Fig. 4.
The values are the percentages of identical residues in the complete length of both compared sequences.
For E_7, only the known residues were compared

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1 *Artemia* E_1	---														1
2 *Artemia* E_7	37.6	---													2
3 *Chironomus thumni* III	14.4	17.2	---												3
4 *Chironomus thummi* IIIa	15.1	11.1	28.1	---											4
5 *Lumbricus terrestris* AIII	16.0	20.7	14.1	13.9	---										5
6 *Glycera dibranchiata* Hb-2	17.9	20.0	16.9	17.6	18.6	---									6
7 *Anadara trapezia* alpha	18.4	14.7	14.0	16.0	17.5	14.9	---								7
8 *Scaepharca inaequivalvis* Hh I	17.9	22.3	11.6	13.8	18.1	13.6	40.9	---							8
9 *Aplysia limacina* myoglobin	14.7	14.8	20.3	16.3	11.7	21.4	18.5	21.0	---						9
10 *Lupinus luteus* leghaemoglobin	13.0	20.7	13.5	17.8	15.5	17.5	12.2	10.4	19.9	---					10
11 *Petromyzon marinus*	17.3	20.8	16.8	14.9	17.6	17.7	19.0	14.8	28.2	14.6	---				11
12 Sperm whale myoglobin	17.7	20.2	18.3	16.5	18.3	20.0	17.6	18.2	21.8	17.0	20.6	---			12
13 Human alpha	16.5	19.3	15.0	14.9	13.8	20.0	19.2	14.7	19.5	14.7	30.2	24.8	---		13
14 Human beta	23.4	23.6	17.6	15.4	16.0	20.5	17.6	18.2	17.2	17.3	22.6	23.4	42.6	---	14

TABLE IV

Comparison of the amino acids identic in myoglobin and haemoglobin with *Artemia* E_1 and E_7, and some globin chains with known atomic structure

Position	Function	Myo/Hb	*Artemia*		*Glycera*	CTT	Lg Hb
			E_1	E_7			
NA1	Beginning of chain	V	T	–	G	–	A
NA2	H-helix contact	L	L	L	L	L	L
A8	H-helix contact ; hydrophobic cluster at bottom of heme pocket	V	I	I	I	V	V
A12	Hydrogen bond to E helix	W	W	W	W	F	W
A14	External salt bridge	K	K	I	D	K	E
A15	Hydrophobic cluster at bottom of heme pocket Spacer between B and G-helices	V	V	L	I	V	F
B6	Close contact with G at E_8 where helices cross	G	G	G	G	P	T
B10	In hydrophobic cluster at right of heme	L	F	L	L	L	F
B12	In Hb : hydrogen bond to F at GH5 and E at B8 In myo : hydrogen bond to water	R	K	K	K	A	L
C2	Sharp turn between B and C helices	P	P	P	P	P	P
C4	Near heme	T	Y	Y	V	K	L
CD1	Packed against heme	F	F	F	F	F	F
CD4	In hydrophobic cluster at right of heme	F	F	F	F	F	F
CD7	In hydrophobic cluster at right of heme	L	V	A	–	–	G
E5	External salt bridge	K	A	K/R	A	E	Q
E7	Interacts with sixth heme ligand	H	H	H	L	H	H
E8	Close contact with G at B6 where helices cross	G	T	L	G	A	A
E11	Packed against heme in right hydrophobic cluster	V	V	V	V	I	V
F4	Packed against heme in left hydrophobic cluster	L	L	L	V	F	L
F8	Fifth ligand to heme	H	H	H	H	H	H
FG2	Unknown	K	R	R	Y	R	K
G16	Packed against A-helix	L	L	L	M	M	I
GH5	In hydrophobic cluster at bottom of heme pocket as a spacer between G and H helices	F	F	F	M	F	W
H10	External salt bridges	K	V	V	A	A	I
H22	External salt bridges	K	I	–	G	K	E
H23	Hydrogen bond to FG5 to stabilize end of H-helix			–			

Myo :	sperm whale myoglobin	Takano (1977)
Hb :	human haemoglobin	Fermi (1976)
Glycera :	*Glycera dibranchiata*	Padlan and Love (1974)
CTT :	*Chironomus thummi* III	Huber *et al.* (1971)
Lg Hb :	Leghaemoglobin from *Lupinus luteus*	Vainshtein *et al.* (1977)

Tryptic peptides were separated by C_{18} reversed-phase chromatography and sequenced manually using the DABITC/PITC method (Chang *et al.*, 1978). The obtained amino acid sequences were aligned with E_1 and the human β chain (Fig. 3).

The primary structure of E_7 is quite different from that of E_1. From the 86 localized residues of E_7, no more than 35 or 40 % are identic. This is comparable with the homology between the human α and β globin chains. The homology between the complete sequences of E_1 and E_7 will probably turn out to be even smaller, because some sequences of tryptic peptides of E_7 occur that have no similarity with any region of E_1, and thus could not be aligned. One of the peptides that is not aligned in Fig. 3 has the sequence DLVDPVTGLTG.

Although it is similar to the amino-terminus of E_1 (seven residues out of 11 or 60 %, Fig. 3), automated sequencing revealed the sequence ALTALEK as the amino-terminus of E_7. The former sequence is possibly representative for the linking region between the structural units within a globin chain. Due to the aspecific cleavage of the native haemoglobin molecule with subtilisin, the linker can remain attached to the amino-terminus of a structural unit (as in E_1) or at the carboxy-terminus. According to this assumption, this peptide must be a carboxy-terminal extension of E_7. It is remarkable that the homology between these linkage regions is much higher than the homology between the structural units themselves (respectively 60 and 40 %).

This may suggest that the linkage regions have an important function in the orientation of the structural units relative to each other. The homology between E_1 and E_7, which will be between 25 and 40 %, is only slightly larger than the homology between globins of unrelated species, or comparable to that of vertebrate myoglobins and haemoglobins (Table III).

Assuming that the invertebrate globins mutated at a rate similar to their vertebrate counterparts, the gen duplications that are responsible for the formation of these two different structural units, must have taken place about 500-850 million years ago (Dickerson and Geis, 1983). Despite the small homology, those residues which are important in determining the tertiary structure and which are identic in vertebrate myoglobin and haemoglobin, are in E_1 and E_7 functionally identic, which suggests the presence of the "myoglobin fold" (Fig. 4, Table IV) (Dickerson and Geis, 1983).

Conclusions

The *Artemia* globin chains contain different structural units. Their amino acid composition shows differences in a limited number of amino acids, but their peptide maps are rather different. The partial amino acid sequence of structural unit E_7 has a homology of 40 % compared to E_1. The linker regions between the structural units are much more conserved (homology 60 %). By estimating the homology between the two complete sequences at 25-40 %, their gene duplication must have taken place about 500-800 million years ago.

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Artemia trypsin-like proteinase a developmentally regulated proteinase

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Abstract

Artemia trypsin-like proteinase has been found previously to be inhibited by lipovitellin and is now shown to require divalent cations for stabilization of the active form. We have also found that the proteinase is inhibited *in vitro* by Gp_4G. After interaction with chaotropic agents, the proteinase becomes insensitive to lipovitellin and Gp_4G and does no longer require divalent cations. The activity and sensitivity to active center-directed inhibitors are, however, maintained. We have found that chaotropic agents induce a concomitant decrease in molecular weight of the proteinase of about 9 000 Da. With the decrease in molecular weight the proteinase also looses the glycoprotein character and the immunorelation with lipovitellin, previously reported as features of the proteinase (Ezquieta and Vallejo, 1985). Altogether the results indicate that *Artemia* trypsin-like proteinase has a region, well differentiated from the active centre, that confers the sensitivity to the inhibitors and the divalent cation, and is related to the yolk origin of the proteinase. This part of the molecule seems to allow the developmental regulation of *Artemia* trypsin-like proteinase. The results suggest that *Artemia* trypsin-like proteinase is a regulatory serine proteinase as it appears to contain a non-catalytic regulatory area.

During the process of activation of the proteinase, the requirement of divalent cations is found to increase. This increase is blocked by the lysosomotropic agent chloroquine in parallel with the developmental activation, suggesting that both the active center and regulatory areas are modified during the process.

Introduction

Literature data suggest that controlled proteolytic mechanisms must be involved in early development but the available data are scarce. *Artemia* seems to be a good system to study the role and regulation of proteinases in development. We have found that *Artemia* trypsin-like proteinase appears to be physiologically regulated by the inhibition of lipovitellin (Ezquieta and Vallejo, 1986ab). In the cyst the proteinase is latent, associated to lipovitellin, and localized in yolk granules. During development, it is activated in parallel with gradual solubleness from the yolk granules in the cytosol and with gradual decrease in molecular weight, from 600 kDa to 30 kDa. The soluble proteinase of nauplii was found to be immunoinhibited by antilipovitellin antiserum, suggesting that it is of yolk origin (Ezquieta and Vallejo, 1985). The activation of the proteinase seems to be produced during the degradation of yolk that in *Artemia* is dependent on the lysosomal system (Perona *et al.*, 1987). The inhibition of the lysosomal function by chloroquine has been found to prevent the activation (Ezquieta and Vallejo, 1986b). We present now that the solubility of the proteinase is also prevented in the presence of this drug. The

process of activation involves changes in the exposure of the active center of the proteinase that can be followed by measuring the susceptibility to active center-directed inhibitors (Ezquieta and Vallejo, 1985). The processing of the proteinase continues in the cytosol with an increase in activity and a decrease in molecular weight (Garesse *et al.*, 1980 ; Ezquieta and Vallejo, 1985).

In this paper we show that *Artemia* trypsin-like proteinase activity can be modulated by divalent cations that are required for the stabilization of its active form. The requirement of divalent cations has been found to increase with development and could be a consequence of the processing of the proteinase. We have observed that chaotropic agents induce a modification of the proteinase with a concomitant decrease in molecular weight. The characterization of the modified proteinase suggests the existence of two different zones in the proteinase. One area appears to be related to the active center, and the other to the regulation of the enzyme. The latter zone is related to the yolk origin of the proteinase and confers the sensitivity to the inhibitors lipovitellin and Gp_4G and the susceptibility to modulation by divalent cations. Regulatory serine proteinases usually contain a catalytic area and a regulatory one (Neurath, 1984 ; Patthy, 1985 ; Rogers, 1985). The results suggest that *Artemia* trypsin-like proteinase behaves as a regulatory serine proteinase.

Materials and methods

Cysts from San Francisco Bay (San Francisco Bay Brand, Newark, California, USA) were used. Chemicals were of analytical grade.

Handling of embryos and larvae

Cysts were subjected to mild dechorionation prior to homogenization and/or incubation. Culture, nauplii synchronization, collection, and counting were as previously described (Vallejo *et al.*, 1979). Homogenization was achieved by using firstly a loose-fitting and secondly a tighter-fitting homogenizer with a Teflon pestle. Cysts were hand homogenized in 3 vol/g and nauplii in 1 vol/g of the "ficoll medium" composed of 0.3 M sucrose, 15 % Ficoll 400 (Pharmacia), 25 mM Hepes buffer, 60 mM potassium chloride, 15 mM sodium chloride, 5 mM magnesium chloride, 0.5 mM calcium chloride, 1 mM sodium borate, adjusted to pH 7.5. This medium has proved to preserve the subcellular structures of *Artemia* cysts (Vallejo *et al.*, 1981 ; Roggen and Slegers, 1985). The particulate and soluble fractions were obtained by centrifuging the homogenates at 150 000 × *g*, 75 min.

Assays

Proteinase activity was determined at 37 °C by the sensitive method of Garesse *et al.* (1979) using protamine sulfate (Calbiochem) as substrate (2 mg/ml) in 50 mM phosphate buffer pH 7.5. In nauplii the proteinase was determined in the presence of 5 mM $MgCl_2$. The reaction was started by addition of the extract or the purified enzyme unless otherwise stated. One unit is equivalent to the activity of 7 ng porcine trypsin (IX Sigma) on protamine for 10 min.

Protein was determined by a modification of the Bradford method (Read and Northcote, 1981).

Determination of the extent of inhibition of *Artemia* trypsin-like proteinase by EDTA, soybean trypsin inhibitor, leupeptin, lipovitellin, Gp_4G, antilipovitellin antiserum, and Concanavalin A

The dependence on divalent cations of *Artemia* trypsin-like proteinase was determined by preincubating the proteinase preparation with EDTA or EGTA (5 mM or 12.5 mM) or 10 mM o-phenantroline for 2-2.5 h at 4 °C. The activity was recovered by a further 1 h-preincubation in the presence of 5 mM excess $MgCl_2$ or $CaCl_2$.

The inhibition by the active center-directed inhibitor soybean trypsin inhibitor (STI) and leupeptin was determined by adding the inhibitor to the reaction mixture before the substrate.

The inhibition by lipovitellin was determined after preincubation (3.5 h at 4 °C) of the proteinase with 414 µg lipovitellin (Ezquieta and Vallejo, 1986a). We have observed that trypsin-like proteinase is inhibited by Gp_4G using protamine as substrate (Ezquieta and Vallejo, 1986c). The inhibition appears to depend on the substrate. With polylysine, inhibition was less marked and no inhibition was observed with histones or cytochrome C. When using a non-basic protein as substrate, bovine serum albumin, no inhibition was obserrved. The inhibitoty effect of Gp_4G was determined with protamine in the presence of 50 µM Gp_4G. The assay in the presence of both lipovitellin and Gp_4G was carried out at 17 °C in parallel with the corresponding control.

The degree of immunoinhibition by lipovitellin antiserum and the reaction of the soluble proteinase with Concanavalin A were determined as described previously (Ezquieta and Vallejo, 1985). The antiserum was a gift of Dr. de Chaffoy de Courcelles.

Purification of *Artemia* trypsin-like proteinase

The proteinase was purified from the cytosol of 26 h-old nauplii by two procedures. All operations were carried out at 4 °C.

Procedure A

Step 1. Obtention of the cytosol

In "ficoll medium" 3 g of nauplii were hand homogenized and the cytosol was obtained by centrifuging the homogenate 105 000 × *g* for 2 h.

Step 2. DEAE-cellulose chromatography

The cytosol was loaded on a column (27 ml) of DEAE-cellulose previously equilibrated with 50 mM potassium phosphate pH 7.5, 20 % glycerol, 5 mM $MgCl_2$ (Buffer A) plus 0.2 M KCl. The column was washed with 50 ml buffer A plus 0.2 M KCl and the proteinase activity eluted with a gradient (80 × 80 ml) of KCl (0.2 M-1.2 M) in buffer A. The fractions containing activity were pooled and the pool used as the source of the proteinase.

Step 3. Gel filtration chromatography on Biogel P-60

A volume from the previous step (2.6 ml) was filtrated through a calibrated column of Biogel P-60 equilibrated in buffer A plus 0.1 M NaCl. The activity eluted as a sharp peak and the molecular weight was calculated as 29 000 Da (Table V).

The scheme for the partial purification (procedure A) of trypsin-like proteinase is presented in Table IV. As can be seen, a 25-fold purification with a yield over 100 % was obtained. This

incidated that in spite of the good kinetics of the proteinase of nauplii (Garesse *et al.*, 1980), some inhibitory material was present that was eliminated by the two subsequent steps of purification.

Procedure B

Step 4. STI-Sepharose affinity chromatography

The active fractions from step 3 were pooled and desalted by filtering through a Sephadex G-25 column equilibrated with 20 mM potassium phosphate buffer pH 7.5 (Buffer B). The fractions containing proteinase activity were applied to a soybean trypsin inhibitor-Sepharose 4B column (10 ml) equilibrated in buffer B and the column was washed with 15 ml of the same buffer. A gradient (30 × 30 ml) of potassim thiocyanate (0-5 M) in buffer B was applied at room temperature and the activity eluted at about 3.5 M salt. Fractions were collected on 5 mM $MgCl_2$. The active fractions were pooled and desalted through a Sephadex G-25 as above (buffer B plus 5 mM $MgCl_2$). The concentration of protein in the preparation was below the detection limit of Bradford's method (Read and Northcote, 1981) and the degree of purification was not calculated. The recovery was 70 %. STI-Sepharose was prepared by coupling the inhibitor (Sigma, type I-S) to CNB-r activated Sepharose 4B (Pharmacia). The elution of digestive trypsins is commonly achieved by shifting the pH from 7.0 to 3.5. In our case this procedure could not be used because *Artemia* trypsin-like proteinase is unstable at acid pH (Ezquieta, 1985). After trying different conditions, the proteinase activity could be eluted as described above.

Results

THE DIVALENT CATIONS DEPENDENCE OF *ARTEMIA* TRYPSIN-LIKE PROTEINASE

We have observed that *Artemia* trypsin-like proteinase becomes unstable during the process of purification. In the search for stabilizing agents, we found that the proteinase required divalent cations for the stabilization of the activity. The binding of divalent cations by *Artemia* trypsin-like proteinase appears to be on a site different from the active center since the specific inhibitor of metallo-proteinases Phosphoramidon (Aoyagi and Umezawa, 1975) did not affect the activity (Table I ; Ezquieta and Vallejo, 1985).

The inhibition of *Artemia* trypsin-like proteinase by depletion of divalent cations can be reverted upon reposition of the cation (Table I). That the divalent cations are required for the stabilization of the activity of the proteinase is supported by the fact that this requirement is observed at 37 °C but not at 17 °C. We have found that both Mg^{2+} or Ca^{2+} can fulfill the requirement and that monovalent cations do not substitute for (Table I). Other divalent cations tested, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, inhibited the proteinase in the μM range (results not shown). The divalent cation appears to be tightly bound to the proteinase since the inhibition by EDTA requires a long preincubation. The binding seems to be loosed during aging of the proteinase and in these preparations a mere dilution reversibly destabilizes the activity (not shown). This observation might be interpreted as mediated by proteolysis of the proteinase produced upon storage. The divalent cation binding appears also to be loosed during the process of activation of the proteinase (Fig. 1). The inhibition by EDTA of the proteinase increased in the soluble and particulate fractions of homogenates of increasing times of development (Table II). The enzyme of cysts appeared insensitive to EDTA as it is to active-center directed

inhibitors (Ezquieta and Vallejo, 1985). After purification of the yolk granules fraction by a sucrose isopycnic gradient, the proteinase in the organelles became, however, sensitive to both types of inhibitors (Ezquieta and Vallejo, 1985) by a figure similar to that observed for the crude particulate fraction of early 7 h-old nauplii. The purification step also improves the accessibility of exogenous substrates allowing the detection of the proteinase in the yolk granules which is not possible in the crude preparation (Ezquieta and Vallejo, 1985). We have detected release of endogenous inhibitors during the purification step (Ezquieta, 1985) that may be related to the improvement in accessibility of substrates and inhibitors. In fact, the accessibility of substrates and inhibitors is found to increase during the physiological activation of *Artemia* trypsin-like proteinase (Ezquieta and Vallejo, 1985) and the decrease in concentration of the inhibitor lipovitellin observed during development probably accounts for the developmental increase in activity of the proteinase (Ezquieta and Vallejo, 1986ab). In this regard we have observed that the sensitivity of the proteinase to Gp_4G, that inhibits *in vitro* the hydrolysis by the proteinase of the routine substrate protamine (Ezquieta and Vallejo, 1986c), increased markedly (about a three-fold) from newly-hatched to late nauplii. Gp_4G has been also found to decrease during development (Warner and Finamore, 1967). This would indicate that at short times of development the enzyme is associated to the endogenous inhibitor(s) and therefore less sensitive to the exogenous addition.

TABLE I

Requirement of divalent cations for the stabilization of the activity of *Artemia* trypsin-like proteinase

	Activity (%)
Control	100
Control +5 mM EDTA	9
Control +5 mM EGTA	10
Control +10 mM o-phenantroline	10
Inactive proteinase +10 mM $MgCl_2$	100
Inactive proteinase +10 mM $CaCl_2$	100
Inactive proteinase +10 mM KCl or NaCl	10
Control +50 M phosphoramidon	100

The particulate fraction of a homogenate from 46 h-old nauplii was used as the source of the proteinase. Activity and the effect of the chelating agents was determined as described in Materials and methods. Data are referred to a control preincubated in the presence of 5 mM $MgCl_2$. The proteinase inactivated by incubation with EDTA or EGTA was reactivated by additional preincubation in the presence of 10 mM $MgCl_2$ or $CaCl_2$ (Materials and methods). The activity in the presence of the specific inhibitor of metallo-proteinases phosphoramidon was determined as indicated (Ezquieta and Vallejo, 1985). The lack of inhibition suggests that the requirement for the divalent cation is not in the active center.

The increased susceptibility to EDTA inhibition found during development can be blocked by chloroquine (Table III) in parallel with the block of the developmental activation of *Artemia* trypsin-like proteinase (Ezquieta and Vallejo, 1986b). It can be seen that chloroquine also inhibited the increase in sensitivity to the active center-directed inhibitor soybean trypsin inhibitor (STI), indicating that these changes in the active center area and in the regulatory area

(see below) are expressions of the process of activation. The block in the increase in sensitivity to inhibitors is observed in the particulate proteinase but it is hardly observed in the soluble proteinase. This may indicate that the changes occur in the yolk granule before the proteinase is made soluble. In fact, chloroquine at low concentrations (<1 mM) was observed to selectively inhibit the solubleness of the proteinase of the yolk granule in the cytosol (Table IV).

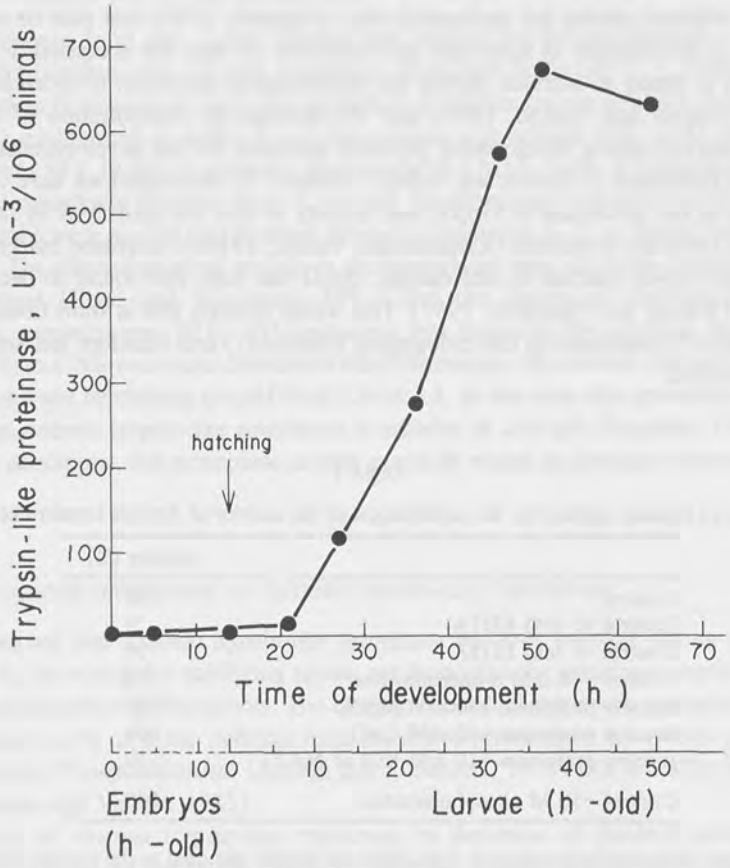

FIG. 1. Developmental activation of *Artemia* trypsin-like proteinase. Cysts and nauplii were incubated for increasing times and the activity was determined in the whole homogenates (Materials and methods). The activity of trypsin-like proteinase is represented *versus* time of development.

CHAOTROPIC AGENTS INDUCE A DECREASE IN MOLECULAR WEIGHT OF ARTEMIA TRYPSIN-LIKE PROTEINASE THAT RESULTS IN AN ACTIVE FORM INSENSITIVE TO INHIBITORS NOT DIRECTED TO THE ACTIVE CENTER

The proteinase preparations obtained by procedure A (Table V) and B were studied by the determination of different characteristics of trypsin-like proteinase. Procedure A gives a partially purified preparation of the proteinase by two purification steps. Procedure B includes an

additional affinity chromatography step on STI-Sepharose of which the elution of the proteinase required the energic chaotropic action of 3.5 M KSCN at room temperature (Materials and methods). As can be seen in Table VI, the proteinase obtained by procedure B appears to be highly modified. The proteinase is much less dependent on divalent cations and has lost the glycoprotein character. The ability to be inhibited by antilipovitellin antiserum and by lipovitellin have been largely decreased. The modification induced by the chaotropic agent also results in a decreased inhibition by Gp_4G. The activity and susceptibility to active center-directed inhibitors were, however, kept in this preparation.

TABLE II

Developmental dependence of the inhibition by EDTA of *Artemia* trypsin-like proteinase

	Inhibition (%)	
	Soluble	Particulate
Cysts	0	0
Nauplii (h)		
7	45	50
13	77	84
22	88	90
32	87	87
37	87	88
46	88	89

Nauplii synchronized immediately after hatching were further incubated for the hours indicated. Cysts and nauplii were homogenized and the soluble and particulate fractions obtained as indicated in Materials and methods. The degree of inhibition by EDTA was determined as described also in Materials and methods.

TABLE III

The lysosomotropic agent chloroquine inhibits the unmasking of *Artemia* trypsin-like proteinase

	Newly hatched nauplii		16 h-old nauplii		16 h-old nauplii plus chloroquine	
	S	P	S	P	S	P
Activity ($U/10^3$ animals)	10	7	167	24	77	9
Inhibition by EDTA (%)	45	50	90	81	84	44
Inhibition by STI (%)	98	63	96	88	87	55

To a culture of cysts, 2 mM chloroquine was added before hatching. In other culture flasks, nauplii synchronized after hatching were allowed to develop further for 16 h, in the absence or presence of 2 mM chloroquine. Nauplii were collected, homogenized, and the soluble (S) and particulate (P) fractions obtained. In the subcellular fractions, the activity and inhibition by EDTA and soybean trypsin inhibitor (STI) were determined as described in Materials and methods.

We have observed (Ezquieta and Vallejo, 1985) that the glycoprotein and vitellin characters of the proteinase are maintained through several intermediate steps introduced in the purification procedure, thus suggesting that these characteristics are not due to contaminants but to an intrinsic part of the proteinase. The results indicate that it is specially during elution from the

affinity chromatography step with the chaotropic agent that the proteinase looses the sensitivity to the physiological effectors. The effects on the proteinase, produced by interaction with STI-Sepharose and posterior elution by the chaotropic agent, have been mimicked by treating a partially purified proteinase preparation with the chaotropic KI. The results suggest that the modification is induced by the chaotropic agents and is probably unrelated to the affinity chromatography step itself.

TABLE IV

Chloroquine at low concentrations inhibits the solubilization to the cytosol of *Artemia* trypsin-like proteinase

	Larval development activity (%)									
	0-16 h					15-30 h				
Chloroquine, mM	0	0.75	1	1.5	2	0	0.25	0.5	1	1.5
Particulate trypsin-like proteinase	100	143	52	41	38	100	111	111	63	42

Larvae were cultured as described (Ezquieta and Vallejo, 1986b) in the presence or absence of the concentrations of chloroquine indicated. The extracts obtained were fractionated into soluble and particulate fractions as indicated in Materials and methods. The activity of the different particulate fractions is given as percentage of the corresponding control. The control activity of the particulate fraction of the 16 h-old larvae was 24 units/10^3 animals and that of 30 h-old larvae, 61 units/10^3 animals.

TABLE V

Partial purification of *Artemia* trypsin-like proteinase

Steps	Protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg proteinase)	Yield (%)
Cytosol	5	23 512	4 702	100
DEAE-cellulose	0.03	1 526	50 866	105
Biogel P-60	0.004	447	111 753	136

The protocol is described in Materials and methods as well as the determination of protein and trypsin-like activity.

The enzyme from step 2 of procedure A was subjected to gel filtration chromatography in the presence of 1.8 M KI. As can been seen in Table VII, the molecular weight of the eluted proteinase activity diminished from 29 kDa to 25 kDa with a shoulder at 20 kDa. The sensitivity to Gp_4G was checked in the pools of the fractions of 25 kDa and 20 kDa. The 25 kDa enzyme retained the capacity of being inhibited by Gp_4G while the 20 kDa enzyme showed a reduced inhibition by Gp_4G, similar or slightly higher than that observed with the enzyme obtained after affinity chromatography (Table VI). Chaotropic agents promote dissociation but if the protein treated is a proteinase it may induce proteolysis. In this regard, it has been described that changes in conformation of a proteinase can induce autoproteolysis (Lah *et al.*, 1984) and that the

covalent bond proteinase-α_2 macroglobulin (Van Leuven, 1982) in the case of tadpole collagenase could be released by treatment with KSCN (Abe and Nagay, 1972). In our case, the loss of the regulatory characteristics of the proteinase induced by the interaction with the chaotropic agent was associated to a decrease in molecular weight. We do not know whether the decrease in molecular weight was due to proteolysis or dissociation. In any case, it seems that the results indicate that the proteinase has lost an area related to its yolk origin which confers on the proteinase the characteristics of glycoprotein, and the capacity of binding the divalent cation and being regulated by the inhibitor(s) lipovitellin and maybe Gp_4G. The behaviour observed by *Artemia* trypsin-like proteinase after treatment with chaotropic agents suggests that it could be considered a regulatory serine, trypsin-like proteinase (see Discussion).

TABLE VI

Modification of *Artemia* trypsin-like proteinase after affinity chromatography and elution with chaotropic agents

	Percentage activity affected	
	Partially purified proteinase	Modified proteinase
EDTA inhibition	45	15
Con A affinity	78	0
Immunoinhibition	70	13
Gp_4G inhibition	86	21
Lipovitellin inhibition	65	18
STI inhibition	96	97
Leupeptine inhibition	90	90

The partially purified enzyme was obtained by procedure A of purification. The modified enzyme was obtained after STI-Sepharose affinity chromatography and elution with KSCN by procedure B. The inhibition by EDTA, Gp_4G, lipovitellin and antilipovitellin antiserum as well as the affinity by Concanavalin A were determined as described in Materials and methods. The inhibition by the active center-directed inhibitors, soybean trypsin inhibitor (STI) and leupeptin, was also determined as described in Materials and methods. The proteinase purified by procedure B had lost sensitivity and we used twice the concentration of antilipovitellin antiserum and Gp_4G to detect immunoinhibition and inhibition by Gp_4G.

TABLE VII

Decrease in molecular weight of *Artemia* trypsin-like proteinase by gel filtration chromatography in the presence of chaotropic agents

	Molecular weight (% distribution of activity)		
	29 000	25 000	21 000
ontrol proteinase	100 %	–	–
I-treated proteinase	–	68 %	32 %

A sample (2.6 ml) of the preparation of trypsin-like proteinase from step 2 was filtrated through a Biogel P-60 column equilibrated as indicated in Materials and methods plus or minus 1.8 M KI. The column was calibrated minus and plus KI with ovoalbumin, chymotrypsinogen, and ribonuclease A. The proteinase filtrated in the absence of KI is the control proteinase.

Discussion

We have observed that *Artemia* trypsin-like proteinase requires divalent cations for stabilization of the active form. The binding of divalent cations to the proteinase appears to be on a site different from the active center (Table I). The requirement for divalent cations is increased during development (Table II) and the increase is blocked by chloroquine (Table III). Chloroquine blocks the developmental activation (Ezquieta and Vallejo, 1986b) and solubleness of the proteinase from the yolk granules in the cytosol (Table IV). The unmasking of the active center, as determined by the accessibility of substrates and inhibitors, is also blocked by chloroquine (Table III). The unmasking of the active center and the increased requirement of divalent cations therefore appear to be expressions of the developmental activation of *Artemia* trypsin-like proteinase.

The Ca-binding area has been sequenced as a part of the regulatory area of serine proteinases (Patthy, 1985 ; Rogers, 1985). In agreement with these data, we have observed that *Artemia* trypsin-like proteinase can lose, without substantial loss in activity, the divalent cations requirement by treatment with chaotropic agents (Table VI). These agents reduce the molecular weight in about 9 000 Da (Table VII). In this regard, many Ca-regulated enzymes can be rendered independent of Ca^{2+} by non-specific proteolytic digestion of their Ca-binding domains (Bremel and Shaw, 1978 ; Mori *et al.*, 1980 ; Manalan and Klee, 1983 ; Krinks *et al.*, 1984 ; Jackson *et al.*, 1985). The proteinase modified by the action of chaotropic agents presented also a reduced sensitivity to lipovitellin and Gp_4G and almost lost the immunorelation with lipovitellin and the character of glycoprotein (Table VI). The loss of the antigenic character may be related to the loss of the glycoprotein components as occurs in some viral proteins (Alexander and Elder, 1984). The proteinase, however, maintained the activity and susceptibility to active center-directed inhibitors (Table VI).

The data obtained with the modified proteinase can be interpreted as there are two well-differentiated areas in the molecule of *Artemia* trypsin-like proteinase, the area related to the active center and that related to the developmental regulation of the activity. Regulatory serine proteinases usually contain two areas, the catalytic portion which is quite conserved evolutively and the non-catalytic portion by which the regulation is exerted and which is specific for the proteinase considered (Patthy, 1985 ; Rogers, 1985). The non-catalytic portion of regulatory serin-proteinases is variable and often includes various domains with particular regulatory physiological functions, apparently incorporated from unrelated genes during evolution (Patthy, 1985 ; Rogers, 1985). The Ca-binding area is found among these domains. On the contrary, the catalytic portion is highly homologous with digestive non-regulated serine proteinases like trypsin (Neurath, 1984 ; Patthy, 1985 ; Rogers, 1985). *Artemia* trypsin-like proteinase behaves as a regulatory serine proteinase adapted to the needs of development. In agreement with the occurrence of a regulatory area in *Artemia* trypsin-like proteinase, the inhibition by lipovitellin is not apparent with a digestive trypsin (Ezquieta and Vallejo, 1986a). Also in agreement, the inhibition of Gp_4G is not competitive (Ezquieta and Vallejo, 1986c) and the binding of divalent cations appears to be in a different site from the active center (Table I).

We have observed that *Artemia* trypsin-like proteinase is inhibited *in vitro* by Gp_4G with a Ki = 0.3 μM (Ezquieta and Vallejo, 1986c). The fact that the concentration of Gp_4G decreases (Warner and McLean, 1968) at the time the proteinase is activated (Ezquieta and Vallejo, 1985) together with the low Ki and the specificity of the inhibition (Ap_4A was not inhibitory ; Ezquieta

and Vallejo, 1986c) suggested that Gp_4G as well as lipovitellin (Ezquieta and Vallejo, 1986ab) could be involved in the regulation of trypsin-like proteinase. Trypsin enzymes cleave by arginine and lysine and we have observed the inhibition with the routine protein substrate protamine which is 90 % arginine. When using polylysine, Gp_4G was rather less inhibitory. With more complex proteins as histones or cytochrome C and non-basic proteins such as bovine serum albumin, the inhibition was not apparent (Ezquieta and Vallejo, 1986c). The results indicate that the inhibition is substrate-dependent. Therefore it is difficult to assign a defined role to Gp_4G in the regulation of *Artemia* trypsin-like proteinase *in vivo*. We have suggested that Gp_4G may regulate *in vivo* the hydrolysis of polypeptides containing clusters of arginine (Ezquieta and Vallejo, 1986c).

The activity of *Artemia* trypsin-like proteinase appears to be controlled during development (Ezquieta and Vallejo, 1986ab). The inhibitor lipovitellin, which is present only in early development (de Chaffoy *et al.*, 1980), is degraded during development by a regulated process (Perona and Vallejo, 1985 ; Perona *et al.*, 1987). Similar characteristics apply to Gp_4G (Warner and McLean, 1968 ; Vallejo *et al.*, 1974). The features of the regulatory mechanism(s) involved in the control of *Artemia* trypsin-like proteinase may suggest a role for the proteinase in early development. Proteinase inhibitors have been proposed as the regulatory control element of protease activity (Holzer, 1976 ; Lenney, 1980). If the regulation is exerted by modification of the proteinase being only active on specific targets, it might be that the proteinase in embryos, although inactive *in vitro* on exogenous substrates (Fig. 1), was active *in vivo* on the physiological substrates.

Conclusions

Artemia trypsin-like proteinase behaves as a regulatory serine proteinase. Two areas can be differentiated in the proteinase, one related to the active center and a regulatory part. The regulatory part that confers the sensitivity to the divalent cation and the inhibitors lipovitellin and Gp_4G appears to be related to the original storage of the proteinase in yolk granules. During the developmental process of activation, the proteinase is modified appearing more susceptible to active center-directed inhibitors and more sensitive to inhibition by chelators and Gp_4G. This suggests that both areas of the proteinase are modified during the process of activation.

Summary

1. *Artemia* trypsin-like proteinase requires divalent cations for stabilization of the active form. The binding appears to be on a site different from the active center. The requirement was observed to increase during the developmental activation of the proteinase. The increased requirement of divalent cations appears an expression of the process of activation of the proteinase in so far as inhibition of the developmental activation by chloroquine inhibited the increase.
2. After treatment with chaotropic agents, the proteinase exhibited a decrease in molecular weight of about 9 kDa. Due to the modification, the proteinase resulted desensitized to the inhibitors lipovitellin and Gp_4G and did not require divalent cations for stability. The modified proteinase, however, remained active and ready to be inhibited by exogenous active center-directed inhibitors. With the decrease in molecular weight, the proteinase lost the

character of glycoprotein and the immunodeterminants common to lipovitellin. The results indicate that *Artemia* trypsin-like proteinase has a regulatory area well differentiated from the active center. The regulatory part seems to be related to the yolk origin of the proteinase and is the area that modulates the activity through divalent cations and the physiological inhibitor(s).

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Developmental changes of chitinolytic enzymes and ecdysteroid levels during the early development of the brine shrimp *Artemia*

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Abstract

The two chitinolytic enzymes – chitinase and N-acetyl-glucosaminidase – as well as the moulting hormones (ecdysteroids) were found during the early development of *Artemia* from dry cysts to stage-I nauplii. The activities of both enzymes showed significant stage specific changes. They increased to high levels at the beginning of the emergence process. This correlation and the delivery of both enzymes into the outer medium suggest that the chitin degrading enzymes in *Artemia* work as hatching enzymes. A further indication of this role of the enzymes is the presence of chitin in the embryonic cuticle. A proof of chitin degradation is still lacking but an indication is given by Morris and Afzelius (1967).

Till now an osmotic pressure built up by glycerol was discussed as a mechanism of the emergence process. We suppose that both enzymatic and osmotic mechanisms are involved in this process and that the emergence process is rather similar to the moulting process of crustaceans.

The level of free ecdysteroids (biologically active) reached maxima during emergence and during hatching. This stage-specific changing indicates that emergence and hatching and also the enzymatic activities might be under hormonal control.

Introduction

Chitin is widely distributed in nature as well in plants, predominantly in fungi, as in animals, especially in arthropods (Jeuniaux, 1963, 1982 ; Muzzarelli, 1977 ; Spindler, 1983). Very high concentrations of chitin are present in the cuticle of arthropods but also in the tissues outside the exoskeleton, *e.g.* in the peritrophic membrane (Peters, 1976). Chitin is present in the exoskeleton of *Artemia* and in addition also in the cysts of the encysted gastrula, as demonstrated by IR-spectroscopy (Spindler, 1983). Chitin is a crystalline polymere of the amino-sugars N-acetyl-glucosamine (GlcNAc) and glucosamine, which are connected by β-1,4-glycosidic bonds. Chitin is always complexed with proteins.

The degradation of chitin is due to the presence of at least two different enzymes, the chitinase and the N-acetyl-glucosaminidase. Chitinase hydrolyses β-(1,4)-acetamino-2-desoxy-D-glycosidic bonds, resulting in dimeres and oligomeres as end products. N-acetyl-glucosaminidase splits the dimeres as well as terminal N-acetyl-glucosamine or glucosamine from polysaccharides, glycoproteins or glycolipids. Chitin-degrading enzymes are distributed from the protozoans up to the mammalians and possess different functions (Jeuniaux, 1963 ; Muzzarelli, 1977 ; Spindler,

1983). These enzymes play a very important role in the moulting cycle of the arthropods. Together with proteolytic enzymes they are involved in the apolysis of the exoskeleton. The end products of the hydrolysis of the chitin are reabsorbed by epidermal cells and can be reused for the synthesis of the chitin of the new exoskeleton (Jeuniaux, 1963 ; Neville, 1975 ; Hepburn, 1976). The complete moulting cycle is under control of the moulting hormones (Gilbert and King, 1973 ; Riddiford, 1980 ; Spindler *et al.*, 1980 ; Granger and Bollenbacher, 1981 ; Stevenson, 1985 ; Skinner, 1985), the so-called ecdysteroids (Goodwin *et al.*, 1978), and a close positive correlation exists between the activity of chitinolytic enzymes and the ecdysteroids (Spindler, 1983 ; Baier and Scheffel, 1984 ; Spindler-Barth *et al.*, 1986).

The presence of chitin in the *Artemia* cysts raises the question, whether chitin-degrading enzymes could be involved in the emergence and hatching processes and whether these processes are under hormonal regulation. For a better understanding of the emergence and hatching mechanism, the composition and structure of the shell and the changes during the early development are important. The cyst shell consists of several layers (Fig. 1) in the following order from outside to inside :

- the outer membrane, which is lost when the cyst dries at the air ;
- the chorion (4.5-7.5 µm thick), which is composed of lipoproteins and which is a tertiary envelope of maternal origin. The chorion contains the pigments and is permeable for solvents and different ions ;
- the outer cuticular membrane, which has several layers and which is the first outer barrier for the permeability ;
- the embryonic cuticle (1.6 µm thick), which is built by the embryo and which resembles the endocuticle of the crustacean exoskeleton ;
- the inner cuticular membrane, which is separated from the embryonic cells by the subcuticular space.

During the early development two additional layers between inner cuticular membrane and outer membrane of the embryo are formed : the hatching membrane and the larval exoskeleton. Morris and Afzelius (1967) identified a membrane in very early developmental stages at the position of these two additional membranes. This membrane remains during further development and is later on replaced by the hatching membrane and the larval exoskeleton. Benesch (1969) described that the hatching membrane is a product of the first embryonic moult and that the embryo builds a new exoskeleton after the moult. The hatching membrane is one of the two layers, which surround the pre-nauplius during emergence. During the hatching phase this layer is ruptured by the embryo. The outer cuticular membrane is already ruptured during the emergence stage, *i.e.* between the stages E_1 to E_2.

Despite a detailed knowledge of the fine structure of the cyst shell, the mechanism of emergence and hatching is not yet unequivocally elucidated (Clegg and Conte, 1980). In this paper we try to shed some light on the following questions :

1. Where is the chitin located within the cysts?
2. Are chitin degrading enzymes present in the cysts?
3. If chitin degrading enzymes are present, do they play a role as hatching enzymes?
4. If there are chitin degrading enzymes and if their activities change during the early development, is it possible that these changes in enzyme levels are hormonally regulated?

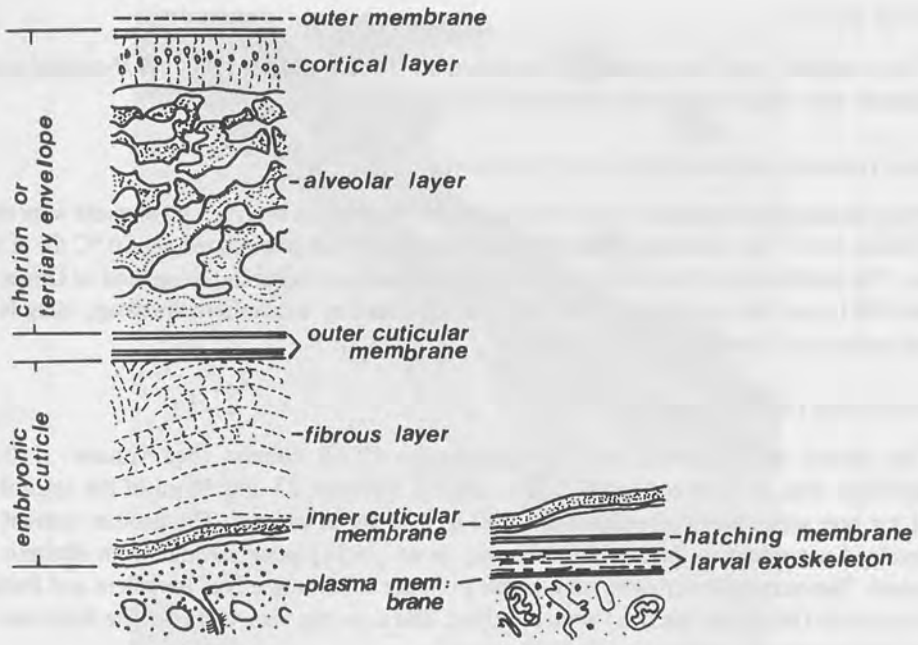

FIG. 1. Diagram of the ultrastructure of the shell (after Morris and Afzelius, 1967). On the left : shell of the dry cyst ; on the right : added envelopes, which are visible just before emergence.

Materials and methods

ANIMALS

Cysts from Metaframe (San Francisco) were used for all the experiments. For the incubation 1 g dry cysts/600 ml medium (400 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$) were used. The cysts were incubated at 30 °C in Erlenmeyer-flasks under continuous shaking (100 rpm).

PREPARATION OF THE CYTOSOL FOR THE ASSAYS

For each incubation time three parallel sets of experiments were performed. After the adequate time an aliquot was taken to determine the percentage of the different stages. The cysts or developmental stages were filtered, deep-frozen at -20 °C, lyophilized, and the dry weight determined. The dry samples were homogenized in sodium citrate phosphate buffer (0.2 M, pH 5.5) with an Ultrasonic power (Branson Sonifier, microtip) for 90 s at 4 °C. After centrifugation (15 min, 4 °C, 10 000 *g*) the pellet was re-extracted in citrate phosphate buffer by an additional treatment with the Branson Sonifier for another 90 s. The combined supernatants were used for the assays. This type of homogenization yielded the highest rates of enzymatic activity and gave the best reproducibility.

Enzyme assays

Chitinase was tested essentially as described by Boden *et al.* (1985), β-N-acetyl-hexosaminidases were tested as already described (Spindler, 1976).

Extraction and determination of ecdysteroids

From the same homogenates which were used for the enzyme assays, 3 ml aliquots were taken and mixed with 7 ml of distilled methanol and the protein was precipitated at -20 °C for at least 1 day. The purification of the ecdysteroids was performed according to the method of Dinan and Rees (1981) and the ecdysteroid concentration was tested by a radioimmuinoassay, using ICT-1 as an antiserum (Spindler *et al.*, 1978).

Ionexchange chromatography

The cytosol was separated on DEAE-Sepharose CL6B column (bed volume: 130 ml), equilibrated with 10 mM phosphate buffer, pH 6.2. Between 25 and 30 ml of the cytosol was used for one separation corresponding to 50 to 120 mg of protein. The protein content was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The enzymatic activities were eluted from the column by a NaCl-gradient and fractions of about 4 ml (80 drops/fraction) were collected. The flow rate was 45 ml/h. The fractions were tested for enzymatic activity as described earlier.

Lysozyme assay

The assay for lysozyme activity was performed as described by Selsted and Martinez (1980).

Results

Localization of chitin

In order to demonstrate chitin in shells of dry *Artemia* cysts, electron microscopical sections were treated with WGA-BSA-gold conjugate. The lectin WGA (wheat germ agglutinin) binds three β-(1,4)-bonds of N-acetyl-glucosamine and therefore has an affinity to chitin. Fig. 1 shows a diagram of the arrangement of the shell. In Fig. 2 the binding sites of the WGA-BSA-gold conjugate are shown. Chitin is localized in the embryonic cuticle and also in the inner cuticular membrane. In the other layers of the shell only a few gold granules were bound unspecifically.

Chitin degrading enzymes and their activities during development from cysts to nauplii

The presence of two different chitinolytic enzymes is not only revealed by the use of two different substrates. The two enzymes, N-acetyl-glucosaminidase and chitinase, can be separated by anion-exchange chromatography as shown in Fig. 3. In addition, the chitinase is further characterized by the appearance of oligomeres of the amino-sugar N-acetyl-glucosamine in the supernatant after an incubation of the enzyme with chitin and also by the fact that lysozyme, which can use chitin as a substrate, could not be detected neither in the medium nor in the homogenates (unpublished). The N-acetyl-glucosaminidase can be separated into two peaks by anion-exchange chromatography after rechromatography.

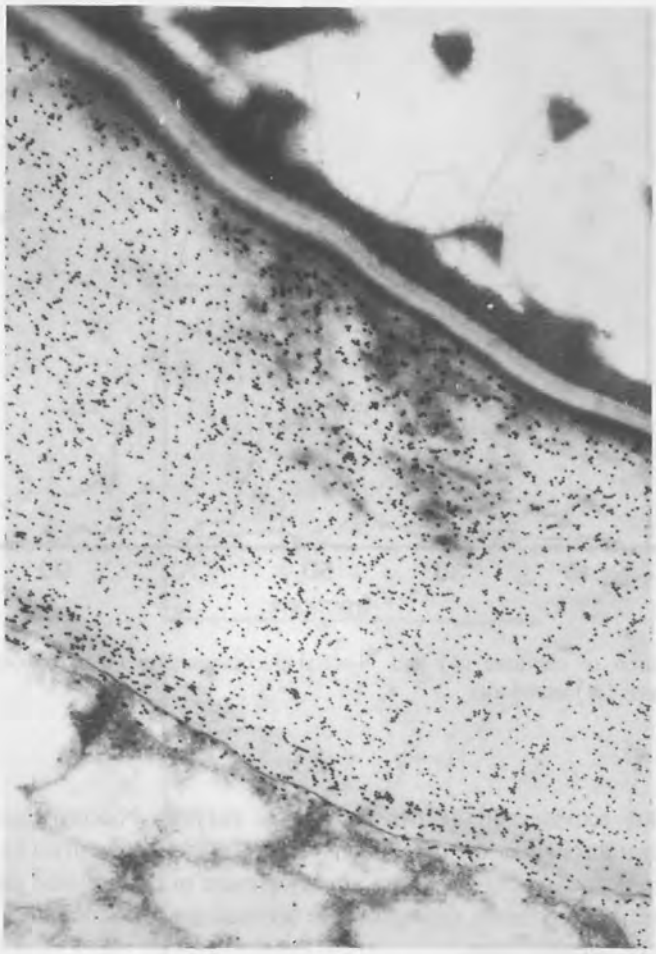

FIG. 2. Localization of chitin in dry cysts. Ultrathin sections of material embedded in Lowicryl were incubated with WGA-BSA-gold conjugate (gold particles with a diameter of 17 nm). The label is nearly exclusively localized on the embryonic cuticle and on the inner cuticular membrane (×28 500). On the upper right : chorion ; on the lower left : cells of the embryo. The figure was kindly provided by Mrs. Krahwinkel, Mrs. Latka, and Prof. Peters (Düsseldorf).

Fig. 4a shows the development of encysted gastrula up to the first nauplius stage and gives the population composition in relation to the incubation time. The first E_1 stages appeared after 9 h of incubation. The transition from E_1 to E_2 was between 9-17 h after the onset of the incubation. After 17 h there was predominantly the transition from E_2 stage to the nauplius and after 21 h of incubation 75 % of the encysted gastrulae had developed to nauplii. Prolonged incubation times did not increase the hatching rate.

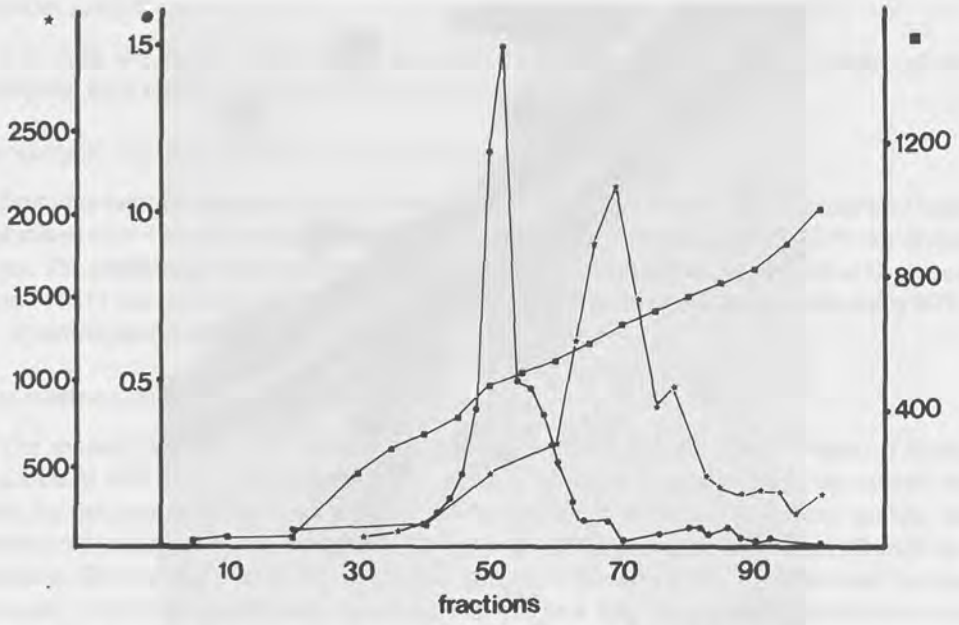

FIG. 3. Separation of chitinase (*) and N-acetyl-glucosaminidase (●) from *Artemia* cysts on a DEAE-ionexchanger. (■) osmolarity.

In Fig. 4bc the activities of the two chitinolytic enzymes N-acetyl-glucosaminidase and chitinase are shown throughout the development from the encysted gastrula to the first nauplius stage. Both chitin-degrading enzymes were already present in the encysted gastrula. The levels of the enzymes remained constant throughout the pre-emergence development and then steadily increased up to the nauplius stage.

We also looked for chitinolytic activity in the medium after 9, 15, 17, and 21 h of incubation. In concentrated media both chitinolytic activities could be detected (Fig. 5). The specific activities of N-acetyl-glucosaminidase and chitinase increase about a five-fold during the emergence phase and reach a maximum, when the maximum of the E_2 stage is reached.

CONCENTRATION OF ECDYSTEROIDS DURING DEVELOPMENT FROM CYSTS TO NAUPLII

In order to determine the concentration of ecdysteroids the same homogenates as those for the determination of the enzyme activities were used. The results of the quantitative determination of the ecdysteroids with the aid of a radioimmunoassay and after separation of conjugated and very polar ecdysteroids on the one hand, and the free ecdysteroids on the other, is shown in Fig. 6. Both two classes of ecdysteroids could be isolated from encysted gastrulae. The concentration of the free ecdysteroids reached a maximum both during the emergence and during the hatching phase, whereas the pattern of the very polar ecdysteroids only showed one maximum in between these two maxima of the free ecdysteroids.

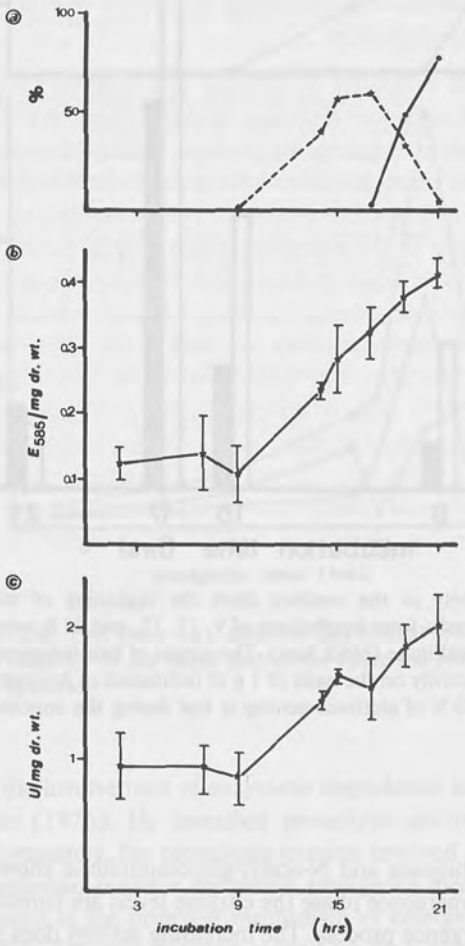

FIG. 4a. Relative composition of the developing population of *Artemia* with increasing incubation time. (∗) E_2 (fully emerged embryo) ; (●) stage-I nauplius.

FIG. 4b. Activity of chitinase during development from the encysted gastrula to the stage-I nauplius.

FIG. 4c. Activity of N-acetyl-glucosaminidase during development from encysted gastrula to stage-I nauplius. The values are means of three different sets of experiments. To determine the enzyme activities 4-6 independent assays per set of experiment were performed. Means ± standard deviations are shown. The dotted line of Fig. 4a represents the onset of stage E_1.

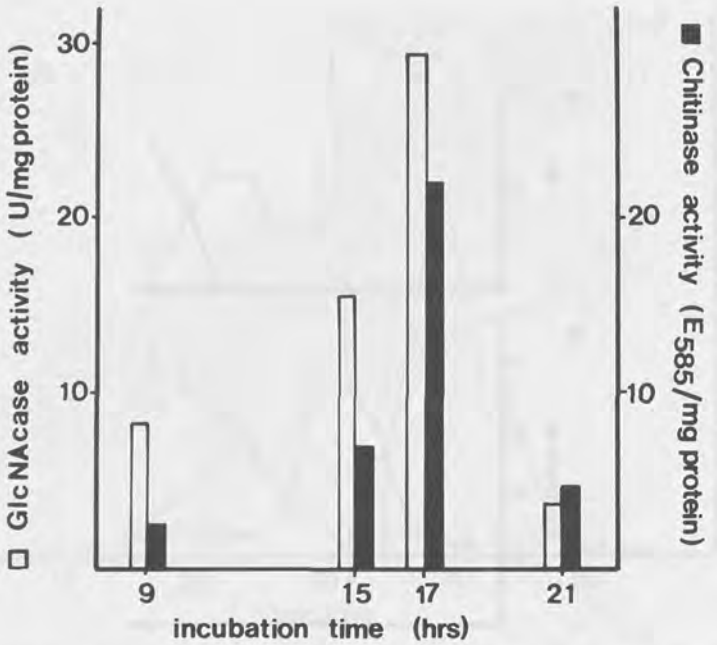

FIG. 5. Chitinolytic activity in the medium from the beginning of stage E_1 to stage-I nauplius. Concentrated and filtrated media from incubations of 9, 15, 17, and 21 h were assayed for N-acetyl-glucosaminidase (open bars) and chitinase (black bars). The means of four independent enzyme assays are given and shown as total specific activity on the basis of 1 g of incubation of dry cysts. The values of the chitinase are too low, since roughly 50 % of chitinase activity is lost during the concentration procedure.

Discussion

The activity levels of chitinase and N-acetyl-glucosaminidase show significant stage specific changes. During the pre-emergence phase the enzyme levels are constant and they both increase with the onset of the emergence process. The increasing activity does not coincide with the first moulting, which takes place within the cyst, but, in our experiments, before 9 h of incubation. The activity increase after 9 and 17 h of incubation therefore presumably correlates with the emergence phase. This indicates that both chitinolytic enzymes may act as hatching enzymes during this developmental stage. A further argument in this direction consists in the detection of both chitin-degrading enzymes in the medium as soon as pre-nauplii are in the emergence phase. The chitinolytic activities must derive from the pre-nauplii, because the activities detected are much too high for activities due to contamination by microorganisms or by damaged cysts. The presence of chitin in the embryonic cuticle also suggests that chitin-degrading enzymes must be involved in the process of emergence. A possible degradation of chitin during early development is currently investigated in our laboratory by the use of WGA-BSA-gold conjugates. An indication that chitin degradation is indeed occurring, is the observation of Morris and Afzelius (1967) that electron optical density in the fibrillar structures in the embryonic cuticle at the stage of emergent larvae diminishes.

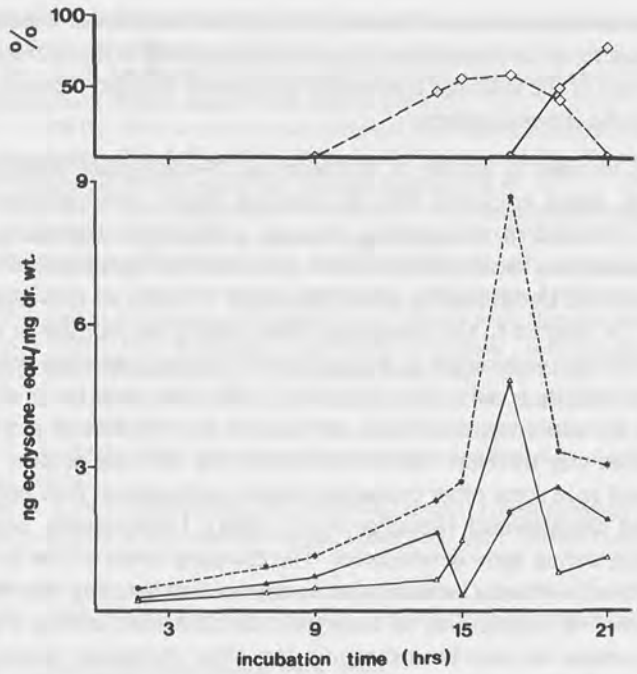

FIG. 6. Concentration of total (∗), polar (Δ), and free (▲) ecdysteroids during development from encysted gastrulae to stage-I nauplius. In the upper part of the figure the percentage of E_2 stages (dotted line) and of stage-I nauplius is shown.

Another indication for the involvement of enzymatic degradation in the transition from stage E_1 to E_2 is given by Sato (1976). He described proteolytic activity in the medium at this developmental stage. Unfortunately, the proteolytic enzyme involved is only poorly defined. In addition to the above mentioned possible enzymatic emergence mechanism the hypothesis is favored that osmotic pressure is the principal mechanism of emergence and hatching (Clegg, 1964 ; Geldiay *et al.*, 1980). Osmotic pressure is built up by a glycerolgradient in the extra-embryonic space of the cyst. Up to the stage E_1, free glycerol accumulates. The chitin-containing layer, which is impermeable for most of the ions, is also impermeable for glycerol. The accumulated glycerol only comes in the outer medium when this layer is ruptured.

The involvement of both osmotic and enzymatic (proteolytic and chitinolytic) mechanisms in the emergence process is very likely and is comparable to moulting of crustaceans. There are indeed very strong parallels between emergence and moulting processes :

1. Proteolytic and chitinolytic degradation of parts of the exoskeleton respectively the embryonic cuticle of the cyst. Similarities in the composition and in the structure of the endocuticle of the exoskeleton and the embryonic cuticle of *Artemia* in the cyst. Correlation of both processes with the levels of chitinolytic enzymes and also the levels of ecdysteroids. A break-up of fibrillar structures of the endocuticle before ecdysis and of the embryonic cuticle before emergence.

2. Increase of the osmotic pressure of the haemolymph and additional contractions of musculature finally leading to the exuviation in a normal moult and to the rupture of the outer cyst shell and later on of the cuticular membrane by glycerol osmotic pressure and by muscular contractions in the *Artemia* embryo.

The continuous increase in activity of chitinase and N-acetyl-glucosaminidase after 17 and 21 h of incubation, which coincides with the hatching phase, could indicate that chitinolytic enzymes are also involved in the hatching process. Chitinolytic degradation of parts of the hatching membrane is likely since chitin should be present in this membrane, which is the exuviae of the embryonic moult. Unfortunately, additional proofs for such an enzymatic mechanism are presently lacking. In contrast to the emergence phase there is no increase in specific activity of chitinase or N-acetyl-glucosaminidase in the medium but instead a decrease before hatching. This decrease in specific activity is only due to an about 7-fold increase in the protein content in the medium, whereas the total enzyme activities are more or less the same as at 17 h of incubation. Chitinolytic enzymes may therefore also be involved in the hatching process.

In *Artemia* as well as in some other crustacean species, ecdysteroids have been detected during embryogenesis and characterized (Spindler *et al.*, 1984). Unfortunately, only little is known about their function during early development. The changing levels of free ecdysteroids during the early development of *Artemia* indicates that emergence and hatching may be under hormonal control. An influence of ecdysteroids on embryonic differentiation and on the secretion of the first embryonic envelope has also been shown in two other crustacean species (Lachaise *et al.*, 1981 ; Lachaise and Hoffmann, 1982 ; Goudeau and Lachaise, 1983) and even more detailed in insects (Hoffmann, 1980). In addition to the influence of ecdysteroids on emergence and hatching in *Artemia*, a regulation of the chitin-degrading enzyme levels at these developmental stages also seems likely. This corresponds quite nicely to the correlation between the rising ecdysteroid titer and the increasing activity of chitin degrading enzymes in pre-moult stages of various arthropods (Spindler, 1983 ; Baier and Scheffel, 1984 , Spindler-Barth *et al.*, 1986).

Conclusions

The mechanism of emergence and hatching in *Artemia* has so far only been discussed in terms of proteolytic enzymes and osmotic pressure. Our results gave conclusive evidence for the presence of chitin, chitin-degrading enzymes, and moulting hormones during the early development of *Artemia*. The correlation between hormone titer, enzyme activity, and developmental stages, suggests a role of chitin-degrading enzymes in emergence and hatching and is also indicative for a hormonal regulation of these processes. Future studies have to show whether the same enzymes are also involved in moulting processes or whether a specific set of chitin-degrading enzymes is only present at a certain developmental stage. A physicochemical characterization of the corresponding enzymes is therefore a mandatory step to better understand the role of chitin degradation during the development of *Artemia*.

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Carbamyl phosphate synthetase activities during *Artemia* development

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Abstract

Two carbamyl phosphate synthetase activities have been characterized in *Artemia* in different stages of its biological cycle, both differing in properties and subcellular localization. The cytosolic one, present in embryos and nauplii, purified to almost homogeneity, resembles the carbamyl phosphate synthetase associated with pyrimidine synthesis (CPSase II). The purified enzyme is activated by phosphoribosyl pyrophosphate and inhibited by several nucleotides of which UDP is the most effective and being unaffected by N-acetylglutamate.

A mitochondrial carbamyl phosphate synthetase activity appears after hatching and increases steadily during larval development, in relation with the morphological, biochemical, and functional changes in the mitochondria. This enzyme utilizes glutamine and/or ammonia as a nitrogen donating substrate but it is not affected by the presence of known CPSase allosteric effectors. On the other hand, the urea concentrations determined in total homogenates during the *Artemia* development are related to the mitochondrial CPSase levels being almost undetectable in embryos.

The properties of this CPSase from *Artemia* mitochondria are consistent with the view that its function is related to urea synthesis, which as the CPAase III may have an osmoregulating function and offers a possible control mechanism involved in environmental adaptation.

A method has been devised to use homogeneous *Artemia* populations in the first stages of development. With the new method, based on different densities and phototropic properties, it has been possible to separate preparative amounts of dry cysts, pre-nauplii larvae, and free-swimming nauplii. These were obtained by incubating dry *Artemia* cysts during different time periods at 30 °C in artificial seawater.

Introduction

The first evidence of the existence of a common precursor for the arginine and pyrimidines biosynthesis was shown by Roopke in 1946. He isolated bacterial mutants with similar requirements for arginine and pyrimidines. This precursor was identified as carbamyl phosphate. The classic work of Grisolia and Cohen (1952), Ratner (1973), and Jones and Lipman (1960) provided considerable information about the formation and utilization of carbamyl phosphate. The enzyme that catalyzes the formation of CP, utilized in the mentioned pathways, is carbamyl phosphate synthetase. The actual knowledge about CPSase has been explored in reviews by Cohen (1962), Gots (1971), and more recently by Ratner (1973).

Different types of carbamyl phosphate synthetase have been found widely distributed in nature. At least three principal classes have been described on the basis of catalytic properties and

metabolic function, namely biosynthesis of pyrimidine nucleotides and arginine and/or urea. The positive effect of N-acetylglutamate on the enzyme activity is inherent to their functions and intracellular localization. CPSase I is found principally in the inner membrane/matrix complex of mitochondria (Gamble and Lehninger, 1973) of ureotelic animal livers, where the ornithine transcarbamylase is also localized. It is assumed that the synthetase I intervenes in the synthesis of arginine and urea. This synthetase utilizes ammonia as nitrogen source and requires N-acetyl-L-glutamate for its activity. CPAase II is found in microorganisms and in the cellular cytoplasm of non-ureotelic tissues of various animals, including experimental tumors, together with aspartate transcarbamylase. It is considered to be responsible for the pyrimidine biosynthesis. This enzyme utilizes glutamin as nitrogen donor and is able to use also NH_4, but it is not activated by N-acetyl-L-glutamate.

The *E. coli* CPAase is glutamin dependent and produces CP for both metabolic pathways (Hager and Jones, 1967). Neurospora (Williams *et al.*, 1971) and *Saccharomyces* (Lacrute, 1968) have two CPSases, one is specific for the arginine and the other for the pyrimidine biosynthesis pathway. Both are glutamin dependent. A peculiar CPSase has recently been discovered in the land snail which uses only glutamin as nitrogen donor and shows an absolute requirement for N-acetyl-1-glutamate (Tramell and Campbell, 1970). This carbamyl phosphate synthetase III is also found in the liver mitochondria of marine elasmobranchs (Casey and Anderson, 1982) and of freshwater teleosts (Anderson, 1980) were it catalyzes the formation of urea for osmoregulation. On the other hand, strong cross-reactivity was observed between CPSase I and III.

In this paper we present the characterization of carbamyl phosphate synthetase activities in different stages of *Artemia* development.

The principal objection to the studies previously done on metabolic changes during the development of *Artemia* is the lack of synchronization of the populations that are used, especially in the first stages of development. For the initial phase of such investigation we therefore have devised a method that permits the obtainment of homogeneous populations of different *Artemia* stages.

Materials and methods

Chemicals and biological material

^{14}C-$BaCO_3$ was obtained from the radiochemical Centre Amersham, and converted to [^{14}C]-KHO_3^-. Nucleotides, amino acids, PRPP, GSH, and bovine serum albumin were supplied by Sigma Chemical Co. Auxiliary enzymes and substrates were provided by Boehringer and Sigma respectively. All other chemicals were of the highest purity commercially available. Dry encysted gastrula (cysts) of *Artemia* were supplied by Longlife Fish Food Product Company. The nauplii obtained by development of the cysts and the adult animals came from Spanish salterns.

Handling of brine shrimp cysts

Dry *Artemia* cysts were resuspended and washed several times with cold distilled water to remove the floating, non viable, cysts. The sedimenting material was treated with 1 % NaClO. The suspension was stirred slowly for 5 min, decanted, and washed until the hypochlorite was

eliminated. The cysts were further treated with 1 % commercial tincture of Mertiolate (Eli Lilly Co) for 5 min and washed again. The hydrated cysts were collected by filtration through cheesecloth and used directly or kept frozen at -70 °C until being used at time zero of development.

The development of *Artemia* was initiated by incubation of freshly-hydrated cysts, 10 g (wet weight) in 2 l flasks containing 1.5 l sterile artificial seawater (Dutrieu, 1960) supplemented with 200 mg streptomycin and 15 000 U penicillin. After 18-20 h of incubation, with slow stirring at 30 °C, in a rotatory shaker, a part of the *Artemia* was found to be in the prenauplii and nauplii stages.

The mixture of these stages, filtrated and washed, was added to a column (9.5 cm × 30 cm) which contained a continuous gradient of NaCl between 0 and 75 % of saturation. The prenauplii and nauplii were transferred to fresh incubation saline medium and grown synchronously. At the appropriate times of development the larvae were harvested by filtration on cloth, washed with distilled water, and kept at -70 °C until being used.

Obtention of cell-free extracts

The frozen samples, hydrated cysts or nauplii, were resuspended in 2 vol of 100 mM Tris-HCl pH 7.0, containing 0.1 mM EDTA, 15 % glycerin and homogenized with a glass hand homogenizer, using a pestle loosely fitted for the cysts and tightly fitted for the nauplii. The homogenate was filtered through glass wool and centrifuged for 10 min at 500 × g. The supernatant was used as soluble extract for the enzymes activity determination.

Preparation of subcellular fractions

Frozen embryos or nauplii were homogenized with 2 vol and 1 vol, respectively, of buffer 25 mM Tris-HCl pH 7.0, containing 0.68 M sucrose, 1 mM EDTA, 2 mM DTE, and 15 % glycerin. The homogenization was carried out by 20-25 hand strokes, in the conditions described above. This preparation, free of cellular debris by filtration through glass wool, will be referred to as the homogenate. The homogenate fraction was centrifuged at 500 × g for 10 min and a pellet, composed of nuclei, yolk platelets and some mitochondria (pellet 1) was obtained. This pellet was washed with the buffer used in the homogenization, and the wash fraction was combined with the original supernatant. Mitochondria were obtained from the resultant supernatant (super 1), by centrifugation at 27 000 × g for 30 min and washed twice with the same buffer. Both pellets were resuspended in one volume of this buffer plus 1 vol of 10 mM bis-tris-acetate pH 7.0.

Enzyme assays, carbamyl phosphate synthetase

^{14}C-bicarbonate fixation

The rate of [^{14}C]-bicarbonate incorporation into carbamyl phosphate was determined using a reaction mixture containing (µmole/ml) : Tris-HCl buffer pH 7.5, 100 ; KCl, 50 ; $MgCl_2$, 20 ; glutathione reduced, 5 ; bovine serum albumine, 0.4 mg ; NH_4Cl, 50 ; ATP, 10 and $KHCO_3^-$, 10 (specific activity 2-3×10^6 cpm/µmole). In the assay of the mitochondrial enzyme, the conditions were the same, except that 100 bis-tris-acetate buffer pH 7.2, NH_4Cl, 10, and triton × 100, 0.01 % were used. The particular assay conditions used in specific experiments are given in the figures and tables. After incubation for 10 min at 37 °C the reaction was terminated by transfer

of a 0.5 ml aliquot to 1 ml of ice-cold water containing two drops of n-octanol and by bubbling CO_2 through the solution for 60 min at 0-2 °C to remove the excess of HCO_3^-. The gassed solution was transferred to a scintillation counting vial containing 0.1 ml of 0.1 N NaOH, and liquid scintillation performed. The ^{14}C-labeled was identified as carbamyl phosphate and the amount obtained under these conditions was the same as if the product of the reaction, before gassing, had been converted to urea (Trotta *et al.*, 1974), and was subsequently gassed as has been described above.

Spectrophotometric assay

The reaction mixture was the same as for ^{14}C bicarbonate fixation, and 2.0 mM phosphoenol pyruvate ; 0.2 mM NADH ; 20 μg of lactate dehydrogenase and 30 μg of pyruvate kinase (ammonium-free) were added.

Glucosephosphate isomerase (E.C.5.3.1.9). The assay mixture contained the following components (μ moles/ml) : imidazol-HCl pH 7.0, 50 ; fructose-6-phosphate, 0.5 ; 2.0 of glucose-6-phosphate dehydrogenase and the enzyme preparation. Increase in absorbance at 340 nm was monitored.

Cytochrome c oxidase (E.C.1.9.3.1). The assay mixture contained the following components (μ moles/ml) : sodium phosphate pH 7.0, 20 ; EDTA, 1.0 ; reduced cytochrome c, 0.1, and the enzyme preparation. Decrease in absorbance at 550 nm was monitored.

Succinate cytochrome c reductase (E.C.1.3.9.9.1). The assay mixture contained the following components (μ mole/ml) : sodium phosphate pH 7.0, 20 ; KCN, 1.0 ; cytochrome c, 0.1 ; sodium succinate, 20, and the enzyme preparation. Increase in absorbance at 550 nm was monitored.

Malate dehydrogenase (E.C.1.1.1.3.7). The assay mixture contained the following components (μ moles/ml) : Tris-HCl pH 7.0, 50 ; KCl, 100 ; $MgCl_2$, 5.0 ; oxalacetate, 1.0 ; NADH, 0.15 ; triton X-100, 0.1 %, and the enzyme preparation. Decrease in absorbance at 340 nm was monitored.

Isocitrate dehydrogenase (NADP-dependent) (E.C.1.1.1.3.7). The assay mixture contained the following components (μ moles/ml) : Tris-HCl pH 7.5, 50 ; sodium D-L-isocitrate, 1.0 ; $MnCl_2$, 2.0 ; NADP, 0.5 ; triton X-100, 0.1 %, and the enzyme preparation. Increase in absorbance at 340 nm was monitored.

Glutamate dehydrogenase (E.C.1.4.1.3). The assay mixture contained the following components (μ moles/ml) : Tris-HCl pH 7.8, 50 ; DTE, 1.0 ; EDTA, 1.0 ; ADP, 1.25 ; $MgCl_2$, 2.5 ; sodium ketoglutarate, 5.0 ; triton X-100, 0.1 ; NADH, 0.1 ; ammonium acetate, 50, and the enzyme preparation. Decrease in absorbance was monitored.

Partial purification of *Artemia* cysts CPAse

The starting material was homogenized as previously described. This homogenate was centrifuged at 500 × *g* for 30 min and gives the supernatant 1. The corresponding pellet, composed as has been mentioned before of yolk platelets and nuclei, was discarded. The large yield obtained in this step with respect to the homogenate was due to difficulties encountered in the assay with the homogenate. At the dilution used this fraction is heavily concentrated and hard to manipulate, therefore the values obtained have an important error. Because of the instability of the enzyme in this step at high dilution, a loss of specific activity occurs when the

enzyme is, however, assayed at a lower protein concentration. The solution obtained in the proceeding step was centrifuged at 27 000 × g for 30 min. The precipitate was eliminated and the supernatant, containing the enzymatic activity, was brought up to 40 % saturation with solid ammonium sulphate, and stirred for 60 min. The protein precipitate was dissolved in the same buffer of homogenization and applied to a Sephadex G-25 column (1.7 cm × 55 cm) equilibrated and eluted with the same buffer. In order, however, to avoid the loss of activity of the diluted enzyme obtained from the column, the protein was precipitated in solid ammonium sulphate and dialyzed again with this buffer overnight before being used. To date, due to the high instability of the enzyme it has not been possible, in any of the attempts made, to extend the grade of purity of the enzyme. Therefore this level of purification was used in the experiments discussed in this paper.

Results and discussion

Obtention of the homogeneous populations of hydrated cysts, prenauplii, and nauplii

After incubating cysts for 15 h there is a mixture of hydrated and nonviable cysts, prenauplii, nauplii, and empty capsules in the salt medium. The alterations produced in the capsule of the *Artemia* eggs during the emergence of the embryos necessarily causes a change in its density. Taking advantage of this property, we thought that the hydrated and nonviable cysts and prenauplii would be able to be separated in a density gradient. The nauplii are easily separated from the other stages by their not well-known phototropic reaction and by their swimming capacity which permits them to move toward any light source and concentrate there.

The mixture of these stages, filtrated and washed, was added to a column that contained a gradient of NaCl as described in "Materials and methods." All of the column was covered with a black paper, except the upper part, which was intensively illuminated. After 30 min under these conditions the components of the heterogeneous mixture put in the column, appeared to be distributed as shown in Fig. 1. The lower band that corresponded to the more concentrated zone of the gradient is composed of hydrated and nonviable cysts (Fig. 1a). In the middle zone the prenauplii concentrated (Fig. 1b), and in the top zone, next to the light source, the nauplii (Fig. 1c). Fractions containing the homogeneous populations were collected separately and each one was filtered and washed until the NaCl was totally eliminated. The enzymatic activity of these samples was measured in their extracts, as has been described in "Materials and methods."

Catalytic and kinetic properties of the cysts CPSase

The carbamyl phosphate synthetase requirements are shown in Table I. When ATP, Mg, L-glutamine, or NH_4 is omitted there is no formation of carbamyl phosphate. The reaction does not require N-acetyl-glutamate for either of the amino donors and the rate is not increased for the presence of this metabolite required for the mitochondrial enzyme (Lusty, 1978). The activity in the presence of both substrates, L-glutamine plus NH_4, is, on the other hand, not additive. In accordance with the CPSase described in eukaryotic tissues (Aitken *et al.*, 1975), this indicates substrates are used for a single enzyme. We have a marked inhibition of the enzymatic activity by N-acetyl-L-glutamate for either of the amino donors. The CPSase reaction requires a divalent cation and the greatest activity is obtained with Mg^{++}. Mn^{++} or Co^{++} can seemingly be

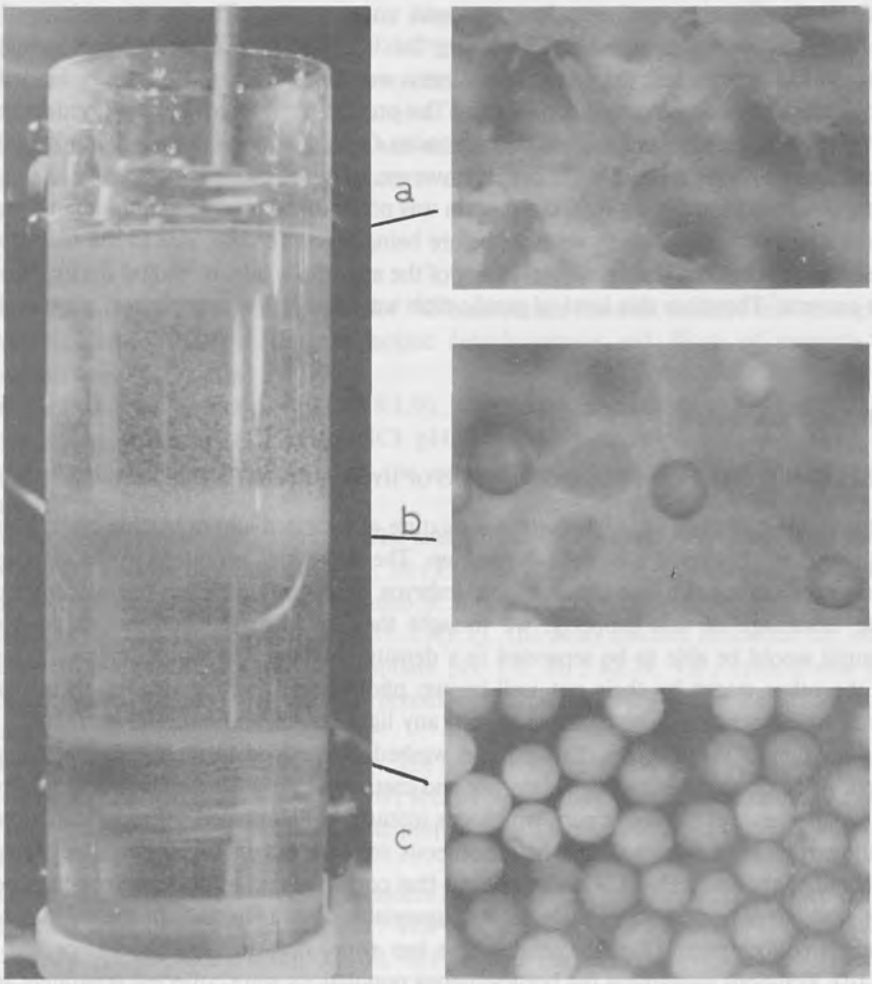

FIG. 1. Separation in NaCl gradient of nauplii, prenauplii and hydrated cysts : a) nauplii ; b) prenauplii and c) cysts. Conditions described in "Materials and methods."

replaced by Mg^{++} to a very limited extent, less than 10 %. The possibility, however, that the radioactivity detected was due to the traces of (^{14}C)-Mn CO_3^- or (^{14}C)-$CoCO_3^-$ is not discarded. Other divalent cations tested are without effect. The stoichiometry of the reaction is shown in Table II. The results suggest that for each mole of ^{14}C-carbamyl phosphate formed, two moles of ADP appear, either with ammonium or L-glutamine. The conversion of ^{14}C-CP into urea, as has been described in "Materials and methods", is total.

The activity of the enzyme was tested at different pH in the presence of NH_4 and/or L-glutamine. The assay conditions were those previously described. Two different buffers were utilized in a pH range from 6.0 to 9.0 ; 0.1 M imidazol buffer from 6.0 to 7.5, and 0.1 M Tris

TABLE I

Requirements for carbamyl phosphate synthesis

Additions to or deletions from the complete system	^{14}C-incorporated into carbamyl phosphate (counts/min)
None [1]	1 085
Minus K^+	297
Minus Mg^+	102
Minus ATP	100
Minus glutamine	132
Minus glutamine + NH_4^+	1 158
Minus glutamine + NH_4^+ + N-ac-glu	1 248
Plus NH_4^+	1 318
Plus NH_4^+ + N-ac-glu	1 215

[1] 0.4 mM glutamine.
The assay conditions were as described in "Materials and methods."

TABLE II

Stoichiometry of *Artemia* CPSase reaction

Nitrogen donor	CP	Urea	ADP	ADP/CP	ADP/Urea
			(mU/ml)		
NH_4^+	1.5	1.7	3.3	2.2	1.9
GLU^+	1.4	1.3	2.6	1.9	2

U=μ moles of the reaction product/h. The amounts of carbamyl phosphate and urea formed were determined by the radioactive assay and the ADP was measured by the spectrophotometric assay as described in "Materials and methods."

from 7.5 to 9.0. The points in Fig. 2 indicate the final pH in the reaction mixture. Under these conditions, maximal activities were observed between pH 7.5 and 8.5 with L-glutamine and NH_4 respectively. However, as can be seen in Fig. 2, beyond ph 8.0 the activity, in the presence of L-glutamine, is less than with NH_4 ; Tris-HCl pH 7.8 was chosen for the studies on this enzyme.

CARBAMYL PHOSPHATE SYNTHETASE ACTIVITIES IN DIFFERENT STAGES OF THE *ARTEMIA* DEVELOPMENT

As mentioned in the introduction, the effect of N-acetyl-L-glutamate on the CPSase activity makes a previous classification of the enzyme within the two main CPSase types (I and II) possible. Thus the CPSase activity was measured in cysts, prenauplii, nauplii, and adult-animal extracts according to the standard radioactive assay. The effect of the N-acetyl-L-glutamate on the activity with both substrate nitrogenous donors, NH_4 and L-glutamine, was also studied in each one of these stages.

As shown in Fig. 3, in the *Artemia* stages studied, CPSase activities are detected which can utilize either NH_4 or L-glutamine as nitrogenous donor. As can be seen in Fig. 4, the enzymatic activity in non-hydrated systs is inhibited by about 25 % with N-acetyl-L-glutamate, in the presence of either of the substrates. The prenauplii enzyme is scarcely inhibited, but in adult

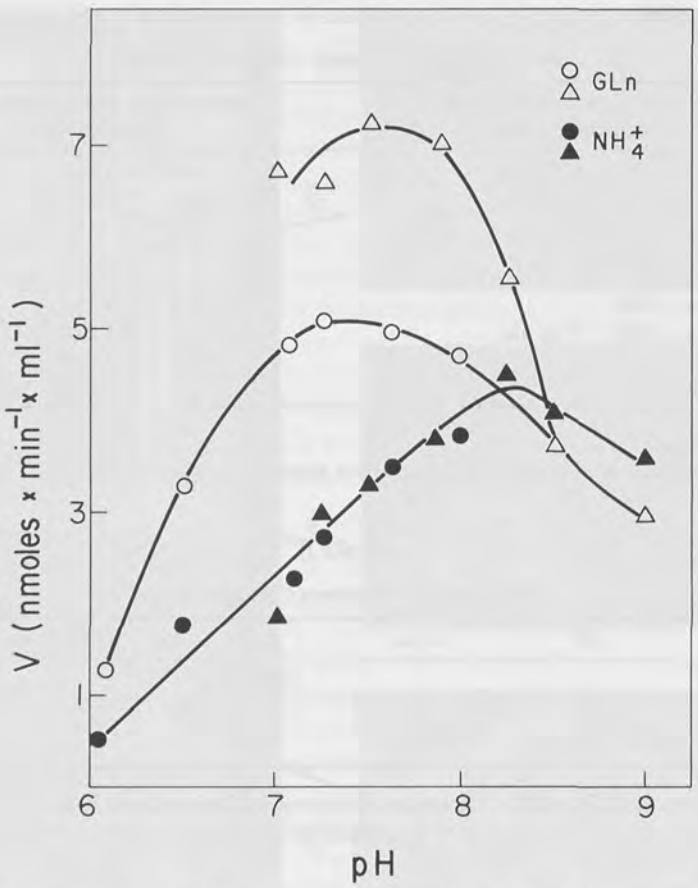

FIG. 2. pH dependence of carbamyl phosphate synthetase activities. Conditions described in "Materials and methods" : (○, Δ) 0.1 Imidazol ; (●, ▲) 0.1 Tris-HCl.

animals and nauplii, even recently hatched, the N-acetyl-L-glutamate has a marked activating effect.

Under all conditions, a decrease in the enzymatic activity was observed in the prenaupliar and naupliar stages, as compared to the level for cysts. In adult animals the activities increased notably. The decrease in the enzymatic activity is probably due to a proteolytic effect caused by some protease induced during the early larval development (Osuna *et al.*, 1977).

The activating effect of N-acetyl-L-glutamate on the enzyme of the nauplii and adult animals may signify either the appearance of a new synthetase during *Artemia*'s development, or an increase in the complexity of the existing enzymes which would endow it with greater functional and regulatory capabilities. The first hypothesis would imply the existence of two synthetases with distinct functions in the cell in the more-developed stages of the biological cycle of *Artemia*, and a relationship with the biochemical and morphological changes described in the *Artemia* mitochondria during their development (Schimtt *et al.*, 1973). The subsequent studies of subcellular localization will permit us to elucidate these problems.

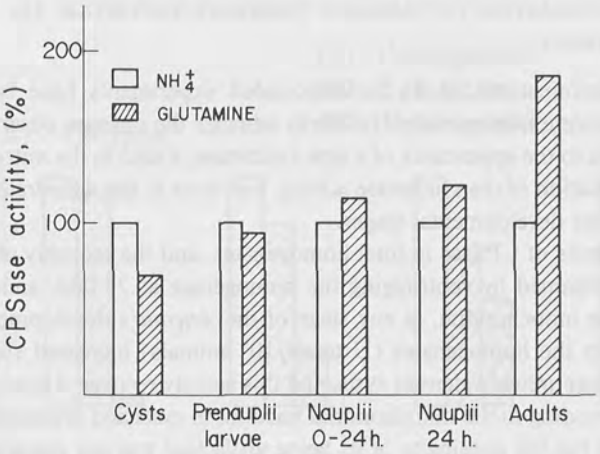

FIG. 3. Carbamyl phosphate synthetase activities in homogenates from different stages of *Artemia*'s early development. Conditions as in "Materials and methods." The activities determinated in presence of L-glutamine represents % of those observed with NH_4^+ (100 %).

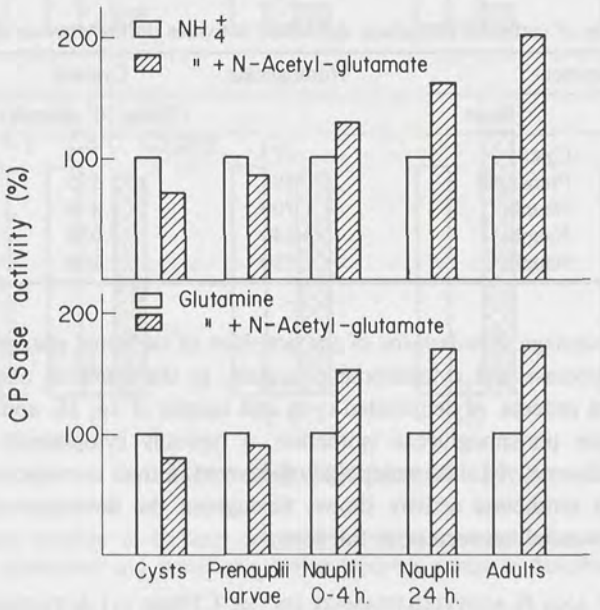

FIG. 4. Effect of N-acetyl-L-glutamate on the activity of CPSases in presence of NH_4^+ and/or L-glutamine. Conditions described in "Materials and methods."

Intracellular localization of carbamyl phosphate synthetase (s) activities during *Artemia* development

The CPSase determinations of all the expounded experiments have been carried out in preparations of complete homogenates. To clarify whether the changes observed with respect to the effector were due to the appearance of a new synthetase, found in the mitochondria, the study of the subcellular location of the synthetase activity was done in the dehydrated cysts, prenauplii, and nauplii of various developmental stages.

In Table III the units of CPSase in total homogenates, and the recovery of this activity in the cytosolic fraction, obtained by centrifuging the homogenate at 27 000, and in the precipitate, which contained the mitochondria, at any stage of the *Artemia*'s development are shown. The synthetase activity in the homogenates (U totals/10^6 animals) increased slightly in prenauplii with respect to the determined values in cysts, and this activity recovered totally in the cytoplasm. The activity corresponding to the mitochondrial fraction, in cysts and prenauplii, was approximately 5 % of the total but the possibility of its being artifactual was not discarded. In the nauplii, newly emerged from the embryonic membrane (24 h), the total activity in homogenates doubled and an important part of the synthetase (30 %) appeared in the fraction which contains the mitochondria. The highest levels of synthetase were detected in unfed nauplii of 36 h development, in which the activity of the homogenate apparently continued to be distributed between the cytoplasmic and mitochondrial fractions, corresponding to some 60 % and 40 % respectively. The decrease in activity observed in nauplii of 48 h development could be explained by the proteolytic phenomena mentioned above, and by the fact that the nauplii were not fed.

Table III

Subcellular location of carbamyl phosphate synthetase activities during *Artemia* early development

Development		Homogenate	Cytosol	Mitochondria
Time (h)	Stage	(Units/10^6 animals)		
0	Cysts	173	175	10
16	Prenauplii	593	575	35
24	Nauplii	1 700	1 450	500
36	Nauplii	3 540	2 050	11 360
48	Nauplii	1 550	1 200	510

In Fig. 5 the percentage distributions of the activities of carbamyl phosphate synthetase(s); phosphoglucose isomerase and cytochrome *c* oxidate, in the fractions obtained through the described fractioning process, of dehydrated cysts and nauplii of 24, 36, and 48 h development are shown. Both the phosphoglucose isomerase, a typically cytoplasmic enzyme, and the mitochondrial cytochrome *c* oxidase were totally recovered in their corresponding fractions. The carbamyl phosphate synthetase activity shows, throughout the development, the distribution pattern already expounded between both fractions.

Effect of the UTP and N-acetylglutamate on the CPSase (s) activities

All of the expounded reasoning is not sufficiently conclusive to justify the existence of a mitochondrial isoenzyme of CPSase in the *Artemia* naupliar stage. In an attempt to differentiate

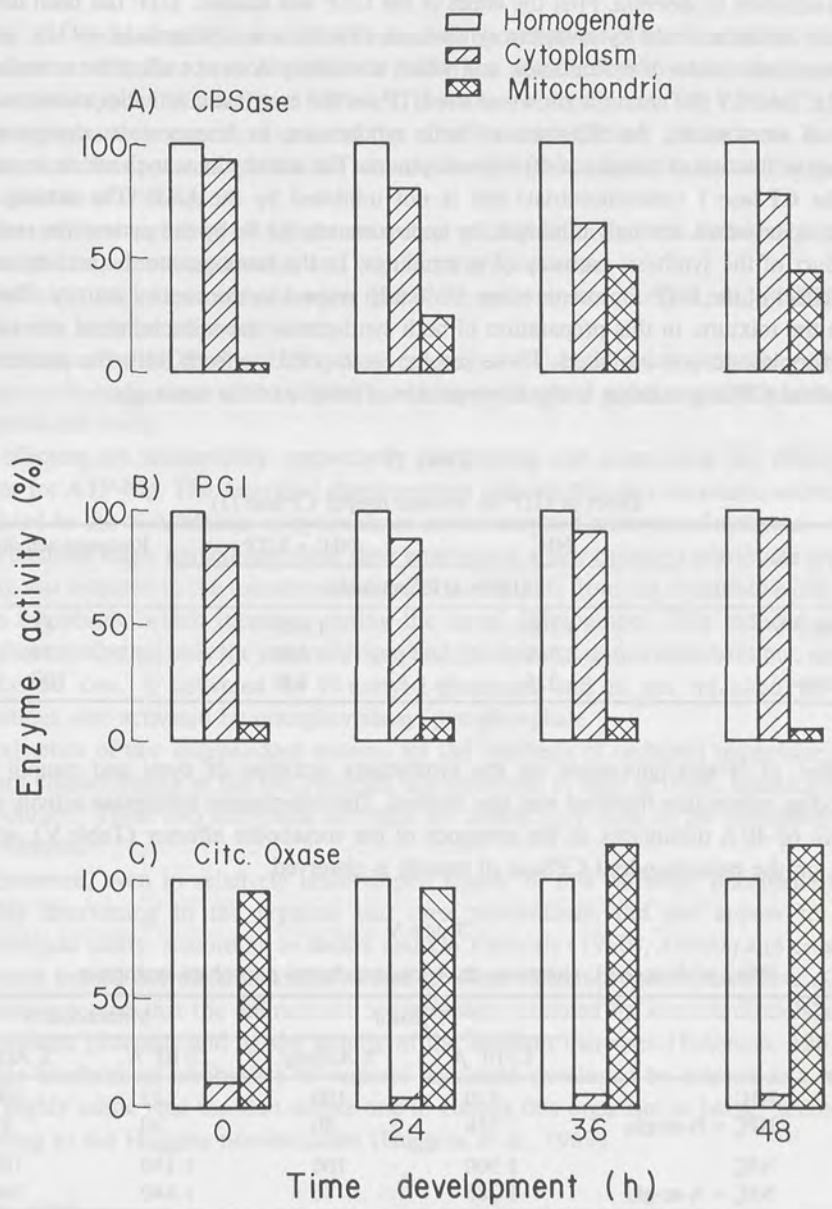

FIG. 5. Intracellular location of CPSases activities during early stages of development of *Artemia*. Carbamyl phosphate synthetase and subcellular marker enzymes assays as described in "Materials and methods."

both activities, a study was carried out with known effectors of the CPSase(s) I and II, on the syspected activities in *Artemia*. First the effect of the UTP was studied. UTP has been described as a potent inhibitor of the cytoplasmic synthetases (Tatibana and Shigesada, 1972), initiators of the biosynthetic route of pyrimidines, and which absolutely does not affect the mitochondrial enzyme. In Table IV the action is shown of the UTP on the enzymatic activities measured in the presence of ammonium, the substrate of both synthetases, in homogenate, cytoplasm, and mitochondrial fraction of nauplii of 40 h development. The activity in mitochondria is similar to that of the CPSase I (mitochondrial) but is not inhibited by the UTP. The activity in the cytoplasm is, however, strongly inhibited, by approximately 85 %, by the pyrimidine nucleotide final product of the synthesis pathway of pyrimidines. In the homogenate the activity resulting from the effect of the UTP represents some 50 % with respect to the control activity. This could be due to the mixture, in this preparation of both synthetases, the mitochondrial one inhibited and the cytoplasmic one inhibited. These results correspond perfectly with the proportion of mitochondrial CPSase existing in the homogenate of nauplii of the same age.

TABLE IV

Effect of UTP on *Artemia* nauplii CPSase (I)

	NH_4^+	NH_4^+ + UTP	Remnant activity (%)
	(Units s/10^6 animals)		
Homogenate	4 200	1 840	45
Cytosol	2 340	330	14
Mitochondria	1 325	1 340	100

The effect of N-acetylglutamate on the synthetases activities of cysts and nauplii in the corresponding subcellular fractions was also studied. The cytoplasmic synthetase activity of cysts and nauplii of 40 h diminishes in the presence of the metabolite effector (Table V), while an activation on the mitochondrial CPSase of nauplii is observed.

TABLE V

Effect of N-acetyl-L-glutamate on *Artemia* carbamyl phosphate synthetase

		Cytosol		Mitochondria	
		U/10^6 A	% Activity	U/10^6 A	% Activity
Cysts	NH_4^+	320	100	32	100
	NH_4^+ + N-ac-glu	256	80	30	97
Nauplii	NH_4^+	2 500	100	1 150	100
	NH_4^+ + A-ac-glu	1 785	70	1 840	160

The enzyme activities were assayed as described in "Materials and methods," nauplii were 40 h old.

The activation by N-acetylglutamate is not very marked, and under the testing conditions this enzyme does not appear to absolutely require the metabolite, as has been described in some systems.

Conclusions

1. During the early development of *Artemia*, two isoenzymes of the carbamyl phosphate synthetase activity have been identified, of which the properties and subcellular location are similar to the types CPSase II and CPSase I, described in vertebrates. The CPSase II, located in the cytoplasm, is constitutional and the mitochondrial CPSase I is induced during the development. Its appearance coincides with the morphogenetic and functional changes in the mitochondria of *Artemia*.
2. In cysts only one carbamyl phosphate synthetase activity exists and it is exclusively cytosolic. Its catalytic requirements, kinetic, and regulatory properties are :
   a) indistinct use of ammonium and/or glutamine as donors of nitrogen, and of ATP-Mg as specific supplier of phosphorus ;
   b) inhibition by UDP and UTP, final products of the *de novo* synthesis pathway of pyrimidines, and activation by PRPP, the intermediary and regulating substrate of the said metabolic route.

   Both effectors act allosterically, respectively diminishing and augmenting the affinity of the enzyme for ATP-Mg. The described characteristics indicate that this enzymatic activity could be related to the biosynthesis of pyrimidines in the encysted embryos of *Artemia*.
3. In the naupliar stage, immediately after their emergence, a new carbamyl phosphate synthetase activity was detected in the mitochondrial fraction. It differs from the constitutive one located in the cytoplasm, which increases during the larval development. This induced carbamyl phosphate synthetase uses the same nitrogen and phosphorus donor substrates but, unlike the cytoplasmic one, is activated by N-acetyl-1-glutamate and is not inhibited by uridilic nucleotides, nor activated by phosphorribosyl pyrophosphate.
4. The existence of two independent systems for the synthesis of carbamyl phosphate and the different characteristics of the two enzymes that intervene in each of them, situate *Artemia* in the evolution. These two enzymatic activities are indeed very close to the systems described in vertebrates.
5. The presence, even in relatively undeveloped stages, of this carbamyl phosphate activity, possibly intervening in the arginine and urea biosynthesis, did not appear to have a physiological utility. According to Bellini and De Viscentis (1960), *Artemia* and crustaceans in general indeed excrete ammonium as final product of the nitrogen metabolism. Although it is also accepted that the ammonium concentration excreted by *Artemia* diminishes as its development proceeds and as the salinity of the medium increases (Emerson, 1967). The possible biochemical mechanism of osmotic regulation developed by *Artemia* as a response to its highly saline vital medium allows one to classify this organism as being "ureosmotic", according to the Huggins nomenclature (Huggins *et al.*, 1969).

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The structure of *Artemia* sp. haemoglobins I. The amino acid sequence of a structural unit

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Abstract

The extracellular haemoglobins (M_r 260 000) of the brine shrimp *Artemia* sp. were cleaved by limited digestion with subtilisin. The resulting structural units of M_r 16 000 (fraction E) were isolated and a single type (E_1) purified to homogeneity. The amino acid sequence of E_1 clearly shows its relationship with the vertebrate globin family. The *Artemia* globins (M_r 130 000) are covalent polymers of eight "myoglobin-like" structural units (M_r 16 000).

Introduction

In the haemolymph of *Artemia* sp., three haemoglobin phenotypes were identified (Bowen *et al.*, 1969). All three have a native M_r of 260 000 and exist as dimers of two globin chains (α and β) of similar size ($M_r \pm 130\,000$). These globin chains have a minimum M_r of 18 000 and a haem content of 1/17 000 M_r suggesting that they contain about eight functional units (Moens and Kondo, 1977, 1978 ; Wood *et al.*, 1981).

The *Artemia* globin may be used as a model of an invertebrate high M_r globin chain. These chains, found in some mollusc and arthropod haemoglobins are, like the haemocyanins, built up as polymers of a variable number of structural and functional units. These functional units, which can be liberated from the intact molecule by limited proteolysis, represent polypeptide fragments with M_r in the range 15 000 - 17 000 containing one haem group. They are able to bind O_2 reversibly with a very high affinity but in a non-cooperative way (Chung and Ellerton, 1979 ; Wolf *et al.*, 1983). They strongly resemble the vertebrate myoglobin type. In this communication we describe the primary structure of a single structural unit isolated from the *Artemia* globin chains.

Materials and methods

Artemia sp. (Salins du Midi, Le Grau-du-Roi, France) haemoglobins were prepared as described by Moens and Kondo (1978). A fraction (E) containing a collection of structural units

(M_r±16 000) was isolated from subtilisin digested haemoglobins by gelfiltration as described previously (Moens *et al.*, 1984). From this a single type of structural unit (E_1) was purified to homogeneity by isoelectric focusing (Moens *et al.*, 1985). Tryptic, chymotryptic, thermolytic and *S. aureus* V_8 protease digestion was carried out on denaturated samples (Allen, 1981). The resulting peptides were separated by HPLC and TLC. Amino acid sequence was determined either automatically using a gas phase sequencer (Applied Biosystems 470A) (Hewick *et al.*, 1981) or manually using the DABITC-PITC double coupling method according to Chang *et al.* (1978).

Results and discussion

Isolation and characterization of a single structural unit

Artemia haemoglobin was subjected to limited subtilisin digestion as described by Dangott and Terwilliger (1980). The resulting cleavage pattern, obtained by SDS-PAGE, is compatible with a random scission of a polymer (M_r 130 000) containing eight monomers of M_r 16 000 (Tanford, 1961). These results, together with the Fe/protein ratio (1/17 000) and the minimum M_r calculated from amino acid composition (M_r 18 000) all confirm that the *Artemia* globin chains are built up by the covalent linkage of eight structural units of M_r±16 000.

The fragment mixture obtained by subtilisin digestion was separated by gelfiltration on a Sephacryl S200 column. Five fractions (A to E) were clearly separated (Fig. 1). The M_r 15 000 - 20 000 fraction (E), representing single structural units, is heterogeneous in M_r and charge. This heterogeneity may result from the aspecific cleavage of a single type of structural unit from "homopolymeric" globin chains or from the existence of several types of structural units within a "heteropolymeric" globin chain or both.

Before further purification becomes possible contaminating subtilisin must be separated from fraction E. This was achieved by chromatography on a DE 52 column at pH 7.6 as described by Schroeder and Huisman (1980). Under these conditions, the fragments were quickly washed free of proteinase activity and were eluted with 250 mM salt as a very sharp band, whereas oxidized material was irreversibly bound.

A pure fraction containing a single structural unit type could only be obtained by isoelectric focusing (pH 4.0-6.5) on a Sephadex G75 layer performed by the method of Radola (1973, 1974) and Frey and Radola (1982) (Fig. 2). Five fractions (E_1-E_5) were clearly separated. Only the most acidic fraction, E_1 is fairly homogeneous (±90 %) as judged by O'Farrell analysis, whereas the other fractions seem to be stable aggregates of polypeptides with different M_r and charges.

Under native conditions E_1 has an M_r of 17 400±200 (n=5) and an apparent pI=4.8. Under denaturing conditions an M_r 15 800±800 (n=6) and a pI=6.4 were determined. The amino acid composition of E_1 shows high contents of D, E, V, and L. C is absent. It is quite similar with that of HbII, confirming that no specific amino acid sequence exists in the intact haemoglobin molecules (Moens *et al.*, 1984). The isolated structural unit is still able to bind dioxygen reversibly. It has a very high oxygen affinity (P_{50} = 0.36±0.045 mm Hg (n=10)) but, as expected, no cooperativity (n = 0.95±0.09 (n=10)) (Moens *et al.*, 1984). E_1 represents not only a structural unit but also a functional entity from which the intact *Artemia* haemoglobin molecule is built up.

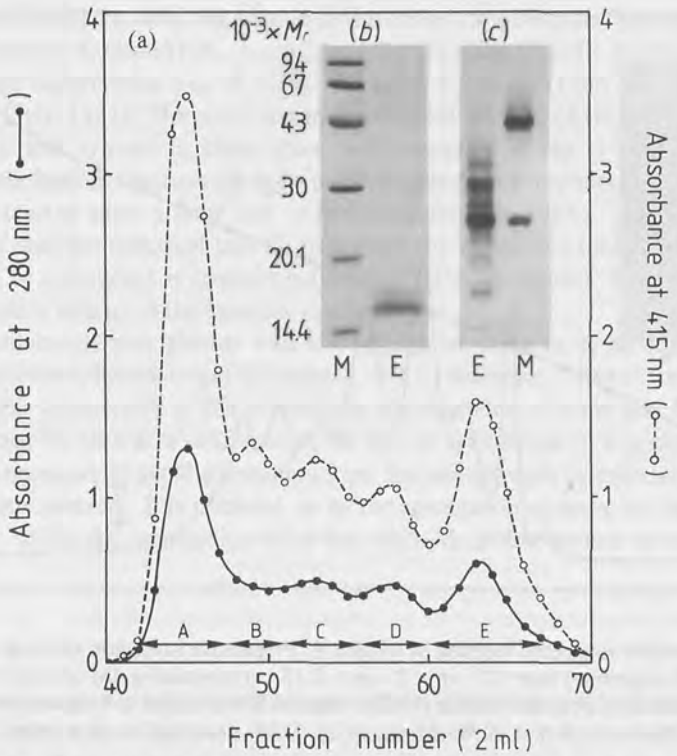

FIG. 1. Separation by gelfiltration and analysis on polyacrylamide gels. (a) Separation by gel filtration of the fragment mixture resulting from limited subtilisin digestion of intact *Artemia* haemoglobins. Digestion was performed at an enzyme-to-substrate ratio of 1:50 for 60 min at 25 °C. The fragments were separated by chromatography on a Sephacryl S-200 column equilibrated in mM-glycine/NaOH (pH 10.5)/2.6 mM-PMSF at a flow rate of 66 ml/h and the fractions pooled as indicated. (b) Analysis on SDS/15 %-polyacrylamide gel as described by Laemmli (1970) of fraction E. (c) Analysis on 7.5 %-polyacrylamide gel as described by Waring *et al.* (1970) of fraction E. M : M_r markers ; E : fraction E from (a) (Moens *et al.*, 1985 ; reprinted by permission from Biochemical Journal).

THE AMINO ACID SEQUENCE OF A STRUCTURAL UNIT

The amino acid sequence of the structural unit E_1 was determined from tryptic, chymotryptic, *S. aureus* V_8, and thermolytic peptides and the proposed sequence aligned with the human β chain (Fig. 3). Compared to the latter, there is an extension of six residues on the amino-terminus and a shortening with two residues on the carboxy-terminus. Most likely this is the result of a proteolytic cleavage of the E_1 structural unit from the intact globin chains. Two insertions (residues : 17-18 ; 81-84) and four deletions (residues 49 ; 97-101 ; 119 ; 124-125) are necessary to obtain maximal homology (38 residues = 26 %).

The alignment suggests that the helical segments, present in all other globin chains, are also present in the E_1 chain. There are three histidines. Residue 63 and 92 seem to be distal and proximal respectively histidine and thus the heme ligands. Histidine at position 35, which is also present in myoglobin and in *Glycera* haemoglobin may be a relict from a cytochrome b_5-like origin (Runnegar, 1984).

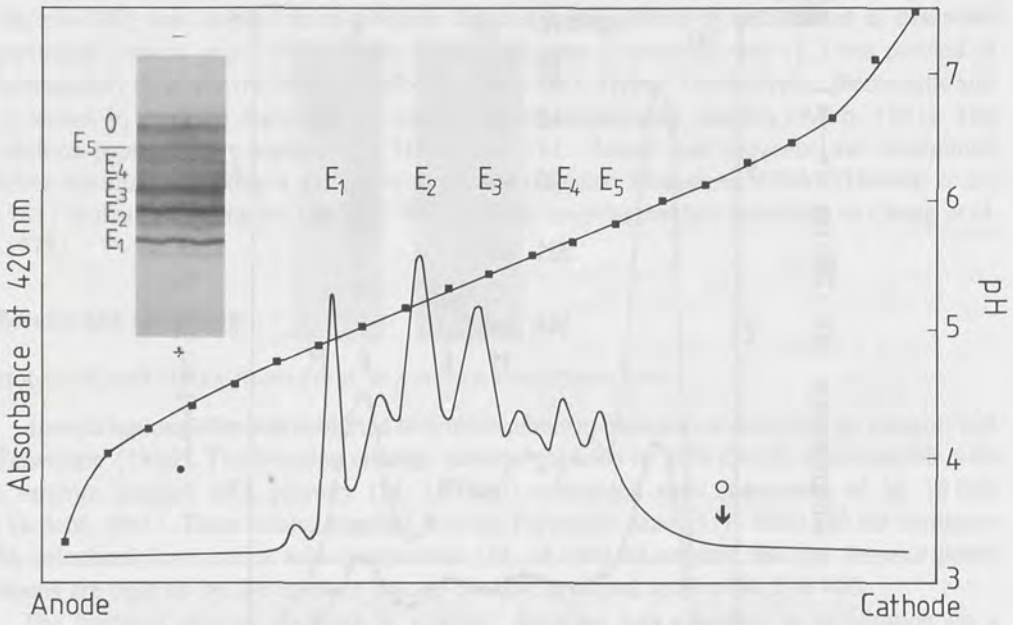

FIG. 2. Preparative isoelectric focusing of fraction E. Preparative isoelectric focusing was performed in a Sephadex G-75 (superfine) layer (21 cm × 25 cm × 0.15 cm) containing 4M-urea and 2 % Ampholines, pH 4-6.5, as described by Frey and Radola (1982). Fraction E was loaded at 5 mg/cm and focused initially at 3 W for 1 h, followed by 4 h at 10 W (Moens *et al.*, 1985 ; reprinted by permission from Biochemical Journal).

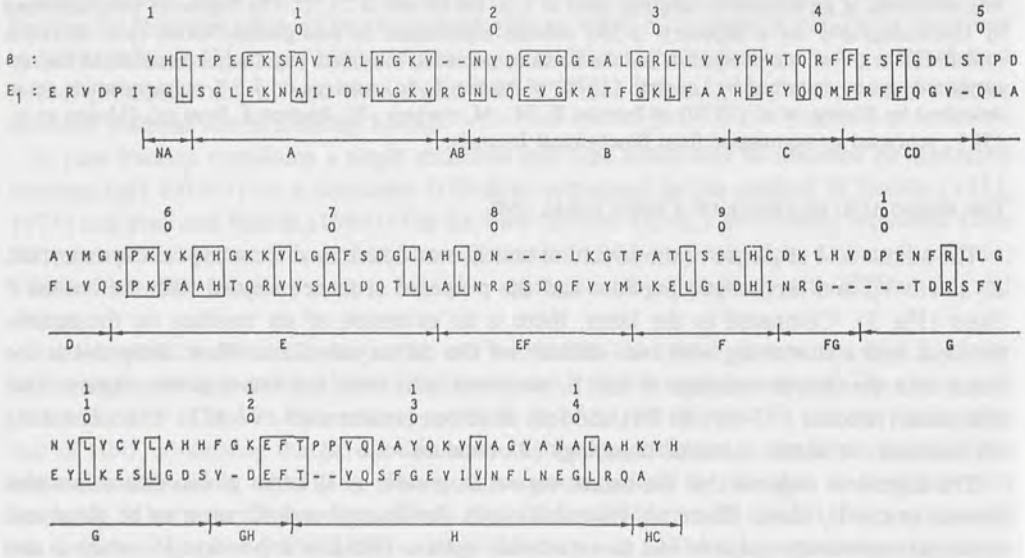

FIG. 3. Comparison of the proposed sequence of *Artemia* E_1 with that of the human β. Helix designations are those of the human β chains.

The majority of residues which are identic in vertebrate myoglobin and haemoglobin are also identic in the *Artemia* E_1 chain (NA_3, I_{12}, A_{14}, A_{15}, B_6, C_2, CD_1, CD_4, E_7, E_{11}, F_4, F_8, G_{16}, GH_5) or they are kept conservative (A_8, B_{10}, B_{12}, C_4, CD_7, E_8, FG_1) (Lesh and Chothia, 1980 ; Dickerson and Geis, 1983). Moreover, the hydrophobicity profiles (Kyte and Doolittle, 1982) of the human β and *Artemia* E_1 chain show, with exception of the G-helix, a good overall similarity. Protein families that have the same pattern of residue hydrophobicity along their amino acid sequences tend to share a same type of folding (Eisenberg, 1984).

This suggests that the structural unit E_1 may show the myoglobin fold. The *Artemia* globin chains can thus be considered as covalent polymers of eight "myoglobin" folded structural units which are definitely related to the classical globin family.

Vertebrate and invertebrate globins with low or high M_r seem to be all structurally related confirming their monophyletic origin (Goodman, 1981 ; Runnegar, 1984). The striking difference in molecular architecture of the invertebrate haemoglobins (Chung and Ellerton, 1979 ; Wood, 1980) may be seen as a difference in the way of aggregation of a common chain type. This high M_r is necessary to avoid elimination from the haemolymph by excretory processes and a too high oncotic pressure. It is obtained, as by the aggregation of many low M_r globin chains as in annelids, or by the aggregation of a few high M_r globin chains as in molluscs and arthropods.

Conclusions

The amino acid sequence of a structural unit (M_r 16 000) of the high M_r globin of *Artemia* (M_r 130 000) clearly shows that it is related in primary and possibly also in tertiary structure to the classical globin family.

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Cis- and *trans*-canthaxanthin levels in *Artemia* cysts of different geographical origin

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Abstract

The carotenoid composition of *Artemia* cysts from 17 geographical strains was studied. All-*trans*-canthaxanthin and *cis*-canthaxanthins were quantitatively determined using a non-aqueous reversed phase chromatographic method. In dehydrated cysts, the average *cis*- and *trans*-canthaxanthin levels (± SD) were 152 ± 52 and 134 ± 42 µg/g, respectively (range 38-227 and 67-201 µg/g). The corresponding concentrations in hydrated cysts were (*cis*) 177 ± 60 µg/g (range 47-271 µg/g) and (*trans*) 112 ± 35 µg/g (range 52-169 µg/g). The *cis/trans* concentration ratios averaged 1.13 (range 0.57-1.47) and 1.60 (range 0.84-2.54) for dehydrated and hydrated cysts, respectively. Differences in total canthaxanthin content in *Artemia* cysts may partly reflect environmental variability (*e.g.* algal supply), whereas the divergence in the relative abundance of *cis*- and *trans*-canthaxanthin may be associated with differences in some physical and biological characteristics of the cysts.

Introduction

All-*trans*-canthaxanthin has been generally considered to be the predominant carotenoid in *Artemia* cysts (Krinsky, 1965 ; Czygan, 1968 ; Hata and Hata, 1969 ; Davies *et al.*, 1970 ; Hsu *et al.*, 1970 ; Soejima *et al.*, 1980). Recently it was, however, demonstrated that a major part of this pigment in fact occurs in a mono-*cis*-configuration (Nelis *et al.*, 1984a). The specific localization of *cis*-canthaxanthins in the eggs and the reproductive system of female animals, their virtual absence in males as well as their disappearance in developing nauplii and their reappearance at the onset of reproduction, have raised questions about the role of these unusual pigments. Some recent evidence (Nelis *et al.*, 1984b) may suggest a possible function in connection with the cryptobiotic process in the cysts. If so, some of the biological variability (with regard to *e.g.* hatching) between cysts of different geographical origin might be partly associated with differences in their carotenoid composition.

Soejima *et al.* (1980) were the first to conduct a comparative study of the canthaxanthin content of cysts from eight geographical strains. Their analytical method did, however, not differentiate between all-*trans* and *cis*-canthaxanthins. Furthermore, the degree of hydration of cysts, which has been shown to significantly affect the relative *cis/trans* abundance (Nelis *et al.*, 1984b), was not taken into account.

The non-aqueous reversed phase liquid chromatographic system reported by Nelis *et al.* (1984a, 1986) permits the separation of all-*trans* and *cis*-canthaxanthins. Using this approach, both isomers were quantitatively determined in dehydrated as well as fully hydrated cysts from 17 geographical strains. The potential value of pigment levels as a biochemical criterion for the characterization of *Artemia* cysts of different geographical origin is presently under investigation.

Materials and methods

Cysts

All *Artemia* cysts came from the collection at the Artemia Reference Center (State University of Ghent, Belgium). Details on the 17 strains (18 batches) studied, and their respective batch numbers are given in Table I. Samples were extra purified using the bipartial flotation technique with brine and freshwater (Sorgeloos *et al.*, 1985). Dehydrated cysts were prepared by drying 10 mg samples in an oven at 40 °C for 24 h followed by cooling in a desiccator. Hydration was achieved by soaking dehydrated cysts in 35 ‰ seawater for 5 h at room temperature, using small plastic filter cups (Beckman). For the determination of individual dry weights, three 200 mg samples of each strain were dried in an oven at 60 °C for 48 h.

Table I

Geographical strains studied

Strain	Origin	Abbreviation	Batch no.	Year
Artemia franciscana				
Manaure	Colombia	MAN	2[a]	1983
Macau	Brasil	MAC1	200[a]	1978
Macau	Brasil	MAC2	971051	1979
Chaplin Lake	Canada	CHL	241[a]	1979
San Francisco Bay	USA	SFB	288-2596	1976
San Pablo Bay	USA	SPB	1628	1978
Great Salt Lake	USA	GSL	285[a]	1978
Artemia Ref. Center	–	RAC	–	1979
Artemia parthenogenetica				
Shark Bay	Australia	SB	299[a]	1980
Tientsin	P.R. China	TIE	242[a]	1978
Lavalduc	France	LVD	–	1980
Tuticorin	India	TUT	215[a]	1978
Izmir	Turkey	IZM	209[a]	1979
Altai-Siberia	USSR	SIB	–	1983
Artemia persimilis				
Buenos Aires	Argentina	BA	223[a]	1979
Artemia urmiana				
Lake Urmia	Iran	URM	338[a]	1982
Artemia tunisiana				
Larnaca	Cyprus	LAR	305[a]	1980
Sfax	Tunisia	SFA	362[a]	1983

[a] Numbers refer to the collection at the Artemia Reference Center.

TABLE II

All-*trans* and *Cis*-canthaxanthin levels (mean values, n = 3) in cysts from 17 geographical strains (18 batches) of *Artemia*

Strain	Dehydration				Hydration			
	Trans-ca (μg/g)	*Cis*-ca (μg/g)	Total (μg/g)	*Cis/trans* ratio	*Trans*-ca μg/g	*Cis*-ca (μg/g)	Total (μg/g)	*Cis/trans* ratio
URM	67	38	105	0.57	56	47	103	0.84
SPB	152	135	287	0.89	118	171	289	1.45
TIE	116	106	222	0.91	108	117	225	1.08
MACl	201	191	392	0.95	162	231	393	1.43
GSL	136	130	266	0.96	117	154	271	1.32
MAN	83	86	169	1.04	69	99	168	1.44
MAC2	176	194	370	1.10	144	228	372	1.58
RAC	193	214	407	1.11	169	261	430	1.54
CHL	124	137	261	1.11	92	160	252	1.74
SB	161	183	344	1.14	141	197	338	1.40
LVD	188	216	404	1.15	154	238	392	1.55
LAR	113	137	250	1.21	94	162	256	1.72
SFA	96	124	220	1.29	84	141	225	1.68
IZM	147	197	344	1.34	123	222	345	1.81
TUT	134	184	318	1.37	108	216	324	2.00
SIB	165	227	392	1.38	137	271	408	1.98
SFB	73	103	176	1.41	52	132	184	2.54
BA	89	131	220	1.47	81	139	220	1.76
Mean (SD)	134(42)	152(52)	286(91)	1.13(0.23)	112(35)	177(60)	289(92)	1.60(0.37)

CAROTENOID DETERMINATION

Details on the analytical procedure are described elsewhere (Nelis *et al.*, in prep.). Basically, all-*trans*-, *cis*-canthaxanthin as well as an internal standard, *i.e.* β-apo-8'-carotenal or β-apo-8'-carotenoic acid ethyl ester are separated by non-aqueous reversed phase chromatography, as reported previously (Nelis *et al.*, 1984a, 1985). The chromatographic system is combined with a simple sample pre-treatment including extraction of cysts (10 mg) with methanol-formic acid (94:6, v/v) (6 ml), neutralization of acid with saturated potassium carbonate (1.5 ml) and dilution with acetonitrile (3 ml). After centrifugation, a 100-µl aliquot of the supernatant is injected into the liquid chromatograph. Calibration is performed by injecting known amounts of the compounds of interest and the internal standard, and plotting the peak height ratios versus the concentrations. Unknown concentrations are determined from the calibration graph. All results are expressed as µg pigment/g dry weight.

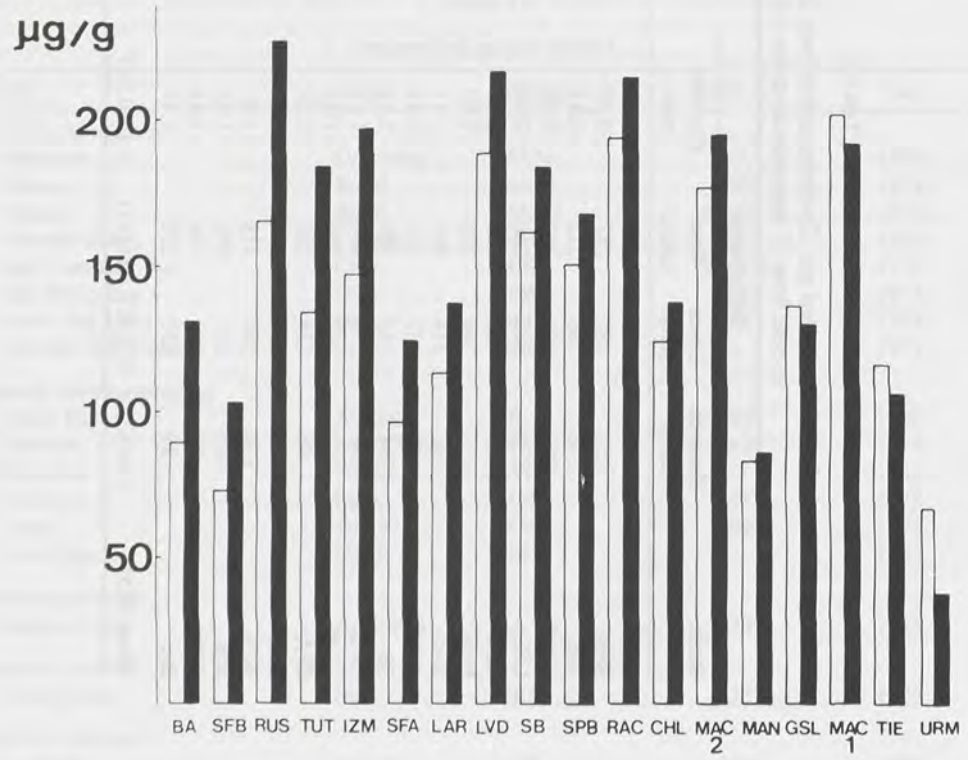

FIG. 1. *Trans*-(□) and *cis*-(■) canthaxanthin levels (µg/g dry weight) in dehydrated cysts of different geographical origin. Abbreviations are explained in Table I.

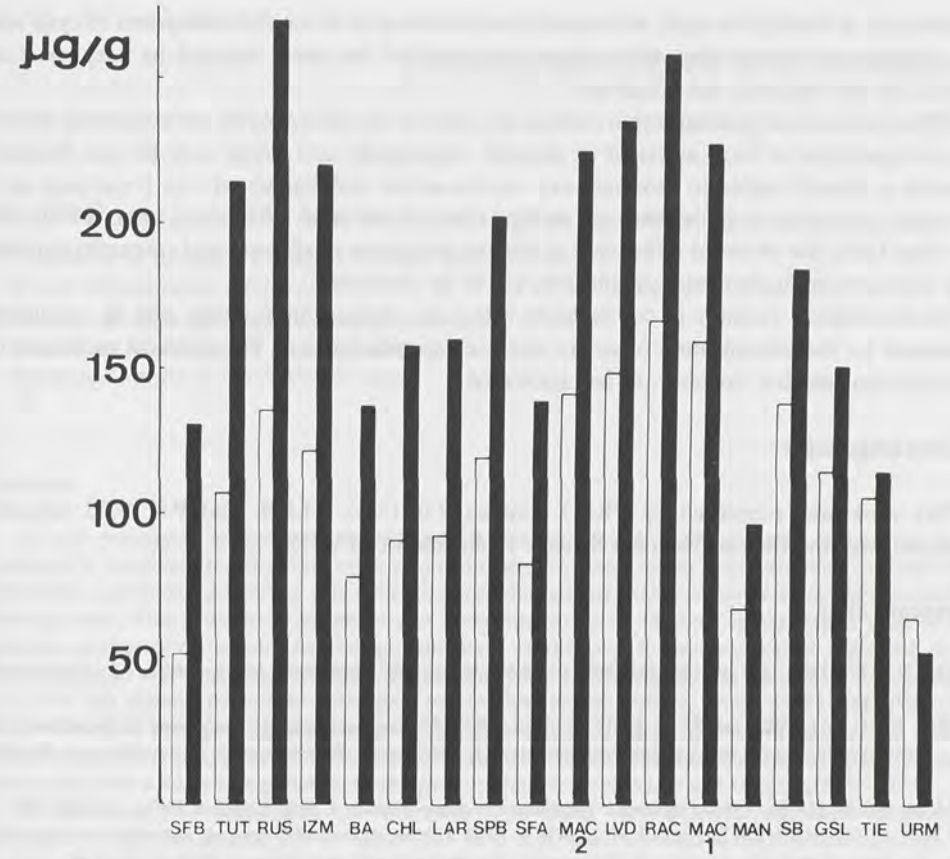

FIG. 2. *Trans*-(□) and *cis*-(■)-canthaxanthin levels (µg/g dry weight) in hydrated cysts of different geographical origin. Abbreviations are explained in Table I.

Results and discussion

The experimental all-*trans* and *cis*-canthaxanthin levels in dehydrated and fully hydrated cysts from 17 geographical strains (18 batches) of *Artemia* are summarized in Table II. The previously observed reversible *cis/trans* conversion associated with hydration/dehydration (Nelis *et al.* 1984b) was confirmed in all strains studied, though to different extents. Upon hydration the *cis*-canthaxanthin concentration increases by approximately 28 % in San Francisco Bay cysts, whereas in Argentinian cysts the corresponding increase is only 6 %. The wide divergence in pigment levels between the different strains becomes evident from a visual representation of the data obtained (Fig. 1 and 2). Soejima *et al.* (1980) observed comparable differences in canthaxanthin content of cysts from eight geographical strains, although their values tend to be somewhat higher. This is obviously due to the lower specificity of their analytical technique, which fails to differentiate between all-*trans* and *cis*-canthaxanthins. Echinenone, the direct precursor of canthaxanthin, reportedly constituted only 1-5 % of the total carotenoid content of cysts. Non-aqueous reversed phase readily separates echinenone from all-*trans* and *cis*-can-

thaxanthins. Although the small echinenone peak showing up in the chromatograms of cysts was not systematically quantitated, the order of magnitude of the levels reported by Soejima *et al.* (1980) for this pigment was confirmed.

Differences in total canthaxanthin content are likely to be influenced by environmental factors (*e.g.* composition of food available to parental population) and hence may be less relevant. *Artemia* is indeed unable to biosynthesize canthaxanthin and has to rely on β-carotene as a precursor, commonly supplied through an algal diet (Davies *et al.*, 1970 ; Hsu *et al.*, 1970). On the other hand, the observed differences in relative abundance of all-*trans* and *cis*-canthaxanthins may have a more fundamental significance, yet to be elucidated.

Cis/trans ratios, possibly in combination with total canthaxanthin levels, may be a valuable parameter for the identification of strains and even specific batches. The practical usefulness of this criterion remains, however, to be established.

Acknowledgements

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The degradation of yolk in *Artemia*

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Abstract

An acid proteinase, characterized as cathepsin B, has been found in *Artemia* cysts and nauplii and is localized in lysosomes together with other acid hydrolases. However, when cysts were homogenized under conditions known to preserve the yolk granules intact, the acid proteinase activity was mainly associated to yolk granules. This association suggested that lysosomes could be involved in the degradation of yolk granules during development. Accordingly the level of the acid hydrolases studied increased during development to reach maximum activity after hatching, when yolk degradation is maximal. The degradation *in vitro* of yolk granules by purified cathepsin B occurs with a specific pattern, similar to the pattern occurring *in vivo* described previously by other authors. The lysosomotropic agents inhibit yolk degradation and can inhibit the progress of development. These results suggested that yolk degradation depends on the lysosomal system and that a correct degradation of the yolk is required for development to proceed.

The process of yolk degradation has been analyzed in detail. Three different populations of lysosomal structures of densities ranging from > 1.3 to 1.18 have been found involved in the degradation of the yolk granule. The electronmicroscopic observation of these structures indicates that the degradation process starts by the fusion of primary lysosomes at the periphery of the yolk granule, that it continues in the resulting phagosome, and probably ends by the exit of the degradation products to the cytosol.

In the presence of a lysosomotropic agent, the phagosomal degradation structures of high density are absent. In parallel, increased lysosomal enzyme activity is found at a lower density. These observations suggested that the phagosomal structures were more fragile when the embryos have been cultured in the presence of the lysosomotropic agent. ATP appears to be involved in the stabilization of the degradation structures and may be involved in the regulation of yolk degradation.

Introduction

Before hatching of the swimming larvae, the dormant gastrula of *Artemia* experiences dramatic morphogenetic changes in the absence of cell division (Clegg and Conte, 1980). The first steps of this developmental program take place in the absence of nutrient supply. The components necessary for the intense synthetic processes are derived from the degradation of maternal reserve sources. As in other embryonic systems, yolk granules in *Artemia* are the major cell component of the dormant cyst and contain at least 80 % of the total protein of the animal (Vallejo *et al.*, 1981). In spite of the fact that yolk granules have been known morphologically for long, the biochemical mechanisms involved in their degradation are unknown. Probably these studies have been handicapped by the fragile stability of these embryonic organelles. We have developed a

procedure that allows the isolation of yolk granules with a good recovery and in a quite intact state (Vallejo *et al.*, 1981). From ultracytomicroscopic evidence it has been suggested that acid hydrolases could be involved in the degradation of yolk although biochemical data were lacking. For example, acid phosphatase has been detected in the yolk of *Drosophila melanogaster* (Sawicki and MacIntyre, 1978) and lysosomes observed in the unfertilized egg of *Xenopus* (Decroly *et al.*, 1979).

In *Artemia*, we have found both by biochemical and electron-microscopic approaches that lysosomes are associated to yolk granules in different stages of degradation. The use of both approaches has allowed to establish that yolk degradation in *Artemia* occurs through the lysosomal system.

Materials and methods

Cysts from San Francisco Bay (San Francisco Bay Brand, Newark, CA 94560, USA) were used. Chemicals were of analytical grade.

Culture, handling, homogenization, and fractionation

Culture and handling of cysts and nauplii were carried out as previously described (Vallejo *et al.*, 1979). Cysts were subjected to dechorionization (Vallejo *et al.*, 1981) prior to homogenization and/or incubation. Homogenization was carried out in "Ficoll medium" (3 volumes/g for cysts and 1 volume/g for nauplii) composed of 0.3 M sucrose, 15 % Ficoll 400 (Pharmacia), 25 mM Hepes buffer, 60 mM potassium chloride, 15 mM sodium chloride, 5 mM magnesium chloride, 0.5 mM calcium chloride, 1 mM sodium borate, and 100 µg/ml soybean trypsin inhibitor adjusted to pH 7.5. To obtain lysosomes, the homogenization of cysts was carried out in 20 volumes/g and that of nauplii in 1 volume of "sucrose medium" composed of 0.68M sucrose, 25 mM Hepes buffer, 60 mM potassium chloride, 15 mM sodium chloride, 5 mM magnesium chloride, 0.5 mM calcium chloride, 1 mM sodium borate, and 100 µg/ml soybean trypsin inhibitor adjusted to pH 7.5. Subcellular fractionation was performed as described by Vallejo *et al.* (1979).

Gradients

Sucrose isopycnic gradients (0.9 M-2.0 M) were spinned at 26 000 rpm for 12 h in a SW27 rotor. The sucrose solutions contained 5 mM EDTA and 100 µg/ml of soybean trypsin inhibitor. The density was determined with a refractometer at 20 °C.

Release of the latency of acid hydrolases in lysosomes

The acid hydrolases activities were released from the mitochondrial-lysosomal fraction by resuspension in 10 volumes of the buffer 15 mM sodium phosphate pH 7.0, 25 mM KCl, 20 % glycerol, 5 mM 2-mercaptoethanol, and 1 % Triton X-100, and incubation at 4 °C for 2 h. The fraction was then centrifuged at 27 000 × *g* for 30 min and the acid hydrolases were found in the supernatant.

When the acid hydrolases were to be assayed in the fractions of sucrose gradients, latency was released by five successive cycles of freezing and thawing.

Solubilization of cathepsin B from the yolk granules fraction

Cathepsin B was solubilized from the yolk granules fraction (500 × *g*, 1 min) obtained in «Ficoll medium» by three different solubilization media. The «Monol medium» (Vallejo *et al.*, 1981) contained 0.2 M Monol, 10 mM sodium citrate, and dissolved about 100 % of the protein of the fraction. The fraction was resuspended (5 mg protein/ml), shaken in the cold for 1 h, and centrifuged at 7 500 × *g* for 30 min.

The "Ballario *et al.* medium" is a modification of the medium described by Ballario *et al.* (1978), and contains 40 % glycerol, 0.2 M KCl, 0.2 M sodium citrate, and 2 mM dithiothreitol pH 7.0. The fraction was resuspended, sonicated and centrifuged at 27 000 × *g* for 30 min. The Triton X-100 containing medium has been described above.

Acid hydrolases assays

Acid hydrolases assays were carried out as previously described (Perona and Vallejo, 1985).

Proteinase activities assays

Proteinase activities assays were carried out by the fluorescamine-based method previously described (Garesse *et al.*, 1979) with protamine (2 mg/ml) as substrate and at pH 3.5 for cathepsin B or pH 7.5, for trypsin-like proteinase with 50 mM acetate or phosphate buffer, respectively.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) in the crude fractions and by the method of Bradford (1976) in the more purified fractions as previously described (Perona and Vallejo, 1982).

Electron microscopy

Samples were prepared for electron microscopy essentially as described previously (Vallejo *et al.*, 1979) and were incubated in Gomori's medium (Beck *et al.*, 1971) to detect the presence of acid phosphatase. In every case, a control without substrate was carried out.

SDS-polyacrylamide gel electrophoresis

Electrophoresis was carried out as previously described (Perona and Vallejo, 1985).

Results

Subcellular localization of cathepsin B-like proteinase in *Artemia*

We have reported that the acid proteinase of *Artemia*, which represents the highest proteolytic activity in the cyst, increases its activity upon resumption of development, reaching a maximum (Perona and Vallejo, 1985) before major degradation of yolk granules. The acid proteinase has been purified from cysts (Perona and Vallejo, 1982) and nauplii (Perona and Vallejo, 1985) and characterized as a cathepsin B-like proteinase. An acid proteinase also characterized as cathepsin

B has been isolated from cysts (Warner and Shridhar, 1980 ; Nagainis and Warner, 1979) and considered to be a cytosol protein. In our experiments, the proteinase was particulate but the percentage and the subcellular fraction to which the proteinase was associated, depended very much on the conditions of homogenization and the composition of the buffer used. We have reported (Perona and Vallejo, 1985) that the cathepsin from cysts sedimented together with the lysosomal marker acid phosphatase, somewhat dispersed by but mostly in the mitochondrial fraction that is known to contain also the lysosomes in other systems (Beaufay, 1972).

We have observed that in nauplii cathepsin B sediments in the same particulate fraction (Table I), when isolated with the same sucrose-containing buffer. Nevertheless, when cysts were homogenized under conditions previously described, to preserve the yolk granules of *Artemia* (Vallejo *et al.*, 1981), most of the particulate activity was found in the heavy fraction mainly composed of yolk granules (Vallejo *et al.*, 1979). These conditions include dechorionization of the cysts to diminish the abrasive effect of the chorion on subcellular organelles, a concentrated homogenate and the use of a Ficoll – containing buffer. However, cathepsin B activity was found in the cytosol in varying proportions up to 50 %. The finding of a trypsin-like proteinase in the yolk granules of cysts (Ezquieta and Vallejo, 1985) suggested that the variable percentage of soluble cathepsin B could be an artifact of isolation related to the presence of this proteolytic activity. In fact the inclusion of soybean trypsin inhibitor (STI) in the homogenization buffer resulted in the obtention of about 95 % of the catheptic activity localized in the particulate fraction. The protective effect of STI can be seen in Fig. 1 where a cysts homogenate obtained in "Ficoll medium" plus STI, was run through a sucrose isopycnic gradient in the presence or absence of STI. In the presence of STI, most cathepsin B activity sedimented to the bottom of the gradient where yolk granules are found (Vallejo *et al.*, 1981).

TABLE I

Subcellular distribution of cathepsin B in Artemia nauplii

Fraction	Cathepsin B	Acid phosphatase	Cytochrome oxidase
500 g, 10 min	18	9	24
7 500 g, 20 min	72	67	70
150 000 g, 75 min	8	19	6
Cytosol	2	5	0

Nauplii were hand-homogenized (1:20 w/v) in "sucrose medium" (see Materials and methods) and the subcellular fractions obtained by centrifugation as indicated. Latency was released from the fractions as described in Materials and methods before determination of cathepsin B and the lysosomal marker acid phosphatase.

Another sample of the homogenate was run in parallel in the absence of STI. Only about 20 % of the activity sedimented at the bottom and the rest appeared dispersed all through the gradient. When the homogenate was obtained in the absence of STI, the activity was lower (about a 3-fold) indicating degradation. The remaining activity was practically only found in the soluble part of the gradient (not shown). The results altogether suggested that the acid proteinase was latent in the particulate fraction and that trypsin-like proteinase was partially releasing the latency during

extraction. In order to solubilize the cathepsin B activity from the particulate fraction, we used different solubilization media and the results are presented in Table II. The Monol-containing medium completely solubilizes the protein (Vallejo *et al.*, 1981) but inactivates the catheptic activity for 90 %. The "Ballario *et al.* medium" solubilizes the majority of protein with a 120 % recovery of cathepsin B. With Triton X-100, much less protein is solubilized and cathepsin B is recovered with about a 400 % yield. This finding clearly indicated that cathepsin B is latent and that Triton X-100 better releases the latency supporting that *Artemia* cathepsin B has a lysosomal location as in mammalian systems (Barrett, 1977 ; Barrett and Kirschke, 1981). In Fig. 2, the protein solubilized by the modification of the Ballario *et al.* (1978) medium and by Triton X-100 has been analyzed by SDS-PAGE. The "Ballario *et al.* medium" solubilizes protein with a pattern typical of lipovitellin, indicating a general solubilization of the particulate fraction. The Monol medium also solubilizes protein with a similar pattern (Vallejo *et al.*, 1981). On the contrary, Triton X-100 solubilizes protein more specifically and no pattern of lipovitellin is evident.

The difference in subcellular localization observed when using "sucrose" *versus* "Ficoll" homogenization buffer appears to depend upon the degree of stabilization of yolk granules (Marco *et al.*, 1981 ; Vallejo *et al.*, 1981). The results were interpreted as cathepsin B being a lysosomal enzyme, but associated to yolk granules probably through lysosomal involvement in yolk degradation.

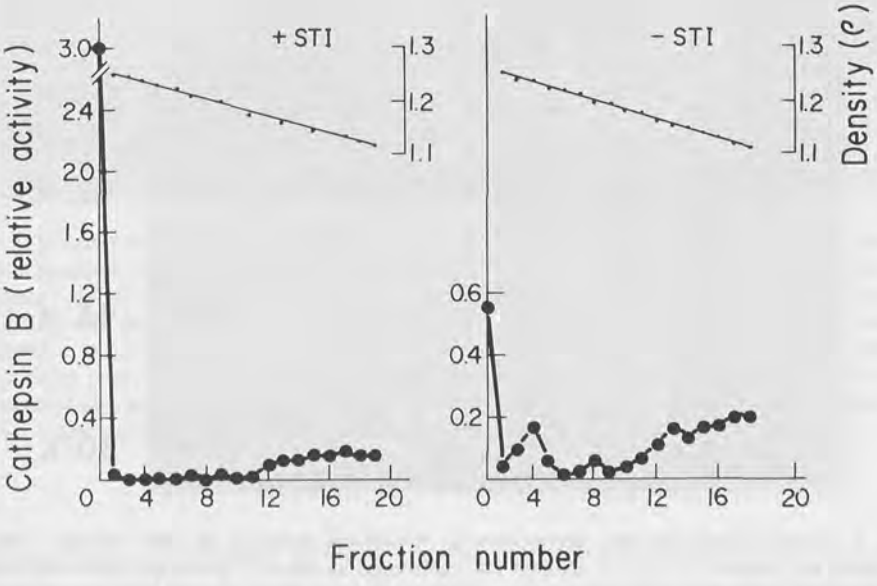

FIG. 1. Protective effect of soybean trypsin inhibitor in the sedimentation of *Artemia* cathepsin B-like proteinase. A homogenate of nauplii (5 h-old) was obtained in "Ficoll medium" and analyzed by a sucrose isopycnic gradient in the presence (a) or absence (b) of STI as indicated in Materials and methods. In the fractions, the refraction index was measured at 20 °C. Cathepsin-B like activity was determined as indicated in Materials and methods.

TABLE II

Effect of different media on the solubilization of cathepsin B

Media	Cathepsin B (% activity solubilized)	Specific activity (U/mg protein)	% Protein solubilized	Trypsin-like proteinase (% activity solubilized)
Monol (Vallejo *et al.*, 1981)	10	0.2	100	–
Ballario *et al.* (1978)	120	4	64	50
Triton X-100	392	500	29	< 0.1

The particulate fraction of a cysts' homogenate obtained in "Ficoll medium" by centrifuging at 150 000 × *g* for 75 min was solubilized by a Monol-containing medium (Vallejo *et al.*, 1981) or by a complex medium described by Ballario *et al.* (1978) and modified. The solubilization with the non-ionic detergent Triton X-100 was carried out as described in Materials and methods.

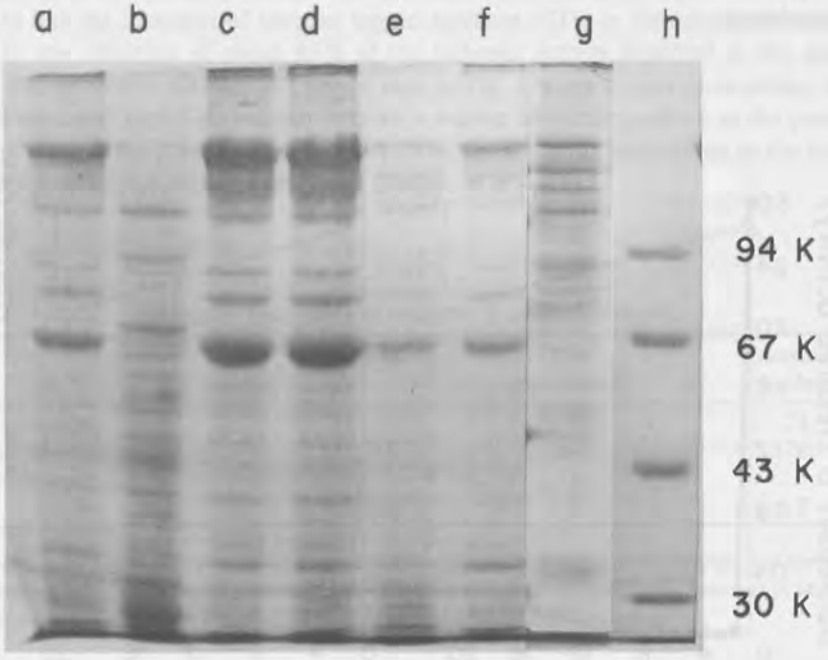

FIG. 2. SDS-polyacrylamide gel electrophoresis of different fractions of cysts extracts. The cysts homogenate was obtained in «Ficoll medium» and centrifuged to obtain a cytosol and particulate fraction. (a) is the pattern observed for the homogenate, (b) for the cytosol, and (c) for the particulate fraction. An aliquot of the particulate fraction was treated with the Ballario *et al.* (1978) medium (Materials and methods) and analyzed (d). After centrifugation, the solubilized material (e) and the remaining particulate material (f) were analyzed. Another aliquot of the particulate fraction was treated with the Triton X-100 medium and the solubilized material analyzed (g). In (h) the molecular weights of the protein markers are indicated. Details are given in Materials and methods.

We ran the mitochondrial-lysosomal fraction obtained in "sucrose medium", through a sucrose isopycnic gradient and determined the activity of seven lysosomal enzymes, in addition to cathepsin B, in the fractions of the gradient (Perona and Vallejo, 1985). All the enzymatic activities migrated as a sharp peak at a density 1.18 which is the density described for lysosomes in other eukaryotic systems (Beaufay, 1972). The fractions comprising the acid hydrolytic activities were pooled and stained for the lysosomal marker acid phosphatase by the method of Gomori (Beck *et al.*, 1971). Electronmicroscopy observations of the stained fractions, obtained from cysts, showed acid phosphatase in vesicles (diameter $\leqq 0.4$ μm) with a morphology resembling primary lysosomes in other systems (Fig. 3). The existence of lysosomes in *Artemia* had not been reported previously. When the fractions obtained from nauplii were analyzed, few primary lysosomes were observed (Fig. 4). Instead, phagosomal degradation structures were found. The difference in morphology of the acid phosphatase-containing structures suggested the occurrence of an intense degradation activity in nauplii. The levels of the eight lysosomal hydrolases studied have been observed to vary with development and to reach a maximum after hatching (Perona and Vallejo, 1985).

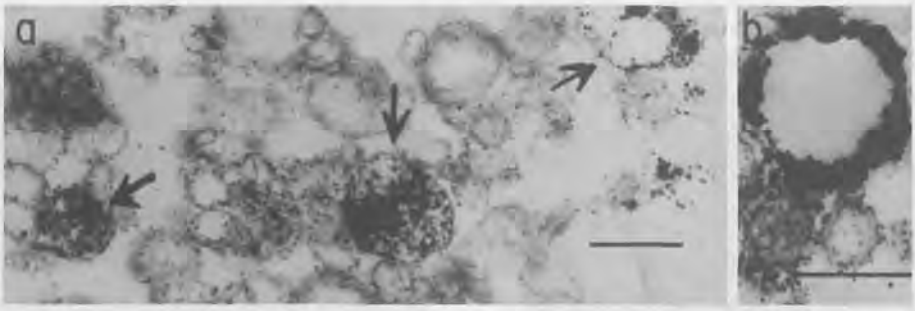

FIG. 3. Electron microscopy of the fractions from cysts that contained acid hydrolase activities in sucrose isopycnic gradients. The mitochondrial-lysosomal fraction (7 500 × *g* for 30 min) was obtained from a cysts homogenate in "sucrose medium" as indicated in Materials and methods. The fraction was analyzed by a sucrose isopycnic gradient. The fractions from the gradient that contained acid hydrolase activities and sedimented at a density of 1.18 were pooled and stained for acid phosphatase activity by the method of Gomori (Beck *et al.*, 1971) and analyzed by electron microscopy. (a) represents a general overview where small vesicles with acid phosphatase activity (arrows) can be observed. (b) is a detail of a stained vesicle at higher magnification. The bar represents 0.33 μm.

The association of lysosomes with yolk granules suggested that lysosomes could be involved in the degradation of yolk in *Artemia*. In this regard, acid hydrolases belonging to the metabolism of carbohydrates, lipids, nucleotides, nucleic acids as well as proteins, have been found to compose the enzymatic endowment of *Artemia* lysosomes (Perona and Vallejo, 1985). This suggests that they could be able to degrade not only the protein but also the other components of *Artemia* yolk (De Chaffoy de Courcelles and Kondo, 1980).

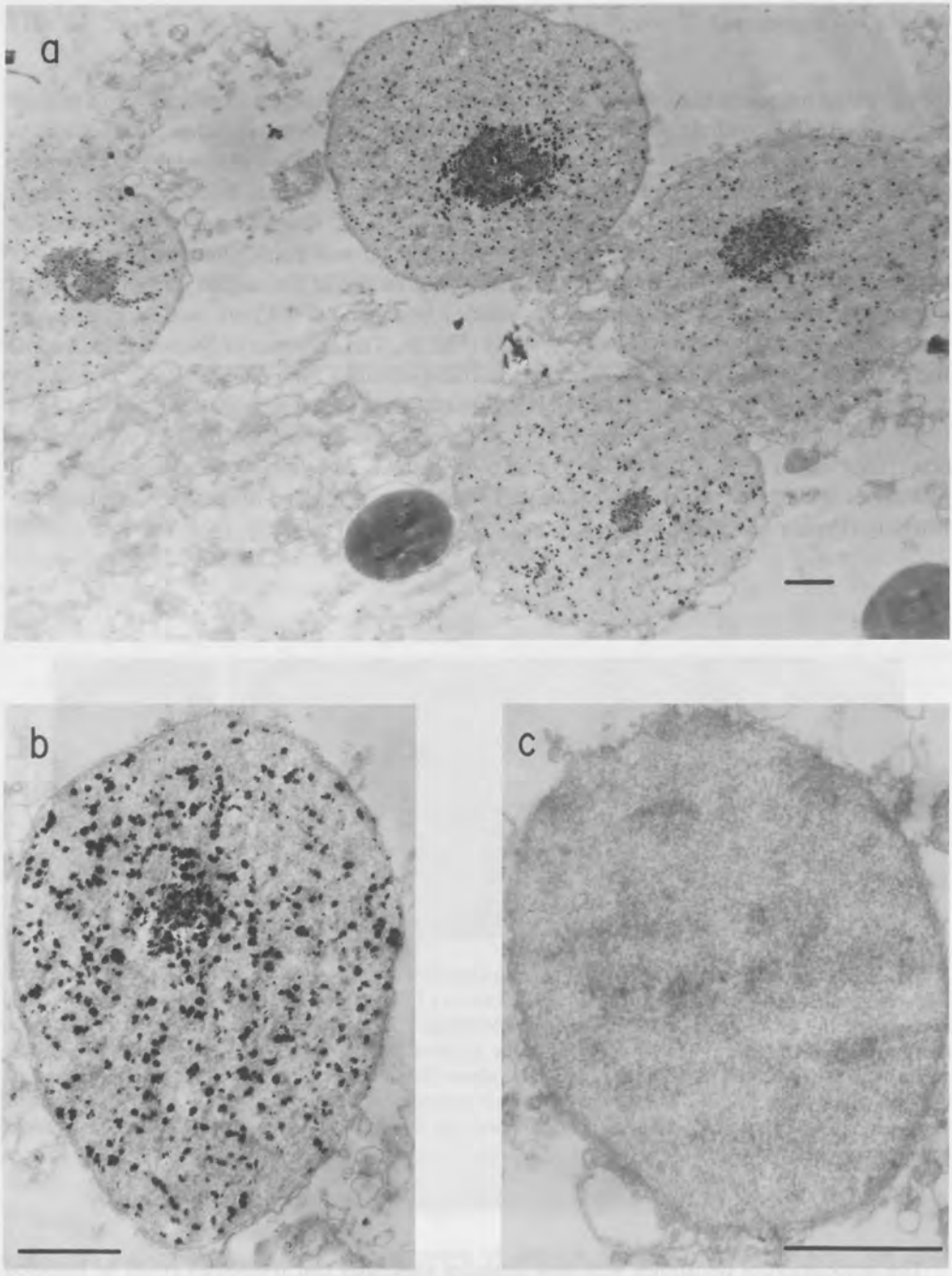

FIG. 4. Electron microscopy of the fractions from nauplii that contained acid hydrolase activities in sucrose isopycnic gradients. The mitochondrial-lysosomal fraction was obtained from a homogenate of nauplii (30 h old) in «sucrose medium» as indicated in Materials and methods and run by a sucrose isopycnic gradient. The active fractions of the gradient were pooled, stained for acid phosphatase activity, and analyzed by electron microscope as in Fig. 3. (a) represents a general overview where doublemembrane, acid phosphatase-containing structures can be observed. (b) is a detail of a stained structure at higher magnification. (c) is a detail of a structure from a preparation that has been incubated in the absence of the substrate for acid phosphatase. The bar represents 1 μm.

CATHEPSIN B DEGRADES SPECIFICALLY THE YOLK GRANULES OF *ARTEMIA*

Cathepsin B has been purified from cysts as well as nauplii to homogeneity (Perona and Vallejo, 1982 ; Perona and Vallejo, 1985). The characterization of the proteinase activity from both stages as a cathepsin B-like proteinase indicated that it was due to the same enzyme. *Artemia* cathepsin B has a molecular weight 68 000, both by denaturing gels and gelfiltration chromatography, indicating that the proteinase is made up of a single polypeptide chain. The proteinase is a glycoprotein, which is in accordance with the suggested lysosomal localization of *Artemia* cathepsin B.

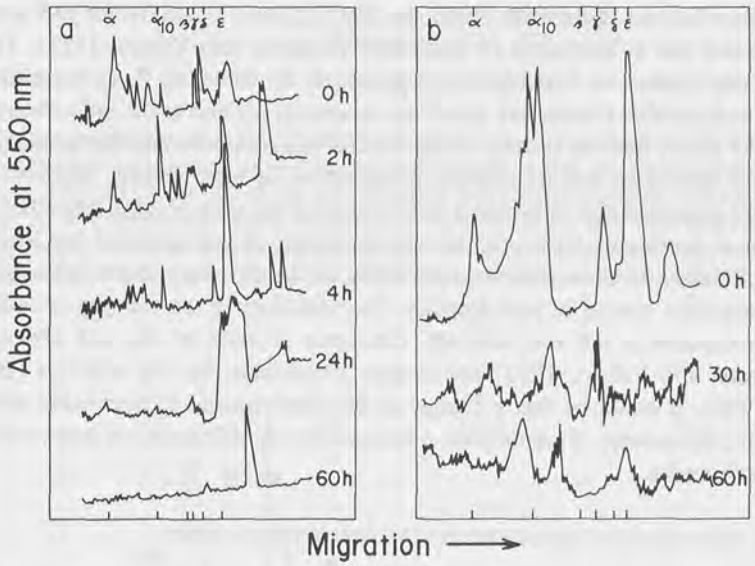

FIG. 5. Degradation pattern of *Artemia* lipovitellin by the acid proteinase of *Artemia* and the trypsin-like proteinase of *Artemia*. (a) A purified yolk granules preparation from cysts was obtained as described previously (Vallejo *et al.*, 1981). An aliquot containing 0.6 mg protein was incubated with 11 units of purified cathepsin B proteinase (Perona and Vallejo, 1982) in 50 mM sodium acetate buffer pH 5.0 and 5 mM 2-mercaptoethanol, at a final volume of 3.5 ml. The incubation was accomplished at 37 °C with agitation and the reaction was stopped at 0 h, 2 h, 4 h, 24 h, and 60 h and their products precipitated by adding tricloroacetic acid up to 10 %. A control without enzyme was carried out in parallel. The samples were processed for analysis in denaturing polyacrylamide disc gels (5.5 %. the gels were stained and their scanning at 550 nm is shown. The time of incubation corresponding to each scanning is indicated. No difference in pattern was found between the control and the 0 h incubation. α1-α10, β, γ, δ, and ε indicate the positions for the corresponding lipovitellin polipeptides. The molecular weight of the apovitellins is α (190 000), α-10 (120 000), β (100 000), γ (90 000), δ (85 000), and ε (68 000). (b) An aliquot of yolk granules obtained as in (a) and containing 2 mg protein as incubated with 2 units of partially purified *Artemia* trypsin-like proteinase (Ezquieta and Vallejo, 1987) in 50 mM sodium phosphate buffer pH 7.5 in a final volume of 3.5 ml. A control without enzyme was also incubated. Incubations were performed as in (a) for 0 h, 30 h, and 60 h. The products of the reaction were also analyzed in SDS disc gels and the scannings are shown. No difference in pattern was found between the control and the 0 h incubation.

De Chaffoy de Courcelles and Kondo (1980) have published the pattern of degradation *in vivo* of lipovitellin. In a first attempt to understand the involvement of lysosomes in the degradation of yolk, we carried out an *in vitro* assay with purified yolk granules (Vallejo *et al.*, 1981) and purified cathepsin B from cysts. The incubation was carried out at acid pH and in the enzyme/substrate ratio calculated to be the *in vivo* one in cysts (Perona and Vallejo, 1982 ; Vallejo *et al.*, 1981). The products obtained at different times of incubation were analyzed by SDS-PAGE. The degradation pattern is shown in Fig. 5a. At short incubation times there is a preferential degradation of α subunit giving a series of products until α_{10}, as well as degradation products of δ, β, and γ subunits. Subsequently, the α_{10} subunit is progressively degraded to products of 68 000-70 000 M_r that tend to accumulate. Finally, by 60 h incubation, a band of M_r 40 000-50 000 is observed. This sequence of intermediates of degradation is similar to the one described *in vivo*, suggesting that cathepsin B could be involved in the degradation *in vivo*. However, we have found a trypsin-like proteinase that in cysts is located in the yolk granules and very inhibited and that is associated to lipovitellin (Ezquieta and Vallejo, 1985). To check if the pattern of degradation we have observed was specific for cathepsin B, we incubated purified yolk granules with purified trypsin-like proteinase at neutral pH and at the ratio observed *in vivo* in cysts. Fig. 5b shows that the pattern of degradation was unspecific, all the subunits appeared degraded at the same rate and no specific accumulation of intermediates was observed. The results therefore suggested that cathepsin B was involved in the physiological degradation of yolk. The hydrolysis of lipovitellin by the trypsin-like proteinase of yolk granules has formerly been observed (De Chaffoy de Courcelles *et al.*, 1980) at an unphysiological pH (pH = 9) and with solubilized lipovitellin instead of yolk granules. The specificity of the splitting of cathepsin and trypsin-like proteinases is not very different. Cathepsin B splits by Arg and preferentially by Arg-Arg (Perona and Vallejo, 1982) and trypsin proteinases, by Arg and Lys (Barrett and McDonald, 1980). It could be that a change in the conformation of lipovitellin produced by alkaline pH and the process of solubilization account for the differences in pattern observed by the two research teams.

Inhibition of yolk granule breakdown by lysosomotropic agents

Altogether the results suggested that lysosomes were involved in the degradation of yolk granules. Accordingly, we have found that the inhibition of the lysosomal function results in the inhibition of the yolk degradation. Lysosomotropic agents are weak basis that accumulate in lysosomes, thus increasing the internal acid pH and preventing the activity of lysosomal hydrolases (Dean *et al.*, 1984). Chloroquine has been used to inhibit the lysosomal function in nauplii, and ammonium chloride in cysts. Cysts are not permeable to chloroquine but are to ammonium. When ammonium chloride was added to the incubation medium, the frequency of hatching was decreased in a concentration-dependent manner (Fig. 6), and hatching was more selectively inhibited than emergence (Table III). Busa and Crowe (1983) have also described an increase in the number of prenauplii that died before hatching in the presence of ammonium chloride. This was interpreted as being due to the activation of pre-emercency development. We have found that the inhibitory effect of ammonium chloride on hatching was partially reversible upon incubation in fresh medium (Fig. 7). Yolk protein represents at least 80 % of the total protein of the cyst (Vallejo *et al.*, 1981). We have determined the protein content of yolk granules by isolating this fraction through a sucrose gradient. The degradation of yolk protein appeared

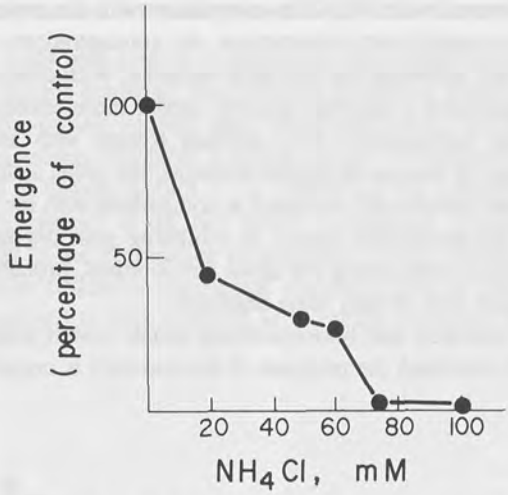

FIG. 6. The lysosomotropic agent NH_4Cl inhibits emergence of *Artemia* nauplii. Cysts were incubated as described in Materials and methods except for the routine medium that was supplemented with different concentrations of NH_4Cl (0, 20, 50, 60, 75, and 100 mM). The pH of the medium was adjusted to pH 8.3 in every case. At 17 h incubation, several aliquots from each incubation flask were taken and the number of nauplii, prenauplii, and cysts were counted under the microscope. Emergence is expressed as the ratio nauplii + prenauplii number/total number of animals in relation with the control (0 mM NH_4Cl) considered to be 100 %.

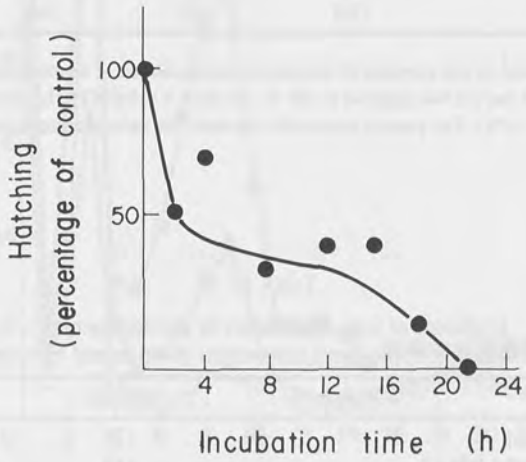

FIG. 7. Reversibility of the inhibitory effect of NH_4Cl on the hatching of *Artemia* nauplii. Cysts were cultured in the presence of 50 mM NH_4Cl (pH 8.3) as indicated in Fig. 5. Animals cultured for the different times indicated were transferred to fresh medium in the absence of NH_4Cl. After 24 h incubation, aliquots were taken and the animals counted under microscope. Hatching is expressed as the ratio nauplii number/total number of animals in relation with the control (no contact with NH_4Cl) considered to be 100 %.

inhibited in the animals treated with NH_4Cl in comparison with the control (Table IV). Similar results were observed in nauplii with chloroquine. At concentrations of about 5 mM, yolk degradation was completely prevented but mortality occurred. With concentrations up to 2 mM, nauplii were viable but exhibited a decrease motility. Under these conditions, yolk degradation was inhibited. When the homogenates from animals treated with ammonium chloride or chloroquine were analyzed by sucrose isopycnic gradients, the pellet sedimenting to the bottom (mainly yolk granules) was considerably enriched in comparison with the controls (see Fig. 10). However, the effect of lysosomotropic agents in inhibiting yolk degradation appeared more intense during the embryonic than during the larval development, probably because in nauplii a percentage of yolk granules have already been degraded.

The results therefore indicated that lysosomotropic agents inhibit yolk degradation and that when yolk degradation is inhibited the progress of development is impeded.

TABLE III

Inhibition of hatching in the presence of ammonium chloride

NH_4Cl (mM)	% prenauplii		
	Incubation time (h)		
	17	20	24
0	9	6	3
50	45	32	16
75	100	100	43
100	100	100	100

As usual cysts were incubated in the presence of increasing concentrations of ammonium chloride and for the time indicated. In the culture flasks the pH was adjusted to pH 8.3 to allow a suitable $[NH_3]$. Cysts, prenauplii, and nauplii were counted (Vallejo *et al.*, 1979). The percent prenauplii expresses the ratio prenauplii/nauplii + prenauplii.

TABLE IV

Inhibition of yolk degradation in developing cysts by the lysosomotropic agent ammonium chloride and by cyanide

	Protein
Control	128
+ 50 mM HN_4Cl	148
+ 100 μM KCN	171

As usual, cysts were incubated for 12 h (Vallejo *et al.*, 1979) and in the presence of either 50 mM NH_4Cl or 100 μM KCN. The homogenates were fractionated by centrifugation through a sucrose isopycnic gradient where the pellets contain mainly the yolk granules (Vallejo *et al.*, 1981). The protein is expressed per million of incubated cysts and is the protein of the pellets of the gradients.

THE INVOLVEMENT OF LYSOSOMES IN THE DEGRADATION OF YOLK GRANULES IN *ARTEMIA*

Homogenates from cysts and nauplii of increasing developmental stages up to 60 h, were analyzed by sucrose isopycnic gradients. Two different lysosomal activities acid phosphatase and cathepsin B as well as the protein content were determined in the fractions of the gradients. The lysosomal activities and protein presented a similar pattern of sedimentation. Three populations of lysosomal structures of density around > 1.3 (bottom of the gradient), 1.25, and 1.18 were identified (Fig. 8). The density of the two heavy populations of phagosomes indicated that lysosomes were involved in the degradation of structures of high density and in the distribution of protein, and that these structures represented the major protein component of the animals. These characteristics pointed to yolk granules as being the structures undergoing degradation.

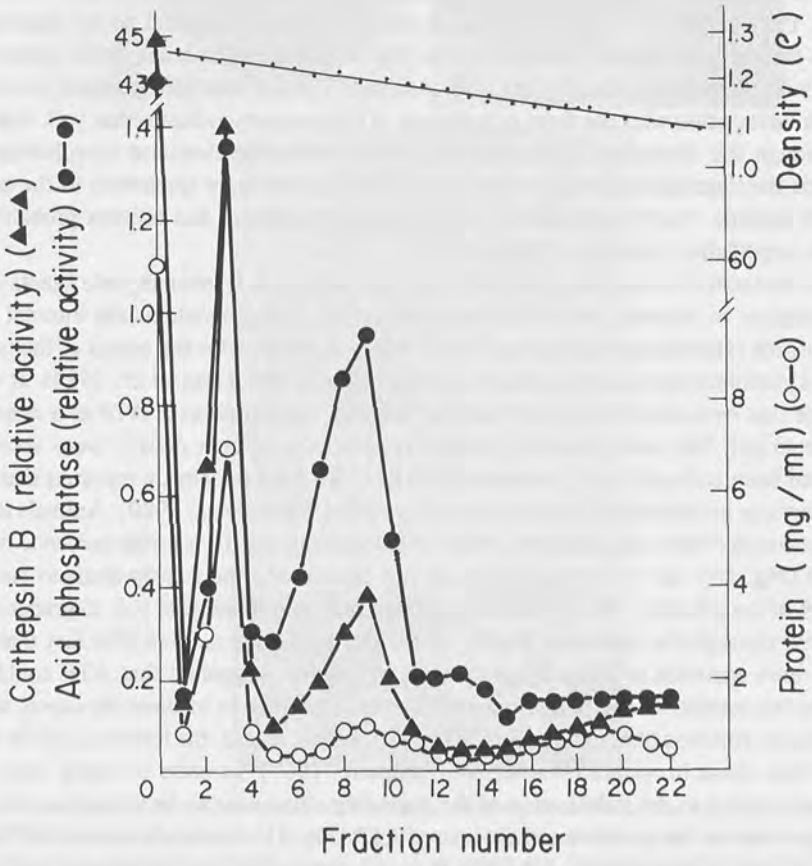

FIG. 8. Isolation of phagosomes in *Artemia* by sucrose isopycnic gradient centrifugation. Nauplii (8 h-old) were homogenized in "Ficoll medium" and the homogenate (2 ml) analyzed by sucrose gradient centrifugation (Materials and methods). The phagosomes were detected by measuring the acid phosphatase and cathepsin B activities as well as the protein (Materials and methods). The density was determined at 20 °C.

Immediately after hydration, most of the activity of the lysosomal enzymes sedimented to the bottom of the gradient and, with development, the activity associated to the three peaks was observed to increase and decrease in an oscillatory pattern (Perona, 1982). The detailed biochemical analysis of the process has indicated that the induction of the degradation of yolk granules occurs at five different times during development. Yolk granules appear to be degraded in five cycles with a duration of about 12 h each (Perona, 1982). The fractions comprising the two banding peaks and the pellet of the gradient of the different developmental stages were analyzed by electron microscopy. The fractions were previously incubated in Gomori's medium to reveal the lysosomal marker acid phosphatase. In Fig. 9, the morphology of the phagosomes sedimenting at the three different densities is presented. In the fraction of density > 1.3 (Fig. 9a) acid phosphatase was observed in vesicles in the process of fusing to the periphery of yolk granules. In the fraction of density 1.25 (Fig. 9b), the structure of the yolk granule is maintained although it is no longer so electrodense and is surrounded by a double phagosomal membrane probably formed from the fusion of primary lysosomes. In the light fraction of density 1.18 (Fig. 9c), the phagosomes have increased in size, retain little material to be degraded, and present an intense phosphatase reaction. In this fraction, double membrane, small empty vesicles (Fig. 9d) with phosphatase reaction are also observed. The fact that yolk granules structures are observed in association with the three populations of phagosomes indicates that yolk degradation occurs through the formation of phagosomes. Both the biochemical and morphological data suggest that the degradation process starts by the fusion of primary lysosomes to the periphery of the yolk granule, that it continues in the resulting phagosome, and finishes probably by the exit of the degradation products to the cytosol.

We were interested in understanding by which mechanism the lysosomotropic agents inhibited yolk degradation in *Artemia*. An ATP-consuming proton pump maintains the internal acid pH of the lysosome (Harikumar and Reeves, 1984) which is required for the action of the lysosomal enzymes. Lysosomotropic agents increase the internal acid pH (Dean *et al.*, 1984). It could be conceivable that lysosomotropic agents induced a higher consumption of ATP as a consequence of the rise in pH. We have observed that the phagosomes of high density were absent when animals had been cultured in the presence of NH_4Cl. We have previously reported that *Artemia* development can be inhibited in the presence of cyanide (Vallejo *et al.*, 1980). Animals incubated with the uncoupler were also analyzed. With cysts incubated for 12 h in the presence of NH_4Cl or cyanide (Fig. 10), the 1.25 phagosomes are not present and the activity tends to band in the lighter part of the gradient. This behavior suggested that the inhibition of yolk degradation could be produced through the increased fragility of the degradation structures. The fact that fragility was even more apparent in the animals cultured in cyanide, suggested that ATP could also be involved in the stability of the phagosomal structures. The fragility of these structures was more conspicuous in embryos than in nauplii (not shown). In this regard, the levels of ATP in embryos are lower than those in nauplii (Warner and Finamore, 1967). In order to check more directly if ATP was involved in the stabilization of the degrading structures, we incubated *in vitro* isolated 1.25 phagosomes in the presence and absence of ATP (Fig. 11). In the absence of ATP and even more so if cyanide was added, cathepsin B was recovered in the incubation medium. The presence of ATP protected the structure of phagosomes especially in short incubations. The data, although preliminary, suggest that ATP is required not only for the maintenance of the internal pH but for the stability of lysosomal structures. Therefore the levels of ATP could be a controlling factor in the regulation of yolk degradation during *Artemia* development.

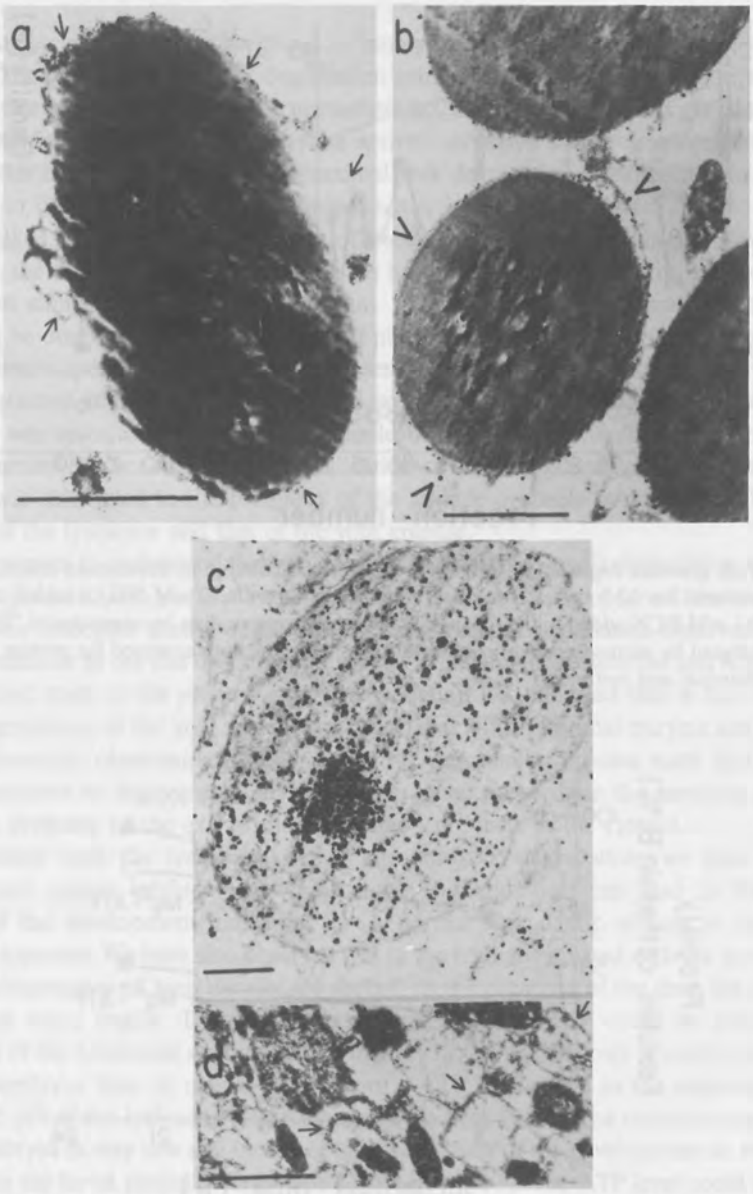

FIG. 9. Morphology of the yolk-degradative lysosomal structure of *Artemia*. The fractions comprising the bottom of the gradient and the two peaks of lysosomal enzyme activities were pooled separately and incubated in Gomori's medium (Beck *et al.*, 1971) to detect the lysosomal marker acid phosphatase. (a) bottom of the gradient ; (b) 1.25 density peak ; (c) 1.18 density peak ; (d) 1.18 density peak. The arrows indicate positive reaction, the arrow heads point to joining versicles and the formation of a double membrane.

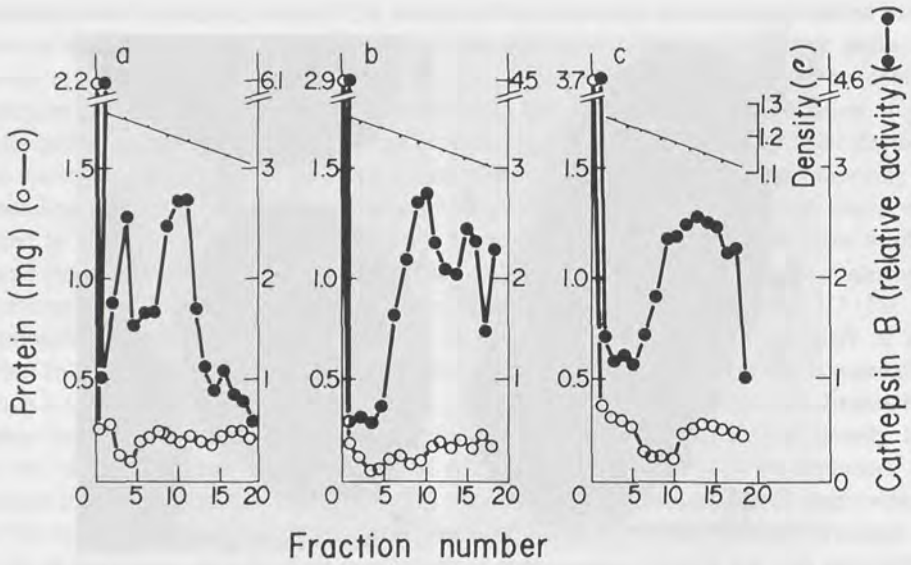

FIG. 10. Yolk granules degradation in nauplii, control and cultured in ammonium chloride or cyanide. Cysts were cultured for 12 h under controlled conditions (a), with 50 mM NH_4Cl added to the medium (b) or with 0.1 mM KCN added to the medium (c). Of the corresponding homogenates in "Ficoll medium" 2 ml were analyzed by sucrose gradient centrifugation and the fractions analyzed for protein and cathepsin B activity (Materials and methods).

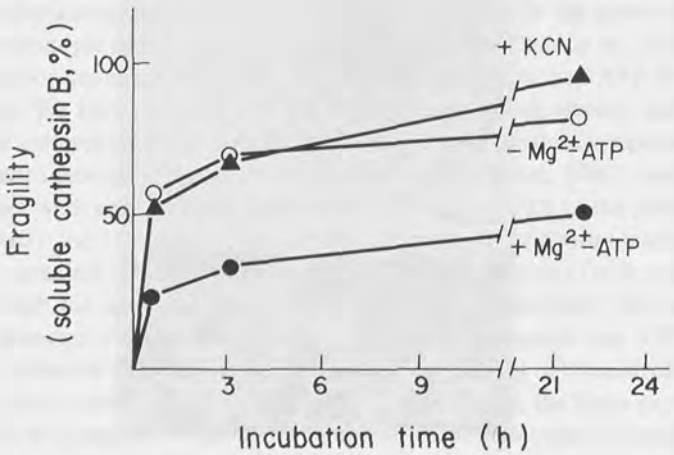

FIG. 11. Effect of ATP on the *in vitro* fragility of *Artemia* phagosomes. Phagosomes of density 1.25 were isolated from 34 h-old nauplii as indicated in Fig. 8. Phagosomes were incubated at room temperature in 50 mM phosphate buffer pH 7.4, 1 mM $MgCl_2$, 0.2 m KCl, 0.3 M sucrose in the absence or presence of 4 mM Mg^{+2}- ATP. At the times indicated, aliquots were taken and centrifuged to obtain the soluble and particulate fractions. Cathepsin B was determined in the fractions. The pellets were resuspended and subjected to three cycles of freezing and thawing in order to release latency. The percentage of cathepsin B activity found to be soluble was considered as a measure of the fragility of the phagosomes.

Discussion

Yolk granules are ubiquitous structures in embryonic systems but the mechanism of degradation is unknown. In *Artemia*, yolk degradation starts upon activation of the dormant cyst. We have found that at this time the major proteolytic activity is due to an acid proteinase that has been characterized as cathepsin B-like. The activity increases during development to reach a maximum after hatching, at the time of maximal yolk degradation. Cathepsin B in *Artemia* has been found to be localized in the lysosomes as it is in mammals (Barrett, 1977 ; Barrett and Kirschke, 1981). As a result, the presence of lysosomes in *Artemia* has been described for the first time. In the dormant embryo the lysosomes have the appearance of primary lysosomes with morphologies similar to mammalian lysosomes. In nauplii, few vesicles with acid phosphatase reaction can be observed and instead abundant phagosomes occur. These findings indicated that active lysosome-dependent degradative processes were taking place in the nauplii. When cysts were homogenized under conditions described previously to stabilize the yolk granule structure, cathepsin β was associated with the yolk granules fraction. The solubilization of the cathepsin from this fraction did not requite the solubilization of the yolk granule. This suggested that the proteinase was associated to the periphery of the granule probably by interaction between the membrane of the lysosome and that of the yolk granule.

In a first attempt to understand the involvement of lysosomes in yolk degradation we incubated *in vitro* yolk granules with purified cathepsin B. We had previously observed that no other acid proteinase was detectable during development. The pattern of degradation observed *in vitro* was in fact very simuilar to the one described *in vivo* by De Chaffoy de Courcelles and Kondo (1980).

The detailed study of the process of yolk degradation has indicated that in fact the intermediates of degradation of the yolk granule are associated with lysosomal enzyme activities. From electronmicroscopic observation it appears that the degradation process starts by the fusion of primary lysosomes to the periphery of the yolk granule, proceeds in the resulting phagosome, and finishes probably by the exit of the degradation products to the cytosol.

In agreement with the lysosomal dependence in yolk degradation, we have found that lysosomotropic agents inhibit yolk degradation. The inhibition can lead to the reversible inhibition of the development indicating that a normal degradation of yolk is required for a normal development. We have also observed that in the embryos, treated with the lysosomotropic agent, the phagosomes of high density are absent. In the presence of the drug the phagosomes appear to be more fragile. The *in vitro* results suggest that ATP could be involved in the stabilization of the lysosomal structures. The fragility of the phagosomes is more conspicuous in developing embryos than in nauplii (not shown). ATP is involved in the maintenance of the internal acid pH of the lysosome (Harikumar and Reeves, 1984). The concentration of ATP in dormant embryos is very low and increases upon activation of the development to reach normal levels later in the larval period (Warner and Finamore, 1967). The ATP level could therefore be a controlling factor in the regulation of the degradation of yolk granules. The regulation is obvious considering the dependence upon a system well differentiated from the yolk granule. The idea that yolk granules can be, in addition to a food reserve, a storage of information for development (Hentschel and Tata, 1976) is supported by the possibility of regulation of the process of degradation.

Conclusions

Yolk granules in *Artemia* appear to be degraded through the lysosomal system. A cathepsin B proteinase is able to degrade lipovitellin with a pattern of degradation similar to the one observed *in vivo*. The enzyme is located in the lysosomes of *Artemia* and together with other lysosomal enzymes increase their levels at the time of yolk degradation. The *in vivo* inhibition of lysosomal activity blocks the degradation of protein from the yolk granules. Biochemical as well as ultracytochemical studies have showed a physical association of lysosomes and yolk granules throughout the process of degradation.

Acknowledgements

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The protein kinase activity associated with the major bis(5′-adenosyl) tetraphosphate-binding protein of *Artemia*

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Abstract

In contrast to what has been observed with mammalian cells, the major bis(5′-adenosyl) tetraphosphate-binding protein of *Artemia* does not copurify with DNA polymerase-α. The partially purified *Artemia* Ap_4A-binding protein possesses an inseparable protein kinase activity which phosphorylates histone 2B on serine residues. This kinase has a native M_r of 93 000 and appears to correspond to the major phosphorylating activity detected in crude larval extracts which has a catalytic subunit of M_r 72 000. In the absence of exogenous protein substrate, the partially purified kinase phosphorylates endogenous polypeptides in the enzyme preparation of M_r 35 000, 40 000, and 42 000 in addition to autophosphorylation of the 72 000 -M_r polypeptide, also on serine residues. The co-sedimentation of all four polypeptides in a sucrose gradient suggests that they are associated in some way in a native complex. Proteolysis may be responsible for generation of the low molecular weight polypeptides.

Histone phosphorylation is unaffected by Ap_4A but is stimulated 2-fold by 1 μM cAMP or cGMP although no binding of the cyclic nucleotides has been detected. 1 μM cAMP and cGMP completely eliminate autophosphorylation of the polypeptides of M_r 42 000 and 40 000 while cGMP additionally suppresses autophosphorylation of the 72 000-M_r and 35 000-M_r polypeptides. The relationship of the kinase to known activities and the possible significance of the binding of Ap_4A is discussed.

Introduction

During the past 20 years many acid-soluble nucleotides have been described which act as specific signal or effector molecules in the regulation of metabolic reactions (Tomkins, 1975 ; Koch and Richter, 1979). One such group of nucleotides of considerable current interest is the bis(5′-nucleosidyl) oligophosphates, in particular bis(5′-adenosyl) tetraphosphate (Ap_4A). Since the first demonstration of its presence *in vivo* (Zamecnik and Stephenson, 1969), Ap_4A has been shown to be a ubiquitous component of living cells (Rapaport and Zamecnik, 1976 ; Zamecnik, 1983 ; Weinmann-Dorsch *et al.*, 1984). It is synthesised *in vitro* and probably *in vivo* by the back reaction of an activated aminoacyl-adenylate with ATP (Zamecnik *et al.*, 1966). This reaction is catalysed with varying efficiency by both prokaryotic and eukaryotic aminoacyl-tRNA synthetases. In certain cases Ap_4A synthesis is strongly dependent on low concentrations of Zn^{2+} ions (Goerlich *et al.*, 1982 ; Blanquet *et al.*, 1983). In eukaryotic cells and tissues the intracellular level of Ap_4A fluctuates in proportion to cellular growth rate within the limits 0.01 μM to 13 μM (Rapaport and Zamecnik, 1976 ; McLennan and Prescott, 1984 ; Weinmann-Dorsch *et al.*, 1984).

Ap_4A has been called a 'pleiotypic activator' of cellular proliferation (Rapaport and Zamecnik, 1976). Evidence in support of this is provided by its possible involvement in a number of important metabolic processes including the initiation of DNA replication (Grummt, 1978, 1983 ; Weinmann-Dorsch *et al.*, 1984), the stimulation of processing of ADP-ribosylated proteins (Surowy and Berger, 1983) and the induction of stress proteins in response to oxidative damage (Varshavsky, 1983 ; Bochner *et al.*, 1984).

Embryonic development of encysted *Artemia* gastrulae up to the emergence of the free-swimming nauplius larvae occurs in the complete absence of DNA replication and cell division (Finamore and Clegg, 1969 ; Iwasaki, 1969). The delay in the onset of DNA synthesis to the point of emergence together with the large quantities of the Ap_4A analogue, Gp_4G, in the dormant cysts (Warner, 1980) has stimulated our interest in *Artemia* as a system in which to study the possible effects of related bis(5′-nucleosidyl) oligophosphates in the control of DNA replication and cellular proliferation.

We have previously reported on the presence of Ap_4A in cysts and larvae of *Artemia* (McLennan and Prescott, 1984). When development is reinitiated, the Ap_4A content of embryos whose endogenous Ap_4A pool has been depleted by storage in brine undergoes a rapid 125-fold increase, reaching a maximum intracellular concentration of 3.3 μM at the point of emergence when DNA replication begins. Thereafter the Ap_4A content declines as development proceeds. These findings are consistent with the suggested role of Ap_4A in the initiation of DNA synthesis.

In order to gain further evidence for such a role in *Artemia* and to learn more about the function of Ap_4A in general, we have sought to identify the intracellular target proteins for this nucleotide. The principal receptor in mammalian cells appears to be a subunit of the holoenzyme complex which is responsible for nuclear DNA replication, DNA polymerase-α (Grummt *et al.*, 1979 ; Rapaport *et al.*, 1981). It has been proposed that Ap_4A which is bound to this subunit can serve to prime the DNA polymerase, possibly at the level of replicon initiation (Zamecnik *et al.*, 1982). This subunit can be resolved as a separate polypeptide (Baril *et al.*, 1983 ; Rapaport and Feldman, 1984), but few other binding proteins have been described so far.

We now report the existence of a major Ap_4A-binding protein in *Artemia* which shows no association with DNA polymerase-α throughout early development. This protein possesses protein kinase activity. Partial characterisation of this enzyme activity is reported here.

Materials and methods

Materials

Great Salt Lake *Artemia* cysts were obtained from the Sanders Brine Shrimp Co., Ogden, Utah, USA in 1980. Phenylmethanesulphonyl fluoride (PMSF), soybean trypsin inhibitor (STI), histone 2B, L-O-phosphoserine, L-O-phosphothreonine, DL-O-phosphotyrosine, guanosine 3′ : 5′-monophosphate (cGMP) and adenosine 3′ : 5′-monophosphate (cAMP) were from Sigma. [^{3}H] Ap_4A (15 Ci/mmol) and [γ-^{32}P]ATP (> 5 000 Ci/mmol) were from Amersham. 0.45 μm nitrocellulose filters were from Millipore.

Hatching of larvae

Decapsulated cysts were prepared as previously described (McLennan and Presscott, 1984) and incubated in artificial seawater (20 ml/g wet wt. cysts) at 28 °C for 21 h. Larvae were

harvested by filtration and washed in ice-cold water followed by buffer A (10 mM potassium phosphate pH 7.5, 10 % glycerol, 1 mM EDTA, 5 mM 2-mercaptoethanol).

Preparation of crude extracts

All procedures were carried out at 4 °C. Washed larvae were immediately suspended in buffer A (5 ml/g wet wt) containing 0.3 M KCl, 1 mM PMSF and 100 µg/ml STI. Breakage was with ten strokes of a loose fitting Potter-Elvehjem homogeniser. After stirring for 30 min, the homogenates were centrifuged (800 g, 10 min), the supernatants removed and then recentrifuged (142 000 g, 1 h). The lipid pellicles were removed, the supernatants dialysed against 2 × 100 vol buffer A and then stored at - 70 °C.

Sucrose density gradient analysis

4.4 ml linear 5-20 % sucrose density gradients were prepared in buffer A. After application of sample, gradients were centrifuged at 40 000 rpm (190 000 g_{av}) for 20 h at 4 °C in a Beckman SW40.1 rotor. Gradients were fractionated upwards from the bottom into equal volume fractions. Bacterial alkaline phosphatase (6.3 S) was included in gradients as a marker when appropriate.

Assays

Ap_4A-binding assays contained binding buffer (30 mM Hepes-NaOH, pH 7.5, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 200 mM sucrose), 0.25 µCi [^{3}H]Ap_4A (2 Ci/mmol) and binding protein in a volume of 75 µl and were incubated at 4 °C for 18 h. 65 µl portions were then filtered under suction through 1.4 cm nitrocellulose discs (pore size, 0.45 µm), washed rapidly with 5 × 1 ml portions of ice-cold binding suffer, dried and the bound radioactivity determined in 2 ml Beckman Ready-solv HP/b scintillation fluid.

DNA polymerase-α was assayed as previously described (Slater and McLennan, 1982).

Protein kinase assays contained 50 µg histone 2B, 1 µCi[γ-^{32}P]ATP (1.4 Ci/mmol) and Ap_4A-binding assay buffer in a volume of 100 µl. Incubation was for 18 h at 25 °C. Acid-insoluble radioactivity was determined by the filter paper method of Gill and Walton (1979). When the autophosphorylation of endogenous polypeptides was determined, histone was omitted from the reaction mixture and the specific activity of the [γ-^{32}P]ATP raised (> 5 000 Ci/mmol).

Gel electrophoresis of phosphorylated products

75 µl portions of protein kinase assay mixtures were equilibrated in 62.5 mM Tris-HCl, pH 6.8, 1 mg/ml bovine serum albumin by buffer exchange on 1 ml columns of Sephadex G-25 (coarse). 50 µl samples were then prepared for electrophoresis and analysed on 10 % polyacrylamide gels in the presence of SDS as described by Hames (1981). After electrophoresis, gels were fixed and washed in 10 % acetic acid, 5 % methanol and the [^{32}P] labelled proteins visualised by exposure of the dried gel to Fuji RX film using a DuPont Cronex Lightning Plus intensifying screen. Molecular weights were calculated from lanes containing standard proteins (Boehringer) stained with Coomassie blue.

Determination of phosphorylated amino acids

50 µg of histone 2B which had been phosphorylated as described above was separated from other phosphorylated contaminants by electrophoresis in an 18 % polyacrylamide gel in the presence of SDS (Thomas and Kornberg, 1975). After location by autoradiography, the histone was extracted from the gel according to Beemon and Hunter (1978). Gel residues were removed by centrifugation and the supernatant lyophilised. The sample was then taken up in 1 ml of nitrogen-bubbled 6 M HCl and hydrolysed *in vacuo* at 110 °C for 2 h. The hydrolysate was lyophilised twice from water and finally redissolved in 200 µl of 11 mM potassium phosphate, pH 3.0. 100 nmol of each O-phosphoamino acid standard was added and a 100 µl sample analysed by high performance liquid chromatography on a 25 × 0.4 cm column of Partisil 10-SAX. Isocratic separation of the O-phosphoamino acids was achieved at 1.5 ml/min with 11 mM potassium phosphate, pH 3.0, 13.5 % methanol (Swarup *et al.*, 1981). Standards were monitored by their absorbance at 206 nm and radioactivity determined by counting 1 ml fractions in 25 % Lumax (LKB Instruments).

Results

The binding of [3H]Ap_4A to crude extracts of both dormant cysts and larvae was readily detected using a nitrocellulose filter-binding assay. For example, sucrose density gradient analysis of the binding activity from a crude preparation of newly hatched larvae (total incubation time, 21 h) is shown in Fig. 1. The major Ap_4A-binding species sedimented at 4.8S (M_r = 93 000). Binding activities of higher molecular weight are also indicated in this profile and other experiments employing ion-exchange separations on DEAE-cellulose or Mono Q (Fig. 2) do suggest several species of binding protein. However, since the separate Ap_4A-binding fractions all co-sedimented at 4.8S in a sucrose gradient (data not shown) the implication is unclear at the moment. Preliminary evidence suggests that this separation is an artefact due to strong protein-protein interactions in the preparations.

Mild extraction conditions were chosen to maximise the possibility of detecting an association of the Ap_4A-binding protein with DNA polymerase-α activity. In contrast to previous findings with mammalian cells, no association was observed on sucrose gradients at any developmental stage examined (*e.g.* Fig. 1) This was despite the fact that the undegraded 9S DNA polymerase-α holoenzyme complex appeared to have been preserved through the use of 1 mM PMSF and 100 µg/ml STI in the extraction buffer. Omission of STI results in a 7.6 S polymerase while the further omission of PMSF further reduces the polymerase to a 6.2 S species (Slater and McLennan, 1982). This lack of association was confirmed by anion-exchange fast protein liquid chromatography (*e.g.* Fig. 2) and a number of other chromatographic techniques (not shown).

In order to allow a more detailed study of the binding protein it was found necessary to remove phosphodiesterases, dinucleoside tetraphosphatase and other enzyme activities which may affect the ligand. A purification scheme was devised which employed chromatography on Matrex-blue gel, DEAE-cellulose and AcA 44 (not shown). The final preparation of Ap_4A-binding activity was found to have co-purified with a protein kinase. Fig. 3 shows the co-migration of histone-phosphorylating activity with the purified Ap_4A-binding protein in a sucrose gradient. Of the proteins tested, histone 2B was the best substrate for the kinase. Protamines were phosphorylated to a lesser extent, but no activity was observed with casein or vitellogenin (not shown).

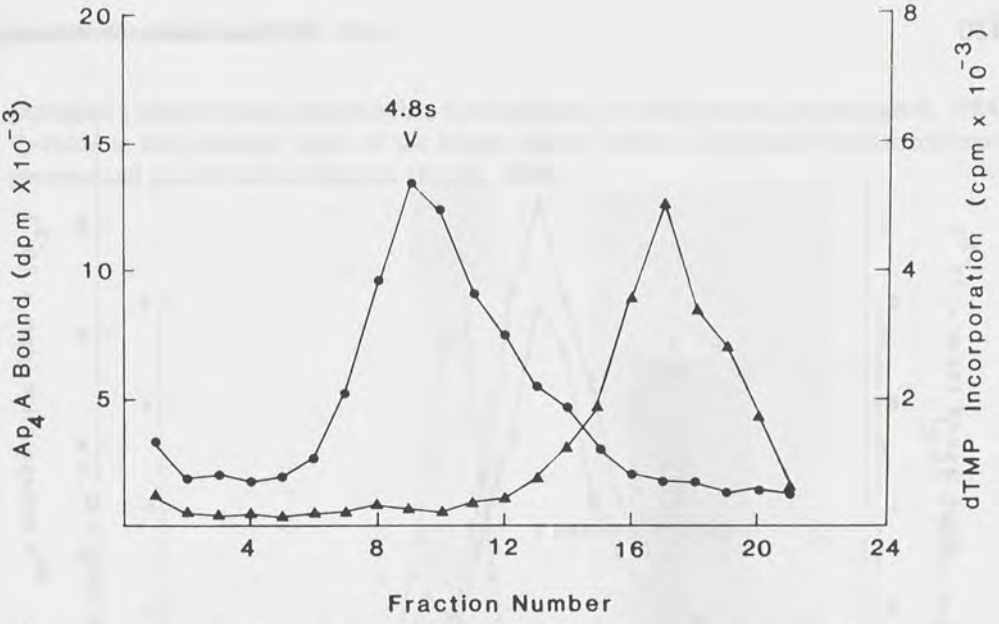

FIG. 1. Separation of Ap_4A-binding protein and DNA polymerase-α activity on a sucrose density gradient. 200 µl of a crude 21 h larval extract in buffer A was subjected to sucrose density gradient analysis as described in Materials and methods. Fractions were assayed for Ap_4A-binding (●) and DNA polymerase-α (▲). The top of the gradient is to the left of the figure.

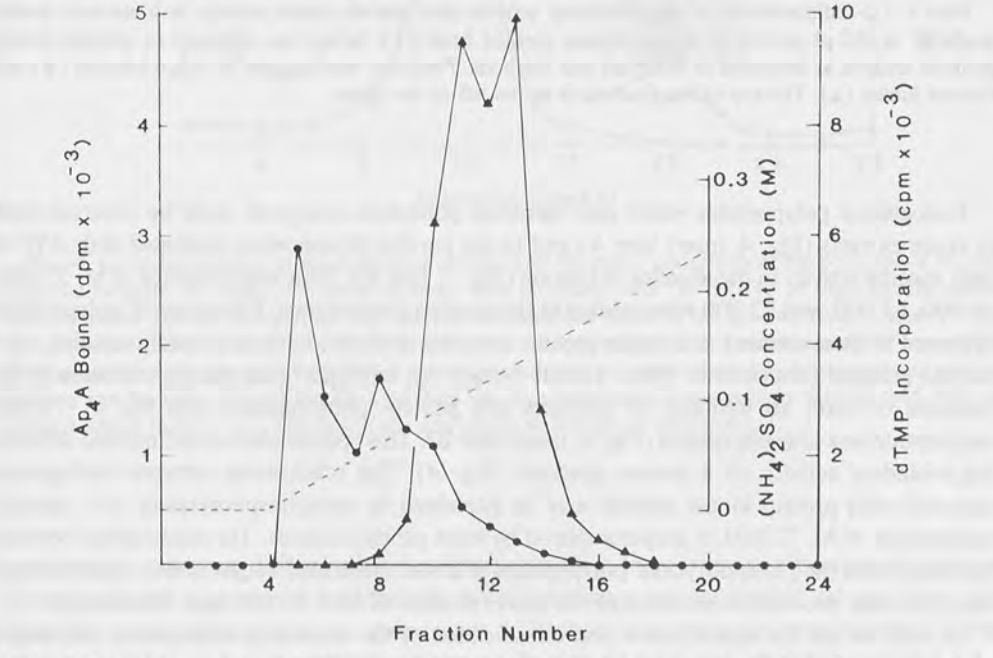

FIG. 2. Separation of Ap_4A-binding protein from DNA polymerase-α by high performance anion-exchange chromatography. 1 ml of crude 21 h larval extract in buffer A was applied to a 1 ml Mono Q HR5/5 column (Pharmacia) equilibrated in 20 mM Tris-HCl, pH 7.5, 10 % glycerol, 1 mM EDTA, 5 mM 2-mercaptoethanol. After elution of the unbound proteins a 20 ml gradient of 0-0.5 M $(NH_4)_2SO_4$ in equilibration buffer was applied and 0.5 ml fractions collected. Fractions were assayed for Ap_4A-binding (●) and DNA polymerase-α (▲). Molarity of $(NH_4)_2SO_4$ is indicated by the broken line.

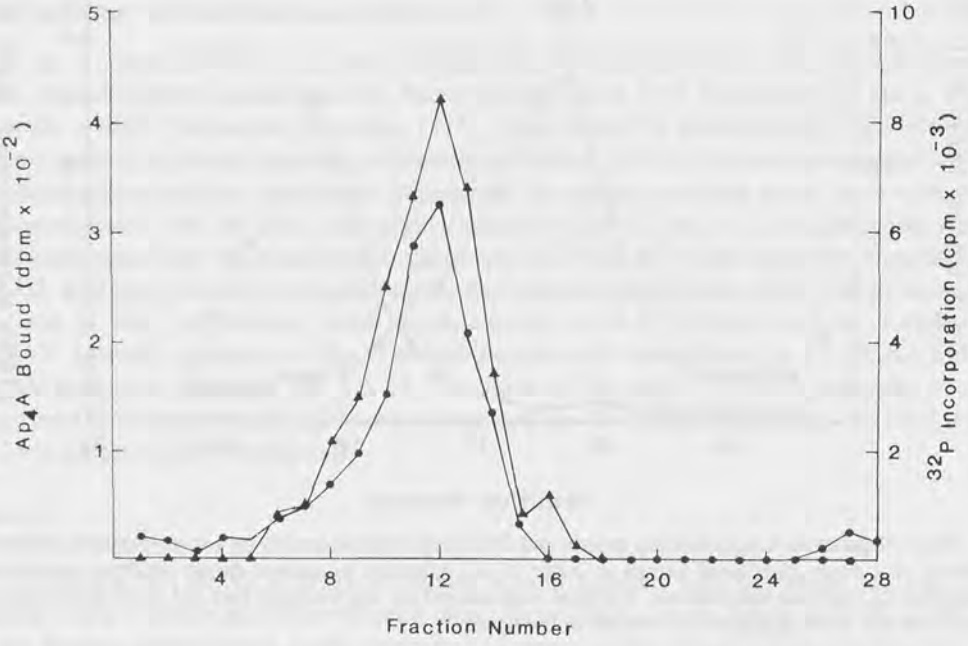

FIG. 3. Co-sedimentation of Ap_4A-binding protein and histone kinase activity in a sucrose density gradient. A 200 µl sample of histone kinase purified from 21 h larvae was subjected to sucrose density gradient analysis as described in Materials and Methods. Fractions were assayed for Ap_4A-binding (●) and histone kinase (▲). The top of the gradient is to the left of the figure.

Endogenous polypeptides which also served as phosphate acceptors could be observed both in crude extracts (Fig. 4, insert lane A) and in the purified kinase when incubated with ATP of high specific activity in the absence of histone (Fig. 7, lane A). Four major species of M_r 35 000, 40 000, 42 000, and 72 000 were evident in the purified preparations. This group of polypeptides appeared to be associated as a single protein complex as shown by their co-sedimentation on a sucrose gradient (not shown). When a crude extract was subjected to autophosphorylation in the presence of NaF, an inhibitor of ATPases and protein phosphatases, only the M_r 72 000 polypeptide was phosphorylated (Fig. 4, insert lane B). This species also co-sedimented with the Ap_4A-binding activity on a sucrose gradient (Fig. 4). The relationship between endogenous acceptors and protein kinase activity may be explained by autophosphorylation of a catalytic polypeptide of M_r 72 000, a property shared by most protein kinases. The relationship between the kinase and the phosphorylated polypeptides of lower molecular weight is less clear, but they may represent proteolytic products of the native protein of M_r = 93 000 (see Discussion).

In order to aid the identification and classification of the *Artemia* protein kinase, the nature of the phosphorylated amino acid was investigated. Several different amino acids may serve as phosphate acceptors, the most common being serine, threonine and tyrosine (Martensen, 1984); less common are thioesters of cysteine and phosphoramidates of lysine and histidine (Fujitaki and Smith, 1984). Type II casein kinase and the cAMP-dependent kinases phosphorylate serine

while type I casein kinases phosphorylate threonine and, to a lesser extent, serine (Roach, 1984). Tyrosine is the principal target of the kinase activity which is associated with transforming proteins and growth factor receptors (Roach, 1984).

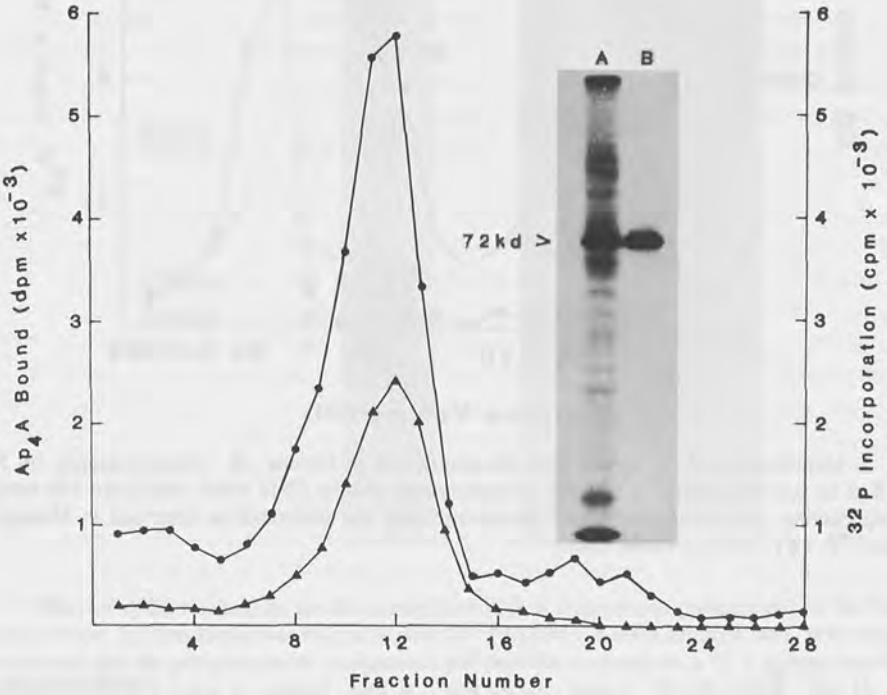

FIG. 4. Co-sedimentation of Ap_4A-binding protein with the major phosphorylatable polypeptide in crude extracts. Samples of crude 21 h larval extracts were incubated with [γ-^{32}P]ATP of high specific activity without added histone as described in Materials and Methods either in the absence (insert, lane A) or in the presence (insert, lane B) of 40 mM NaF and the phosphorylated products analysed on a 10 % SDS-polyacrylamide gel. A further 200 µl sample of crude extract was subjected to sucrose density gradient analysis and fractions assayed for Ap_4A-binding (●) and for protein kinase activity with 40 mM NaF and without added histone (▲). The top of the gradient is to the left of the figure.

A sample of histone 2B phosphorylated by the purified *Artemia* kinase was separated from the phosphorylated endogenous polypeptides on a denaturing polyacrylamide gel and then acid-hydrolysed to yield its constituent amino acids. [^{32}P]phosphoamino acids were separated by high performance ion-exchange chromatography on Partisil 10-SAX and identified with the aid of verified standards. The major modified amino acid was found to be O-phosphoserine (Fig. 5). A similar treatment of endogenous acceptor polypeptides also showed O-phosphoserine to be the major product (not shown).

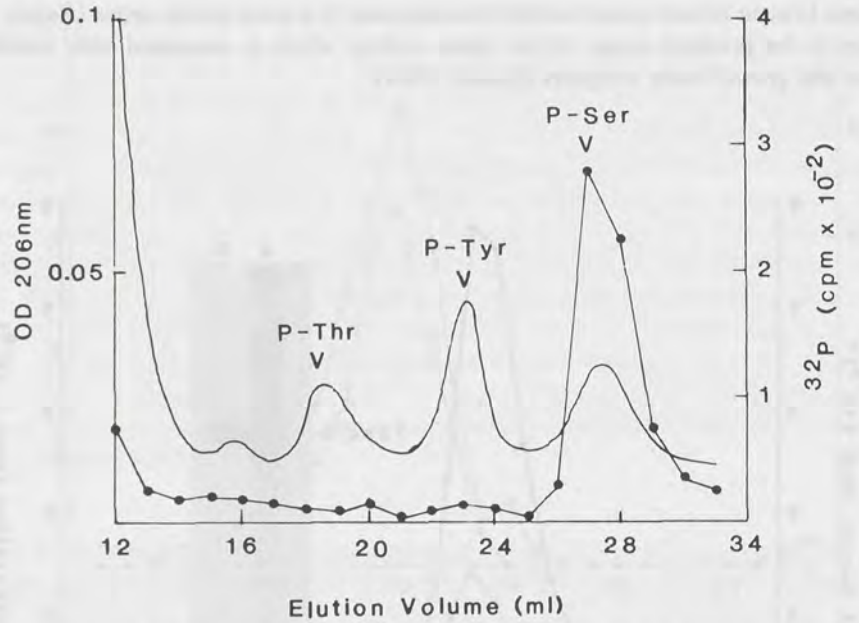

FIG. 5. Identification of the amino acid phosphorylated in histone 2B. Chromatography on Partisil 10-SAX of an acid hydrolysate of isolated, phosphorylated histone 2B to which was added 100 nmol each of phosphoserine, phosphothreonine and phosphotyrosine was performed as described in Materials and methods. ^{32}P, (●) ; O.D.$_{206}$, (solid line).

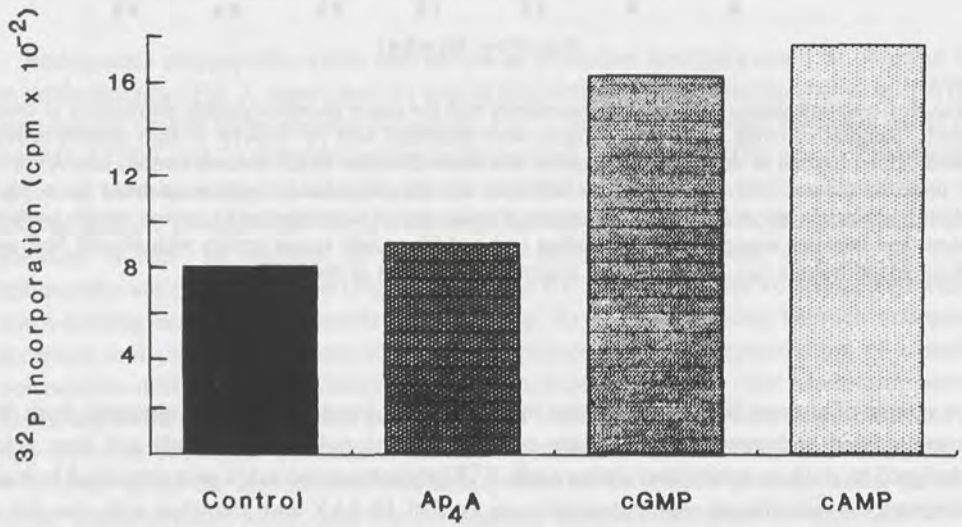

FIG. 6. Effect of cyclic nucleotides and Ap_4A on histone kinase activity. Purified kinase was assayed for histone phosphorylation in the absence (control) and the presence of 1 μM nucleotide as indicated. Acid-insoluble radioactivity was determined as described in Materials and methods.

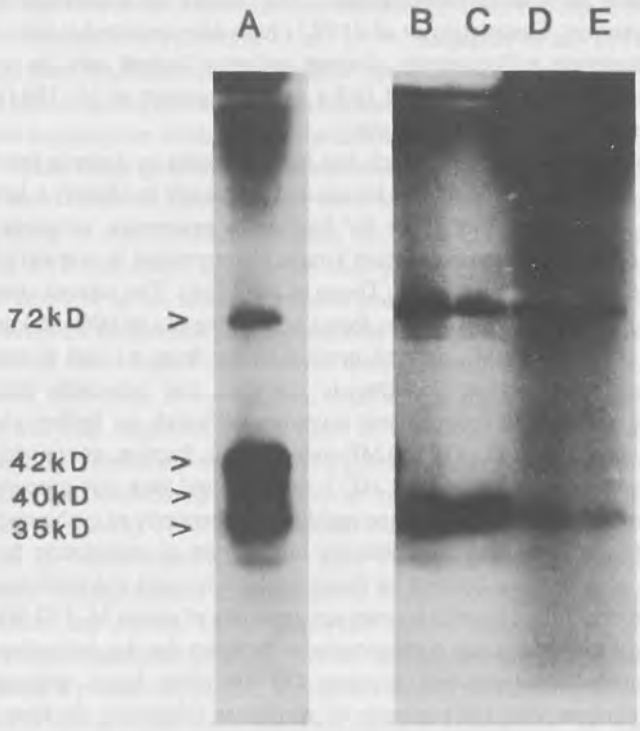

FIG. 7. Effect of cyclic nucleotides on the phosphorylation of endogenous polypeptides in the histone kinase preparation. Purified kinase was incubated with [γ-^{32}P]ATP (< 5 000 Ci/mmol) and cyclic nucleotides as indicated and the phosphorylated endogenous polypeptides analysed on a 10 % polyacrylamide gel in the presence of SDS. Lane A, control ; lane B, 1 µM cAMP ; lane C, 10 µM cAMP ; lane D, 1 µM cGMP ; lane E, 10 µM cGMP.

Protein kinases may also be classified according to the effector molecules which modify their activity (Roach, 1984). Stimulation of histone phosphorylation was observed in the presence of 1 µM cAMP or cGMP (Fig. 6). Ap_4A on the other hand had no detectable effect ; nor did Ca^{2+} and/or calmodulin (not shown). The effect of cyclic nucleotides on the phosphorylation of endogenous acceptors was also examined. 1 µM cAMP or cGMP completely suppressed phosphorylation of the polypeptides of M_r 42 000 and 40 000 while cGMP also reduced phosphorylation of the 72 000 -M_r and 35 000 -M_r polypeptides (Fig. 7). cAMP and cGMP exert their regulatory influence on the cyclic nucleotide-dependent protein kinases by binding to a specific site, however no significant binding of either nucleotide could be detected with the purified preparation (not shown).

Discussion and conclusions

Our initial investigations of the target proteins for Ap_4A in *Artemia* have shown no detectable binding of this nucleotide to any component associated with DNA polymerase-α throughout

pre-emergence and early larval development. The variety of techniques employed strongly support this conclusion. Castroviejo *et al.* (1982) have also reported a lack of affinity of Ap_4A for the DNA polymerase α-like activity of wheat embryos. Indeed only the α-polymerases from calf thymus (Grummt *et al.*, 1979) and HeLa cells (Rapaport *et al.*, 1981) have so far been shown to possess an Ap_4A-binding subunit.

The major Ap_4A-binding protein which has been detected in *Artemia* has been shown to be a protein kinase. Attempts to classify the kinase and ultimately to identify it have so far been only partly successful. The strong preference for histone as exogenous substrate distinguishes this kinase from the only other *Artemia* protein kinase characterised in any detail so far, the cyclic nucleotide-independent casein kinase II (Thoen *et al.*, 1984). The subunit composition of casein kinase II is also different, comprising two forms containing α (36 000 -M_r) and β (28 000 -M_r) subunits and α, α′ (33 000 -M_r, derived proteolytically from α) and β subunits respectively. Histone phosphorylating activity in *Artemia* embryos has previously been separated into cAMP-dependent and cAMP-independent fractions, although no further characterisation was reported (Sierra *et al.*, 1977). The cAMP-independent fraction contained an eIF-2 kinase, however no phosphorylation of rat liver eIF-2 was detected with our preparation.

Phosphorylation of histone 2B on serine residues is a property of cyclic nucleotide-dependent protein kinases (Roach, 1984). The relatively low degree of stimulation by both cAMP and cGMP, the lack of detectable binding of these nucleotides and the molecular size differences (cyclic nucleotide-dependent protein kinases are generally of native M_r 150 000-180 000) would appear at first sight to rule out any correspondence between the Ap_4A-binding kinase and either class of cyclic nucleotide-dependent enzyme. On the other hand, although extraction and purification was performed in the presence of proteinase inhibitors, we have generally found it difficult to eliminate completely the proteolysis of *Artemia* proteins during isolation (Slater and McLennan, 1982 ; McLennan and Miller, this volume). It is therefore possible that partial proteolysis of a cyclic-nucleotide dependent kinase has led to the loss of affinity for effector molecules and so to misinterpretation of results. There are several known examples of the alteration in properties of protein kinases consequent upon proteolysis during purification (Roach, 1984). For example, cGMP-dependent protein kinase is a dimer of native M_r 150-160 000 and is particulary prevalent in arthropods (Kuo and Shoji, 1982). Proteolysis during extraction can yield a catalytic subunit of 75 000 -M_r and even catalytically active fragments of lower molecular weight *e.g.* 34 000 -M_r. The ability to bind and to be strongly stimulated by cGMP is lost upon proteolysis. Furthermore, autophosphorylation of the 75 000 -M_r subunit is suppressed by cGMP and by histone (de Jonge and Rosen, 1977). Several of our results could therefore be explained if the kinase which we have isolated is a proteolysed form of cGMP-dependent protein kinase.

An unusual histone kinase has been isolated from developing grasshoppers which is stimulated 2 to 3-fold by both cAMP and cGMP (Vardanis, 1980). It has a native M_r of 180 000 and a high affinity for cyclic nucleotides, hence any correspondence with the *Artemia* enzyme would also have to imply proteolytic modification. Further work is necessary to investigate these possibilites in full.

The importance of Ap_4A-binding to the phosphorylation reaction is unclear. No significant effect of the nucleotide on the kinase could be detected at a level which would be expected to give a response if it were indeed acting as a regulatory molecule. This may indicate that Ap_4A binds to the active site. Indeed we have evidence that ATP and ADP, but not AMP, can compete

with Ap_4A for binding. This further distinguishes this protein from the Ap_4A-binding subunit of mammalian DNA polymerase-α (Grummt *et al.*, 1979 ; Rapaport *et al.*, 1981). Other investigations have shown that Ap_4A and the homologue Ap_5A can act as competitive multisubstrate inhibitors of rabbit muscle adenylate kinase (Purich and Fromm, 1972 ; Lienhard and Secemski, 1973). Binding and competitive inhibition of the activity of the tyrosine-specific protein kinase activity of $pp60^{v\text{-}src}$ from Rous sarcoma virus-transformed cells but not of the cellular homologue $pp60^{c\text{-}src}$ has also been observed (Barnekow, 1983 ; Maness *et al.*, 1983). It may therefore be argued that the binding of Ap_4A, which generally occurs at intracellular concentrations much lower than those of ATP and ADP, could be of little physiological significance. However since the ability to bind Ap_4A is by no means a general property of protein kinases (Maness *et al.*, 1983) or of other adenine nucleotide-binding proteins and since the intracellular concentration of Ap_4A may in fact be much higher than normal during encystment (McLennan and Prescott, 1984), a role for this nucleotide in the reaction catalysed by the kinase is still possible.

Precisely where this role might be, is still open to question. The lack of association of the Ap_4A-binding protein and therefore of the protein kinase with *Artemia* DNA polymerase-α does not directly support an involvement for Ap_4A in priming DNA replication as previously suggested. However Varshavsky (1983) has pointed out that a secondary role for Ap_4A which would predominate at concentrations higher than those required by replication is in the alterations in patterns of transcription and translation which accompany the exposure of cells to stress. Protein kinases are known to be important in the regulation of protein synthesis, particularly at the level of initiation (Ochoa, 1983). An Ap_4A-regulated kinase would form an intriguing link between a product of protein synthesis (Ap_4A) and the alterations in the patterns of mRNA translation which must accompany encystment (translation of stress proteins and suppression of normal translation) and redevelopment (re-initiation of protein synthesis). This area of nucleotide-mediated regulation is open to detailed investigation in the future.

Summary

Bis(5'-adenosyl) tetraphosphate (Ap_4A) is a member of a recently discovered family of nucleotides which are synthesised by aminoacyl-tRNA synthetases. In mammalian cells, Ap_4A has been reported to increase in concentration during the G1 phase and to reach a maximum during S-phase. As a ligand of the replicative DNA polymerase-α, it may be involved as an initiating molecule during DNA replication. Although Ap_4A also increases during the period of pre-emergence development in *Artemia*, it does not bind to the *Artemia* DNA polymerase-α. A 4.8S (M_r = 93,000) Ap_4A-binding protein is present in extracts of cysts and larvae however, but it is separable from DNA polymerase-α by several methods. This protein was purified on Matrex-blue gel, DEAE-cellulose and Ultrogel AcA44 and was found to have protein kinase activity. The preferred exogenous substrates for this activity were histone 2B followed by protamines. The lack of activity with casein distinguishes it from the *Artemia* casein kinase II. H.p.l.c. analysis showed that histone 2B was phosphorylated on a serine residue. Endogenous polypeptides of M_r = 35 000, 40 000, 42 000, and 72 000 in the kinase preparation also served as phosphate acceptors. These polypeptides co-sedimented with the Ap_4A-binding activity on a sucrose density gradient. The 72 000-M_r polypeptide was the only one phosphorylated in crude extracts incubated with [γ-^{32}P]ATP and NaF, an inhibitor of ATPases and protein phosphatases.

1 μM cAMP or cGMP stimulated histone phosphorylation 2-fold and completely eliminated the autophosphorylation of the polypeptides of M_r 42 000 and 40 000. cGMP also suppressed the autophosphorylation of the 35 000-M_r and 72 000-M_r polypeptides. Ap_4A, Ca^{2+} and calmodulin had no effect on the activity of the kinase. Since the kinase did not bind either cAMP or cGMP, it is unlikely to be related to the cyclic nucleotide-dependent protein kinases unless partial proteolysis during purification has altered its properties. The precise role of this kinase and the effect of the binding of Ap_4A remain to be determined.

Acknowledgement

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Histochemical and biochemical study of alkaline phosphatase (ALP) activity in developing embryos and larvae of *Artemia* [1]

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Abstract

ALP activity was investigated during the brine shrimp development from embryos to juvenile stages by quantitative, histochemical, and electrophoretic methods, the inhibitors L-phenyalanine (LPA) and (+)p-bromotetramisole oxalate (BTO) being added to controls. Before hatching, a low ALP activity, a weak cytoplasmic histochemical staining that is partly associated with yolk, and slowly migrating electrophoretic enzyme bands were detected. At emergence, ALP activity increases rapidly and becomes most evident in the differentiating midgut and antennal glands, at the same time as new electrophoretic bands appear. The enzyme activity and the histochemical staining increase further in the naupliar and metanaupliar stages, as the midgut and the antennal glands become functional and the maxillary glands differentiate. Cytologically, the most ALP activity is associated with plasma membrane specializations, *i.e.* microvilli and channel-like membrane infolds. The gastric caeca and the middle midgut, as well as different portions of the antennal and maxillary glands, show differences in the intensity and distribution of their ALP reaction. When localized in the midgut cells, the latter is more sensitive to ALP inhibitors, particularly LPA, than in the antennal and maxillary glands. The electrophoretic ALP bands differ from one another in their inhibitor sensitivity.

The results demonstrate that during the development of *Artemia*, ALP activity consists of several enzyme forms. In early stages, some of them may participate in the cell metabolism, later, other alkaline phosphatases may be related to the morpho-functional differentiation of the digestive and excretory-osmoregulatory organs, playing a major role in active transport processes, at plasma membrane specializations. ALP of the midgut is different from the enzyme of the antennal and maxillary glands and has the characteristics of an "intestinal" microvillous ALP. Individual portions of the midgut and the glands appear to be differently specialized.

Introduction

Alkaline phosphatase (ALP) is one of the typical brush-border enzymes (Oda *et al.*, 1969) and can also be associated with other membrane specializations, such as the channel-like infolds close to mitochondria which are characteristic of cells performing active ion transport (Zaccone and Licata, 1981 ; Zaccone, 1983). The same cell with microvillous ALP can show cytoplasmic ALP activity depending on other phosphatases (Fuller and Owczarzak, 1967 ; Ono, 1975 ; Young *et al.*, 1981ab). Cytoplasmic and/or membrane ALP is also localized in several different

[1] This research was partially supported by a grant (60 %) of the MPI.

cell types which, as a common feature, have high metabolic activities, such as differentiating embryonic cells (Kaltenbach *et al.*, 1977 ; Whittaker, 1977 ; Swartz, 1982). The actual roles of the different alkaline phosphatases are still unclear. Membrane-bound ALP seems to participate in active transmembrane transport-mechanisms (Kaplan, 1972 ; Birge and Avioli, 1981). The brush-border enzyme also plays a role in contact digestion (Fuller and Owczarzak, 1967 ; Carone *et al.*, 1979 ; Ferreira and Terra, 1980). Cytoplasmic ALP is likely to correspond to several individual phosphatases which perform various functions (Lojda *et al.*, 1979 ; Swartz, 1982).

Developmental studies of ALP have been carried out mainly on vertebrates, where the enzyme is considered as a marker of the brush-border differentiation in the small intestine and the nephron (Moog, 1962 ; Webster and Harrison, 1969 ; Hugon, 1970 ; Ono, 1975 ; Calvert *et al.*, 1981 ; Neiss and Klein, 1981). Results comparable with those found in vertebrates have, however, been obtained from the digestive and excretory-osmoregulatory organs of larval and adult invertebrates (Arvy, 1969 ; Ferreira and Terra, 1980 ; Ferreira *et al.*, 1981 ; Raineri, 1985), inter alia crustaceans (Fuller and Owczarzak, 1967 ; Falugi and Raineri, 1978 ; Hryniewiecka-Szyfter and Tyczewska, 1980), and allow to carry morpho-functional cytological similarities up to the histoenzymic level. Many enzyme activities have been the object of biochemical investigations during *Artemia* development (Clegg and Conte, 1980), but the corresponding histochemical data are still very scarce. Such information can, however, integrate the biochemical findings to obtain a better knowledge of the brine shrimp developmental physiology, because it allows to correlate structure with function. For instance, aminopeptidase and acetylcholinesterase histochemically detected in *Artemia* embryos and larvae, have been proved to be good differentiation markers of microvilli-bearing cells and neuro-muscular cells respectively (Falugi, 1978 ; Falugi *et al.*, 1979 ; Raineri and Falugi, 1983). In view of these observations and considering that most microvillous aminopeptidase activities correlate with those of ALP, the present quantitative, histochemical, and electrophoretic study of ALP in brine shrimp embryos and larvae has been undertaken to follow the differentiation of structures specialized in active transport functions, such as the digestive tract and the excretory-osmoregulatory glands. As shown in other invertebrates (Morrill, 1973 ; Pfohl, 1975 ; Whittaker, 1977), the developmental quantitative/qualitive changes of ALP activity may be useful to investigate genic expression and its regulation. Partial preliminary results of this research have been published elsewhere (Falugi and Raineri, 1978).

Materials and methods

Cysts supplied by San Francisco Bay Brand, Inc., Menlo Park, Ca, USA, were used to obtain *Artemia* larvae. These were cultured in seawater and fed with *Chlorella* and yeast ; a few were raised up to the adult stage. The embryonic stages of morula, blastula, early and late gastrula, and prenauplii (Na O-Na 6) (Benesch, 1969) were obtained by dissecting the brood pouches of viviparous females followed by histochemical examination to localize ALP activity. Dehydrated and 18-h rehydrated cysts, early and late emergence (E_1 and E_2) (Nakanishi *et al.*, 1962), nauplii I and II, metanauplii I, II, III, and IV (Anderson, 1967), and the following six juvenile stages before the development of the leaflike gills, were investigated by both histochemical and biochemical procedures.

Homogenates

Rehydrated cysts were picked up with a small glass shovel, the emergent and larval stages with a glass pipette under a stereomicroscope. Excess water was eliminated by centrifugation at 1 500 × *g* for 5 min. Specimens (150-500) of the same developmental stage – fresh or stored for maximum a week at - 30 °C – were homogenized for 10 min at 4 °C in 1.0-1.5 ml of pasteurized seawater. The cyst shells were discarded by centrifugation at 2 000 × *g* for 10 min. To the crude homogenates 0.5 % Triton X-100 was added followed by homogenization for 10 min to prepare the samples for electrophoresis.

Quantitative assay

ALP activity of crude homogenates was determined colorimetrically following the procedure of Bessey *et al.* (1946), as modified by the Sigma Chemical Co. (1963) with p-nitrophenyl-phosphate as substrate, at pH 10.5. The absorbance was measured with a Bausch and Lomb Spectronic 20 at 410 nm wavelength, and the enzyme activity was expressed in Sigma Units (1 SU = 1 µM of p-nitrophenol released under the method's conditions) per mg of protein nitrogen, as determined by the xanthoproteic method of Millon-Nasse (Oser, 1965).

ALP histochemistry

Embryos and larvae, the latter after a prefixation in cold acetone for 15 min, were fixed either in 4 % calcium-formalin, or in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h at 4 °C. Then they were washed thoroughly in three changes of the same cacodylate buffer and stained by one of the following procedures : 1) Gomori's calcium-cobalt method, according to Barka and Anderson (1963), substrate Na β-glycerophosphate, at pH 9.2 ; 2) Burstone's (1961) simultaneous azo-coupling method, with naphthol AS-MX phosphate as substrate and Fast blue RR salt as azo-dye, both at concentration 0.5 mg/ml in 0.1 M veronal acetate buffer, pH 8.6 ; 3) McGadey's (1970) tetrazolium procedure, with 5-bromo-4-chloro-3-indoxyl phosphate, toluidine salt, as substrate, and Nitro BT as tetrazolium salt, at final concentrations 0.15 mg and 0.50 mg/ml respectively, in 0.1 M veronal acetate buffer, at pH 7.5, 8.2, 8.6 or 9.2. Controls were incubated in a medium without substrate, or pre-incubated for 30 min either in 5-25 mM L-phenylalanine (LPA), an inhibitor of intestinal microvillous ALP (Watanabe and Fishman, 1964), or in 0.1-0.5 mM (+)p- bromotetramisole oxalate (BTO), an inhibitor of most other types of ALP (Borgers and Thoné, 1975) ; the same inhibitor concentration was then added to the histochemical media. After staining, the embryos and larvae were embedded in methacrylate (JB-4, Polysciences, USA) and cut into serial 2 or 4 µm sections, using a LKB Pyramitome equipped with a glass knife ; a few specimens of each larval stage were mounted *in toto*.

Disc electrophoresis

It was made on 7.5 % polyacrylamide gel, according to a previously described procedure (Raineri and Falugi, 1983). Concentrated Triton X-100 extracts (0.8-1.2 mg protein N/ml) were centrifuged at 12 000 × *g* for 15 min at 4 °C in a B-20 International Refrigerated Centrifuge and the supernatants, diluted 1 : 1 with 40 % sucrose, were used as samples (200 µl for each tube). A current of 2.5-4 mA per tube at 4 °C was applied until the tracking dye (bromophenol blue) reached the end of the gels. Alkaline phosphatase from the small intestine of chickens (Type V,

Sigma Chem. Co., St. Louis, Mo (USA), 100 µg/ml in 40 % sucrose, was subjected to electrophoresis as control. The gels were then fixed for 15 min in 80 % ethanol at 4 °C, washed thoroughly in distilled water, and stained by Burstone's (1961) histochemical method for ALP, changing the medium after 40 min.

Results

Developmental quantitative changes of ALP activity

The enzyme activity was low in dehydrated cysts (0.44 SU/mg N) and increased during rehydration, being 1.28 and 2.55 SU/mg N after 18 and 24 h (= E_1-E_2 stage) respectively. At hatching the rise in ALP activity became faster and continued almost linearly during the naupliar stages (4.8 SU/mgN in the nauplius I and 12.5 SU/mg N in the nauplius II). The rate of increase was enhanced further starting from the metanauplius I stage (18.9 SU/mg N). Metanauplii IV showed an ALP activity (64.3 SU/mg N) that is about a 13-fold greater than nauplii I. These results are summarized in Table I and Figure 1.

Histochemical distribution of ALP activity

In the embryonic and early prenaupliar (Na O-Na 4) stages, a very faint ALP reaction was distributed in the different cell lines without being significantly different from one another. The histochemical product was mainly localized in the cytoplasm, most of it being associated with a few yolk platelets. In Na 5-Na 6 ALP activity was stronger in all the embryonic tissues, though it was more evident in the differentiating endodermal cells (rudiment of the midgut), where it was also associated with the plasma membranes. At emergence, both the midgut and the antennal glands showed a more intense histochemical staining (Fig. 2). In the midgut epithelium, a moderate ALP activity was associated with the baso-lateral plasma membranes. In the cytoplasm, granules of the histochemical product were found at the nuclear envelope and the yolk platelets, but they were much more numerous and similar to vesicles in their appearance at the apex of the cells. Here, a fully differentiated brush-border was not evident (Fig. 12). Each antennal gland consisted of two large, bean-shaped cells which face one another as to form the rudiment of the coelomic sac, and of a few smaller outer cells. The cells of the coelomic sac showed an ALP activity that was moderate at the nuclear envelope and the plasma membranes lining the lumen, that appears as a cleft. It was stronger where the coelomic cells come into very close contact with the outer cells at the extremities of the lumen. Numerous granules of the histochemical product were found in the cytoplasm, part of them being associated with yolk platelets (Fig. 10).

At hatching and during the nauplius I stage (Fig. 3 and 4), the histochemical staining increased further and showed changes in its cytological distribution both in the midgut and the antennal glands at the same time when these organs differentiated. As the yolk platelets decreased in number and eventually disappeared, the related enzyme reaction became less and less evident. In the midgut epithelium, vitellolysis occurred together with a rise and later a decrease in the number of the ALP-active granules or vesicles at the apex of the cells. Contemporaneously a brush border developed and showed an increasing histochemical staining (Fig. 13 and 14). ALP activity was stronger at the basal plasma membranes, where some infolds developed, but became fainter at the lateral membranes. In the antennal glands, ALP-active granules were less abundant

TABLE I

Alkaline phosphatase activity during embryonic and larval development

Hours of rehydration	Developmental stage	Number of determinations	SU/mg N Averages ± SD
0	Encysted gastrula	5	0.44 ± 0.07
18	Early differentiating nauplius (Na O-Na 4)	4	1.28 ± 0.45
24	Emergence (E_1-E_2)	2	2.55
32- 36	Hatching	3	3.60 ± 0.10
36- 50	Nauplius I	8	4.80 ± 0.57
50- 60	Nauplius II	7	12.50 ± 1.54
60- 80	Metanauplius I	9	18.90 ± 1.55
80-100	Metanauplius II	9	37.40 ± 4.60
100-120	Metanauplius III	8	53.07 ± 5.12
120-150	Metanauplius IV	6	64.31 ± 4.07

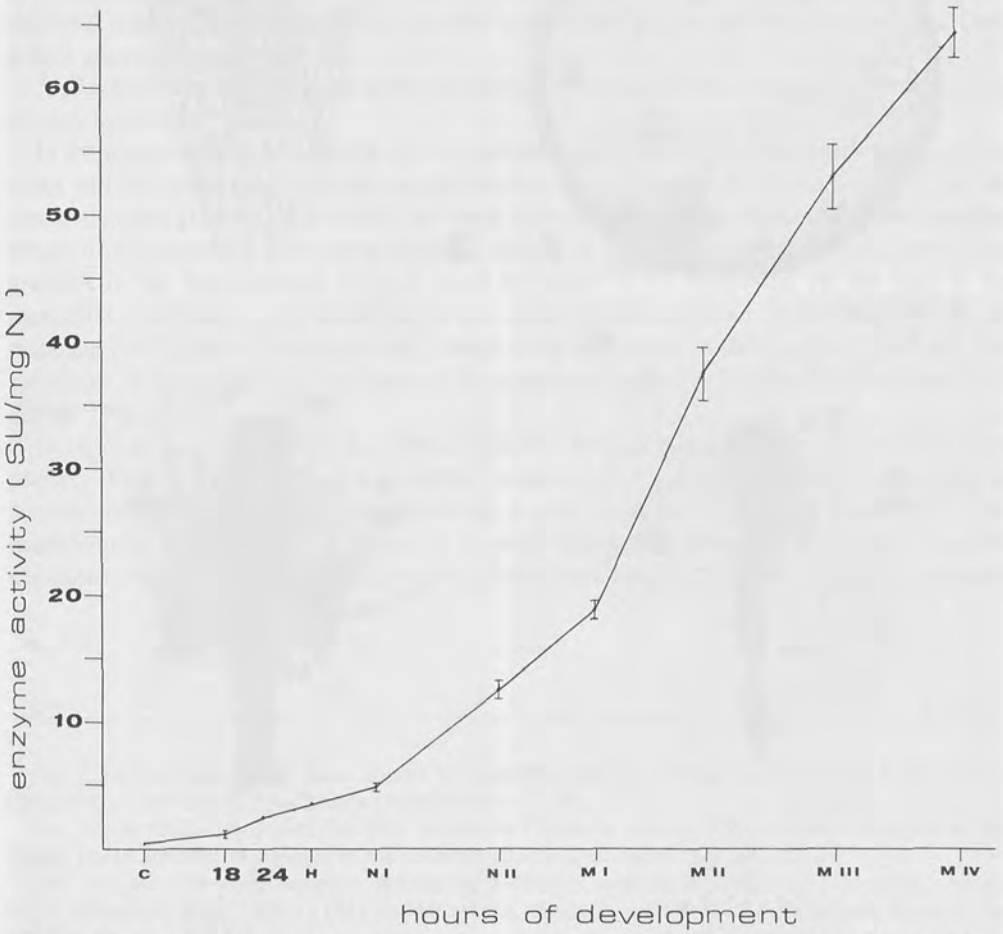

FIG. 1. Alkaline phosphatase activity during *Artemia* development. (C) : dehydrated cyst ; (H) : hatching ; (N I, N II) : first- and second-instar nauplius ; M I -M IV) : first-fourth-instar metanauplius. Each point is the mean of the determinations and the bars represent the standard deviations. (see "Materials and methods" and Table I).


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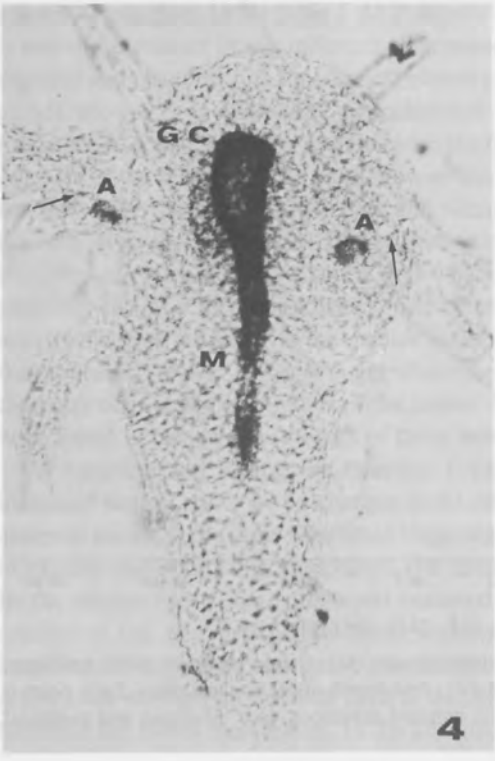
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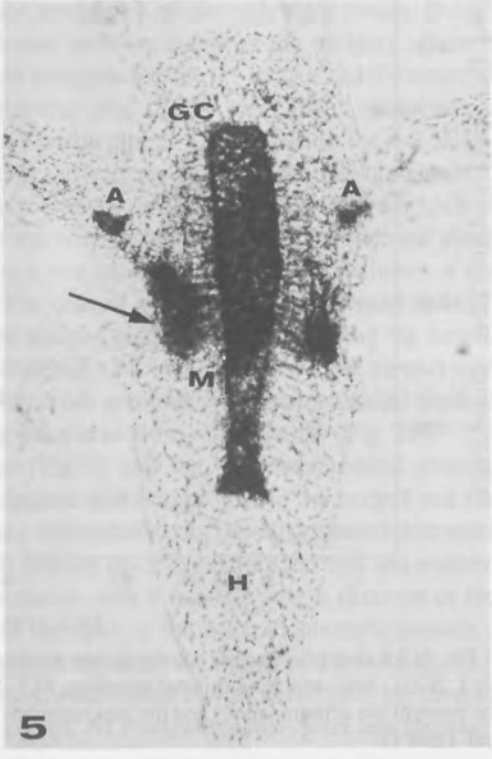
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in the cytoplasm, while a greater amount of the histochemical product was associated with the plasma membranes. A striation occurred that corresponds most probably to membrane infolds which are more developed in the outer cells, lining the early beginning of the excretory duct (Fig. 11).

In the nauplius II (Fig. 5) some differences in ALP activity distribution along the midgut were distinguished. The caudal portion had a lower brush border and was less histochemically stained. The strongest ALP reaction was shown by the middle midgut : most enzyme activity was localized in the brush border, particularly at its tip, and in the cell basal region, and appeared as a vertical striation. ALP-active granules were more numerous in the sovranuclear cytoplasm (Fig. 15). The gastric caeca had a lower brush-border with a more delicate appearance and a fainter histochemical staining. The ALP-active basal striation was less pronounced than in the middle midgut (Fig. 16). In the antennal glands, ALP staining was more evident at the plasma membrane infolds of the outer cells, which bend around the coelomic sac and line the U-shaped proximal tract of the excretory duct. Its distal portion has on the contrary, a thinner wall with a faint enzyme reaction (Fig. 8).

ALP activity was also localized in the rudiments of the gonads and a cluster of cells at the base of each mandible (Fig. 5).

In the metanauplius I and II (Fig. 6), the morpho-histochemical differences among the gastric caeca and the middle and posterior midgut became more pronounced (Fig. 15 and 16). In the antennal glands (Fig. 9) ALP activity was weak in the coelomic sac and stronger in the proximal tubule of the excretory duct, being localized mainly at the cell membrane infolds. Only a few granules of the histochemical product could be found in the cytoplasm. At the base of the mandibles, ALP-active cells developed as two bands which extend in caudal direction. In the metanauplius II a few of them enclosed a small cavity at the level of the maxillary segments. The rudiments of the gonads and the buds of the post-naupliar segments showed a moderate ALP activity (Fig. 6).

In more advanced larval stages, while the gastric caeca developed further as two deep outer pockets (Fig. 7), the same basic distribution pattern of ALP activity was seen in the midgut. In juvenile forms, the antennal glands contained a larger number of cytoplasmic granules of the histochemical product ; they had, however, no more detectable ALP activity in the last two stages examined here. As the maxillary glands differentiated they showed increased histochemical

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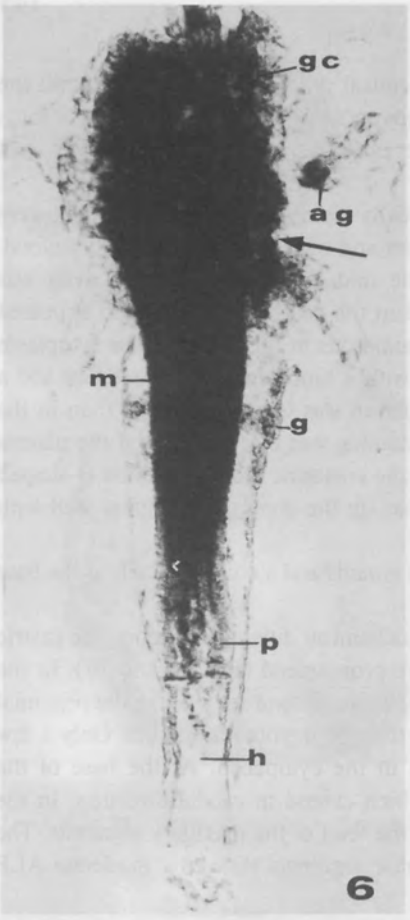
FIG. 2. Late emergence (E_2) stage stained by Gomori's method. ALP activity is stronger in the midgut (large arrow) and the antennal glands (small arrows). × 140.

FIG. 3. Just hatched first-instar nauplius stained by Gomori's method. ALP activity is moderate in the midgut (large arrow) and stronger in the antennal glands (small arrows). × 200.

FIG. 4. Late first-instar nauplius stained by Gomori's method for ALP. (A) antennal glands ; (GC) developing gastric caeca ; (M) middle midgut. Arrows : rudiments of the excretory ducts of the antennal glands. × 200.

FIG. 5. Nauplius II stained by Gomori's method. (A) antennal glands ; (GC) gastric caeca ; (M) middle midgut ; (H) hindgut. Arrow : early rudiments of the gonads and other ALP-active cells (mandibulary glands ? See text). × 180.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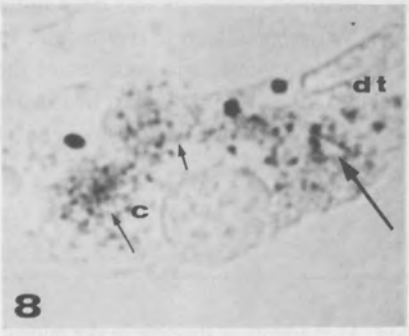
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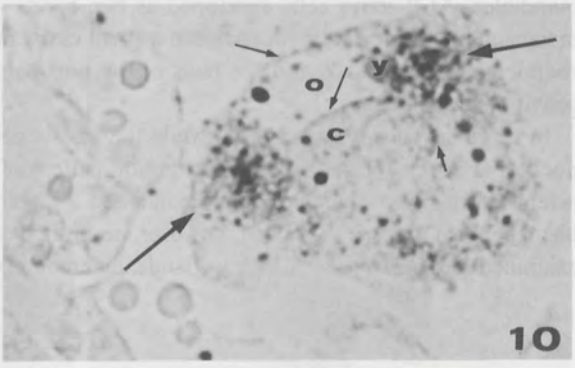
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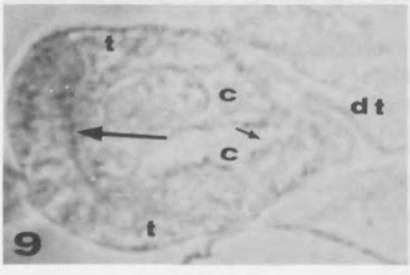
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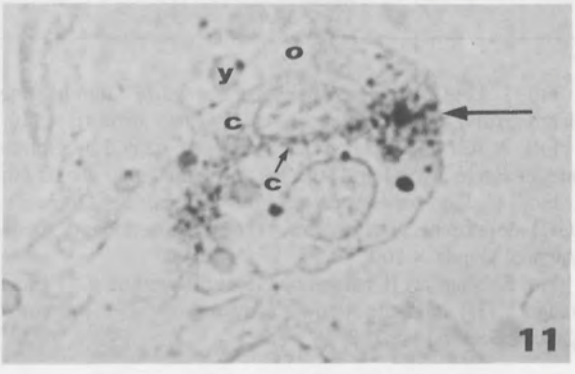
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staining. In juvenile stages (Fig. 17) their coelomic sac had a thin wall with a moderate ALP activity, while the proximal portion of the excretory duct, which is coiled around the sac, consisted of taller, more intensely stained cells. Their ALP activity was mainly localized at the luminal surface, where a brush border seemed to exist, and in the basal region, where a striation could be distinguished. The lumen was much wider than in the antennal glands and in many cases contained roundish concretions (Fig. 18). In the distal portion of the duct the cells were lower, with no evident brush border nor basal striation, and showed a faint histochemical staining (Fig. 17).

In late metanauplii and juvenile stages, ALP activity was localized in branched, neuron-like cells which formed a network inside the upper lip. A few were associated with the mouth and the foregut wall (Fig. 19 and 20).

The three histochemical methods gave comparable results. The best ones, however, have been obtained with McGadey's (1970) procedure, as it revealed more sites of ALP activity with sharper localizations of the histochemical product. The Gomori method gave rise to some aspecific diffuse staining of the nuclei, the yolk platelets, and the cuticle. These non-enzymic localizations were not considered in the above description.

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Fig. 6. Metanauplius I stained by McGadey's method for ALP. (ag) antennal gland ; (g) rudiments of the gonads ; (gc) gastric caeca ; (h) hindgut ; (m) middle midgut ; (p) posterior midgut. Differences in the enzyme distribution along the alimentary tract can be observed ; the differentiating metanaupliar segments show a moderate ALP activity. × 130.

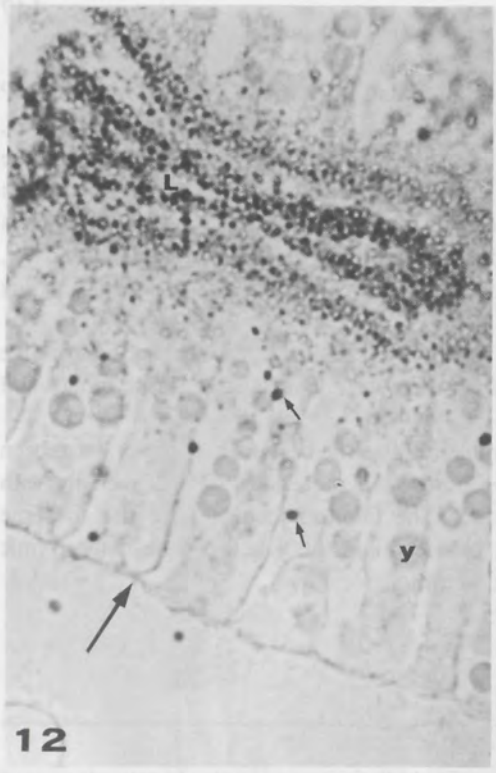
Fig. 7. Third juvenile stage, oblique 4 µm section through the cephalic region stained by McGadey's method. (e) paired eye ; (g) nervous ganglion. The gastric caeca are cut at different levels, the upper one in the figure at its cephalic end, the lower one at the confluence with the midgut ; a difference in their ALP distribution is observed. Large arrow, brush border ; small arrows, basal ALP-active striation (membrane channel-like infolds). × 550.

Fig. 8. Antennal gland of a second-instar nauplius, 2 µm section stained by McGadey's procedure. (c) cell of the coelomic sac, which shows a large nucleus and a moderate amount of cytoplasmic ALP-active granules ; (dt) distal tubule of the excretory duct. Large arrow, proximal tubule transversely cut and its lumen ; middle arrow, proximal tubule tangentially cut : a striation (plasme membrane infolds) is distinguished ; small arrow, luminal cleft of the coelomic sac. × 1500.

Fig. 9. Antennal gland of a second-instar metanauplius. 2 µm section stained by Burstone's method for ALP. (c) cells of the coelomic sac, whose plasma membranes (small arrow) line the lumen ; (dt) distal tubule of the excretory duct ; (t) proximal tubule, which is bent around the coelomic sac and shows ALP-active plasma membrane infolds (large arrow). × 1300.

Fig. 10. Antennal gland of an emerging nauplius, 2 µm section stained by McGadey's method. (c) cell of the coelomic sac and its nucleus, which shows some ALP activity at its envelope (small arrow) ; (o) outer cell (early rudiment of the proximal tubule) with a moderate enzyme staining at the plasma membrane (middle arrows) ; (y) yolk platelet with ALP-active granules at its boundary. Large arrows, early beginning of the proximal tubule of the excretory duct. A conspicuous amount of histochemically stained granules is seen in the cytoplasm. × 1500.

Fig. 11. Antennal gland of a first-instar nauplius, 2 µm section stained by McGadey's method. (c) cells of the coelomic sac ; (o) outer cell and its nucleus ; (y) ALP-active yolk platelet. Small arrow, ALP-active plasma membranes lining the lumen of the coelomic sac ; large arrow, early beginning of the proximal tubule of the excretory duct. × 1500.



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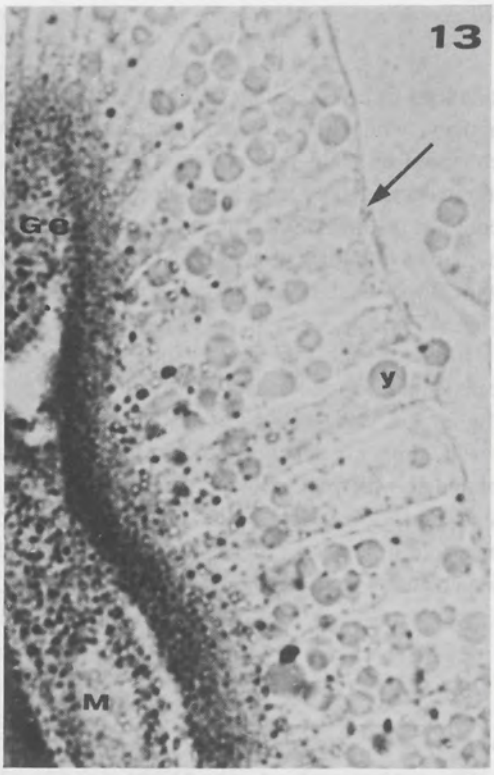

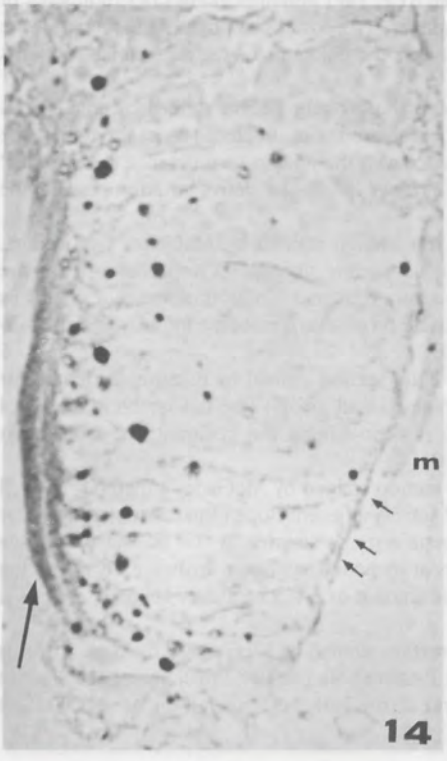

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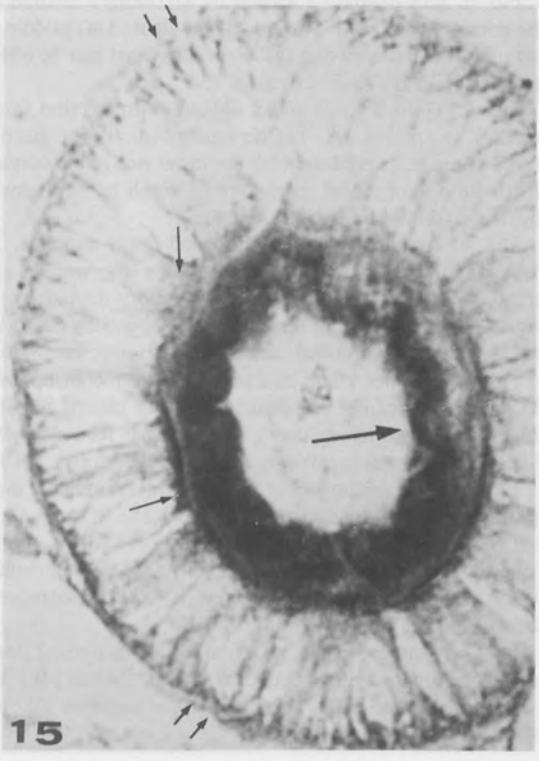

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At pH 7.5-8.2, ALP reaction of the midgut cells was less evident in the brush border than at higher pH. The cytoplasmic staining was slightly diminished, though it did not show apparent variations at the yolk platelets. Such a yolk-bound histochemical reaction is inhibited partly by 0.5 mM BTO, and not at all by LPA. In Na 5- Na 6, 25 mM LPA significantly decreased the enzyme staining of the endodermal cells. In the midgut of the larvae it completely inhibited ALP reaction in the brush border, and to a large extent in the cytoplasm. Any brush border ALP staining in the gastric caeca was abolished by 5 mM LPA, but a less evident effect was observed in the middle midgut. Such a difference was more pronounced at the highest pH. In the cytoplasm of the midgut cells, ALP reaction was significantly inhibited by 0.5 mM BTO, but less in the brush border. The inhibition was less pronounced with 0.1 mM BTO, and almost inexistent at pH 7.5. At the higher concentrations, both inhibitors partially decreased the enzyme reaction of the maxillary glands, and even less that of the antennal glands. In juvenile stages the latter was almost insensitive to LPA and BTO.

Electrophoretic developmental pattern of ALP activity

In both dehydrated and rehydrated cysts, a single electrophoretic region (A) of ALP activity migrated near the cathode. In most cases, it consisted of two bands (A', A'') which were very close to one another. At hatching, a second, faster migrating band B was detected by employing the most concentrated extracts, and became more and more evident in the first- and second instar nauplii also with more diluted samples. In some cases, a third, more anodal band C was seen in nauplii II though it was more often detected and was stained more intensely in metanauplii. In metanauplii IV and in the early juvenile stages the fastest band D could be distinguished. Bands A'-A'' and B, however, were the only ones to be stained in late juvenile forms, and in some cases bands C and D of metanauplii had not been visualized even by using concentrated extracts.

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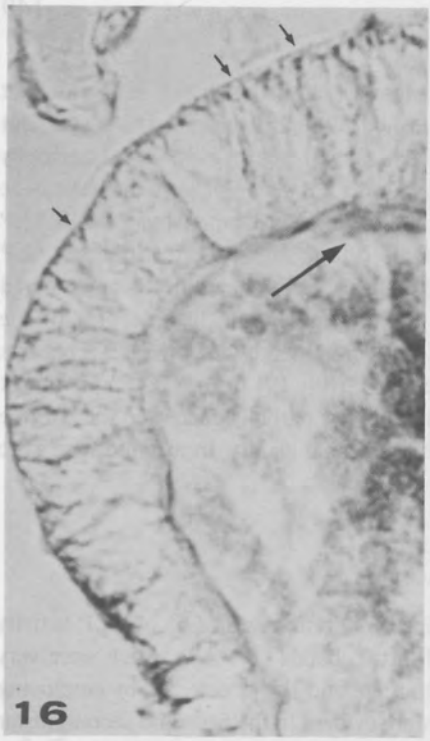
Fig. 12. Midgut epithelium of an emerging nauplius, 2 µm section stained by McGadey's procedure. A narrow lumen (L), but no well-defined brush-border is seen. Many ALP-active, vesicle-like structures are localized at the apex of the cells, and other histochemically stained granules (small arrows) are found deeper in the cytoplasm, part of them being associated with yolk (y). Large arrow : ALP activity at the basal and lateral cell membranes. × 1500.

Fig. 13. Midgut epithelium of a just hatched nauplius, 2 µm section stained by McGadey's method. (GC) gastric caeca ; (M) middle midgut ; (y) yolk platelet. Arrow : ALP activity at the basal plasma membranes. Compare with Fig. 12 to note the stronger enzyme staining at the apex of the cells and the wider lumen of the gut. × 1500.

Fig. 14. Midgut epithelium of a late first-instar nauplius, 2 µm section stained by McGadey's method. ALP activity at the apex of the cells is strongly diminished, while a brush border (large arrow) is distinguished ; it is lacking in the posterior midgut next to the future confluence with the hindgut (bottom of the figure). Small arrows : developing basal plasma membrane infolds. (m) muscle fibre. × 1500.

Fig. 15. Transversal 4 µm section through the middle midgut of a metanauplius II, stained by McGadey's procedure. Large arrow : strongly ALP-active brush border ; middle arrows : ALP activity at the apex of the cells ; small arrows : basal plasma membrane infolds. A very similar enzyme distribution pattern is found in the midgut epithelium of nauplii II – metanauplii I, which, however, still contains some yolk. × 1500.



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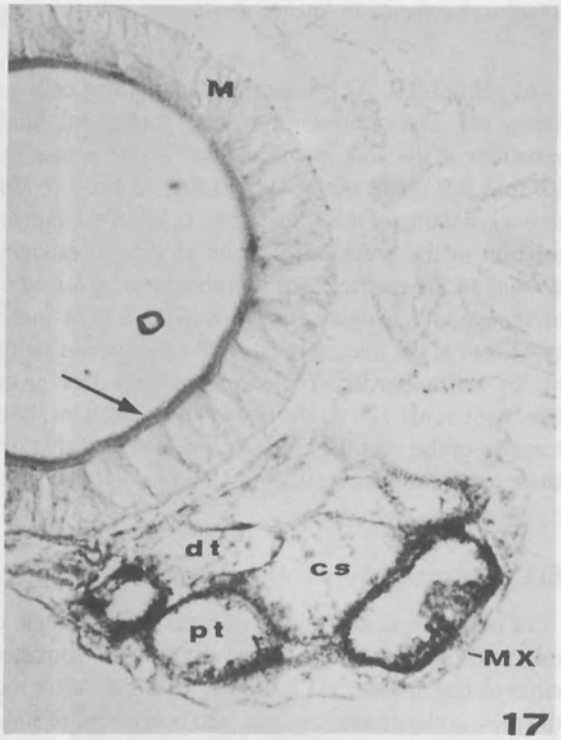
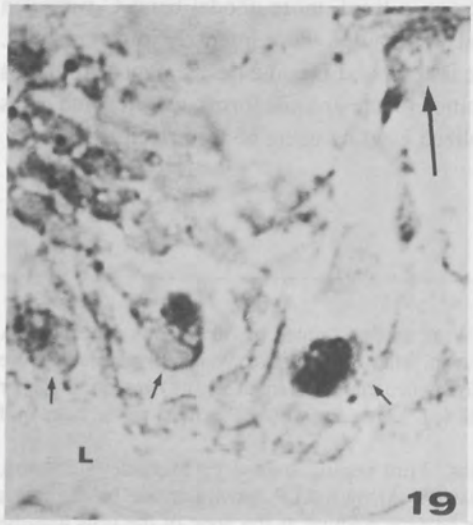
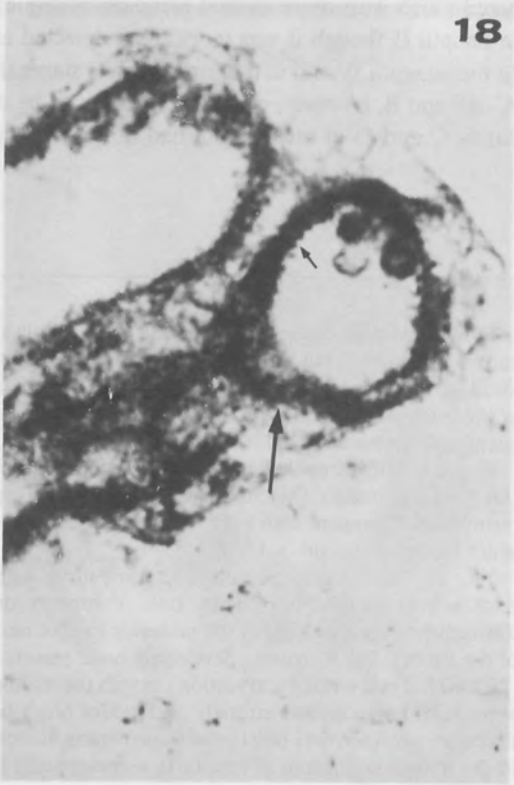


17



19



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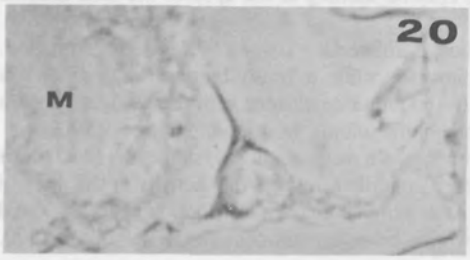

20

Band B was completely inhibited by 25 mM LPA and most of its staining was suppressed at 5 mM concentration. No evident effect was noted on bands A'-A". Bands A'-A" were strongly inhibited by 0.5 mM BTO, which was not the case for band B. The staining of bands C and D was slightly diminished by both inhibitors at the higher concentrations without being significantly different from one another. These results are shown in Fig. 21.

Type V Sigma chicken intestinal phosphatase separated two bands : the more conspicuous one migrated just in the same region as band B, the other was thinner and slightly more cathodal.

Discussion

The results show that various alkaline phosphatases are active in different developmental stages of *Artemia*, and that the marked increase in the enzyme activity which starts about at hatching together with the occurrence of new electrophoretic bands, correlates with a histochemical localization of most ALP in cells specializing in active transport functions.

In early embryos ALP activity is low and corresponds to less electrophoretically mobile bands and to a faint cytoplasmic histochemical staining that is spread in all the embryonic areas. In other early invertebrate embryos ALP activity shows similar characteristics and consists of several enzyme forms. Most of them appear to be bound to cell membranes, such as the endoplasmic reticulum, the Golgi apparatus and Golgi-derived vesicles, owing also to a lesser inactivation by fixatives in comparison with soluble alkaline phosphatases (Morrill, 1973 ; Pfohl, 1975 ; Whittaker, 1977). This "early" embryonic ALP (Pfohl, 1975) may participate in metabolic processes related to histogenesis (Maekawa and Yamana, 1975 ; Kaltenbach *et al.*, 1977), or be a precursor of the plasma membrane enzyme that becomes more evident later in development (Mulnard and Huygens, 1978 ; Swartz, 1982). In *Artemia*, similar functions may be ascribed to ALP activity both of early embryonic tissues and the rudiments of the metanaupliar segments and the gonads. As for the latter, ALP in vertebrates is considered a marker of the primordial germ cells, its functions, however, are still unknown (Swartz, 1982). Other phosphatases with an acidic

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FIG. 16. Portion of a gastric caecum in a second-instar metanauplius, 4 µm section stained by McGadey's procedure. Large arrow : brush border with a moderate ALP activity ; small arrows : basal plasma membrane infolds. × 1500.

FIG. 17. Late juvenile stage, 4 µm section stained by Burstone's method. (M) : middle midgut with a strongly ALP-active brush border (arrow) ; (MX) maxillary gland, whose proximal tubule, that is seen here in the lower portion of the gland (pt), is stained more intensely than the coelomic sac (cs) and the distal tubule (dt). × 400.

FIG. 18. Late juvenile stage, 4 µm section through the coiled proximal tubule of a maxillary gland ; Burstone's method for ALP. Large arrow : basal striation (= plasma membrane infolds) ; small arrow : brush border. Roundish bodies are found into the lumen. × 1800.

FIG. 19. Metanauplius IV, 2 µm section through the upper lip and the foregut ; McGadey's staining for ALP. Three cells (small arrows) having a pyriform body, slender processes, and containing many ALP-active granules, are associated with the foregut's wall ; (L) lumen of the foregut. Another similar, histochemically stained cell is observed above (large arrow). × 1600.

FIG. 20. Juvenile stage, 2 µm section through the mouth region ; McGadey's staining for ALP. A histochemically stained, branched cell is associated by its processes with the mouth opening (M). × 1700.

optimum pH can be associated with yolk platelets and have a role in the hydrolysis of their components (Cone and Eschenberg, 1966 ; Tsusaka, 1967) and possibly as well in the genesis of biomembranes (Moon and Morrill, 1979). The yolk platelets of *Artemia* can give rise to membrane-bound organelles (Marco *et al.*, 1980) and the breakdown of their storage molecules, such as threhalose, can involve a phosphorylytic cleavage (Clegg and Conte, 1980). Their phosphatase staining, which increases in early differentiating cells when yolk is utilized for morphogenesis, may also depend on phosphatases other than ALP, since it is evident at pH 7.5 and nearly insensitive to ALP inhibitors. The platelets stain, indeed, more intensely for acid phosphatase activity (Falugi and Raineri, 1978), that might be visualized, together with other phosphatases, by the histochemical methods employed here (Mulnard and Huygens, 1978 ; Lojda et al., 1979).

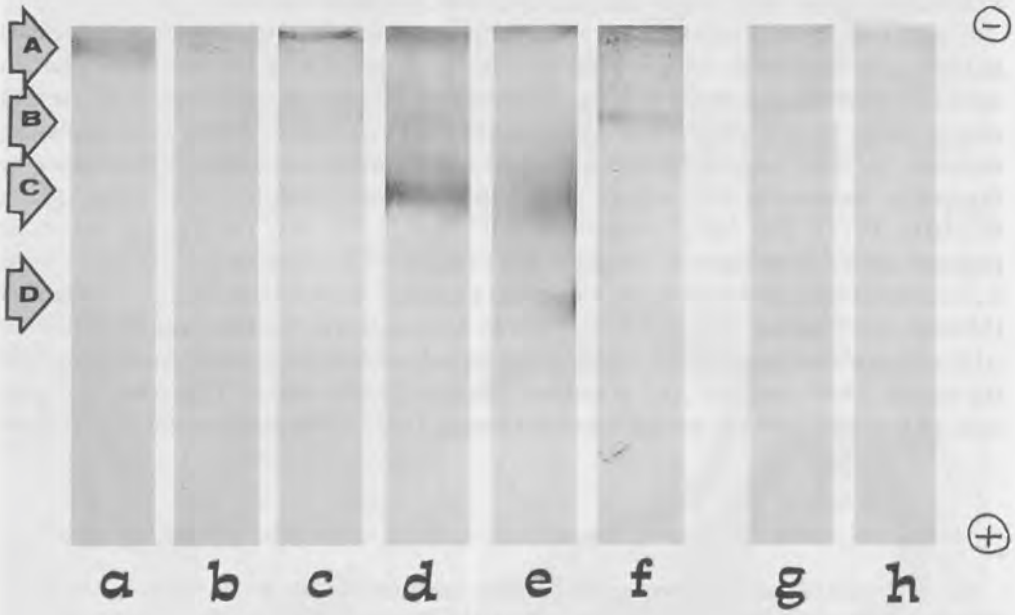

FIG. 21. Acrylamide gels after electrophoresis and staining by Burstone's procedure for ALP. (a) dehydrated cyst ; (b) late emergence – hatching ; (c) nauplius I ; (d) metanauplius II ; (e) metanauplius IV ; (f) late juvenile stages, and controls of the same with the inhibitors 0.5 mM BTO (g) and 25 mM LPA (h). (A, B, C, D) regions where electrophoretically mobile bands of ALP migrate in the different developmental stages.

At emergence, together with a rapid rise of ALP activity and the appearance of the electrophoretic band B, the developing midgut and antennal glands show a stronger ALP reaction that is increasingly associated with plasma membranes. Alkaline phosphatases from various species and tissues differ in a number of properties (Chang and Moog, 1972), so that a detailed comparison cannot be made. In agreement with the present findings, the development of ALP activity shows,

however, similar characteristics in *Artemia* as in other invertebrates and even vertebrates (Urbani, 1962). Other basically similar results have been obtained in invertebrates (Morrill, 1973 ; Pfohl, 1975 ; Whittaker, 1977) and vertebrates (Maekawa and Yamana, 1975). In the latter, ALP activity increases rapidly at the end of gastrulation, while the endodermal cells express a new ALP molecular form. The highest ALP activity becomes localized in the differentiating midgut and excretory-osmoregulatory glands (Arvy, 1969), being associated mainly with their plasma membrane specializations. In *Artemia* the appearance of a new, "intestinal-like" ALP is indicated by the sensitivity to LPA shown by both the midgut enzyme staining and band B, and possibly also by the electrophoretic mobility of the latter, which is very similar to that of the chick small intestinal phosphatase. In early nauplii, "intestinal" ALP may be, partly at least, in a precursor form, cytochemically visualized as the ALP-active granules or vesicles at the apex of the midgut cells, since they have not yet a fully differentiated brush-border. In developing small intestinal cells of vertebrates, ALP-active apical vesicles can reflect either exocytosis of new enzyme molecules bound to Golgi-derived membranes, inserted at the cell surface (Kaltenbach *et al.*, 1977 ; Aihara *et al.*, 1982), or absorption of nutrients (Ono, 1975). They decrease in number together with other changes in ALP cytochemical distribution, as cell differentiation goes on. In the brine shrimp larval midgut, comparable variations of ALP histochemical pattern can be related to the morpho-functional differentiation of the cells, particularly at the plasma membrane level. In the second-instar nauplius, when the alimentary tract is completed and feeding begins (Anderson, 1967), most ALP activity is indeed associated with microvilli and basal membrane infolds, *i.e.* cytological specializations for active transport functions. Microvillous ALP, that is co-distributed with aminopeptidase (Falugi, 1978), may play a digestive role and participate in the final breakdown and absorption of nutrients. As found in other intestinal cells (Lojda *et al.*, 1979 ; Young *et al.*, 1981ab) and suggested here by the different inhibitor sensitivity, some cytoplasmic ALP activity can depend on enzymes other than the microvillous one. At the apex of the cells, ALP may be associated with vesicles related to absorption and/or secretion (Hootman and Conte, 1974 ; Schrehardt, 1987). At the basal membrane infolds the enzyme may be involved in transmembrane transport related to osmoregulation (Hootman and Conte, 1974 ; Conte, 1984). Other osmoregulatory structures, such as the rectal pads of isopods (Hryniewiecka-Szyfter and Tyczewska, 1980) and tardigrades (Raineri, 1985), and the ionocytes of fish (Zaccone and Licata, 1981 ; Zaccone, 1983) show ALP activity at their channel-like membrane infolds. No ALP reaction is, however, detected in the salt gland of *Artemia*, although it is the major larval osmoregulatory organ together with the midgut and has similar cytological specializations (Conte, 1984). The reasons of such a discrepancy are not clear. The actual roles of different phosphatase activities in transport mechanisms are indeed not fully understood. In some osmoregulatory cells, for instance, nitrophenylphosphatase but no ALP, is histochemically detected at the plasma membrane channels (Zaccone, pers. commun.).

The present results also show that the midgut and the antennal and maxillary glands of *Artemia* have different ALP activities. Moreover, various cytological features and ALP distribution in individual portions of these same organs suggest a local specialization.

Some differences of the histochemical pattern along the developing midgut may depend on differentiation, since it proceeds in caudal direction involving both the hydrolysis of yolk and the appearance of the ALP-active plasma membrane specializations. Morpho-histochemical peculiarities of the gastric caeca are, however, most evident in late metanauplii and juvenile forms, therefore seemingly corresponding to different functions. The gastric caeca actually differ from

the middle midgut in a number of cell characteristics and are the equivalent of the hepatopancreas or digestive glands of other crustaceans (Hootman and Conte, 1974 ; Schrehardt, 1987). Because the latter also show ALP activity (Snyder and Green, 1970 ; Krysztofiak and Klepke-Van Beckhoven, 1980), the enzyme histochemically detected in *Artemia* may be related to comparable functions, such as among others secretion of digestive enzymes, storage, and metabolic transformations of nutrients (Schrehardt, 1987). As suggested by its inhibitor sensitivity and optimum pH, ALP of the gastric caeca might be different from the middle midgut enzyme. A better quantitative/qualitative characterization of their ALP activities is, however, necessary, since different intensities of the histochemical staining have also been observed in the tests.

The higher quantitative increase in ALP activity which starts at the metanauplius I stage, as well as the appearance of the electrophoretic bands C and D which are no more detected in juvenile forms, may reflect further differentiation of the midgut and the antennal glands, and the development of the post-naupliar segments with their structures, particularly the maxillary glands. Both in vertebrates and invertebrates, comparable morphogenetic processes occur together with a rise of ALP activity and changes in the phosphatase isozyme patterns (Webster and Harrison, 1969 ; Morrill, 1973 ; Pfohl, 1975). In the brine shrimp larvae, however, the latter may also depend on physiological events, as molting (Chang and O'Connor, 1983), involving an increase in soluble, more electrophoretically mobile phosphatases. A study of the relationships between ALP activities and physiological conditions of the larvae requires a better synchronized material and more carefully controlled culturing conditions. Because acid phosphatase bands of metanauplii have the same electrophoretic mobilities as bands C and D (Falugi and Raineri, 1978), an interference between these enzymes cannot be excluded (Lojda *et al.*, 1979). Several phosphatases can coexist in the antennal and maxillary glands, which show histochemically detectable acid phosphatase (Falugi and Raineri, 1978), and are likely to have ATPase activity, as found at the membrane infolds of other osmoregulatory organs of *Artemia* (Conte, 1984). This would explain the relative insensitivity of their enzyme staining to ALP inhibitors and the lack of a clear electrophoretic equivalent. Similar results have been obtained in the antennal gland of a crayfish : acid phosphatase is localized in the coelomic sac, both acid and alkaline phosphatases are, however, found in the labyrinth, and it is difficult to draw a sharp distinction here between the two enzymic activities by light-microscopic histochemistry (Fuller and Owczarzak, 1967). In agreement with ultrastructural findings (Tyson, 1968, 1969), in the antennal and maxillary glands of *Artemia* both ALP histochemical pattern and cytological features indicate that the excretory duct in its proximal region is more specialized for active transport functions than the coelomic sac and the distal tubule. The presence of concretions into the lumen, and of aminopeptidase activity in the brush border (Falugi, 1978), seem to indicate an excretory role involving the breakdown of molecules, besides the osmoregulatory function (Conte, 1984).

Further ultrastructural studies would allow a deeper insight in the physiology of ALP-active structures in *Artemia*. They could for instance show, if the histochemically stained cells that appear at the base of the mandibles, correspond either to functional mandibulary glands (Conte, 1984), or to the earliest mesenchimal primordium of the maxillary glands which will develop caudally. In juvenile stages, the regression of the antennal glands might involve a shift from alkaline to acid phosphatase activity, corresponding to the appearance of histochemically stained granules in the cytoplasm. The ALP-active branched cells associated with the mouth and the foregut wall might be neuro-sensory organs, as found in other invertebrates (Raineri, unpubl.).

As shown previously (Falugi, *et al.*, 1979 ; Raineri and Falugi, 1983), the present research confirms that a combined biochemical and histochemical approach to study some enzyme activities, which are good differentiation markers, can be useful for obtaining a better knowledge of the brine shrimp developmental physiology. As in other invertebrate embryos and larvae (Morrill, 1973 ; Pfohl, 1975), the major increase in ALP activity in *Artemia* appears to depend on new enzymic molecular forms to be related to the function rather than to the early differentiation of the organs where they are localized. As generally accepted, the appearance of stage-specific enzymes can reflect selective gene expression (Bagshaw, 1980) in the brine shrimp development. It is, however, necessary to ascertain if the appearance of the "intestinal" ALP at the end of pre-emergence development depends on the transcription of specific genes in the endodermal cells, or on the translation of a preformed corresponding mRNA as found, for instance, in the endodermal ALP of ascidian embryos (Whittaker, 1977). The synthesis of a protein molecule can moreover precede the appearance of its enzyme activity. The development of ALP activity can be modulated by intracellular orthophosphate and other microenvironmental conditions, and require the enzymic protein to be bound to carbohydrate groups and be assembled into cell membranes at the level of the Golgi apparatus (Wachsmuth and Thorhorst, 1974 ; Pfohl, 1975 ; Bjerknes and Cheng, 1981).

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Occurrence and properties of the glycoprotein artemocyanin

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Abstract

Artemocyanin, found in adult *Artemia*, is a glycoprotein with a blue-green colour due to a bound bilatriene pigment. Artemocyanin I has been found in aspirated haemolymph and artemocyanin II in homogenised shrimps. Artemocyanin II is a trimer approximately 25 nm in diameter, $s^{o}_{20,w}$ = 27.3S, M_r = 1 200 000. The two artemocyanins give a reaction of identity on fused rocket immunoelectrophoresis.

Artemocyanin *in vivo* comprises three monomers each containing two M_r 190 000 polypeptides. Specific cleavages lead to the observation of additional species in preparations *in vitro*. Thus, isolated artemocyanin I contains M_r 190 000 and some 104 000 and 87 000 polypeptides. Principal components of artemocyanin II from homogenates are M_r 104 000, 87 000, and 67 000.

The M_r 67 000 subunit has proteolytic activity and is responsible for degradation of *Artemia* haemoglobin preparations and for uncertainty about haemoglobin structure. The proteolytic activity is potentiated by exposure to SDS and is displayed by the artemocyanin II trimer, dimer, or monomer. This M_r 67 000 subunit appears to be derived from the M_r 87 000 subunit.

Artemocyanin I is not proteolytic. Its M_r 190 000 subunit has been experimentally cleaved with proteases of *Artemia* origin to yield, ultimately, a subunit of M_r 67 000 without proteolytic activity.

Electron microscopy, dissociation studies in the analytical ultracentrifuge and non-dissociating gel experiments indicate a trimeric structure, whereas the 190 000 M_r of the major polypeptide indicates the presence of six molecules. Models incorporating these relationships are discussed.

Introduction

Artemocyanin was discovered in 1980 (Krissansen *et al.*, 1980) when it was identified as the agent responsible for proteolytic degradation of haemoglobin while the latter was being isolated from *Artemia* homogenates. Degradation was thus shown to be the basis of previous uncertainty as to the subunit structure of *Artemia* haemoglobin (Krissansen *et al.*, 1981a). The responsible protease was itself recognised as being an unusual and potentially interesting molecule, having a blue colour, a high molecular weight and a requirement to react with SDS before becoming proteolytic (Krissansen *et al.*, 1981b). We have therefore studied its structure, the nature of its chromophore, its origin and activation. Two different forms of the molecule have been distinguished (Krissansen *et al.*, 1983a). Artemocyanin I is the molecule as found in uncontaminated haemolymph, whereas the related molecule isolated from homogenised whole *Artemia* we call artemocyanin II. Artemocyanin I can, however, be recovered from homogenates by taking special precautions and working swiftly.

Materials and methods

Isolation of artemocyanin II

A detailed protocol for the preparation of artemocyanin II has been published by Krissansen *et al.*, (1983b). A brief summary follows. Adult *Artemia* (300 g) were homogenised in 300 ml buffer and 0.3 mg/ml soybean trypsin inhibitor at 0-4 °C, filtered and centrifuged for 30 min at 16 000 × *g*. By fractional precipitation with polyethylene glycol 6 000, artemocyanin was separated from glutinous orange material and recovered, first in a 6-8 % (w/v) cut and then in 3-6 % fractions. It was applied to DEAE-Sephadex A-50 and eluted in 50 mM Tris-Cl, 250 mM NaCl, pH 7.6, then concentrated to 9.5 ml by ultrafiltration. At this stage the material could be stored at - 80 °C.

Further purification was achieved on preparative polyacrylamide gels (Gabriel, 1971) containing 8 ml of 10 % acrylamide and 16 ml of 5 %, at 4 °C. Visible artemocyanin was excised, eluted, and applied to a ConA-Sepharose 4B column, then desorbed with 50 mM Tris-Cl, pH 7.6, 125 mM NaCl, 200 mM α-methyl-D-glucoside (3 ml/h) and reconcentrated. A polyacrylamide gel electrophoresis step was repeated.

Isolation of artemocyanin I

Haemolymph was extracted into buffer containing soybean trypsin inhibitor by inserting a glass needle into *Artemia* tails under a binocular microscope. The sex of the shrimps was noted. Haemocytes were centrifuged off. A micro-scale purification of up to 50 aspirates was performed by ConA-Sepharose 4B chromatography and electrophoresis.

Results and discussion

Observation of the degradative effects of artemocyanin

Adult *Artemia* haemoglobin, during purification, suffered extensive degradation that could be prevented by the presence of stabilisers and proteolysis inhibitors, *e.g.*, thioglycollate (10 mM) with soybean trypsin inhibitor (0.2 mg/ml) and KCN (2 %) (Krissansen, 1983). Nevertheless, haemoglobin purified with proteolysis inhibitors present and appearing intact on non-dissociating polyacrylamide gels, still appeared extensively degraded when run on SDS-containing denaturing gels. This could have indicated earlier nicking, but the anti-proteolytic precautions and the intactness of the haemoglobin on non-dissociating gels (Fig. 1a) argued against it. A crucial finding was that haemoglobin excised accurately from a non-dissociating gel and then run on an SDS gel remained intact.

A triplet of minor bands (A, B, C in Fig. 1a) from the non-dissociating gel running slower than haemoglobin was shown to contain the haemoglobin-degrading activity, but this was only expressed on SDS gels or after contact with denaturant. The proteolytic activity had an optimum at pH 8 (range 7-11) and was lost within 30 min if heated to 50 °C with SDS. The dependence of proteolysis on the concentration of SDS or other denaturants is shown in Fig. 2.

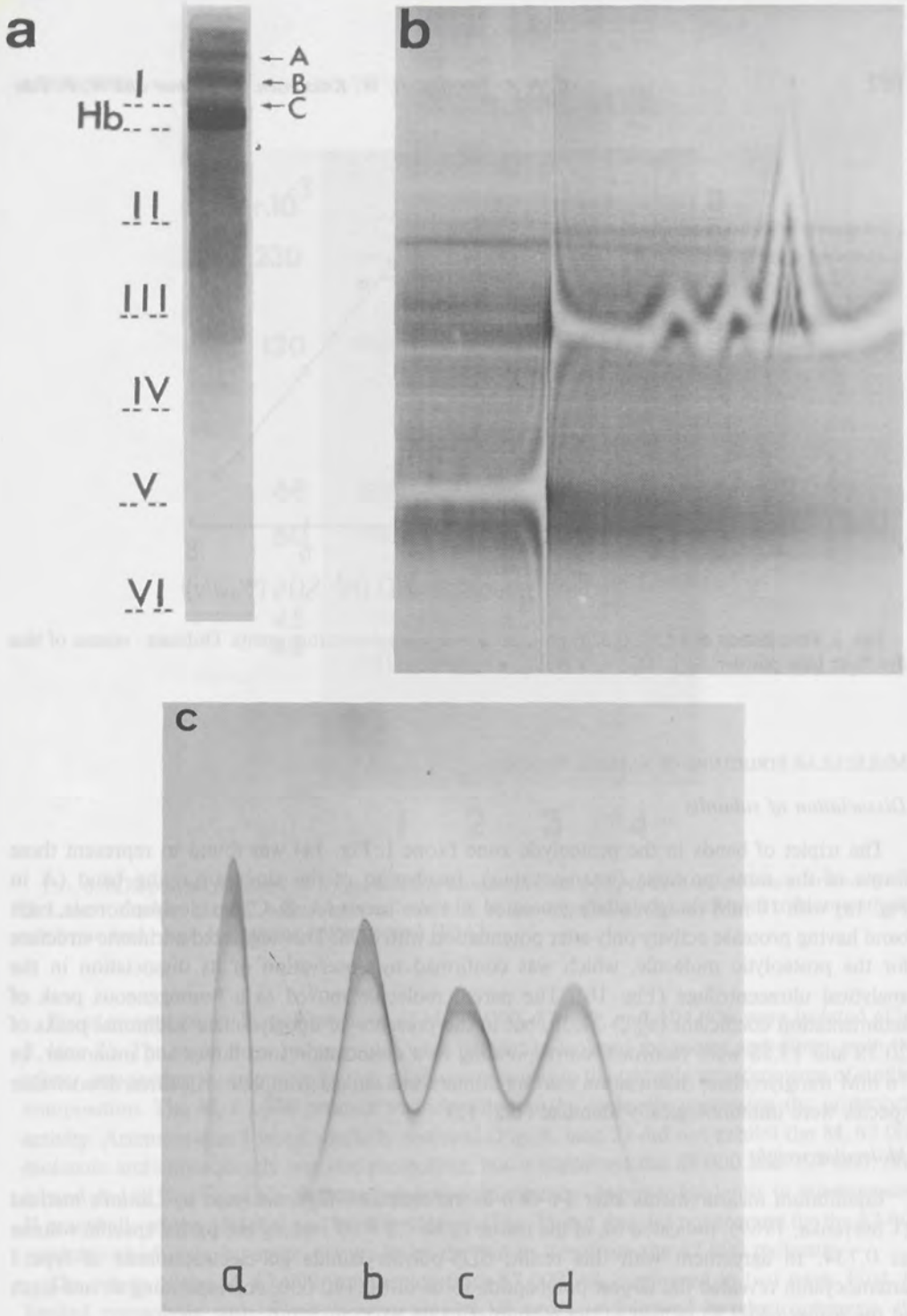

FIG. 1. a. Non-dissociating 10 % polyacrylamide gel of haemoglobin previously purified in the presence of stabilizers, heavily loaded to show artemocyanin proteases (A, B, C, being trimer, dimer, monomer). Zones I-VI identify gel slices tested for SDS-potentiated proteolytic activity. b. Analytical ultracentrifugation of artemocyanin partially dissociated in the presence of 19 mM thioglycollate, showing (left to right) monomer, dimer, and trimer, photographed after 26 min at 46 560 rev/min in a double-sector cell. c. Rocket immunoelectrophoresis showing immunological relationship between trimer (a and b), dimer (c), and monomer (d). Wells contained about 1 µg protein. After 23 h diffusion, agarose containing rabbit anti-artemocyanin II serum was poured alongside to provide the electrophoresis gel.

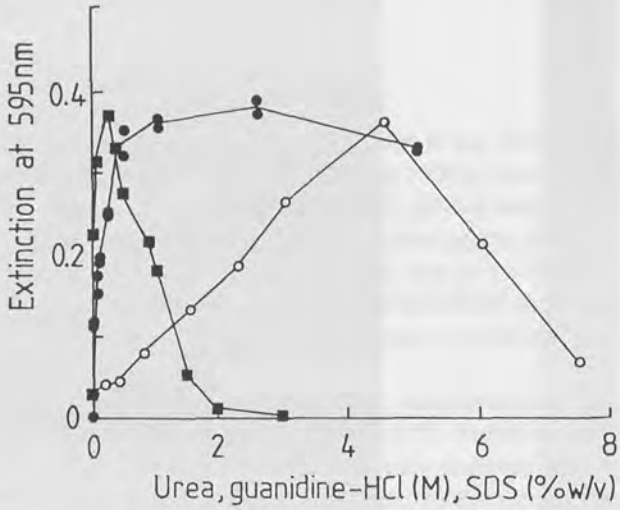

FIG. 2. Potentiation of artemocyanin protease activity with denaturing agents. Ordinate : release of blue dye from hide powder. (●) SDS ; (○) urea ; (■) guanidine-HCl.

MOLECULAR STRUCTURE OF ARTEMOCYANIN

Dissociation of subunits

The triplet of bands in the proteolytic zone (zone I, Fig. 1a) was found to represent three forms of the same protease (artemocyanin). Incubation of the slowest-running band (A in Fig. 1a) with 10 mM thioglycollate generated all three bands (A, B, C) on electrophoresis, each band having protease activity only after potentiation with SDS. This suggested a trimeric structure for the proteolytic molecule, which was confirmed by observation of its dissociation in the analytical ultracentrifuge (Fig. 1b). The parent molecule moved as a homogeneous peak of sedimentation coefficient ($s^{o}_{20,w}$) 27.3S, but in the presence of thioglycollate additional peaks of 20.5S and 13.5S were recorded, corresponding to a dissociation into dimer and monomer. In 76 mM thioglycollate, dissociation into monomers was almost complete. All three dissociation species were immunologically identical (Fig. 1c).

Molecular weight

Equilibrium measurements after 14-48 h in the ultracentrifuge, analysed by Lamm's method (Chervenka, 1969), indicated M_r of the trimer to be 1.2×10^6, taking the partial specific volume as 0.734. In agreement with this result, SDS-polyacrylamide gel-electrophoresis of type I artemocyanin revealed the largest polypeptide to be of M_r 190 000, corresponding to one-sixth of the trimer (27.3 S) or half of the monomer (13.5 S).

Polypeptide composition

The various polypeptides found on electrophoresis of artemocyanins I and II have all been shown to derive from the degradation of the M_r 190 000 molecule found only in artemocyanin I.

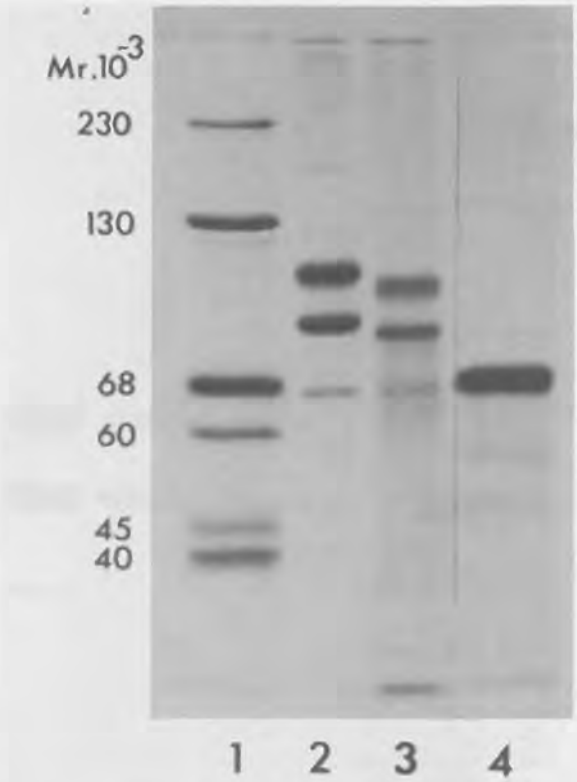

FIG. 3. SDS-polyacrylamide (10 %) gel-electrophoresis showing polypeptides isolated from artemocyanin II. Lane 1, markers ; lane 2, artemocyanin II ; lane 3, artemocyanin deglycosylated with trifluoromethanesulphonic acid ; lane 4, deglycosylation control (BSA).

From artemocyanin II, polypeptides of M_r 67 000, 87 000, and 104 000 were isolated (Fig. 3, lane 2). The same three polypeptides were present in isolated monomer and dimer as in the trimer, supporting the proposition that all monomers within the trimeric structure were of similar composition. The M_r 67 000 product was identified as the molecule possessing the proteolytic activity. Artemocyanin I when similarly analysed (Fig. 4, lane 2) did not exhibit the M_r 67 000 molecule and consequently was not proteolytic, but it contained the 87 000 and 104 000, plus a band at 190 000 from which those two had originated by cleavage. Evidently in artemocyanin II practically all the 190 000 had been so cleaved (Fig. 3), but that did not account for the 67 000 product, which was, however, shown to be derived in turn from the 87 000 molecule.

The origin of the M_r 67 000 polypeptide from 87 000 was confirmed in two ways. First, by limited proteolysis with *Staphylococcus aureus* V8 protease followed by electrophoresis, the 67 000 and 87 000 molecules yielded mostly the same peptides while the 104 000 molecule gave a completely different set. Second, amino acid analysis of these three molecules (Krissansen *et al.*, 1983a), treated statistically to assess compositional divergence (Harris and Teller, 1973), indicated a high degree of relatedness between the 67 000 and 87 000 (D = 0.0183).

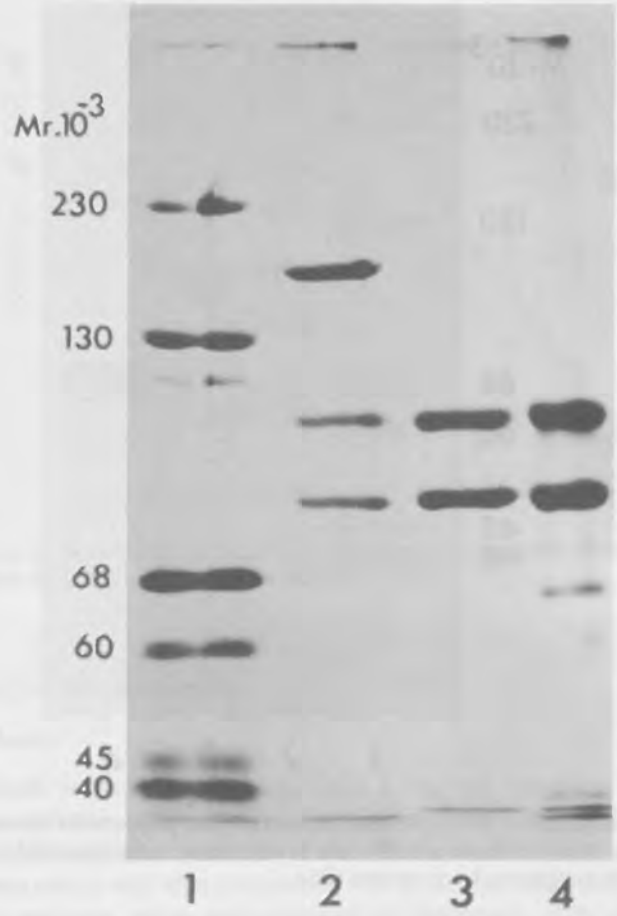

FIG. 4. SDS-polyacrylamide (10 %) gel-electrophoresis showing polypeptides isolated from artemocyanin I. Lane 1, markers ; lane 2, artemocyanin I purified from female haemolymph ; lane 3, as (2), incubated with trace homogenate of *Artemia* ; lane 4, artemocyanin II.

The high degree of commonality between artemocyanins I and II is illustrated in Fig. 5, where it can be seen that a reaction of immunological identity exists between artemocyanin I extracted from either male or female shrimp haemolymph and artemocyanin II, but that the reaction with the latter is characterised by a small spur which qualifies the identity as slightly imperfect.

We conclude that artemocyanin II as isolated from homogenised *Artemia* is a proteolytically altered form of the artemocyanin I found in pure haemolymph. However, this conversion has not been precisely reproduced in the laboratory. Fig. 4, lane 3, shows the effect of *Artemia* homogenate in cutting artemocyanin I to generate the M_r 104 000 and 87 000, but not 67 000 products. Further work (Krissansen *et al.*, 1985) has isolated two pure proteases from *Artemia* that apparently reproduce the generation of 67 000 from 87 000 but curiously the product then has no proteolytic activity and a further modification is evidently necessary.

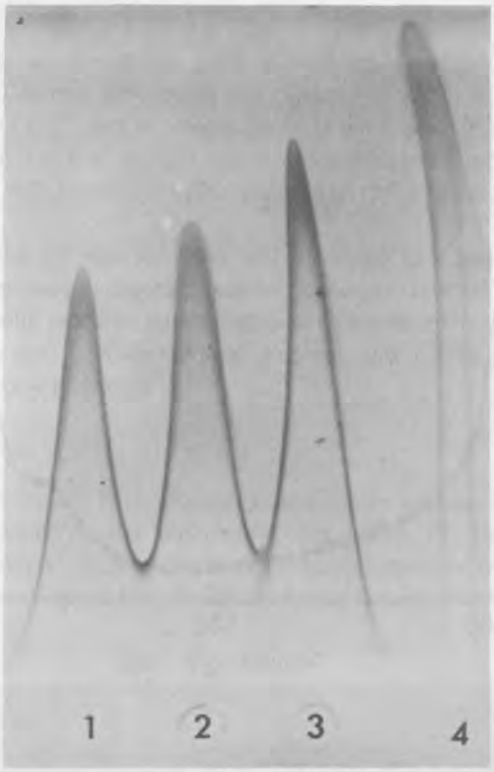

FIG. 5. Rocket immunoelectrophoresis showing immunological relationship between artemocyanin I from male (1) and female (2), and artemocyanin II (3 and 4). Experimental details as in Fig. 1c.

NON-PROTEIN LIGANDS

Carbohydrate

Retention of artemocyanin on concanavalin A-Sepharose 4B indicated glycosylation. The carbohydrate content was 8 % by weight. By staining on SDS-polyacrylamide gels with the periodic acid-Schiff reagent and scanning at 520 nm, the M_r 104 000 and 87 000 polypeptides were found to be glycosylated, but not the 67 000. This was further confirmed as shown in Fig. 3, where in lane 3 deglycosylation has increased the mobility of the two larger molecules, corresponding now to M_r 98 000 and 85 000, whereas the 67 000 has not changed.

Lipid content

A significant lipid content was extractable by the method of Bligh and Dyer (1959). Phospholipid was a major component, the phosphorus content of 0.8 μg per mg protein equating with about 2 % phospholipid. Thin layer chromatography identified mainly phosphatidylcholine, with some phosphatidylethanolamine. Several neutral lipids were also detected.

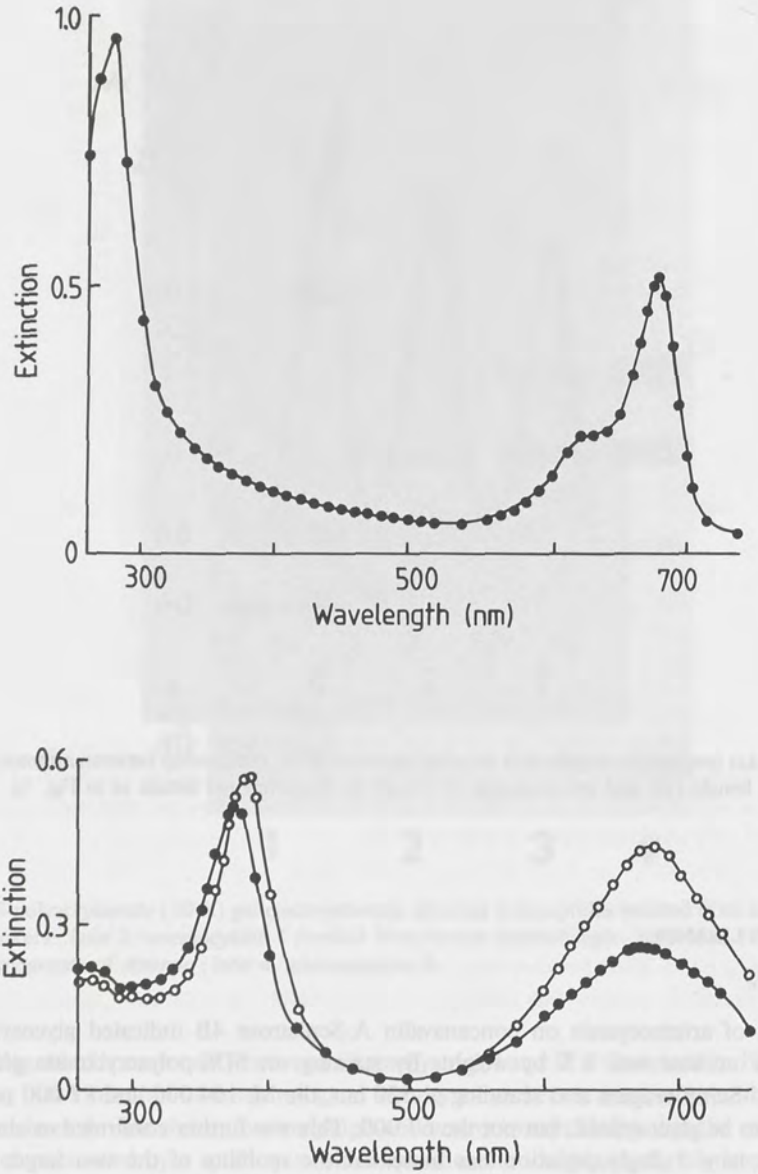

FIG. 6. Extinction spectra of artemocyanin II. Above : in 50 mM Tris-Cl, pH 7.6. Below : (●) the isolated chromophore dissolved in chloroform after extraction with 5 % HCl-methanol, compared with biliverdin (○).

Blue chromophore

Artemocyanin II has an absorption peak at 680 nm, E (1 %) = 6.0, corresponding to $E_M = 7.2 \times 10^5$ (Fig. 6). Atomic absorption and electron paramagnetic resonance spectra both identified the presence of Cu^{2+} ions in artemocyanin II but it was clear that the copper was not the source of this colour. The Cu^{2+} content varied widely between samples (0.01 %-0.13 %), and neither removal of Cu^{2+} with cyanide or dithionite, nor its replacement, significantly changed the spectrum (Fig. 6).

The chromophore (Fig. 6) was extracted and identified as a bilatriene bile pigment by a sequence of spectral responses to chemical tests as previously reported (Krissansen *et al.*, 1984) including a positive Gmelin reaction, haematinic acid and methyl vinyl maleimide generation on oxidation with chromic acid, and an oxidised zinc complex having the red fluorescence and characteristic spectrum of a bilatriene.

MOLECULAR ARCHITECTURE

Examination of artemocyanin II molecules in the electron microscope with negative staining revealed a three-fold symmetry with remarkable clarity (Fig. 7). The maximum diameter is 25 nm. Techniques developed for the analysis of ribosomal structure from electron micrographs (Kastner *et al.*, 1982) were applied to the artemocyanin images (Fig. 8).

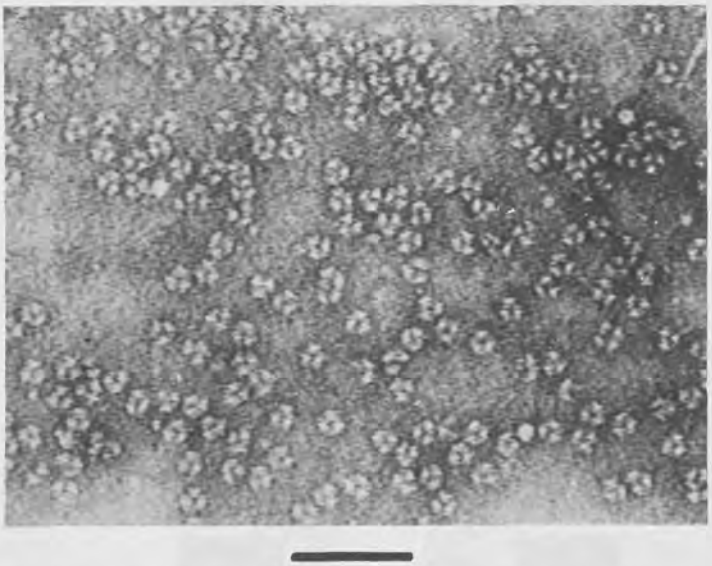

FIG. 7. Artemocyanin II negatively stained with phosphotungstate ; bar = 100 nm.

We can construct hypothetical models incorporating the known features of artemocyanin. For consistency with literature reviewed above, the model will be discussed in terms of a trimeric structure in accordance with its appearance on non-dissociating gels and its dissociation in

thioglycollate. Since artemocyanin I further dissociates in SDS to yield six apparently identical polypeptides of M_r 190 000 we can postulate that these subunits associate first in pairs, forming the monomer. The implication is that each M_r 190 000 subunit has two different types of binding site. The monomer is formed by the association of two subunits at a binding site of relatively high affinity, whereas a lower affinity interaction between monomers stabilizes the trimer.

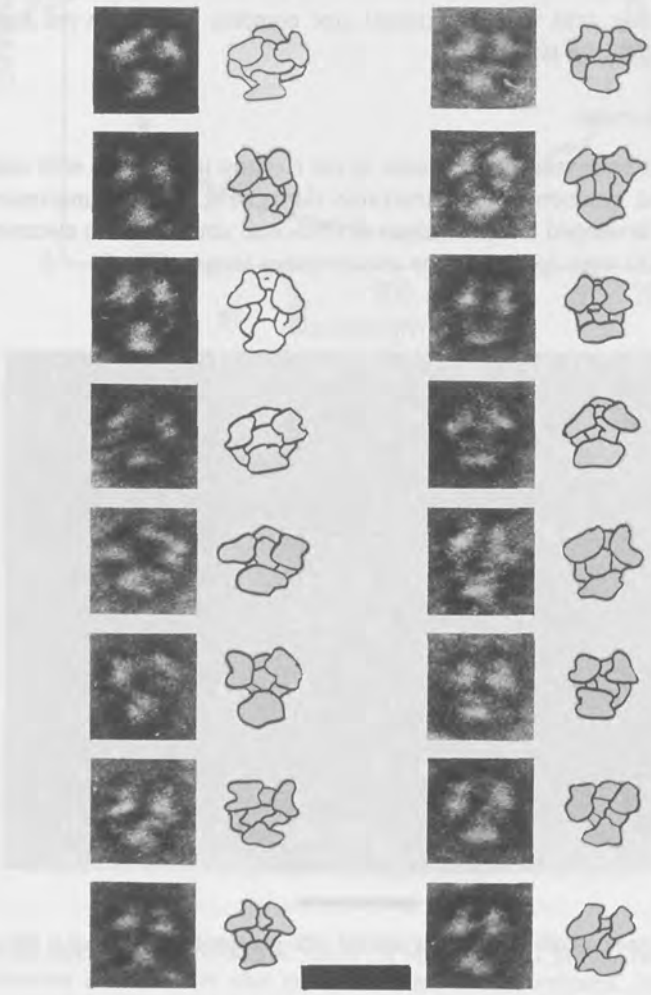

FIG. 8. A gallery of outlines drawn around typical images of artemocyanin, employing an electron microscope magnification of 40 000 and photographic enlargement of 20. Subsequently reduced; bar = 30 nm.

Two ways of interpreting the electron micrographs have been considered (Fig. 9). One interpretation is that the monomer, comprising two similar subunits, is asymmetric in shape with an elongated bridge to its neighbour. An alternative interpretation allows the subunits to be more globular, on the assumption that in the electron micrographs all six are visible, alternately below the plane of the molecule where they contrast clearly, and above it where they are more obscured by underlying stain. The two alternative assumptions have the same formal basis, namely that M_r 190 000 subunits associate to form monomers which then associate into trimers. The polypeptides of smaller M_r than 190 000 originate proteolytically.

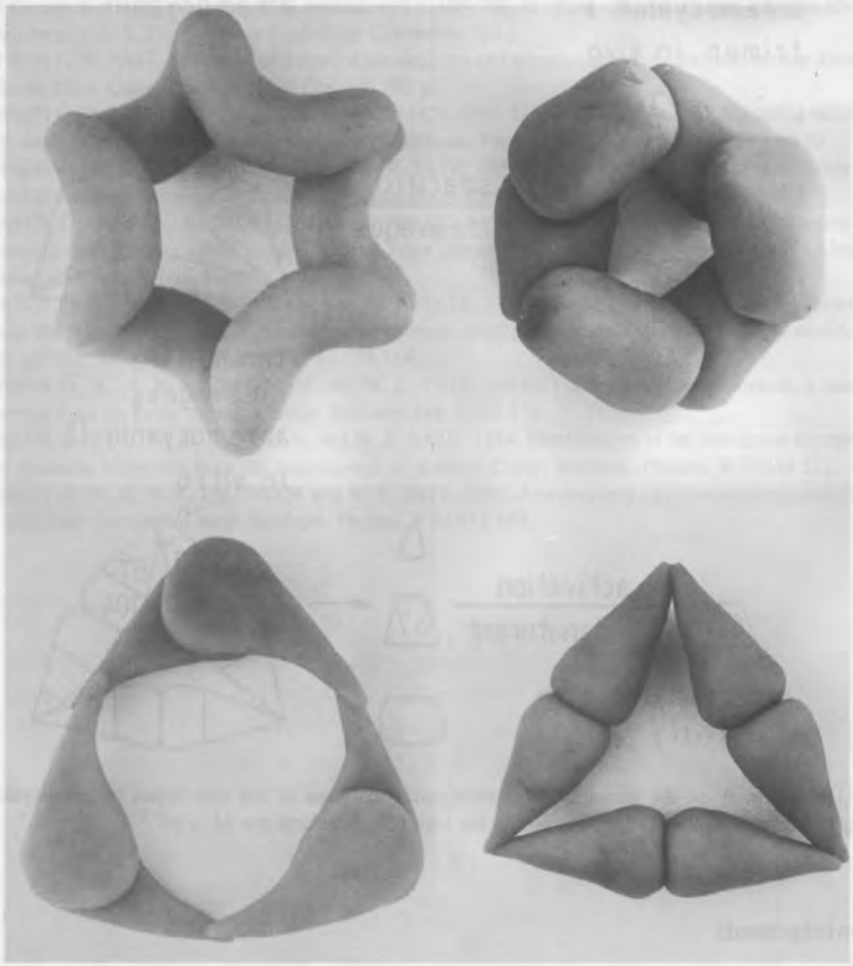

FIG. 9. Four hypothetical models of artemocyanin ; above, with subunits having bilateral symmetry ; below, with less symmetrical subunits. Each model portrays an assembly of six M_r 190 000 polypeptides.

Conclusions

Fig. 10 summarises our conclusions on the basis of one of the alternative structural models described above. According to these models only the M_r 190 000 subunit exists in artemocyanin *in vivo*. During extraction the smaller polypeptides arise by proteolysis, which is more extensive when shrimps are homogenised in quantity than when they are bled singly. No purified protease has been found both to generate the M_r 67 000 polypeptide and to confer SDS-activated proteolytic activity on it. Although the various cleavages are artefactual, the possibility that similar degradation forms part of the process for disposal of artemocyanin *in vivo* is not excluded. If the potentiation of the proteolytic activity does not occur *in vivo*, this raises interesting questions as to the origin of this domain in the molecule.

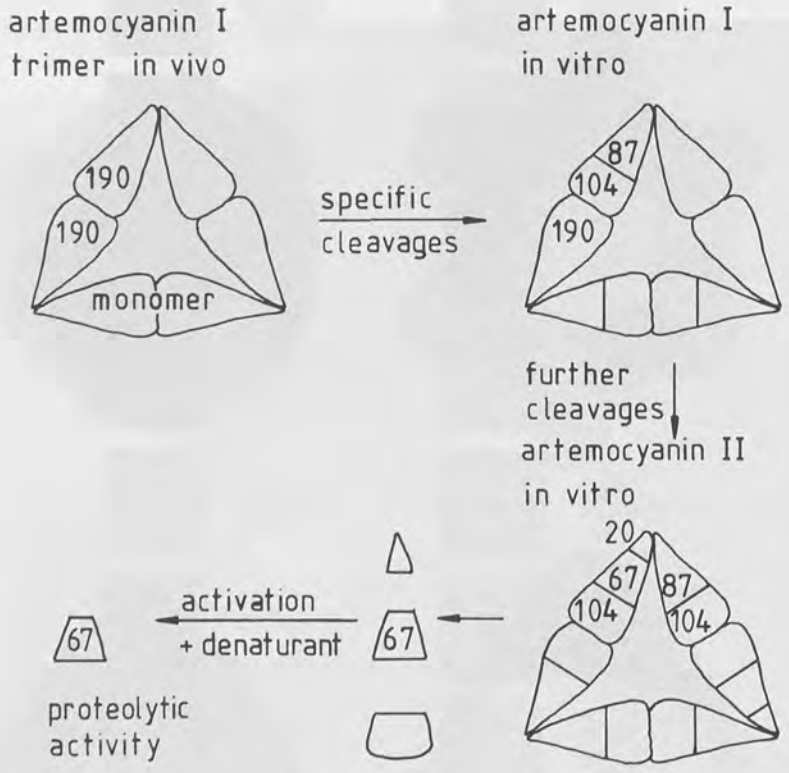

FIG. 10. Summary of the structure of artemocyanin in terms of the four types of polypeptide found, showing the relationship between artemocyanins I and II. Numbers are $M_r \times 10^{-3}$.

Acknowledgements

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activation
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Moulting-hormone activity in adult *Artemia*

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Abstract

The *Artemia* strain from Great Salt Lake (GSL 375) was used. Total body extracts and hemolymph of adult *Artemia* were purified by solvent partition and column chromatography. Moulting-hormone concentrations were then determined by a specific radioimmunoassay (RIA). Results are expressed in ng ecdysteroid activity/g fresh weight (or per ml). Initially, the RIA data on extracts of adults were highly variable. The use of a more accurate sampling technique based on the reproductive cycle of the females allowed to determine a hormone activity pattern.

In females, moulting cycles alternate with vitellogenic cycles. The lowest values of ecdysone (3-6 ng ecdysone equivalents/g fresh body weight or ml hemolymph) occur during previtellogenesis and when lateral sacs containing ripe eggs are present. Peak values (±20 ng/g) are reached every vitellogenic cycle at periods corresponding to the most intensive yolk accumulation.

The exact timing of developmental progress in the adult stage is much more difficult in males than in females. Ecdysteroid peak values (±10 ng ecdysone equivalents/g fresh body weight) are present during the copulation period.

Introduction

Moulting hormones (MH's) play a predominant role in the growth of Crustacea. Considerable endocrinological research has been done especially on crabs, lobsters, shrimps, isopods, and amphipods (Kleinholz, 1942 ; Knowles and Carlisle, 1956 ; Passano, 1960 ; Adiyodi and Adiyodi, 1970 ; Kleinholz and Keller, 1979 ; Spindler *et al.*, 1980).

A central idea in this field of malacostracan research is that substances originating from the eyestalk, mediate various processes such as Ca-metabolism, limb-regeneration, moulting and moulting cycles, vitellogenesis, and reproduction.

Until now, information is scarce about the complexity of the endocrine system in lower Crustacea, such as *Artemia* sp. (Anostraca). Ultrastructural studies have been conducted on the nauplius eye and adjacent organs of adult *Artemia* (Elofsson and Lake, 1971 ; Rasmussen, 1971 ; Anadon and Anadon, 1980). Neurosecretion has been demonstrated by Hentschel (1963, 1965), Baid and Ramaswami (1965), and Van den Bosch de Aguilar (1974, 1976, 1977). No exact data are, however, available on moulting-hormone concentrations.

The appearance of new appendages after moulting is a good indication of the life stage of the larval or preadult animal. When the adult stage is reached this criterion can no longer be used. Observations on individual animals demonstrated that moulting does occur in adults at regular time intervals as has been mentioned by Bowen (1962) in the Utah race of Great Salt Lake and the Californian race of San Francisco Bay. This moulting pattern has been described more in detail by Metalli and Ballardin (1972) in *Artemia persimilis*.

In order to find a possible relationship between moulting cycles and vitellogenic cycles, we measured ecdysteroid activities in adult females. This paper deals with primary results of ecdysone titers in adult *Artemia*, and their possible function is discussed.

Materials and methods

Animals

The *Artemia* Great Salt Lake strain (GSL 375) was used. Individuals were taken from dense populations raised in culture tanks of 1 m^3 at the Artemia Reference Center, Gent, Belgium.

Sampling method

Males in riding position were separately collected from the free-swimming ones. Female animals were selected microscopically according to their reproductive stage. Each vitellogenic cycle could be divided into successive stages (Table I), which could be easily followed thanks to the transparency of the body wall. The animals were rinsed with destilled water and blotted dry. Groups of 20 individuals were frozen in liquid nitrogen and stored at -20 °C until use.

Table I

Summary of the successive stages in vitellogenic cycle

Stage OV-	No vitellogenesis, ovary fully transparent
Stage OV±	A few oocytes begin yolk accumulation
Stage OV+	Yolk accumulation oocytes are found over the entire length of the ovary
Stage OV++	Oocytes (opaque, due to the presence of lipovitellin) arranged in a thick, white double strand
Stage L.S.	Ripe eggs in lateral sacs

To obtain hemolymph, adult *Artemia* were put on a clean slide and a few legs were removed. Hemolymph was then sucked up with a micropipette (2 or 5 µl, Blaubrand) and collected in an Eppendorf tube, hanging in liquid nitrogen, and stored at -20 °C.

Extraction procedure

The samples were weighed and homogenized with an Ultra-Turrax (IKA-Werk, Staufen) in methanol : aceton (1:1, v/v, both from Merck, p.a.). The hemolymph aliquots were treated in the same way. After overnight incubation at 4 °C, all samples were centrifuged for 20 min at 15 000 rev/min at 4 °C in a Sorval RC-5B. The supernatants were evaporated under vacuum in a waterbath at 54 °C with a Buchi Rotavapor.

Further purification was achieved by solvent partitioning in water : n-hexane (from Carlo Erba, 1:1, v/v). The cleanup of the aqueous phases was done with column chromatography (Sep-pak

C_{18} cartridges from Waters Associates). Ecdysteroids were eluted with methanol, which was evaporated under a stream of nitrogen, the residues redissolved in ethanol (Merck p.a.), and stored at 4 °C.

Determination of ecdysteroids

Ethanol was removed from the stored samples by a stream of nitrogen. The residue was redissolved in 500 µl phosphate buffered saline (10 mM PBS, pH 7.4). 100 µl aliquots were used for radioimmunoassay (Walgraeve *et al.*, 1986). The antiserum (DUL-2) used was a gift from Dr. J. Koolman, and was specific for ecdysone.

Results

As it was very difficult to accurately determine the developmental stages of females before they started yolk deposition, sampling was started from the moment that yolk accumulating oocytes appeared, just after the last preadult moult. From this stage on, all successive stages could easily be recognized.

In Fig. 1 the ecdysone activities in whole body extracts of females during the first two vitellogenic cycles are shown. A peak value of about 12 ng ecdysone equivalents/g fresh body weight was found at the onset of yolk deposition, as well in the first as in the second vitellogenic cycle. The lowest values, about 3 ng/g, were always found during the lateral-sac stage. Females moulted just before this stage.

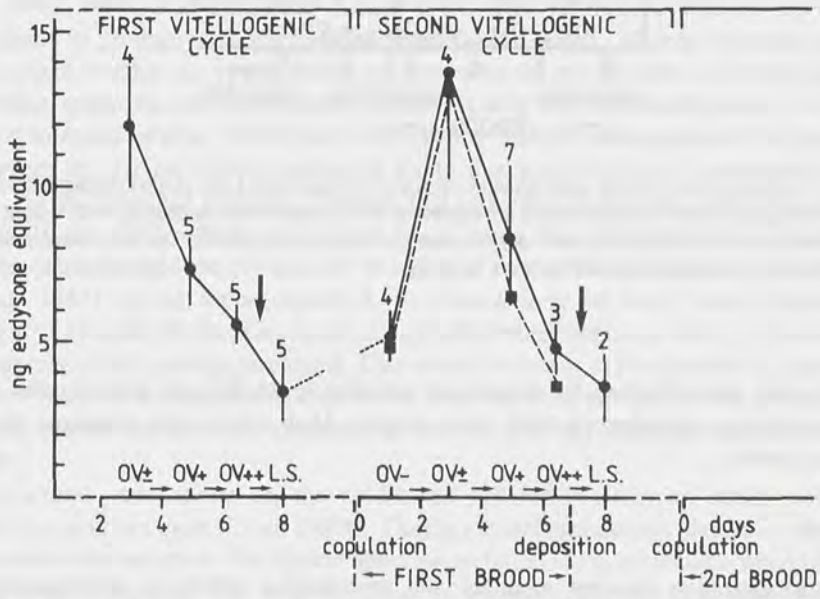

FIG. 1. Ecdysteroid activity in adult females of *Artemia* (Great Salt Lake strain) during different stages of the first two vitellogenic cycles. Concentrations are expressed in ng ecdysone equivalents/g body weight (●—●, with SD) and per ml hemolymph (■--■). Arrows (↓) indicate moults. The data correspond to sets of 20 individuals ; an approximate time scale is given.

A similar pattern was observed in data obtained with hemolymph extracts : the peak value (13.1 ng/ml) was measured at a period of the most intensive yolk accumulation by the oocytes.

In following vitellogenic cycles (Fig. 2) the same general moulting hormone pattern was found (peak value of 21 ng/g during vitellogenesis, lowest value of 1.8 ng/g when oocytes were loaded with lipovitellin). A small peak (6.6 ng/g) was, however, observed after egg deposition and before moulting. The value of 3.1 ng/g at the lateral-sac stage could be compared with the data from the corresponding stage in the first two cycles. No hemolymph samples were collected.

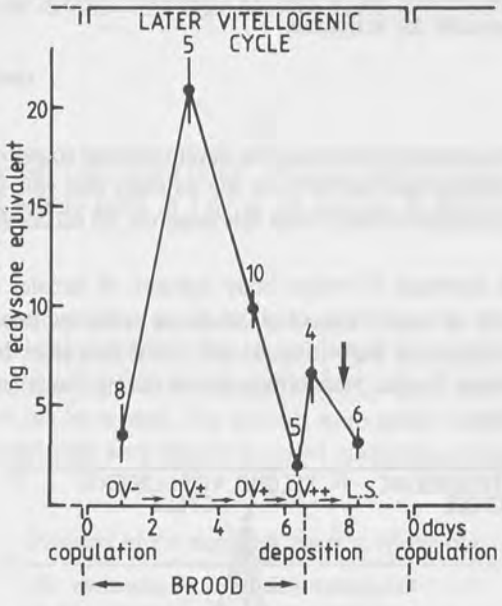

FIG. 2. Ecdysteroid activity in adult females of *Artemia* (Great Salt Lake strain) during different stages of a later vitellogenic cycle. Concentrations are expressed in ng ecdysone equivalents/g body weight (●—●, with SD) and per ml hemolymph (■--■). Arrows (↓) indicates a moult. The data correspond to sets of 20 individuals ; an approximate time scale is given.

Variable data were obtained for ecdysteroid activities in whole body homogenates of males (5-15 ng ecdysone equivalents/g fresh body weight). High values were measured during the copulation period.

Discussion

The synchronization between moulting and reproductive activity is well-documented in Decapoda (Adiyodi and Aiyodi, 1970). This suggests that the same hormonal mechanisms may be involved in the control of both processes.

In *Orchestria gammarella* (Amphipoda) the relationship between vitellogenin synthesis and moulting has been described by Meusy *et al.* (1977). In this species, extensive research on the

control of vitellogenesis has been carried out. Blanchet *et al.* (1979) measured ecdysteroid levels in reproductive females during the course of the moult cycle and found a peak of 20-OH ecdysone at the end of the D_2 stage, just before moulting. Ecdysone (20-OH) appears to be necessary for vitellogenin synthesis as cauterization of the Y-organs (moult glands) depresses vitellogenin synthesis (Meusy *et al.*, 1977). The onset of vitellogenin synthesis seems to require a low titer of 20-OH ecdysone, as is found at the beginning of the moult cycle, since this process is inhibited when 20-OH ecdysone is introduced in crystalline form, just after moulting (Blanchet *et al.*, 1975).

Presently, the endocrinology of vitellogenesis is better documented in insects than in crustaceans. In all insects studied so far, ecdysone titers of the same magnitude as we measured in *Artemia* were found in vitellogenic females. Only in Diptera has it been shown, however, that the moulting hormone is the inductor of vitellogenin synthesis by the fat body. This hormone acts as a female sex hormone. When injected in adult males, vitellogenin appears (Briers and Huybrechts, 1984). In the other insect orders moulting hormone is also present in higher concentrations in females than in males but here, injection in males does not elicit vitellogenin synthesis. In insects, the ovary is a site of moulting-hormone synthesis but it also accumulates this hormone. There are also other sites of synthesis (Romer, 1971).

To date, it is not known whether the ovaries are generally a major source of moulting hormone in Crustacea. Since the highest activities and titers are found during the period of active yolk-deposition, the situation might be similar to that in insects.

Junera *et al.* (1977) prove that in *Orchestia gammarella* vitellogenin synthesis is controlled by an ovarian hormone, namely the "vitellogenin-stimulating ovarian hormone" or VSOH (Meusy, 1980 ; Meusy and Charniaux-Cotton, 1984). This VSOH stimulates the sub-epidermal adipose tissue to produce vitellogenin (Charniaux-Cotton, 1980 ; Blanchet-Tournier, 1982).

The problem whether the yolk proteins are formed by the oocyte itself or whether they are of extra-ovarian origin like in most animal species, is still not completely solved for *Artemia*. According to Anteunis *et al.* (1964) and Criel (1980a) yolk formation starts in the center of an oocyte where an atypical vitellin nucleus is found that probably forms proteinaceous vitellin granules. We also obtained evidence, however, that exogenous vitellogenin synthesis takes place in *Artemia* (Criel, 1984). Preliminary experiments, using immunocytochemical localization of lipovitellin, demonstrated the presence of this protein in the "fat-storage cells" (Lockhead and Lockhead, 1941) of the limbs (unpubl.). In *Branchinecta packardi* Pearse, Gilchrist and Zagalsky (1983) found "connective-tissue storage cells" which appear to be the extra-ovarian site of biosynthesis of yolk-protein precursors. This would be similar to the situation in insects where the bulk of vitellogenin is synthesized by the fat body and trace amounts by the ovarian follicles themselves. Further research will be needed to find out if 20-OH ecdysone controls vitellogenin synthesis.

Ultrastructural observations on the oviduct of *Artemia* (Californian strain) showed the existence of a secretory cycle (Criel, 1980b). During previtellogenesis the glandular cells produce a scarce merocrine secretion. The typical apocrine secretion starts at the time when the highest ecdysone level is reached. This is at the onset of vitellogenesis, and lasts until ovulation. It remains to be demonstrated whether ecdysone controls the activity of these glandular cells.

No exact data on hormone activity in reproducing males are available. In males, moulting occurs at regular time intervals and always after copulation (pers. unpubl. observ.). We measured high ecdysone activities during the copulation period but this does not prove that this hormone

triggers the copulation behavior. When an accurate sampling technique for male *Artemia* will have been worked out, the role of ecdysteroids in the male reproduction system will be studied.

Our results are the first precise data on ecdysteroid activities and titers in female adults of Great Salt Lake *Artemia*. Our next goal is to elucidate the role of this and other hormones in reproduction. We expect that the moulting hormone will only be one out of a series of hormonal factors controlling the reproduction. Research on the titers and the role of testosterone, progesterone, and neuropeptides in the reproduction of females and males is already in progress.

Acknowledgements

We thank Dr. J. Koolman for the antiserum and the staff of the Artemia Reference Center for providing the animals. This work has been supported by an FKFO grant of the Belgian National Science Foundation to E.V.B. and a grant of the IWONL to H.W.

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An action spectrum for light-induced hatching of *Artemia* cysts

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Abstract

The influence of light on the hatching induction of *Artemia* cysts is well known. Our objective was to determine the absorption characteristics of the responsible photoreceptor by action spectroscopy. The results suggest the involvement of a haemopigment located in the gastrula. Haematin, the haemopigment of the cyst shell is demonstrated to have a light-screening function towards the photoreceptor.

Introduction

The influence of light on the development resumption of hydrated *Artemia* cysts has been previously demonstrated (Sorgeloos, 1973 ; Royan, 1976 ; Sorgeloos *et al.*, 1976 ; Spektorova and Syomik, 1979). The hatching results after exposure to different light intensities and the light sensitivity of cysts of different *Artemia* strains was examined by Vanhaecke *et al.* (1981). We have demonstrated a correlation between light intensity, exposure time, and hatching percentage. Exposure to light of the wavelength region between 400 and 600 nm was proved to be most effective in triggering hatching (Van der Linden *et al.*, 1985). Any information on the photoreceptor system involved in light induced hatching is, however, lacking.

The work reported in this paper concerns the *in vivo* determination of the absorption properties of the initial photoreceptor. Therefore action spectra for light-induced hatching of cysts and decapsulated cysts were constructed. This enabled us to draw some conclusions about the nature and localisation of the photoreceptor pigment.

Materials and methods

Action spectroscopy

Wavelength specific dose-response curves for light induced hatching of cysts were determined and action spectra constructed. Reciprocity was tested for each wavelength by examining the light-induced response for two different combinations of light intensity (I) and exposure time (t) which product (I×t) is constant. These reciprocal light condition combinations always resulted in the same hatching response. All experiments concerning the same action spectrum were performed with cysts of the same batch (cysts : San Francisco Bay Brand (SFBB) 2149 ;

decapsulated cysts : Great Salt Lake (GSL) NA-401). Hydrated cysts were decapsulated by treating them with sodium hypochlorite, which causes oxidation of the chorion (Sorgeloos *et al.*, 1983). This procedure was carried out under red-infrared light, which has a negligible influence on the hatching (Van der Linden *et al.*, 1985).

A monochromator (Ernst Leitz GMBH Wetzlar Lampenhaus 250, 5482) was used. The equipment was mounted in such a way that the bottom of a test tube, coated with a single layer of about 150 hydrated cysts, was illuminated from underneath. The fluence (total incident light energy per unit area) was varied by changing the exposure time or using layers of cambric. The fluence rate (μEinst/m^2 s) was measured with a quantum sensor (Li-1905B) connected to a quantum meter (Li-188B). After light exposure the cysts were transferred to test tubes filled with seawater. The test tubes were fixed on a rotating bar and kept for 48 h in the dark at favorable hatching conditions (salinity 8 ‰, pH 8, temperature 25 °C). After the incubation period, the content of the test tubes was fixed with lugol and the hatching percentages were calculated on the results of four replicates.

The dose-response data obtained were log-logit transformed and χ^2 values were calculated. Abbott's formula was used to correct for the hatching response in the dark (Brown, 1978). Finally, action spectra were obtained by plotting the reciprocal values of the fluence, which give a constant response for all wavelengths, versus wavelength (Shropshire, 1972).

Absorption spectroscopy

Haematin was extracted from empty cyst shells by overnight stirring in cold 1 % NaOH (Gilchrist and Green, 1960). The carotenoids in the gastrulae were extracted by grinding decapsulated cysts in methanol with a Potter Elvejhem homogenizer. The supernatant was used for absorption spectroscopy. Spectra of cysts, decapsulated cysts, and their extracted pigments were determined with an Aminco DW2a dual-wavelength double-beam spectrophotometer, linked to a microcomputer Digital Minc Declab 23.

Results

Action spectrum for hatching induction of cysts

The dose-response curves obtained for exposure of hydrated cysts to different wavelenghts between 400 and 700 nm are shown in Fig. 1. Based upon these data, action spectra for 27 % and 50 % hatching response were constructed (Fig. 2). In the present case, the dose-response curves were not parallel. This may be due to self-screening effects and/or interaction of two or more pigment systems (Shropshire, 1972). In spite of this, the action spectra were very similar and showed two peaks with maxima at 525 and 575 nm (Fig. 2).

Before drawing conclusions about the congruence between the action spectrum and the absorption spectrum of the photoreceptor, more information on possible light-screening pigments is needed. Their presence is, however, suggested by the deviation from parallelism of the dose-response curves. The external location of the dark pigment haematin suggests a possible screening role. The absorption spectrum of this pigment is shown in Fig. 3. It's possible screening role was examined by comparing the influence of light on the hatching response of cysts and of decapsulated cysts. After removing the pigmented chorion from the cysts (decapsulation), they were still sensitive to light. This confirms the results of Vanhaecke *et al.* (1981) who

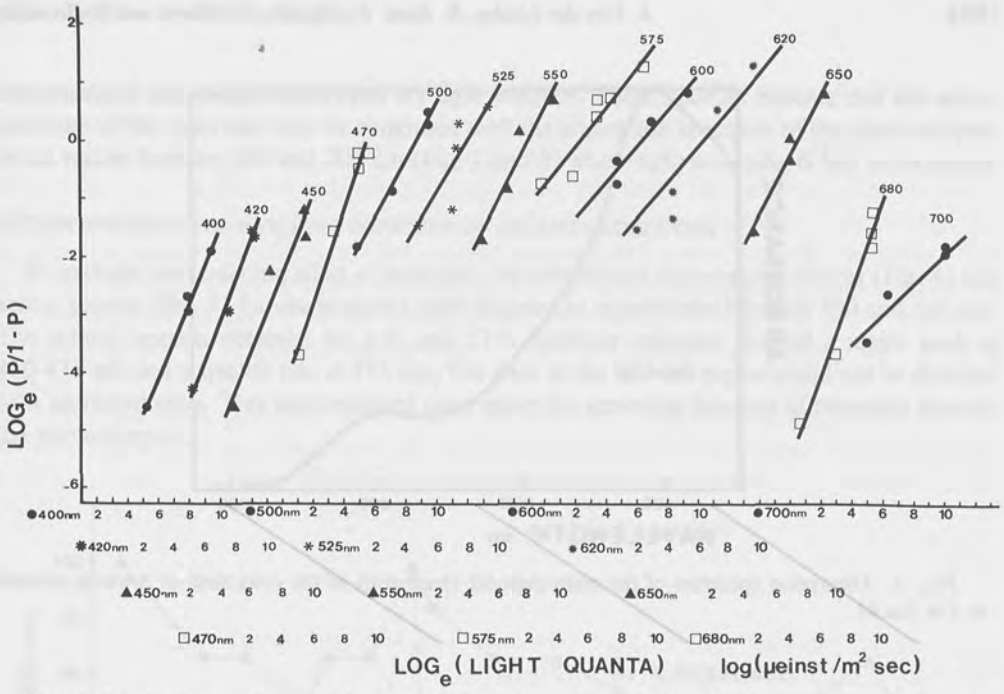

FIG. 1 Log-logit transformed dose-response curves for hatching percentage of *Artemia* cysts (SFBB-2149). The cysts were exposed for different periods to the wavelengths indicated. The hatching percentages were assayed after 48 h. (P) hatching probability for Abbott corrected hatching percentages.

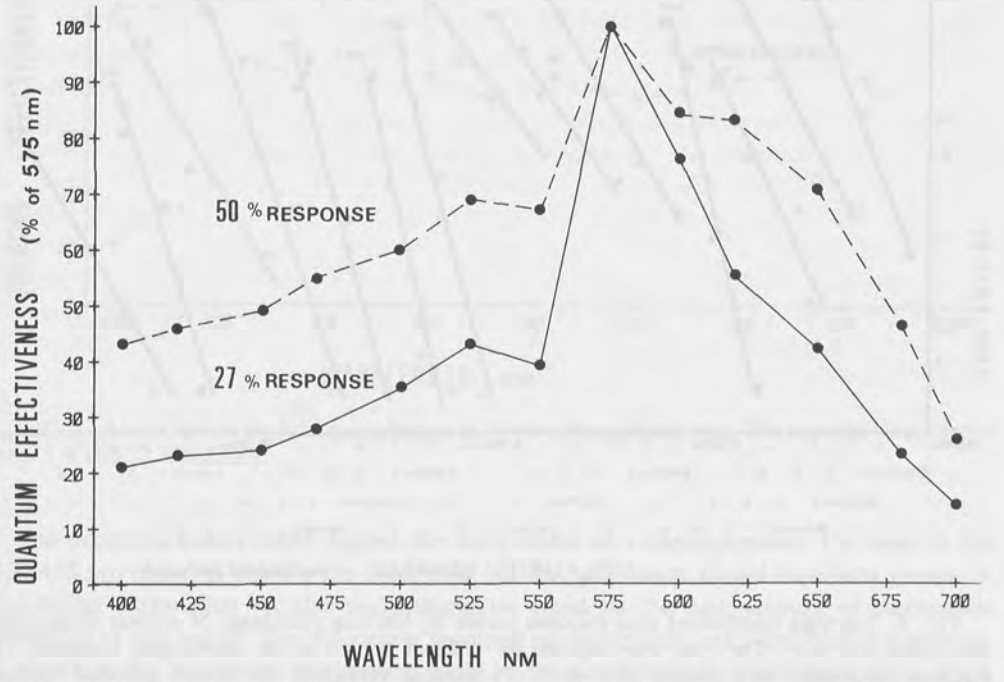

FIG. 2. Action spectra for hatching induction of *Artemia* cysts. The response sizes selected were : 27 % and 50 % hatching.

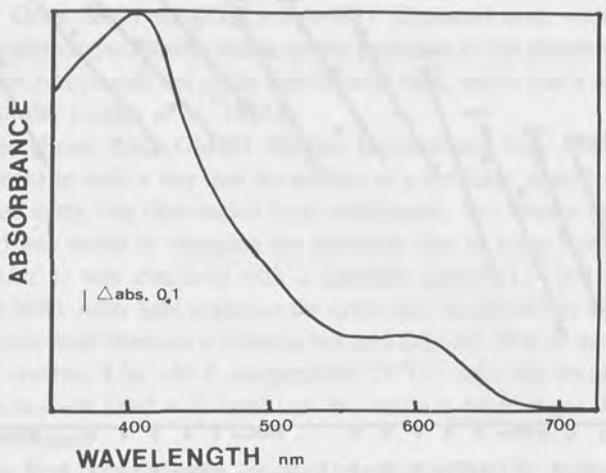

FIG. 3. Absorption spectrum of the main pigment (haematin) of the cysts shell of *Artemia*, extracted in 1 % NaOH.

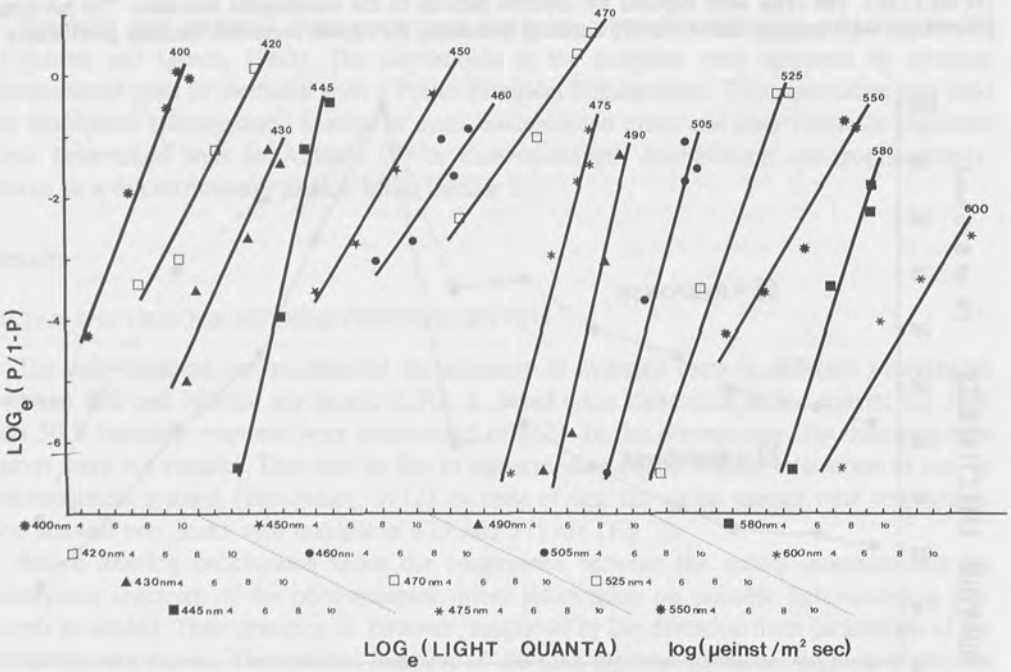

FIG. 4. Log-logit transformed dose-response curves for hatching percentage of *Artemia* decapsulated cysts (GSL-NA-401). The cysts were exposed for different periods to the wavelengths indicated. The hatching percentages were assayed after 48 h. (P) hatching probability for Abbott corrected hatching percentages.

demonstrated that decapsulated cysts are light sensitive. These findings indicate that the action spectrum of the cysts can only be congruent with the absorption spectrum of the photoreceptor in the region between 500 and 700 nm (Fig. 2 and 3) where light screening is less pronounced.

ACTION SPECTRUM FOR HATCHING INDUCTION OF DECAPSULATED CYSTS

To exclude the screening effect of haematin, we determined dose-reponse curves (Fig. 4) and action spectra (Fig. 5) for decapsulated cysts exposed to wavelengths between 400 and 600 nm. The actions spectra obtained for 5 % and 27 % hatching response showed a major peak at 450-475 nm and a smaller one at 575 nm. The peak in the 450 nm region could not be detected with untreated cysts. This demonstrated once more the screening function of haematin towards the photoreceptor.

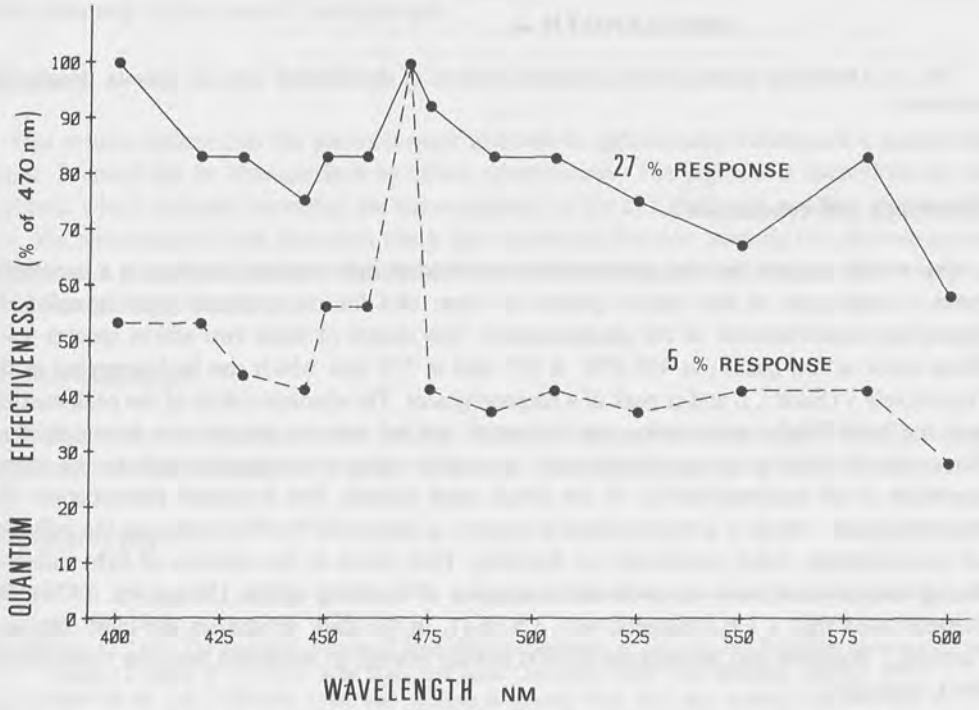

FIG. 5. Action spectra for hatching induction of *Artemia* decapsulated cysts. The response sizes selected were 5 % and 27 % hatching.

The occurring action peaks suggest the involvement of a haemopigment. The peak in the 450-475 nm region is possibly the Soret peak but the peak height should have been greater. A convenient explanation for this underestimation could be the high amount of carotenoids absorbing in this region. The absorption spectrum of carotenoids, extracted from the gastrula, is shown in Fig. 6. Another explanation could be the harmful effect of high light doses which underestimates the response size of the normally more protected photoreceptor.

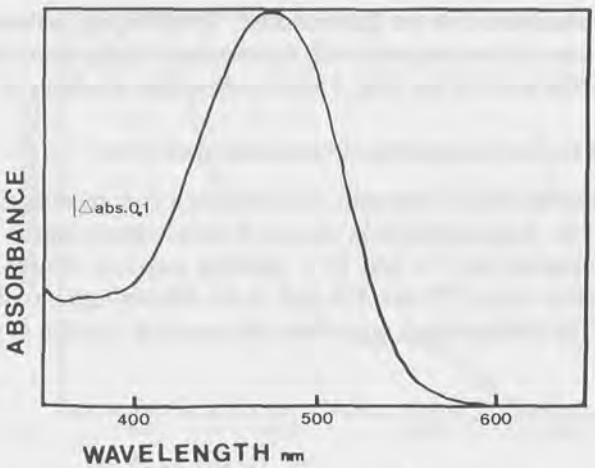

FIG. 6. Absorption spectra of the carotenoid content of decapsulated cysts of *Artemia*, dissolved in methanol.

Discussion and conclusions

Our results suggest that the photoreceptor involved in light-induced hatching is a haemopigment. Comparison of the action spectra for cysts and for decapsulated cysts revealed the absorption characteristics of the photoreceptor. The results of these two action spectra show three major action peaks : at 450-470, at 525 and at 575 nm, which can be interpreted as the respectively γ (Soret), β and α peak of a haemopigment. The absolute values of the peak maxima and the peak height proportions can, however, not be used to identify the haemopigment. Screening or binding to macromolecules can easily cause a wavelength shift in the action spectrum or an underestimation of the actual peak surface. The proposed participation of a haemopigment, which is a redox-sensitive system, is supported by the results on the influence of environmental redox conditions on hatching. First, there is the absence of light influence during anoxic conditions or upon administration of reducing agents (Sorgeloos, 1976). We demonstrated that a 1 h treatment with 3 % H_2O_2 in the dark substitutes the light treatment (unpubl.). Bogatova and Schmakova (1980) already proved an enhanced hatching yield with an H_2O_2 treatment.

The light sensitivity of decapsulated cysts, the screening effect of haematin towards the photoreceptor, and the distortion of the action spectrum for decapsulated cysts indicate that the photoreceptor is located in the gastrula and not in the cyst shell.

The difference between the two action spectra are evidence for the screening role of haematin. Since haematin and the photoreceptor are both haemopigments with similar absorption characteristics, a hatching delaying or photoreceptor-protective function can be attributed to haematin. Photoreceptor protection seems plausible because high doses of light energy in the 400-500 nm range do not have the expected hatching-improving effect on decapsulated cysts and probably even become harmful. On the other hand, the oxidation-reduction capacities of haematin suggest that prolonged exposure to light in humid circumstances causes oxidation or bleaching. Thus,

more light can penetrate the cyst shell and reach the photosensitive pigment inside the gastrula. In this way, immediate excitation of the photoreceptor under unfavorable hydration conditions is prevented. A similar hatching-delaying function was suggested by Shan (1970) in *Pleuroxus denticulatus*, a branchiopod exhibiting light-induced hatching. He proposed that the dark pigmentation of the ephippium, which surrounds the resting eggs, constitutes a mechanism for retarding the development until hatching can be accomplished under favorable conditions. The dark color of the ephippium is first bleached by the sunlight to allow further light penetration and development. Bleaching of the ephippia with hypochlorite results also in an improved hatching response to light of the resting eggs of *Daphnia* (Pancella and Stross, 1963 ; Stross, 1966).

To conclude, we suggest that the photoreceptor involved in light-induced hatching of *Artemia* cysts is a haemopigment located inside the gastrula and shielded by the pigment of the cyst shell, namely haematin. Future work will focus on the determination of light-induced changes in the cysts resulting in the onset of development.

Summary

Our results suggest that the photoreceptor involved in light-induced hatching is a haemopigment. It could not be characterized by action spectroscopy. The pigment is located inside the gastrula, which excludes haematin, the haemopigment of the cyst shell, as a possible photoreceptor. We demonstrated that haematin has a light-screening function towards the photoreceptor. Therefore it possibly constitutes a mechanism to delay hatching or to protect the photoreceptor from harmful light doses.

Acknowledgements

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The function of messenger RNA associated poly(A)-binding proteins

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Abstract

In eukaryotic cells, it is generally accepted that messenger-like RNA is associated with specific proteins in ribonucleoprotein particles (mRNP). The protein moiety of mRNP depends on the cellular localization. An exchange of proteins has been observed after its transport from the nucleus to the cytoplasm and after its entry into polysomes. One of the main protein binding sites is the poly(A)-sequence at the 3' end of mRNA. It is assumed that this homopolymer segment regulates mRNA transport from nucleus to cytoplasm, mRNA translation and mRNA stability, and that these phenomena can be modulated by the poly(A)-binding protein. In *Artemia* sp. cryptobiotic embryos the main protein associated with the poly(A)-sequence of non-polysomal mRNP has a M_r of 38 000 (P 38). The protein is purified and is characterized by the existence of at least seven ionic species with a pI ranging from 9.2 to 6.6 due to post-translational phosphorylation. A sequence-independent stoichiometry of one protein per 8-12 nucleotides is measured and specificity for poly(A) is demonstrated in mixing experiments. The protein contains dimethylarginine, is devoid of cysteine and has a high glycine content similar to the basic hnRNP proteins of M_r 28 000-38 000 found in various organisms. P38 is phosphorylated by a mRNP associated kinase in serine and threonine residues, and can be phosphorylated by *Artemia* sp. cytosol phosphatases. From our present results we may conclude that the poly(A)-binding protein is a multifunctional protein involved in mRNP-associated phenomena taking place in the nuclear and cytoplasmic compartments. Since phosphorylation-dephosphorylation alters the poly(A)-binding capacities, this post-translational modification might be an important regulatory factor in mRNA metabolism.

Introduction

After the detection of mRNP in the cytoplasm of fish embryos by Spirin (1969), their existence has been reported in many organisms. In the past few years evidence has been presented that mRNP proteins are of great importance in cellular events such as mRNA maturation, nucleocytoplasmic transport and protein synthesis (Beyer *et al.*, 1981 ; Palayoor *et al.*, 1981 ; Schmid *et al.*, 1983). The protein moiety of mRNP depends on the cellular localization and an exchange of proteins has been observed after nucleocytoplasmic transport and mRNP entry into polysomes (Preobrazhensky and Spirin, 1978). Even the protein composition of polysomal poly(A)-containing mRNP is constantly changed according to the physiological conditions (Greenberg and Carroll, 1985). This dynamic property of the protein moiety makes it difficult to identify the *in vivo* -mRNP proteins since isolation procedures may alter the protein composition. So far, only a few proteins seem to be universal for all mRNPs of a specific cellular localization. A typical set of proteins with M_r 29 000 to 42 000 is associated with heterogeneous nuclear RNP (Karn *et al.*, 1977) while a poly(A)-binding protein of M_r 73 000-78 000 is a major

constituent of most polysomal mRNPs (Greenberg, 1980). A small number of other proteins such as initiation factors and protein kinases have been demonstrated on mRNP and their function in mRNA metabolism has been discussed (De Herdt *et al.*, 1981 ; Thoen *et al.*, 1984a).

The poly(A)-sequence at the 3' end of the majority of the mRNAs is a universal feature of all organisms and is one of the main protein binding sites. It is postulated that the poly(A)-protein complex is important in the regulation of protein synthesis (Müller *et al.*, 1978). The length of the poly(A)-sequence is modulated by endo- and exonuclease activities and by poly(A) polymerase. The susceptibility of the poly(A)-sequence for these enzymes is regulated by the poly(A)-protein interaction (Bergmann and Brawerman, 1977 ; Palatnik *et al.*, 1984). In this view it is clear that all factors interfering with the poly(A)-binding protein – poly(A) interaction will affect protein synthesis.

In this communication we describe the isolation and characterization of the M_r 38 000 dalton poly(A)-binding protein (P38) isolated from non-polysomal poly(A)-containing mRNP from cryptobiotic gastrulae of *Artemia* sp. This protein represents 60-70 % of the total amount of mRNA associated protein and is extensively phosphorylated by the mRNP associated casein kinase II (Thoen *et al.*, 1984a). The poly(A)-binding protein has a comparable molecular weight, amino acid composition and protein : poly(A)-stoichiometry as HD40, a helix-destabilizing protein, isolated by Szer and collaborators from the ribosomal fraction and from hnRNP of *Artemia* sp. (Marvil *et al.*, 1980). We also examined the protein composition of ribosome preinitiation complexes of cysts and polysomal poly(A)-containing mRNP of nauplii. The absence of P38 from the latter mRNP will be reported and a hypothesis is presented concerning the functionality of the poly(A)-binding proteins.

Materials and methods

Fractionation

The postmitochondrial supernatant (PMS) is prepared from 200 g (dry weight) of cryptobiotic gastrulae of *Artemia* sp. (Macau, Brazil ; obtained from the *Artemia* Reference center, University of Ghent, Belgium) as described by Slegers *et al.* (1981). After differential centrifugation of the postmitochondrial supernatant in a Beckman R60 rotor for 17 h at 4 °C (250 000 × *g*), a postribosomal supernatant and a ribosome-containing pellet are obtained. Poly(A)-containing mRNP is prepared from the postribosomal and ribosome fraction by affinity chromatography on oligo(dT)-cellulose. The poly(A)-binding protein P38 is isolated by a 1 M KCl wash of oligo(dT)-cellulose-bound mRNP (Slegers *et al.*, 1981). After dialysis against 20 mM potassium phosphate pH 6.2, 50 mM KCl, 0.4 mM EDTA, 5 mM 2-mercaptoethanol, 10 % glycerol (buffer A) proteins are fractionated on phosphocellulose P11. The bound material is eluted with a salt gradient from 50 to 400 mM KCl in buffer A. P38 elutes at 200 mM KCl and is further purified on poly(A)-Sepharose 4B as described (De Herdt *et al.*, 1982). The helix-destabilizing protein HD40 is prepared according to Marvil *et al.* (1980).

Polysomal poly(A)-containing mRNP is isolated from the postribosomal supernatant of nauplii. 90 g of cryoptobiotic gastrulae are grown in artificial seawater (30 g/l) at 30 °C for 36 h. Nauplii are collected, washed with distilled water and homogenization buffer (25 mM Tris/HCl pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, 150 mM sucrose, 0.001 TIU/ml aprotinin ; buffer B). After homogenization in a Potter-Elvehjem apparatus, a postmitochondrial supernatant is prepared by

two consecutive centrifugations in a Beckman JA20 rotor at 4 °C for 30 min (12 000 × *g*). PMS is loaded on 8 ml cushions of 50 % sucrose in buffer B and centrifuged for 2 h at 4 °C in a Beckman R60 rotor (250 000 × *g*). The polysome pellets are rinsed with buffer B and stored at - 70 °C until further use. Polysomal poly(A)-containing mRNP is dissociated from the ribosome subunits by dissolving the pellets in 10 mM Hepes pH 7.2, 250 mM KCl, 5 mM 2-mercaptoethanol, 0.1 mM PMSF (buffer C – 1 ml/pellet) and heating in the presence of 5 mM EDTA at 50 °C for 5 min. After dilution with buffer C (EDTA concentration below 1 mM) poly(A)-containing mRNP is isolated by affinity chromatography on oligo(dT)-cellulose equilibrated with buffer C. Thermal elution of the column results in the release of poly(A)-containing mRNP. mRNP is concentrated by Amicon PM30 ultrafiltration and applied to a Sepharose 4B column equilibrated with 10 mM Hepes pH 7.2, 100 mM KCl, 5 mM mercaptoethanol, 10 % glycerol (buffer D).

Assays

Poly(A)-binding protein-RNA complex formation is measured in 5 mM Tris/HCl pH 7.4 and 100 mM KCl. The reaction mixture (40 µl) contains ± 0.5 µg RNA and 0.5 – 5 µg P38. After incubation for 1 min at 4 °C the reaction is stopped by dilution with 1 ml ice-cold buffer and filtration through nitrocellulose membranes (Millipore HAWP, 0.45 µm). Before use filters are washed with 0.5 N KOH (30 min), rinsed with distilled water and equilibrated with 5 mM Tris/HCl pH 7.4 and 100 mM KCl.

Phosphorylation of the M_r 38 000 poly(A)-binding protein and HD40 is performed in 10 mM Tris/HCl pH 8.2, 12.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 3.3 mM [γ - ^{32}P]ATP (3 000 Ci/mmol ; Amersham International, Buckinghamshire, UK), 1 µM ATP and 75 units of protein kinase (1 unit will transfer 1.0 pmol phosphate from [γ - ^{32}P]ATP to casein/min). The protein kinase is isolated from non-polysomal mRNP as described (Thoen *et al.*, 1984a).

Dephosphorylation of poly(A)-binding mRNP proteins is performed in 17.5 mM Tris/HCl pH 7.0, 0.03 mM EGTA, 0.03 % (v/v) 2-mercaptoethanol at 30 °C using two *Artemia* sp. cytosol phosphatases (Thoen *et al.*, 1984b). Both phosphorylation and dephosphorylation reactions are terminated by TCA precipitation.

Protein synthesis is in the nuclease-treated rabbit reticulocyte lysate exactly as described by Pelham and Jackson (1976).

Transfer of proteins to nitrocellulose by electroblotting and the protein-RNA binding assay is performed according to Richter and Smith (1983). The poly(A)-binding capacity is determined with 3' end labelled poly(A). Labelling with [5' - ^{32}P]pCp is as described by England *et al.* (1980). Other techniques used *e.g.* double immunodiffusion and 10 % (w/v) polyacrylamide/dodecylsulfate gels are described by De Herdt *et al.* (1984), two-dimensional gel-electrophoresis by O'Farrell (1975) and analysis of tryptic digests by one-dimensional gel-electrophoresis by Cleveland *et al.* (1977).

Results

The M_r 38 000 poly(A)-binding protein of *Artemia*

Non-polysomal poly(A)-containing mRNP of cryptobiotic embryos is characterized by the presence of a discrete number of proteins with M_r of 87 000, 76 000, 65 000, 50 000, 45 000,

38 000, and 23 500. The M_r 38 000 and 23 500 proteins represent more than 70 % of the protein content. Electroblotting of mRNP proteins and subsequent incubation with [^{32}P]pCP labelled poly(A) reveals that the latter two polypeptides are the only poly(A)-binding proteins of the mRNP (Fig. 1). The M_r 38 000 and 23 500 proteins as well as a M_r 73 000 poly(A)-binding protein are also present in a free state in the cytosol, but they cannot be detected in the membrane fraction. Marvil *et al.* (1980) isolated a M_r 40 000 helix-destabilizing protein (HD40) from a crude ribosomal fraction of cryptobiotic gastrulae and from the nuclear fraction of *Artemia* sp. nauplii and stated that HD40 can only be detected in the nucleus after development to the nauplius stage (Thomas *et al.*, 1981). In addition, De Herdt *et al.* (1982) demonstrated that HD40 is not a constituent of 80S ribosomes, but a component of ribosome associated mRNP. Both the M_r 38 000 protein and HD40 are characterized by a high glycine content, the presence of dimethylarginine and the absence of cysteine, indicating a possible relationship between both proteins and hnRNP proteins.

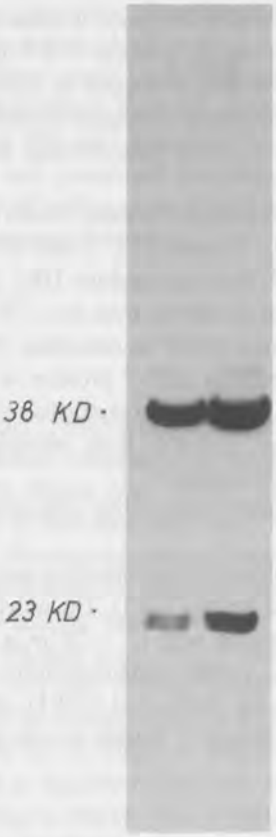

FIG. 1. Identification of poly(A)-binding proteins in cytoplasmic non-polysomal mRNP. mRNP proteins are analyzed on a 10 % (w/v) polyacrylamide sodium dodecylsulfate gel and electroblotted on nitrocellulose membranes (Richter and Smith, 1983). [^{32}P]pCp labeled poly(A) (specific activity 45 000 cpm/μg) is used to detect the poly(A)-binding proteins by incubation of the nitrocellulose blot with 1-2 × 10^6 cpm [^{32}P]pCp labelled poly(A) for 3 h at room temperature. The blot is washed, dried and autoradiographed.

The possible identity of HD40 and the M_r 38 000 protein is further strengthened by the identical mobility in sodium dodecylsulfate polyacrylamide gels, their comparable nucleic acid-binding characteristics, tryptic peptide maps and from immunodiffusion experiments (Fig. 2). It is clear that although isolated from different cellular fractions, the M_r 38 000 poly(A)-binding protein and HD40 represent the same protein. A large number of nucleic acid-binding proteins, isolated from various organisms exhibit the same characteristics and there is no doubt that they are structurally and/or functionally related to each other. The presence of the poly(A)-binding protein of M_r 38 000 (or HD40) in different cellular localizations indicates that the latter protein may have an important function in mRNA metabolism.

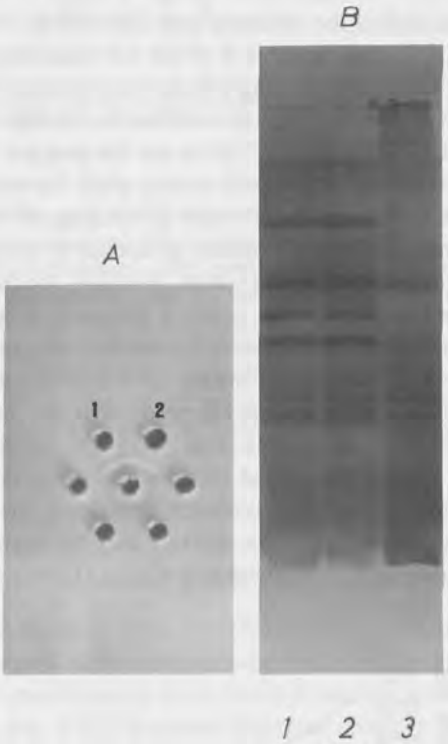

FIG. 2. Identity between the poly(A)-binding protein P38 and the helix-destabilizing protein HD40. A. Double immunodiffusion with anti-P38 antiserum. The central well contains 1 mg anti-P38 antiserum. Well 1 and 2 contain 10 μg HD40 and 8 μg P38 respectively. B. Analysis of tryptic digests of P38 and HD40 by one-dimensional gel electrophoresis according to Cleveland *et al.* (1977). Lane 1, 5 μg P38 + 0.5 μg trypsin ; lane 2, 5 μg HD40 + 0.5 μg trypsin ; lane 3, 0.5 μg trypsin. Incubation was for 3 h at 37 °C. (Reproduced from De Herdt *et al.*, 1984).

Interaction of the M_r 38 000 poly(A)-binding protein with nucleic acids

The nucleic acid-binding properties of the M_r 38 000 protein are reported in detail by De Herdt *et al.* (1982, 1984) and Nowak *et al.* (1980). The protein interacts with natural and synthetic nucleic acids and a stoichiometry of 10-12 nucleotides/protein is calculated. The

interaction is fast (completed within 5 s) and polyadenylic acid is preferred over all other nucleic acids. The helix-destabilizing character is demonstrated by melting of double stranded poly(A + U).

The pH dependence of the interaction is studied from pH 5.5 to pH 10 (Fig. 3). No pH optimum is observed and the retention of poly(A) on nitrocellulose filters increases linear as a function of the pH. This can be explained by the existence of several ionic species of the poly(A)-binding proteins (see below). At pHs above 9.2 (the highest pI value of P38) there is virtually no binding. Lowering of the pH changes the overall charge of the protein and binding to poly(A) is initiated. The lower the pH, the more P38 iso-species are able to bind to ribonucleic acid. The optimal concentration of monovalent cations is found to depend on the pH value of the assay mixture. A gradual shift of the optimum from 200 mM KCl to 50 mM KCl is observed when the binding assay is performed at pH 8.5 or pH 5.0 respectively (Fig. 4a). This may be explained by the fact that the poly(A)-binding protein itself becomes more and more positively charged at low pH values. It is also important to note that the binding of protein to RNA requires a minimal concentration of 20-30 mM KCl. This is not the case for divalent cations which are not necessary to observe interaction of P38 with nucleic acids. Nevertheless, poly(A)-binding is dependent on the Mg^{++} concentration. Above 2 mM the binding of P38 to poly(A) is drastically inhibited (Fig. 4b). It is clear that in the isolation procedure of cytoplasmic mRNP Mg^{++}-ions have to be excluded from the homogenization buffers. However, for the purification of polysomal mRNP Mg^{++} ions are necessary to obtain intact polysomes. In the latter purification the concentration of Mg^{++}ions may not exceed 2 mM in order to avoid loss of proteins from mRNP.

In this laboratory a pairwise repetition of the M_r 38 000 dalton protein on the poly(A)-sequence with a periodicity of 20 ± 2 nucleotides has been calculated (De Herdt *et al.*, 1982). Baer and Kornberg (1983) reported similar results for a M_r 75 000 dalton protein isolated from rat liver. Nowak *et al.* (1980) have demonstrated that the nucleotide/protein ratio determines the physical organization of the protein-RNA complex. Increasing the concentration of protein results in the formation of „beads". It is not unlikely that the *in vivo* situation is even more complex than can be deduced from *in vitro* binding studies (Glowacka *et al.*, 1984).

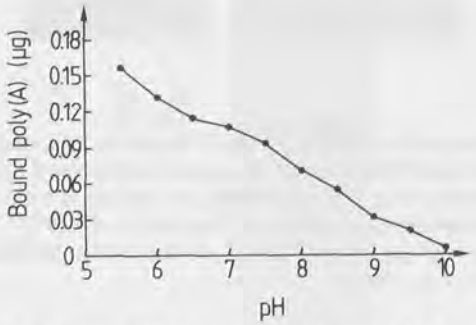

FIG. 3. pH dependence of the poly(A)-P38 interaction. 85 pmol P38 is mixed with 1.8 nmol poly(A) (130 000 cpm/µg) in assay buffers of pH 5.5 to 10.0 containing 125 mM KCl. 20 mM Hepes (pH 5.0 to 8.0) and 20 mM Tris (pH 7.0 to 10.0) are used as buffers. After incubation at 4 °C for 1 min, the reaction is stopped by addition of ice-cold buffer of the corresponding pH and immediately filtered over nitrocellulose filters. Before use, nitrocellulose filters are washed for 30 min in 0.5 N KOH and rinsed with distilled water. Control incubations (no poly(A)-binding protein added) are performed at each pH value.

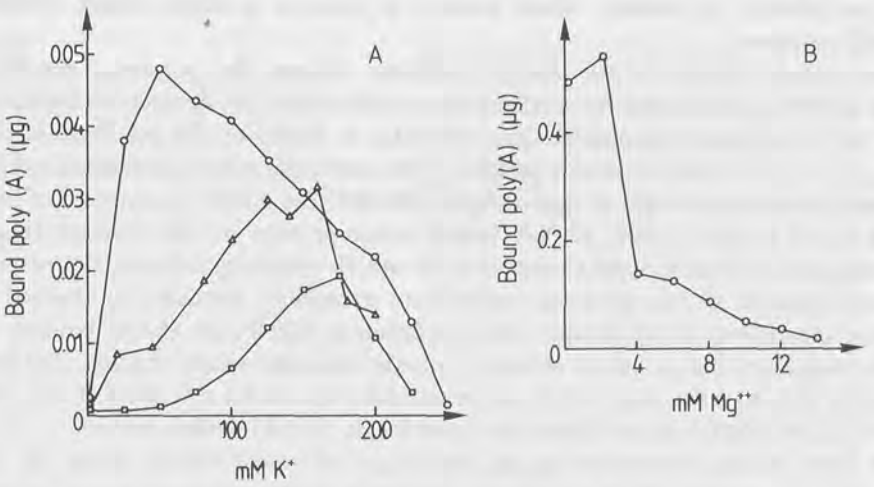

FIG. 4. A. Dependence of the poly(A)-P38 interaction on monovalent cations at different pH values. 52 pmol poly(A)-binding protein is mixed with 14 nmol poly(A) at pH 5.0, 7.5 or 8.5 in 20 mM Tris and a variable potassium chloride concentration. After incubation at 4 °C for 1 min, the reaction is stopped by addition of ice-cold buffer of the corresponding pH value and salt concentration, and immediately filtered over nitrocellulose filters. Before use filters are washed for 30 min in 0.5 N KOH and rinsed with distilled water. Control incubations (no poly(A)-binding protein added) are performed at 50 mM and 250 mM KCl. (○——○) salt dependence at pH 5.0 ; (△——△) at pH 7.5 ; (□——□) at pH 8.5. B. Dependence of the poly(A)-P38 interaction on divalent cations. 100 pmol poly(A)-binding proteins is mixed with 2.0 nmol poly(A) in 20 mM Tris-HCl pH 7.5, 125 mM KCl and incubated as described in A. Control incubations are performed at each Mg^{++}-concentration.

Absence of the M_r 38 000 poly(A)-binding protein from polysomal poly(A)-containing mRNP

To evaluate whether the presence of the 38 000 protein is restricted to stored non-polysomal mRNP, we isolated polysomal poly(A)-containing mRNP from nauplii. The integrity of the polysomes is checked by centrifugation on a 20-55 % sucrose gradient in buffer B (Beckman SW27 rotor, 125 000 × *g*, 6 h, 4 °C). A typical polysome profile is obtained in which messenger RNP is complexed with one to seven 80 S ribosomes (data not shown).

Comparison of the poly(A)-content of non-polysomal mRNP from cryptobiotic gastrulae and polysomes from nauplii derived from the same amount of gastrulae reveals that both fractions contain about the same amount of polyadenylic acid. However, only 22 % of the polysomal poly(A)-sequences can be bound to oligo(dT)-cellulose. Proteins with M_r 85 000, 73 000, 55-57 000, 38 000, and 22 000 are detected in the polysomal mRNP bound to oligo(dT)-cellulose. The latter proteins are also minor constituents of the unbound fraction. In the latter fraction, the main proteins have an M_r of 95 000 and 33 000. Analysis of the unbound fraction by sucrose gradient centrifugation reveals the presence of a poly(A)-containing 17 S mRNP with the same protein composition as mRNP present in the oligo(dT)-cellulose bound fraction. The majority of the poly(A)-sequences as well as the M_r 33 000 and 95 000 proteins sediment in the 2-4 S region. These results suggest that the poly(A)-sequence of polysomal mRNP is either very

short or shielded by proteins which prevent, at least to a certain extent, binding to oligo(dT)-cellulose.

After thermal elution of the oligo(dT)-cellulose columns the polysomal poly(A)-containing mRNP is concentrated by ultrafiltration and subjected to gel filtration on Sepharose 4B (Fig. 5a). In a typical preparation about 40 OD_{260} is loaded on the gel filtration column ($OD_{260}/_{280}$ = 1.38). From the elution position of the polysomal poly(A)-containing mRNP we calculated a molecular weight of approximately 800 000. The mRNP is composed of proteins with a M_r of 85 000, 73 000, 55 000. Several minor proteins are also detected (Fig. 5b). Although poly(A)-sequences can be assayed in the mRNP containing fractions, they never reach the level measured for non-polysomal mRNP from cryptobiotic gastrulae. As observed in gel filtration experiments of cytoplasmic poly(A)-containing mRNP, the elution position of the poly(A)-sequences is also slightly shifted to a lower molecular weight position. The purified polysomal poly(A)-containing mRNP can be translated in a rabbit reticulocyte lysate. The M_r 73 000 and Mr 55 000 dalton proteins are identified as poly(A)-binding proteins.

The most striking observation is the absence in polysomal mRNP of the M_r 38 000 poly(A)-binding protein which is a major constituent of non-polysomal poly(A)-containing mRNP. Double immunodiffusion experiments of crude polysomes, purified polysomal mRNP and anti-P38 antiserum produce no precipitin lines. Furthermore the protein composition of polysomal poly(A)-containing mRNP analysed by polyacrylamide gel electrophoresis is different from the protein composition of non-polysomal mRNP. From these results we conclude that it is not unlikely that the transfer of stored mRNP to polysomes is accompanied by a drastic change in protein composition. The M_r 38 000 poly(A)-binding protein is exchanged by M_r 55 000 and 73 000 proteins which are reported to be typical polysomal poly(A)-binding proteins (Greenberg, 1980). Preliminary experiments indicate that the M_r 38 000 poly(A)-binding protein is absent from cytoplasmic poly(A)-containing mRNP from nauplii, suggesting that the function of P38 in the metabolism of cytoplasmic mRNP is restricted to the cryptobiotic stage.

Post-translational phosphorylation of P38

Although the M_r 38 000 poly(A)-binding protein and HD40 are identical an interesting difference is observed by two-dimentional polyacrylamide gel electrophoresis (O'Farrell, 1975). A preparation of the M_r 38 000 poly(A)-binding protein shows at least seven ionic species with a pI ranging from 6.6 to 9.2. On the contrary HD40 purified from the ribosomal fraction of *Artemia* sp. as described by Marvil *et al.* (1980) only contains the two most basic isoforms with a pI of 9.2 and 8.5 (Fig. 6). The pI values of 6.6 to 9.2 are similar to those published by Fuchs *et al.* (1980) for hnRNP proteins. The charge heterogeneity is a consequence of post-translational phosphorylation. When basic isoforms are subjected to phosphorylation by the mRNP-associated casein kinase II the acidic species are generated. Autoradiography demonstrates the existence of at least four ionic species with a pI of 7.3, 7.0, 6.8 and 6.7 (Fig. 6). These results indicate that the poly(A)-binding protein is subjected to multisite phosphorylation, and that HD40 consists of a specific subset of basic isoforms. The reaction can be reversed by dephosphorylation. Two protein phosphatases are identified in the cytosol of *Artemia* sp. cryptobiotic gastrulae that are able to dephosphorylate the poly(A)-binding protein. It is tempting to suggest that the reversible phosphorylation-dephosphorylation reaction is the regulating mechanism for the interaction of the M_r 38 000 poly(A)-binding protein with RNA. Evidence for this hypothesis is obtained from studies of the effect of phosphorylation-dephosphorylation on the poly(A)-binding capacity of

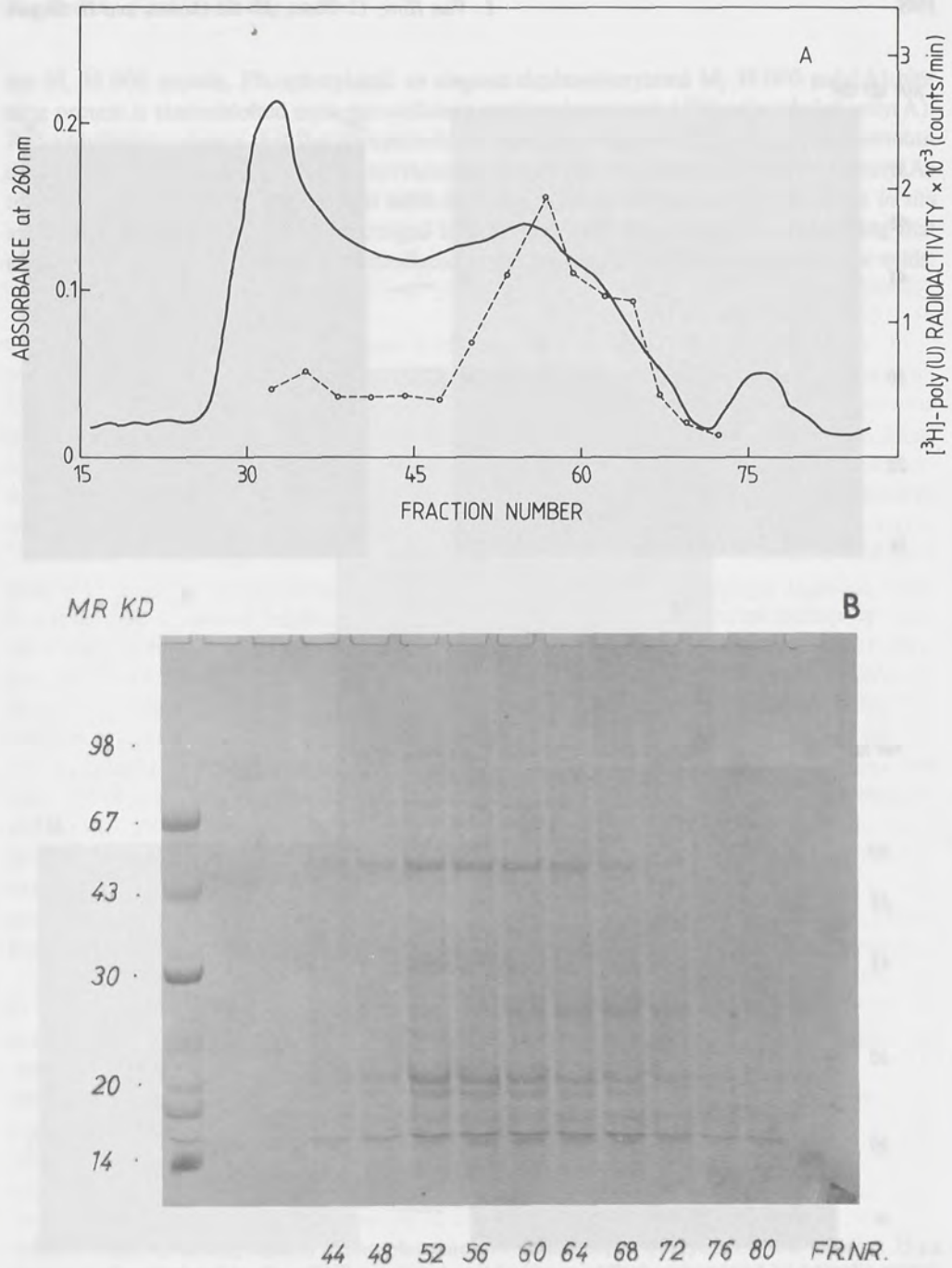

FIG. 5. Characterization of polysomal mRNP. A. Poly(A)-containing mRNP eluted from oligo(dT)-cellulose is concentrated by ultrafiltration and subjected to gel filtration on a Sepharose 4B column in buffer D. Fractions of 1.9 ml are collected (——) absorbance at 260 nm ; (○----○) hybridization with [^{3}H]poly(U) as described by De Herdt *et al.* (1982). B. The protein composition of the poly(A)-containing mRNP purified by gelfiltration is analyzed by 10 % (w/v) polyacrylamide sodium dodecylsulfate gel-electrophoresis.

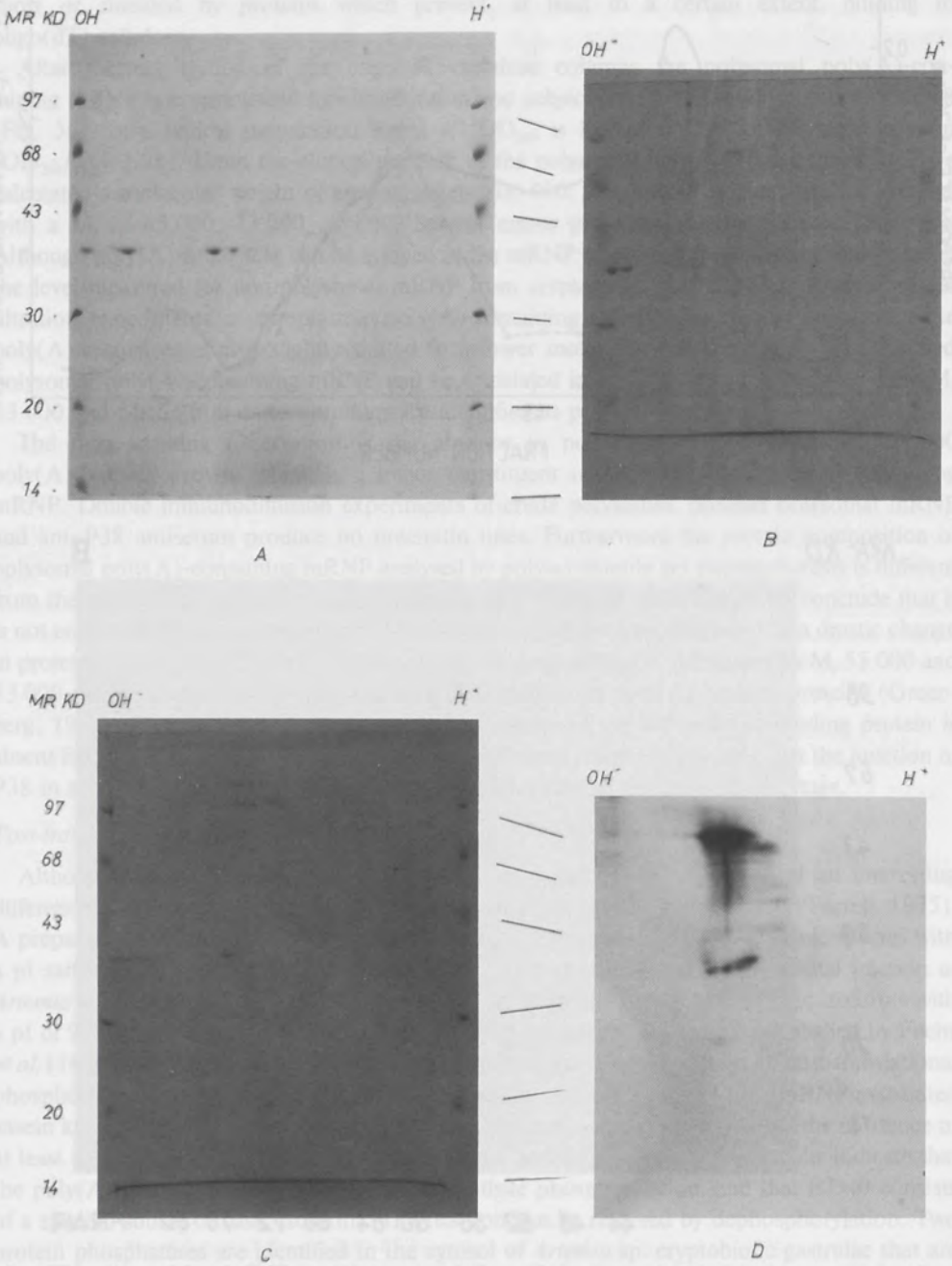

FIG. 6. Analysis of P38 and HD40 by two-dimensional gel electrophoresis. Protein samples of 150 µg are analysed as described by O'Farrell (1975). (1) P38 ; (B) HD40 prepared as described by Marvil *et al.* (1980) ; (C) HD40 phosphorylated by mRNP-associated protein kinase ; (D) autoradiogram of phosphorylated HD40 (reproduced from De Herdt *et al.*, 1984).

the M_r 38 000 protein. Phosphorylated, *in situ* and dephosphorylated M_r 38 000 poly(A)-binding protein is electroblotted onto nitrocellulose and incubated with [^{32}P]-pCp labeled poly(A). Autoradiography reveals that the phosphorylated protein is capable of binding a larger amount of poly(A) than *in situ* and dephosphorylated proteins (Fig. 7). When the amount of poly(A) retained by phosphorylated proteins is taken as 1, the capacity of poly(A)-binding of the *in situ* and dephosphorylated protein is decreased to 0.37 and 0.03 respectively. It is surprising that although more negative charges are introduced in the protein, it binds stronger to nucleic acids.

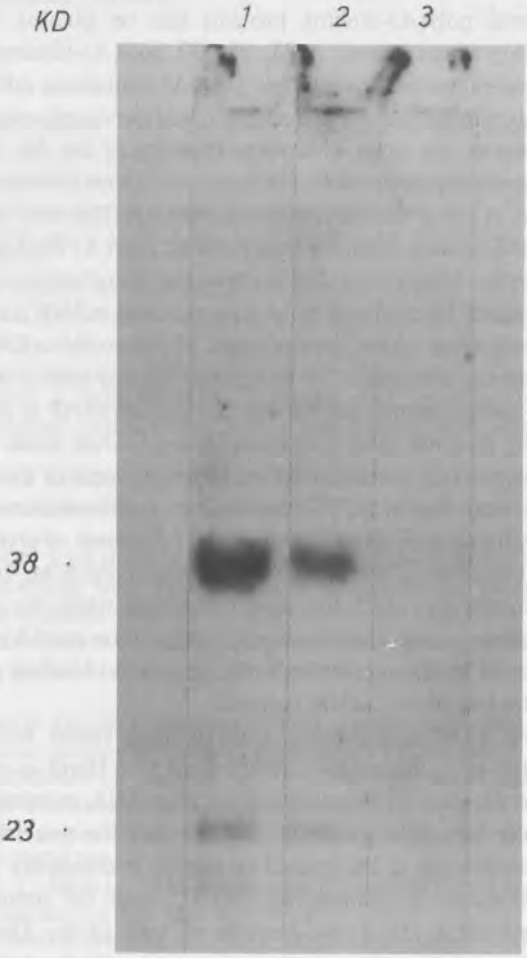

FIG. 7. Poly(A)-binding capacity of phosphorylated, *in situ* and dephosphorylated mRNP proteins. 35 µg protein is phosporylated by the mRNP associated protein kinase or dephosphorylated by cytosolic protein phosphatases of *Artemia* sp. (0.6 U/ml) as described in methods. After electrophoresis on 10 % (w/v) polyacrylamide sodium dodecylsulfate gels, proteins are electroblotted onto nitrocellulose and incubated with [^{32}P]-pCp labeled poly(A) (specific activity 12 000 cpm/µg) for 3 h at room temperature (Richter and Smith, 1983). Blots are washed, dried and autoradiographed. Lane 1, phosphorylated mRNP proteins ; lane 2, *in situ* mRNP proteins ; lane 3, dephosphorylated mRNP proteins.

Discussion

The presence of a poly(A)-protein structure at the 3'-end of mRNA, is a universal property of messenger ribonucleoproteins. In the past few years many reports have been published concerning the functional significance of the latter structure. Although the efficiency of mRNA translation, regulation of mRNA stability, nucleocytoplasmic mRNA transport and binding to the nuclear matrix are influenced by the properties of the poly(A)-protein structure, its function remains unknown (Palatnik *et al.*, 1980, Beyer *et al.*, 1981 ; Palayoor *et al.*, 1981 ; Schmid *et al.*, 1983).

In *Artemia* sp. several poly(A)-binding proteins can be isolated from different cellular localizations and developmental stages. A M_r 38 000 poly(A)-binding protein is the major constituent of stored non-polysomal cytoplasmic poly(A)-containing mRNP. The protein is the target of reversible post-translational phosphorylation and dephosphorylation and the degree of phosphorylation determines the poly(A)-binding capacity of the M_r 38 000 protein. When development of the cryptobiotic gastrulae is resumed, polysomes are formed and the M_r 38 000 poly(A)-binding protein is lost from the cytoplasmic mRNP. This may be a consequence of the dephosphorylation of the protein resulting in a reduced poly(A)-binding capacity. This hypothesis is strengthened by the observation that in ribosome-preinitiation complexes only the most basic iso-species are present. Furthermore poly(A)-containing mRNP contains a M_r 73 000 and a M_r 55 000 protein which are typical components of polysomal mRNP in other organisms. However, a possible identity remains to be established. In any case it is clear that the protein composition of stored non-polysomal mRNP and polysomal mRNP is completely different and that the poly(A)-binding proteins differ to a certain extent in their mode of action. It is possible that modification of the poly(A)-protein structure by replacement of a poly(A)-binding protein by another initiates the next step in mRNP metabolism. An illustration of such a modification is the observation of Schmid *et al.* (1983) that in the cytoplasm of erythropoietic mouse cells induced by Friend leukemia virus, 50 % of the mRNP contains a M_r 76 000 poly(A)-binding protein and is incorporated into 48 S initiation complexes while the remaining 50 % of the mRNP, lacking this protein, is not. Also the susceptibility of the poly(A)-segment for enzymatic hydrolysis could be altered by the replacement of one poly(A)-binding protein by another and this could determine the fate of an mRNA species.

The M_r 38 000 poly(A)-binding protein, isolated from stored non-polysomal mRNP of cryptobiotic gastrulae has been thoroughly characterized (De Herdt *et al.*, 1982, 1984). Based on its characteristics a function in the early stages of mRNA metabolism can be proposed. Moreover, recent results from this laboratory indicate that the amount of M_r 38 000 dalton poly(A)-binding protein present in the cytosol of nauplii is drastically decreased suggesting a specific cryptobiotic function on cytoplasmic mRNP. Since the protein is present in large quantities in stored mRNP a stabilizing function is very likely. The observation that the poly(A)-binding protein inhibits protein synthesis in rabbit reticulocyte lysate (De Herdt *et al.*, 1984), as well as the observed accumulation of the M_r 38 000 protein in the nucleus of *Xenopus laevis* oocytes (De Herdt *et al.*, 1983) suggests that this protein may be involved in several mRNA related phenomena. However, other cytoplasmic and/or nuclear functions cannot be excluded.

The multifunctionality of the poly(A)-binding protein may be regulated by mechanisms that affect the protein-poly(A) interaction. The observed effect of phosphorylation/dephosphorylation

on the poly(A)-protein interaction suggests that the functionality of the poly(A)-binding protein is regulated by the latter mechanism.

Acknowledgements

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The role of proteases and their control in *Artemia* development

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Content

Introduction

In recent years *Artemia* embryos have served as an important organism in the study of the biochemistry of development (see monographs edited by Bagshaw and Warner, 1979 and Persoone *et al.*, 1980). Outstanding papers now appear regularly in international journals on the purification and characterization of enzymes from different developmental stages in an attempt to gain insight into the mechanism by which the developmental program is expressed in *Artemia* as an invertebrate model. Of fundamental importance to these studies, and others concerned with the control of developmental events, is an understanding of the proteases and their inhibitors in *Artemia* embryos since these proteins are believed to play important roles in the regulation of developmental events that require proteolytic initiation.

In most tissues studied proteases are involved in protein turnover through the inactivation of proteins and enzymes, but numerous examples exist which show proteases involved in activation reactions as well (Reich *et al.*, 1975 ; Wolf, 1985). In order to exert biological control over its proteins most cells contain a wide variety of proteases including amino- and carboxypeptidases and endoproteases. In encysted embryos of *Artemia*, few proteases have been detected thus far, while still others may be present in inactive or masked forms. Moreover, since changes in the activity and/or molecular size of several proteins and enzymes of *Artemia* have been observed in extracts from developing embryos around the time of hatching (*e.g.*, modification of elongation factor 1 (EF-1_H), Twardowski *et al.*, 1976 ; elongation factor 2 (EF-2), Yablonka-Reuveni and Warner, 1979 ; RNA polymerase I, Osuna *et al.*, 1977b ; and RNA polymerase II, Cruces *et al.*, 1984), an understanding of protease activity at different stages of development is necessary to assess the importance of these protease-induced changes in the regulation of development.

In the following pages, I intend to review what is currently known about proteases and their control in *Artemia* embryos. In addition I present some recent findings from my laboratory, yet to be published, on the *in vitro* activation of a new protease which is inactive in extracts of dormant *Artemia* embryos, and on work to characterize two thiol protease inhibitors in the postribosomal fraction of encysted embryos.

Characterization and properties of thiol proteases from dormant gastrulae of *Artemia*

Dormant embryos and early gastrulae of *Artemia* contain a cathepsin B-like protease which represents over 90 % of the total protease activity in crude homogenates. Using dormant cysts from Utah as starting material (about 50 % hatchability, Warner *et al.*, 1979) a cathepsin B-like thiol protease has been purified to homogeneity from the postribosomal fraction (Warner and Shridhar, 1985). The protease has a molecular weight of 55 000 ± 4 200 by gel filtration and is composed of subunits of 31 500 ± 559 and 25 867 ± 1087 as determined by SDS-polyacrylamide gel electrophoresis. On isoelectric focusing gels two discrete bands have been detected, one at pH 4.6 and the other at pH 5.1. In the pH range 3.5 to 4.5 the protease catalyzes the complete hydrolysis of a variety of proteins including protamine, bovine serum albumin, *Artemia* lipovitellin, hemoglobin and *Artemia* acidic proteins including the protein synthesis elongation factor, EF-2. At pH 6.0-6.5, conditions in which virtually all *Artemia* cytosol proteins are resistant to hydrolysis by the protease, the enzyme has been shown to have a high degree of specificity for *Artemia* elongation factor 2 (EF-2), and probably explains the reduced capacity for protein synthesis and the appearance of fragments of EF-2 in partially purified protein preparations from dormant cysts and developing embryos (Yablonka-Reuveni and Warner, 1979). The data in Fig. 1 demonstrate the effect of this protease on *Artemia* EF-2 at pH 6.0-6.5 and show that virtually all other major acidic cytosol proteins are resistant to proteolytic modification by this enzyme under these conditions. In addition the *Artemia* cyst cytosol protease catalyzes the hydrolysis of BANA and N-Cbz-Arg-Arg-NA, well known cathepsin B substrates but K_{cat} data for various substrates show that the protease prefers proteins as substrates especially protamine which is rich in arginine groups (Warner and Shridhar, 1985).

The cytosol protease is sensitive to a variety of thiol protease blocking reagents such as leupeptin, antipain, chymostatin, Ep-475, iodoacetate, Cu^{2+} and Hg^{2+}, but it is relatively insensitive to pCMB and NEM. Moreover, kinetic data for leupeptin and Ep-475 show that equimolar amounts of these inhibitors (with enzyme) produce 50 % inhibition suggesting that the enzyme is a thiol protease containing two binding sites for these agents which must be filled to completely inactivate the protease. Confirmation of this view will require the use of radiolabeled inhibitors in inhibitor binding experiments. The cytosol protease is uneffected by pepstatin, ovomucoid, soybean trypsin inhibitor, and PMSF but easily inactivated by exposure to 0.1 % SDS, 7 M urea, and incubation at pH 8 and above at 40 °C.

An important property of the encysted embryo cytosol protease is its ability to catalyze the hydrolysis of yolk proteins under physiological conditions. Using lipovitellin and thiol protease isolated from dormant gastrulae of *Artemia* we have observed a pattern of lipovitellin hydrolysis *in vitro* which closely resembles that found in intact gastrulae and nauplii (De Chaffoy de Courcelles and Kondo, 1980 ; Vallejo *et al.*, 1980). The sequential hydrolysis of LV- α1 (M_r 185 000) to smaller lipovitellins and eventually to LV-α10 (M_r 120 000) by the thiol protease

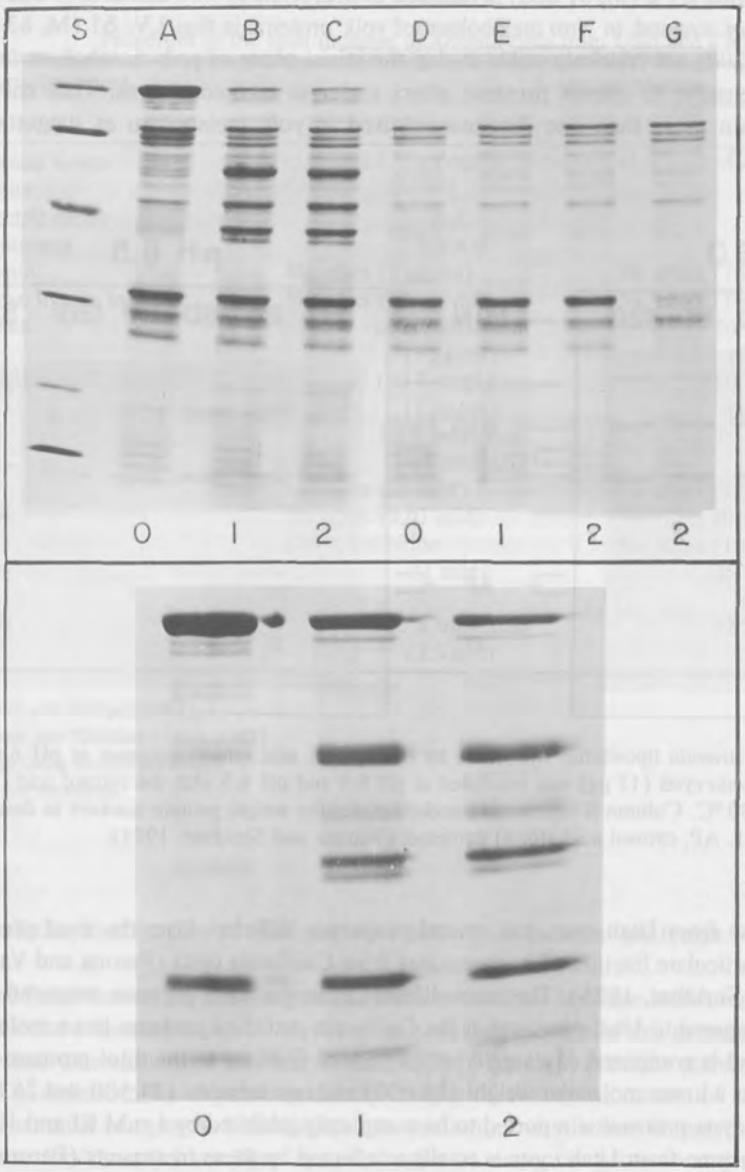

FIG. 1. Hydrolysis of *Artemia* elongation factor 2 by the cytosol acid (thiol) protease at pH 6.0-6.5. Top panel. Incubation of *Artemia* soluble acidic proteins (60 μg) with cytosol acid (thiol) protease (4.5 μg) and added EF-2 (8 μg) (A-C) or without added EF-2 (D-G) at 30 °C and pH 6.5. Column G did not contain added acid (thiol) protease (AP). Column S represents standard molecular weight proteins. From top to bottom they are : phosphorylase (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), and α-lactalbumin (M_r 14 400). Bottom panel. Incubation of EF-2 (8 μg) with the cytosol acid (thiol) protease (4.5 μg) only at 30 °C and pH 6.0. (Warner and Shridhar, 1985).

is shown in Fig. 2 while LV-ε (M_r 61 000) is resistant to hydrolysis *in vitro* and *in vivo*. The main difference between *in vivo* and *in vitro* metabolism of yolk proteins is that LV- δ1 (M_r 83 500) and LV-δ2 (M_r 73 200) are relatively stable during the initial phase of yolk metabolism *in vivo*, whereas they are sensitive to cytosol protease attack under *in vitro* conditions. Thus encysted embryos may contain more than one protease involved in yolk metabolism as suggested by Vallejo *et al.*, (1980).

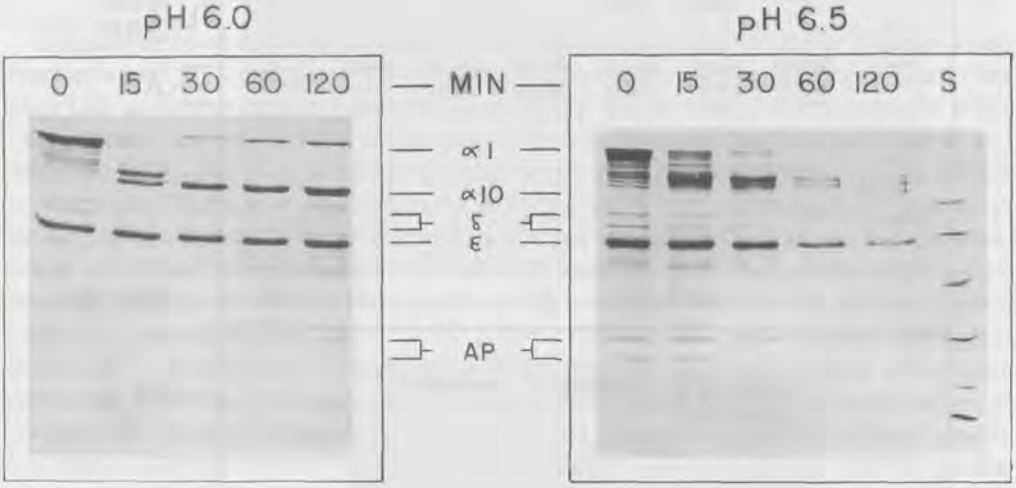

FIG. 2. Pattern of *Artemia* lipovitellin hydrolysis by the cytosol acid (thiol) protease at pH 6.0-6.5. Lipovitellin from *Artemia* cysts (12 μg) was incubated at pH 6.0 and pH 6.5 with the cytosol acid (thiol) protease (1.9 μg) at 30 °C. Column S represents standard molecular weight protein markers as described in the legend to Fig. 1. AP, cytosol acid (thiol) protease. (Warner and Shridhar, 1985).

The thiol protease from Utah cysts has several properties different from the thiol protease purified from the particulate fraction of homogenates from California cysts (Perona and Vallejo, 1982 ; Warner and Shridhar, 1985). The main differences in the thiol protease prepared from California cysts compared to Utah cysts is that the California cyst thiol protease has a molecular weight of 68 000 and is composed of a single polypeptide in contrast to the thiol protease from Utah cysts which has a lower molecular weight (55 000) and two subunits (31 500 and 25 867). Also the California cysts protease is reported to be completely inhibited by 1 mM KI and 10 μM pCMB while the enzyme from Utah cysts is totally uneffected by these treatments (Perona and Vallejo, 1982 ; Warner and Shridhar, 1985). Otherwise the two enzymes share similar properties including the fact that both thiol proteases are glycoproteins. The major properties of these thiol proteases are compared in Table I.

While there appear to be differences in the properties of the thiol protease isolated from Utah and California cysts, a final conclusion on these differences may require that the thiol protease be isolated and purified by identical procedures, avoiding the use of strong oxidants such a NaOCl to sterilize the cysts which we have shown causes artifacts in activity measurements of proteins rich in SH-groups (Warner, 1979 ; Warner and Shridhar, 1985).

TABLE I

Properties of the thiol protease in *Artemia* cysts from Utah and California

Property/treatment	Source of enzyme	
	California cysts[a]	Utah cysts[b]
Molecular weight	68 000	55 000
Subunits	1	2
Isoelectric forms	n.d.	2
pH optimum	3.5-4.0	3.5-4.5[c]
Pepstatin	No effect (1 µg/ml)	No effect (10 µg/ml)
Soybean trypsin inhibitor	No effect (10 µg/ml)	No effect (1 mg/ml)
Antipain	100 % inhibition (1 µg/ml)	50 % inhibition (0.5 µg/ml)
Leupeptin	100 % inhibition (1 µg/ml)	50 % inhibition (0.2 µg/ml)
Cu^{2+}	90 % inhibition (1 mM)	95 % inhibition (0.1 mM)
Ca^{2+}	No effect (10 mM)	No effect (10 mM)
PMSF	No effect (0.01 mM)	No effect (0.13 mM)
KI	100 % inhibition (1 mM)	No effect (1 mM) 25 % inhibition (10 mM)
pCMB	100 % inhibition (2.5 µM)	12 % inhibition (1 mM)

[a] Perona and Vallejo (1982).
[b] Warner and Shridhar (1980, 1985).
[c] Range using several substrates.
n.d. = not measured.

Subcellular distribution of thiol protease activity in dormant gastrulae

Cell fractionation studies designed to localize the thiol protease activity in young gastrulae of *Artemia* have been equivocal in showing the site(s) of storage of this protease in these embryos. Published data show that between 18 and 86 % of the thiol protease activity in crude homogenates is sedimentable depending on the origin of the cysts, nature of the homogenization medium, ratio of cysts to homogenization medium, and whether or not the endogenous thiol protease inhibitor(s) have been removed prior to assay for protease activity in each fraction (Marco *et al.*, 1980 ; Perona *et al.*, 1981 ; Warner and Shridhar, 1980, 1985). Homogenization media containing divalent cations and/or Ficoll enhance the sedimentation properties of the protease, while those media lacking these components, or ones containing EDTA show a greater proportion of protease in the non-sedimentable or soluble fraction. The distribution of thiol protease activity in homogenates prepared with different media is shown in Table II. It has also been reported that the amount of thiol protease in the cytosol increases with developmental age (Marco *et al.*, 1980), and preliminary data from our laboratory suggest that anoxia, which causes a reversible decrease in the internal pH of the embryos, may also affect the subcellular distribution

of the thiol protease (Busa *et al.*, 1982 ; Bernaerts, unpubl. observ.). Perona and coworkers (Perona *et al.*, 1981 ; Perona and Vallejo, 1982) have suggested that the sedimentable thiol protease is associated with the lysosome fraction, but this view remains to be confirmed using immunohistochemical techniques to localize the enzyme. The presence of a potent thiol protease inhibitor(s) in the postribosomal fraction may also lead to an underestimate of the thiol protease content of the cytosol if care is not taken to remove the inhibitor from this fraction before assay for protease activity (Nagainis and Warner, 1979 ; Warner and Shridhar, 1985). Since the role of the thiol proteases in protein metabolism will depend, to a large extent, on their localization in the embryo, *in situ* localization studies of this protein should be conducted on developing embryos as well as on embryos subjected to environmental stress such as high/low salt, temperature, and varying oxygen levels to obtain a more complete picture of this enzyme than is currently available.

TABLE II

Distribution of thiol protease activity
in various cell fractions of *Artemia* gastrulae

Cell fraction	Percent activity in various buffers[e]			
	STKMD[a]	STE[b]	PK[c]	SFHMC[d]
500 × *g* pellet	n.d.	n.d.	n.d.	46
700 × *g* pellet	19.8	2.8	n.d.	n.d.
2 000 × *g* pellet	n.d.	n.d.	n.d.	30
15 000 × *g* pellet	19.0	14.5	14.6	n.d.
15 000 × *g* supernate	61.2	82.7	n.d.	n.d.
27 000 × *g* pellet	n.d.	n.d.	n.d.	10
150 000 × *g* pellet	n.d.	n.d.	4.0	n.d.
150 000 × *g* supernate	n.d.	n.d.	81.4	14

[a] 0.25 M sucrose, 0.03 M Tris-HCl, pH 7.3 (22 °C), 5 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT (Warner and Shridhar, 1985).

[b] 0.25 M sucrose, 0.03 M Tris-HCl, pH 7.3 (22 °C), and 1 mM EDTA (Warner and Shridhar, 1985).

[c] 0.015 M potassium phosphate, pH 7.0 (22 °C) and 0.1 M KCl (Warner and Shridhar, 1985).

[d] 0.30 M sucrose, 15 % Ficoll 400, 25 mM Hepes buffer, 5 mM $MgCl_2$, and 0.5 mM $CaCl_2$ adjusted to pH 7.5 (Marco *et al.*, 1980).

[e] Buffers a, b, c were used with Utah cysts while buffer d was used with California cysts.

n.d. = not measured.

Thiol protease levels in *Artemia* embryos during development

Measurements of the thiol protease levels in extracts from *Artemia* embryos at different stages of development have been complicated by the presence of protease inhibitors in embryos extracts and the possible compartmentalization of the proteases (Nagainis and Warner, 1979 ; Marco *et al.*, 1980). In 1980 two reports showing conflicting results appeared in the literature. In one paper Marco and co-workers (1980) presented data showing a 4 to 5-fold increase in "acid" (thiol) protease activity of California embryos at the time of hatching (about 23 h incubation)

which then decreased to about twice the dormant cyst level by 60 h development. In contrast, Warner and Shridhar (1980) reported that the acid (thiol) protease level of California cysts decreased by at least 50 % prior to hatching and does not increase at hatching. The latter measurements were made on embryo extracts which had been fractionated on DEAE-cellulose columns to remove the endogenous inhibitor(s). More recently we have examined these differences in protease activity levels using California cysts, and we find that the low levels of acid protease activity observed in prehatch embryos by some workers is mainly the result of the use of NaOCl as a sterilizing agent which reduces the apparent acid (thiol) protease levels of protein preparations by 56-82 % depending on the protein fraction assayed for thiol protease activity (Warner and Shridhar, 1985). As well, we found that the presence of high levels of endogenous acid (thiol) protease inhibitor(s) in the cytosol of prehatched embryos lowers the total measurable acid (thiol) protease activity by at least 30 % if not removed prior to the assay for protease activity. A reported shift in the subcellular distribution of thiol protease activity at the time of hatching also makes interpretation of earlier results difficult (Marco *et al.*, 1980). The deleterious effects of the NaOCl sterilization procedure on enzyme measurements has been observed by other workers, but it is still not clear why residues of this reagent (on cysts) cannot be neutralized, while no effect has been observed on nauplii obtained from NaOCl-treated cysts. The advantage of NaOCl treatment is that it improves the extent and synchrony of hatching as well as the efficiency of cyst homogenization.

Avoiding the use of NaOCl as a sterilizing agent of cysts, we have determined the acid (thiol) protease levels in *Artemia* embryos at various times of incubation up to 36 h development at 30 °C. In place of NaOCl we have used 0.1 % benzalkonium chloride in the hydration medium to sterilize the cysts, and 0.005 % in the incubation medium along with a revised dose of antibiotics (Yablonka-Reuveni *et al.*, 1983). In these experiments we used a homogenization medium containing 0.1 M KCl buffered with 0.015 M potassium phosphate, pH 7.0 which we found to solubilize at least 80 % of the measurable thiol protease activity in embryo homogenates irrespective of the stage sampled between 0 and 36 h development (Warner and Shridhar, 1985). The results of four separate experiments with California cysts are shown in Fig. 3A. These data show that the acid (thiol) protease activity is highest in extracts from dormant cysts and declines nearly exponentially during development until shortly after hatching begins after which time the (total) protease level remains constant for at least 20 h additional development. The total acid (thiol) protease measurements represent enzyme free of endogenous inhibitors plus enzyme bound or inhibited in the extract, while the free acid (thiol) protease data represent the level of enzyme not complexed with inhibitor protein(s). The data in Fig. 3B shows that about 40 % of the solubilized acid (thiol) protease in extracts from prehatched embryos is inactive (inhibited), but this fraction decreases markedly coincident with emergence of prenauplii. It is our view that these results represent the actual state of acid (thiol) protease activity in developing embryos of *Artemia* excluding as yet unresolved problems of subcellular localization.

Nature and ontogeny of other proteases during development in *Artemia*

The most dramatic changes in protease activity in *Artemia* occur following hatching of nauplii when at least four proteases become readily measurable (Osuna *et al.*, 1977a). These proteases have been designated as protease A, B, C, and D and their order of appearance is shown in Fig. 4. All four proteases have been isolated from 60 h nauplii, purified to near homogeneity and

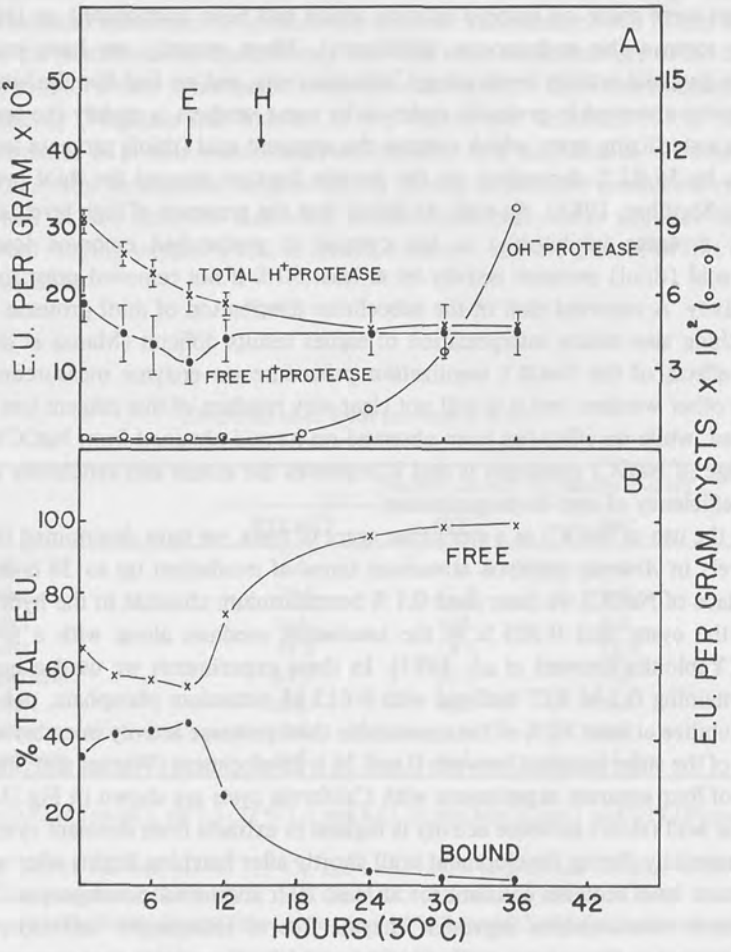

FIG. 3. Protease activity in biochemical preparations from *Artemia* embryos at various stages of development. A. Level of free acid (thiol) protease, total acid (thiol) protease, and "alkaline" protease activity at various stages of development. E and H represent the onset of emergence and hatching, respectively. Enzyme units are expressed in terms of wet weight (fully hydrated) of starting cysts and each point represents the average of four determinations. B. percent acid (thiol) protease in the "free" and "bound" form in the postribosomal fraction calculated from the data in A. (Warner and Shridhar, 1985).

partially characterized (Olalla *et al.*, 1978 ; Sillero *et al.*, 1980 ; Burillo *et al.*, 1982). Protease A has a molecular weight of 38 000, is inhibited by PMSF and STI but does not hydrolyze TAME. Its selectivity for CLNE as substrate and relative inactivity in the hydrolysis of yolk platelets indicate that this enzyme may be primarily an esterase. Protease B has a molecular weight of 33 000, is inhibited by STI and TLCK, and utilizes BAPNA, TAME, and casein as substrates. Of all proteases in 60 h nauplii, protease B shows the most activity on *Artemia* yolk platelets *in vitro*, and in many respects, this protease is a trypsin-like enzyme. Protease C has a molecular weight of 34 000, is inhibited by STI and TLCK, hydrolyzes BAPNA, TAME, and casein as substrates and is moderately active in the hydrolysis of yolk proteins *in vitro*. Protease D has a molecular weight of 36 000, is not inhibited by any of the above mentioned inhibitors, but it is partially (50 %) inhibited by 0.7 mM dithioerythritol. It hydrolyzes casein and azocasein and has weak activity on yolk platelets, but it is inactive on several synthetic substrates (Burillo *et al.*, 1982). All four proteases described above show maximum activity in the pH range 7.5 to 8.5, and all appear to be localized in the cytosol.

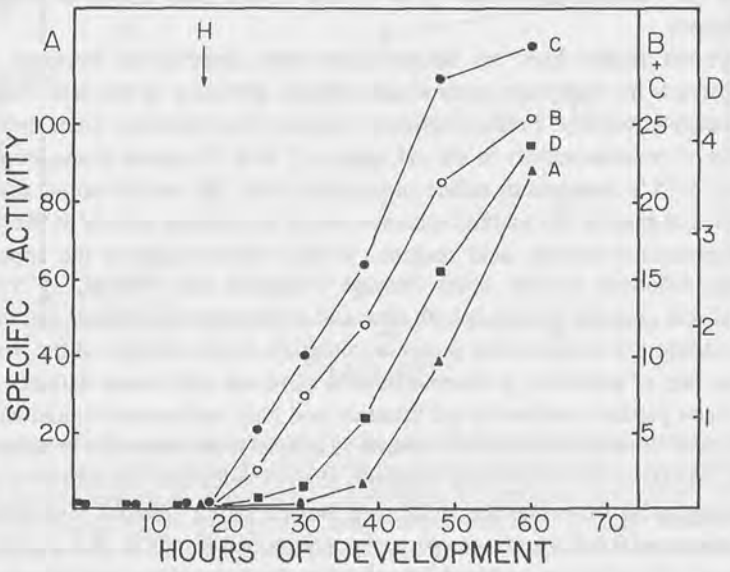

FIG. 4. Induction of "alkaline" proteases during early development of *Artemia*. The specific activity of enzyme A (▲) was assayed with CLNE ; protease B (○) and C (●) were assayed with BAPNA, and protease D (■) was measured with casein in the presence of 100 µg/ml of soybean trypsin inhibitor. H, onset of hatching. (redrawn from Osuna *et al.*, 1977a).

The role of these proteases in yolk granule and protein metabolism during early development is not known. However, since at least 50 % of the embryo yolk platelets have been degraded before these proteases show significant activity in embryo extracts, it remains unclear as to whether these enzymes are involved in yolk platelet metabolism, at least in the initial stages of yolk metabolism. The appearance of these proteases shortly after hatching may signify an aspect

of molecular differentiation of cells lining the gut which are clearly distinguishable by 12-16 h incubation of the cysts at 30 °C (Beharry, unpubl. observ.). As in the case of the thiol proteases, the role of these enzymes in development will require an immunohistochemical approach to determine the location of these enzymes in developing embryos to more accurately ascertain their probable function.

While proteases A, B, C, and D have been shown to appear sequentially beginning around 24 h incubation at 30 °C based on activity measurements, Garesse *et al.* (1980) have suggested that these enzymes are present in early nauplii (prior to their appearance as active enzymes) in a masked, high molecular weight complex (about 125 000 daltons). This conclusion was based on the use of high concentrations of chaotropic agents such as KI to dissociate the proteases from inhibitors and other macromolecules in preparations from developing embryos. Thus the increase in protease activity with an alkaline pH optimum during posthatch development may be the result of an unmasking process rather than enzyme induction. However, it still remains unclear to this reviewer whether or not proteases A, B, C, and D are present in the multiprotein complex sensitive to KI treatment since this treatment releases proteases of molecular weights 21 000 to 25 000 while the values for proteases A, B, C, and D have been shown to range from 33 000 to 38 000 daltons.

In some recent studies from my laboratory we have detected the presence of an inactive protease in extracts from dormant cysts which requires activation by the acid (thiol) protease to unmask its catalytic activity. Freshly prepared extracts from dormant Utah cysts contain only trace amounts of protease activity in the pH range of 7 to 8. However, if one incubates a protein preparation (25-75 % ammonium sulfate precipitate) from the postribosomal fraction at 30 °C and pH 4.0-4.3, a gradual but marked increase occurs in protease activity at pH 7.5 as detected by the trinitrobenzene sulfonic acid reagent (TNBS) which measures the appearance of free amino groups following peptide bond cleavage (Nagainis and Warner, 1979). The rate of appearance of this protease activity is both time and temperature dependent and inhibited by the presence or addition of *Artemia* thiol protease inhibitors. In the absence of the *Artemia* protease inhibitors the rate of activation is faster than that observed with crude extracts. The activated enzyme has been partially purified by gel filtration and high performance liquid chromatography on a reverse phase column and shown to hydrolyze a class of soluble acidic proteins from *Artemia* embryos (M_r 50 000 to 100 000) more efficiently than proteins and peptides from other sources. It is moderately active on protamine sulfate and bovine serum albumin. The activated enzyme has a pH optimum of 7.5-8.0 and a temperature optimum of 40-45 °C. The pattern of activation of this protease (P_3) is shown in Fig. 5 compared to the level of the acid (thiol) protease (P_2) and another protease which utilizes CLNE as substrate (P_1). The extent of activation varies from 10 to 30-fold the basal level depending on the initial activity in the non-activated preparation. The results in Fig. 6 show the chromatographic pattern of the activated protease (P_3) on a column of Sephadex G-150 after 90 min activation compared to proteases P_1 and P_2. The elution position of P_3 along with other results suggest that P_3 has a molecular weight of between 5 000 and 10 000, which makes it unique among proteases. Chromatography of non-activated (0 treatment) protein preparations show very low levels of P_3 while P_1 and P_2 appear in the same elution position shown in Fig. 6 (data not shown). Experiments to characterize protease P_3 shows it to be unaffected by temperatures up to 90 °C, treatment with 0.1 N HCl, iodoacetate (1 mM), leupeptin and PMSF (2-25 µg/ml), or by STI and ovomucoid (2 µg/ml). It is slightly inhibited by TLCK (14-19 %) at 0.2 mM) and completely inhibited by 1 % SDS. The appearance

of P_3 during incubation at pH 4 may also explain the observation of Marco *et al.* (1980) who found a shift in the pH optimum of various preparations of the acid (thiol) protease upon incubation of various enzyme preparations at pH 3.5 for a short time.

Although considerable work remains to be done to characterize protease P_3 and determine its role in protein metabolism during early development, it is clear that this protease is unique among the wide variety of proteases known to exist in cells and worthy of further study.

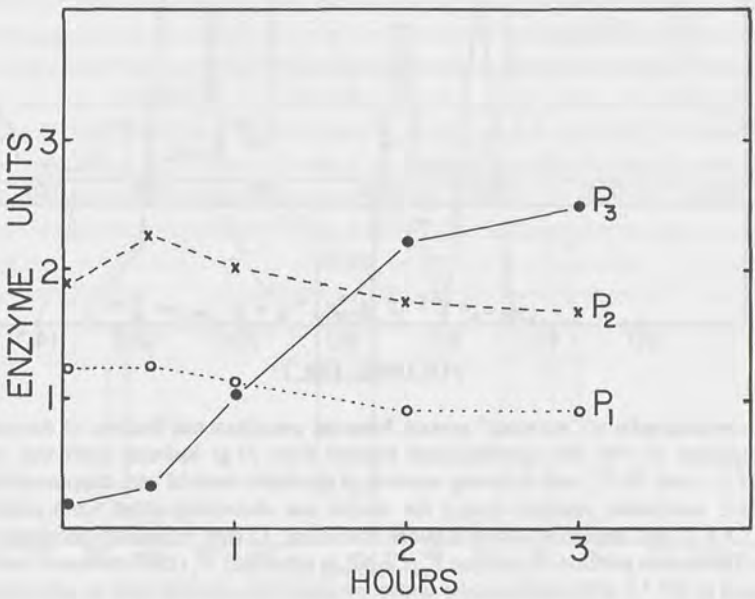

FIG. 5. Activation of a "neutral" protease (P_3) in the postribosomal fraction of *Artemia* cysts. A sample of protein (25-75 % ammonium sulfate fraction) from the postribosomal fraction was incubated at pH 4.2 and 30 °C and aliquots assayed at various times of incubation for protease activity at pH 7.5 with CLNE as substrate (P_1) and acidic cytosol proteins from *Artemia* as substrate (P_3). The acid (thiol) protease activity was measured using bovine serum albumin as substrate (P_2). Enzyme units are defined as nanomoles of free amino groups released per minute per aliquot of "activated" enzyme.

Characterization of two thiol protease inhibitors from dormant gastrulae

In our first paper on proteases in *Artemia* embryos, we described the presence of a protease inhibitor in the postribosomal fraction of dormant cysts which could be separated from the acid protease by chromatography on DEAE-cellulose columns (Nagainis and Warner, 1979). We have now succeeded in purifying the inhibitor to near homogeneity, and we have initiated studies to determine the role of this inhibitor during early development.

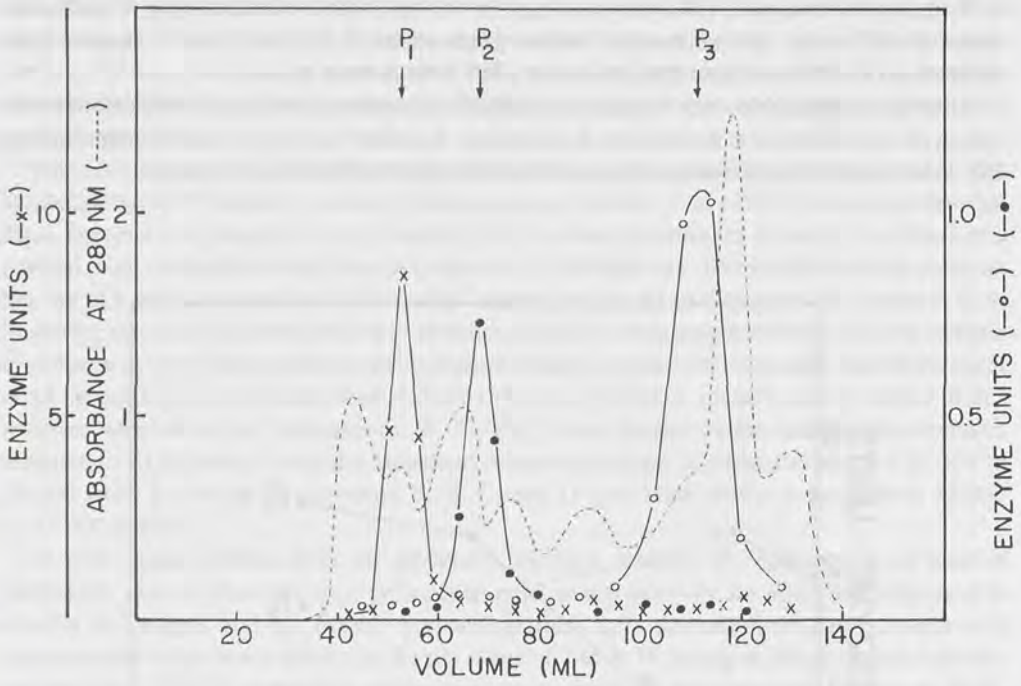

FIG. 6. Chromatography of "activated" protein from the postribosomal fraction of dormant cysts on a column of Sephadex G-150. The postribosomal fraction from 25 gr hydrated cysts was "activated" for 90 min. at pH 4.2 and 30 °C, and following removal of insoluble material and concentration by pressure filtration (YM-2 membrane, Amicon Corp.) the sample was chromatographed on a column of Sephadex-G-150 (1.5 × 77 cm, superfine) using a buffer containing 15 mM potassium phosphate and 25 mM KCl, pH 6.8. The elution position of protease P_1 (CLNE as substrate), P_2 (thiol protease), and P_3 (activated protease assayed at pH 7.5 with soluble acidic cytosol proteins from *Artemia* cysts as substrates) are shown.

Previously (Fig. 3B) it was shown that about 40 % of the total acid (thiol) protease activity in early embryos is bound or complexed to a macromolecule(s) which inhibits its activity on proteins. We have now partially purified the protease inhibitor from the postribosomal fraction by a combination of ion-exchange and filtration chromatography. The results in Fig. 7 show two steps in the purification of the inhibitor. The data in panel A show that the thiol protease inhibitor elutes from a CM-cellulose column as two peaks of activity (I_a and I_b) in the presence of a dilute saline buffer at pH 5.0. Subsequent chromatography of inhibitor Ia and Ib on separate columns of Sephadex G-50 yields sharp peaks of activity (panels B and C) at positions corresponding to molecular weights of M_r 8 700-9 300. When the purified acid (thiol) protease is incubated at pH 4.0 and 40 °C with either Ia or Ib, the inhibitory effect is found to be temporary only since the inhibitor is eventually hydrolyzed or inactivated under these conditions. When the pH of the incubation buffer is raised to between pH 5.0 and 6.5, the inhibitor becomes resistant to hydrolysis by the acid (thiol) protease and effective in the inhibition of the acid (thiol) protease, especially on EF-2 which is very sensitive to limited proteolysis at pH 6.0-6.5. Additional

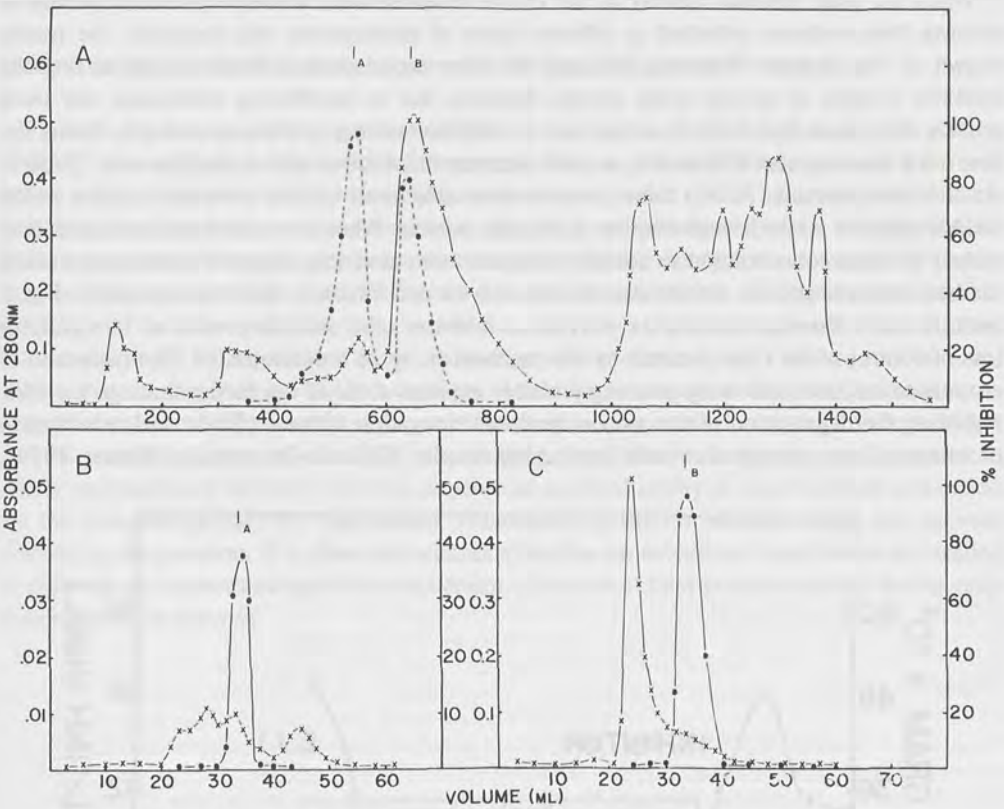

FIG. 7. Chromatography of thiol protease inhibitors on carboxymethyl cellulose and Sephadex G-50. Panel A, the DEAE-cellulose was through proteins from a 25-75 % ammonium sulfate fraction of the postribosomal supernatant from 50 g Utah cysts (dry weight) were adjusted to pH 5.0 with acetic acid then applied to a column of CM-cellulose (2.2 × 50 cm) equilibrated with 25 mM sodium acetate, pH 5.0. After washing the sample on the column, the protease inhibitor proteins were eluted with the starting buffer containing 50 mM KCl. After 900 ml had washed through the column, the remainder of the protein was eluted with a linear gradient of KCl. None of the proteins to elute with the KCl gradient showed inhibitor activity. (X——X) absorbance at 280 nm ; (●-----●), protease inhibitor activity. Panel B. The fractions from the CM-cellulose column containing Ia were pooled, concentrated by pressure filtration, then filtered through a column of Sephadex G-50 (1.1 × 60 cm, superfine) using a buffer containing 15 mM potassium phosphate, pH 6.1, and 50 mM KCl. (X——X), absorbance at 280 nm ; (●——●) thiol protease inhibitor activity. Panel C. The fractions from the CM-cellulose column containing Ib were pooled, concentrated by pressure filtration, then filtered through a column of Sephadex G-50 as for Ia in panel B.

findings on the purification and characterization of the *Artemia* embryo thiol protease inhibitor (Ia and Ib) will be presented in more detail elsewhere.

When the total inhibitor activity in the DEAE-cellulose wash through (protein) fraction of extracts from embryos collected at different times of development was measured, the results shown in Fig. 8 were obtained. Although we have experienced difficulty in quantifying the inhibitor content of various crude protein fractions due to interferring substances, our most reliable data show that there is an increase in inhibitor activity in *Artemia* embryos during the first 6-9 h development followed by a rapid decrease in inhibitor level during the next 27-30 h. At 36 h development (30 °C) there is barely detectable levels of thiol protease inhibitor in the DEAE-cellulose wash-through fraction of embryo extracts. When one calculates the ratio of acid (thiol) protease concentration to inhibitor concentration at various stages of development using the best available specific activity data for the enzyme and inhibitor, the results shown in Fig. 8 indicate that following hatching the enzyme to inhibitor ratio exceeds a value of 16 signifying loss of control of the thiol protease by this mechanism by 36 h development. The high ratio of enzyme to inhibitor following hatching probably explains some of our earlier findings (*in vitro* regarding the degradation of the protein synthesis elongation factor 2 (EF-2) and reduction in protein synthesis capacity of extracts from young nauplii (Yablonka-Reuveni and Warner, 1979).

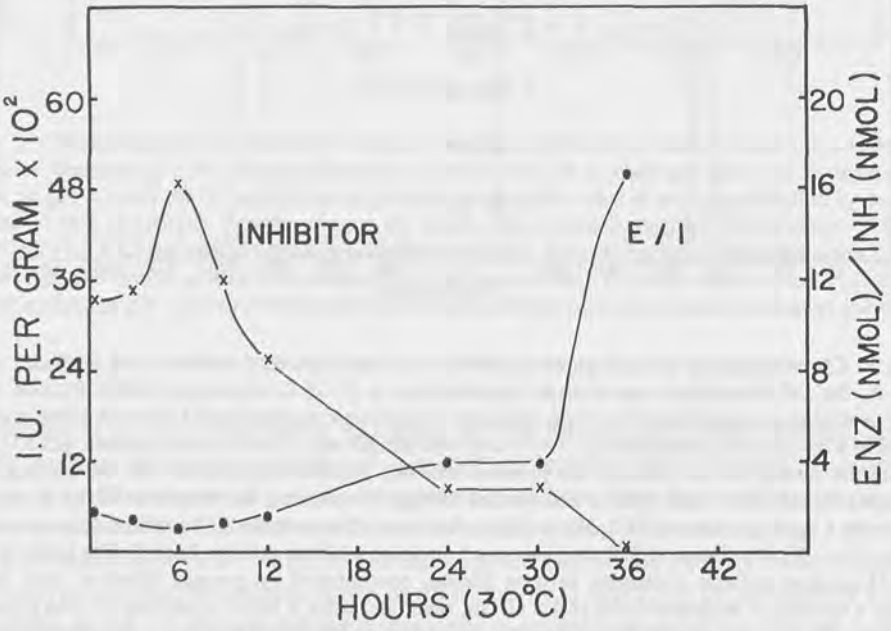

FIG. 8. Thiol protease inhibitor activity in *Artemia* embryos at various times of development. The content of thiol protease inhibitor activity (X—X) was measured in the DEAE-cellulose wash-through fraction of postribosomal fractions prepared from embryos at different times of development using extraction buffer "PK" in Table I. One Inhibitor Unit (I.U.) is defined as the amount of protein producing 1 % inhibition of thiol protease activity on the linear part of the inhibitor concentration curve, and I.U. is expressed per gram wet weight starting cysts (fully hydrated). The curve labeled E/I represents the estimated molar ratio of acid (thiol) protease to inhibitor protein based on protease measurements made on the same extracts after elution from DEAE-cellulose.

While some of our early unpublished data suggest that inhibitor Ia and Ib may be different conformational forms of the same protein molecule, the origin of Ia and Ib and their importance in development remains to be established.

Control of thiol protease activity in *Artemia* embryos during early development

Control of protease activity in *Artemia* embryos is probably exerted at multiple sites and by a variety of conditions in the embryo. In encysted embryos the acid (thiol) protease described herein is the major protease and its activity appears to be under control of several mechanisms including the ionic environment, intracellular pH (pH_i), compartmentalization, and presence of inhibitory proteins. Since we now know that the pH of *Artemia* embryos is at least pH 7.9 during aerobic development (Busa *et al.*, 1982) how are enzymes such as the acid (thiol) protease able to carry out their function ? As well, how is the acid (thiol) protease protected *in vivo* against inactivation at pH 8.0, conditions known to inactivate the protease *in vitro* (Warner and Shridhar, 1980 ; Perona and Vallejo, 1982) ? One way would be to sequester the thiol protease in an organelle with a more favourable environment such as in lysosomes (Perona and Vallejo, 1982) or yolk platelets (Schuel *et al.*, 1975). Alternatively, the inhibitor proteins I_a and I_b, present in nearly stoichiometric amounts with the protease in encysted embryos, could provide protection for the protease against OH^- inactivation. Attachment to the cytoskeleton could also provide stability to the protease. It is clear that additional studies on subcellular localization are needed to enhance our understanding of the mechanism of control of thiol protease activity during early development in *Artemia.*

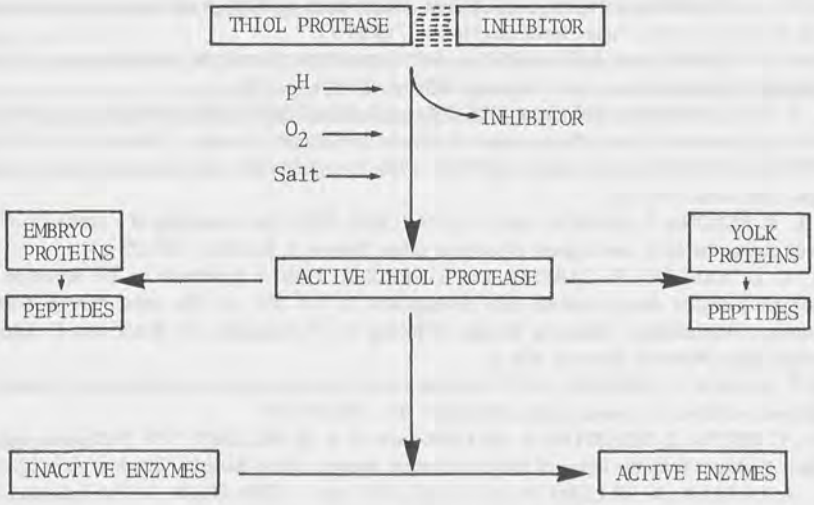

FIG. 9. Proposed mechanism of thiol protease control and activation in early embryos of *Artemia.*

An interesting adaptation of encysted embryos of *Artemia* is their ability to withstand long periods of anoxia (months) during which time the pH_i probably fall to near or below pH 6.0 (Busa *et al.*, 1982). This resistance to anoxia is lost shortly after hatching concomitant with a possible change in the subcellular storage site of the thiol protease (Marco *et al.*, 1980) and reduction in protease inhibitor levels (see Fig. 8). These intracellular changes may lead to uncontrolled activation of the thiol protease such that several critical enzymes/proteins are inactivated leading to cytolysis and death of young nauplii.

In the diagram shown in Fig. 9, a mechanism is proposed for the control of the thiol protease activity in the cytosol of young embryos based on currently available data. This model shows the thiol protease as having a central role in embryo protein metabolism, which for now, serves as our working hypothesis for protease involvement in control of development.

Summary and conclusions

In this brief review of the role of proteases and their control during development in *Artemia*, I have attempted to include the major aspects of our current knowledge of the various proteases known to exist in *Artemia* embryos. The major unresolved problem, as I see it, in regards to our understanding of the function of the proteases *in vivo* is the site of localization of these enzymes in the developing embryo. It appears that the best approach to solving this problem is to use labeled antibodies prepared against the various proteases to localize the proteases immunohistochemically. Until these experiments are completed, speculation will continue on the role of these proteases during development.

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Study of the oxygen binding properties of *Artemia* hemoglobins

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Abstract

The physiological properties of total hemoglobin samples from *Artemia* populations of different geographical origin (Tientsin, Great Salt Lake, Chaplin Lake, Macau, Galera Zamba) have been studied *in vitro*. Measurements carried out with the diffusion chamber technique at different pH-values and temperatures, revealed significant differences in oxygen affinity (p50 values between 1.25 and 16.33 mm Hg), cooperativity (Hill coefficients between 1.07 and 2.18) and free oxygen-binding energy (between - 34.7 and - 56.2 Kj/mol). All the strains showed a decrease of oxygen affinity with increasing temperature, and a positive Bohr effect. Tientsin containing a considerable amount of Hbl (36.6 %), shows the lowest oxygen affinity, while Macau and Galera Zamba (except at pH 7.65) containing most of Hb3 show the highest affinity.

The highest cooperativity is found for Tientsin, and the lowest for Galera Zamba. Submission of the adult *Artemia* strains to cyclic hypoxic stress resulted in the production of hemoglobin. Hb2 was always the main pigment. Next to Hb2, Tientsin and Great Salt Lake produced preferentially Hbl, and Galera Zamba and Macau Hb3. Chaplin Lake produced the three types of hemoglobin.

Introduction

Earlier studies on the respiratory physiology of the adult brine shrimp *Artemia* have shown that this branchiopod adapts to low oxygen levels by hemoglobin synthesis in order to facilitate respiration (Decleir *et al.*, 1980). Bowen *et al.* (1969) have shown the existence of three types of hemoglobin in *Artemia*, which according to their electrophoretic mobility have been called Hbl, 2 and 3. „Total hemoglobin" extracts of a San Francisco Bay strain (Vos *et al.*, 1979) cultivated in laboratory circumstances (25 °C and 32 ‰ salinity) mainly contain Hb2 and small amounts of Hbl, while Hb3 is electrophoretically not detectable. This Hb composition can be influenced by submitting the animals to hypoxic stress. It has been demonstrated that responses to long-term hypoxic conditions include next to changes in reproductive behaviour (Dutrieu, 1960 ; Sorgeloos, 1975), a modified energy balance (Gilchrist, 1956) and an increase of the hemoglobin concentration, alterations in the procentual composition of the three hemoglobin types (Vos *et al.*, 1979). The observation, that the induced Hb3 shows oxygen-binding properties which are very suited for survival in a low oxygen environment (Van den Branden *et al.*, 1978), illustrates the existence of adaptional mechanisms to cope to a certain degree with changing environmental oxygen availability.

The work presented here is part of a program destined to examine and compare the oxygen-binding properties of the hemoglobins from a number of *Artemia* populations of different geographical origin. This paper describes the first results of the *in vitro* oxygen-binding properties of total Hb extracts of two North American, two South American, and one Asian population. In all the cases it was necessary to submit the *Artemia* cultures to cyclic hypoxic stress in order to obtain sufficient hemoglobin for extraction. The following functional properties of the Hb extracts were determined : the oxygen affinity (p50), the temperature and Bohr effect, the cooperativity (Hill coefficient n), the free oxygen-binding energy, and the Bohr factor.

Materials and methods

Cysts of *Artemia* populations originating from five geographical sites (Tientsin, China (T), Chaplin Lake, Canada (CH) ; Great Salt Lake, USA (GSL) ; Macau, Brazil (M) ; Galera Zamba, Columbia (GZ), were used. Mass cultures were run (at 25 °C and 35 ‰ salinity) as described by Sorgeloos (1979) and Versichele and Sorgeloos (1980).

Hemoglobin induction in adult *Artemia* was obtained by submitting them to cyclic hypoxic stress (Lavens and Sorgeloos, 1984). The hypoxia sensitivity of each particular population mostly showed considerable variability. After harvesting the animals were thoroughly washed with distilled water and frozen in flat layers of ± 1.5 cm thickness at - 70 °C until hemoglobin extraction. Total hemoglobin was obtained as described previously (Moens and Kondo, 1977).

The physiological properties of the hemoglobin samples were measured with the diffusion chamber technique (Sick and Gersonde, 1969 ; Van Pachtebeke *et al.*, 1982) at five temperatures and four pH values.

Electrophoresis was carried out on cellulose acetate strips or on 1 % agarose gels. The presence of hemoglobin was demonstrated using benzidine-H_2O_2 reagent, respectively 0.5 % Amido Black. Densitometric scanning was performed with a Kipp Microdensiscan (KS3).

Results

Table I presents the procentual hemoglobin composition in the five *Artemia* strains studied. The hemoglobin extracts of all the populations mainly contain Hb2 (Fig. 1). The induction of Hb1 is most pronounced in Tientsin and Great Salt Lake, and of Hb3 in Galera Zamba and Macau. Chaplin Lake contains the three types of hemoglobin. All the registrated oxygen-dissociation curves had a sigmoidal shape, which is conform with the findings of Van den Branden *et al.* (1978).

TABLE I

Procentual composition of *Artemia* hemoglobin extracts

	% Hb1	Hb2	Hb3	(n)
Tientsin	36.3 ± 3.3	63.7 ± 3.3	0	8
Great Salt Lake	22.2 ± 3.1	77.8 ± 3.1	0	24
Chaplin Lake	10.1 ± 1.8	82.6 ± 3.7	7.3 ± 2.1	12
Macau	0	91.4 ± 3.5	8.6 ± 3.5	10
Galera Zamba	0	73.4	26.6	3

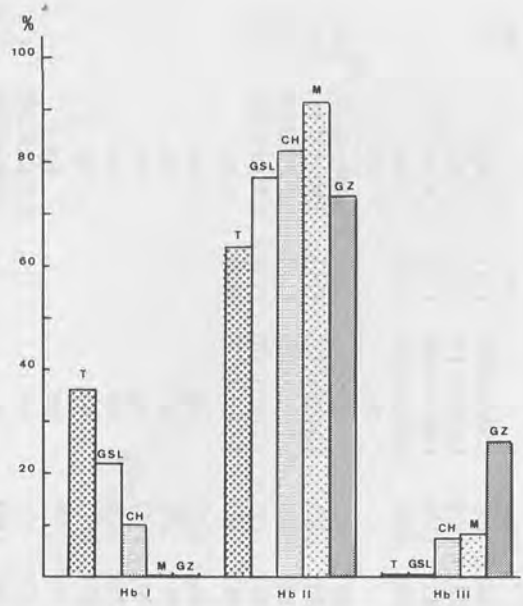

FIG. 1. Procentual distribution of the three types of *Artemia* hemoglobins. Tientsin (T) ; Great Salt Lake (GSL) ; Chaplin Lake (CH) ; Macau (M) ; Galera Zamba (GZ).

In Table II the data on oxygen affinity (p50 values) are presented. The five populations show a decrease in affinity with increasing temperature. In Fig. 2a, 3a, and 4a, oxygen affinity is plotted in relation to changing pH and temperature for respectively Tientsin, Great Salt Lake, and Chaplin Lake. These three populations show a positive Bohr effect which is more pronounced at higher temperatures, except for Chaplin Lake where at the highest temperature and the lowest pH values, a negative Bohr effect is observed. Tientsin hemoglobins show the highest pH sensitivity.

Table III contains the results on the cooperativity (Hill coefficients). The highest cooperativity is found for Tientsin (n = 2.14 at pH 8.25 and 8.60) and for Great Salt Lake (n = 2.18 at pH 8.60) and the lowest for Macau (n = 1.07 at pH 8.60) and Galera Zamba (n = 1.15 at pH 8.60).

In Fig. 2b, 3b, and 4b the evolution of cooperativity is plotted in function of pH and temperature for the same three populations mentioned before. Cooperativity in Great Salt Lake shows a maximum that shifts from 22 °C at lower pH values to 26 °C for increasing pH values. Tientsin shows no marked maximum for changing temperatures, and for Chaplin Lake a slight increase is noticed around 28 °C. Cooperativity of the last two strains seems to be more influenced by pH than by temperature. Insufficient measuring points made it impossible to realize similar graphics for Macau and Galera Zamba.

The mean values of free oxygen-binding energy (ΔH) and of the Bohr factors (Φ) are presented in Table IV. The ΔH ranges between - 34.7 and - 56.2 Kj/mole, differring as follows : Tientsin <Great Salt Lake <Chaplin Lake <Macau <Galera Zamba. The Bohr factors range between - 0.13 and - 0.45.

TABLE II

P-50 values of total hemoglobin extracts of *Artemia* populations from different geographical origin

		Tientsin	n	Great Salt Lake	n	Chaplin Lake	n	Macau	n	Galera Zamba	n
	T°										
pH 7.25	10	n.v.		2.65(± 0.11)	(8)	1.82(± 0.34)	(9)	1.58(± 0.07)	(11)	n.v.	
	18	n.v.		5.81(± 0.30)	(10)	4.11(± 0.33)	(13)	3.53(± 0.17)	(11)	n.v.	
	22	n.v.		6.92(± 0.33)	(8)	6.23(± 0.43)	(13)	4.90(± 0.38)	(13)	n.v.	
	26	n.v.		8.40(± 0.41)	(11)	7.25(± 0.54)	(10)	6.16(± 0.21)	(11)	n.v.	
	34	n.v.		n.v.		n.v.		n.v.		n.v.	
pH 7.65	10	2.80(± 0.21)	(8)	2.15(± 0.09)	(11)	1.55(± 0.09)	(9)	n.v.		1.44(± 0.11)	(12)
	18	6.61(± 0.25)	(8)	4.75(± 0.27)	(10)	4.27(± 0.20)	(10)	3.34(± 0.13)	(11)	4.97(± 0.51)	(10)
	22	9.01(± 0.22)	(11)	6.74(± 0.20)	(10)	5.79(± 0.18)	(12)	5.13(± 0.22)	(11)	5.23(± 0.26)	(7)
	26	11.17(± 0.42)	(15)	7.96(± 0.17)	(8)	7.08(± 0.41)	(9)	5.71(± 0.40)	(12)	8.48(± 0.64)	(11)
	34	n.v.		13.56(± 0.25)	(8)	11.43(± 0.91)	(9)	10.39(± 0.52)	(10)	n.v.	
pH 8.15	10	2.48(± 0.12)	(9)	2.16(± 0.13)	(8)	1.25(± 0.14)	(12)	n.v.		n.v.	
	18	6.23(± 0.28)	(14)	3.95(± 0.29)	(17)	3.26(± 0.11)	(8)	n.v.		n.v.	
	22	7.26(± 0.21)	(9)	5.99(± 0.27)	(8)	4.47(± 0.30)	(11)	n.v.		n.v.	
	26	9.22(± 0.26)	(10)	7.60(± 0.22)	(11)	5.80(± 0.29)	(13)	n.v.		n.v.	
	34	16.33(± 0.31)	(9)	12.32(± 0.37)	(11)	12.40(± 0.63)	(11)	n.v.		n.v.	
pH 8.60	10	n.v.		n.v.		n.v.		n.v.		n.v.	
	18	4.35(± 0.19)	(9)	3.88(± 0.16)	(10)	2.89(± 0.17)	(7)	n.v.		1.51(± 0.10)	(7)
	22	6.04(± 0.13)	(8)	5.08(± 0.27)	(10)	3.16(± 0.26)	(10)	n.v.		2.07(± 0.23)	(7)
	26	7.18(± 0.24)	(8)	6.83(± 0.24)	(10)	4.78(± 0.18)	(11)	n.v.		3.08(± 0.14)	(7)
	34	13.70(± 0.16)	(8)	11.66(± 0.47)	(10)	8.49(± 1.14)	(9)	n.v.		5.95(± 0.48)	(7)

n.v. = no values

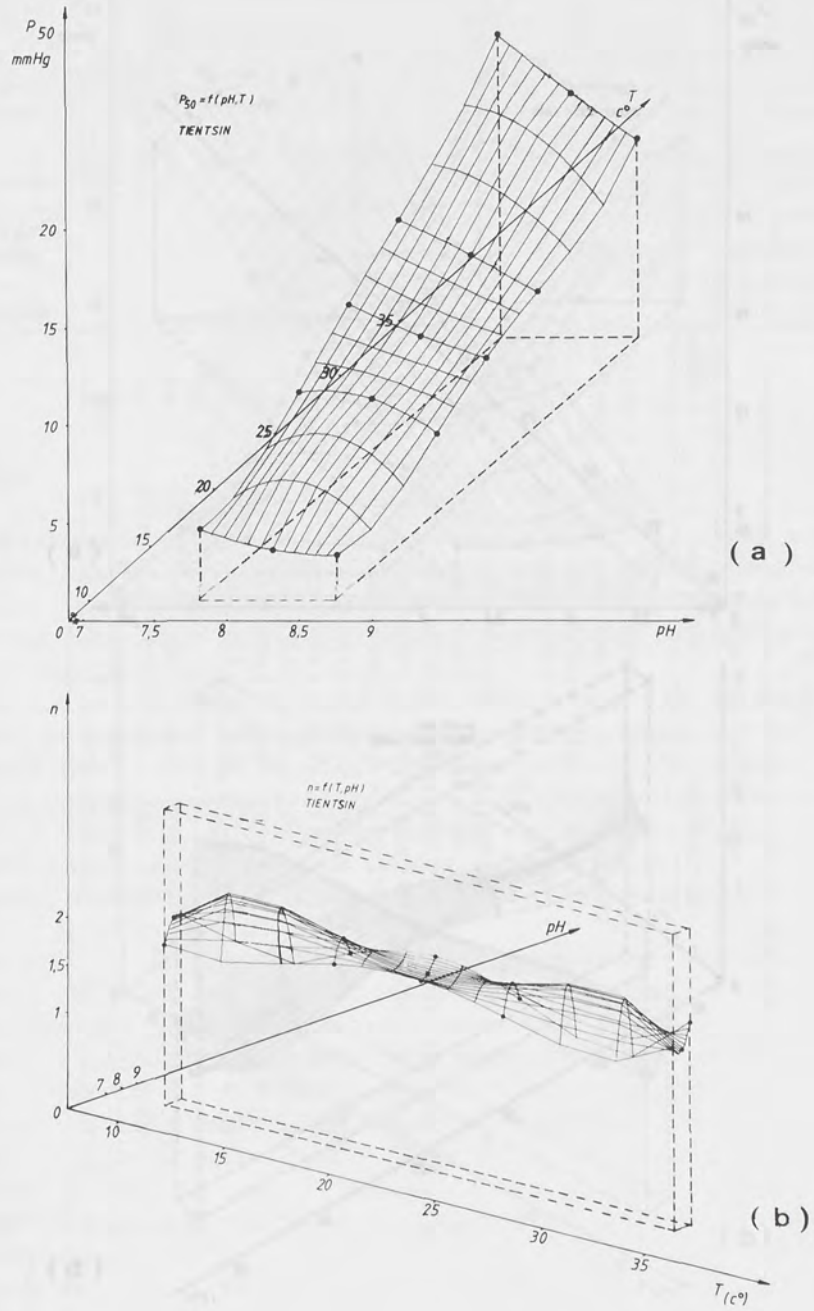

FIG. 2. Influence of pH and temperature on the oxygen affinity (a) and cooperativity (b) of hemoglobin extracts of Tientsin.

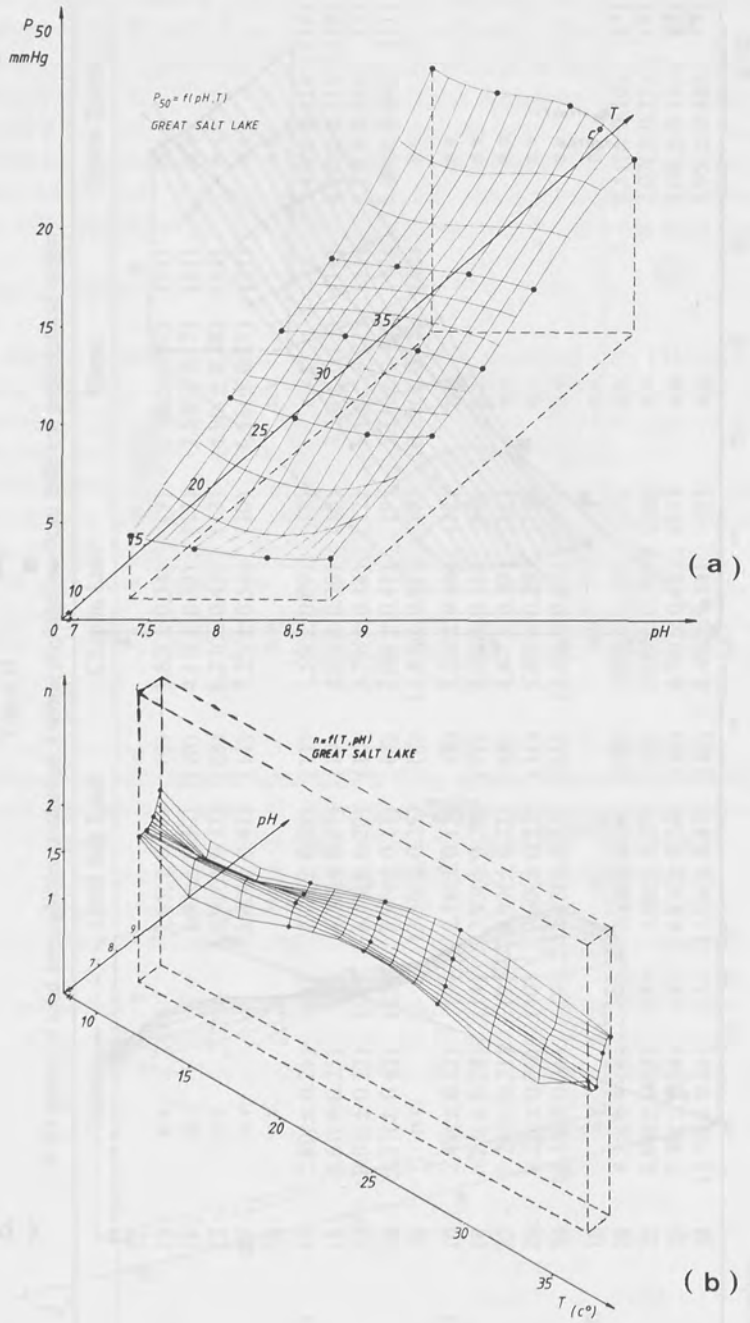

FIG. 3. Influence of pH and temperature on the oxygen affinity (a) and cooperativity (b) of hemoglobin extracts of Great Salt Lake.

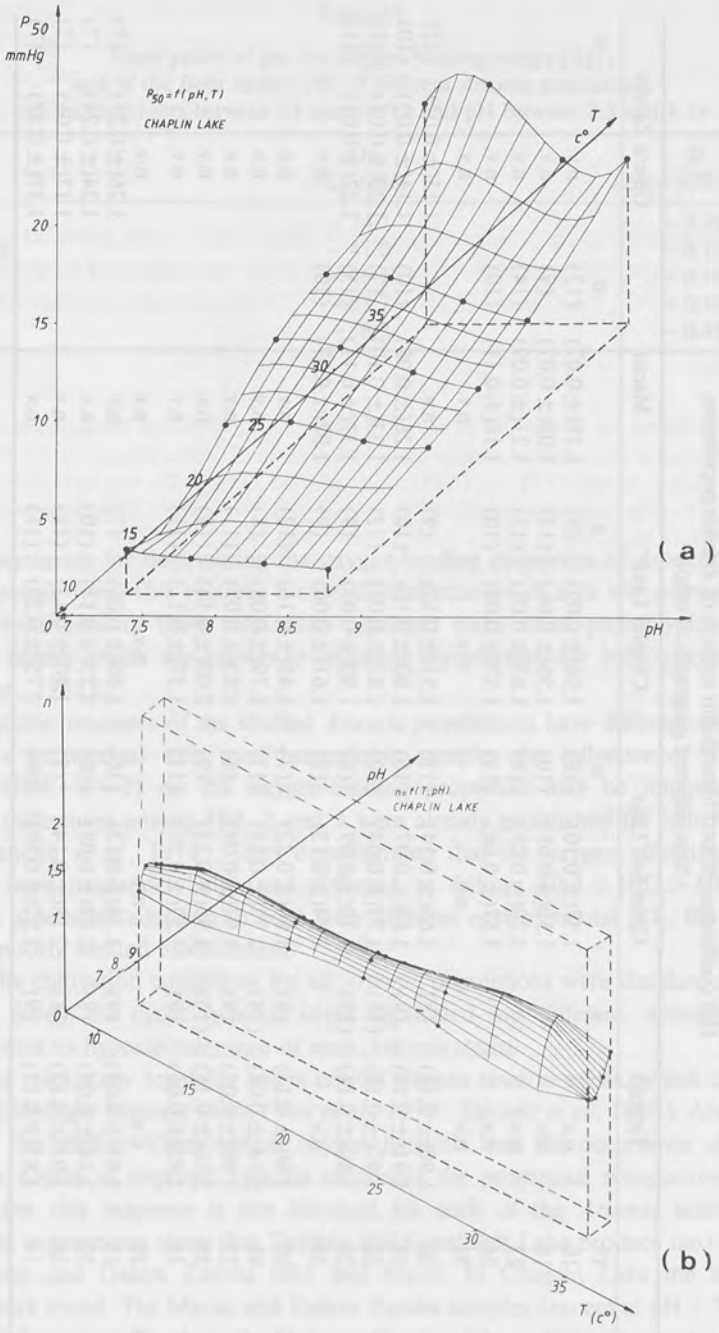

FIG. 4. Influence of pH and temperature on the oxygen affinity (a) and cooperativity (b) of hemoglobin extracts of Chaplin Lake.

TABLE III

Hill coefficients of total hemoglobin extracts of *Artemia* populations from different geographical origin

		Tientsin		Great Salt Lake		Chaplin Lake		Macau		Galera Zamba	
	T°		n		n		n		n		n
pH 7.25	10	n.v.		1.57(± 0.10)	(8)	1.56(± 0.09)	(9)	1.15(± 0.07)	(12)	n.v.	
	18	n.v.		1.50(± 0.08)	(10)	1.56(± 0.06)	(13)	1.08(± 0.07)	(9)	n.v.	
	22	n.v.		1.70(± 0.05)	(8)	1.67(± 0.08)	(13)	1.21(± 0.09)	(14)	n.v.	
	26	n.v.		1.57(± 0.06)	(11)	1.57(± 0.04)	(10)	1.18(± 0.11)	(10)	n.v.	
	34	n.v.		n.v.		n.v.		n.v.		n.v.	
pH 7.65	10	1.90(± 0.16)	(8)	1.66(± 0.14)	(11)	1.51(± 0.12)	(9)	n.v.		1.13(± 0.07)	(12)
	18	1.91(± 0.14)	(8)	1.69(± 0.07)	(10)	1.80(± 0.10)	(10)	1.26(± 0.08)	(10)	1.14(± 0.09)	(10)
	22	1.97(± 0.15)	(11)	1.73(± 0.04)	(10)	1.82(± 0.08)	(12)	1.33(± 0.10)	(7)	1.20(± 0.08)	(7)
	26	1.80(± 0.14)	(15)	1.70(± 0.09)	(8)	1.90(± 0.07)	(9)	1.32(± 0.11)	(15)	1.22(± 0.08)	(11)
	34	n.v.		1.64(± 0.13)	(8)	1.61(± 0.06)	(9)	1.07(± 0.07)	(10)	n.v.	
pH 8.15	10	1.90(± 0.17)	(9)	1.80(± 0.22)	(7)	1.45(± 0.15)	(12)	n.v.		n.v.	
	88	2.14(± 0.08)	(14)	1.72(± 0.13)	(17)	1.79(± 0.06)	(8)	n.v.		n.v.	
	22	1.98(± 0.05)	(9)	1.92(± 0.09)	(8)	1.83(± 0.07)	(11)	n.v.		n.v.	
	26	2.10(± 0.15)	(10)	1.93(± 0.13)	(9)	2.00(± 0.14)	(13)	n.v.		n.v.	
	34	1.92(± 0.06)	(9)	1.52(± 0.11)	(11)	1.57(± 0.11)	(11)	n.v.		n.v.	
pH 8.60	10	n.v.		n.v.		n.v.		n.v.		n.v.	
	18	1.95(± 0.08)	(9)	1.78(± 0.13)	(10)	1.69(± 0.08)	(7)	n.v.		1.26(± 0.25)	(7)
	22	2.14(± 0.04)	(8)	2.04(± 0.08)	(10)	1.73(± 0.11)	(10)	n.v.		1.24(± 0.24)	(7)
	26	1.91(± 0.12)	(8)	2.18(± 0.14)	(8)	1.90(± 0.13)	(11)	n.v.		1.17(± 0.06)	(7)
	34	1.85(± 0.11)	(9)	1.82(± 0.19)	(10)	1.75(± 0.20)	(11)	n.v.		1.15(± 0.17)	(7)

n.v. = no values

TABLE VI

Mean values of the free oxygen binding energy (ΔH) and of the Bohr factors (Φ) of different *Artemia* populations (all measurements between 18 and 26 °C, and pH between 7.3 and 8.5)

	ΔH (Kj/mole)	Φ (Δlog p50/ΔpH)
Tientsin	- 34.7	- 0.19
Great Salt Lake	- 41.0	- 0.13
Chaplin Lake	- 46.5	- 0.19
Macau	- 53.7	- 0.19
Galera Zamba	- 56.2	- 0.45

Discussion

All the experiments for determining the oxygen-binding properties of *Artemia* hemoglobins were carried out *in vitro*. This requires a careful interpretation in a *in vivo* context. Maybe the extracellular occurrence of these respiratory pigments make them probably less sensitive to specific local factors which are known to influence the intracellular hemoglobins to a larger extend (Moens, 1981).

The hemoglobin mixtures of the studied *Artemia* populations have different oxygen-binding properties. As we worked with total hemoglobin samples the influence of the procentual composition (Hbl + 2 + 3) on the oxygen-binding properties may be important since the physiological differences among Hbl, 2 and 3 were already established for San Francisco Bay (Van den Branden *et al.*, 1978). They demonstrated that the oxygen affinities of the three hemoglobins were moderately high and differred as follows Hb3 > Hb2 > Hb1. This was interpreted as a possible adaption to cope with different environmental pO_2, Hb3 being more efficient in a poorly aerated environment.

Although the cultivation conditions for all *Artemia* populations were standardized (temperature, salinity, food), the cyclic hypoxic stress applicated was different, which is due to the different response to hypoxic tolerance of each *Artemia* strain.

Artemia is a respiratory regulator and a critical oxygen tension exists, which decreases with acclimation to stronger hypoxic stress (Vos *et al.*, 1979 ; Decleir *et al.*, 1980). An interrelationship between this shift to lower critical oxygen tensions, and the occurrence of Hb3 is very probable. The degree of imposed hypoxia influences the procentual composition of the total hemoglobin, but this response is not identical for each of the *Artemia* strains used. The electrophoretic experiments show that Tientsin and Great Salt Lake produce next to Hb2, Hb1, while in Macau and Galera Zamba Hb3 was found. In Chaplin Lake the three types of hemoglobin were found. The Macau and Galera Zamba samples (except at pH 7.75) containing the highest Hb3 percentages show the highest affinity, while in Tientsin samples, containing a considerable amount of Hb1, the lowest oxygen affinity was observed. Our results suggest that hypoxic tolerance among these *Artemia* populations decreases as follows : Galera Zamba > Macau > Chaplin Lake > Great Salt Lake > Tientsin.

The relation between the procentual hemoglobin composition and the oxygen-binding properties seems to confirm that the conclusions of Van den Branden *et al.* (1978) can be extrapolated to other *Artemia* strains than San Francisco Bay, but the role of the genetical background of the hemoglobin synthesis of each population remains to be investigated.

Acknowledgements

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Enzymes related to nucleotide and nucleic acid metabolism

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W. Decleir, L. Moens, H. Slegers, E. Jaspers, and P. Sorgeloos (Eds). Universa Press, Wetteren, Belgium. 562 p.

An increased dietary tryptophan requirement induced by interference with purine interconversion in *Artemia*

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Abstract

When *Artemia* larvae are reared in a phosphate-rich medium containing,in addition to albumin and starch particles, amino acids and sugars, their purines are freely interconverted because their dietary purine requirement can be met by one of the nucleotides (IMP, AMP, or GMP), by one of the nucleosides (IR, AR, or GR), or by adenine or by hypoxanthine. The reduced efficiency of guanine can not be explained by a reduced GMP reductase activity, because guanosine and guanylic acid are efficiently utilized. Their pyrimidines are also freely interconverted since their pyrimidine requirement can be met by one of the nucleosides (CR or UR) or by one of the nucleotides (CMP or UMP).

When *Artemia* larvae are reared in a medium lacking the amino acids and sugars normally included in the liquid phase, they develop the symptoms of a folic-acid deficiency in spite of a standard folic-acid supply. Dietary tryptophan relieves the symptoms, as does dietary thymidine without tryptophan. Without thymidine, folinic acid, contrary to folic acid, can ensure a normal morphogenesis. The suspected impairement of dihydrofolate reductase and thymidylate synthetase activities by a tryptophan deficiency is subordinated to the quality of purine and pyridimine compounds used to meet the growth requirements. The results indicate that a requirement for additional tryptophan arises as a consequence of an AMP:GMP imbalance.

Introduction

Artemia postembryonic development is anamorphic, meaning that it is progressing without any cataclysmic event through a number of larval stages, successively adding new segments with appendages coming gradually into service. Next to nothing is known about the processes which control the anamorphic development pattern which raises in simple terms the fundamental problem of growth regulation and differentiation. Dietary studies can provide some clues to understand how this pattern is established and controlled. I agree with Fryer's (1983) statement the limb-bearing trunk segments are designated as thorax and the succeeding apodous segments as abdomen for descriptive convenience. The existence of mosaics combining genitalian and phyllopodan tissues suggests the homology between the thoracic and genital segments (Bowen *et al.*, 1966). On the basis of experimental evidence, gained by nutritionally induced morphogenetic effects, I have proposed (Hernandorena, 1985a) that during *Artemia* postembryonic development, the programme of appendicular morphogenesis can be interpreted in more or less integral versions. The wild-type phenotype, actually "normal", with appendages on 11 segments would represent a regressive evolution with respect to the archaic phenotype with appendages

on every single segment. The archaic phenotype would correspond to the integral version of the programme.

Contrary to most living organisms, the Utah strain of *Artemia* is incapable of synthesizing the purine ring *de novo* (Clegg *et al.*, 1967 ; Warner and McClean, 1968). This incapacity should facilitate the elucidation of the metabolic significance of the dietary requirement for folic acid, a vitamin which is usually required for the biosynthesis of the purine ring and for the biosynthesis of thymidylate. In *Artemia*, dietary folic-acid deletion has no morphogenetic incidence on larvae reared in a nutritive medium containing thymidine. The double folic acid plus thymidine deletion induces a precocious metamorphosis which results in an abortive appendicular morphogenesis with 3, 6, or 8 instead of 11 pairs of thoracic appendages in service before the onset of metamorphosis (Fig. 1). The precocious metamorphosis induced by a double folic acid plus thymidine deletion can be related to a reduced thymidylate biosynthesis, resulting from the exhaustion of endogenous folic acid. Since the only metabolic fate of dietary thymidine which restores a normal morphogenesis on larvae reared in a medium lacking folic acid, is its incorporation into DNA, the precocious metamorphosis can be related to a reduced DNA biosynthesis (Hernandorena, 1985a).

FIG. 1. Abortive appendicular morphogenesis.

These metabolic data fit the biochemical data relating metamorphosis to a high RNA : DNA ratio in *Crangon crangon* (Regnault, 1977) and in *Palaemon serratus* (Van Wormhoudt and Sello, 1980). Campillo (1979) showed that during the development of *P. serratus*, the quantity of DNA stops increasing during two very precise periods, namely, the zoe-mysis transformation and metamorphosis. If metamorphosis is indeed the result of a reduced DNA biosynthesis, a mechanism capable of preventing DNA biosynthesis must exist in *Artemia* larvae reared in a folic-acid containing medium which triggers the onset of metamorphosis after the morphogenesis of 11 pairs of thoracic appendages. This mechanism would be upset under nutritional conditions, *i.e.* reduced purine : pyrimidine ratio, inducing the morphogenesis of supernumerary genitalia on otherwise apodous abdominal segments. In view of the present results, it is important to recall that the most efficient inducer of abdominal genitalia is deoxyuridine, the precursor of deoxyuridylate which is the substrate of thymidylate synthetase (Hernandorena and Kaushik, 1985).

The synthetic semi-defined nutritive medium, defined by Provasoli and d'Agostino (1969) for the axenic culture of *Artemia* (Utah strain), is composed of a liquid phase containing mineral salts, amino acids, sugars, nucleic acid components, vitamins, a pH buffer, organic phosphate, and by a fine particulate phase consisting of precipitated egg albumin, gelled rice starch, and cholesterol. According to the authors, phagotrophy is the most efficient way to satisfy the bulk nutritional requirements of *Artemia* and the amino acids and sugars incorporated in the liquid phase are dispensable. During experiments to test the influence of the amino acids of the liquid phase on the ammonia excretion rates of *Artemia*, it appeared that, under special circumstances, some component of the amino acid-sugar mixture was essential. Larvae reared in a nutritive medium lacking the amino acid-sugar mixture, developed the symptoms of a folic acid deficiency in spite of a standard folic acid supply. This observation prompted the present investigation.

Material and methods

The Utah strain and the method developed by Provasoli and d'Agostino (1969) for the axenic culture of this strain were used.

A standard concentration of 20 mg albumin/100 ml nutritive medium (20 mg %), and one of 100 mg starch/100 ml nutritive medium (100 mg %), were used throughout the experiments. Until now, I have been using medium "100" with tryptophan included in the amino acid-sugar mixture as formulated by Provasoli and d'Agostino prior to their 1969 publication. The composition of the amino acid-sugar mixture is given in Table I. During the present experiments, the mixture was added to the nutritive medium or omitted with or without individual components of the mixture being added separately in respective concentrations as indicated in Table I.

The nucleic acid components included into the liquid phase differed both qualitatively and quantitatively from those proposed by Provasoli and d'Agostino (1969). The thymidine requirement is not essential for larvae reared in a folic-acid containing medium (Hernandorena, 1970). Therefore thymidine was omitted unless otherwise stated. Contrary to purine and pyrimidine nucleotides, purine bases and purine and pyrimidine nucleosides are efficiently utilized by *Artemia* if an additional phosphate supply is provided (Hernandorena, 1985b ; Hernandorena and Kaushik, 1985). The sodium β-glycerophosphate (pentahydrate) concentration which stands at 50 mg % as proposed by Provasoli and d'Agostino (1969) is now routinely increased up to 250 mg % whether the requirements are met by bases, by nucleosides, or nucleotides. The quality

and quantity of nucleic acid components utilized to satisfy the purine and pyrimidine requirements are given in Results and discussion. Nucleotides were used to meet the purine requirement because differences among efficiencies of nucleotides are minimal compared to differences among efficiencies of bases and nucleosides (Hernandorena, 1985b). Nucleosides and nucleotides were used to meet the pyrimidine requirement.

The standard folic acid concentration established at 1.4 mg % by Provasoli and d'Agostino (1969) was used unless otherwise stated, although it exceeds largely the minimal requirement which is lower than 0.1 mg % (Hernandorena, unpubl.).

Morphogenesis was checked by a microscopical examination of anesthesized animals. Data on survival rates are given when additional information was gained.

TABLE I

Composition of medium "100", with tryptophan included in the amino acid-sugar mixture. Concentrations are expressed in mg/100 ml nutritive media

Substance	Concentration
Glucose	300
Saccharose	300
L. glutamic acid	100
L. serine	40
L. histidine	20
L. threonine	20
L. phenylalanine	10
L. tryptophan	10

Results and discussion

In the first set of experiments, the purine requirement was met by inosinic acid (IMP= 100 mg %) and the pyrimidine requirement by uridine (UR=40 mg %). Table II shows that larvae reared in a thymidine-free, folic-acid containing nutritive medium presented an abortive appendicular morphogenesis when the amino acid-sugar mixture was omitted from the liquid phase. The only component of the mixture capable of restoring a normal morphogenesis was tryptophan. Double supplementation including tryptophan plus any one of the components of the mixture, resulted in normal morphogenesis. Without additional tryptophan, dietary thymidine (TdR= 10 mg %) restored a normal morphogenesis but sexual maturity was not reached. Young adults suffered from black disease characterized by the blackening of the gills (Fig. 2). These results indicated that when larvae are reared in a nutritive medium lacking additional tryptophan, folic acid is incapable to ensure thymidylate biosynthesis.

An essential step in the production of methylenetetrahydrofolate, the cofactor of thymidylate synthetase, is the reduction of folic acid. This reduction is inhibited by aminopterin. The results reported in Table III show that dietary tryptophan did not relieve the killing effect of aminopterin on larvae reared in a medium lacking the amino acid-sugar mixture and containing 0.1 mg % folic acid. Thymidine (TdR=10 mg %) supplementation relieved the effect of aminopterin during larval life but the mortality increased sharply during the juvenile stage. The double thymidine plus tryptophan supplementation, however, prevented larval and juvenile mortalities. These results

TABLE II

Normal (N) or abortive (A) appendicular morphogenesis induced on larvae reared in nutritive media supplemented or not with the amino acid-sugar mixture components. Concentrations are expressed in mg/100 ml of nutritive media. Larvae fed IMP=100, UR=40, standard folic acid supply

	Included	Amino acid-sugar mixture									
		Omitted									
Additions	None	None	Glucose	Saccharose	Glutamic acid	Serine	Histidine	Threonine	Phenyl alanine	Tryptophan	Thymidine
Concentrations	–	–	300	300	100	40	20	20	10	10	10
Appendicular morphogenesis	N	A	A	A	A	A	A	A	A	N	N

TABLE III

Effect of thymidine and tryptophan supplementation on the survival of larvae reared in nutritive media lacking the amino acid-sugar mixture and containing aminopterin. Concentrations expressed in mg/100 ml of nutritive media. Larvae fed IMP=100, UR=40, folic acid=0.1

	Aminopterin 0.1 mg				Aminopterin 0.25 mg			
Additions	None	Tryptophan	Thymidine	Tryptophan + thymidine	None	Tryptophan	Thymidine	Tryptophan + thymidine
Concentrations	–	10	10	10 + 10	–	10	10	10 + 10
Survival %								
8th day	0	0	100	100	0	0	100	96
18th day			45	88			12	80

suggested a duality in the role of folic acid, involved in thymidylate biosynthesis and in sexual maturity.

Folinic acid (N^5-formyl-tetrahydrofolate) bypasses the reductive step required for the production of methylene-tetrahydrofolate. The results in Table IV show that folinic acid, contrary to folic acid, enabled the realization of a normal appendicular morphogenesis and sexual maturity of larvae reared in a medium lacking the amino acid-sugar mixture. These results suggested that the dihydrofolate reductase activity and consequently the thymidylate synthetase activities are impaired by deletion of tryptophan.

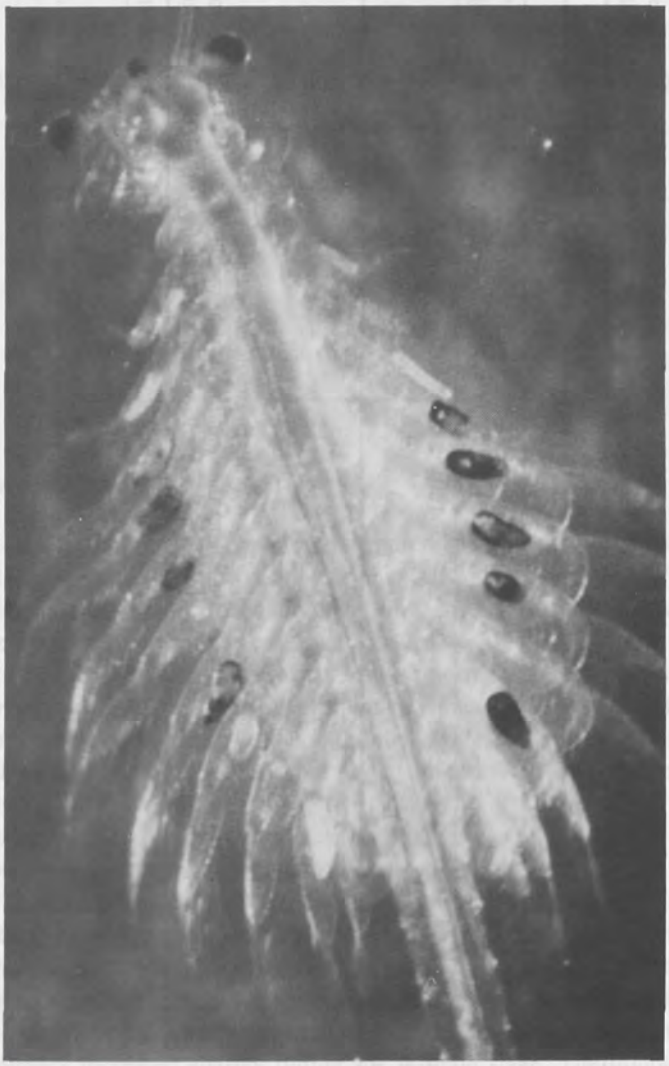

FIG. 2. Black disease, note the blackening of the gills.

TABLE IV

Effect of folic acid or folinic acid supplementation on appendicular morphogenesis and survival of larvae reared in nutritive media lacking the amino acid-sugar mixture and lacking folic acid. Concentrations are expressed in µg/100 ml of nutritive media. Larvae fed IMP=100, UR=40

	Additions						
	None	Folic acid			Folinic acid		
Concentrations	–	5	10	25	5	10	25
Appendicular morphogenesis	A	A	A	A	A	A	N
Survival percentage							
8th day	96	96	84	88	92	96	100
18th day	0	0	0	0	35	80	92

TABLE V

Effect of quality of dietary pyrimidines on appendicular morphogenesis of larvae reared in nutritive media lacking the amino acid-sugar mixture. Concentrations are expressed in mg/100 ml of nutritive media. Larvae fed IMP=100, standard folic acid supply

	Additions			
	None	Tryptophan	Aspartate	Malate
Concentrations	–	10	10	10
UR = 40	A	N		
CR = 40	N	N	A	A
CMP = 60	N	N	A	A

It can be concluded that, in spite of a standard folic acid supply, the rate of DNA biosynthesis can be modulated by the rate of thymidylate biosynthesis at the level of folic acid reduction. How relevant this conclusion may be to control the *Artemia* postembryonic development is unknown.

In the next experiment, larvae were reared in a medium lacking the amino acid-sugar mixture. Their purine requirement was met by inosinic acid (IMP=100 mg %) and their pyrimidine requirement by cytidine (CR=40 mg %). Both nucleosides CR or UR were found to be capable of satisfying the pyrimidine requirement of larvae reared in phosphate-rich medium containing the amino acid-sugar mixture (Hernandorena and Kaushik, 1985). Larvae reared in a medium lacking the amino acid-sugar mixture, of which the pyrimidine requirement was met by CR instead of by UR, presented a normal appendicular morphogenesis. If CR was replaced by cytidylic acid (CMP=60 mg %), morphogenesis was also normal. In this case tryptophan supplementation had no effect but aspartate or malate supplementation induced an abortive appendicular morphogenesis (Table V).

These results were tentatively interpreted to mean that the amino acid-sugar deletion interferes with the nitrogen transfer reactions required for nucleotide interconversions. When the pyrimidine requirement is met by UR, the entire cytidine series is derived from the uridine series through one single reaction catalyzed by CTP synthetase. This enzyme requires glutamine as amino donor. When the pyrimidine requirement is met by CR or by CMP, deaminative reactions give rise to the corresponding uridine compounds. Glutamine is involved not only in the interconversion of pyrimidine nucleotides but also in the interconversion of purine nucleotides :

the production of GMP from IMP requires glutamine. Transfer of the amino group of aspartate provides an amino nitrogen for the production of AMP from IMP. Adenylosuccinate synthetase has been characterized in *Artemia* (Faraldo *et al.*, 1983). If the hypothesis of the implication of nucleotide interconversion is correct, the quality of the purine compound, used to meet the purine requirement, should interfere with the morphogenetic effect of a tryptophan deletion. The uniqueness of *Artemia* is that its purine requirement can be met by GMP as well as by IMP or AMP (Hernandorena, 1972, 1985a) because, contrary to most eukaryotes, it is equipped with a GMP-reductase (Renart and Sillero, 1974). When larvae are reared in a medium containing the amino-acid sugar mixture, their purines are freely interconverted.

In the next experiment, larvae were reared in a medium lacking the amino acid-sugar mixture. Their purine requirement was met by adenylic acid (AMP=100 mg %) or by guanylic acid (GMP=100 mg %). Their pyrimidine requirement was met by uridine compounds (UR= 40 mg % or UMP=60 mg %) or by cytidine compounds (CR=40 mg % or CMP=60 mg %). Results of Table VI show that the morphogenesis of larvae fed AMP was normal whether their pyrimidine requirement was met by uridine or by cytidine compounds. Larvae fed GMP died whether their pyrimidine requirement was met by uridine or by cytidine compounds. When reaching the larval stage corresponding to the morphogenesis of six pairs of thoracic appendages (growth index 7), they suffered from black disease. Dietary thymidine had no beneficial effect on the mortality of larvae fed GMP, whereas dietary tryptophan restored normal growth and morphogenesis.

It is clear that when the amino acid-sugar mixture is omitted from the liquid phase, the morphogenetic effect of a tryptophan deletion depends on the quality of the purine compound used to meet the growth requirements. Tryptophan supplementation is essential for larvae fed GMP and not essential for larvae fed AMP. When larvae are fed IMP, the common precursor of AMP and GMP, tryptophan supplementation is less stringent and becomes circumstancial depending on the quality of the compound used to meet the pyrimidine requirement and on the thymidine supplementation. Experiments limited to nutritional studies do not reveal how much AMP and GMP are produced from IMP by larvae fed IMP and reared with or without the amino acid-sugar mixture. Xanthosine monophosphate (XMP) is a very strong inhibitor of GMP-reductase in *Artemia* cysts (Renart *et al.*, 1976). The metabolic fate of endogenously produced GMP from IMP through XMP and the metabolic fate of exogenously supplied GMP may be different. A clear indication of the importance of general nitrogen metabolism was supplied by the significant differences in ammonia-excretion rates recorded in larvae reared in media supplement or not with the amino acid-sugar mixture. Paradoxically the ammonia-excretion rates of larvae reared in media lacking the amino acid-sugar mixture are much higher than those of larvae reared in media supplemented with the mixture (Hernandorena and Kaushik, unpubl.).

Conclusions

The suspected impairement of dihydrofolate reductase and thymidylate synthetase activities by a tryptophan deletion is subordinated to the quality of purine and pyrimidine compounds used to meet the growth requirements. The metabolic significance of the increased tryptophan requirement remains unknown. Even if the interpretation of purine interconversion is correct, it is not clear why an imbalanced AMP:GMP production should interfere with folic acid reduction and thymidylate biosynthesis. Besides being incorporated into proteins, tryptophan in *Artemia* is metabolized to ommochromes characterized in the compound eyes (Kiyomoto *et al.*, 1969)

TABLE VI

Effect of quality of dietary purines and pyrimidines on appendicular morphogenesis of larvae reared in nutritive media lacking the amino acid-sugar mixture. Concentrations are expressed in mg/100 ml of nutritive media. Standard folic acid supply

	Purines											
	AMP = 100						GMP = 100					
	Pyrimidines						Pyrimidines					
	UR = 40 or UMP = 60			CR = 40 or CMP = 60			UR = 40 or UMP = 60			CR = 40 or CMP = 60		
Additions	None	Thymidine	Tryptophan	None	Thymidine	Tryptophan	None	Thymidine	Tryptophan	None	Thymidine	Tryptophan
Concentration	–	10	10	–	10	10	–	10	10	–	10	10
Appendicular morphogenesis	N	N	N	N	N	N	†	†	N	†	†	N

and to serotonin (Aramant, 1980). The amount of tryptophan supplied by albumin particles does not limit the rate of protein biosynthesis because larvae, reared in a nutritive medium lacking additional tryptophan, can grow as fast as those reared in a medium containing additional tryptophan when fed AMP, whatever the compound used to meet the pyrimidine requirement or when fed IMP, provided uridine is not used to meet the pyrimidine requirement. Excess dietary tryptophan induces in *Artemia* the phenocopy of the *garnet* mutation which affects both eye-colour and structure (Hernandorena, 1984). Tryptophan deletion has, however, apparently no effect on eye pigmentation. Serotonin biosynthesis might be a good target for further experiments because biopterin mediates tryptophan hydroxylation, the first step in serotonin biosynthesis.

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De novo purine biosynthesis in developing *Artemia* nauplii

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Abstract

The existence of a complete classical pathway of *de novo* purine synthesis in *Artemia* nauplii was assessed at three different levels. Glutamine-dependent phosphoribosylpyrophosphate amidotransferase (PRPPA-Tase) is present in nauplii, increasing its activity during the larval development. The early enzymatic steps from the *de novo* purine biosynthetic pathway was determined by measuring the (U-^{14}C)-glycine incorporation, in the presence of azaserine, into formylglycinamide ribonucleotide (FGAR). The overall purine biosynthesis was assessed measuring the incorporation by nauplii of radiolabelled precursors into cellular purines, and posterior HPLC analysis of the nucleotides or from bases from acid-soluble fractions and RNA.

Introduction

In early reports, the lack of detectable *de novo* purine biosynthesis in *Artemia* sp. was established (Clegg *et al.*, 1967), suggesting that during its development, *Artemia* converts large amounts of guanine compounds, contained in encysted embryos (Finamore and Warner, 1963), into adenine and guanine nucleotides (Clegg *et al.*, 1967 ; Warner and Finamore, 1967 ; Warner and McClean, 1968). This view continues to be accepted by many authors (Hernandorena, 1975 ; Hernandorena and Koushik, 1981). Although purine interconversion has been systematically studied (Clegg and Conte, 1980 ; Warner, 1980), as well as the enzymes involved in the metabolic pathway from GMP to AMP (Renart *et al.*, 1976 ; Faraldo *et al.*, 1983 ; Pinto *et al.*, 1983), such a purine interconversion has not beel fully elucidated.

Several studies performed in this laboratory in recent years have indicated that *Artemia* nauplii are able to synthesize purine nucleotides using the *de novo* pathway. Partial supporting evidence was communicated previously (Llorente *et al.*, 1980 ; Liras and Llorente, 1981, 1983) and a full account is presented here.

Materials and methods

Materials

Artemia cysts were obtained from San Francisco Bay Brand Co., Division of Metaframe Co., Menol Park, CA ; (U-^{14}C)-glycine and (^{14}C-HCO_3 were purchased from the Radiochemical Center Amersham. Azaserine was obtained from Calbiochem. Nucleotides, enzymes and other

chemicals were from Sigma or Biochemical. DEAE-Sephacel from Pharmacia. GP_4G was a gift from Dr. A. Sillero.

A Waters Assoc. HPLC System equipped with a U6K injector, a 440 UV detector at a fixed wavelength of 254 nm and a μBondapak C_{18} column (300×4 mm, 10 μ particle) and another arrangement composed of a M-45 pump and a U6L injector, a 2138 Uvicord 3 UV detector, at a fixed wavelength of 254 nm (LKB) and Polygosyl (Macherey Naget) C_{18} column (200×4 mm, 5 μ particle) were used for the HPLC analysis. Chromatographic profiles were registered in a Omniscribe recorder. Spectrophotometric measurements and UV spectra were carried out in a Spectronic 2000 (Bausch & Lomb).

Methods

Handling of brine shrimp cysts

The cysts were hydrated and washed as described by Llorente and Cardaba (1987). Development of *Artemia* was initiated by incubation of freshly-hydrated cysts in the same conditions as described, except that the medium used (Dutrieu, 1960), was diluted a 5-fold. After about 20 h of incubation, with slow stirring at 30 °C in a rotatory shaker, the nauplii were isolated and transferred to fresh incubation saline medium and grown synchronously. At the appropriate times of development the larvae were harvested by filtration on cloth, washed with distilled water, and kept at -70 °C until used for *in vitro* studies, or incubated under the conditions described for precursors incorporation studies. All experimental procedures were carried out under strict sterile conditions.

PRPPTase preparation and assay

For the studies reported here, glutamine phosphoribosylpyrophosphate amidotransferase was partially purified from *Artemia* nauplii as described by Tsuda *et al.* (1979) with some modifications. Frozen or freshly-obtained nauplii were homogenized, with 2 vol of 50 mM Tris-HCl buffer pH 7.5, containing 0.2 M sucrose, 20 mM 2-mercaptoethanol, 2 mM $MgCl_2$ and soybean trypsin inhibitor (0.2 mg/ml), at 4 °C. The homogenate was centrifuged at 10 000 rpm for 10 min, the precipitate was discarded, and the solution was centrifuged at 40 000 rpm for 30 min. The supernatant fraction was adjusted, with stirring, to pH 5.0 with 5 % acetic acid. After standing for 2 min, the precipitate was removed by centrifugation at 10 000 rpm for 10 min and the supernatant fluid was neutralized with 1 N KOH, brought up to 55 % saturation with solid ammonium sulphate, and allowed to stir for 120 min. The precipitated protein was collected by centrifuging at 10 000 rpm for 45 min and this fraction was suspended in 0.5 M Tris-HCl pH 7.5. The enzyme preparation obtained by this purification procedure was not quite free of interfering enzymes, mainly glutaminase. The conversion of (^{14}C)-glutamine into (^{14}C)-glutamate was used as assay procedure (Hill *et al.*, 1969 ; Holmes *et al.*, 1973). The standard assay consisted of 50 mM Tris-HCl pH 7.5 ; 5 mM $MgCl_2$, 5 mM KF, 1 mM DTE, 4 mM L-glutamine, 2.5 mM phosphoribosylpyrophosphate (PRPP) and the enzyme preparation, in a total volume of 0.1 ml. The reaction was initiated with the addition of (^{14}C)-glutamine following incubation for 30 or 60 min at 37 °C. A 20 μl aliquot was spotted, on Whatman no. 3 MM chromatography paper with 5 μl of L-glutamate and L-glutamine as a carrier (0.5 μmoles each). Glutamate was separated by high voltage electrophoresis at 4 000 volts for 60 min in 50 mM sodium borate buffer, pH 9.0. The spots were identified with ninhydrin, cut out and counted for radioactivity.

FGAR determination

A modification of the Henderson (1962) and Rosenbloom *et al.* (1968) methods was employed to quantify the formation of radioactive N-formylglycinamide ribonucleotide (FGAR) in the presence of (U-^{14}C)-glycine and azaserine. The nauplii were homogenized (1/1) in a modificated Krebs-Ringer solution : 0.1 M phosphate buffer pH 7.4, 22 mM glucose, 8 mM L-glycine, 15 mM L-glutamine and 1 mM DTE. Of the nauplii extract 0.1 ml (about 30 mg protein/ml) was incubated, in a final volume of 0.5 ml, with 50 mM Tris-HCl pH 7.5, 0.1 M KCl, 10 mM glucose, 2 mM L-glycine, 10 mM $MgCl_2$, 5 mM L-glutamine, 1 mM DTE and after 10 min of preincubation 2.5 µCi (U-^{14}C)-glycine were added, and after 45 min at 37 °C the reaction was stopped by the addition of 2 ml of 0.4 M cold PCA and allowed to stand at 4 °C for 2 min. The acid soluble supernatant fractions was adjusted to pH 7.0-8.0 with 1 N KOH, and the precipitate of potassium perchlorate was removed by centrifugation. The supernatant was placed on 1×3 cm Dowex (1×8 formate) columns, washed previously with 5 M formic acid, H_2O until neutrality, and equilibrated with 0.5 M formic acid. Each column was washed with 20-30 ml of 0.5 M formic acid, and the accumulated (^{14}C)-FGAR was eluted with 10 ml of 4 M formic acid. Fractions of 0.2 ml were collected and counted for radioactivity. FGAR was characterized by thin-layer chromatography on cellulose plates developed in butanol-acetic acid-H_2O (2:1:1) with FGAR as the reference compound (Rf 0.26) (Reem, 1973).

Purine biosynthesis "de novo"

A total of 0.5 g nauplii obtained in sterile conditions, at different times of development, were incubated in 25 ml artificial seawater, composed of 75 % Dutrieu's Medium (1/5 diluted), 25 % Eagle's Minimum Essential Medium, 5 mM glucose, 5 mM L-glutamine, 5 mM aspartic acid, penicillin (13.0 U/ml), and streptomycine (0.4 mg/ml). After preincubation at 30 °C for 30 min, the labeled purine precursors, (^{14}C)-HCO_3^- (final concentration 2 mM ; specific activity 60 µCi/µmol) or (U-^{14}C)-glycine (final concentration 0.15 mM ; specific activity 56 µCi/µmol) were added. After 3 h of incubation at 30 °C, the nauplii were collected by filtration, washed with 10 mM $NaHCO_3$ or 10 mM glycine, and the incubation medium frozen at -70 °C until processing for high pressure liquid-chromatography (HPLC) analysis. Purine synthesis was gauged by the incorporation of the labeled precursors into acid-soluble and RNA nucleotides or bases.

The acid-soluble fraction was obtained by homogenization of frozen nauplii in 0.4 M $HClO_4$, following centrifugation and neutralization of the soluble material with KOH and filtration to remove $KClO_4$. Nucleotides were separated by anion-exchange chromatography on DEAE-Sephacel columns equilibrated with water. After sample application and washing with H_2O until no absorbance at 260 nm, the nucleotides were eluted by 0.5 M $(NH_4)_2CO_3$, treated with concentrated HCOOH until pH 2.0, lyophylized, and the residue redissolved in water and stored frozen at -40 °C until analysis.

RNA was extracted from acid insoluble material as described by Tyner *et al.* (1953). RNA was hydrolized by adding 0.3 M KOH and incubating at 37 °C for 20 h. Hydrolyzed RNA was separated from DNA by acid precipitation until pH 1.0-2.0 and centrifugation. The supernatant, containing (2'P) and (3'P) RNA nucleotides, was neutralized and frozen at -40 °C until HPLC processing.

Two HPLC methods were used : (2′P) and (3′P) RNA nucleotides were separated by reverse phase HPLC on the μBondapak C_{18} column isocratically eluted, at a flow rate of 2 ml/min, with 0.1 M KH_2PO_4 buffer pH 4.0, containing 1 % methanol. The starting buffer was run for 13 min, and then a second buffer was applied (0.1 M KH_2PO_4, pH 4.0, 10 % methanol) to elute (2′P)AMP. Bases obtained from nucleotides hydrolized in 1 N HCl at 100 °C for 60 min were chromatographes on a Polygosyl C_{18} column isocratically eluted with 0.2 M KH_2PO_4, pH 3.65, with a flow rate of 1.0 ml/min.

All HPLC runs were monitored at 254 nm, and fractions collected every 20 s and counted for radioactivity.

Results and discussion

Phosphoribosylpyrophosphate amidotransferase activity

PRPPTase catalyzes the synthesis of phosphoribosylamine metabolite, which can be considered the first specific precursor of purine biosynthesis. The reaction catalyzed by PRPPATase has received considerable attention as the main site of regulation of this pathway, since, until now, there have been no other similar rate limiting reactions known in *de novo* purine synthesis (Wyngaarden, 1972, 1973). The enzyme from a wide variety of organisms is sensitive to inhibition by purine nucleotides, as has been demonstrated in birds (Caskey *et al.*, 1964), bacterial systems (Nierlich and Magasanik, 1965 ; Messenger and Zolkin, 1973 ; Wood and Seegmiller, 1973). Changes in rates of *de novo* purine biosynthesis can be due to variations in the levels of substrates, different amounts or activities of PRPPATase, including its sensitivity to end products, and varying concentrations of inhibitory ribonucleotides. Less is known about the mechanisms which control the rate of synthesis and degradation of PRPPATase.

Fig. 1 shows the PRPPATase activities in *Artemia* nauplii at different stages of development. The activity levels detected in newly-hatched nauplii increased 5 times in 3-day-old unfed nauplii, but remained at the same level in 6-day-pld fed nauplii.

Some of the properties of the enzyme, which had been partially purified by acid precipitation and ammonium sulphate fractionation from nauplii extracts, were studied in 50-60 h-old nauplii. The enzyme preparation was unstable, and since attempts to stabilize it were not successful, it was assayed immediately after the ammonium sulphate fraction was dialyzed (see "Materials and methods"). Under standard conditions, the enzymatic reaction was linear with time over the periods assayed, and proportional to the protein concentration added. The purified PRPPATase exhibited normal kinetic for saturation by PRPP, with the K_m at about 0.5 mM (K_m values for PRPP ranged from 0.05 to 0.5 mM in various systems ; Wyngaarden, 1979), K_m values for glutamine could not be determined due to a contaminating glutaminase in the preparation (Liras and Llorente, 1981).

The enzyme is inhibited by end products of the pathway, and the effect of adenine and guanine mono- and di-nucleotides on the PRPPATase can be seen in Table I. Under the conditions of reaction utilized, 5 mM PRPP and 1 mM nucleotide, the highest inhibition is observed with ADP. The effect of GP_4G on the enzyme was also tested, and its inhibitory effect was in the same range as of the AMP and GMP. These results agree with the $(I)_{50}$ values for adenine and guanine nucleotides reported, although the PRPPATase sensitivity to purine ribonucleotide inhibition varies with the grade of purification of the enzyme.

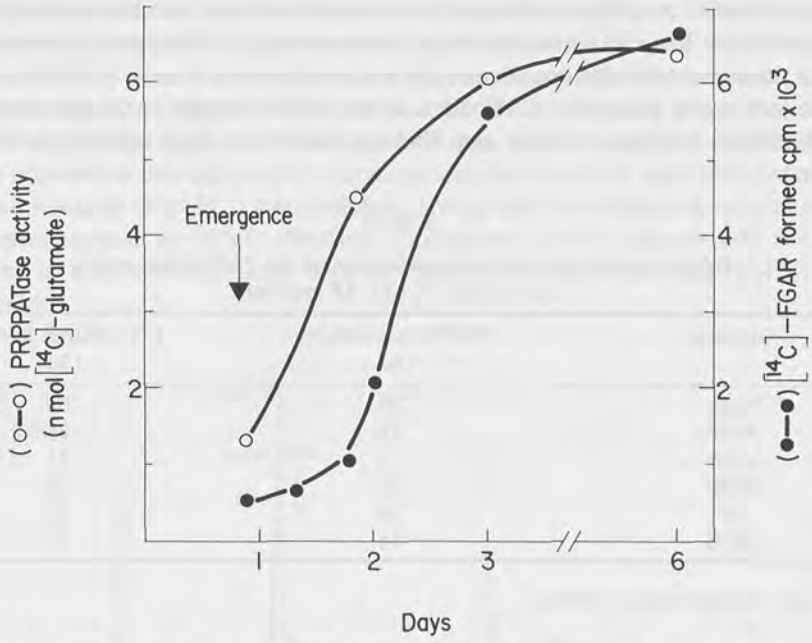

FIG. 1. Comparison of the rate of the early steps of *de novo* purine synthesis measured by the incorporation of (U-^{14}C)-glycine into FGAR, with the activities of PRPPATase, at different stages in the development of *Artemia* nauplii.

MEASUREMENT OF THE EARLY STEPS OF *DE NOVO* PURINE PATHWAY

The presence and functioning in *Artemia* nauplii of the early enzymatic steps of the purine *de novo* pathway has been established using the *in vitro* technique developed by Henderson (1962). The method is based on the measurement of the incorporation of the labeled *de novo* purine precursors into FGAR in the presence of azaserine at concentrations that inhibit formylglycinamidine ribonucleotide synthetase but not PRPPATase. Under such conditions it was assumed that the quantity of (^{14}C)-FGAR formed, pointed to the initial rate of purine nucleotide biosynthesis, which has been frequently utilized to evaluate the rate of purine synthesis *in vitro*. Fig. 1 shows the accumulation in the conditions established (Llorente *et al.*, 1980) and described in "Methods", of the (^{14}C)-FGAR in *Artemia* during nauplii development. The amount of (^{14}C)-FGAR formed increased with the age of the nauplii in a qualitatively similar form to that obtained in PRPPATase activity. On the other hand, the rate of (U-^{14}C)-glycine incorporation into FGAR was decreased by the same compounds known to inhibit purine synthesis by affecting the PRPPATase activity (Table I).

DE NOVO PURINE NUCLEOTIDE SYNTHESIS

The overall work of *de novo* purine biosynthesis pathway was demonstrated by determining the incorporation *in vivo* of (^{14}C)-HCO_3^- and (U-^{14}C)-glycine into acid-soluble cellular purines and into those incorporated into RNA (see "Methods". The pattern of labelling of the RNA

nucleotides obtained by alkaline hydrolysis from nauplii incubated with the labelled precursors, is shown in Fig. 2. The HPLC profiles indicate that in using (U-^{14}C)-glycine, radioactivity only appears in the adenine and guanine nucleotides, while radioactivity from (^{14}C)-HCO_3^- is incorporated into purine and pyrimidine nucleotides. In the HPLC analysis of the free bases released by acid hydrolysis of the acid soluble and RNA nucleotides the same radioactivity distribution is observed.

TABLE I

Inhibitory effect of purine ribonucleotides on the PRPPATase activity and on the (^{14}C)-FGAR synthesis

Additions	PRPPATase activity (%)	(^{14}C)-FGAR formed (%)
None	100	100
AMP	57	86
ADP	5	11
GMP	38	20
DP	24	20
GP_4G	44	25

See "Methods" for experimental conditions.

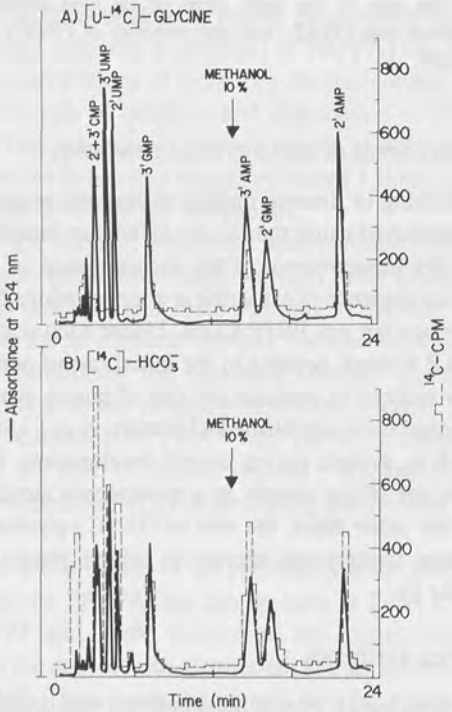

FIG. 2. HPLC analysis of nucleotides obtained by alkaline hydrolysis of RNA from 30 h-old nauplii incubated with : A) (U-^{14}C)-glycine and B) (^{14}C)-HCO_3^-.

The bases obtained by acid hydrolysis of nucleotides labeled with (U-^{14}C)-glycine were isolated by HPLC, and the fractions corresponding to adenine and guanine were collected ; after concentration, they were used as substrates of adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT), obtained from 48-h-old *Artemia* nauplii. The products of the enzymatic reaction were, in turn, analyzed by HPLC with the resulting observation that radioactivity then coincides with the AMP and GMP peaks, products of the said reactions (Fig. 3). It was, therefore, proven that the radioactivity associated with the purine bases isolated by HPLC effectively corresponds to (^{14}C)-adenine and (^{14}C)-guanine formed by *de novo* synthesis, since both are substrates of the APRT and HGPRT enzymes, respectively.

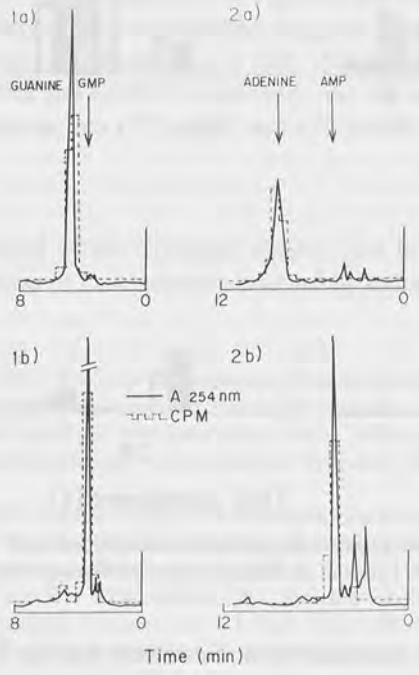

FIG. 3. Confirmation of the nature of radioactivity associated to purines from *Artemia* nauplii incubated with (U-^{14}C)-glycine. Guanine and adenine peaks were isolated from acid hydrolyzates of nucleotides by HPLC and concentrated ; aliquots of these purine base preparations were treated with partially purified APRT and HGPRT activities (Rottlàn *et al.*, 1985). 1a and 2a are controls, showing the absorbance and radioactivity profiles of guanine and adenine peaks before enzymic treatment ; 1b and 2b show the absorbance and radioactivity profiles of guanine and adenine samples after completion of enzymic reaction ; radioactivity is now associated to nucleotide peaks.

The labelling observed in recently hatched nauplii can be clearly observed in pyrimidines and difficulty in purines with (^{14}C)-HCO_3^- as precursor (Fig. 4). With (U-^{14}C)-glycine, only the purines are labelled and radioactivity associated with them is not detected until the nauplii attain a 20 h development after hatching. However, it must be noted that the level of incorporation of

the labelled precursors depends on the saline concentration in the medium, notably increasing at low salt concentration. Similar observations have been done concerning incorporation of exogenous purines (Rotllán *et al.*, 1987).

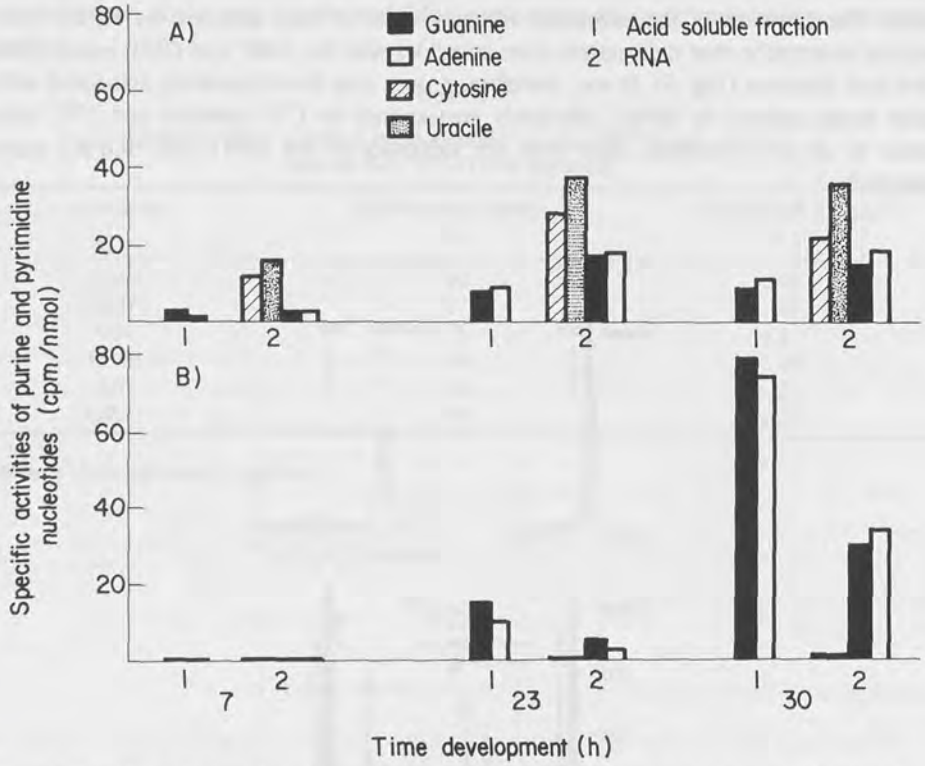

FIG. 4. Specific activities of purine and pyrimidine nucleotides from *Artemia* nauplii incubated with : A) (^{14}C)-HCO_3^- and B) (U-^{14}C)-glycine at different stages of development.

The net purine synthesis calculated from acid-soluble fraction, RNA and DNA, parting from (U-^{14}C)-glycine, in nauplii of 23 and 30 h development after hatching is 2.5 and 24.0 nmoles/g respectively, in the described conditions (see "Methods"). These values are close to those described in systems of which the *de novo* purine synthesis capacity has been discussed (Allsop and Watts, 1983 ; Wang and Simashkevich, 1981 ; Sheehan and Tully, 1983). On the other hand, the ratio of the synthesis of the purine nucleotides from labelled bases, (^{14}C)-hypoxanthine, (^{14}C)-adenine, and (^{14}C)-guanine, reported by this same group in this symposium, with respect to *de novo* synthesis is approximately 5 to 1. Reported values by Zoref-Shani *et al.* (1984) for human skin fibroblasts, rat skeletal muscle, and rat cardiomyocytes are similar.

Conclusions

The results of the present study bring evidence of the capacity of *Artemia* nauplii to synthesize *de novo* purine nucleotides. This conclusion is supported by the following facts : 1) the enzyme

which catalyzed the first committed step of the *de novo* pathway, PRPPATase, has been detected, increasing its activity during the naupliar development. Partially purified enzyme exhibited normal kinetics for saturation by PRPP and sensitivity to end product inhibition by purine nucleotides, including GP_4G ; 2) the early enzymatic steps from the *de novo* purine biosynthetic pathway were determined by measuring the (U-^{14}C)-glycine incorporation into FGAR, in the presence of azaserine. This incorporation was qualitatively diminished by the same inhibitors of PRPPATase and rose in parallel with the enzyme activity ; 3) the overall purine biosynthesis was assessed measuring the uptake by nauplii, which had been grown and incubated under optimal conditions, and incorporation of (U-^{14}C)-glycine and (^{14}C)-HCO_3^- into cellular purines. HPLC analysis of the nucleotides or from bases from acid-soluble fractions and RNA were performed and the chromatographic profiles demonstrated purine and pyrimidine radiolabelling from (^{14}C)-HCO_3^- and exclusively in purines when (U-^{14}C)-glycine was the precursor.

The pyrimidine labelling occurred in newly-hatched nauplii while the radioactivity in purines, from both precursors, appeared approximately 20 h later. HPLC-isolated adenine and guanine were used as substrates of APRT and HGPRT respectively, and the radioactivity associated with them was converted quantitatively into (^{14}C)-AMP and (^{14}C)-GMP as their chromatographic profiles indicated.

Acknowledgements

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Artemia DNA polymerases : activity gel analysis of DNA polymerase-α and -γ polypeptides and immunological detection of a polypeptide related to DNA polymerase-β

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Abstract

When cysts and larvae of *Artemia* are extracted in various ways and analysed on glycerol density gradients, no activity corresponding to a typical low molecular weight DNA polymerase-β is observed, only a 9-10S DNA polymerase-α. This lack of a DNA polymerase-β activity is confirmed by the "activity gel" technique whereby polymerases are renatured and assayed autoradiographically *in situ* after electrophoresis in a polyacrylamide gel containing SDS. However, on probing a nitrocellulose blot of soluble proteins from cysts and newly hatched larvae fractionated on a SDS-polyacrylamide gel with an anti-chick DNA polymerase-β antibody, a strongly cross-reacting band of M_r 48 000 is seen. Crude nuclear fractions do not contain this species and it is completely absent from older larvae. The size and antigenic cross-reactivity of this polypeptide suggest that it is a catalytically inactive, cytoplasmic form of DNA polymerase-β.

Activity gels of soluble fractions do reveal major DNA polymerase-α catalytic polypeptides of 112 000 M_r and 82 000 M_r with minor species of 100 000 M_r and 74 000 M_r. Greatest activity is seen in 7 h cysts but no activity remains beyond 48 h in contrast to what is found with the conventional polymerase assay. This is due to the extreme sensitivity of this technique to contaminating proteinases. A DNA polymerase-γ polypeptide of M_r 118 000 is also observed which increases in content in the particulate fraction during larval development. Activity gel analysis of cysts and larvae solubilised directly in 1 % SDS in the presence of very high concentrations of proteinase inhibitors (2.5 mM phenylmethanesulphonyl fluoride and 500 µg/ml soybean trypsin inhibitor) show that cysts and larvae contain virtually the same amount of each DNA polymerase-α catalytic polypeptide. Previously observed developmental changes are probably due to changes in extractability and proteolytic enzymes.

Introduction

The cleavage stages of early embryogenesis in many organisms are characterised by rapid DNA replication and increase in cell number (Kornberg, 1980). To achieve the necessary rate of cell growth and division, oocytes and embryos of organisms such as sea urchins (Shioda *et al.*, 1980), *Drosophila* (Banks *et al.*, 1979), and *Xenopus* (Zierler *et al.*, 1985) contain large stockpiles of DNA polymerases sufficient for the synthesis of some 10-100 000 nuclear equivalents of DNA. After rapid cleavage, when the specific activity of the DNA polymerases are once more similar to those found in dividing adult somatic cells, the synthesis of new enzyme recommences in pace with the subsequent increases in cell number that are part of further development through larval stages to the adult.

Post-cleavage gastrular cysts of *Artemia* are unusual among embryonic systems in that DNA replication is prematurely arrested by the process of encystment. Furthermore nuclear DNA synthesis does not resume immediately upon rehydration as do RNA and protein synthesis but remains inactive throughout the period of pre-emergence development, resuming only upon emergence of the pre-nauplius larva (Finamore and Clegg, 1969 ; Iwasaki, 1969 ; Olson and Clegg, 1978). Nothing is known about the timing of the onset of mitochondrial DNA replication in *Artemia* but the immature state of the mitochondria in cysts suggests that re-initiation will be similarly retarded (Marco *et al.*, 1981).

Precisely why DNA replication is delayed until emergence is not known but it may be that actively replicating chromosomes cannot be protected from lethal breakage during the repeated cycles of rehydration and dehydration which a cyst is likely to experience in its natural environment. In addition the delay may permit the repair of lesions introduced into the DNA as a result of such cycles. Nor is anything known about the factors which control the timing of re-initiation of DNA replication or to what extent pre-emergence development may be analogous to the G1 phase of the cell cycle with emergence signalling the onset of an 'S phase'-equivalent. One such factor may be the nucleotide P^1,P^4-bis (5′-adenosyl) tetraphosphate (Ap_4A) which accumulates during pre-emergence development in a manner similar to that observed during a mammalian cell G1 phase and reaches a maximum at the point of emergence (McLennan and Prescott, 1984). The target protein for this nucleotide in mammalian cells is a subunit of the replicative DNA polymerase-α (Grummt *et al.*, 1979 ; Rapaport *et al.*, 1981) although this may not be the case in *Artemia* (Prescott and McLennan, 1987).

In order to learn more about DNA replication and its control in *Artemia* we have undertaken a study of the DNA polymerases present in cysts and larvae. The DNA polymerases of multicellular animals are of three major types (Scovassi *et al.*, 1980 ; Hübscher, 1983) : 1) DNA polymerase-α, a large heterogeneous multisubunit complex responsible for nuclear DNA replication and for some repair reactions ; 2) DNA polymerase-β, a small chromatin-bound enzyme required for the repair of certain classes of lesion ; and 3) DNA polymerase-γ, the enzyme responsible for mitochondrial DNA replication. A further enzyme, DNA polymerase-δ has been reported in calf thymus and rabbit bone marrow and may be a specialised form of DNA polymerase-α (Byrnes, 1984).

In a previous study we showed that DNA polymerase-α was readily detectable in extracts of dormant cysts and increased 2 to 3-fold during pre-emergence development. Maximum activity was reached at the onset of the DNA synthetic phase (Slater and McLennan, 1982). The mitochondrial DNA polymerase-γ was also present in the cyst and attained maximum activity 8 h after rehydration, some 8 h before DNA polymerase-α. A further increase was observed between 48 h and 72 h after the reinitiation of development.

Two interesting questions arose from this initial work. Firstly, despite repeated attempts, no activity corresponding to a DNA polymerase-β was ever detected. Since insects were also believed to lack this enzyme, an evolutionary explanation appeared likely. Secondly, we were not certain whether the increases in DNA polymerase-α and DNA polymerase-γ activity observed during pre-emergence development represented new enzyme synthesis as in a normal cell cycle or the reassembly and release of enzyme preserved from the earlier stages of cleavage and rapid DNA synthesis. We can now report that *Artemia* cysts do in fact possess a polypeptide which is antigenically related to DNA polymerase-β but which is catalytically inactive and is located in

the cytoplasm, and that the content of active DNA polymerase-α polypeptides originally present in the cyst changes little, if at all, during development.

Materials and methods

Materials

Great Salt Lake cysts were supplied by the Sanders Brine Shrimp Co., Ogden, UT, USA in 1980 and were decapsulated and incubated as described previously (McLennan and Prescott, 1984). Phenylmethanesulphonyl fluoride (PMSF), soybean trypsin inhibitor (STI), and bovine fibrinogen were from Sigma, and nitrocellulose sheets, SDS, and the Immun-blot (GAR-HRP) assay kit were purchased from Bio-Rad. dATP, dCTP, dGTP, TTP, and protein molecular weight standards were obtained from Boehringer while pre-stained protein molecular weight standards were from Bethesda Research Laboratories. [^{3}H]TTP (49 Ci/mmol) and [α-^{32}P]TTP (410 Ci/mmol) were products of Amersham International.

Homogeneous chick embryo DNA polymerase-β and a monospecific rabbit anti-chick DNA polymerase-β were the generous gifts of Dr. Akio Matsukage (Yamaguchi *et al.*, 1982).

Glycerol density gradient analysis

Cysts, larvae, logarithmic phase HeLa cells and normal adult rat liver were suspended in 4 vol 0.2 M potassium phosphate pH 7.2, 5 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 10 mM $NaHSO_3$, 50 µg/ml STI at 4 °C. When freshly added this combination of proteinase inhibitors was found to be effective in preventing the appearance of low molecular weight degradation products of DNA polymerase-α on glycerol gradients at all developmental stages. Suspensions were homogenised with ten strokes of a Potter-Elvehjem homogeniser then stirred for 1 h. Homogenates were centrifuged for 1 h at 105 000 *g*, dialysed overnight against 20 mM potassium phosphate pH 7.2, 0.5 M KCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM $NaHSO_3$ and 200 µl samples centrifuged through 10-30 % (v/v) glycerol density gradients made up in the same buffer (McLennan and Keir, 1975).

DNA polymerase assays

Artemia DNA polymerase activity in glycerol gradient fractions was assayed using the DNA polymerase-α assay without dideoxy-TTP (a β-polymerase and γ-polymerase inhibitor) (Slater and McLennan, 1982). The Mg^{2+} concentration was increased to 7 mM for the assay of HeLa cell and rat liver DNA polymerases.

Activity gel analysis

Cysts and larvae at the appropriate developmental stage were homogenised as above in 4 vol homogenising buffer (20 mM potassium phosphate pH 7.2, 0.4 M sucrose, 4 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM PMSF, 10 mM $NaHSO_3$, 50 µg/ml STI). The homogenates were filtered through four layers of cheesecloth then centrifuged at 5 000 *g* for 20 min. The pellets, which comprised mainly nuclei and yolk platelets but also many mitochondria which are sequestered within the platelets (Marco *et al.*, 1981), were washed with homogenising buffer, then resuspended in 4 vol homogenising buffer containing 0.2 M potassium phosphate pH 7.2,

0.5 M KCl and stirred for 4 h. These extracts (the particulate fraction) and the first 5 000 *g* supernatants (the soluble fraction) were centrifuged for 1 h at 105 000 *g* then dialysed against 62.5 mM Tris-HCl pH 6.8, 5 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM $NaHSO_3$. PMSF was added to a final concentration of 1 mM and the extracts concentrated 5-fold in an Amicon B15 Minicon concentrator. Samples containing 200 μg protein were incubated for 3 min at 37 °C in sample buffer immediately before electrophoresis.

SDS-polyacrylamide slab gels (7.5 %) were prepared and electrophoresed as described by Blank *et al.* (1983). Stacking gels were polymerised with riboflavin and resolving gels (10×14×0.15 cm) contained 150 μg/ml activated DNA and 50 μg/ml bovine fibrinogen. Gels were stacked at 250 V and run at 350 V at 10 °C for a total of 2.5 h. After electrophoresis lanes containing protein standards were removed and stained with Coomassie blue while the remainders of the gels were washed with shaking at room temperature for 3×20 min periods in 25 vol 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 1 mM 2-mercaptoethanol, 20 % (v/v) redistilled propan-2-ol (Blank *et al.*, 1983) followed by 2×20 min washes with 25 vol 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 1 mM 2-mercaptoethanol. The gels were then incubated at 4 °C for 3 h in 50 vol 15 % (v/v) glycerol, 1 mM 2-mercaptoethanol containing either 50 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$ (for DNA polymerase-α) or 50 mM Tris-HCl pH 8.4, 10 mM $MgCl_2$ (for DNA polymerase-γ). They were then incubated in sealed plastic bags at 37 °C for 18 h in assay buffers : 15 % (v/v) glycerol, 5 mM 2-mercaptoethanol, 15 μM each of dATP, dGTP, dCTP, 1 μCi/ml [α-^{32}P]TTP (410 Ci/mmol) containing either 50 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM $MgCl_2$ (for DNA polymerase-α) or 50 mM Tris-HCl pH 8.4, 80 mM KCl, 10 mM $MgCl_2$ (for DNA polymerase-γ).

The assay medium was removed and the gels washed in 50 vol each time of 5 % TCA, 1 mM $Na_4P_2O_7$ for 4×15 min periods at room temperature followed by 6 h, 16 h and 2×1 h washes at 4 °C. The stained sections were equilibrated in 5 % TCA, the gels reassembled and dried down onto Whatman 3 MM paper. Autoradiography was at −70 °C with Fuji RX film and a DuPont Cronex Lightning Plus intensifying screen.

Protein blotting and immunological detection

Samples of soluble and particulate fractions (200 μg) prepared as described above were electrophoresed in 10 % SDS-polyacrylamide gels (Laemmli, 1970) for 5 h at 150 V. Proteins were electrophoretically transferred overnight to nitrocellulose at 30 V, 150 mA in 25 mM Tris, 192 mM glycine, pH 8.3, 20 % methanol, 0.1 % SDS. The blot was incubated for 24 h at room temperature with a 1:200 dilution of anti-chick DNA polymerase-β in 20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.01 % thimerosal and the bands visualised using the Bio-Rad Immun-blot (GAR-HRP) kit following the manufacturer's instructions. The second antibody was used at a dilution of 1:2 000.

Results

DNA polymerase-β is readily extracted from most tissues by 0.2 M potassium phosphate buffer and can be distinguished from DNA polymerase-α by its resistance to inhibition by N-ethylmaleimide and by its size (single polypeptide of M_r 40-50 000) (Chang, 1976). The results of applying this extraction procedure to adult (non-proliferating) rat liver and rapidly

dividing HeLa cells are shown in Fig. 1A and B. The 3.4S DNA polymerase-β activity is easily separated from the high molecular weight DNA polymerase-α. By way of contrast, no activity with properties characteristic of a DNA polymerase-β was detected in extracts of dormant *Artemia* cysts or 24 h-old nauplius larvae (Fig. 1C and D). Only a high molecular weight, 9-10S DNA polymerase-α activity was evident. Such a size is characteristic of the undegraded holoenzyme complex (Grosse and Kraus, 1981 ; Masaki *et al.*, 1982) and is achieved only in the presence of both PMSF and STI. Omission of STI results in a 7.6S DNA polymerase-α while the further omission of PMSF produces a 6.2S species (Slater and McLennan, 1982). DNA polymerase-γ was not detected with the α-specific assay. The use of either more or less rigorous extraction conditions involving different ionic strengths and salts, non-ionic detergents, different extraction times, altered assay conditions or non-decapsulated cysts failed to reveal a DNA polymerase-β activity from any developmental stage examined up to 72 h-old larvae.

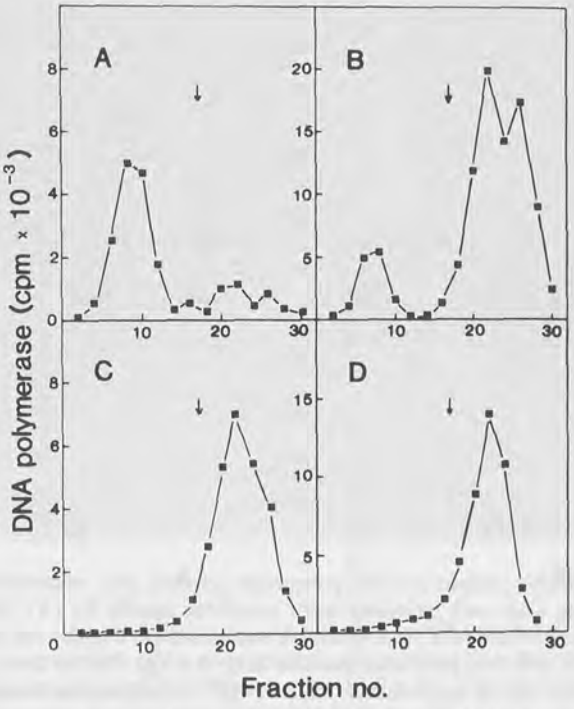

FIG. 1. Glycerol density gradient analysis of crude cell extracts for DNA polymerase activities. (A) Rat liver ; (B) HeLa cells ; (C) *Artemia* cysts, and (D) *Artemia* nauplius larvae. The arrows indicate the position of the marker enzyme lactate dehydrogenase (7.2S). The tops of the gradients are to the left.

One possible reason for the lack of an assayable activity of low molecular weight could be the complexing of the DNA polymerase-β with a cofactor of high molecular weight or with a specific inhibitor which reassociates with the enzyme during the dialysis of crude extracts. Inhibitors of both DNA polymerase-α and β have been described in *Xenopus* ovaries and embryos (Fox *et al.*, 1980 ; Smith *et al.*, 1983). Such inhibitors may be of importance in the developmental regulation

of polymerase activity. In order to ensure the freedom of any possible DNA polymerase-β activity from such non-covalent associations, the total polymerase activity of soluble and particulate fractions of different developmental stages was examined by the 'activity gel' technique (Spanos *et al.*, 1981). In this method samples are incubated in SDS and separated in dissociating polyacrylamide gels. After renaturation, enzyme activity is determined *in situ*.

When measured under conditions optimised for DNA polymerase-α, the polymerase activity of the soluble fraction of unincubated cysts revealed major bands of $M_r \sim 112\,000$ and 82 000 with minor bands at 118 000, 100 000, and 74 000-M_r (Fig. 2A, lane a). By 7 h all species other than that of M_r 118 000 had increased markedly in activity, returning to original levels by 24 h (lanes b, c). At 48 h and 72 h after rehydration, the only bands visible were of M_r 118 000 and 112 000 (lanes d, e). These were also the only two bands extracted from the particulate fractions which consist of nuclei, yolk platelets, and mitochondria (lanes f-j).

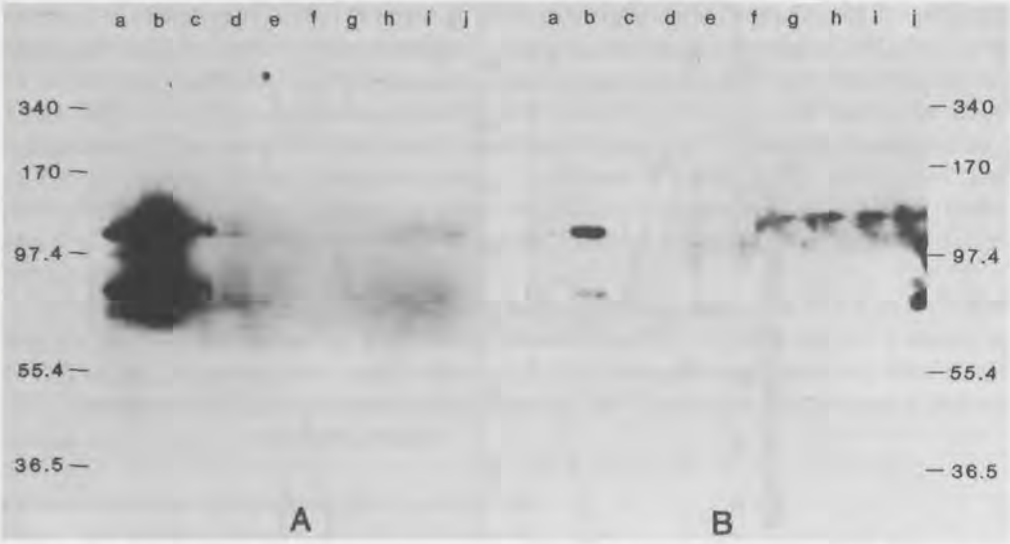

FIG. 2. Autoradiographic analysis of DNA polymerase activities after renaturation and assay *in situ* in SDS-polyacrylamide gels. Gels were incubated under conditions specific for (A) DNA polymerase-α and (B) DNA polymerase-γ. Extracts were made of soluble fractions at (a) 0 h, (b) 7 h, (c) 24 h, (d) 48 h, and (e) 72 h of development and from particulate fractions at (f) 0 h, (g) 7 h, (h) 24 h, (i) 48 h, and (j) 72 h of development. Numbers refer to the molecular masses ($\times 10^{-3}$) of polypeptide standards. Autoradiography was for 7 days.

When assayed under conditions which favour DNA polymerase-γ activity and which, in terms of pH and ionic composition might also be expected to favour DNA polymerase-β, no previously undetected bands were found in either soluble or particulate fractions (Fig. 2B). The time-dependent increase in the activity of the 118 000 M_r band in the particulate fraction (lanes f-j) and its preference for DNA polymerase-γ assay conditions suggest that it is the DNA polymerase-γ described previously (Slater and McLennan, 1982). High activity of DNA polymerase-γ in older larvae may reflect increased mitochondrial content and activity in muscle tissue. The remaining bands have molecular weights characteristic of DNA polymerase-α polypeptides from

many species as visualised on activity gels (Hübscher *et al.*, 1981 ; Scovassi *et al.*, 1982). At no time was a DNA polymerase activity of low molecular weight observed which suggests that complexed forms of DNA polymerase-β are not present in these extracts. The autoradiographs were deliberately overexposed to ensure the detection of minor bands.

An alternative method for the detection of a DNA polymerase-β which does not rely on the demonstration of enzyme activity is by immunoassay. Such an approach is possible in this case since the evolutionary conservation of the DNA polymerase-β polypeptide structure and therefore the cross-reaction of the enzyme with a heterologous antibody is high (Chang *et al.*, 1982 ; Tanabe *et al.*, 1984).

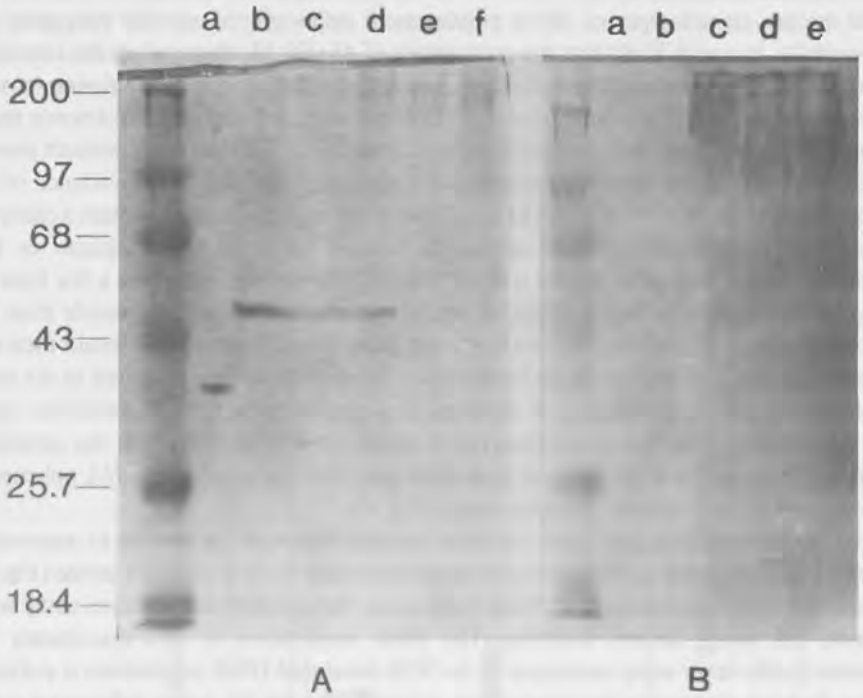

FIG. 3. Nitrocellulose blots of *Artemia* proteins separated by SDS-polyacrylamide gel electrophoresis and probed with rabbit anti-chick DNA polymerase-β. (A) Chick DNA polymerase-β control (a) and extracts of soluble fractions prepared from *Artemia* after (b) 0 h, (c) 7 h, (d) 24 h, (e) 48 h, and (f) 72 h of development. (B) Extracts prepared from particulate fractions after (a) 0 h, (b) 7 h, (c) 24 h, (d) 48 h, and (e) 72 h of development. Numbers refer to molecular masses ($\times 10^{-3}$) of pre-stained polypeptide standards.

Nitrocellulose blots of soluble and particulate protein fractions separated by dissociating polyacrylamide gel electrophoresis were probed with a monospecific antibody raised against homogeneous chick embryo DNA polymerase-β (Yamaguchi *et al.*, 1982). The blots were visualised with horseradish peroxidase-linked goat anti-rabbit IgG. Pure chick embryo DNA polymerase-β appeared as a single band of $M_r \sim 38\,000$ (Fig. 3A, lane a). This is close to the figure of 40 000 reported (Yamaguchi *et al.*, 1982). Fig. 3A also shows quite clearly the presence

of a cross-reacting polypeptide of $M_r \sim 48\,000$ in soluble extracts of *Artemia* at 0, 7, and 24 h of development (lanes b-d). The continuity of the bands is due to the heavy protein loading used. The 48 000 M_r band is absent at later times (lanes e, f). The size of the polypeptide strongly suggests that it is a form of DNA polymerase-β, yet it is present exclusively in the soluble cytoplasmic fraction. Extraction of crude nuclear pellets with 0.2 M potassium phosphate pH 7.2, 0.5 M KCl (Fig. 3B, lanes a-d), ammonium sulphate, non-ionic detergents or the preparation of a non-histone chromatin protein fraction with 5 M urea, 0.6 M guanidinium hydrochloride (data not shown) failed to reveal any cross-reacting material. The light staining near the top of the gel is due to the trapping of antibody in a precipitate of high molecular weight apolipovitellin polypeptides.

Whilst proper classification of DNA polymerase-β relies on the specific properties of its enzyme activity, it is very likely that the polypeptide of 48 000 M_r observed on the immunoblot is indeed a DNA polymerase-β based on its size and the fact that the evolutionary structural conservation of this enzyme is such that if a DNA polymerase-β did exist in *Artemia* then the blotting experiment would have detected it. Also a cytoplasmic location is not without precedent (see "Discussion"). Since close examination of the activity gels shows no evidence of DNA polymerase activity at M_r 48 000 it can be concluded that : a) *Artemia* cysts contain a catalytically inactive DNA polymerase-β ; b) the protein is located either in the cytoplasm or in the nucleoplasm from which it can readily leak out ; and c) the protein disappears a few hours after hatching of the nauplius larvae. It might be argued that the loss of the polypeptide from larvae is due to proteolytic degradation *in vitro* by the gut proteinases, however one would then expect degradation products to appear on the immunoblot. While cyst extracts prepared in the absence of proteinase inhibitors do generate a cross-reacting species of M_r 22 000 (data not shown), neither this nor any other band was observed in larval extracts. Furthermore, the combination of PMSF and STI used is quite sufficient to prevent extensive degradation of DNA polymerase-α when measured by conventional solution assay (Fig. 4).

On the other hand this particular inhibitor cocktail seems to be unable to guarantee the detection of DNA polymerase-α by the activity gel technique in 48 h and 72 h larvae (Fig. 2 and 4). Substantial activity is apparent at these times using the conventional solution assay whereas the activity gel barely detects anything. The likely explanation of this discrepancy is the requirement by the latter assay technique for an SDS-denatured DNA polymerase-α polypeptide to reform its active site during the renaturation process. Whereas the native polymerase complex may be little affected by the breakage of a single susceptible peptide linkage away from the active site, such mild proteolysis may completely prevent the proper refolding of the cleaved, denatured structure and so abolish all activity.

The ability to detect DNA polymerases after incubation in SDS offers an opportunity to determine whether or not changes in extractability by salt are the cause of the fluctuations in DNA polymerase-α activity observed previously (Slater and McLennan, 1982). Cysts and larvae were homogenised directly in buffer containing 1 % SDS in order to strip all polymerase polypeptides from subcellular structures. In view of the increased susceptibility of denatured polypeptides to proteolytic degradation even in the presence of SDS, different concentrations of PMSF and STI were tried in the homogenising buffer in order to preserve activity. Without these inhibitors, the recovery of all DNA polymerase-α polypeptides from unincubated cysts was very low (Fig. 5, lane c). At 7 h (lane f) and 24 h (lane i) of development the 112 000 M_r and 100 000 M_r species were more evident but by 48 h (lane l) they decreased again without the

protection of proteinase inhibitors. The magnitude of the effect of SDS-resistant proteolysis at 0 h is quite striking, but it was readily overcome by the inclusion of PMSF and STI at the concentrations normally used (1 mM and 50 μg/ml respectively) (lane b). Under these conditions, the recoverable activity of the high molecular weight polypeptides was virtually the same at 7 h (lane e), 24 h (lane h), and 48 h (lane k) as at 0 h, suggesting that *de novo* synthesis of this group had not taken place. In order to preserve activity of the lower molecular weight polypeptides of M_r 82 000 and 74 000, very high concentrations of PMSF (2.5 mM) and STI (500 μg/ml) were required in conjunction with the SDS-extraction technique at all developmental stages (lanes a, d, g, and j). Again there appears to be little change in the activity of this group during development (the slight increase at 7 h is not reproducible). The smaller species appear to be very sensitive to proteolysis when assayed by the activity gel technique, yet even the extreme conditions required to preserve their catalytic activity failed to reveal anything which could correspond to a DNA polymerase-β, thus confirming the previous results.

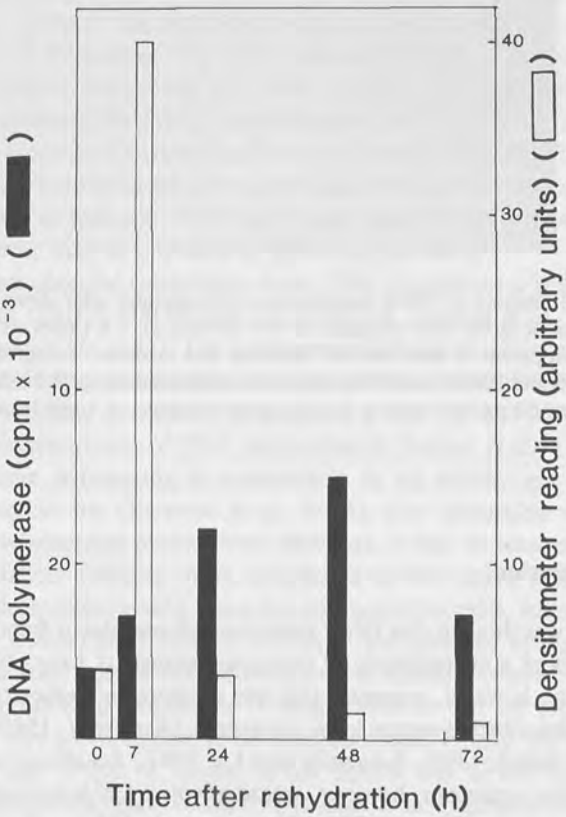

FIG. 4. Comparison of DNA polymerase-α activities determined by solution assay and activity gel assay. The activity of DNA polymerase-α in the soluble extracts of *Artemia* cysts and larvae was determined with the conventional solution assay (filled bars). The activities of the separated DNA polymerase-α polypeptides in Fig. 2A were quantitated with a Joyce-Loebl densitometer and summed for each developmental stage (open bars).

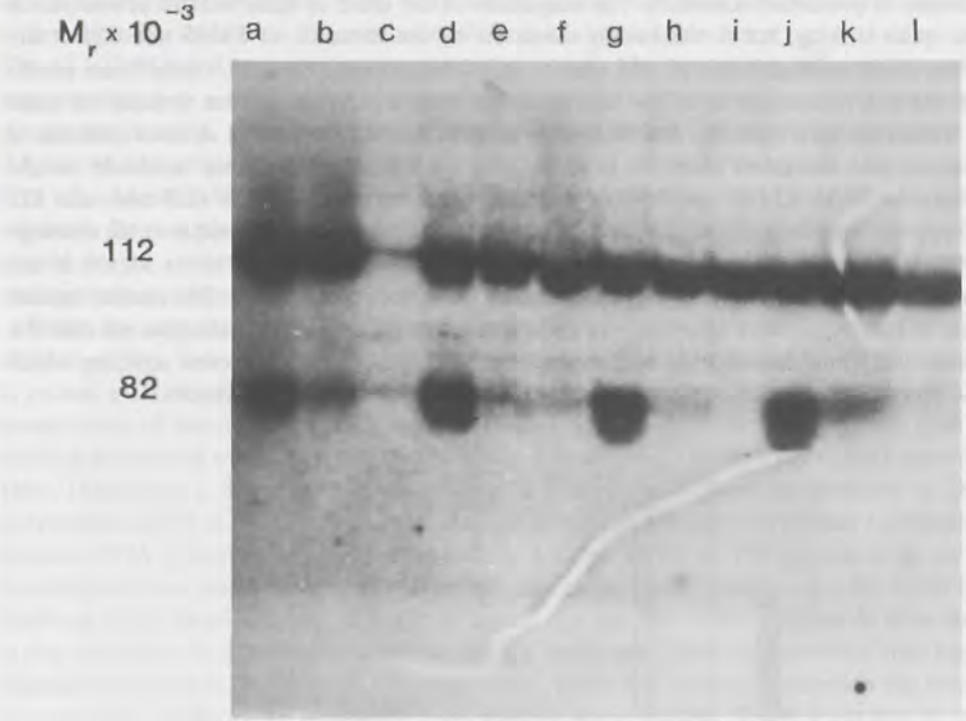

FIG. 5. Activity gel analysis of DNA polymerase-α polypeptides after direct solubilisation in SDS. Soluble extracts of cysts or larvae were prepared at 0 h (lanes a-c), 7 h (lanes d-f), 24 h (lanes g-i), and 48 h (lanes j-l) of development as described in "Materials and methods" except that the homogenisation buffer included 1 % SDS and PMSF and STI at respective concentrations of 2.5 mM and 500 μg/ml (lanes a, d, g and j), 1 mM and 50 μg/ml (lanes b, e, h, k) or zero (lanes c, f, i and l). Autoradiography was for 48 h.

Discussion and conclusion

For many years it was thought that DNA polymerase-β was absent from protozoa, fungi, and plants and was therefore a characteristic of metazoan animals (Chang, 1976 ; McLennan and Keir, 1977). Recently, however, enzymes with one or more properties typical of DNA polymerase-β have been described in several lower organisms. (Baril *et al.*, 1980 ; Chang *et al.*, 1980, 1982 ; Schiebel and Rafael, 1980 ; Sakaguchi and Lu, 1982 ; Stauder *et al.*, 1983).

One group of higher organisms, however, where a typical DNA polymerase-β is not readily demonstrated is the insects (Chang, 1976 ; Scovassi *et al.*, 1983), although a low molecular weight polymerase has been detected in cockroach testes (Scovassi *et al.*, 1982) and a β-like 5S activity in adult *Drosophila* (Furia *et al.*, 1979). This latter activity may be sensitive to extraction conditions which may explain the failure of others to detect it (Dusenbery and Smith, 1983). This discrepancy among the insects also arises within another group of arthropods, the Crustacea,

where the apparent absence of an active DNA polymerase-β from *Artemia* embryos may be contrasted with its presence in lobster gonads (Chang, 1976).

A cytoplasmic location for DNA polymerase-β as well as DNA polymerase-α has been reported in unfertilised sea urchin eggs. These stored pools of enzymes are attached to the rough endoplasmic reticulum and are translocated into the new nuclei during early embryogenesis. In the case of *Hemicentrotus pulcherrimus*, the translocation of both enzymes is complete by late blastula (Shioda *et al.*, 1980) whereas Hobart and Infante (1980) have reported that 75 % of the DNA polymerase-β of *Strongylocentrotus purpuratus* remains cytoplasmic at this stage. The total activity of both enzymes remains constant as does the content per nucleus. Whilst we cannot exclude the presence of a small amount of DNA polymerase-β in the nuclei of *Artemia* gastrulae, it is apparent from our results that the bulk of it is isolated in a freely soluble and catalytically inactive form at all stages examined up to hatching.

DNA polymerase-β is generally a predominant activity in activity gels (Scovassi *et al.*, 1982) even under conditions optimised for DNA polymerase-α, yet its presence in *Artemia* could not be detected with this technique. It is possible that the *Artemia* enzyme is very sensitive to extraction conditions, however the persistent cytoplasmic location of the protein suggests that the essential function of the enzyme may have been served by the time the gastrulae desiccate. It may then be inactivated and transported back to the cytoplasmic fraction for subsequent degradation. The requirement for the dry packaging of the DNA may also dictate the removal from the nucleus of non-essential non-histone chromosomal proteins. The dispensation with DNA polymerase-β after embryogenesis is consistent with the view that organisms of limited lifespan need not invest unnecessary resources in a full range of DNA repair systems. Essential repair replication activity may be provided by DNA polymerase-α.

Several workers have observed catalytically active DNA polymerase-α polypeptides on activity gels in the size ranges 110-120 000 M_r and 70-80 000 M_r (Spanos *et al.*, 1981 ; Karawya and Wilson, 1982 ; Scovassi *et al.*, 1982). The lower molecular weight species are generally thought to be proteolytically derived *in vitro* from the higher molecular weight polypeptides which in turn may be degradation products of a native polypeptide in the 150-190 000 M_r range, which is obtained from immunoprecipitates of DNA polymerase-α (Kaguni *et al.*, 1983 ; Karawya *et al.*, 1984). Since this large polypeptide is undetectable in an activity gel, presumably due to difficulties in refolding *in situ* (Karawya *et al.*, 1984), it is impossible to say how much it contributes to the developmental profiles here. However, it may be reasonably inferred that no change in its degradation products infers no change in the native polypeptide itself. It is interesting that such degradation products are still obtained when cysts, whose proteinase content is relatively low, are homogenised directly in SDS in the presence of high concentrations of PMSF and STI. This may suggest that proteolysis has already occurred *in vivo* as part of a biologically relevant processing programme (Chang, 1980) or that the proteinases responsible for the conversion *in vitro* are insensitive to the inhibitors used. Finally, the intriguing suggestion that the 120 000 M_r and 70 000 M_r species in calf thymus may be the translation products of different mRNAs (Detera-Wadleigh *et al.*, 1984) adds a new dimension to our still limited understanding of the composition of and relationship between DNA polymerase-α species. What we have shown in *Artemia* is that the content of detectable and therefore probably all catalytic polypeptides of DNA polymerase-α does not *per se* control the timing of DNA synthesis. This must be the result of other factors which are generated during pre-emergence development.

Acknowledgements

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Occurrence of a nucleotidase activated by ATP in *Artemia* cysts extracts

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Abstract

An activity has been found in *Artemia* cysts extracts which hydrolyzes GMP to guanosine being stimulated by ATP. This activity was recovered in the 25-45 % $(NH_4)_2SO_4$ fraction of the 100 000×*g* supernatant and presented a molecular weight around 180 000. The activation constant for ATP was 2.5 mM. The *K*m values for GMP found in the absence and presence of ATP were 10 and 0.4 mM, respectively. This activity may correspond to a cytosolic 5′-nucleotidase activated by ATP, already described in other tissues.

Introduction

Several enzymes of the purine nucleotide interconversion cycle have been purified and characterized in *Artemia* cysts extracts : GMP reductase (EC 1.6.6.8) (Renart *et al.*, 1976), denylosuccinate synthetase (EC 6.3.4.4) (Faraldo *et al.*, 1983), adenylosuccinate lyase (EC .3.2.2) (Pinto *et al.*, 1983), and IMP dehydrogenase (EC 1.2.1.14) (Canales *et al.*, 1985). The ransformation of XMP to GMP is catalyzed by GMP synthetase (EC 6.3.4.1), an enzyme which equires ATP and NH_3 as co-substrates. While studying this enzyme we observed that the C-labelled XMP used as substrate was transformed into xanthosine in the presence of ATP, but ot significantly, in the absence of this nucleotide. The presence of NH_3 in the reaction mixture id not affect the rate of XMP hydrolysis. This activity is probably related to the cytosolic '-nucleotidase already described in several other tissues (Van den Berghe *et al.*, 1977 ; Itoh *et al.*, 1978 ; Worku and Newby, 1983).

Materials and methods

Plastic-backed (20 cm × 20 cm) sheets of PEI-impregnated cellulose (MN-Polygram, CEL 300 PEI/UV_{254}), manufactured by Macherey-Nagel & Company, Düren, Fed. Rep. Germany, were used. The sheets were soaked for at least 15 min in 2.0 M NaCl, rinsed in several changes of deionized water, and dried. They were then developed in deionized water and dried before use. [U-^{14}C] guanosine 5′-monophosphate (NH_4^+) was obtained from the Radiochemical Centre, Amersham, England. Nucleotides and nucleosides were obtained from Boehringer.

Artemia eggs were from Bio-Marine Research, Hawthorne, CA, USA.

Preparation of extracts

Cysts washed as previously described (Faraldo *et al.*, 1983) were disrupted in a cold mortar with 10 vol of buffer A (20 mM Tris-HCl buffer pH 7.5, 0.5 mM EDTA, 1 mM 2-mercaptoethanol). The homogenate was filtered through glass wool and centrifuged at 100 000×g for 1 h. The resulting supernatant was filtered again through glass wool.

Enzymatic assays

The hydrolytic activity was followed with two methods, using either labelled or unlabelled GMP as substrates. In the first case, the reaction mixture contained in a final volume of 50 µl the following components : 50 mM Tris-HCl buffer pH 7.5, 6 mM $MgCl_2$, 0.24 mM [U-^{14}C] GMP, enzyme and ATP as indicated. The reaction was initiated by the addition of the enzyme. After incubation at 37 °C the reaction was terminated by immersing the reaction vessels in a boiling water bath for 1 min. Subsequently they were cooled in ice and centrifuged for 1.5 min at about 8 500×g in a Beckman Microfuge B centrifuge. Aliquots of 5 µl were subjected to thin layer chromatography (TLC) on PEI-cellulose, using 0.5 M LiCl as ascendent eluant. After chromatography, the plate was monitored on a TLC linear analyzer (Model LB 283 from Labor Berthold), with the entrance window (250 mm × 15 mm) covered with thin foil 1.5 mg/cm^2 polyethylene. The count pulses were collected and stored in a multichannel analyzer. Total radioactivity of individual peaks was calculated by integrating selected regions of interest of the radioactive profile displayed at the screen of the display unit. The percentage contribution of each region to the total measured radioactivity was obtained after background substraction, and was used as a measure of the enzymatic activity. Controls with labelled GMP and guanosine were carried out in parallel. All measurements were performed with a gain of 3. One unit (U) is the amount of enzyme able to produce 1 µmol of guanosine/min in the above described conditions.

The hydrolytic activity on GMP was also measured through evaluation of the inorganic phosphate liberated (Malpartida and Serrano, 1981). The reaction mixture contained, in a final volume of 0.2 ml the following components : 50 mM Tris-HCl buffer pH 7.5, 6 mM $MgCl_2$, 3 mM ATP, GMP as indicated and enzyme. After incubation at 37 °C for 30 min, the reaction was stopped by addition of 0.85 ml of a reagent containing 0.29 N sulfuric acid, 0.4 % ammonium molybdate and 0.4 % sodium dodecylsulfate. Colour was developed by the addition of 70 µl of 1 % ascorbic acid. The absorbance at 750 nm was measured after 5 min of standing at room temperature. Controls without GMP and without ATP were run in parallel. This method allows the determination of phosphate in the presence of ATP.

Hydrolysis of p-nitrophenyl phosphate

The reaction mixture contained the following components in a final volume of 0.5 ml : 50 mM Tris-HCl buffer pH 7.5, 6 mM $MgCl_2$, 12 mM p-nitrophenyl phosphate, and enzyme. The reaction was started by the addition of enzyme, incubated for 1 h at 37 °C, stopped with 0.8 ml of 1 N NaOH and the absorbance determined at 405 nm.

Catalase was assayed as described by Martin and Ames (1961). Protein was determined by the method of Bradford (1976).

Results and discussion

As stated in the introduction, the nucleotidase activity reported here was detected using labelled XMP as substrate. This nucleotide, unavailable from commercial sources, was obtained in our laboratory by deamination of [U-^{14}C] GMP by methods previously described (Vallejo *et al.*, 1976). For obvious reasons we tested other nucleotides, and GMP proved to be a good substrate of the reaction. The experiments described below were carried out with this nucleotide.

To characterize this activity, we firstly tested a salt fractionation of the 100 000×*g* supernatant. As shown in Table I, most of the hydrolytic activity on GMP, activated by ATP, sedimented between 25-45 % saturation of $(NH_4)_2$ SO_4. In this fraction a 12-fold activation by ATP was obtained. The hydrolytic activity on GMP was linear with both time and amount of extract (Fig. 1). In this figure the effect of ATP on the hydrolysis of GMP can easily be appreciated, *i.e.* after 10 min of incubation 2.85 and 0.21 nmoles of GMP were hydrolyzed in the presence and absence of ATP, respectively. The linearity with the amount of extract was followed in the presence of 3 mM ATP. The reaction was linear up to at least 35 µg of protein. In both cases the enzymatic activity was measured after TLC chromatography of the reaction mixture and determining both the disappearance of labelled GMP and the appearance of a molecule that chromatographed in the same position as a marker of guanosine.

TABLE I

Partial purification of an ATP dependent hydrolytic activity on GMP

Step	Volume (ml)	Protein (mg)	Total activity (mU)		Specific activity (mU/mg)		Yield (%)	
			-ATP	+ATP	-ATP	+ATP	-ATP	+ATP
100 000×g supernatant	120	263	202	2 011	0.8	7.6	100	100
$(NH_4)_2SO_4$ fractionation								
0-25 %	3.0	3	4.6	34.2	1.5	11.4	2	2
25-45 %	3.8	111	153.6	1 887.0	1.4	17.0	76	94
45-60 %	4.4	89	20.4	21.4	0.2	0.2	10	1
60-80 %	5.8	34	4.6	1.4	0.13	0.04	2	1

As described in "Materials and methods", 7 g of *Artemia* cysts were used and processed. Fractionation with $(NH_4)_2SO_4$ was carried out as previously described (Faraldo *et al.*, 1983). After desalting each fraction on Sephadex G 25 M, the hydrolytic activity on [U-^{14}C] GMP (0.24 mM, 10 µCi/µmol) was assayed in the absence and presence of 3 mM ATP. Concentration of $MgCl_2$ in the reaction mixture was 6 mM. Volumes and protein were measured after desalting.

The activation constant of ATP was calculated in the presence of a fixed amount of GMP and variable amounts of ATP. An activation constant of around 2.5 mM was obtained for ATP in those experimental conditions (Fig. 2). At 3 mM ATP, the activity was around a 10-fold of that obtained in the absence of ATP.

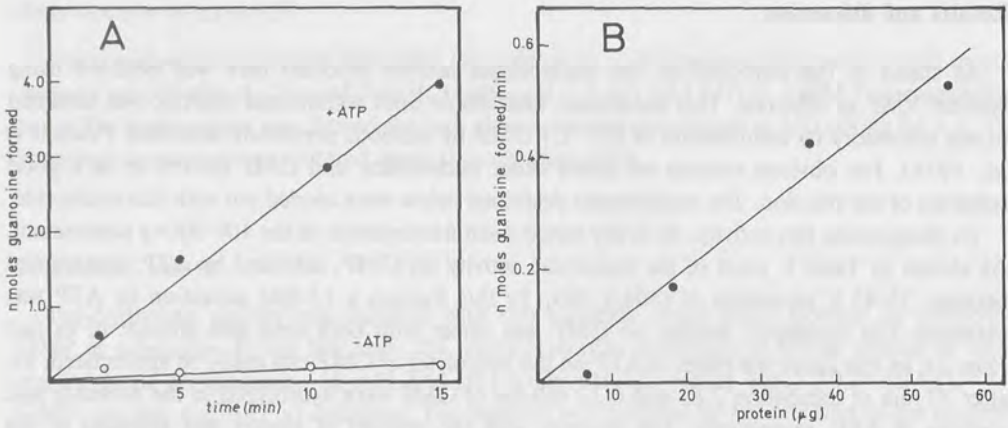

FIG. 1. Hydrolytic activity on GMP. Linearity with both time (A) and amount of extract (B). The 25-45 % ammonium sulfate fraction (Table I) was used : 18 μg of protein in (A) and as indicated in (B). In this case the incubation time was 15 min. The concentration of [U-^{14}C] GMP was 0.24 mM (10 μCi/μmol). In B, and when indicated in (A), the reaction mixtures were supplemented with 3 mM ATP. In all experiments the $MgCl_2$ concentration was 6 mM. Enzymatic activity was determined after chromatography of the reaction mixtures on PEI-cellulose as described in "Materials and methods."

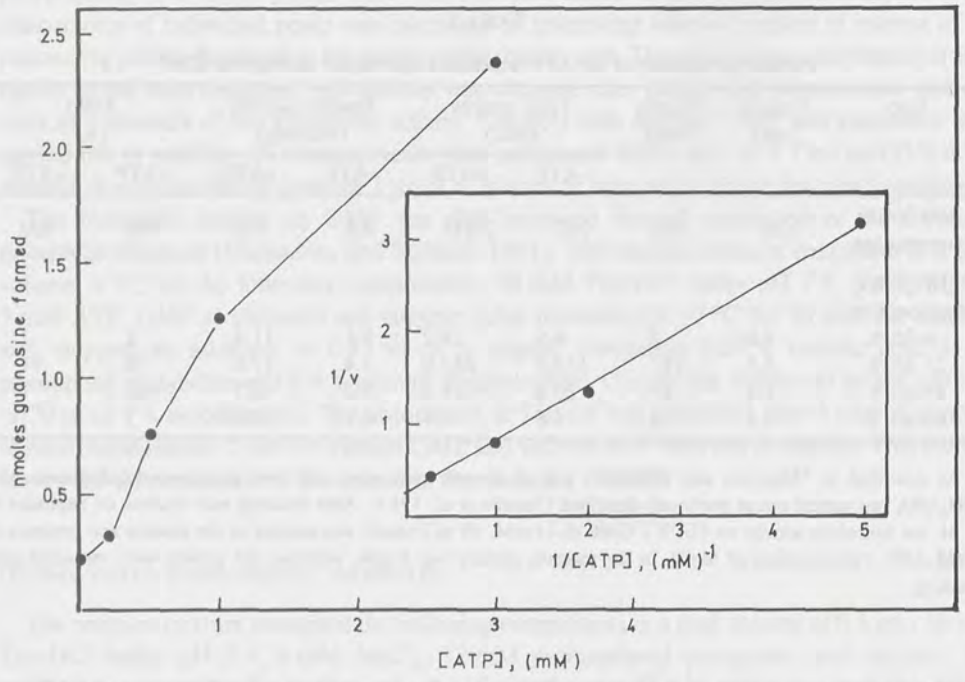

FIG. 2. Activation of the hydrolysis of GMP by ATP. The reaction mixture contained, in a final volume of 50 μl, 50 mM Tris-HCl pH 7.5, 1 μl of the 25-45 % $(NH_4)_2SO_4$ fraction (Table I), 0.4 mM [U-^{14}C] GMP (5 μCi/μmol) and ATP as indicated. $MgCl_2$ concentration was 2 mM in excess of that of ATP. In the insert it is the Lineweaver-Burk representation of the result.

The nucleotidase activity from the 25-45 % $(NH_4)_2SO_4$ fraction was further purified by sucrose gradient centrifugation (Fig. 3). The ATP stimulated hydrolysis of GMP sedimented as a peak clearly different from the profile obtained for the hydrolysis of p-nitrophenylphosphate, a substrate of other (unspecific) phosphatases. From the position of a marker (catalase), a molecular weight of around 180 000 can be assigned to this activity. Using the enzyme obtained from the sucrose gradient, the influence of substrate concentration on the reaction velocity was tested in the presence and absence of ATP. The assay was carried out measuring phosphate liberated from GMP, as described in "Materials and methods." In those conditions the *K*m values obtained for GMP were 0.4 and 10 mM, respectively. ATP did not seem to alter the Vmax of the reaction (Fig. 4). A *K*m value of 0.3 mM for GMP was also obtained in the presence of 3 mM ATP and using the radioactive method (result not shown).

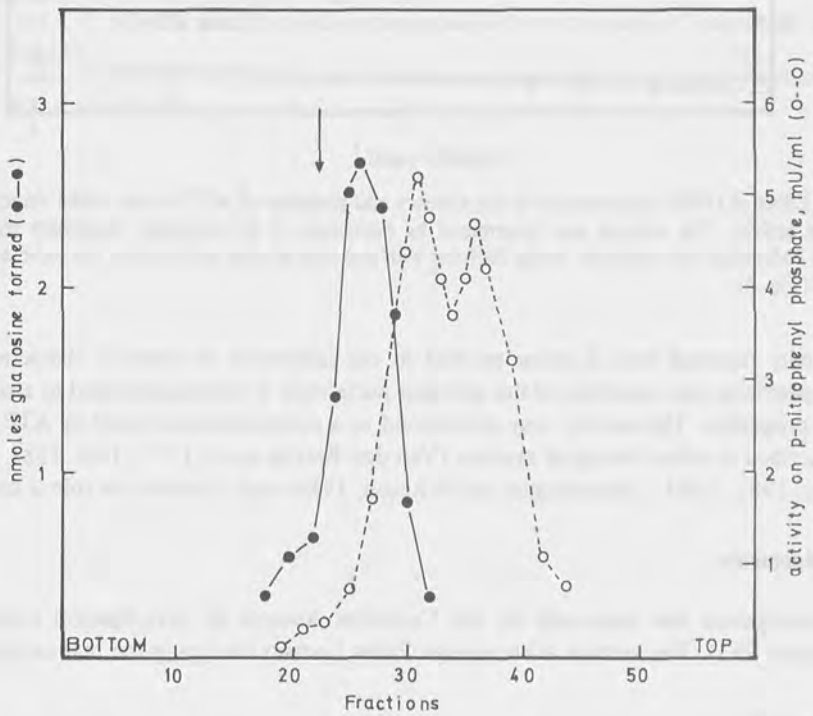

FIG. 3. Sucrose gradient centrifugation of the hydrolytic activity on GMP. 350 µl of the 25-45 % $(NH_4)_2SO_4$ fraction were layered on the top of 10 ml of a 10-30 % sucrose gradient in 20 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1 mM 2-mercaptoethanol. Centrifugation was carried out for 15.5 h at 2 °C and 39 000 rpm using a Beckman Model L5-65 centrifuge and a SW-41 rotor. Samples of nine drops (0.2 ml) were collected by suction from the bottom. Enzymatic activity was measured by the radioactive method in the presence of 0.24 mM [U-^{14}C] GMP (10 µCi/µmol) and 3 mM ATP. The activity on p-nitrophenyl-phosphate (o) was measured as described in "Materials and methods." Marker catalase sedimented in the position indicated by the arrow.

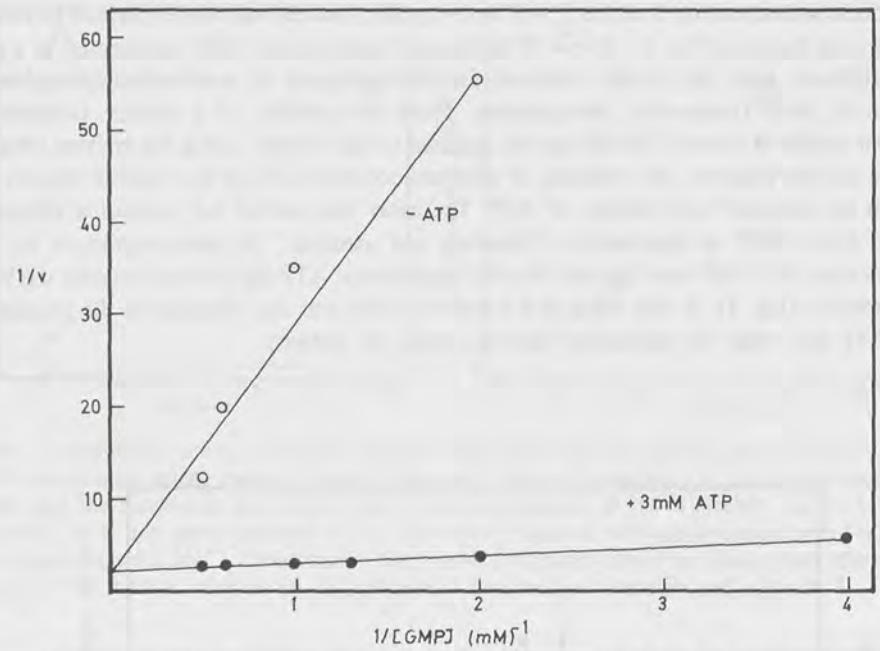

FIG. 4. Effect of GMP concentration in the absence and presence of ATP, on the initial velocity of the nucleotidase activity. The velocity was determined by evaluation of the inorganic phosphate released as described in "Materials and methods" using fractions with maximal activity pooled from the sucrose gradient experiment (Fig. 3).

The activity reported here is being purified in our laboratory in order to characterize the substrate specificity, the specificity of the activator nucleoside 5′-triphosphate and to study other molecular properties. This activity may correspond to a nucleotidase activated by ATP, similar to that described in other biological systems (Van den Berghe *et al.*, 1977 ; Itoh, 1981 ; Worku and Newby, 1982, 1983 ; Tjernshaugen and Fritzson, 1984) and of which the role is unknown.

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FIG. 4. Effect of GMP concentration in the absence and presence of ATP, on the initial velocity of the nucleotidase activity. The velocity was determined by evaluation of the inorganic phosphate released as described in "Materials and methods" using fractions with maximal activity pooled from the sucrose gradient experiment (Fig. 3).

The activity reported here is being purified in our laboratory in order to characterize the substrate specificity, the specificity of the activator nucleotide 5'-triphosphate and to study other molecular properties. This activity may correspond to a nucleotidase activated by ATP, similar to that described in other biological systems (Van den Berghe *et al.*, 1977 ; Itoh, 1981 ; Worku and Newby, 1982, 1983 ; Tjernshaugen and Fritzson, 1984) and of which the role is unknown.

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Biosynthesis of nucleotides from exogenous purines in *Artemia* nauplii

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Abstract

The metabolic fate of labelled purine bases, adenine, guanine, and hypoxanthine has been studied in *Artemia* nauplii. These bases are incorporated into acid soluble nucleotides as well as into RNA and DNA nucleotides. Higher incorporation was found in RNA. [^{14}C]-hypoxanthine enters into adenine and guanine nucleotide pools [^{14}C]-adenine enters also into both adenine and guanine nucleotides, but [^{14}C]-guanine labelling is restricted practically to guanine nucleotides. Specific activities of DNA nucleotides are significantly decreased in comparison to RNA or acid soluble nucleotides. Purine salvage enzymes, adenine phosphoribosyltransferase and hypoxanthine, guanine phosphoribosyltransferase, and adenosine kinase have been found in cytosolic extracts of *Artemia* nauplii and both phosphoribosyltransferase activities have been purified.

Introduction

Purine biosynthesis and interconversion of nucleotides in *Artemia* sp. have received considerable attention because of some unique features associated with the larval development of this crustacean.

The finding of large quantities of the unusual nucleotide diguanosine tetraphosphate (Gp4G) in the encysted embryos of *Artemia* sp. (Finamore and Warner, 1967) and its progressive disappearance through development, together with the apparent inability of *Artemia* nauplii to incorporate *de novo* purine precursors (Clegg *et al.*, 1967 ; Warner and McClean, 1968) into nucleotides and nucleic acids led to the formulation of the hypothesis that the stored Gp4G serves as a source of ATP and other required nucleotides for nucleic acids led to the formulation of the hypothesis that the stored Gp4G serves as a source of ATP and other required nucleotides for nucleic acid synthesis, mostly dATP (Finamore and Clegg, 1969) implying an important conversion from guanine to adenine compounds. For a review see Clegg and Conte (1980) or Warner (1980).

Nevertheless, until now, the precise molecular mechanism of such purine interconversion still remains to be clarified.

In this paper we report the result of the incorporation of exogenous purines into nucleotides and nucleic acids in *Artemia* nauplii as well as some properties of some purine salvage enzymes involved.

Materials and methods

Chemicals and materials

Bases, nucleosides, nucleotides, AMP-sepharose and GMP-agarose were from Sigma Chemical Co., DEAE-sephacel from Pharmacia. Other chemicals, were of the highest commercially available purity. Dry encysted embryos of *Artemia* were supplied by San Francisco Bay Brand Co. [8-^{14}C] labelled purines were obtained from Amersham.

A HPLC setup was used composed of a 6 000 A pump, a U6K injector, a 440 UV detector at a fixed wavelength of 254 nm, and a Bondapak C18 column (300 mm × 4 mm, 10 μ particle), from Waters Assoc. Another arrangement was composed of a M-45 pump and a U6K injector (Waters Association), a 2 138 Uvicord S UV detector at a fixed wavelength of 254 nm (LKB), and a Polygosil C 18 column (200 × 4 mm, 5 μ particle) from Macherey-Nagel. Each analytical column was protected by a Guard-Pak module (Waters) packed with C18 material. Chromatographic profiles were registered with a Omniscribe recorder (Houston Inst.). Spectrophotometric measurements and UV spectra were carried out in a Spectronic 2 000 (Bausch and Lomb).

Growth and incubation of nauplii.

Nauplii were obtained and incubated as described by Llorente *et al.* (1985). About 0.3 g of nauplii obtained in sterile conditions were incubated in 15 ml of a medium composed of 75 % five-fold diluted Dutrieu medium and 25 % Eagle's Minimum Essential Medium ; and fortified with 5 mM glucose, penicillin 13 U/ml and streptomycin 0.4 mg/ml. After pre-incubation at 30 °C for 30 min the desired labelled purine was added (5 μM final concentration, specific activity = 54 Ci/mol) ; incubation was maintained at 30 °C for 3 h. Then, the animals were rapidly collected by filtration, washed with cold diluted Dutrieu medium, and frozen at - 70 °C.

Sample preparation

Acid soluble fraction

Acid soluble fraction was obtained by homogenization of frozen nauplii in 0.4 N $HClO_4$, insoluble material was separated by centrifugation and acid extracts were neutralized with KOH as described in standard procedures. Part of the neutral extract was fractionated by anion-exchange chromatography on DEAE-Sephacel columns. The column was equilibrated with water and, after sample application, was eluted with water until no absorbance at 260 nm (fraction I) occurred. Nucleotides were eluted by 0.5 M $(NH_4)_2CO_3$ (fraction II). This fraction was treated with concentrated HCOOH to pH 2. This was done because in our lyophilization system ammonium formate is better eliminated than ammonium carbonate. Fractions I and II were lyophilized, the residue redissolved in water and stored frozen at - 40 °C until analysis.

Nucleic acids

Acid insoluble material was washed twice with cold ethanol, delipidated by extraction with ethanol/ether and finally extracted with hot 10 % NaCl to obtain a nucleic acid fraction as described by Tyner *et al.* (1953). RNA and DNA were precipitated by cold ethanol at 0 °C overnight, recovered by centrifugation and redissolved in water. RNA was selectively hydrolyzed by adding 0.3 M KOH and incubating at 37 °C for 20 h. Hydrolized RNA was separated from

undigested DNA by acid precipitation with HCl to pH 1-2 and centrifugation. The supernatant containing 2' and 3' RNA nucleotides was neutralized and frozen at - 40 °C. The precipitated DNA was washed twice with cold ethanol, redissolved in water, and frozen.

HPLC procedures

All eluents were degassed by vacuum filtration and samples were filtered through Millex HV_4 filters (Millipore) before injection.

Acid soluble nucleotides

Nucleotides contained in fraction II were separated and identified by reverse phase HPLC on the μBondapak C18 column isocratically eluted with 0.2 M KH_2PO_4 pH 6.0 (buffer 1), flow 1.3-1.5 ml/min. After 10 min of isocratic elution, buffer 2 (0.2 M KH_2PO_4, 10 % methanol, pH 6.0) was isocratically applied for 10 min, to elute the strongly retained materials. The column was re-equilibrated after 15 min with buffer 1 before the next injection. Under these conditions the elution order of nucleotides is : GTP, GDP, GMP, IMP, ATP, ADP, Gp4G, Gp3G, and AMP. Fractions of 20 s were collected and counted for radioactivity. For more rapid analysis fraction II was hydrolyzed in 1N HCl at 100 °C for 1 h, neutralized, and chromatographed to separate and quantify free purine bases on the Polygosil C18 column isocratically eluted with 0.2 M KH_2PO_4 pH 3.65, flow 1 ml/min. Under these conditions purine bases were eluted in the order : guanine, hypoxanthine, xanthine, and adenine. Fractions of 20s were collected and counted for radioactivity.

RNA nucleotides

The 2' and 3' nucleotides were analyzed by reverse phase HPLC on the μBondapak column isocratically eluted with buffer 1 (KH_2PO_4 0.1 M, 1 % methanol pH 4.0, flow 2.0 ml/min). After 13 min of injection, buffer 2 (KH_2PO_4 0.1 M, 10 % methanol pH 4.0) was applied to elute strongly retained 2'AMP. Nucleotides elute in the order l: 3'CMP, 2'CMP, 3'UMP, 2'UMP, 3'GMP, 3'AMP, 2'GMP, and 2'AMP. Fractions of 20s were collected and counted for radioactivity. The column was re-equilibrated after 15 min with buffer 1.

DNA bases

DNA was hydrolyzed in 98% formic acid at 180 °C for 1 h (Wyatt and Cohen, 1953) and, after hydrolysis the acid was eliminated under reduced pressure at 50 °C and the residue redissolved in the buffer used to elute the HPLC column (Polygosil C18). Separation of DNA bases was done under isocratic conditions, the eluent being 10 mM KH_2PO_4, 5 % methanol pH 4.0, flow 1.0 ml/min. Bases eluted in the order : cytosine, guanine, thymine, and adenine. For counting the radioactivity, 30s fractions were collected.

ENZYME ASSAYS

Obtention of cytosolic extracts

Nauplii were homogenized with 2 vol. of Tris- HCl 50 mM pH 7.5, 0.1 M KCl, 1 mM $MgCl_2$, and soybean trypsin inhibitor 0.2 mg/ml at 0-4 °C. The crude homogenate was centrifuged at 100 000 g for 1 h and clear supernatants were used for enzyme assays and purifications.

Adenine phosphoribosyltransferase (APRT) assay

Assay mixture contained 50 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM phosphoribosylpyrophosphate (PRPP) 50 μM[8-^{14}C]-adenine (2 Ci/mol), 20 mM NaF, and appropiate enzyme preparation in a final volume of 100 μl. Incubation was done at 37 °C for 10 min and terminated by 2 min inmersion in a boiling water bath. Estimation of formed AMP was made by retention of the nucleotide in DEAE-cellulose filters as described (Rotllán and Miras-Portugal, 1985a).

A sample from 40-80 % ammonium sulphate fractionation, previously equilibrated through a Sephadex G-25 column equilibrated with 10 mM Tris-HCl, 5 mM $MgCl_2$, 20 % glycerol, and 1 mM dithioerythritol pH 6.0 (buffer A) was applied to a GMP – agarose column (0.6 cm × 8 cm) equilibrated in buffer A. Elution was at a flow rate of 10 ml/h until no absorbance at 280 nm occurred, weakly bound proteins were eliminated by 0.2 M KCl in elution buffer. HGPRT activity was eluted by 5 mM PRPP in the elution buffer. The eluent, containing unbound protein from GMP-agarose column was applied to an AMP-sepharose column (0.6 cm × 8 cm) equilibrated in buffer A. Washing and elution of APRT activity was carried out in the same way as explained for the GMP-agarose column. Enzyme activities are expressed as cpm of nucleotides formed (AMP or GMP) by aliquots of each fraction under standard assay conditions.

A 30 μl aliquot of cytosolic extract diluted 10-fold with homogenization buffer was incubated in a reaction mixture of 300 μl final volume and, at indicated times, 50 μl samples were taken for analysis of labelled AMP.

Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) assay

Assay mixture was the same as that for APRT assay except that purine was [8-^{14}C]-hypoxanthine or [8-^{14}C]-guanine. Incubation at 37 °C for 20 min was terminated with boiling water bath. The nucleotide formed was estimated in the same way as in APRT assay.

Adenosine kinase assay

This enzyme activity was measured as described by Rotllán and Miras-Portugal (1985b). A unit of enzyme activity is the amount of enzyme that transforms 1 μmol of substrate/min under the described assay conditions. APRT and HGPRT activities were determined by correcting for the initial burst synthesis of nucleotide (see Results).

Protein determination

Proteins were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Results

Distribution of radioactivity from labelled purine bases

Radioactivity from [8-^{14}C] labelled adenine, guanine, and hypoxanthine is found in the acid soluble fraction as well as in the acid insoluble fraction incorporated into the nucleic acids. Results of *in vivo* labelling with the three before-mentioned bases are summarized in Table I. Acid soluble purines and nucleic acids are labelled and the most important incorporation of radioactivity takes place in RNA. DNA labelling is very low in comparison with RNA. Fraction-

ation of acid soluble fraction by a DEAE-Sephacel column shows that an important portion of radioactivity is found in fraction I. It contains non-phosphorylated derivatives, depending on the nature of the administered bases. The radioactivity level in fraction I increases in the order : adenine < hypoxanthine < guanine. Fraction II which contains nucleotides, reflects the inverse relationship, the resulting adenine is the best precursor of nucleotides.

TABLE I

Distribution of radioactivity from labelled purine bases in *Artemia* nauplii

		8^{14}C-Adenine	8^{14}C-Guanine	8^{14}C-Hypoxanthine
Acid soluble fraction	I	40 (70)	30 (86)	30 (82)
	II	16 (30)	5 (14)	7 (18)
Acid insoluble fraction	RNA	70 (91)	40 (90)	90 (92)
	DNA	7 (9)	5 (10)	8 (8)

Data in nmoles/g protein. Numbers ( ) indicate % radioactivity with respect to the total in each fraction.

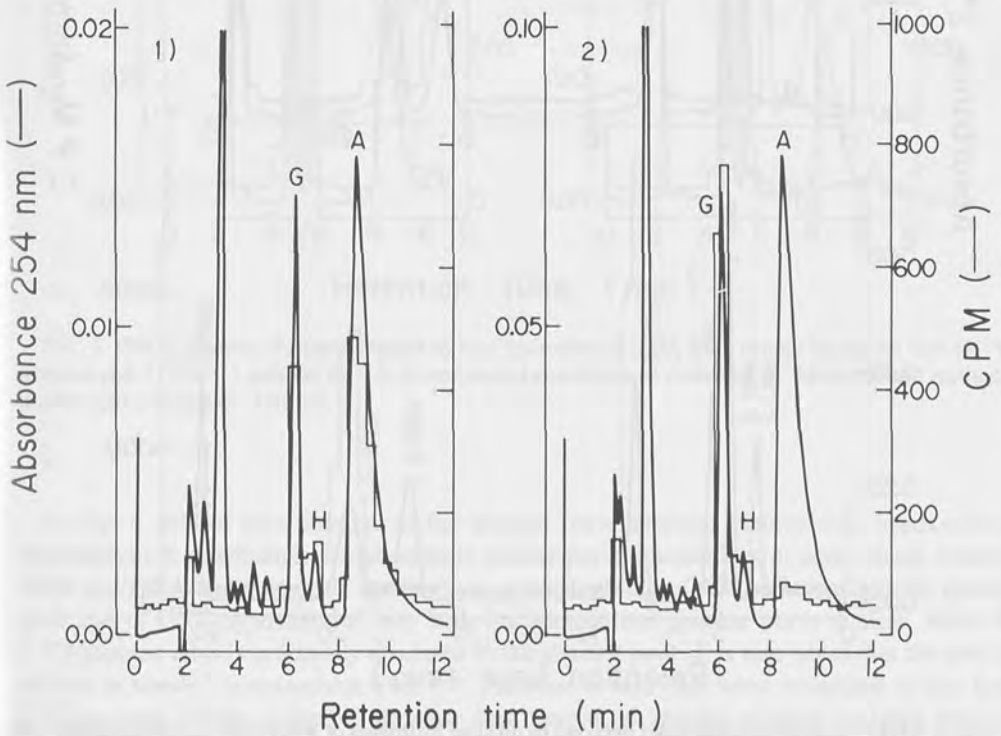

FIG. 1. HPLC analysis of purine bases released by acid hydrolysis of acid soluble nucleotides from nauplii incubated with labelled purine bases : 1) [8-^{14}C] adenine and 2) [8-^{14}C] guanine.

METABOLIC FATE OF LABELLED PURINE BASES INTO SOLUBLE NUCLEOTIDES, RNA AND DNA

Fraction II concentrated by lyophilization can be used for quantification of individual nucleotides by HPLC (see "Materials and methods"). For rapid analysis it was, however, preferred to detect the free purine bases released by acid hydrolysis of purine nucleotides. In Fig. 1 the chromatographic profiles of acid hydrolyzates of fraction II obtained from 30 h old nauplii incubated for 2 h with [^{14}C]-adenine and [^{14}C]-guanine respectively are shows. A significant labelling in guanine from [^{14}C]-adenine and a low label in adenine from [^{14}C]-guanine are evident.

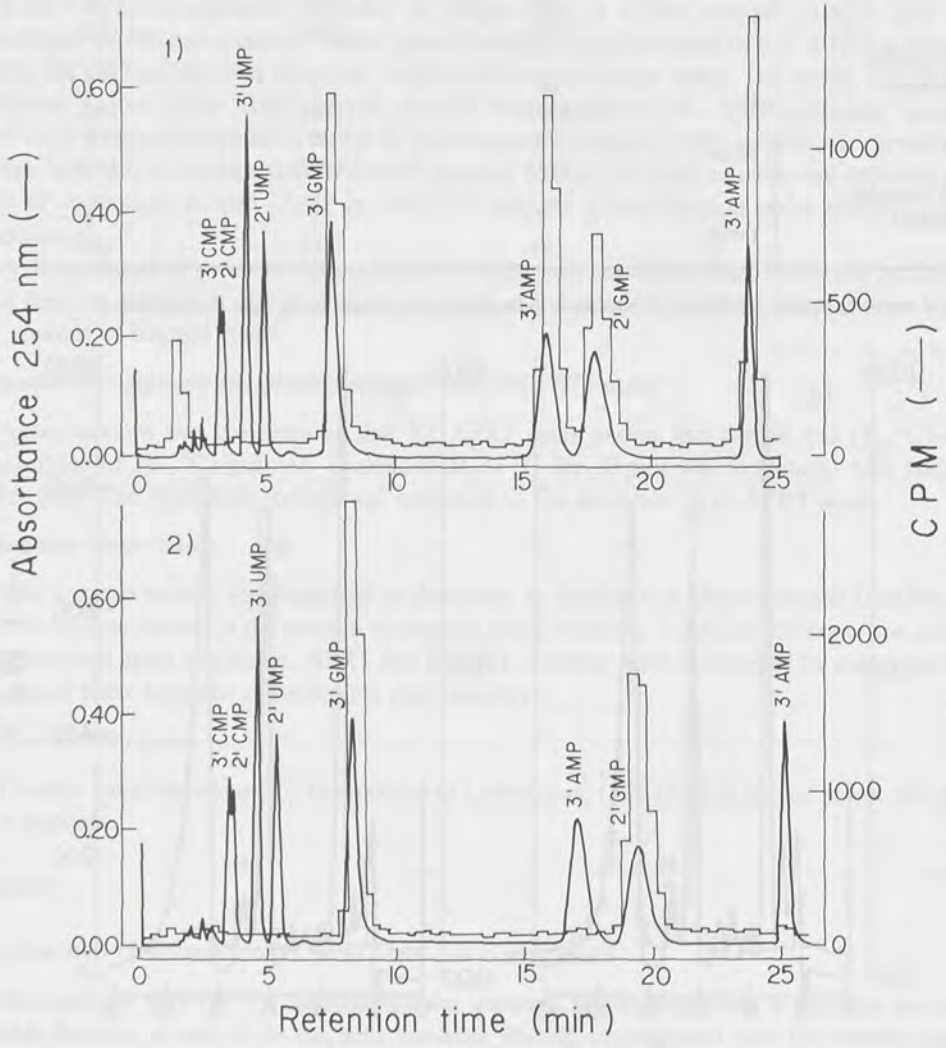

FIG. 2. HPLC analysis of nucleotides obtained by alkaline hydrolysis of RNA from nauplii incubated with 1) [8-^{14}C] adenine and 2) [8-^{14}C] guanine for 3 h. Experimental conditions as described in „Materials and methods ; growth and incubation of nauplii".

Incorporation of labelled adenine and guanine into nucleic acids is shown in Fig. 2 and 3 which represent the chromatographic profiles of 2' and 3' RNA nucleotides released by alkaline hydrolysis and DNA bases obtained by acid hydrolysis respectively. In both cases an important labelling in RNA and DNA guanine from ^{14}C-adenine and very low or even undetectable label in nucleic acid adenine from [^{14}C]-guanine are again observed.

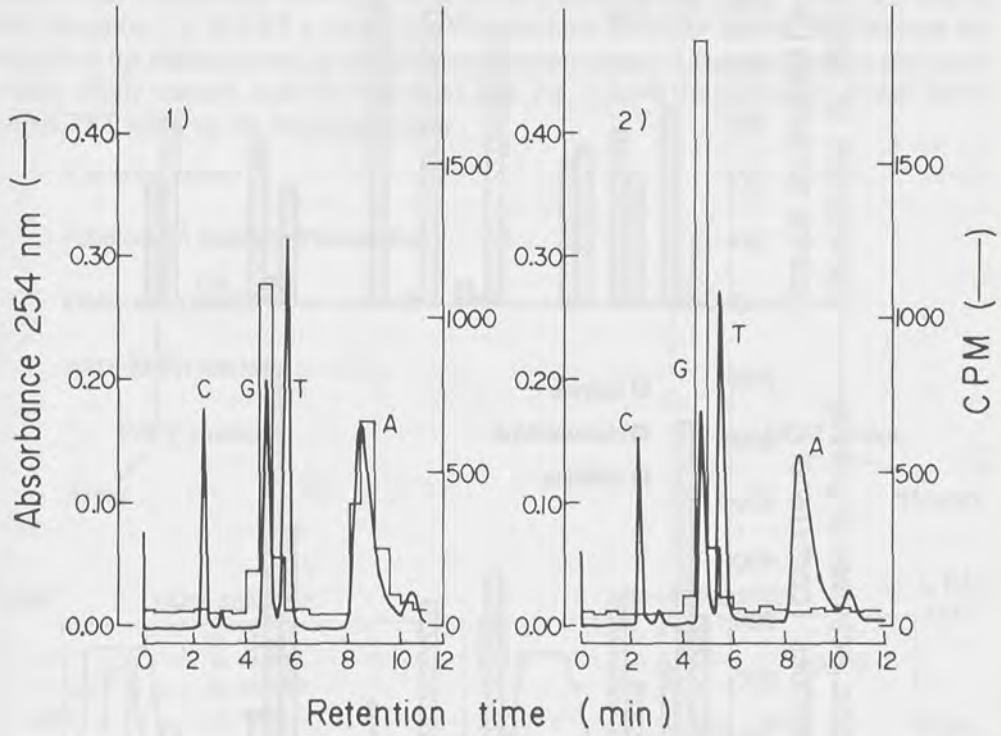

FIG. 3. HPLC analysis of bases obtained by acid hydrolysis of DNA from nauplii incubated with [8-^{14}C] adenine and 2) [8-^{14}C] guanine for 3 h. Experimental conditions as described in "Materials and methods ; growth and incubation of nauplii".

In Fig. 4 data of incorporation of the labelled bases adenine, guanine, and hypoxanthine, expressed in % distribution of radioactivity among purine nucleotides in acid soluble fraction, RNA and DNA and in specific activities are given. Entering of [^{14}C]-adenine into the guanine pool and of [^{14}C]-hypoxanthine into both the adenine and guanine pools is clear, while the [^{14}C]-guanine label is practically restricted to the guanine pool. It is also noted that the specific activity in labelled hypoxanthine from [^{14}C]-adenine is very high when compared to that from [^{14}C]-guanine. Finally a marked decrease was noticed, in specific activities of DNA bases in comparison with acid soluble or RNA nucleotides, the adenine decrease being the most evident one.

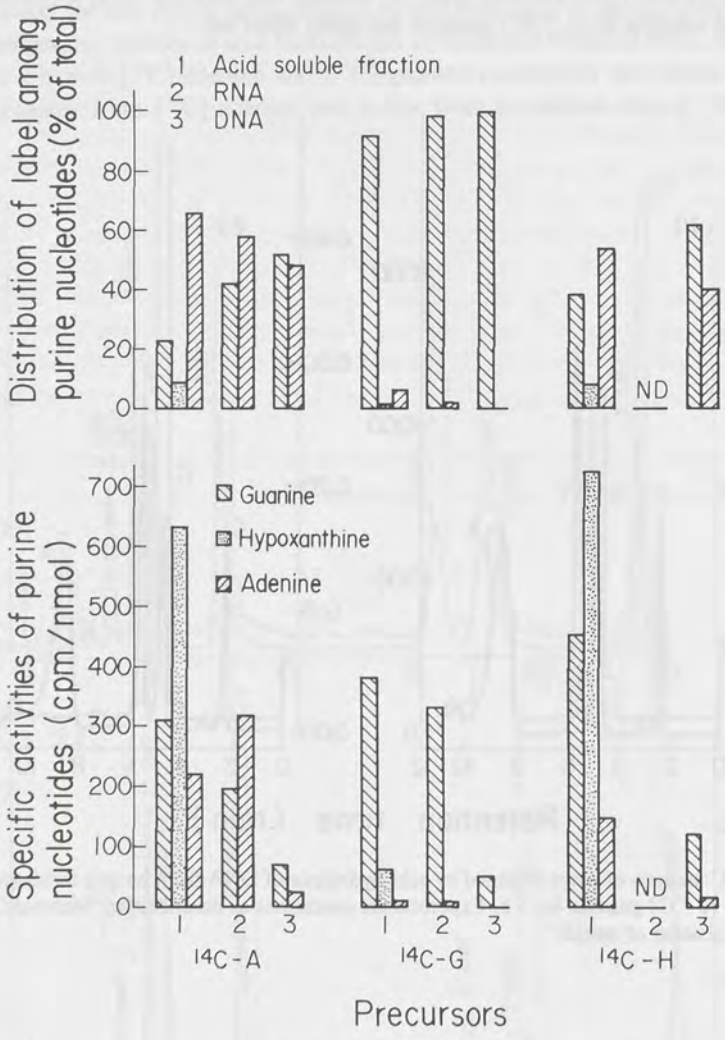

FIG. 4. Distribution of radioactivity from 8-^{14}C labelled adenine, guanine, and hypoxanthine into acid soluble, RNA and DNA purine nucleotides. At the top, distribution of radioactivity among purine nucleotides (% of total). At the bottom specific activities of purine nucleotides: ^{14}C-A, [8-^{14}C]Adenine, ^{14}C-G, [8-^{14}C] guanine, ^{14}C-H, [8-^{14}C] hypoxanthine; ND stands for not determined.

PURINE SALVAGE ENZYMES

Purine phosphoribosyltransferases and adenosine kinase activities are present in cytosolic extracts of *Artemia* nauplii. Adenine phosphoribosyltransferase (APRT) activity is around 7.0 mU/mg of protein, but the hypoxanthine-guanine phosphoribosyltransferase results are very low, about 0.5 mU/mg. Adenosine kinase has been estimated to be low, 0.1 mU/mg. The three enzymes precipitate between 40 and 80 % of ammonium sulphate saturation and attempts to achieve a major purification were done using affinity chromatography. APRT binds itself only to AMP-Sepharose but HGPRT is bound to AMP-Sepharose and GMP-agarose. The enzymes are eluted from the affinity column by phosphoribosylpyrophosphate. Adenosine kinase is not bound to these affinity supports under the conditions used. Fig. 5 shows the purification of both APRT and HGPRT based on the following scheme :

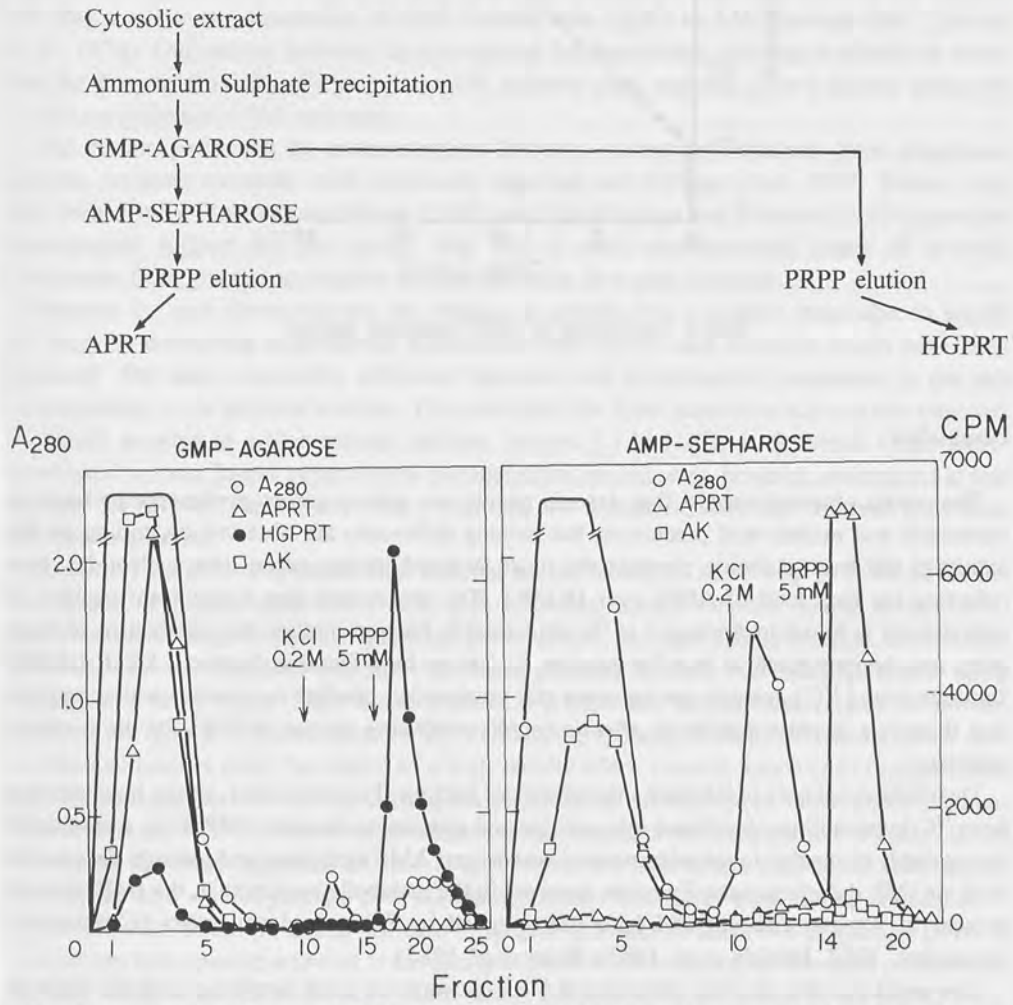

FIG. 5. Sequential purification of APRT and HGPRT activities from *Artemia* nauplii by affinity chromatography.

Adenosine kinase activity is recovered in the unbound protein fraction of the AMP-sepharose column. Both purine phosphoribosyltransferases exhibit an initial burst synthesis of nucleotide which is completed in the first minute of reaction even at 0 °C, after burst, formation of product becomes linear with respect to time (Fig. 6).

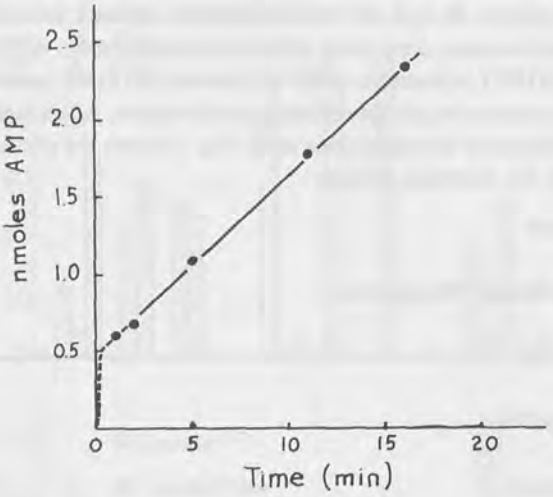

FIG. 6. Time course of APRT catalyzed reaction.

Discussion

The results obtained suggest that *Artemia* nauplii are able to use exogenous purine bases as nucleotide and nucleic acid precursors, but striking differences are observed depending on the nature of the base. Adenine presents the most favoured incorporation into nucleotides, thus reflecting the high level of APRT over HGPRT. The observation that a significant quantity of radioactivity is found in fraction I of the acid soluble fraction implies that catabolism of these bases may be important, at least for guanine. In fact we have found in fraction I highly labelled xanthine from [^{14}C]-guanine among other still unidentified labelled metabolites which suggests that there is a guanase activity in *Artemia* nauplii competing against HGPRT for the common substrate.

Distribution analysis of labelling into purines of fraction II (nucleotides), shows how labelling from ^{14}C-hypoxanthine distributes into adenine and guanine nucleotides, IMP is not accumulated but probably channeled to the adenine pool via succinyl AMP synthetase and towards the guanine pool via IMP dehydrogenase. Enzymes involved in the metabolic branching at the IMP level are present in *Artemia* embryos and have been studied by Sillero and coworkers (Canales and Fernández, 1982, Faraldo *et al.* 1983 ; Pinto *et al.* 1983).

The metabolic fate of [^{14}C]-adenine and [^{14}C]-guanine are most surprising. Indeed, while an important labelling in guanine nucleotides from labelled adenine is detected, the conversion of [^{14}C]-guanine into adenine is much less evident.

The fact that specific activity of IMP of nauplii incubated with [^{14}C]-adenine is similar to that obtained when nauplii were incubated with [^{14}C]-hypoxanthine and about ten times higher than IMP specific activity in nauplii labelled with [^{14}C]-guanine, points to the fact that the metabolic flux from adenine to guanine could operate via AMP deaminase. This also suggests a severe limitation in the conversion from guanine to adenine nucleotides. This is supported, by the observations of Hernandorena and Kaushik (1983) who have shown that ammonium excretion by *Artemia*, when growing in the presence of adenylic purines, is clearly higher than when growing with guanylic purines. Therefore, work in progress is now directed towards the study of AMP deaminase from *Artemia* nauplii.

The presence of GMO reductase, a key enzyme involved in the conversion of guanine to adenine nucleotides in cells, has been reported in *Artemia* embryos (Renart and Sillero, 1974). Their kinetic properties, *in vitro* have been extensively studied. It was suggested that the enzyme can play a role in the channeling of GMP coming from Gp4G to AMP through IMP, (Renart *et al.*, 1976). Our results, however, do not support this hypothesis, although it should be noted that the enzyme studied is the one present in *Artemia* cysts, and that little is known about the *in vivo* regulation of GMP reductase.

Our results pertaining to interconversion between nucleotides obtained from exogenous purines, contrast markedly with previously reported one (Clegg *et al.* 1967, Warner and McClean, 1968). Finamore and Glegg (1969) and Van Dembos and Finamore (1974) gave the experimental support for the current idea that at early developmental stages of *Artemia*, conversion from guanine to adenine nucleotides must be highly favoured.

Reasons for such divergence are not obvious at present, but it appears reasonable to search for them by comparing experimental conditions under which such opposite results have been obtained. The most remarkable difference between both experimental procedures is the salt concentration in the external medium. The noticeable flux from guanine to adenine was observed in nauplii growing in a high salinity medium, around 1.3 M NaCl, as described in the aforementioned reports. In our experimental manipulations nauplii were, however, maintained at low salinity, 0.1 M NaCl, and under these conditions the interconversion flux observed goes from adenine to guanine.

From this point it is apparent that external salinity could in some way be involved in the modulation of internal metabolic events, such as purine interconversions. Ewing *et al.* (1980) have presented strong evidence in this sense showing how, in a high salinity medium, intracellular pools of UTP are decreased and RNA synthesis seriously limited, with maximal effects being reached at 1 M or higher NaCl concentrations. Furthermore, similar observations on protein synthesis are reported (Conte *et al.*, 1973). Perhaps the apparent inability of *Artemia* for *de novo* synthesis of purines could be related to a high salinity effect, because appreciable incorporation of *de novo* precursors into nucleotides and nucleic acids can be detected in a low salinity medium (Llorente *et al.*, 1987). Whether external salinity can modulate the mobilization of Gp4G is not known but has been suggested (Conte *et al.*, 1980). We have found indications that, at low salinity, the acid soluble guanine pool falls more rapidly than under high salinity conditions.

We, however, found a marked decrease in specific activities of adenine and guanine DNA in comparison with specific activities in the acid soluble or RNA nucleotides. The most pronounced decrease being observed in adenine. These results indicate the contribution of the Gp4G pool, not or poorly labelled to DNA adenine and guanine, and agrees well with the observations of Finamore and Clegg (1969). These authors proposed Gp4G to play a key role as a source of

required dATP for the synthesis of DNA. It is, however, worth noting that in spite of the strong experimental support for the hypothesis that Gp4G serves as a source of DNA purines, details of the mechanism involved in such conversion are still unknown.

The incorporation of free bases into nucleotides in cells is accomplished by the action of purine phosphoribosyltransferases. From our results, it is evident that *Artemia* has, at least two of such enzymatic activities : one for adenine (APRT) and the other for hypoxanthine and guanine (HGPRT). Whether there is another enzyme for xanthine (Tutle and Krenitsky, 1980) or if the same enzyme that uses hypoxanthine and guanine could also exist (Wang and Simashkevich, 1981) has not been investigated.

As described for other phosphoribosyltransferases by Thomas *et al.* (1973), Kenimer *et al.* (1975) and Rotllán and Miras-Portugal (1985a), *Artemia* enzymes exhibit a rapid initial burst synthesis of nucleotide before reaching a steady state phase. Despite efforts made to clarify the mechanism for such behaviour (Kenimer *et al.* 1975), no clear explanation has been given.

The nucleoside kinase specific for adenosine, adenosine kinase, is also present in cytosolic extracts of *Artemia*. It is assumed that it is used in the salvage of adenosine, although incorporation of adenosine into nucleotides through alternative pathways (nucleosidases, phosphorylases or deaminases) cannot be excluded. The presence of adenosine deaminase isozymes in *Artemia* and induction of one of them in early development has recently been reported (Fernández *et al.*, 1984). This kinase activity appears to be low compared to APRT or HGPRT. The enzyme is not bound to any of the affinity gels used in this study, contrasting with the behaviour of adenosine kinases found in mammals which are retained in AMP-sepharose (Rottlán and Miras-Portugal, 1985b).

Acknowledgements

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Interconversion of purine nucleotides in *Artemia* : a review

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Content

Introduction

Artemia presents two special peculiarities concerning the metabolism of purine nucleotides : it is apparently unable to synthesize *de novo* the purine ring but stores diguanosine tetraphosphate (Gp_4G) during a certain period of its life cycle. The commercially available encysted grastulae contain 5 µmol of Gp_4G per 100 000 animals ; considering a content of 250 000 cysts per g, the concentration of Gp_4G is around 12 mM at this stage (Finamore and Clegg, 1969 ; Vallejo *et al.*, 1974 ; Gilmour and Warner, 1978). This figure represents 1-2 % of the cysts dry weight and about half their content in purine nucleotides (Gilmour and Warner, 1978). The level of Gp_4G decreases to 25 % of the original value after 3 days of development to larva (Finamore and Clegg, 1969 ; Sillero and Ochoa, 1971). In male adult animals, this nucleotide is almost undetectable (Warner and McClean, 1968). The situation is different in the females. They start to accumulate Gp_4G, exclusively, in the developing ovarian eggs, for a span of 3-4 days (Warner and McClean, 1968). Studies are lacking concerning in which of the different ovarian cell types Gp_4G is synthesized (Lochhead and Lochhead, 1967 ; Iwasaki, 1973). The mature oocyte

migrates to the ovisac where fertilization seems to happen. After development to gastrula, the emerging cyst contains Gp_4G at the concentration above stated, and the female becomes depleted of this nucleotide.

Besides Gp_4G, other dinucleoside polyphosphates such as diguanosine triphosphate (Gp_3G) (Warner and Finamore, 1965a), diguanosine diphosphate (Gp_2G) (Gilmour and Warner, 1978), diadenosine tetraphosphate (Ap_4A) (McLennan and Prescott, 1984) and guanosine 5'-triphosphate 5'-adenosine (Gp_3A) (Gilmour and Warner, 1978), are also present in *Artemia* cysts. Very little is known on the metabolism and function of these nucleotides and hence they will be only mentioned here. A complete review (Clegg and Conte, 1980) on cellular and developmental biology of *Artemia*, covering also significant aspects of nucleotide metabolism, and another more specific one on dinucleoside polyphosphates (Warner, 1980) were presented at the First International Symposium on *Artemia*.

Here we shall refer to recent advances on the enzymes more directly involved in purine interconversion and to the problems still pending on Gp_4G metabolism. To situate *Artemia* in a wider perspective, references to purine nucleotide metabolism in other biological systems will also be made.

Biosynthesis de novo of purine nucleotides

Work from several laboratories indicates that *Artemia* is incapable of synthesizing purines *de novo* (Clegg *et al.*, 1967 ; Warner and McClean, 1968). After incubation of nauplii during 4 h with either [^{14}C]-labeled bicarbonate, formate or glycine, both separately or in combination, radioactivity was recovered in acid soluble and nucleic acid pyrimidines, but not in the purines of the same fractions (Clegg *et al.*, 1967). Strangely, neither female *Artemia* carrying oviduct eggs incorporated [^{14}C]-labeled bicarbonate into acid soluble purine nucleotides after 21 h of incubation, just in a period when they were actively synthesizing Gp_4G (Warner and McClean, 1968). Similar results were obtained with both nauplii and mature male *Artemia*. On the other hand, when nauplii and either mature or inmature females were challenged with [^{3}H]guanosine, in the same conditions as above, synthesis of labeled Gp_4G and other guanine and adenine nucleotides was observed. Curiously, mature male and young female *Artemia*, contrary to nauplii, did not synthesize labeled Gp_4G from [^{3}H]guanosine (Warner and McClean, 1968).

The above experimental facts led to the conclusion that *Artemia* required preformed purines to carry out nucleotide and nucleic acid synthesis during their life cycle. In this line, the experiments of Hernandorena (Hernandorena, 1972, 1975 ; Hernandorena and Kaushik, 1983) clearly point to the strict requirement of purines in the medium for the correct growth of *Artemia*.

On the contrary, and concerning the question of whether *Artemia* is able to synthesize purines *de novo*, Liras and Llorente (1982) and Llorente *et al.* (1984) incubated nauplii with [U-^{14}C]glycine and isolated labeled adenine and guanine nucleotides both from the pool of free nucleotides and from RNA lysates. These experiments are in contradiction with the previously reported findings (Clegg *et al.*, 1967 ; Warner and McClean, 1968). At present the reason for the discrepancy is not clear to the reviewers although the use of different strains of *Artemia* and the application of more sensitive methods of isolation of nucleotides (HPLC) could nevertheless be mentioned as important experimental differences. Llorente *et al.* (1984) also mentioned the occurrence in nauplii extracts of P-ribosyl-PP-amido transferase, the first enzyme of the *de novo* pathway of purine nucleotides.

Other biological systems are unable to synthesize purines *de novo*. This parameter is usually measured either through evaluation of the incorporation of labeled glycine and/or formate into purine nucleotides or by measuring the dependence of preformed purines for growth. The absence of purine biosynthesis *de novo* has been clearly established in mature erythrocytes from rabbit (Lowy and Williams, 1960 ; Lowy *et al.*, 1961), mouse (Fontenelle and Henderson, 1969) and human (Lowy *et al.*, 1962). In these three cases, incorporation of labeled formate into purine nucleotides takes place if the medium is supplemented with the precursor 5-amino-4-imidazole carboxamide, showing that the erythrocytes possess the two last enzymes of the purine biosynthetic pathway. In addition human, but not rabbit and mouse, erythrocytes do not synthesize AMP from IMP due to the lack of adenylosuccinate synthetase or adenylosuccinate lyase, or both (Williams, 1962).

Human leukocytes (Scott, 1962, Williams, 1962) and blood platelets (Holmsen and Rozenberg, 1968) are also defective in this metabolic route. It is significant that whereas leukocytes from chronic granulocytic leukemia (Williams, 1962), polycythemia vera, chronic lymphocytic, and chronic myelocytic leukemias do not incorporate [^{14}C] formate into adenine or guanine, leukocytes from acute myeloblastic leukemia do (Scott, 1962). This is an old observation that presumably deserves more attention. Again in all types of leukocytes the addition of 5-amino-4-imidazole carboxamide improves the incorporation of labeled glycine into purine nucleotides. Minimal alterations in the *in vitro* growth conditions of animal cells tend to change the rate of the *de novo* pathway. The addition of glucose or uridine, increases the rate of purine biosynthesis *in vitro* under aerobic conditions (Thomson *et al.*, 1960), whereas the presence of adenine stops the incorporation of [^{14}C]glycine into purine nucleotides (McFall and Magasanik, 1960). These authors made the observation that mouse fibrocyte strain L cells, which had been cultivated in the presence of adenine or guanosine for several generations, suppressed the synthesis of purines *de novo* and derived their purines from the exogenous compounds, whereas cells which had been grown in the absence of purines continued to synthesize adenine and guanine *de novo* for at least 24 h, when either adenine or guanosine were added to the medium (McFall and Magasanik, 1960). They suggested that adenine or guanosine in the growth medium might inhibit endogenous synthesis by repressing the formation of an enzyme essential for purine biosynthesis.

Non hepatic tissues other than placenta appear capable of only limited synthesis of purines *de novo* (Wyngaarden and Kelley, 1978) and rely on the circulating purines produced by the liver. The rabbit bone marrow cells are a good example of that. Rabbits injected intravenously with [^{14}C]-formate incorporate radioactivity into the adenine of those cells, in normal, but not in hepatectomized animals (Lajtha and Vane, 1958). The absence of the *de novo* pathway in this pluripotent cell line is rather surprising. The possibility that enzymes of this pathway are repressed by the circulating purines has not been explored.

Finally some trypanosomid parasites (Kidder and Dutta, 1958 ; Marr *et al.*, 1978) require the addition of preformed purines for growth.

This pathway is in need of a systematic search for all the enzymes, and/or intermediary substrates, involved in the formation of the purine ring in chosen biological model systems, both able or unable to synthesize purines *de novo*. The question of the repression or inhibition of some of these enzymes by preformed purines (McFall and Magasanik, 1960) deserves more attention. For the above reasons, *Artemia* could be one of those models. This would be a painstaking, although worthy investigation.

Synthesis of diguanosine tetraphosphate. GTP : GTP guanylyltransferase

Once GMP is synthesized, its transformation into GTP is possible through the action in sequence of nucleoside mono and diphosphate kinases. As shown below, both enzymes are present in *Artemia* extracts. An enzyme activity, named GTP : GTP guanylyltransferase or Gp_4G synthetase, has been described in *Artemia* (Warner and Huang, 1974 ; Warner *et al.*, 1974 ; Warner, 1979, 1980). This synthetase, purified from a yolk platelet enriched fraction, utilizes GTP as substrate yielding Gp_4G and PP_i as products, has a pH optimum of around 6 and requires Mg^{2+} and dithiothreitol for optimal activity. The Michaelis constant values for GTP, Gp_4G, and PPi are 2.2, 1.1, and 0.8 mM, respectively. The enzyme also catalyzes, at a lower rate (8 %), the synthesis of Gp_3G and GTP from equivalent amounts of GDP and Gp_4G. It is located in the yolk platelets (80 %) and in the mitochondrial fraction. An estimated molecular weight of 490 000 was obtained by exclusion chromatography on Sepharose 6B. In summary, Warner's group has reported the following two reactions, both reversible, catalyzed by this enzyme, although the possibility that two different enzymes are involved in the synthesis of these nucleotides has not been ruled out (Warner and Huang, 1974). (As shown below, the letters on the arrows indicating a reaction, refer to the enzyme noted with the same letter in Fig. 1.

1) $2\ GTP \overset{u}{\rightleftharpoons} Gp_4G + PPi$

2) $GDP + Gp_4G \overset{u}{\rightleftharpoons} Gp_3G + GTP$

This interesting enzyme deserves further work. In the meantime, some speculations can be forwarded. Data on the maximum velocity of this enzyme per g of cysts, are not explicitly stated. Warner and Huang (1974) reported that 383 mg of protein from the 700 × *g* sediment contain 241 mU. In our experience, that amount of protein is present in the 700 × *g* precipitate obtained from $4.2\ 10^5$ embryos or around 2 g of dry cysts. From that, a maximum velocity of around 150 mU/g is calculated for the synthetase. The corresponding figure for the Gp_4G hydrolase is about half, 80 mU/g. Although the actual velocities of both enzymes inside the cell are not known, the presence in cysts of two enzymes with potential antagonic effects on Gp_4G is motivating. However, the possibility that the physiological effect of the synthetase during development to larva is to yield GTP from Gp_4G cannot be disregarded (Warner and Huang, 1974). In addition Warner (1980) proposes a complex cycle in which Gp_4G is both synthesized and hydrolyzed, by the synthetase, simultaneously with the entry of GDP and GTP into, and the exit of Gp_3G from, yolk platelets. Although with the data available, it seems difficult to us to visualize how the exit and entry of those molecules into yolk platelets can take place, the possibility that the enzyme is bound to the outer part of the yolk particle can be raised as another alternative for that proposed cycle to be functional. It would also be interesting to follow the fate of the synthetase after incubation of the cysts for a few days, when most of the yolk platelets have been degraded and/or processed, to see whether the enzyme is degraded or liberated to the cytosol.

Diadenosine tetraphosphate, also present in *Artemia* cysts (McLennan and Prescott, 1984), could be synthesized by this enzyme, by the aminoacyl s-RNA synthetases (Zamecnik *et al.*, 1966 ; Plateau *et al.*, 1981 ; Blanquet *et al.*, 1983 ; Wahab and Yang, 1985) or by other enzyme systems. Related to that it would be worthy to ascertain whether ATP is also substrate of the Gp_4G synthetase.

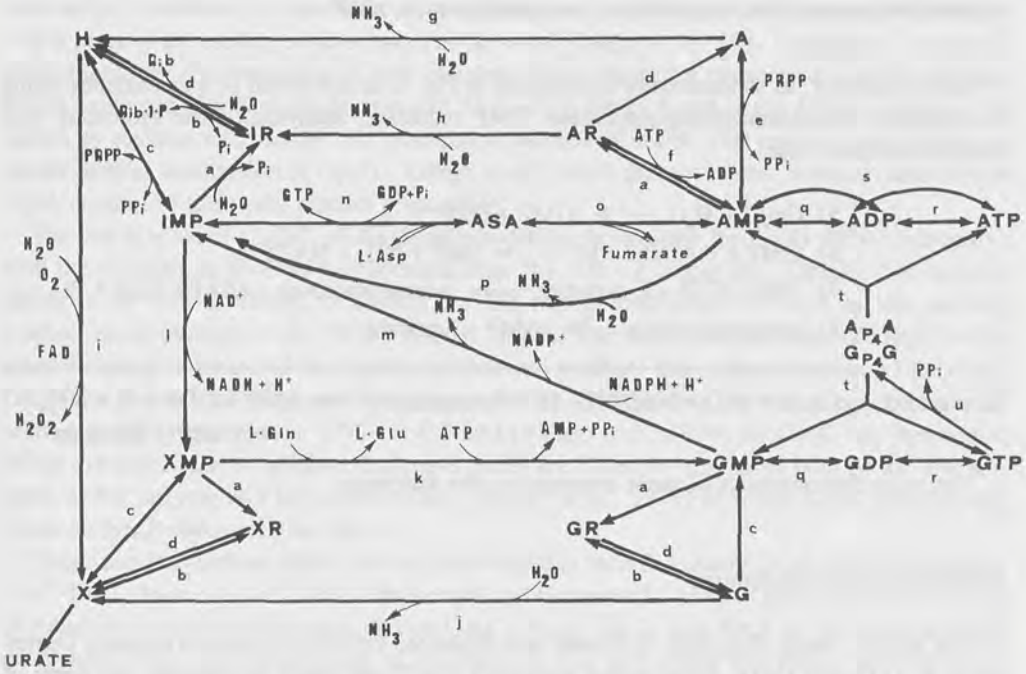


FIG. 1. Interconversion of purine nucleotides. The letters on the figure refer to the following enzymes. a. 5'-nucleotidase EC 3.1.3.5 ; b. Purine-nucleoside phosphorylase EC 2.4.2.1 ; c. Hypoxanthine phosphoribosyltransferase EC 2.4.2.8 ; d. Nucleosidase EC 3.2.2.1 ; e. Adenine phosphoribosyltransferase EC 2.4.2.7 ; f. Adenosine kinase EC 2.7.1.20 ; g. Adenine deaminase EC 3.5.4.2 ; h. Adenosine deaminase EC 3.5.4.4 ; i. Xanthine oxidase EC 1.2.3.2 ; j. Guanine deaminase EC 3.5.4.3 ; k. GMP synthetase EC 6.3.4.1 ; l. IMP dehydrogenase EC 1.2.1.14 ; m. GMP reductase EC 1.6.6.8 ; n. Adenylosuccinate synthetase EC 6.3.3.4 ; o. Adenylosuccinate lyase EC 4.3.2.2 ; p. AMP deaminase EC 3.5.4.6 ; q. Nucleosidemonophosphate kinase EC 2.7.4.4 ; r. Nucleosidediphosphate kinase EC 2.7.4.6 ; s. Adenylate kinase EC 2.7.4.3 ; t. Diguanosinetetraphosphatase EC 3.6.1.17 ; u. Guanosinetriphosphate guanylyltransferase EC 2.7.7.45.

A different guanylyltransferase, which catalyzes the capping reaction of newly synthesized mRNA, has also been described in *Artemia* (Yagi *et al.*, 1984). This enzyme consists of a single polypeptide chain of 73 kDa, with an associated RNA triphosphatase activity which removes the γ-phosphoryl group of the nascent RNA, before the capping reaction occurs.

3) pppN- $\xrightarrow{\text{RNA triphosphatase}}$ ppN- + Pi

4) pppG + ppN- $\xrightarrow{\text{guanylyltransferase}}$ GpppN- + PPi

Similar guanylyltransferases have been described in other systems (Shuman *et al.*, 1980 ; Venkatesan *et al.*, 1980 ; Smith and Furuichi, 1982 ; Wang and Shatkin, 1984). The enzyme forms a nucleotidyl intermediate by phosphoamide linkage of GMP. The covalently bound GMP can also be transferred *in vitro* to different nucleotides, *i.e.* ATP, ADP, GTP, UDP yielding dinucleoside polyphosphates such as Ap_4G, Ap_3G, Gp_4G, or Up_3G, respectively (Wang and Shatkin, 1984).

A possible pathway from diguanosine tetraphosphate to AMP

Such a pathway, as schematically represented in Fig. 1, is composed of four enzymes acting in sequence : dinucleosidetetraphosphatase, GMP reductase, adenylosuccinate synthetase, and adenylosuccinate lyase.

5) $Gp_4G + H_2O \xrightarrow{t} GMP + GTP$

6) $GMP + NADPH + H^+ \xrightarrow{m} IMP + NH_3 + NADP^+$

7) $IMP + GTP + \text{L-aspartate} \overset{n}{\rightleftharpoons} \text{adenylosuccinate (ASA)} + GDP + Pi$

8) $\text{adenylosuccinate} \overset{o}{\rightleftharpoons} AMP + \text{fumarate}$

Sum of 5-8 $Gp_4G + H_2O + NADPH + H^+ + \text{L-aspartate} \longrightarrow AMP + GDP + Pi + NH_3 + NADP^+ + \text{fumarate}$

The main characteristics of these enzymes are the following.

Dinucleosidetetraphosphatase

This activity, firstly described by Warner and Finamore (1965b) in *Artemia* extracts, cleaves Gp_4G to GTP and GMP. Since at that time, only Gp_4G was tested as substrate, the name of diguanosinetetraphosphatase was adopted for this enzyme. Later, the name of dinucleosidetetraphosphatase was proposed, as the enzyme cleaves this type of nucleotides, irrespective of the nature of the nucleotide base (Vallejo *et al.*, 1974, 1976). The enzyme (cytosolic) and the substrate have different subcellular distribution. It is well established that Gp_4G is present in the 700 × *g* precipitate of *Artemia* extracts (Sillero and Ochoa, 1971 ; Warner *et al.*, 1972) composed mainly of nuclei and yolk platelets (Warner *et al.*, 1972). In our view, the exact localization of this nucleotide is still unsolved. Paradoxically, the separation of these two particles proved a difficult task. After three years of unpublished experimental work (1972-1974) we arrive, among others, to the conclusion that one reason for that difficulty was that once the 700 × *g* precipitate is resuspended, clusters of nuclei and yolk platelets are easily formed. Both the fragility of nuclei and the presence of Gp_4G play a role in that process, this last point being easily demonstrated by *in vitro* experiments (unpubl. observ.). From these clusters it is troublesome to separate nuclei and yolk platelets by differential centrifugation. Actually we tried, unsuccessfully, a variety of such methods. We have also reproduced the experimental procedures developed by Warner *et al.* (1972), but measuring DNA in all the intermediate purification stages. In their aqueous method, the purified yolk platelets obtained after the second sucrose gradient (Table I in Warner *et al.*, 1972) contained, in our hands, 32, 37, and 33 % of the total yolk platelets, DNA and Gp_4G, respectively, present in the 700 × *g* precipitate. One is faced with the disjunctive of admitting that either yolk platelets actually contain at least 37% of the total DNA content of the cysts or that the preparation of yolk platelets is strongly contaminated by nuclei remnants. We are more in favor of the second possibility. Based on these grounds it is for us uncertain to ascribe a nuclear, as originally postulated by Sillero and Ochoa (1971), or yolk platelets localization (Warner *et al.*, 1972) for this nucleotide. The existence of DNA in yolk platelets, at lower amounts, can

however be considered, in view of the results of Marco *et al.* (1981), pointing to the yolk platelets as the place of processing of mitochondrial particles. Vallejo *et al.* (1981) developed a method, using Ficoll, for the separation of yolk platelets. They obtained a preparation of yolk granules which contained 100 % of the protein and 75 % of the Gp_4G found in the 500 × *g* fraction. As shown by electron microscopy, this preparation was free of nuclei. This result strongly points to a yolk platelet localization of Gp_4G ; Vallejo *et al.* (1981) did not report, however, data on the DNA content of their yolk platelet preparation.

The low K*m* value (2 μM) of the dinucleosidetetraphosphatase for Gp_4G allows interaction with the substrate as soon as it is liberated from the 700 × *g* precipitate. Actually, this enzyme seems to be well in excess to account for the rate of hydrolysis of Gp_4G *in vivo*, around 2 nmol/min/g (Vallejo *et al.*, 1974 ; Warner, 1980). The exact concentration of Gp_4G in the cytosol cannot be measured by classical biochemical methods then, after disruption of the cysts, Gp_4G can be artificially liberated from the precipitate. Disregarding this fact, a concentration of around 1 mM (Warner *et al.*, 1972) or 0.2 mM (Vallejo *et al.*, 1974) for Gp_4G has been found in the cytosol. It can be inferred that these values are lower *in vivo*, based both in the low K*m* value of the enzyme and the concentration (Renart *et al.*, 1976) at which Gp_4G activates the cytosolic GMP reductase (see below).

Dinucleosidetetraphosphatase is widely distributed in nature (Lobatón *et al.*, 1975 ; Cameselle *et al.*, 1982 ; Moreno *et al.*, 1982 ; Jabubowski and Guranowski, 1983 ; Ogilvie and Antl, 1983). The enzyme from several sources cleaves Gp_4G, Ap_4A, Up_4U, and Xp_4X to the corresponding nucleoside mono and triphosphates, at the same rate and with similar K*m* values, and is strongly inhibited (K*i* = 10 - 100 nM) by nucleoside 5'-tetraphosphates. Most probably this enzyme hydrolyzes Ap_4A, also present in *Artemia* extracts (McLennan and Prescott, 1984).

Guanosine monophosphate (GMP) reductase

GMP reductase was firstly described in microorganisms by Mager and Magasanik (1960). Its presence in human and rabbit erythrocytes was demonstrated by the ability of these cells or hemolysates to incorporate either guanosine or GMP into IMP (Hershko *et al.*, 1963). The enzyme was firstly well characterized in eukaryotes, in the cytosolic fractions of calf thymus and *Artemia* (Stephens and Whittaker, 1973 ; Renart and Sillero, 1974 ; Renart *et al.*, 1976). The *Artemia* enzyme (50 mU/g dry cysts) showed two apparent K*m* values of 5 and 50 μM towards GMP. Xanthosine 5'-phosphate (XMP) was a very strong inhibitor (K*i* = 10 nM) of the reaction. In the presence of 1.5 μM XMP hyperbolic kinetics were found, with a unique K*m* value of around 50 μM. Gp_4G activated the enzyme (K*a* = 30 nM), concomitantly changing to hyperbolic the kinetics of the enzyme, with a unique K*m* value of about 5 μM. Gp_4G also counteracted very effectively the inhibition of the enzyme by XMP. Other structural analogues (Ap_4A, Gp_3G), tested at micromolar concentration had no detectable effect on the enzyme. GTP (mM) was also able to counteract the inhibition of GMP reductase by XMP. The effect of Gp_4G on the enzyme is then very specific. The complex kinetic and the existence of allosteric effectors at physiological concentration, together with our lack of success in resolving two isozymes makes it likely that GMP reductase presents negative cooperativity towards its substrate. Practically the same properties were found for GMP reductase from human erythrocytes, although a different interpretation was given to the kinetics of the enzyme (Spector *et al.*, 1979).

Adenylosuccinate synthetase

The enzyme with an activity of around 50 mU/g dry cysts was purified from the 27 000 × *g* supernatant (Faraldo *et al.*, 1983). After chromatography on DEAE-cellulose, two enzyme forms were apparent. One of them was not retained by the column (form I) and the other eluted with a linear gradient of KCl (form II). The isoelectric points for forms I and II were 6.5 and 6.2, respectively. The molecular weights of forms I and II determined by gel filtration were 90 000 and 83 000, respectively. The K*m* values were as follows : form I of the enzyme towards IMP, GTP, and aspartate, 26, 29, and 600 μM, respectively ; form II for the same substrates 20, 25, and 700 μM, respectively. GMP and GDP were competitive inhibitors towards GTP. *Ki* values found were, for forms I and II : (80 and 10 μM) and (65 and 10 μM), respectively. Gp_4G was uncompetitive inhibitor (K*i* = 50-60 μM) towards GTP, for both forms of the enzyme, when the concentration of IMP and aspartate were fixed at 0.25 and 4 mM, respectively. Gp_4G behaved as competitive inhibitor (K*i* = 80-100 μM) towards GTP, also for both forms of the enzyme, at concentrations of 0.1 and 1 mM of those substrates, respectively. Fructose 1,6-bisphosphate was found to be competitive inhibitor towards IMP with a K*i* value of 22 μM for both forms of adenylosuccinate synthetase.

The presence of two isozymes of adenylosuccinate synthetase has only been previously reported in rat liver (Matsuda *et al.*, 1977). The liver isozymes (termed M and L) presented slight kinetic differences. Matsuda *et al.* (1977), pointed that isozyme L was important in the regulation of purine nucleotide synthesis, whereas isozyme M, participated in the regulation of glucolysis. Compared to rat liver isozymes, the physiological significance between both forms of *Artemia* adenylosuccinate synthetase is not clearly different, due to the similarity in their kinetic properties. Adenylosuccinate synthetase has been studied in several eukaryotic sources such as placenta (Van der Weyden and Kelly, 1974), rat (Ogawa *et al.*, 1976, 1977) and rabbit muscle (Muirhead and Bishop, 1974) and rat liver (Matsuda *et al.*, 1977). The enzyme from *Artemia* seems more similar to the enzyme from placenta : both have relatively lower K*m* values for IMP and higher for aspartate.

Adenylosuccinate lyase

This enzyme splits adenylosuccinate into fumarate and AMP and represents the last step in the synthesis of this adenine nucleotide from Gp_4G. The enzyme from *Artemia* (Pinto *et al.*, 1983) presented drastic differences in molecular weight depending on the pH of the medium. Forms of 200 kDa and larger than 500 kDa were detected after chromatography on Sepharose CL-6B using elution buffers of pH 8.7 and 6.5, respectively. The existence of other forms could be observed at intermediate pH values. The 200 kDa form obtained by chromatography at pH 8.7 did not revert to a larger size upon re-chromatography at pH 6.6 ; however the higher molecular mass form obtained at pH 7.1 passed to 200 kDa form after re-chromatography at pH 8.7. This could mean, among other possibilities, that the 200kDa form is the more stable form of the enzyme or that upon chromatography on Sepharose CL-6B, a factor is lost which is essential for the conversion of the 200 kDa form to higher molecular forms. Such conversion is possible in the 27 000 × *g* supernatants (Pinto *et al.*, 1983). It is worthy to note that the highest change in the molecular forms of the enzyme occurs in the pH range 7-7.5. Changes in molecular forms depending on pH have not been described for the enzyme from other sources.

The purified enzyme from *Artemia* cysts (Pinto *et al.*, 1983), had a molecular mass of 200 kDa and a single subunit of 56 kDa as revealed by electrophoresis on sodium dodecylsulfate polyacrylamide gel. The 200 kDa form could represent a tetramer, and larger molecular forms could be due to association of monomers with or without participation of a factor essential for the association but not for the catalytic activity. K*m* values for adenylosuccinate, AMP, and fumarate were 1, 36, and 350 μM, respectively. The enzyme exhibits a Uni-Bi-ordered mechanism, is maximally active at a pH value of 8.0-8.5, and the isoelectric point determined by chromatofocusing was 5.04. Maximum velocity of this enzyme in *Artemia* cysts is around 180 mU/g (Pinto *et al.*, 1983).

Other degradative routes for diguanosine tetraphosphate

Van Denbos and Finamore (1974) proposed a different pathway for the utilization of Gp_4G represented essentially by the following two reactions (the asterisk denotes position of the [^{14}C]label, see below).

9) $\overset{*}{\underset{\text{(adenine)}}{A}} + Gp_4G \longrightarrow \overset{*}{A}p_4G + \text{Guanine}$

10) $\overset{*}{A}p_4G \xrightarrow{t} \overset{*}{\text{ATP}} + \text{GMP}$

The experimental evidence for such pathway is an indirect one and is based on a study of the distribution of radioactivity when *Artemia* nauplii were supplied with either [U-^{14}C] adenosine or [U-^{14}C] guanosine. In both cases the ratio ^{14}C-purine base to ribose of the labeled commercial products was around 0.9. When [U-^{14}C] adenosine was administered, that ratio was determined in AMP, ADP and ATP. The corresponding values after 15 min of incubation were 0.8, 1.3 and 9.5, respectively. The disproportionate labeling pattern for ATP could be explained, as proposed by Van Denbos and Finamore (1974), by the following set of reactions.

11) $\underset{\text{(adenosine)}}{\overset{**}{AR}} \underset{a}{\overset{f}{\rightleftharpoons}} \underset{\text{(AMP)}}{\overset{**}{ARP}} \overset{q}{\rightleftharpoons} \underset{\text{(ADP)}}{\overset{**}{ARPP}} \overset{j}{\rightleftharpoons} \underset{\text{(ATP)}}{\overset{**}{ARPPP}}$

12) $\underset{\text{(AMP)}}{\overset{**}{ARP}} + PPi \overset{e}{\rightleftharpoons} \underset{\text{(adenine)}}{\overset{*}{A}} + \underset{\text{(phosphoribosylpyrophosphate)}}{\overset{*}{PRPP}}$

The labeled adenine produced in step 12 could yield ATP, through reactions 9 and 10, exclusively labeled in its adenine moiety. ATP molecules so produced, plus those generated through the reactions listed in 11) could generate a pool of ATP with a ratio [^{14}C]adenine/[^{14}C]ribose = 9.5. When nauplii were incubated in the presence of [U-^{14}C] guanosine, the isolated GMP, GDP, GTP, Gp_4G, AMP, and ADP had ratios of labeled base/ribose similar to that of the labeled guanosine precursor. ATP however showed preferential labeling in its ribose moiety. Also in that case, a set of reactions, also implying the synthesis of Ap_4G (step 9) were proposed (Van Denbos and Finamore, 1974) to explain these results, but we shall not go through them here.

Inspired by the results of Van Denbos and Finamore (1974), and by the presence, recently described, of Ap_4G in other biological systems (Lee *et al.*, 1983 ; Garrison and Barnes, 1984), the occurrence in *Artemia* extracts of the enzyme catalyzing the above reaction 9 was investigated, so far without success, in our laboratory. If this enzyme is not actually present in *Artemia*, the

interesting results of Van Denbos and Finamore (1974) have to be interpretated in a different way.

Another possible degradative pathway for Gp_4G is through the Gp_4G synthetase. As described above (reaction 1) this enzyme also catalyzes the cleavage of Gp_4G by PPi; in that case the enzyme acts as a pyrophosphorylase producing 2 moles of GTP per mol of Gp_4G. A study of the level of PPi in yolk platelets, mitochondria and cytosol could be of interest to have a quantitative approximation to the pyrophosphorolytic activity of this enzyme on Gp_4G (Warner, 1975). Related to this, a phosphorolytic activity on dinucleosidetetraphosphates has been described in yeast extracts cleaving these compounds to the corresponding nucleoside di- and triphosphates (Guranowski and Blanquet, 1985).

Synthesis of GMP from IMP

IMP is the center of an important metabolic crossroads diverging towards AMP or GMP. Theoretically, the study of the first pathway is more attractive in *Artemia*, and therefore attention was paid to it first, in our laboratory. Nevertheless, and in order to get a more complete picture of the interconversion of nucleotides, the pathway IMP $\xrightarrow{1}$ XMP $\xrightarrow{k}$ GMP has also been considered.

Inosine monophosphate (IMP) dehydrogenase

This enzyme catalyzes the conversion of IMP into XMP using NAD^+ as cofactor. As happened with adenylosuccinate lyase (Pinto *et al.*, 1983), IMP dehydrogenase also presented different molecular forms in *Artemia* (Canales *et al.*, 1985). The enzyme from the supernatant had a molecular size of around 450 kDa. After being recovered in the 30-40 % saturation ammonium sulfate fraction, it showed an heterogeneous size ranging from around 450 kDa to more than 1 000 kDa, as determined by gel filtration on Sepharose 4B. In the presence of 2 M NaCl a single form of around 260 000 molecular weight was obtained. This anomalous behaviour could be due to ionic interactions between enzyme molecules either alone or plus the contribution of other macromolecules, probably favored after sequestration of water molecules from the microenvironment of the protein by ammonium sulfate. The smallest active form observed had a molecular mass of around 130 kDa. The enzyme is probably composed of 55 kDa subunits which tend to form aggregates.

Artemia IMP dehydrogenase was purified near homogeneity (Canales *et al.*, 1985) and the following properties were found : the K*m* values for IMP and NAD^+ were 15 and 200 µM, respectively. GMP, XMP, GTP guanosine 5'-tetraphosphate, and Gp_4G were competitive inhibitors of the reaction towards IMP with K*i* values of 140, 180, 175, 120, and 87 µM, respectively. Maximum velocity of the enzyme is around 5 mU/g dry cysts.

ATP activated nucleotidase

The transformation of XMP to GMP is catalyzed by GMP synthetase. While studying this enzyme it was observed that the [^{14}C]-labeled XMP used as substrate was transformed into xanthosine in the presence of ATP, but not significantly in the absence of this nucleotide (Sillero *et al.*, 1984 ; Pinto *et al.*, 1987). The presence of either NH_3 or glutamine in the reaction mixture did not affect the rate of XMP hydrolysis. This activity, probably related to the cytosolic 5'-nucleotidase already described in several other tissues (Van den Berghe *et al.*, 1977 ; Itoh,

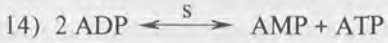
1981 ; Worku and Newby, 1983) hydrolyzes GMP, IMP, and XMP to the corresponding nucleosides. The activation constant for ATP was 2.5 mM. The K*m* values for GMP found in the absence and presence of ATP were 10 and 0.4 mM, respectively. Little is known on the physiological role of this activity.

Due to the interference of this enzyme, the properties of GMP synthetase were not further investigated, although preliminary results pointed to a maximum velocity of less than 5 mU/g dry cysts (unpublished results from this laboratory).

Synthesis of nucleoside di- and triphosphates

Interconversion between nucleoside mono-, di- and triphosphates is catalyzed by nucleoside monophosphate and diphosphate kinases.

Dismutation of ADP is catalyzed by adenylate kinase



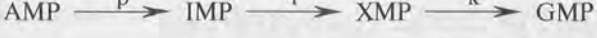
These three enzymes (q,r,s), usually present in relatively high maximum velocities in biological systems, catalyze non limiting steps in the interconversion of nucleotides. Maximum velocity of nucleoside diphosphate kinase, (50 U/g dry cysts) is well in excess (around a thousand fold) over the enzymes which transform Gp_4G into AMP. Nucleoside diphosphate kinase has been purified 1 900-fold from *Artemia* cysts (Renart *et al.*, 1984). As happens with the enzyme from other sources (Garces and Cleland, 1969), the purified enzyme exhibits a ping-pong mechanism of reaction. The calculated K*m* values for ADP, GTP, ITP, UTP, and CTP were 0.04, 0.17, 0.25, 0.27, and 0.62 mM, respectively (Renart *et al.*, 1984).

In addition, net synthesis of ATP occurs chiefly in mitochondria, as these organelles are active shortly after incubation of the cysts (Schmitt *et al.*, 1973 ; Vallejo *et al.*, 1979).

By either of the above pathways, there is experimental evidence that shortly after incubation of adult animals with $[^{32}P]H_3PO_4$, the label is incorporated preferentially into ATP and GTP (Kobayashi *et al.*, 1972).

On the conversion of adenine precursors to guanine nucleotides

The reluctance of *Artemia* cysts/larvae to carry out such a conversion is an old observation from several laboratories (Warner and McClean, 1968 ; Van Denbos and Finamore, 1974). What follows is a possible interpretation of that finding. A pathway for the synthesis of GMP from AMP, involving a minimum of intermediates is :



We have mentioned previously that the level of GMP synthetase (k) is very low in *Artemia* cysts extracts. The same happens with the first step of this pathway catalyzed by AMP deaminase (p). We have been unable to detect this activity using a method involving measurement of the

ammonia liberated from AMP ; with high pressure liquid chromatographic techniques a maximum velocity of around 0.25 mU/g has been measured (unpublished results from this laboratory). The very low levels of AMP deaminase, IMP dehydrogenase, and GMP synthetase in cysts could explain the difficulty of *Artemia* to synthesize GMP from adenine precursors. AMP deaminase is a complex enzyme. It presents frequently, sigmoidal kinetics, has different regulatory properties in different organs (Coffee and Solano, 1977 ; Yun and Suelter, 1978 ; Wheeler and Lowenstein, 1979 ; Ashby and Holmsen, 1983) and may be present at high (rat skeletal muscle 200 U/g, Aragón *et al.*, 1981) or undetectable levels (insect flight muscle (Aragón *et al.*, 1981) and *Artemia*). The enzyme from rat muscle is activated by Ap_4A (Fernández *et al.*, 1984b).

Related to the interconversion between adenine and guanine nucleotides, one report should be mentioned. Llorente *et al.* (1984) observed in larvae, that it was easier to get labeled guanine nucleotides from adenine precursors than the reverse, *i.e.* adenine nucleotides from guanine. This result is in contradiction with those presented above (Warner and McClean, 1968 ; Van Denbos and Finamore, 1974). As stressed previously (Clegg and Conte, 1980), the geographical origin, and the experimental conditions may explain apparently conflicting results. In relation to this it is worthy to mention the finding of Hernandorena (1985) showing that the dietary efficiency of purine bases and nucleosides can be significantly increased by increasing the phosphate supply.

Ammonia excretion

Ammonia is produced chiefly by deamination of amino acids and by degradation/interconversion of nucleotides (Lowenstein, 1972 ; Weil-Malherbe, 1974 ; Goodman and Lowenstein, 1977 ; Aragón and Lowenstein, 1980 ; Aragón *et al.*, 1981). *Artemia* is considered an ammoniotelic organism. The relationship between ammonia excretion and purine nucleotide metabolism in adult *Artemia* has been measured by Hernandorena and Kaushik (1983).

The so-called purine nucleotide cycle, or Lowenstein cycle, is very well documented in rat muscle and is composed by the following reactions.

$$15)\ AMP + H_2O \xrightarrow{p} IMP + NH_3$$

$$7)\ IMP + L\text{-aspartate} + GTP \overset{n}{\rightleftharpoons} ASA + GDP + Pi$$

$$8)\ ASA \overset{o}{\rightleftharpoons} AMP + fumarate$$

$$\text{Sum } 15,7,8 \quad L\text{-aspartate} + GTP + H_2O \longrightarrow fumarate + GDP + Pi + NH_3$$

Due to the very low level of AMP deaminase activity, this cycle does not seem to be operative in *Artemia*.

A theoretically very similar pathway for the evolution of NH_3 could be the following

$$16)\ IMP + NAD^+ + H_2O \xrightarrow{l} XMP + NADH + H^+$$

$$17)\ XMP + L\text{-glutamine} + ATP + H_2O \xrightarrow{k} GMP + L\text{-glutamate} + AMP + PPi$$

$$6)\ GMP + NADPH + H^+ \xrightarrow{m} IMP + NADP^+ + NH_3$$

$$\text{Sum } 16,17,6 \quad NAD^+ + L\text{-glutamine} + ATP + NADPH + 2H_2O \longrightarrow NADH + L\text{-glutamate} + AMP + PPi + NADP^+ + NH_3$$

As the Lowenstein cycle, this one may also produce intermediates of the Krebs cycle through additional transamination of glutamate, it liberates NH_3 from amino acids, and consumes one mol of nucleoside triphosphate per mol of ammonia evolved. In addition, a transhydrogenation between NAD^+ and NADPH takes place. From a theoretical point of view, however, the low levels of GMP synthetase detected in cysts may also question the functionality of this cycle in *Artemia*. In any case, NH_3 is also produced in *Artemia* by GMP reductase (Renart *et al.*, 1976) and in the course of uricolysis (Hernandorena and Kaushik, 1983).

There is another enzyme, adenosine deaminase, participating in the interconversion of nucleosides, and which may play an important role in the liberation of ammonia.

Adenosine deaminase

The activity of this enzyme increases steadily during the first 45 h of development, reaching at that time a 15-fold increment in specific activity in the 145 000 × *g* supernatant extract (Fernández *et al.*, 1984a). Gel filtration of these supernatants on a Sephacryl S-300 column showed that, at least, two different molecular forms were present in the extracts, and that the increase in adenosine deaminase activity was accounted for the increase of the higher molecular weight form. As estimated by gel filtration, the molecular weight of both forms was 30 000 and 135 000. In addition, the smallest form consisted of three isozymes (termed II, III, and IV) as showed by chromatofocusing. Isozyme I corresponded to the 135 000 molecular weight protein present almost exclusively in the incubated cysts. The pI values for isozymes I, II, III, and IV were 4.2, 4.9, 5.0, and 5.2, respectively. Referred to 10^6 animals (cysts or larvae) the sum of the activities for isozymes II, III, and IV were 0.7, 1.1, 0.7, 0.3, and 0.2 units at 0, 15, 24, 39, and 47 h of incubation, respectively. The corresponding values for isozyme I were 0, 0.1, 3.1, 3.8, and 4.2 units, respectively.

The occurrence of adenosine deaminase in *Artemia* may explain that the quantity of ammonia liberated is higher when the dietary requirements are met by AMP than when supplied by either IMP or GMP (Hernandorena and Kaushik, 1983). These nucleotides are probably transported inside the cell as the corresponding nucleosides, and the deaminase may liberate ammonia as the first step in the utilization of adenosine. The other nucleosides, inosine or guanosine, do not have a first deaminative step in their utilization.

Concluding remarks

Purine nucleotide interconversion represents a very complex array of reactions whose regulation is, in general, far from being understood, as data are lacking on both the exact concentration of substrates and effectors and on the catalytic properties of the participating enzymes. In addition, it is frequent that extrapolations are made from one to another biological system. This may be particularly incorrect in the case of interchange of enzyme data between pro and eukaryotes. The drastic differences in the regulatory properties of GMP reductase from both sources is an illustrative example (Mager and Magasanik, 1960 ; Renart *et al.*, 1976).

It can also be emphasized that, with the exception of adenosine deaminase, we have not observed significative changes in maximum velocity in any of the enzymes related to nucleotide interconversion, during development from cysts to larvae : dinucleosidetetraphosphatase, GMP reductase, IMP dehydrogenase, adenylosuccinate synthetase, adenylosuccinate lyase and ATP

activated nucleotidase. The cysts seem to be actually afforded with the enzyme machinery needed to carry out interconversion of purine nucleotides. On the other hand, the strong regulation of the tetraphosphatase and GMP reductase point to these two enzymes as physiologically relevant in the synthesis of IMP from Gp_4G. The information available on purine interconversion in *Artemia* is still fragmentary, *i.e.* very little is known on enzymes of the salvage pathway of purine bases and nucleosides. This is a field that we would like to further pursue in order to collect data directed to the design of computer simulation programs that could help to the quantification and understanding of this cycle (Franco and Canela, 1984).

Related to *Artemia*, two qualifications are frequently applied to diguanosine tetraphosphate : major source of both purine nucleotides and phosphate bond energy during development from encysted gastrula to larva. Whereas the first statement seems to be correct, the second one is, in our view, misleading for the following reasons :

a) Although at first sight the amount of Gp_4G (12 µmoles/g dry cysts) seems very high, the figure of soluble purine nucleotides content in *Artemia* is not very far from that of other cells. As an example, rat liver contains around 4 µmoles of adenine nucleotides (AMP, ADP, ATP) per g wet weight (Williamson and Brosnan, 1974). Moreover, as shown by Clegg *et al.* (1967) Gp_4G is transformed preferentially to adenine nucleotides without change in the total purine concentration.

b) It is well known that the amount of nucleoside triphosphates present in the steady state represents only a minimal fraction of that being continuously recycled.

c) Although the actual pathway for the utilization of Gp_4G is not definitively established, three possibilities have been above pointed. Cleavage to GMP and GTP, followed by the transformation of GMP into AMP ; in that case no net synthesis of nucleoside triphosphate occurs. The cleavage of one mole of Gp_4G through the Van Denbos and Finamore (1974) pathway, or by pyrophosphorolysis (Warner and Huang, 1974) produces one mole of ATP, or two moles of GTP, respectively. In any case, a maximum of around $12 \times 2 = 24$ µmoles of nucleoside 5'-triphosphate would be synthesized from the Gp_4G stored in one g of cysts.

d) This is a very low figure in relation to the comparatively huge energy reserves present in the cysts in form of protein, trehalose, glycogen, glycerol, and lipids. For example, only during the first 24 h of development 150 mg of protein, from yolk platelets, are degraded per g of cysts (Olalla *et al.*, 1977). Most probably, this represents the liberation of 1 500 µmoles of amino acids. Assuming that only two carbon atoms, from each amino acid, are oxidized in the Krebs cycle, this represents the synthesis of about $1\,500 \times 12 = 18\,200$ µmoles of ATP from ADP, in the first 24 h of development. The content of trehalose plus glycogen is around 160 mg/g dry cysts (Clegg, 1962, 1965). This is roughly equivalent to 900 µmoles of stored glucose. If only 10 % of it were oxidized, the synthesis of around $90 \times 36 = 3\,240$ µmoles of ATP are generated. Related to that, the oxygen consumption during the first 4 of development is around 140 µl/30 mg cysts (Clegg, 1964). This is enough to support the synthesis of 1 250 µmoles of ATP/g dry cysts. We are aware that those are bulky figures, and that both elimination of nutrients to the medium and interconversion between trehalose, glycogen, and glycerol seems to take place (Clegg, 1965). Nevertheless, we try only to point out the big difference between the quantity of

high energy phosphate bonds trapped in Gp_4G and the potentiality for the synthesis of ATP present in the non nucleotide material of the cysts. Gp_4G may have some energetic role only in the very first time of development, in the case that not enough functionally active mitochondria are available in the cell.

In our view, the stored Gp_4G, besides being a source of purine rings, also plays a regulatory role during development. Actually one clear example of that has already been described (Renart *et al.*, 1976). One may recall that an analogous nucleotide, diadenosine tetraphosphate, is being investigated in several laboratories and thought to be a regulatory molecule by several criteria (Zamecnik, 1983 ; Sillero *et al.*, 1985). *Artemia* may well be a kind of present nature has offered us to help to unravel the role in biology of dinucleoside polyphosphates.

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The cAMP-independent protein kinase activity associated with non-polysomal cytoplasmic messenger ribonucleoproteins of cryptobiotic gastrulae of *Artemia* sp.

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Abstract

The association of a protein kinase with cytoplasmic non-polysomal messenger ribonucleoprotein is demonstrated by sucrose gradient centrifugation, gel filtration on Sepharose 4B and sucrose isopycnic centrifugation. The native enzyme has a molecular weight of 136 000 and is composed of subunits with M_r 36 500 (α) and 28 000 (β). The enzyme has a $\alpha_2\beta_2$ structure, is cyclic nucleotide-independent and is identified as a casein kinase II. The enzymatic activity has an optimum at pH 7.75 and is dependent on the presence of divalent ions. The enzyme is inhibited by heparin, poly(L)-glutamic acids and caffeine. The exogenous substrates of the enzyme are eIF2α and the poly(A)-binding mRNP proteins. The main endogenous phosphate acceptor is the M_r 38 000 poly(A)-binding protein. A function of the mRNP associated protein kinase in the regulation of the repression of stored mRNP is proposed.

Abbreviations

Hepes : 4-(2-hydroxyethyl)-1 piperazine ethane sulphonic acid.
PMSF : phenylmethanesulphonyl fluoride.
EDTA : ethylenediaminetetraacetic acid, disodium salt.
mRNA : messenger ribonucleic acid.
mRNP : messenger ribonucleoprotein.
iRNA : inhibitor RNA.
eIF2 : eukaryotic initiation factor 2.
poly(A) : poly(riboadenylic acid).
poly(U) : poly(ribouridylic acid).
oligo(dT) : oligo (deoxyribothymidylic acid).

Introduction

Protein phosphorylation has often been mentioned as a general mechanism for the regulation of cell metabolism (Cohen, 1980). Factors involved in gene expression and protein synthesis are therefore possible candidates for regulation by phosphorylation-dephosphorylation (Ochoa and De Haro, 1979 ; Traugh, 1981). The phosphorylation of ribosomal proteins, initiation- and elongation factors of protein synthesis and mRNP is well documented. However, despite

considerable efforts in many laboratories the function of phosphorylation in protein synthesis is largely unknown.

Initially research was mainly focused on the phosphorylation of ribosomal proteins. The major phosphoprotein is S6 (for a review see Gordon *et al.* 1982). The latter protein is phosphorylated under different physiological conditions and a correlation between phosphorylation and increase in protein synthesis has often been observed (Duncan and McConkey, 1982 ; Thomas *et al.*, 1982). However, phosphorylation of S6 is also induced in conditions that decrease protein synthesis and highly phosphorylated S6 is not a prerequisite for activating the translational machinery of cells (Ballinger and Hunt, 1982 ; Kruppa and Clemens, 1984). In the majority of cell types the rate of protein synthesis is affected by changes in external conditions *e.g.* mitosis, serum factor deprivation, stress, etc. In the sequential events of protein synthesis initiation is most likely involved in translational control. eIF2, eIF3 eIF4B, and eIF5 are the phosphorylated initiation factors (Reichert and Issinger, 1981). The most extensively studied is the phosphorylation of eIF2 by the heme regulated protein kinase (heme controlled repressor) and the double stranded RNA activated protein kinase (Clemens, 1980 ; Levin *et al.*, 1981). The phosphorylation of eIF2 inhibits the exchange of GDP for GTP and impairs the ability of eIF2 to recycle. The phosphorylation of eIF2β, eIF3 and eIF4B seems to be of minor importance for translational regulation (Jagus *et al.*,1981) although a function in the preferential translation of a subset of mRNAs has been proposed (Duncan and Hershey, 1985).

Less is known about the function of phosphorylation of mRNP proteins. In eukaryotic cells messenger RNA is associated with proteins. The latter proteins probably have a function in translational control. The presence of phosphoproteins in mRNP has been demonstrated in many eukaryotes *e.g.* duck erythroblasts (Gander *et al.*, 1973), Hela cells (Auerbach and Pederson, 1975), embryonic chicken muscle (Bag and Sells, 1979), rat liver (Cardelli and Pitot, 1980), mouse plasmacytoma cells (Egly *et al.*, 1981) reticulocytes (Rittschof and Traugh, 1982). The possible function of mRNP proteins and their phosphorylation has been reviewed (Egly *et al.*, 1979). Up to now, the physiological significance of the latter phosphorylation remains unknown.

Artemia sp. is very suitable for the study of the regulation of mRNP translation. In the cryptobiotic phase no polysomes have been detected and their rapid formation in development is due to the presence of stored mRNP. A fraction of the latter RNP exists in a repressed form by association with an inhibitor ribonucleoprotein (Slegers *et al.*, 1981 ; Piot *et al.*, 1984 ; De Herdt *et al.*, 1985). The mRNA associated proteins have been identified previously. The M_r 38 000 poly(A)-binding protein has been characterized as a multifunctional protein and eIF2 is present on a distinct fraction of non-polysomal mRNP (De Herdt *et al.*, 1984 ; De Herdt *et al.*, 1985).

In order to resolve the function of phosphorylation in the regulation of mRNP translation, the mRNP associated cAMP-independent protein kinase has been purified and its substrates identified. The results presented in this report demonstrate that phosphorylation of mRNP proteins is involved in the regulation of the repression of stored non-polysomal mRNP.

Experimental procedures

Materials

Artemia sp. cryptobiotic embryos (Macau, Brasil) were obtained from the *Artemia* Reference Center (State University of Ghent, Belgium) ; oligo(dT)-cellulose from Collaborative Research

(Waltham, MA, USA) ; Sepharose 4B, low molecular weight marker proteins and Pharmalyte carrier ampholytes from Pharmacia Fine Chemicals (Uppsala, Sweden) ; Whatman GF/C filters from Whatman (Maidstone, UK) ; [^{3}H]-poly(U) (20-72 Ci/mmol) and [γ - ^{32}p]-ATP (3 000 Ci/mmol) from Amersham International (Buckinghamshire, UK) ; heparin, poly(L)-glutamic acid, cAMP and histones from Sigma (St. Louis, USA).

Buffers

buffer A : 10 mm Hepes pH 7.2, 100 mM KCl, 0.1 mM dithiothreitol ;
buffer B : 20 mM Tris/HCl pH 7.6, 6 mM magnesium acetate, 7 mM 2-mercaptoethanol, 0.1 mM EDTA, 10 % glycerol.

Fractionation

100-200 g (dry weight) of cryptobiotic gastrulae of *Artemia* sp. were separated from sand and metal impurities by flotation in saturated NaCl, washed with distilled water and homogenization buffer A. The washed gastrulae were ground at 4 °C in a motor-driven mortar in the presence of a small amount of homogenization buffer (50 µl/g gastrulae) supplemented with 0.5 mg/ml heparin, 0.1 mM PMSF and 150 mM sucrose. The homogenate was diluted to 400 ml and filtered through cheese cloth. The postmitochondrial supernatant was prepared by two successive centrifugations of the filtrate at 27 700 × *g* for 30 min at 4 °C in a Beckman JA 20 rotor.

Poly(A)-containing mRNP was prepared by affinity chromatography on oligo(dT)-cellulose type T2 in buffer A containing 0.1 mM PMSF and 250 mM KCl as described (Slegers *et al.*, 1981). Proteins of mRNP were prepared by a 1.5 M KCl salt wash of oligo(dT)-cellulose bound RNP. The M_r 38 000 poly(A)-binding protein was purified from mRNP proteins according to De Herdt *et al.* (1984). Poly(A)-containing mRNP was analyzed on 10-30 % sucrose gradients in buffer A. Conditions of centrifugations are described in the figure legends. Analysis of poly(A)-containing mRNP by gel filtration was on Sepharose 4B. mRNP, concentrated with crystalline sucrose, was applied to a 1.4 × 100 cm column of Sepharose 4B previously equilibrated with buffer A containing 10 % glycerol.

Buoyant Density Centrifugation

For isopycnic centrifugation in sucrose, samples were placed on three layers (3 ml each) of sucrose solutions (30 %, 50 % and 70 % w/w) in buffer A. Centrifugation was in a Beckman SW 41 rotor at 251 800 × *g* for 90 h and at 4 °C.

The fractions of sucrose isopycnic gradients were collected by sucking through a stainless-steel needle from the bottom of the centrifuge tubes. Densities were determined by weighing samples in a calibrated 100 µl constriction pipette.

Protein kinase activity assay

The mRNP associated protein kinase was purified as described (Thoen *et al.*, 1984). The standard assay was in 10 mM Tris/HCl pH 8.2, 12.5 mM $MgCl_2$, 50 mM KCl and 7 mM 2-mercaptoethanol (kinase buffer), 75 µg casein and an appropriate amount of the protein kinase containing fraction. Reaction mixtures of 200 µl were incubated at 37 °C after addition of 1 µM[γ - ^{32}p]-ATP (10 Ci/mmol). After incubation for 30 min, the reaction was terminated by the addition of trichloroacetic acid to a final concentration of 10 % (w/v). After 30 min the

acid-precipitable radioactivity was collected on Whatman GF/C filters. Filters were washed with 10 ml of 5 % (w/v) trichloroacetic acid, 10 ml of ethanol, dried and counted in 10 ml scintillation cocktail.

One unit of kinase activity will transfer 1.0 pmol of phosphate from [γ - ^{32}p]-ATP to casein per min.

Assay volumes were increased to 1.0-1.5 ml for gel electrophoresis. After incubation for an appropriate time at 37 °C, trichloroacetic acid was added to a final concentration of 10 % (w/v). The precipitate was pelleted, washed with ethanol and ether, respectively, and dried.

Polyacrylamide Gel Electrophoresis

Analysis of proteins by electrophoresis on 10 % (w/v) polyacrylamide dodecylsulphate gels was as described (Slegers *et al.*, 1981). Two-dimensional gel electrophoresis was performed according to the procedure of O'Farrell (1975).

After the staining procedure, the phosphorylated proteins were visualised by autoradiography. Destained gels were dried under vacuum and autoradiographed on Kodak Ortho-G film.

Molecular weights were estimated using phosphorylase b (94 000), serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (21 500) and α-lactalbumin (14 000).

Miscellaneous

Poly(A)-sequences were localized by hybridization with [^{3}H]- poly(U) as described previously (Slegers and Kondo, 1977).

Protein concentrations were determined by alkaline hydrolysis and reaction with ninhydrin (Allen, 1981), using bovine serum albumin as a standard.

Columns used in gel filtration were calibrated with 40 S (1.4×10^6) ribosomal subunits, thyroglobulin (660 000) and ferritin (449 000).

Results

Evidence for the association of a protein kinase activity with mRNP

Poly(A)-containing mRNP was purified by affinity chromatography on oligo(dT)-cellulose and analyzed by centrifugation on a 10-30 % sucrose gradient (Fig. 1). The main particle sedimented at 17S. The integrity of the mRNP was checked by hybridization with [^{3}H]-poly(U). More than 60 % of the poly(A)-sequences cosedimented with the 17S RNP and approximately 28 % was located at the top of the gradient indicating a partial cleavage of the poly(A)-tail from mRNP. Protein kinase activity was measured in each fraction of the gradient with the standard assay using casein (Fig. A1) or endogenous proteins as substrates (Fig. 1B). The protein kinase activity was coincident with the distribution of mRNP poly(A)-sequences if casein was used as substrate. On the contrary the endogenous phosphorylation has a different distribution with a maximum between 17S mRNP and the top of the gradient. The observed difference may be explained by an overlap of either the distribution of the mRNP associated enzyme and proteins copurified with mRNP or free enzyme copurified with mRNP. It will be demonstrated below that the former supposition is valid.

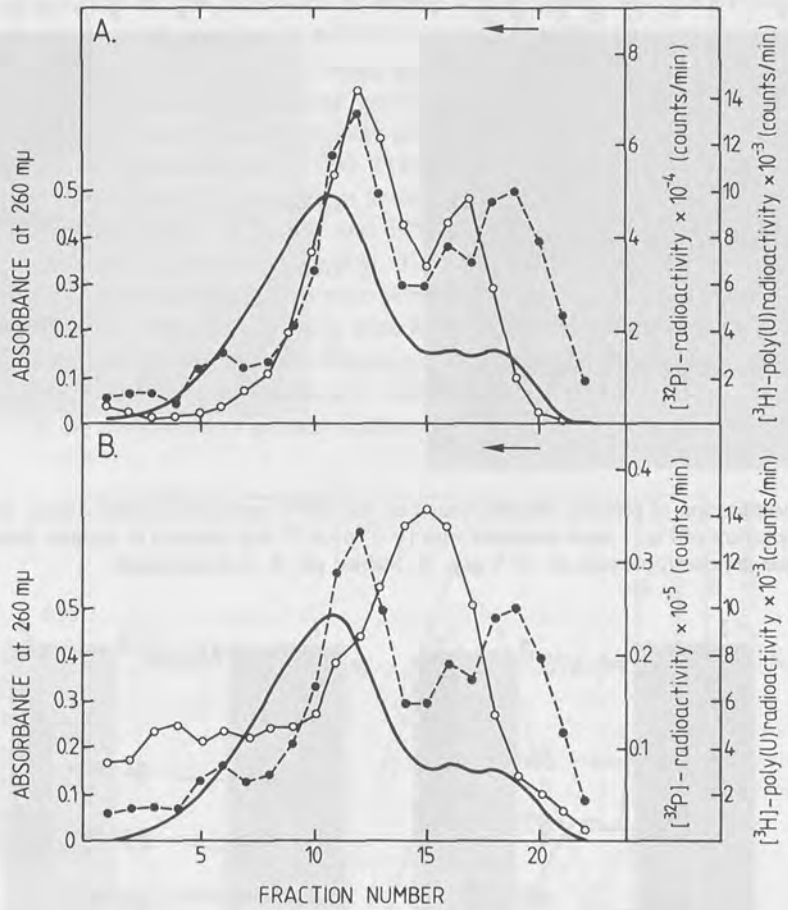

FIG. 1. Sucrose density gradient centrifugation of mRNP. Poly(A)-containing mRNP was analyzed on a 10 %-30 % (w/v) sucrose gradient in buffer A. Centrifugation was at 103 000 × *g* for 21 h in a Beckman SW27 rotor. The direction of centrifugation is indicated. Absorbance at 280 mμ (——). Hybridization with [^{3}H]-poly(U) (●----●). Protein kinase activity was measured by the standard assay with casein (A) and endogenous proteins (B) as phosphate acceptors (○——○).

The endogenous phosphate acceptors were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis of the 17 S mRNP containing gradient fractions and by autoradiography (Fig. 2). The protein composition of the 17S mRNP was identical to the ones described previously (Slegers *et al.*, 1981). Proteins with M_r of 112 000, 87 000, 76 000, 65 000, 38 000, 26 000 and 23 500 were found to be endogenous substrates of the protein kinase present in the sucrose gradient fractions. One additional phosphate acceptor with a molecular weight of 43 000 was observed in mRNP prepared by affinity chromatography on oligo(dT)-cellulose in a slightly lower salt concentration (100 mM KCl instead of 250 mM KCl).

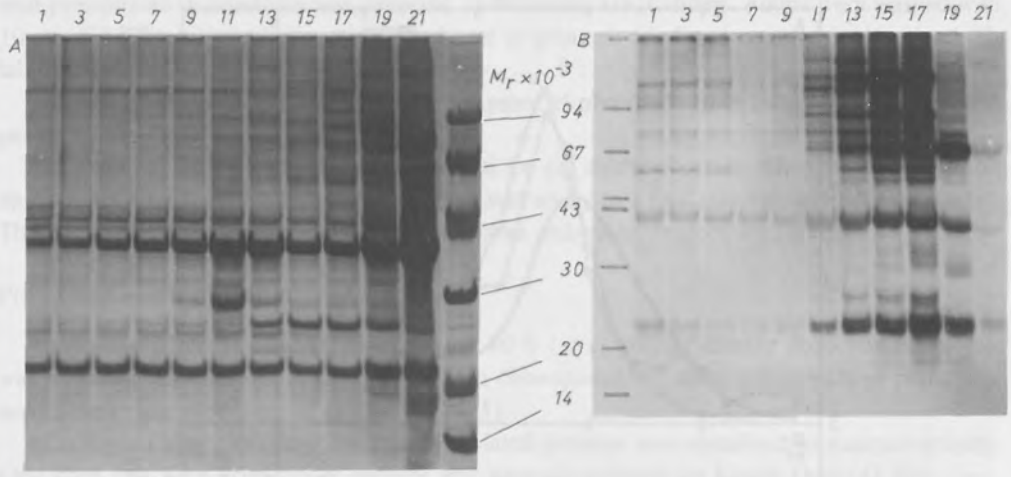

FIG. 2. Identification of proteins phosphorylated by the mRNP associated protein kinase. Fractions of the sucrose gradient of Fig. 1 were incubated with [γ - ^{32}p]-ATP and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10 % gels. A. Stained gel. B. Autoradiogram.

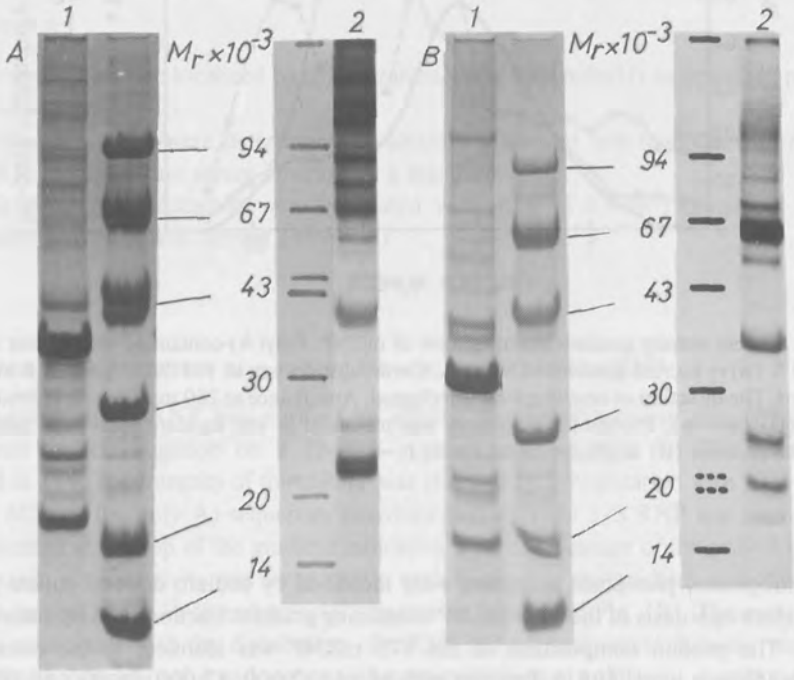

FIG. 3. Comparison of protein composition and endogenous phosphate acceptors of mRNP purified by sucrose density gradient centrifugation and gel filtration. Proteins were analyzed on 10 % sodium dodecyl sulfate polyacrylamide gels. A. mRNP purified by sucrose density gradient centrifugation (fraction 11 of Fig. 1). B. mRNP purified by gel filtration on Sepharose 4B. (see fraction 104, Thoen *et al.*, 1984, Fig. 4). Lane 1, stained gel ; lane 2, autoradiogram.

Additional evidence for the association of protein kinase activity with mRNP was obtained from the analysis of poly(A)-containing mRNP by gel filtration. The same results were obtained as with sucrose gradient centrifugation (Thoen *et al.*, 1984). The protein composition and the endogenous phosphate acceptors of mRNP purified by gel filtration (Fig. 3) were comparable to those of mRNP purified by sucrose density gradient centrifugation with the exception that some additional proteins with M_r of 125 000, 112 000, 105 000, 87 000 and 76 000 were more phosphorylated by the protein kinase present in the sucrose gradient fractions (Fig. 3A). On the other hand, two proteins with M_r 26 000 and 68 000 were phosphorylated to a greater extent in mRNP purified by gel filtration (Fig. 3B). The fractions obtained by gel filtration which contained maximal protein kinase activity were subsequently analysed by sucrose density gradient centrifugation (Fig. 4). Poly(A)-sequences were localized by hybridization with [^{3}H]-poly(U) and protein kinase activity was assayed using casein as a substrate. The poly(A)-sequences and the protein kinase activity were completely coincident with the absorbance at 280 mμ in agreement with the association of protein kinase with mRNP.

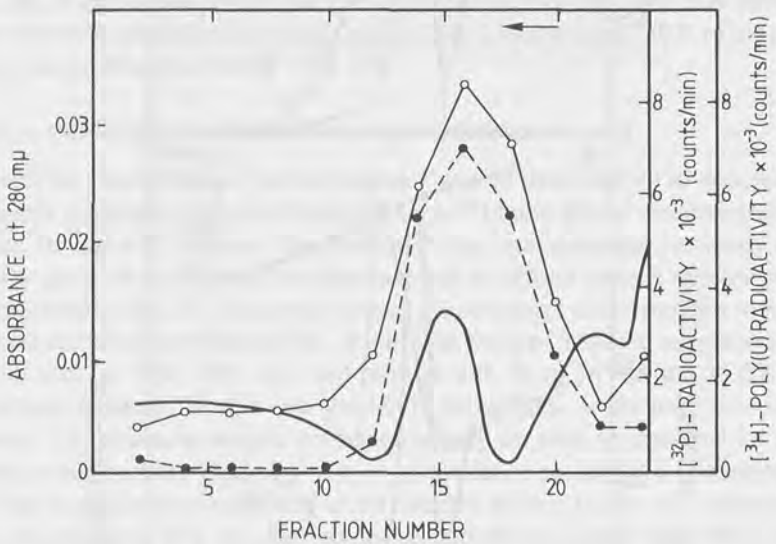

FIG. 4. Sucrose density gradient centrifugation of mRNP purified by gel filtration. Poly(A)-containing mRNP was chromatographed on Sepharose 4B as described by Thoen *et al.*, 1984. Fractions with maximal protein kinase activity were centrifuged on a 10-30 % (w/v) sucrose gradient in buffer A. Centrifugation was at 110 000 × *g* for 16 h in a Beckman SW41 rotor. Absorbance at 280 mμ (——). Hybridization with [^{3}H]-poly(U) (●----●). Protein kinase activity measured by the standard assay with casein as phosphate acceptor (○——○). The direction of sedimentation is indicated.

The integrity of 17S poly(A)-containing RNP was further demonstrated by estimation of the buoyant density by sucrose isopycnic centrifugation of RNP previously purified by gel filtration on Sepharose 4B. The poly(A)-containing mRNP was localized by measurement of the absorbance at 280 mμ and by hybridization with [^{3}H]-poly(U) (Fig. 5A). Protein kinase activity was measured with the standard assay with casein as exogenous substrate (Fig. 5B). The mRNP is

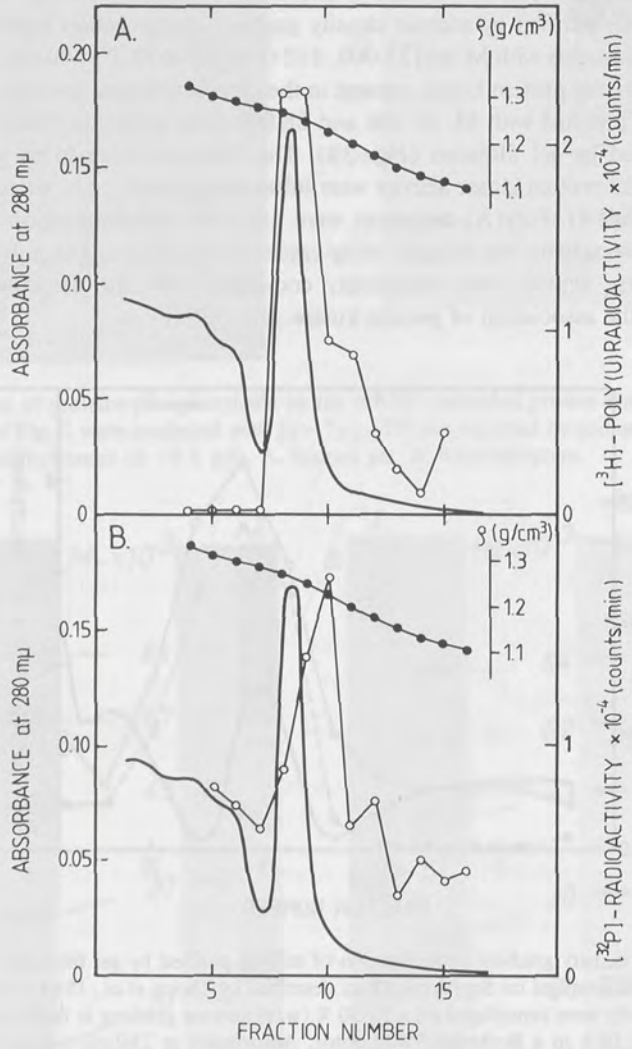

FIG. 5. Isopycnic centrifugation of mRNP purified by gel filtration. Poly(A)-containing mRNP purified by gel filtration on Sepharose 4B (Thoen *et al.*, 1984) was subjected to isopycnic centrifugation in sucrose. Centrifugation was at 251 800 × *g* for 90 h in a Beckman SW41 rotor. Absorbance at 280 mμ (——). Sucrose density (●——●). A. Hybridization with [^{3}H]-poly(U) (○——○). B. Protein kinase activity measured by the standard assay with casein as phosphate acceptor (○——○).

banded at a density of 1.25-1.26 g/cm^3 in agreement with previous results (Slegers *et al.*, 1981). The [^{3}H]-poly(U) radioactivity and absorbance at 280 mμ were coincident indicating the integrity of mRNP particles. The protein kinase activity is slightly displaced to a lower density of 1.24 g/cm^3. Apparently the enzyme is dissociated from mRNP in the latter conditions. However further experiments demonstrated that this conclusion is premature. Fractions of the isopycnic sucrose density gradient with the maximal absorbance at 280 mμ (fraction 8, Fig. 5A) and with the maximum of protein kinase activity (fraction 10, Fig. 5A) were further analyzed by centrifugation on 10-30 % (w/v) linear sucrose density gradient (Fig. 6). Both gradients were assayed for poly(A)-sequences and protein kinase activity as above. The poly(A)-sequences were associated with the 17S mRNP. However, two peaks of protein kinase activity were observed, one associated with the mRNP complex and one sedimenting more slowly, probably corresponding to protein kinase dissociated from 17S mRNP (Fig. 6A). The buoyant density fraction with maximal protein kinase activity (Fig. 6B) showed that the majority of the enzyme sedimented with a lower velocity and only a small fraction of the enzyme was still associated with the poly(A)-containing mRNP. We may conclude that protein kinase activity is associated with the 17S poly(A)-containing mRNP but due to the strong centrifugal force in isopycnic centrifugation the enzyme is partially dissociated from mRNP. Approximately 50 % of the protein kinase activity remained bound to mRNP (Fig. 6A).

Enzymatic properties of mRNP associated protein kinase

Two enzyme conformations (protein kinase 1 and 2) were purified to near homogeneity by ion exchange chromatography on phosphocellulose P11 and affinity chromatography on casein-Sepharose 4B and ATP agarose. The purification has been described previously (Thoen *et al.*, 1984). The purity of the enzymes was demonstrated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Fig. 7). The protein kinase 1 is composed of two proteins with M_r of 36 500 and 33 000 and a minor protein of M_r 28 000. The three proteins are autophosphorylated after incubation with [γ - ^{32}p] ATP. Only two proteins with M_r of 36 500 and 28 000 are observed in the protein kinase 2. In this case the M_r 28 000 protein is the main autophosphorylated component. The molecular weights are approximately the same as measured for the α, α' and β subunits of other protein kinases. α' may be a degradation product of α (Hathaway and Traugh, 1982). The sedimentation coefficient of the purified protein kinase was measured by sucrose gradient centrifugation (Fig. 8). The enzyme sedimented as a single peak with a sedimentation coefficient of 8.2 S $\pm$ 0.3 S. The latter value is equivalent with a molecular weight of approximately 136 000 $\pm$ 4 000 dalton. A molecular weight of 150 000 $\pm$ 5 000 dalton was estimated by high pressure liquid chromatography on Spherogel TSK G3000 SW (Fig. 9). Experiments have been carried out to determine optimal conditions for protein kinase activity.

The kinase reaction has an absolute requirement for either Mg^{2+} or Mn^{2+} as a divalent cation (Fig. 10A). A plateau of maximum activity was obtained at about 10-12 mM. Approximately the same maximum activity was obtained with either Mn^{2+} and Mg^{2+}.

The activity of the mRNP kinase is inhibited by KCl (Fig. 10B). This inhibition may be explained by a decreased affinity of the enzyme for the protein substrates or alternatively, the salt may alter the integrity of the kinase. The enzyme showed little activity at a pH of less than 6.5 and above a pH of 9. Optimal enzymatic activity was found in the range of 7.5 - 8.5 with a maximum at pH = 7.75 (Fig. 10C). A similar pH-dependence has also been described by Cardelli and Pitot (1980) for a polysomal mRNP associated protein kinase.

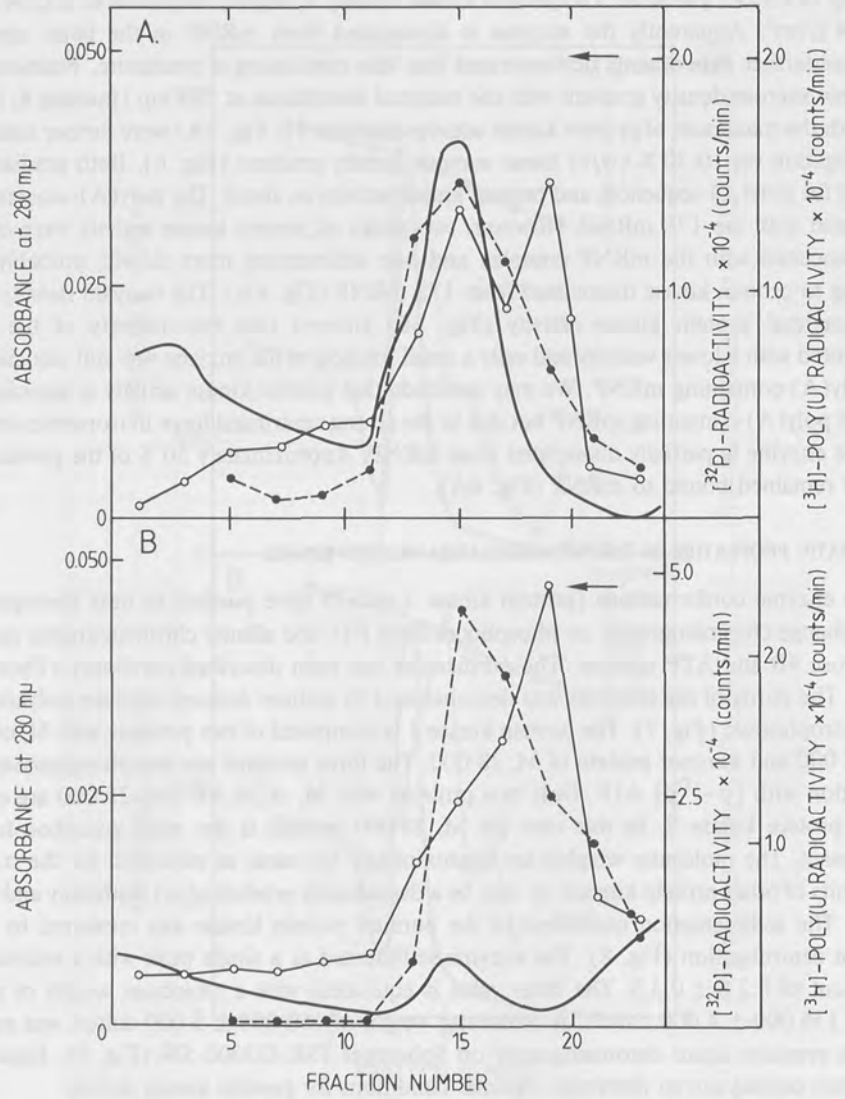

FIG. 6. Sucrose density gradient centrifugation of mRNP purified by isopycnic centrifugation. Fractions from the experiment of Fig. 5 with maximal absorbance (fraction 8) (A) and maximal kinase activity (fraction 10) (B) were centrifuged on a 10 %-30 % (w/v) sucrose density gradient in buffer A. Centrifugation was at 110 000 × *g* for 16 h in a Beckman SW41 rotor. The direction of centrifugation is indicated. Hybridization with [^{3}H]-poly(U) (●----●). Protein kinase activity was measured by the standard assay with casein as phosphate acceptor (○——○).

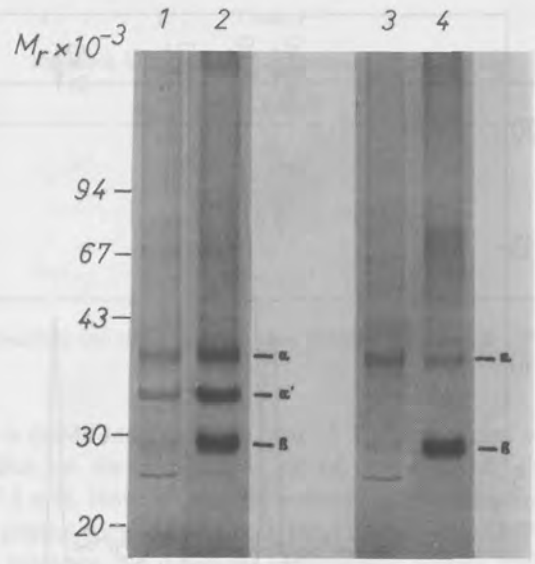

FIG. 7. Composition of mRNP associated protein kinase. The protein kinase was purified as described (Thoen *et al.*, 1984) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane 1, stained gel of protein kinase 1 ; lane 2, autoradiogram of the autophosphorylated protein kinase 1 ; lane 3, stained gel of protein kinase 2 ; lane 4, autoradiogram of the authophosphorylated protein kinase 2. Phosphorylation was in the standard kinase assay.

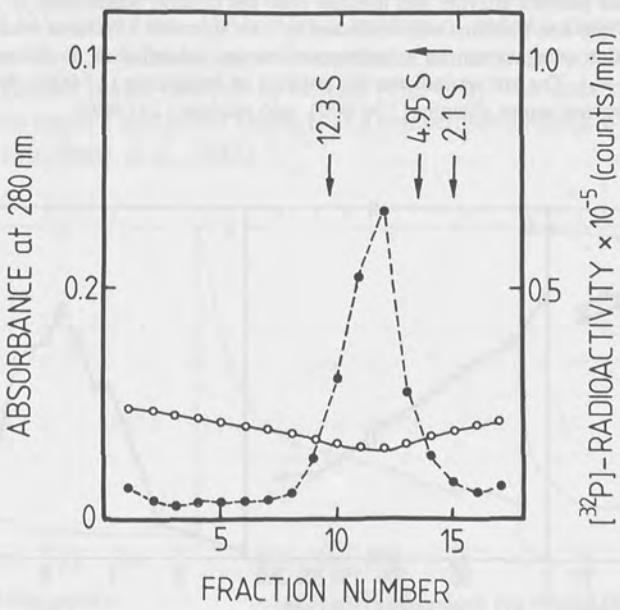

FIG. 8. Sedimentation of protein kinase. mRNP associated protein kinase was centrifuged on 10 %-30 % (w/v) sucrose gradients. Centrifugation was in the Beckman SW40 rotor at 155 000 × *g* for 16 h. Positions of protein standards are indicated : catalase (12.3 S), ovalbumin (4.9 S), and cytochrome C (2.1 S). Protein kinase activity (●----●). Absorbance at 280 mμ (○——○).

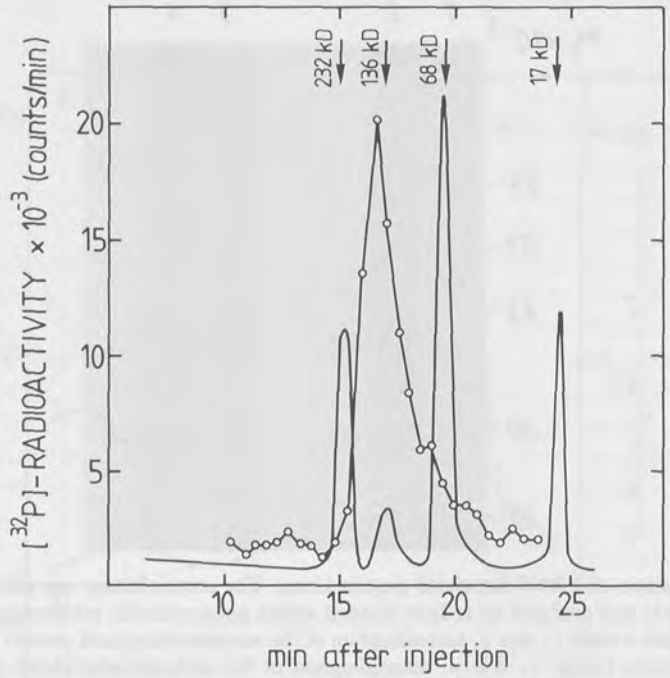

FIG. 9. Gel filtration of protein kinase on a TSK G3000 SW HPLC column (60 cm × 7.5 mm). An aliquot (100 µl) of the purified enzyme was injected onto the column equilibrated at 20 °C in buffer. The flow rate was 1.0 ml/min and fractions were collected at 30 s. intervals. Fractions were assayed in standard kinase assay conditions using casein as exogenous substrate. Absorbance at 220 nm (——), [^{32}P] – incorporation (○——○). The arrows indicate the position of myoglobin (17 000), bovine serum albumin (68 000), dimer of bovine serum albumin (136 000), and catalase (232 000).

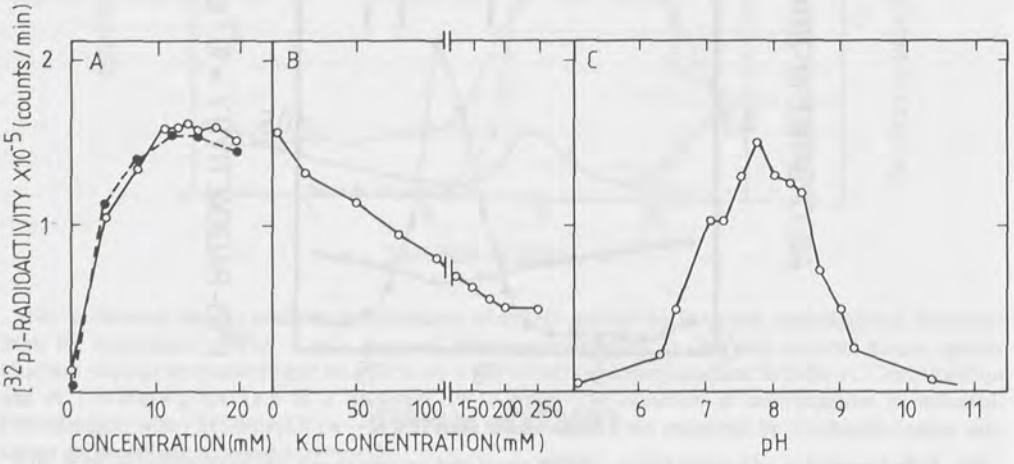

FIG. 10. Enzymatic properties of mRNP associated protein kinase. The effect of (A) Mg^{2+} (○——○) and Mn^{2+} (●----●), (B) KCl and (C) pH was measured in the standard kinase assay using casein as phosphate acceptor.

TABLE I

Influence of cyclic AMP on protein kinase activity

Exogenous substrate	mM cAMP	% ^{32}p incorporated
Casein	0	100 [1]
	$0.5.10^{-6}$	108
	$0.5.10^{-3}$	104
	0.5	87
Histone	0	7
	$12.5.10^{-3}$	7

[1] The incorporation of radioactivity into casein in the absence of cAMP was taken as 100 %.

The protein kinase is cAMP-independent (Table I). Concentrations of cAMP up to 0.5 µM had no detectable effect on the phosphorylation of casein. cAMP is even inhibitory at a concentration above 0.5 mM. However, the latter concentration is higher than the cellular level and probably has no physiological significance. The influence of cAMP was also tested using histone as exogenous substrate. No difference in enzymatic activity could be detected with or without the cyclic nucleotide.

The protein kinase associated with mRNP has been identified as a casein kinase II by the use of specific inhibitors of the enzyme (Fig. 11). Heparin and poly(L)-glutamic acid are found to be potent inhibitors of casein kinase II and do not affect casein kinase I (Hathaway *et al.*, 1980 ; Meggio *et al.*, 1983). The mRNP associated protein kinase is completely inhibited at a heparin concentration of 0.375 µg/ml (Fig. 11A). Also a mixture of poly(L)-glutamic acids strongly inhibited the enzyme and 50 % inhibition is observed at a concentration of 10 µM (Fig. 11C). The activity of the enzyme is also inhibited by caffeine (Fig. 11B). The latter substance is known to inhibit both casein kinase I and casein kinase II as a competitive inhibitor with respect to ATP (Lecomte *et al.*, 1980, Plana *et al.*, 1982).

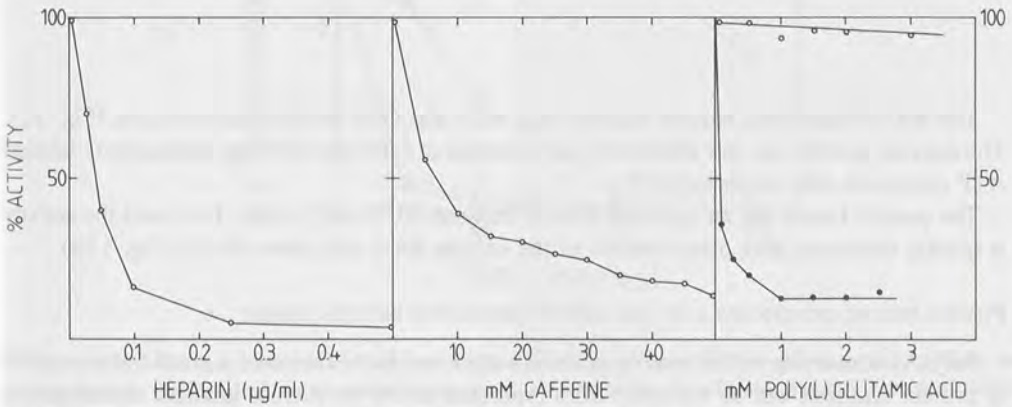

FIG. 11. Identification of the protein kinase as a casein kinase II. The inhibition of the enzyme by heparin (A), caffeine (B) and poly(L)-glutamic acids (C) was measured in the standard kinase assay with casein as phosphate acceptor.

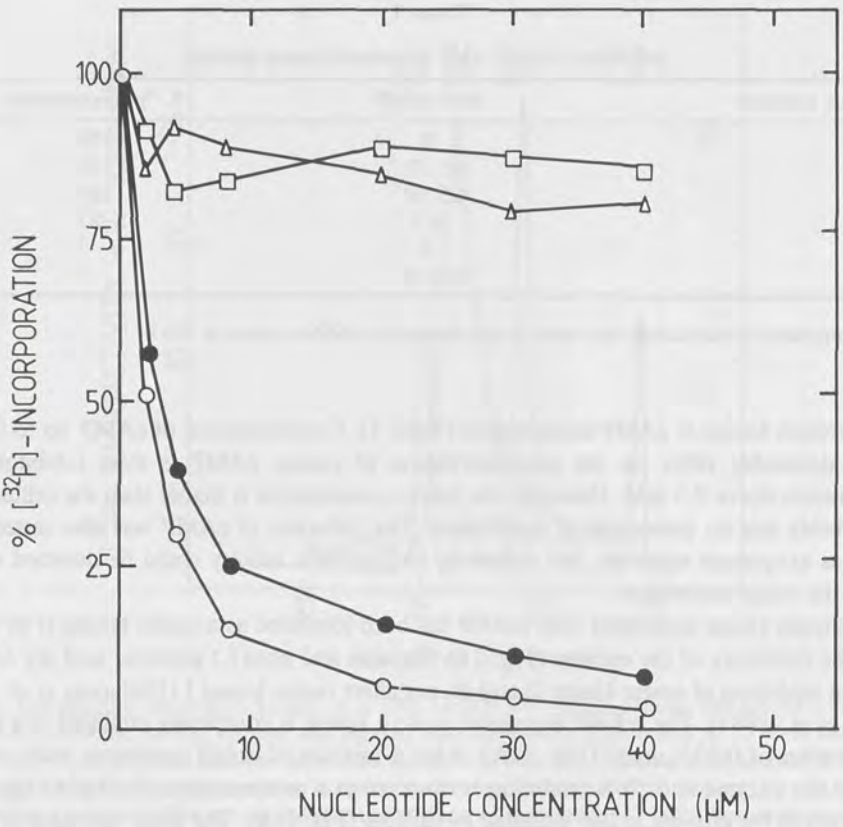

FIG. 12. Identification of the nucleotide phosphate donors used by the protein kinase. Competition between [γ - ^{32}p]-labelled ATP (1 μM) and a varying concentration of unlabelled ATP (○——○), GTP (●——●), CTP (△——△), and UTP (□——□) was investigated in standard assay conditions.

The mRNP associated enzyme utilized both ATP and GTP as phosphoryl donors (Fig. 12). The enzyme activity was not affected by the presence of CTP and UTP but radioactivity labeled ATP competed with unlabeled GTP.

The protein kinase has an optimum activity between 30 °C-40 °C (Fig. 13A) and the activity is quickly destroyed after preincubation of the enzyme for 5 min above 56 °C (Fig. 13B).

PHYSIOLOGICAL SIGNIFICANCE OF THE mRNP ASSOCIATED PROTEIN KINASE

Poly(A)-containing mRNP mainly exists in a repressed form. However, a small fraction active in protein synthesis can be separated from repressed mRNP by sucrose gradient centrifugation or by gel filtration on Sepharose 4B (Thoen *et al.*, 1984 ; De Herdt *et al.*, 1985). The translational activity is correlated with the presence of eIF2. mRNP purified by gel filtration was pooled, concentrated by ultrafiltration and translated in a rabbit reticulocyte lysate. The observed

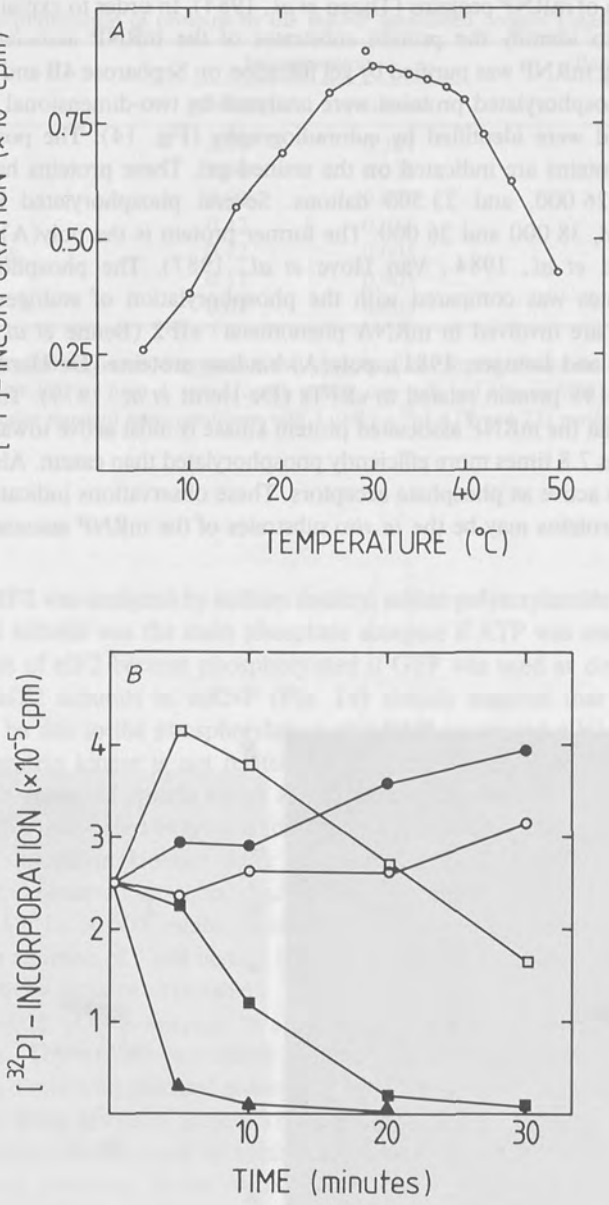

FIG. 13. Stability of the protein kinase. A. The temperature optimum for the enzymatic activity was measured in the standard kinase assay using casein as a substrate. Incubation was for 30 min at varying temperatures. B. The enzyme was preincubated for 5 min at 37 °C (○——○), 45 °C (●——●), 50 °C (□——□), 52 °C (■——■), and 56 °C (▲——▲). The remaining activity of the enzyme was subsequently measured in standard assay conditions using casein as a substrate.

translational activity was completely inhibited after preincubation of mRNP with ATP and is due to phosphorylation of mRNP proteins (Thoen *et al.*, 1984). In order to explain this phenomenon it was necessary to identify the protein substrates of the mRNP associated protein kinase. Poly(A)-containing mRNP was purified by gel filtration on Sepharose 4B and was incubated with [γ - ^{32}p] ATP. Phosphorylated proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis and were identified by autoradiography (Fig. 14). The positions of the main phosphorylated proteins are indicated on the stained gel. These proteins have M_r of 125 000, 65 000, 38 000, 26 000, and 23 500 daltons. Several phosphorylated ionic species were observed for the M_r 38 000 and 26 000. The former protein is the poly(A)-binding protein of mRNP (De Herdt *et al.*, 1984 ; Van Hove *et al.*, 1987). The phosphorylation of several exogenous substrates was compared with the phosphorylation of endogenous proteins. The assayed substrates are involved in mRNA phenomena : eIF2 (Benne *et al.*, 1979), ribosomal proteins (Reichert and Issinger, 1981), poly(A)-binding proteins (De Herdt *et al.*, 1984) and the RNA-binding 19S protein related to eEFTs (De Herdt *et al.*, 1979). The results presented in Table II show that the mRNP associated protein kinase is most active towards eIF2. The latter initiation factor was 7.8 times more efficiently phosphorylated than casein. Also the poly(A)-binding proteins were active as phosphate acceptors. These observations indicate that eIF2 and the poly(A)-binding proteins may be the *in vivo* substrates of the mRNP associated protein kinase.

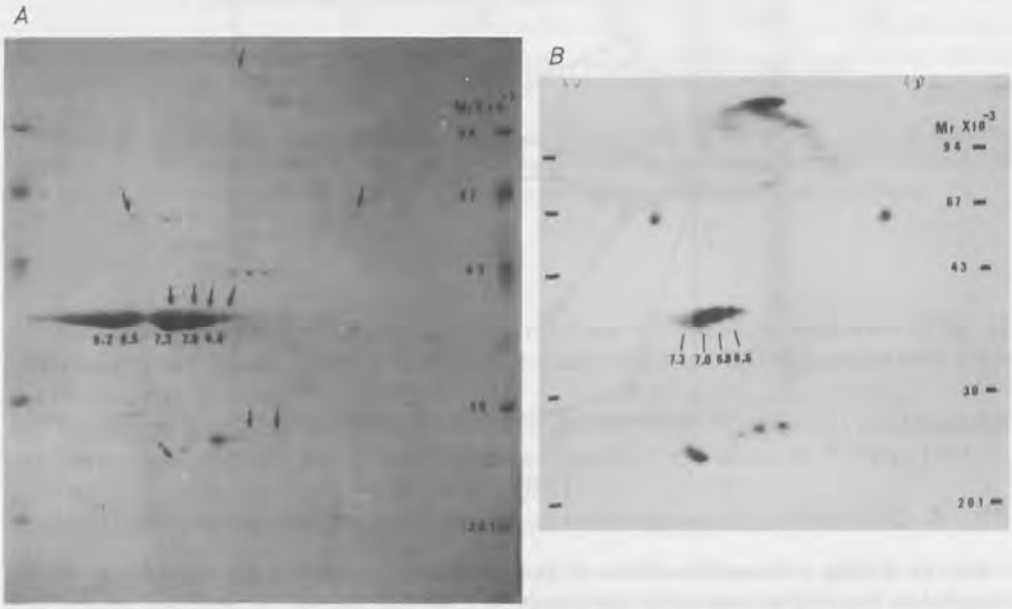

FIG. 14. Identification of endogenous phosphorylated mRNP protein by two-dimensional gel electrophoresis. Electropheresis is as described by O'Farrell (1975). The stained gel (A) is dried and autoradiographed (B).

TABLE II

Phosphorylation of proteins by the mRNP associated protein kinase

Acceptor	Incorporation		Percentage [1]
	pmol/μg	mol/mol	
eIF2	2.14	0.301	782
Casein	1.30	0.038	100
P23	0.90	0.020	47
P38	0.42	0.016	36
80 S ribosomal proteins	0.12	0.005	8
Histone	0.17	0.005	5
19 S protein	0.07	0.005	5

[1] The radioactivity incorporated into casein, expressed as mol phosphate/mol protein is taken as 100 %. eIF2, P23, P38, 80 S ribosomal and 19 S protein are from *A. salina*, casein is from cow milk and histone from calf thymus. Phosphorylation was performed under standard assay conditions with 1 μM[γ - ^{32}p]-ATP and 2 U purified protein kinase.

Phosphorylated eIF2 was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 15). The α subunit was the main phosphate acceptor if ATP was used as donor while the α and β subunits of eIF2 became phosphorylated if GTP was used as donor. The absence of phosphorylated eIF2 subunits in mRNP (Fig. 14) already suggests that the inhibition of translation may not be due to the phosphorylation of mRNP-associated eIF2. Furthermore the mRNP associated protein kinase is not related to the heme regulated protein kinase and the double stranded RNA activated protein kinase known as the enzymes which phosphorylate eIF2.

Although the mRNP associated enzyme is inhibited by hemin (Fig. 16) as the heme regulated protein kinase, the inhibition kinetics differ significantly. A 50% inhibition of the hemin-controlled repressor is observed at around 5 μM hemin and the activity is completely inhibited at 10 μM (Traugh, 1981). A 50% inhibition of the mRNP – associated protein kinase can be accomplished by the addition of 7 μM hemin, but even at a concentration of 80 μM hemin 43 % of the enzymatic activity remains detectable.

The molecular weight of both enzymes is approximately the same but they have a different quaternary structure. The mRNP associated enzyme has a $\alpha_2\beta_2$ structure while the heme regulated kinase is a dimer with identical subunits of Mr 80 000-105 000. The non-identity with the double stranded RNA activated protein kinase is obvious from the results of Fig. 17. The mRNP associated enzyme is inhibited by synthetic double stranded RNA.

So the most likely substrate of the mRNP associated protein kinase is the M_r 38 000 poly(A)-binding protein. This protein exists in several ionic forms with a pI of 9.2, 8.5 and 7.3 (De Herdt *et al.*, 1984). The phosphorylation of the latter protein has been studied in detail and results in the generation of several acidic species with a pI of 8.5, 7.3, 7.0, 6.8, and 6.6 (De Herdt *et al.*, 1984 ; Van Hove *et al.*, 1987). The acidic species were not observed in mRNP-ribosome pre-initiation complexes suggesting that phosphorylation/dephosphorylation of the poly(A)-binding protein is involved in the regulation of mRNP translation.

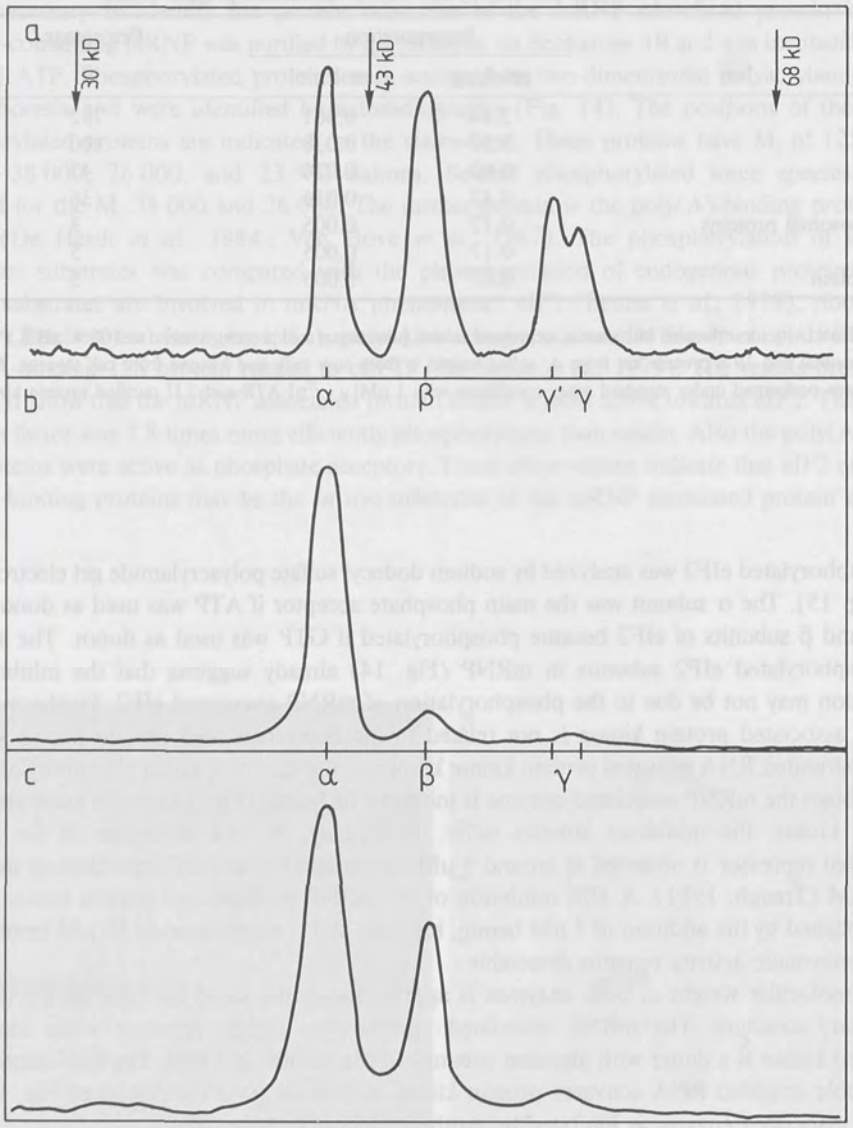

FIG. 15. Analysis by sodium dodecyl sulfate polyacrylamide gel-electrophoresis of the phosphorylation of eIF2 by the purified mRNP associated protein kinase. 25 µg eIF2 was phosphorylated in standard conditions using ATP and GTP as phosphoryl donors. A. Laser densitograms of stained gel. B. Autoradiogram of phosphorylated eIF2 with ATP as donor. C. Autoradiogram of phosphorylated eIF2 with GTP as donor.

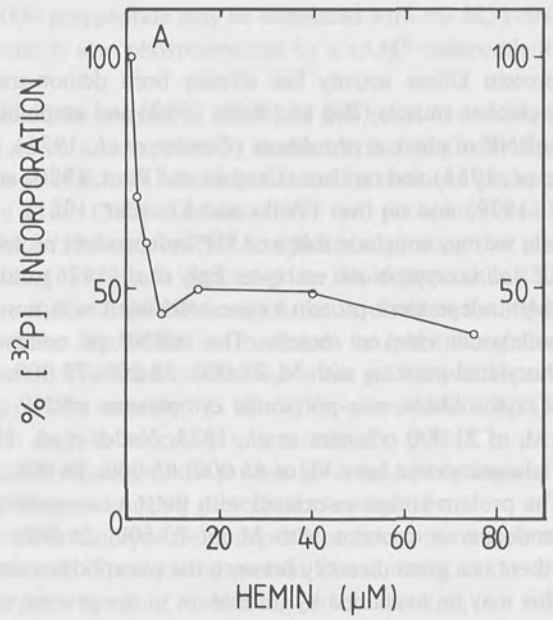

FIG. 16. Effect of hemin on protein kinase activity. The effect was measured in the standard protein kinase assay using casein as a substrate.

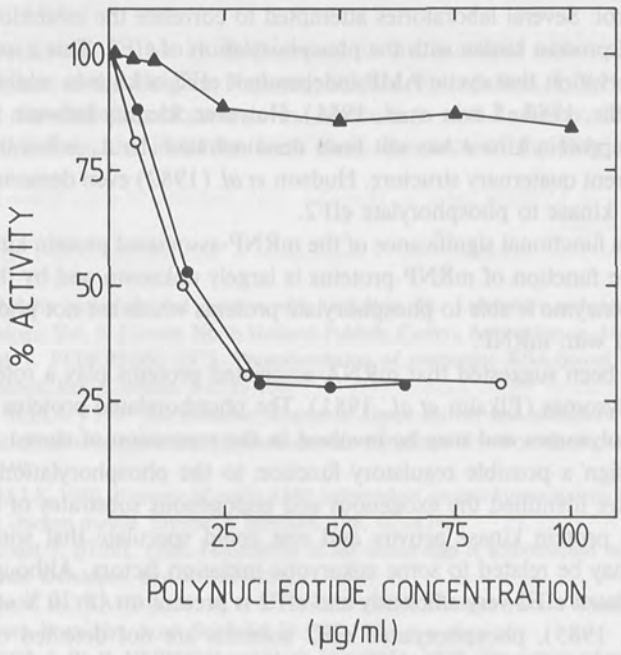

FIG. 17. Effect of synthetic ribonucleic acids on protein kinase activity. Varying concentrations of poly(A) : poly(U) (○——○), poly(G) : poly(C) (●——●) and poly(A) (▲——▲) were added to the standard protein kinase assay. Casein was used as a substrate.

Discussion

The presence of protein kinase activity has already been demonstrated in RNA-binding proteins of embryonic chicken muscle (Bag and Sells, 1980) and amphibian oocytes (Stepanov *et al.*, 1982), in free mRNP of chick erythroblasts (Gander *et al.*, 1973), Hela cells and mouse plasmacytoma (Egly *et al.*, 1981) and rat liver (Cardelli and Pitot, 1980) and in hnRNP of HeLa cells (Periasamy *et al.*, 1979) and rat liver (Wilks and Knowler, 1981).

From the present data we may conclude that a cAMP-independent protein kinase is associated with stored mRNP of *A. salina* cryptobiotic embryos. Egly *et al.* (1976) and Bag and Sells (1979) also have found a cAMP-independent protein kinase associated with non-polysomal mRNP of plasmacytoma and embryonic chicken muscle. The mRNP of embryonic chicken muscle contained four phosphorylated proteins with M_r 27 000, 38 000, 73 000, and 75 000 (Bag and Sells, 1979). In avian erythroblasts, non-polysomal cytoplasmic mRNP contained at least one phosphoprotein with M_r of 21 000 (Gander *et al.*, 1973, Nudel *et al.*, 1973). Phosphorylated proteins in mRNP of plasmacytoma have M_r of 45 000, 65 000, 76 000, 90 000, and 125 000 (Egly *et al.*, 1976). The protein kinase associated with poly(A)-containing mRNP of *A. salina* phosphorylated five endogenous proteins with M_r of 23 500, 26 000, 38 000, 65 000, and 125 000. Apparently there is a great diversity between the phosphoproteins identified in mRNP of different origins. This may be explained by differences in the protein composition of mRNP due to tissue specificity or to differences in the purification procedure.

It has been proposed that the mRNP-associated protein kinase is involved in the regulation of mRNA translation. In protein synthesis the step of initiation is most likely involved in translational control. Several laboratories attempted to correlate the inhibition of translation by mRNA-associated protein kinase with the phosphorylation of eIF2. This hypothesis was mainly based on the observation that cyclic AMP-independent eIF2-α kinases inhibited the translation of mRNA (Clemens, 1980 ; Levin *et al.*, 1981). However, identity between these enzymes and mRNP-associated protein kinase has not been demonstrated. In *A. salina* these enzymes have a completely different quaternary structure. Hudson *et al.* (1982) even demonstrated the inability of mRNP protein kinase to phosphorylate eIF2.

The study of the functional significance of the mRNP-associated protein kinase is complicated by the fact that the function of mRNP proteins is largely unknown and by the observation that in a free state the enzyme is able to phosphorylate proteins which are not phosphorylated by the enzyme associated with mRNP.

Recently it has been suggested that mRNA-associated proteins play a role in the passage of free mRNP to polysomes (Elkaim *et al.*, 1981). The phophorylated proteins of free mRNP are not observed in polysomes and may be involved in the repression of stored mRNP.

In order to assign a possible regulatory function to the phosphorylation in the repression mechanism we have identified the exogenous and endogenous substrates of the non-polysomal mRNP-associated protein kinase activity and one could speculate that some phosphorylated mRNP proteins may be related to some eukaryotic initiation factors. Although purified protein kinase phosphorylated eIF2 very efficiently and eIF2 is present on 15-30 % of the stored mRNP (De Herdt *et al.*, 1985), phosphorylated eIF2 subunits are not detected on mRNP by two-dimensional gel electrophoresis. Although this result indicates that the enzyme is not functional in translational control by phosphorylation of eIF2 it is not unlikely that eIF2 is dissociated from mRNP in the purification procedure of the phosphorylated mRNP proteins.

Only the M_r 125 000 polypeptide may be compared with the M_r 110 000-120 000 subunit of eIF3. The latter subunit is also phosphorylated by a cAMP-independent protein kinase (Benne *et al.*, 1978).

The main substrate of the enzyme is the M_r 38 000 poly(A)-binding protein. This protein is separated in at least seven ionic species by isoelectric focusing with a pI of 6.6 up to 9.2. The acidic species with a pI of 6.6 to 7.3 are generated by multisite phosphorylation (De Herdt *et al.*, 1984 ; Van Hove *et al.*, 1987). The most basic non-phosphorylated species with a pI of 8.5 and 9.2 were observed in ribosome-mRNP complexes suggesting that phosphorylation of the M_r 38 000 poly(A)-binding protein is restricted to the repressed mRNP. The protein kinase associated with non-polysomal mRNP seems to be involved in the regulation of the repression of stored mRNP. This regulation is mediated by phosphorylation of the M_r 38 000 poly(A)-binding protein. The exact mechanism is not yet known. However, phosphorylation of the poly(A)-binding protein increased its affinity for ribonucleic acids (Van Hove *et al.*, 1987). The latter association may be necessary to maintain the mRNP in a repressed state. Dephosphorylation reduced the capacity of the protein for poly(A)-binding and could be a prerequisite for the formation of ribosome-mRNP preinitiation complexes.

In *A. salina* cryptobiotic embryos two independent mechanisms seem to control the repression of stored mRNP ; the mRNP associated protein kinase acting at the level of mRNP-ribosome association phosphorylates the poly(A)-binding protein resulting in a reduced binding of mRNP to ribosomes and mRNP associated iRNA acting at the level of initiation (De Herdt *et al.*, 1985).

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Periodicity in elongation of oligo (A) tracts of RNA in developing embryos of *Artemia*: the role of RNP associated poly (A) polymerase

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Abstract

Analysis of chain lengths of the 3'oligo(A) stretches of poly(A)RNA species from the subcellular fractions of the dormant and developing embryos of *Artemia* has revealed an interesting periodicity of about 30 nucleotides in their chain lengths. The pattern of labelling of the newly synthesized oligo(A) tails of the membrane and nuclear RNAs also revealed a similar periodicity of their formation. The increase in poly(A) RNA levels of the membrane fraction, a feature characteristic of early development, is accompanied by an increase in the level of the major polyadenylating enzyme, poly(A) polymerase in these membranes.

Poly(A) polymerase from the membranes has been shown to be present as a RNP complex, which is resistant to RNases and is in firm association with the 3' terminal oligo(A) stretches (about 30 residues) of RNA. This observation along with the characterization of the product length of the *in vitro* reactions of poly(A) polymerase tend to implicate this enzyme in the step-wise elongation of the poly(A) tails of the pre-formed and newly synthesized RNAs. Polyadenylation may thus be an important process for the activation of dormant RNA species during reinitiation of development.

Introduction

Activation of the dormant cysts of *Artemia* results in the reinitiation of arrested development in the absence of any DNA and RNA synthesis (Nakanishi *et al.*, 1962 ; Clegg and Golub, 1969). Thus this system has provided us with a model for the study of the mechanism of utilization of the pre-formed and stored messengers.

The presence of latent messengers has been demonstrated in a variety of developmental systems such as sea urchins and plant seeds. The stored and newly synthesized mRNAs are associated with proteins ("informosomes") that govern their utilization in protein synthesis (Preobrazhensky and Spirin, 1978). The location of latent mRNAs in association with membrane fractions of the dormant embryos of *Artemia* has been demonstrated (Simons *et al.*, 1978 ; Nilsson and Hultin, 1982). While it has been clearly established that reinitiation of development of the cryptobiotic embryos is accompanied by an active mobilization of the latent messengers to polysomes (Hultin and Morris, 1968), the precise nature of the mechanism of activation of the messengers remains unclear.

The presence of translatable poly(A) RNA in the dormant cysts of *Artemia* has been unequivocally established (Felicetti *et al.*, 1975 ; Nilsson and Hultin, 1975). On account of the impermeability of *Artemia* cysts to metabolic precursors of macromolecules, it has been difficult to follow the turnover of these molecules on activation. We have successfully used the addition

of carrier-free 32P-phosphate to the activation medium, to label the macromolecules (Susheela and Jayaraman, 1976). We have earlier demonstrated that the reinitiation of development of the cryptobiotic embryos of *Artemia* is accompanied by an active labelling of the poly(A) stretches of the pre-formed RNAs (Jeyaraj *et al.*, 1982). This was especially true of the RNAs present in the membrane fraction, comprising cytoplasmic and nuclear membranes. This fraction harboured about 60 % of the newly labelled poly(A) RNA at 4 h of activation and the label was located exclusively in the 3' oligo(A) stretches of the RNA indicating that the polyadenylation of the pre-formed RNA in these fractions is important for development.

Early development of *Artemia* is characterized by a steady increase of poly(A) RNA levels (James and Tata, 1980 ; Jeyaraj *et al.*, 1982). Much of this increase has been shown by us to be due to the increase in the membrane poly(A) RNA levels. Since during early development, the levels of the major polyadenylating enzyme poly(A) polymerase has been shown to register a sharp increase in its levels (Jeyaraj *et al.*, 1980 ; Sastre and Sebastian, 1980, 1983). We decided to look into the mechanism of polyadenylation of the pre-formed RNA species of *Artemia*, since this may lead us to an understanding of the mechanisms of capacitation of stored mRNAs of the cryptobiotic embryos.

Methods

Embryos – subcellular fractions – RNA isolation

Encysted *Artemia* embryos (a parthenogenetic strain) were collected from the salt pans near Tuticorin, on the southeastern coast of India. The processing of the embryos prior to their storage and their activation in 1 % saline have been described earlier (Susheela and Jayaraman, 1976 ; Raja *et al.*, 1983).

RNA was prepared from the subcellular fractions of the embryos activated to the desired stage in presence of carrier-free 32P-phosphate (BARC, Bombay, India) as described earlier (Jeyaraj *et al.*, 1982). The poly (U) binding activity of the RNA samples was determined essentially as described by Sheldon *et al.* (1972).

Preparation of RNP particles

Dormant or activated embryos were homogenized in buffer 'A' (0.01 M Tris-HCl pH 7.6, 0.01 M KCl, 1.5 MM magnesium chloride, 0.0065 % β-mercaptoethanol, 0.13 % Triton X-100, 0.1 % diethyl pyrocarbonate and 2 units/ml of heparin) and the subcellular fractions obtained as described before (Jeyaraj *et al.*, 1982). The addition of Mg^{++} at a final concentration of 30 mM precipitated the RNP particles. (Jeffery 1977 ; Palmiter, 1974). The RNPs from the particulate fractions were obtained after sonication of these particles and sedimentation of the insoluble particles at 10 000 x *g* for 10 min prior to Mg^{++} addition. The RNPs were suspended in TKM buffer containing 50 mM Tris-HCl, pH 7.6, 50 mM KCl, and 1 mM magnesium chloride. The Mg^{++} precipitate was salt washed by adjustment of the KCl concentration to 500 mM and incubation on ice for 20 min. The salt washed RNPs were further purified by centrifugation through 5 ml of 20 % sucrose for 2 h at 36 000 rpm.

The purified RNP particles were suspended in TKM buffer and were fractionated in a 20-50 % discontinuous sucrose gradient in TKM by centrifugation at 30 000 x *g* for 2 h. Fractions of 0.2 ml were collected and analyzed for their absorbancy at 260 nm and 280 nm, poly(U) binding

capacity of RNA isolated from these fractions (Sheldon *et al.*, 1972) and poly(A) polymerase activity (Hadidi and Sethi, 1976).

Purification and estimation of poly(A) polymerase from the subcellular fractions of embryos

The nuclear and particulate fractions of the dormant and activated cysts were suspended in TME buffer (0.02 Tris-HCl pH 8.0, 0.5 mM EDTA, 0.2 mM β-mercaptoethanol) sonicated for 2 min at 0 °C, centrifuged at 5 000 rpm for 10 min and the supernatant used as the crude enzyme source.

As the majority of the PAP activity from the crude extract of the particulate fraction was not retained on the DEAE cellulose column (Jeyaraj *et al.*, 1982 ; Rose *et al.*, 1979) these extracts were mixed with DEAE cellulose (3 g cellulose/200 mg protein) and incubated in ice for 1 h after which the cellulose was spun down at 10 000 rpm for 10 min. The supernatants exhibiting poly(A) polymerase activity were pooled, lyophilized and loaded on to a phosphocellulose column (2.5 cm × 13 cm) pre-equilibrated with TME. The column was washed with 3 column volumes of the same buffer and the sample eluted with a linear salt gradient from 0.0 M-0.4 M KCl in TME. 4 ml fractions were monitored for absorbancy at 280 nm and assayed for poly(A) polymerase activity (Hadidi and Sethi, 1976). The protein samples were subjected to SDS-PAGE (Laemmli, 1970).

RNA binding activity of PAP

RNA binding activity of poly(A) polymerase was determined according to the method of Richter and Smith (1983). Purified PAP (1 µg protein) was mixed with different concentrations (1-5 µg) of labelled RNA in a total volume of 0.1 ml of binding buffer (10 mM Tris-HCl pH 7.0, 50 mM NaCl, and 1 mM EDTA) and incubated at 30 °C for 30 min. The solution was filtered through nitrocellulose filters (0.45 µm) under low suction, washed with binding buffer and after drying with ethanol the label retained was determined.

Poly(A) tract analysis

To characterise the lengths of poly(A) stretches of RNA, samples (approx. 5-10 absorbancy at 260 nm) in 200 µl of 0.01 M Tris-HCl pH 7.5, 0.2 M NaCl, and 0.02 M EDTA were digested with T1 RNase (4 units/ml) and pancreatic RNase A (4 µg/ml) for 10 min at 37 °C. The samples were treated with Sevag's mixture and the undigested oligo(A) stretches were precipitated by ethanol. The poly(A) tracts were analysed in a 10 % acrylamide-6M urea gel (1.5 mm × 160 mm × 120 mm) prepared in TEB buffer (10.8 g/l Tris 5.5 g/l boric acid, and 0.3 g/l EDTA, pH 8.3). The samples prepared in TEB buffer containing 0.005 % bromophenol blue and 10 % glycerol were heated at 60 °C for 5 min, immediately chilled on ice and loaded. Electrophoresis was performed at 140 volts till dye marker reached 3/4 th of the gel. The gel was stained and photographed under a short range UV source of 254 nm (Newman and Martin, 1982). The standard poly(A) markers (Miles) were used to calibrate the gel.

Isolation of poly(A) specific proteins

Proteins bound to poly(A)tail were isolated from the fractions showing poly(A)polymerase activity from DEAE cellulose column by poly(A) Sepharose 4B column by the procedure described by Sheldon *et al.* (1972).

Separation of RNase resistant subparticles of RNP

The gradient purified RNPs were treated with pancreatic RNase A (5 μg/ml) and T1 RNase (5 units/ml) and were layered on a 15-40 % linear sucrose gradient in TKM buffer and centrifuged at 41 000 rpm for 4 h. Fractions of 0.2 ml were collected and the absorbancy at 280 nm and poly(A) polymerase activity were determined after dialysis of the fractions. RNA was extracted from different peak fractions of the gradient and the length of the poly(A) tracts was analyzed on 10% acrylamide-6 M urea gels as described earlier.

Immunological detection of PAP

Antiserum against purified PAP was raised in rabbits by injecting the 46-48KD band of the PAGE gel. To quantify the amount present in the RNP particles 450 mg of agarose in barbitone buffer containing 2 ml of PAP antiserum and 8 ml of PBS was poured on a 21 × 7.5 cm glass plate. Sucrose gradient fractions after dialysis were lyophilized and loaded in the wells. Rocket immuno electrophoresis was carried out at 5 to 10 volts/cm for 2 h. After 72 h the plate was stained with 0.5 % Coomassie Brilliant Blue in 43 % methanol and 7 % acetic acid.

End labelling of RNA

The RNA extracted from the gradient fractions was dephosphorylated with alkaline phosphatase and end labelled with γ32P-phosphate ATP (Specific activity 3 000 ci/mM) using T4 poly nucleotide kinase (Maniatis *et al.*, 1982).

Results

Distribution of poly(A) RNA in the total and RNP particles of the membrane fraction during development

In order to know whether poly(A) RNAs labelled in the membrane fraction during early periods of activation of the dormant embryos, are present in a free or bound state, we had obtained RNP particles from these fractions after sucrose density gradient. The distribution of poly(A) RNA in the total and RNP fractions is outlined in Table I. The majority of the particulate poly(A) RNAs were present as RNP complex. The RNP was purified through a sucrose gradient to characterize the particles maximally enriched in poly(A) RNA (Fig. 1).

Table I

Poly(A)RNA content of the particulate fraction and RNP particles of the activated embryos of *Artemia*

Fraction	Cpm/mg RNA	Cpm bound to poly(U)filter per mg RNA	% poly (A)RNA
30 000 x*g* pellet	145 063	9 066	6.2
RNP particle	67 601	7 763	11.5
Gradient purified RNP	101 526	16 970	16.7

Cysts (500 mg) were activated in presence of (250 μci) carrier-free 32P-phosphate for 4 h. The RNA was extracted from the total particulate fraction and the RNP particles. The poly(A)RNA content was estimated as cpm/mg RNA bound to poly(U) filters/mg RNA × 100.

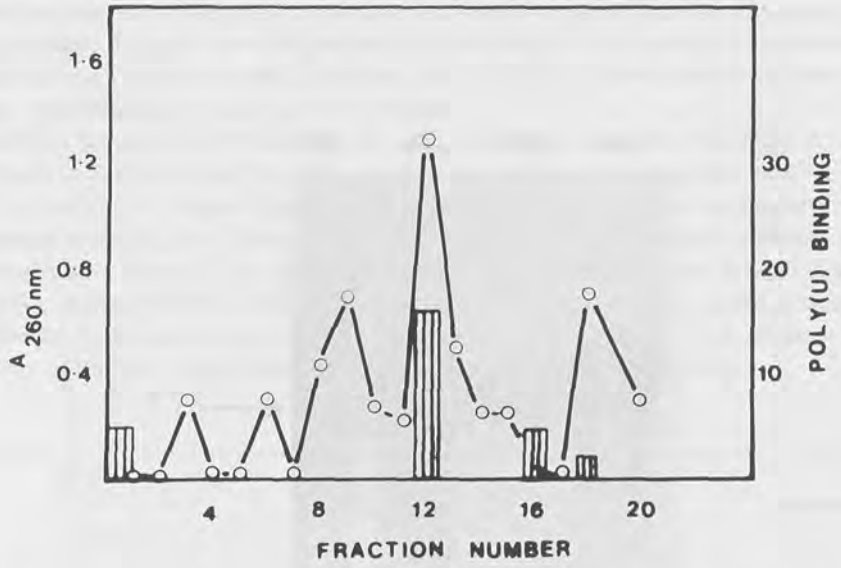


FIG. 1. Sucrose density gradient profile of RNP particles. Labelled RNP particles obtained from the membrane fractions of embryos activated for 4 h in presence of 32-phosphate, were layered on a 20-50 % discontinuous sucrose gradient and centrifuged at 30 000 x*g* for 2 h. Individual fractions (0.2 ml) were monitored for their absorbance at 280 nm. Labelled RNA extracted from these fractions were estimated for the poly(A) content by the ability to bind to poly(U) filters.

CHARACTERIZATION OF 3'OLIGO(A) TRACTS OF THE POLY(A) RNAS SYNTHESIZED DURING EARLY DEVELOPMENT

Labelled RNA species from the nuclear and cytoplasmic membrane fractions, analyzed in agarose-acrylamide gels revealed that two major species of RNA (27S and 29S), were predominantly labelled both in the total and RNP fractions (Fig. 2). Since it has been earlier demonstrated by us that the majority of the incorporated 32P-phosphate label (about 80 %) was present in the 3' oligo(A) stretches of the RNA which were resistant to pancreatic and T1 RNAses (Jeyaraj *et al.*, 1982), we followed the labelling kinetics of the oligo(A) tracts of these RNAs by separating the RNase resistant stretches in a 10% acrylamide- 6 M urea gel.

On activation of the embryos for 90 min, the poly(A) stretches of 25-35 residues of the membrane RNA were predominantly labelled (Fig. 3). At 120 min of activation, the label had declined in these stretches and the labelling of higher oligo(A) tracts *i.e.* A90, A120, and A160 increased. With nuclear RNA, higher oligo(A) tracts, (especially A120) were initially labelled, followed by the labelling of both shorter (A30) and longer (A90) oligo(A) stretches. However, in both the fractions the label was distributed in oligo(A) tracts that differed from each other by a periodicity of about 25 residues.

Analysis of the oligo(A) chain lengths of the unlabelled RNA of the various subcellular fractions of dormant and developing embryos has also revealed this interesting feature of periodicity of the oligo(A) stretches. The RNA from all fractions of the dormant embryos

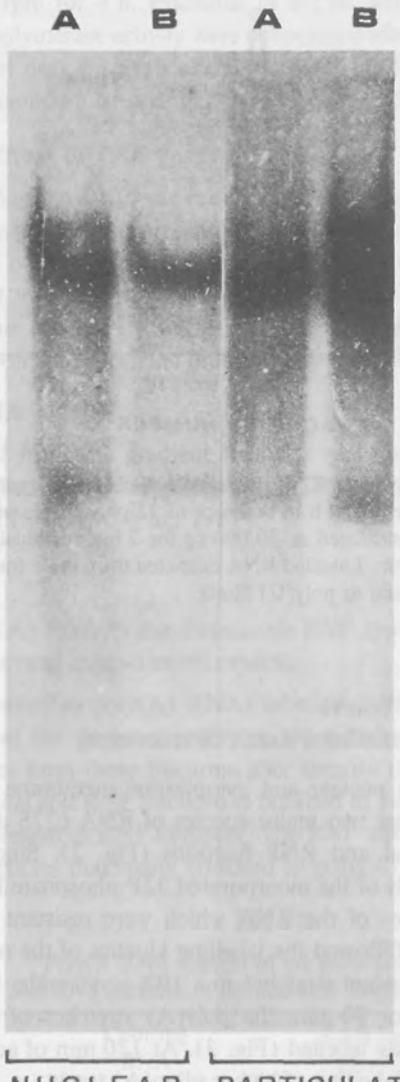


FIG. 2. Autoradiograph of labelled RNA from RNP particles of the nuclear and membrane fractions of the developing embryos. Labelled RNA obtained from the total and RNP particles of nuclear and membrane fractions of activated embryos were analysed in an acrylamide (2 %)-agarose (0.5 %) composite gel. RNA from lane A : nuclear : lane B : nuclear RNP : lane A : membrane ; lane B : membrane RNP.

revealed the presence of oligo(A) tracts from A25-A160 lengths (Fig. 4A). Ethidium bromide staining revealed that apart from the periodicity of the lengths of these oligo(A) stretches, which differed from each other by about 25 residues, the abundance of these species was also different with the predominance of A25 and A35 stretches.

In embryos activated for 90 min there was a sharp decline in the levels of all oligo(A) stretches except for A25-A35 stretches (Fig. 4B). At 3 h or later periods of development until pre-nauplius stage, the levels of the longer oligo(A) tracts increased, and the pattern was similar to those of the dormant cysts (Fig. 4). These results indicated that poly(A) RNA with different oligo(A) tract lengths were present in all the subcellular fractions of dormant cysts. A rapid degradation of the RNA species with larger oligo(A) tracts occurred during the early hours of activation. This was followed by the appearance of RNA with larger oligo(A) tracts. It is apparent that the periodicity of the tract lengths was being maintained throughout their synthesis.

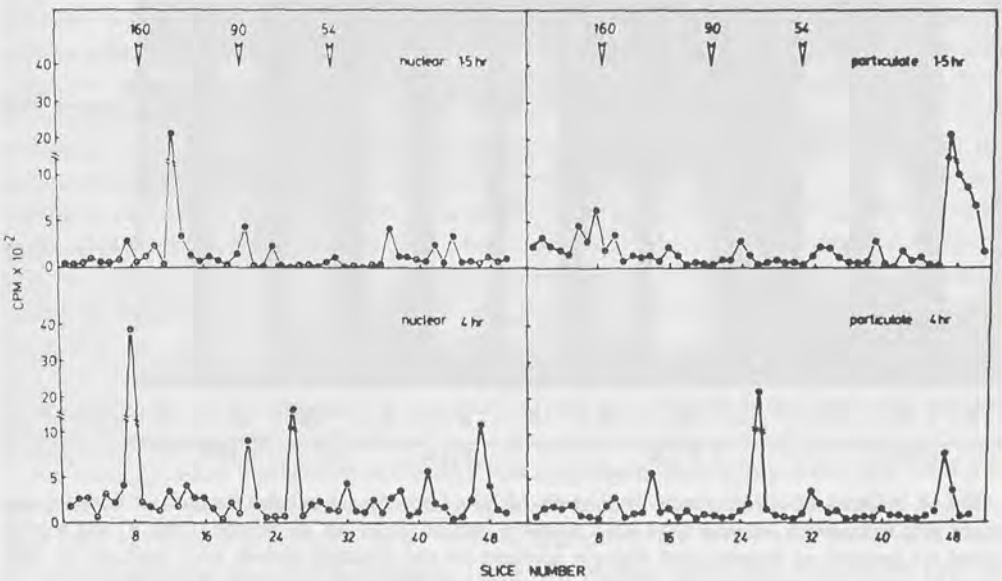


FIG. 3. Analysis of oligo(A)tracts of labelled poly(A) RNA samples. The nuclease resistant stretches of labelled RNA extracted from the nuclear and membrane fractions of embryos activated for the indicated periods were analyzed in a 10 % acrylamide-6 M urea gel. After the completion of the electrophoretic run (140 volts for 4-5 h), the gels were sliced in 3 mm pieces, solubilized with 30 % hydrogen peroxide and the radioactivity measured.

LEVELS OF POLY(A) POLYMERASE ACTIVITY IN THE SUBCELLULAR FRACTIONS AND RNP PARTICLES IN THE DEVELOPING EMBRYOS OF *ARTEMIA*

In order to evaluate the possible role of the major polyadenylating enzyme, poly(A) polymerase (PAP), in the process of adenylation of RNA, we had determined the levels of PAP in the dormant and developing embryos. It can be seen from the results outlined in Table II that

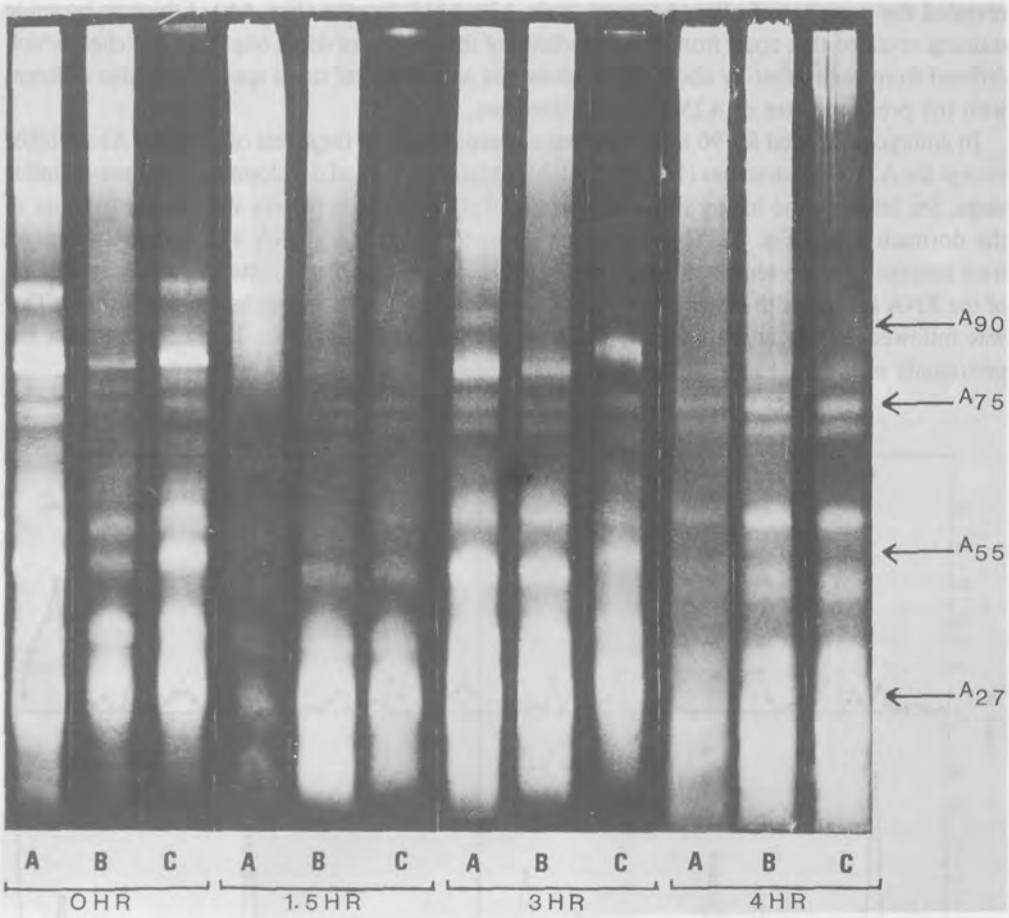


FIG. 4. Analysis of oligo(A)tracts of unlabelled RNAs from the sub-cellular fractions of dormant and developing embryos of *Artemia*. Total RNA from the nuclear (lanes A), membrane (lanes B) and soluble (lanes C) fractions of dormant and embryos activated for the indicated periods were analyzed for their oligo(A) tracts after treatment with RNases. The tracts were visualized with ethidium bromide.

TABLE II

Poly(A)polymerase activity in the total and RNP particles of the subcellular fractions of the developing embryos of *Artemia*

Stage of development	PAP activity [1]					
	Nuclear		Membrane		Soluble	
	Total	RNP	Total	RNP	Total	RNP
Dormant	104	n.d.[2]	n.d.	n.d.	296	8
Activated (4 h)	1 737	753	598	57	–	–

[1] Expressed as p moles of tritiated ATP incorporated/mg protein/60 min.

[2] n.d. = not detectable. The minimal detectable level of activity is 6 p moles of tritiated ATP.

all the subcellular fractions of the dormant embryos exhibited very low levels of PAP activity. However, upon activation for 4 h there was a marked increase in the levels of this enzyme in all fractions with a more pronounced increase in the membrane fraction. Interestingly, the RNP particles isolated from the membrane fractions, which were enriched in labelled poly(A) RNAs (Table I), contained all of the PAP activity as well even after these RNP particles were purified through a sucrose density gradient (Table II and Fig. 10).

Thus a parallelism was observed between the active labeling of the poly(A) RNA in the total membrane and the RNP particles with the increase in PAP activity in these particles.

Purification and properties of PAP from the membrane fraction

Activated embryos

We decided to characterize PAP from the membrane fractions further in order to understand the reasons behind the observed level increase of this enzyme. At all stages of purifications, the various criteria of PAP assays, such as primer dependence, substrate specificity and inhibitor effect, etc. were monitored (Rose *et al.*, 1979). After an initial passage of the sonicated extracts of the membrane fraction through DEAE cellulose column, the break-through material which exhibited PAP activity, had several proteins (Fig. 5, lane A, B). After concentration of the proteins by lyophilization they were fractionated through a phosphocellulose column. There were two peaks of PAP activities, one with a considerably higher activity level (peak I) than the other (peak II) which eluted closely at 0.21 M KCl and 0.26 M KCl concentration respectively (Fig. 6). However, both the peaks exhibited the presence of a single polypeptide of identical molecular weight of 48 KD. (Fig. 5, lane C, D). However, there was a difference in their primer specificity.

The more active form of PAP (peak I) exhibited a higher reaction rate with poly(A) as primer in comparison to t-RNA whereas the second form (peak II) exhibited a basal level with both the primers (data not shown).

Interestingly, when purification of the PAP was undertaken from embryos activated for 4 h in presence of 32P-phosphate, the active form (peak I) of PAP was extensively labelled and very little label was present in peak II (Fig. 6). Auto-radiography of the SDS-PAGE gels also revealed an intense labelling of the 48KD peptide (data not shown). This result has shown that the activated form of PAP is highly phosphorylated.

Dormant embryos

Although the membrane fractions of the dormant embryos did not exhibit any PAP activity, we subjected them to similar purification steps to eliminate the possibility of some associated inhibitor. At no stage of purification we could demonstrate PAP activity. However, the peptides eluted at 0.21 M and 0.26 M KCl concentrations from phosphocellulose column, resembled in molecular weight and total content (data not shown) to the peptides obtained from activated embryos. In addition, both total membrane fraction and the peptides eluted from phosphocellulose column were immuno-reactive with antiserum raised against purified PAP of activated embryos at significant dilutions (data not shown). These results clearly show that the membrane fractions of the dormant embryos did indeed harbour the inactive form of PAP.

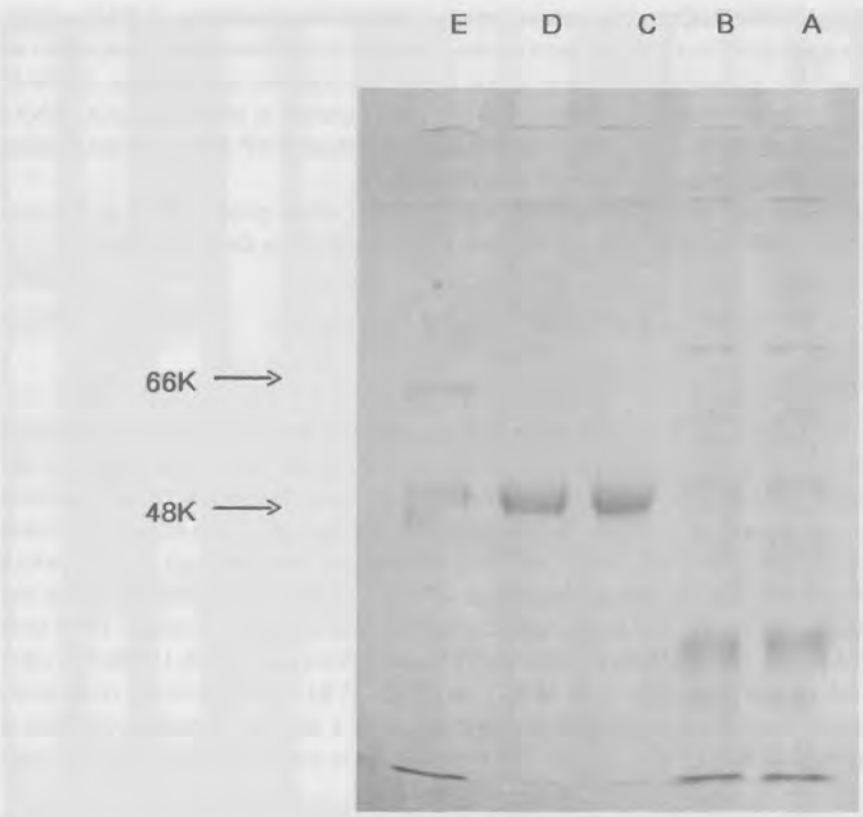


FIG. 5. SDS-page analysis of the purification steps of poly(A) polymerase from the membrane fractions of *Artemia* embryos. Lane A, DEAE breakthrough materials ; lane B, DEAE breakthrough materials ; lane C, phosphocellulose peak I ; lane D, phosphocellulose peak II ; lane E, standard proteins.

RNA BINDING PROPERTIES OF PAP

Since a rather firm association of PAP with the poly(A) RNP particles was seen (Table II and Fig. 10), it was of interest to test whether the purified enzyme exhibited a similar affinity to the poly(A) RNA from these fractions. Accordingly, labeled RNAs from different subcellular fractions of *Artemia* embryos, activated for 4 h were isolated and the efficiency of purified PAP to bind these RNA species was tested by its ability to retain labelled RNA on nitrocellulose filters (Richter and Smith, 1983). It can be seen from the results outlined in Fig. 7 that maximum retention was observed with membrane RNA followed by nuclear and soluble RNA species, which have been shown to have 61 %, 36 %, and 3 % poly(A) RNA content, respectively (Jeyaraj *et al.*, 1982). Labelled yeast RNA was not retained at all, reiterating the specificity of this binding. Moreover, the interaction between labelled membrane RNA and PAP was competed only by unlabelled RNA from membrane fraction (Fig. 7).

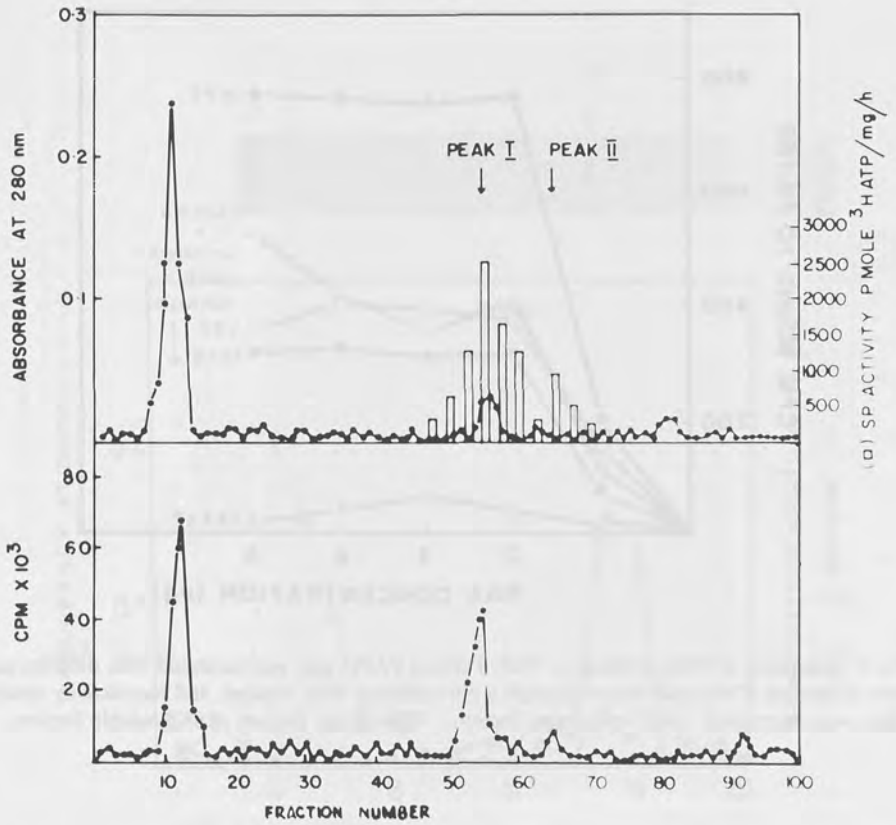


FIG. 6. Phosphocellulose column purification of PAP. The breakthrough materials of DEAE cellulose column were lyophilized and loaded on a phosphocellulose column (2.5 × 13 cm), equilibrated with TME buffer. The proteins were eluted with a 0.0-0.4 MKCl gradient in TME buffer. Fractions (4 ml) were monitored for absorbance at 280 nm and PAP activity. In a parallel run, the labelled proteins obtained from cysts activated in presence of 32P-phosphate were monitored for the radioactivity.

POLY(A) SEPHAROSE COLUMN CHROMATOGRAPHY

Affinity of PAP to poly(A) RNA was clearly demonstrated by RNA binding studies. To confirm the same, fractions showing PAP activity from DEAE cellulose column (necessary to remove the endogenous RNA) were passed through poly (A)Sepharose column. PAP activity was detected only in the bound fractions eluted by 1 M KCl. Polypeptide pattern of the same showed the presence of 48 kD peptide (Fig. 8).

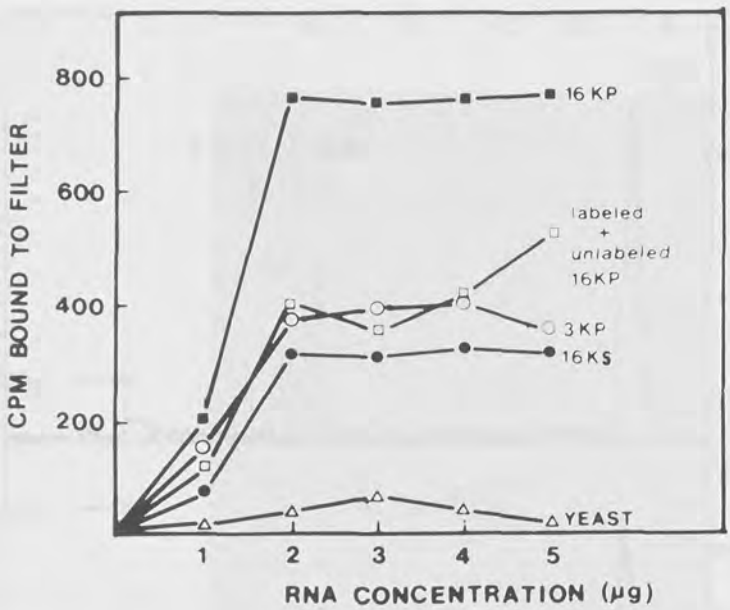


FIG. 7. Specificity of RNA binding by PAP. Purified PAP(1 µg) was incubated with different concentrations of labelled RNAs and filtered through a nitrocellulose filter, washed, and radioactivity retained on the filter was determined. 16KP-membrane fraction ; 3KP-nuclear fraction ; 16KS-soluble fraction.

CHARACTERIZATION OF *IN VITRO* PRODUCTS OF PAP

When t-RNA was used as primer with purified PAP, the reaction proceeded linearly up to 30 min. Electrophoretic characterization of the product(s) showed them to be of sizes larger than tRNA and a trail of products indicated their size variability (Fig. 9, lane C, D). Upon digestion of the products with pancreatic A and T1 RNases, the major oligo(A) stretch detected was about A25-A35 residues long (Fig. 9, lane E-F).

DEMONSTRATION OF THE ASSOCIATION OF PAP WITH OLIGO(A) TRACTS OF POLY(A)RNA

In view of the high specificity exhibited by PAP to poly(A) RNA, it was of interest to find out the domain of association of PAP with poly(A) RNA. Accordingly, the gradient purified RNP particles were subjected to RNase treatment and re-fractionated through a 15 to 40 % sucrose gradient. While the untreated particles sedimented mostly near the bottom of the gradient, (Fig. 10, panel A) the RNase treated particles generated several discrete species of sub-particles, which have majority of PAP activity and are immunoreactive to PAP antiserum, the majority of which were slow sedimenting and floated on the top of the gradient (Fig. 10, panel B).

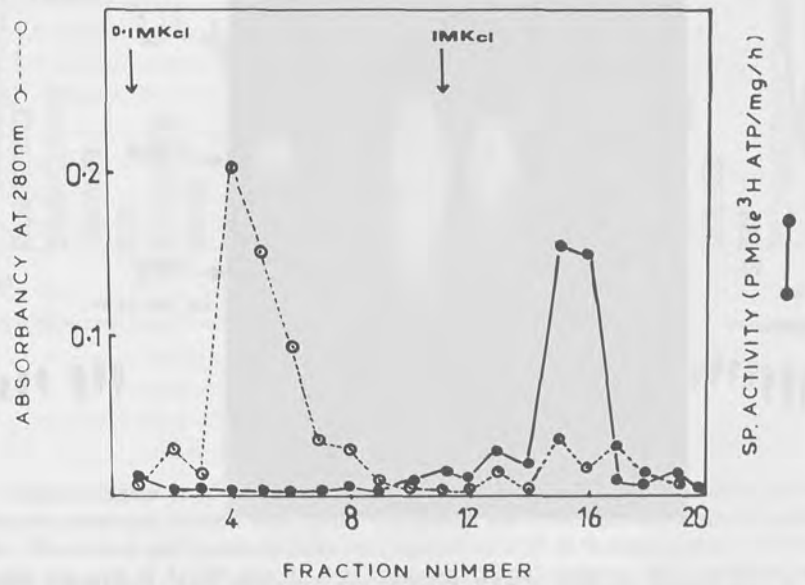


FIG. 8. Poly(A) sepharose column chromatography for poly(A) specific proteins. Fractions having poly(A) polymerase activity from DEAE cellulose column were pooled and loaded on a poly (A) sepharose column (1 × 7 cm). Poly(A) specific proteins were eluted by 1 M KCl. Individual fractions (2 ml) were collected and monitored for absorbance at 280 nm, PAP activity and polypeptide makeup by SDS PAGE.

PAP activity and the cross-reaction to PAP antiserum of these fractions also followed a similar shift of pattern (Fig. 10, panel A and B). Thus treatment of RNP particles with RNases resulted in the generation of low-density particles, which contained most of the PAP actoivity of these RNP particles.

Oligo (A) tract analysis of the RNAs present in the RNP particles revealed that the oligo (A) tracts were of A25-A160 residue lengths, with species differing with each other by 25-35 residues. In contrast, the low density particles released by RNase digestion contained RNA species of short chain lengths (Fig. 11). RNase digestion of these RNA species, resulted in only one major oligo(A) stretch of about 30 residues. (Fig. 11, panel A). Autoradiographs of the end labelled RNase resistant core stretches clearly reveal the exclusive presence of short oligo(A) stretch (Fig. 11, panel B).

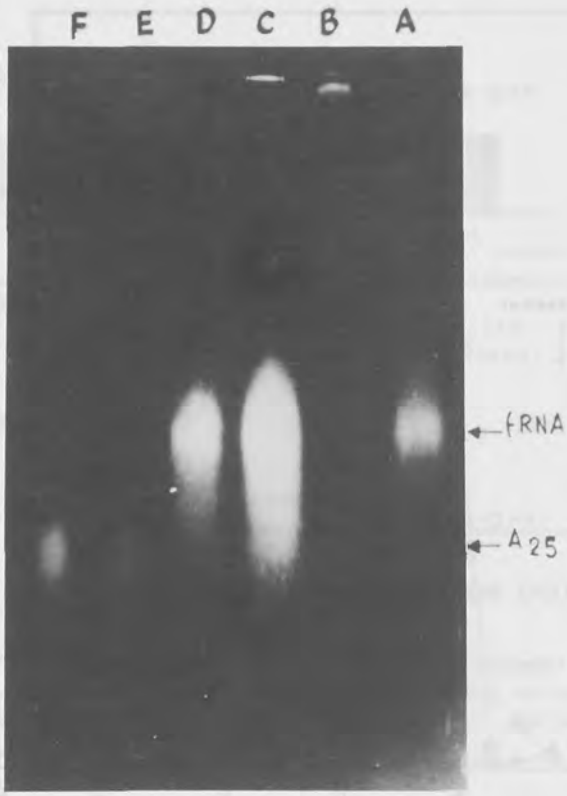


FIG. 9. *In vitro* reaction products of PAP. Reaction mix containing PAP (1 µg) and t-RNA (200 µg/assay) were incubated with unlabelled ATP and other requirements. The products of the reaction before and after RNases digestion were analysed in a 10 % acrylamide-6M Urea gel. Lane A, t-RNA ; lane B, t-RNA + RNases ; lane C and D reaction products ; lane E and F : reaction + RNases.

Discussion

The ubiquitous presence of poly(A) RNA in eukaryotic systems has provoked several studies on their synthesis, turnover and function (Brawerman, 1981). Poly(A) mRNAs have been shown to be present as mRNP particles, in association with several proteins. The dynamic association or dissociation of some of the proteins of the RNP particles has been implicated in the translatability and the turnover of these poly(A) RNAs (Preobrazhensky and Spirin, 1978 ; Vincent *et al.*, 1981). Existence of stable and compact cytoplasmic RNP particles containing repressed poly(A) mRNAs has been established. However, many of these studies have been confined to cytoplasmic RNP complexes.

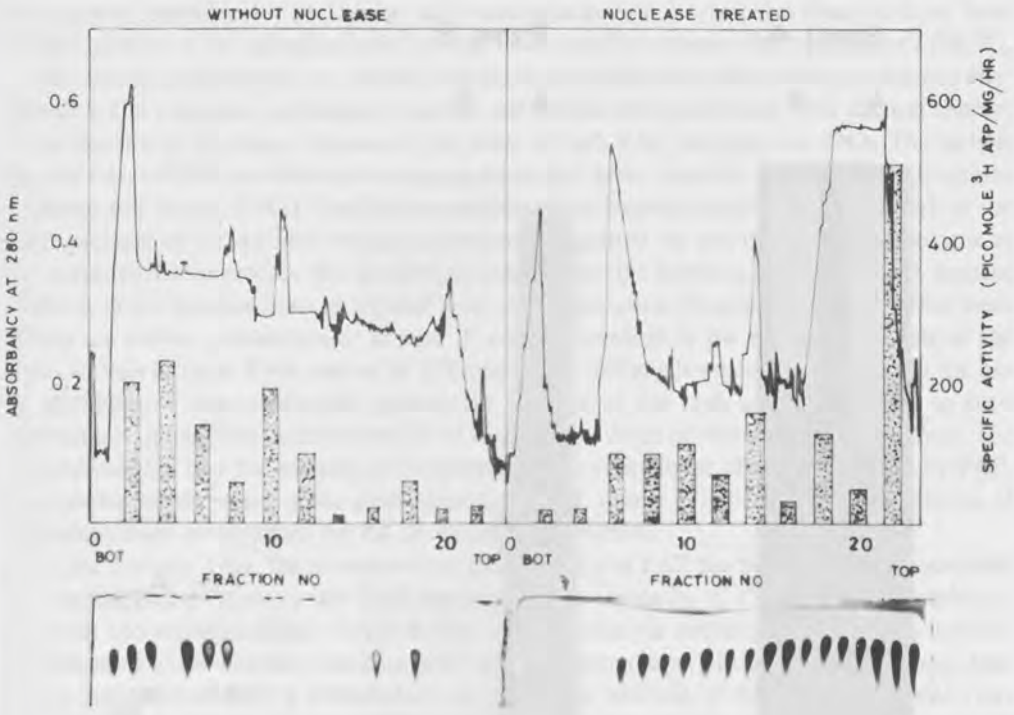


FIG. 10. Characterization of RNP sub-particles obtained by RNase treatment. Gradient purified RNP particles from the membrane fraction were treated with PancA and T1 RNases under similar conditions to that of RNA. The control and treated particles were layered on a 15-40 % linear sucrose density gradient in TKM buffer and spun at 41 000 rpm for 4 h. Fractions (0.2 ml) collected and monitored at 280 nm through an autoscan system. They were assayed for PAP activity and immunoreactivity against PAP antiserum. (——)OD 280 nm ; (▨) PAP activity.

The post-transcriptional addition of adenosine residues at the 3' end of mRNA is yet another facet of poly(A) RNAs that is poorly understood in molecular biology. While it is commonly accepted that poly (A) polymerase (PAP) is the major polyadenylating enzyme in eukaryotic systems, there appear to be other steps involved prior to this adenylation process. For example poly(A) addition has been shown to be a site-specific reaction involving processing of the RNA species by splicing precisely at sequences downstream to a highly conserved hexanucleotide sequence, 'AAUAAA' (Proudfoot and Brownlee, 1974 ; Moore and Sharp, 1984).

There are reports of diverse forms of PAP in eukaryotic systems, with different molecular identities (Edmonds, 1982). A direct correlation between the levels of cytoplasmic poly(A) polymerase and the increase of poly(A) RNA has been demonstrated soon after fertilization in sea urchin (Slater *et al.*, 1972 ; Wilt, 1973, 1977) and also in cAMP stimulated differentiation of neuroblastoma (Simontov and Sachs, 1974).

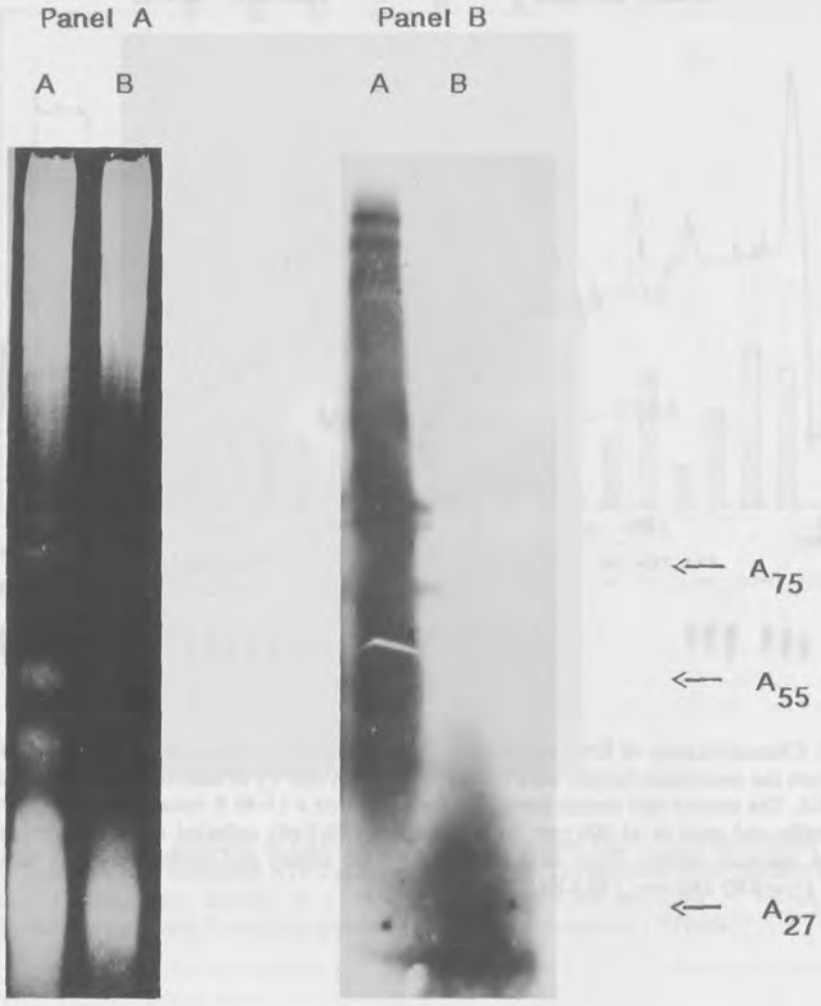


FIG. 11. Analysis of oligo(A) tracts of the RNA form RNP subparticles released by RNase treatment of membrane RNP particles. RNA extracted from the top and bottom of the gradient obtained after nuclease treatment of the RNP particles (fraction 6 and 20 of Fig. 10) was digested with RNases and the tracts analyzed as outlined in methods. They were end labelled after RNase treatment with γ32P-phosphate-ATP and polynucleotide kinase as outlined in methods. Panel A, ethidium bromide staining of the gel ; panel B, autoradiograph of the same gel. A, RNA from bottom of the gradient ; B, RNA from top of the gradient.

A correlation between the increase in poly(A) RNA levels and that of the enzyme poly(A) polymerase in the developing *Artemia* embryos has been observed earlier (Jeyaraj *et al.*, 1980 ; Sastre and Sebastian, 1980, 1983). However, since much of the increase in PAP levels was recorded in the nuclear fraction and the poly(A) RNP particles of the cytosol exhibited negligible levels of PAP activity, no direct role was ascribed to PAP in the process of polyadenylation

(Sastre and Sebastian, 1983). During early hours of activation, we had also observed a low level of PAP activity in the cytoplasm and an even lower level in cytosolic RNP particles (Table II).

Our studies with *Artemia* have tended to concentrate on the membrane fraction, obtained after Triton X-100 treatment, consisting of nuclear and cytoskeletal membranes. This fraction appears to be the site of the major increase of the levels of both PAP and poly(A) RNA. The earliest translatable mRNA populations in *Artemia* have also been traced to the membrane fractions (Nilsson and Hultin, 1982). The firm association of the labelled poly(A) RNA and PAP in the RNP particles of the *Artemia* embryos has strongly suggested the involvement of this enzyme in the polyadenylation process. We had demonstrated earlier the presence of poly(A) RNA in these particles in the dormant cysts by tritiated poly(U) hybridization (Jeyaraj *et al.*, 1982). That these RNAs are further polyadenylated at their 3' ends, is revealed by the exclusive labelling of the poly(A) tails of these RNA species by 32P-phosphate. Although we could not resort to the use of inhibitors of macromolecular synthesis in *Artemia*, as the cysts are impermeable to such compounds, it has been demonstrated by us in a related system of cryptobiotic development, *i.e.*, *Streptocephalus*, that the addition of cordycepin, a known inhibitor of polyadenylation by PAP, hinders the development of the cysts (Jeyaraj, unpubl. observ.), indicating that the process of polyadenylation is important for the developmental program.

In the dormant cysts, the presence of an inactive form of PAP has been clearly demonstrated by immunoprecipitation by the PAP antiserum. The similarity in the molecular weights of dormant and activated forms of PAP further indicates that the activation involves only a minor modification of the enzyme. The possibility that phosphorylation of the inactive form may lead to the activation of PAP is indicated by the significant labelling of the PAP in activated cysts (Fig. 6), which shows enhanced affinity to poly(A) template. Earlier, we had demonstrated the availability of labeled high energy phosphate donor, 1,3 disphosphoglycerate, synthesized during the early development of *Artemia* (Raja *et al.*, 1983). Evidence for the *in vivo* and *in vitro* activation of PAP by cAMP mediated phosphorylation of the enzyme has been observed earlier (Corti *et al.*, 1976 ; Rose and Jacob, 1980 ; Tsiapalis *et al.*, 1982).

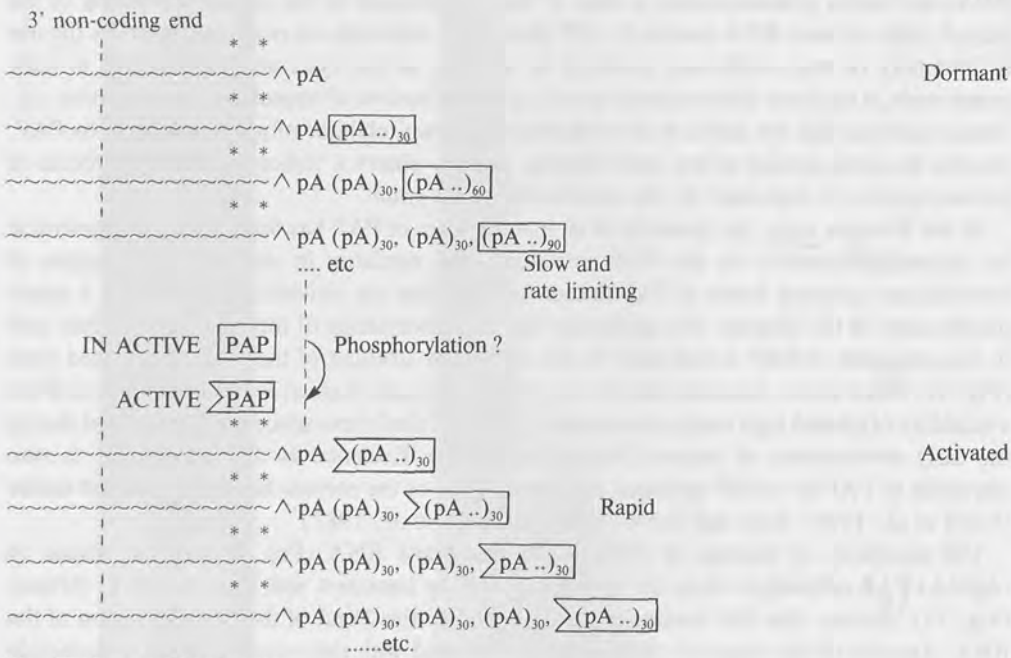
The specificity of binding of PAP to the membrane RNA (Fig. 7) and the release of oligo(A)-PAP subparticles from the membrane RNP by treatment with Panc A and T1 RNases (Fig. 10), indicate that this enzyme is tightly bound to the 3'end of the poly(A) region of the RNA. Analysis of the oligo(A) chain lengths associated with this enzyme, reveal a nucleotide length of about A25-A35 residues (Fig. 11). The previously reported values of about A25-A35 residues of oligo(A) stretches synthesized *in vitro* by PAP (Rose and Jacob, 1976 ; Adolf and Swetly, 1978), as well as the product lengths observed by us (Fig. 9) strongly suggest that the size of oligo(A) stretches that can be in firm association with PAP is of this order.

The presence of 48 kD peptide with PAP activity in the poly(A)Sepharose fractions clearly demonstrates the affinity of PAP to poly(A)tail (Fig. 8).

Our observations on the periodicity of the poly(A) chain lengths of both pre-formed and newly synthesized mRNAs from dormant and developing cysts, is in line with several reports that have appeared on the periodicity of the 3' oligo(A) chain lengths of mRNA species, observed either as a result of protection of these stretches by some proteins or the periodicity of their synthesis. Earlier it has been shown that the chain lengths of newly synthesized globin mRNA, differed by about 25 oligo(A) residues (Kelly and Cox, 1982). Digestion of the poly(A) RNP particles from Hela cells, with RNase T2, resulted in fragments with regular multiplicites of 27 oligo(A) residues (Baer and Kornberg, 1980). It was suggested that the periodicity observed was a result

of protection by some poly(A) binding proteins. Another model for the organization of poly(A) protein complex in mRNP, had suggested that about 45 residues were bound to a protein from the 5'end of the poly (A) (Adams *et al.*, 1980). More recently it was shown that the nuclease resistant sites of the 3' non coding regions of the globin mRNAs were distinguished by a complex loop of structures that precede the protected oligo(A) stretches with a periodicity of about 20-25 AMP residues. (Krowczynska and Brawerman, 1986).

With the view to understand the mechanism(s) behind the periodicity of the oligo(A) stretches observed, we outline the following model for the polyadenylation of RNAs observed on re-initiation of development in *Artemia.*



** = Nuclease resistant cores

Summary and conclusion

Our results have shown that PAP is strongly associated with a single species of about 30 oligo(A) residues, it implicates this protein in the periodicity observed in the chain elongation and its association only with the growing 3' termini. The association of inactive form of PAP with RNP particles of dormant embryos is activated by phosphorylation.

The understanding of the mechanism of polyadenylation, may enable us to look on the one hand at the mobilization of the stored messengers and on the other at the role played by the oligo (A) termini of mRNA in protein synthesis.

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Genome structure and expression

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Molecular characterization of *Artemia* mitochondrial DNA : cloning, physical mapping, and preliminary gene organization

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Abstract

To extend to the crustacean class the increasing available information on the organization of mitochondrial DNA in the animal kingdom, the complete *Artemia* mitochondrial genome (10.1 megadaltons) has been cloned in pBR322. The entire *Artemia* mt DNA molecule is contained in two different recombinant plasmids, one with a 9.6 kilobase insert and the other with a 5.7 kb insert. A physical map of the *Artemia* mt DNA from North America has been constructed using six restriction enzymes. The genes coding for 16s rRNA and cytochrome oxidase subunit I have been localized in the molecule, using both Southern (1975) blots and partial sequencing analysis. They are oriented in opposite directions and organized in a similar way to *Drosophila*, and different from sea urchin and mammalian mt DNA. This study provides useful specific probes for further investigation of the genome organization of *Artemia* mitochondrial DNA.

Introduction

The mitochondrial genome of animal cells consists of a double stranded circular DNA molecule of 15 to 19.5 kb pairs (Wallace, 1982). In recent years, considerable attention has been devoted to the study of the organization and expression of mitochondrial DNA, and in some cases (mammals) the complete nucleotide sequence of the molecule is known (Anderson *et al.*, 1981 ; Bibb *et al.*, 1981). It shows a compact organization with no introns and few and short intergenic spaces, codifying for 2 rRNAs, 22 tRNAs, and 13 proteins. In addition, some variations in the genetic code with respect to the previously known, universal one, have been detected. Transcription mapping studies (Clayton, 1984) and the use of antibodies raised against synthetic oligopeptides have shown that all the mitochondrial genes are expressed *in vivo* (Michel *et al.*, 1984). In addition, 12 out of the 13 presumptive proteins have been identified and assigned to well known components of the respiratory chain located in the mitochondrial membrane (Chomyn *et al.*, 1985). Therefore, the mitochondrial DNA plays a crucial, although minor role in the biogenesis of mitochondria.

Much less is known about the mitochondrial DNA in other animals, although the results obtained so far clearly indicate that the genetic information is the same in all of them. However, the partial sequencing of sea urchin mt DNA (Roberts *et al.*, 1983) and specially the sequence of *Drosophila* mt DNA (de Bruijn, 1983 ; Clary and Wolstenholme, 1983) have provided new

information. The organelle genome organization in these organisms is rather different and in addition there are new changes in the genetic code when compared with the universal and the mammalian mt DNA ones.

These results are consistent with the fast evolution rate assigned to the mitochondrial genome (Brown *et al.*, 1979 ; Brown, 1980), and clearly indicate that this molecule is endowed with a greater flexibility and adaptability at the molecular level, but with a very high conservation at the level of the genetic information encoded in it.

In order to know the degree of change in the organization of the mitochondrial genome through the animal kingdom, much more information from different organisms is necessary. In this context, the data available hitherto on the class Crustacea are surprisingly scanty, since only very few studies on their mitochondrial DNA have been reported (*e.g.* Schmitt *et al.*, 1974).

The special advantages of *Artemia* as a model system in biological studies (Vallejo *et al.*, 1981) makes it a very suitable organism to perform these studies. We have initiated the characterization of mt DNA from *Artemia*, cloning the entire mitochondrial genome in a plasmid vector, pBR322, constructing a physical map and locating some of the genes in the molecule. The results obtained indicate that in crustaceans, the mt DNA has a genome organization similar to that found in insects and different to the mammal and sea archin mt-DNA organization.

Materials and methods

Brine shrimp cysts were obtained from San Francisco Bay. Recombinant plasmid 62F9 was a kind gift from Dr. W. Gehring. It contains 1.6 kb of *Drosophila melanogaster* mitochondrial DNA (Steward and Gehring, 1981). Recombinant plasmid H was a kind gift from Dr. M. Renaud. It contains 4.9 kb of *Drosophila melanogaster* mt DNA almost coincident with the region sequenced by de Bruijn (1983).

^{32}P-labeled nucleosides triophosphate, restriction enzymes, and DNA modifying enzymes were from Amersham, England. Other reagents were of analytical grade.

Mitochondrial DNA preparation

Artemia mitochondrial DNA was prepared by a procedure slightly modified from that described by Schmitt *et al.* (1974), from 12-14 h developed cysts homogenized under disruptive conditions (Vallejo *et al.*, 1981) to isolate as many as possible free mitochondria. The homogenates were initially centrifuged at 2 000 × *g* × 10 min to sediment the nuclei and the remaining yolk granules. The supernatant was centrifuged at 10 500 × *g* × 30 min to prepare a crude mitochondrial fraction. This fraction was further purified through a discontinuous sucrose gradient to recover the free mitochondria at the appropiate density. After a short DNase I treatment, to digest contaminating external DNA, the mitochondria were washed and lysed. The supercoiled mitochondrial DNA was finally purified in ethidium bromide CsCl gradients.

Cloning of *Artemia* mitochondrial DNA

A complete *Bam*HI restriction digestion of *Artemia* mt DNA was cloned into the *Bam*HI site of pBR322. The recombinants were selected in the appropriated antibiotic containing plates and further characterized by restriction enzyme analysis of the plasmids obtained from mini-prepa-

rations (Holmes and Quigly, 1981). Large scale purification was performed by a slightly modified alkaline procedure (Maniatis *et al.*, 1982).

Restriction digestion and electrophoretic conditions

Digestions with restriction enzymes were carried out as indicated by the manufacturers, except in double digestions in which the appropriate low, medium or high salt buffers were used (Maniatis *et al.*, 1982).

DNA was electrophoresed on 0.7 to 1.2 % agarose gels using TBE buffer (Tris 100 mM, boric acid 100 mM, EDTA 2 mM) at room temperature. Usually 1 to 3 µg of restriction digested DNA were loaded in each lane.

Blotting and hybridization

The DNA fragments separated in agarose gel were blotted onto nitrocellulose filters according to Southern (1975). Nick translated DNA probes (specific activity 10^8 cpm/µg) were hybridized in 4×SSC, 0.1 % SDS, 1× Denhart solution, 50 % formamide and 100 µg/ml denatured salmon sperm DNA at 42 °C for 48 h.

DNA sequencing

Partial sequencing of DNA fragments was performed by the method of Sanger *et al.* (1977). The selected DNA was cloned in M13 mp8 and both extremes sequenced using nucleotide chain terminating inhibitors. The data were compared with the bovine (Anderson *et al.*, 1982) and *Drosophila melanogaster* (de Bruijn, 1983 ; Garesse, unpubl.) mt DNA sequences, using different computer programs developed by Staden (1982).

Results

Cloning of *Artemia* mitochondrial DNA

North America *Artemia* mitochondrial DNA is cleaved into two fragments by digestion with *Pst*I or *Bam*HI. *Eco*RI cleaves this DNA in six fragments, *Ava*I in seven, and *Hin*dIII in nine. *Pvu*II has only one target site, since the digestion with this enzyme linearizes the molecule. It has also been observed that *Sma*I does not cleave the *Artemia* mt-DNA.

The molecular weight of the different fragments is shown in Table I. For each enzyme their sum consistently indicates that the molecular weight of *Artemia* mt-DNA is 15.3 kb (10.1 Md), that is in the range of other animal mt-DNAs (10 to 13 Md).

To study the organization of the mitochondrial genes, we cloned the two *Bam*HI fragments in pBR322. *Artemia* mt-DNA was completely digested with *Bam*HI and ligated with pBR322 linearized with the same enzyme and dephosphorylated with alkaline phosphatase. Several recombinant plasmids were selected for further analysis and two of them, containing the large (MA-2) and small (MA-3) fragments were purified in large scale (Fig. 1). No other recombinants were obtained from this or similar experiments, suggesting that there are no other small fragments generated in the *Bam*HI digestion that would have escaped the UV detection in agarose gels. Thus we conclude that MA-2 and MA-3 should contain the entire *Artemia* mitochondrial DNA molecule.

TABLE I

Restriction fragments of North American *Artemia* mitochondrial DNA (kb)

Restriction enzyme	*Pvu*II	*Pst*I	*Bam*HI	*Ava*I	*Eco*RI	*Hin*dIII
	15.3	8.9	9.6	6.0	5.8	3.8
		6.4	5.7	3.3	3.4	2.35
				3.25	2.7	2.3
				0.9[1]	1.5	1.85
				0.8	1.0	1.7
				0.2	0.9	1.4
						1.2
						0.5
						0.2
Total (kb)	15.3	15.3	15.3	15.35	15.3	15.3

[1] Double bands, as deduced by the enhanced ethidium fluorescence.

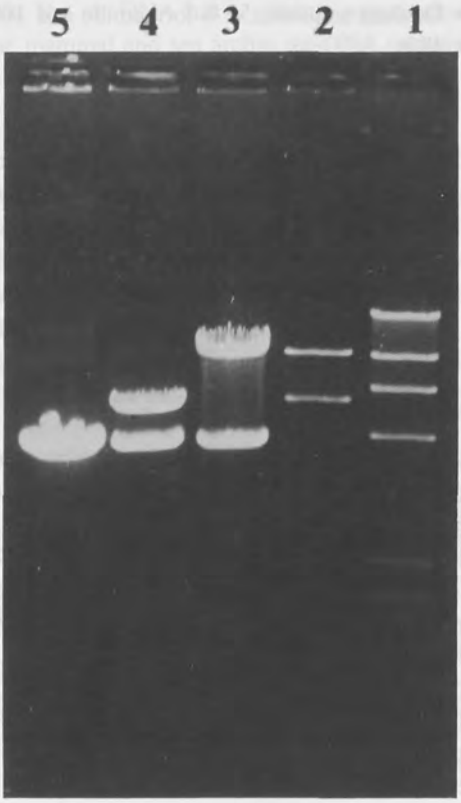

FIG. 1. Cloning of *Artemia* mitochondrial DNA. Clones MA-2 and MA-3 xwere digested with *Bam*HI and electrophoresed in a 1 % agarose gel, showing clearly that MA-2 contains the 9.6 kb *Bam*HI fragment and MA-3 the 5.7 kb *Bam*HI fragment. Both clones together contain the entire *Artemia* mitochondrial DNA. Lane 1, λ × *Hin*dIII ; lane 2, *Artemia* mt-DNA × *Bam*HI ; lane 3, MA-2 × *Bam*HI ; lane 4, MA-3 × *Bam*HI ; lane 5, pBR322 × $Bam H_1$.

Restriction mapping of *Artemia* mitochondrial DNA

We have determined the restriction sites for several enzymes (*Pvu*I, *Pst*I, *Bam*HI, *Eco*RI, *Ava*I, and *Hin*dIII) within the inserted mitochondrial DNA in the plasmids MA-2 and MA-3. Their locations were determined using well-established methods, involving single and combined digestions. In some cases, the location of a restriction site was further confirmed cloning the region of interest in M13 mp8 and digesting the purified recombinant DNA with the appropriated enzyme.

*Hin*dIII, *Eco*RI, and *Ava*I

*Bam*HI restricted MA-2 or MA-3 DNA was digested with either *Eco*RI, *Hin*dIII, or *Ava*I, and with different combinations of these enzymes in double digestion experiments as described in Materials and methods. The results obtained are summarized in Table II, in which the molecular weights of the different fragments expressed in kilobases are presented.

For the different restriction enzymes tested, we have also identified the fragments located at both ends of the MA-2 and MA-3 and their orientation within the molecule of pBR322. This was straightforwardly detected from the digestion of the original recombinant plasmid (without *Bam*HI cleavage) with the different restriction enzymes. Only the fragments located at the ends change their position compared with a similar digestion of the *Bam*HI restricted recombinant DNA.

*Pst*I and *Pvu*II

One *Pvu*II and two *Pst*I sites were also mapped in MA-2 or MA-3. They can be easily localized in double digestions with *Eco*RI, *Hin*dIII, and *Ava*I.

*Pvu*II cleaves the 3.4 kb *Eco*RI fragment of MA-2 in a 2.8 kb and 0.6 kb fragments, and the 2.4 kb *Hin*dIII fragment of MA-2 in a 1.9 kb and a 0.5 kb fragment. These results localize unambiguously the *Pvu*II site into the MA-2. A similar strategy to map the *Pst*I sites was used. They are located in the 1.4 kb *Hin*dIII fragment of MA-2 and the 1.8 kb *Hin*dIII fragment of MA-3.

Using the data generated in these experiments, we have aligned progressively the different fragments and constructed a restriction map of both clones, as shown in Fig. 2. The results of this study indicate that the mitochondrial sequences had been maintained integrally in the recombinant plasmids, since all the restriction sites determined in the mitochondrial DNA were present in the DNA purified from the clones. Furthermore, there was a complete correspondence in the size of fragments generated from the whole mt DNA and the two cloned fragments.

Genetic organization of *Artemia* mt DNA

Location of the 16s rRNA

The 3′ end of the mt 16s rRNA sequences is well conserved among different animal species. In fact, this region is the more homologous one to the 23s rRNA of *E. coli*. Several experiments (Eckermann and Symons, 1978 ; Blanc *et al.*, 1981) suggest that this sequence is involved in the binding of the 3′ terminus of aminoacyl tRNA to the ribosomal A site, and in mammals (Glotz *et al.*, 1981) and in *Drosophila* (Clary and Wolstenholme, 1985) a peculiar secondary structure is also well maintained there.

TABLE II

Restriction fragments of the inserted mitochondrial DNA in the plasmids MA-2 and MA-3

MA-2 (kb)						MA-3 (kb)					
*Eco*RI	*Hin*dIII	*Ava*I	*Eco*RI + *Hin*dIII	*Eco*RI + *Ava*I	*Hin*dIII + *Ava*I	*Eco*RI	*Hin*dIII	*Ava*I	*Eco*RI + *Hin*dIII	*Eco*RI + *Ava*I	*Hin*dIII + *Ava*I
3.4	2.8	3.3	2.35	2.9	2.7	5.0	1.85	3.9	1.75	3.15	1.7[1]
2.7	2.35	3.25	2.2	2.25	2.25	0.75	1.7	0.9	1.6	0.75	0.75
1.5	2.3	2.2	1.5	1.5	1.5		1.0	0.75	1.0	0.7[1]	0.6
1.0	1.4	0.9	0.9	0.85	0.85		0.6	0.2	0.6	0.2	0.5
0.9	0.6		0.8	0.7	0.7		0.5		0.5		0.2
0.15			0.6	0.6[1]	0.6		0.2		0.2		0.15
			0.4[1]	0.15[1]	0.3				0.1		0.1[1]
			0.2[1]		0.2[1]						

[1] Double fragments.

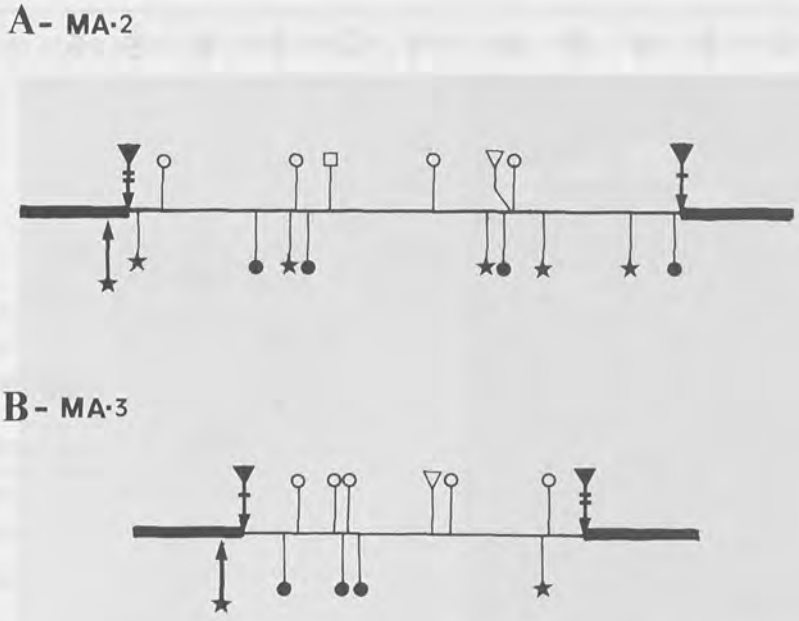

FIG. 2. Physical map of the recombinant plasmids MA-2 and MA-3. Both recombinants are drawn in linearized form and oriented with respect to the single *Eco*RI-site of pBR322. The thin line represents the inserted DNA and the thick line the vector DNA. ⥯ and ⥯ denote the joining points of both inserted DNA. (⎍) *Hin*dIII ; (⎍) *Eco*RI ; (⎍) *Ava*I ; (⎍) *Pst*I ; (⎍) *Pvu*II.

Thus it makes sense to think that in *Artemia* mt-DNA, this region could also be conserved, and is easily detectable using as radioactive prove a homologous region. To check this possibility, we have hybridized MA-2 and MA-3 DNA restricted with several enzymes with a nick translated recombinant DNA containing this region from *Drosophila melanogaster*. This plasmid also contains the $tRNA^{Leu}_{UUR}$ and 350 bp of URF-1, although they are poorly conserved in mammals and *Drosophila melanogaster* mt-DNA (Garesse, unpubl.).

The results are shown in Fig. 3. In addition to the expected cross-hybridization with the pBR322 bands, the probe also hybridizes with some mitochondrial bands. A positive signal is clearly detected in the 0.75 kb *Eco*RI-*Bam*HI (MA-3), 0.6 kb *Hin*dIII-*Bam*H_1 (MA-3), and 1.7 kb *Hin*dIII (MA-3) fragments. The 3.9 kb *Ava*I (MA-3) fragment is not visible because this region of the gel was excised to eliminate the strong hybridization signal produced by the 4 kb pBR322 fragment (see legend of Fig. 3). These hybridization data allow to localize the 16s rRNA in the *Pst*I-*Bam*H_1 region of MA-3. In order to rule out the formal possibility that the positive signals were produced by other extra-ribosomal sequences also contained in the 62F9 plasmid (see above), the result was confirmed using an alternative strategy. We cloned the three positive fragments in M13 mp8 and sequenced their extremes using the dideoxy method. The high level of homology when compared with the bovine (Anderson, 1982) or *Drosophila melanogaster* (Garesse, in prep.) 16s rRNA region was detected at one end of the 1.7 *Hin*dIII fragment (Fig. 4A).

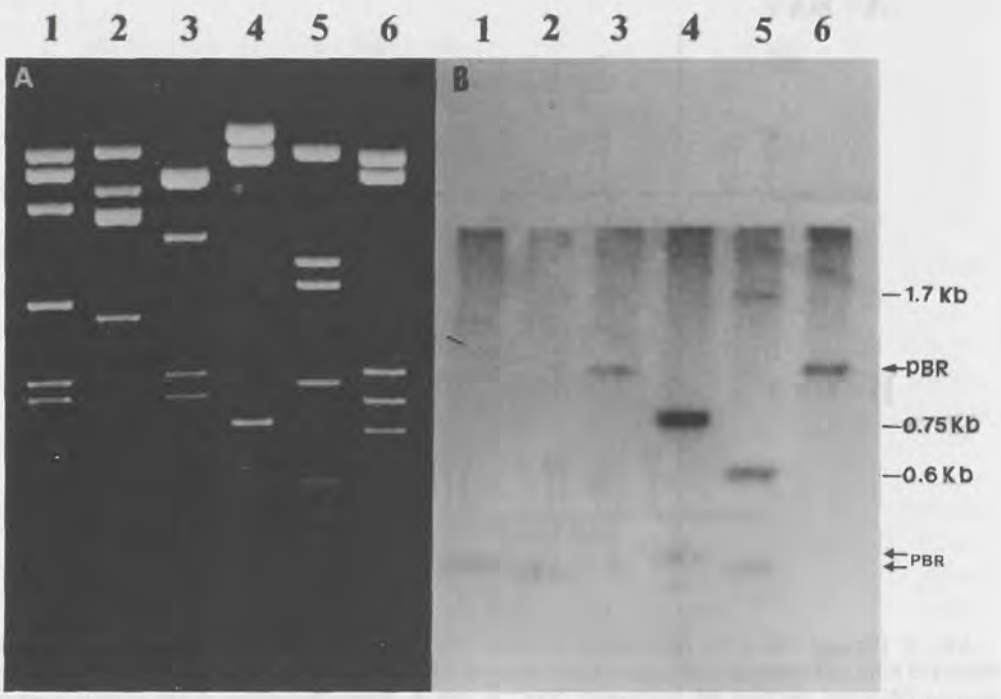

FIG. 3. Southern (1975) blot of MA-2 and MA-3 hybridized with the *Drosophila* 16s rRNA probe. A Southern blot containing MA-2 and MA-3 DNA digests with *Eco*RI, *Hin*dIII or *Ava*I was hybridized with a probe containing the 16s rRNA from *Drosophila melanogaster*. Panel A shows the Ethidium bromide stained 1 % agarose gel. Lane 1, MA-2 × *Eco*RI ; lane 2, MA-2 × *Hin*dIII ; lane 3, MA-2 × *Ava*I ; lane 4, MA-3 × *Eco*RI ; lane 5, MA-3 × *Hin*dIII ; lane 6, MA-3 × *Ava*I. Panel B shows the autoradiography of the Southern blot from the gel A, using the 16s rRNA probe. In addition to the pBR322 bands (arrows) that cross-hybridize with the probe, a positive signal is detected from the 0.6 kb *Bam*HI - *Hin*dIII (MA-3), 1.7 kb *Hin*dIII (MA-3), and 0.75 kb *Eco*RI - *Bam*H_1 fragments. The upper region of the gel was excised before transfer in order to eliminate the strong hybridization produced by the 4 kb pBR322 fragments generated in the plasmid digestions.

→

FIG. 4. Homology at the nucleotide and amino acid level among the bovine, *Drosophila*, and *Artemia* mitochondrial sequences. The three sequences are shown aligned in the 5′-3′ direction. Panel A corresponds to the same homologous 16s rRNA region in bovine (nucleotides 2 356 to 2 475 as described in Anderson *et al.* (1982) and *Drosophila melanogaster* (Garesse, in prep.) mitochondrial DNA. Panel B correspond to the central region of Co I gene in both bovine (nucleotides 6 364-6 538) and *Drosophila melanogaster* sequences. Asterisks between two sequences indicate the positions of identical nucleotides. In some cases, spaces have been introduced in order to maximize the homology. The comparison was carried out using the programs developed by Staden (1982). Panel C corresponds to the translation products of the sequence of cytochrome oxidase subunit I shown in panel B. Boxes denote places in which amino acid variations appear, with asterisks indicating non conservative substitutions.

A. HOMOLOGY AT THE NUCLEOTIDE LEVEL AMONG THE 16S rRNA FROM COW, *ARTEMIA*, AND *DROSOPHILA* MT-DNA

```
Bovine        TTTAACGGCCGCGGTATCCTGACCGTGCAAAGGTAGCATAATCATTTGTTCTCTAAATAA
              *  ** ***** ***** ****** *** ***************** *   * * * *
Artemia       TAAAAGGGCCGTGGTATACTGACCATGCGAAGGTAGCATAATCATTAGCCTTTTGATTTG
              * *** * **  *   *             * * ****  ********* ***** ***
Drosophila    TTAAATGCCCCAGTATTTTGACTGTGCAAAGGTTAGCTAAATCATTAGTCTTTTAATTGT

Bovine        GGACTTGTATGAATGGCCGCACGAGGGTTTTACTGTCTCTTACTTCCAATCAGTGAAATT
               * ** * ********    ***** * *   *********  *  ***    *  * *
Artemia       AGGCTGAATGAATGGTTTGACGAGAGATGGTCTGTCTCTTCGATTAAATTGAAGTTAAT
              ******************* ***** * **   **** ** *  *  *  ** *    *
Drosophila    AGGCTGGAATGAATGGTTGGACGAAATATTAACTGTTTCATTTAAAATTTTTATAGAATT
```

Total homology (%)		
	Bovine/*Artemia*	67
	Drosophila/*Artemia*	59

The *Artemia* sequence was obtained from a subclone of the 1.7 kb *Hind* III fragment of MA-3.

B. HOMOLOGY AT THE NUCLEOTIDE LEVEL OF COW, *ARTEMIA*, AND *DROSOPHILA* CYTOCHROME OXIDASE SUBUNIT I

```
Bovine        AGACCCTATTCTATATCAACACTTATTCTGATTCTTTGGACACCCCGAAGTCTATATTTT
               ** ** ** ** ******** ***** ***** ***** ** ** ***** ********
Artemia       GGATCCCATCCTTTATCAACATTTATTTTGATTTTTTGGCCATCCTGAAGTGTATATTTT
               ***** **  * ************************** ** ******** ********
Drosophila    AGATCCTATTTTATATCAACATTTATTTTGATTTTTTGGTCACCCTGAAGTTTATATTTT

Bovine        AATCTTACCTGGGTTTGGAATAATCTCTCATATCTGGACCTACTACTCAGGAAAAAAAGA
              *** ******** ** ** *** * ** ** **    * * *  *   *** ** ** **
Artemia       AATTTTACCTGGATTCGGGATAGTGTCCCACATTATTAGCCAAGAAAGAGGTAAGAAGGA
              *************** ** *** * ** ** ******** ******  *** ** *****
Drosophila    AATTTTACCTGGATTTGGAATAATTTCTCATATTATTAGACAAGAATCAGGAAAAAAGGA

Bovine        ACCATTCGGATATATGGGAATAG-TTTGGGCTATAATGTCAATCGATTTCTAGGT
              * **** **     * ** **** *** ****** *  **** **  **** ***
Artemia       AGCATTTGGTACATTAGGTATAGATTTATGCTATACTTGCAATTGGTATTCTTGGT
              * * ****** * * *** ***  *  ******** * ** *****  *  * **
Drosophila    AACTTTTGGTTC-TCAGGAATAATT-GATGCTATATTAGCTATTGGATTATTAGGA
```

Total homology (%)		
	Bovine/*Artemia*	71
	Drosophila/*Artemia*	79

The *Artemia* sequence was obtained from a subcloned, 0.9 kb *Bam* HI - *Eco* RI fragment of MA-2.

C. HOMOLOGY AT THE AMINO ACID LEVEL AMONG BOVINE, *DROSOPHILA*, AND *ARTEMIA* CYTOCHROME OXIDASE SUBUNIT I

```
                                                    Homology (%)
                                                                  **      *  **
Bovine        DPILYQHLFWFFGHPEVYILILPGFGMISHIVTYYSGKKEPFGYMGM
                                                        83
Artemia       DPILYQHLFWFFGHPEVYILILPGFGMMSHIITQESGKKEAFGTLGM
                                                      93.5
Drosophila    DPILYQHLFWFFGHPEVYILILPGFGMISHIISQESGKKETFGSLGM
```

* Asterisks indicate the positions in which non conservative amino acid substitutions have occurred.

In addition to this verification, the sequencing data allow to deduce the orientation of the 16s rRNA gene in the *Artemia* mt-DNA molecule. It is oriented 5′ → 3′ in the direction *Pst*I → *Bam*H_1 (MA-3). Assuming a similar length for the bovine and *Artemia* 16s rRNA the total region that it expands can be calculated.

Localizing the cytochrome oxidase subunit I gene in Artemia *mt-DNA*

To determine additional gene position in *Artemia* mt-DNA we have also used the same strategies described, *i.e.* sequencing some M13 mp8 clones containing specific mt-DNA fragments and hybridizing *Artemia* mt-DNA with nick translated *Drosophila* clones.

In Fig. 4B and C the results obtained in a sequencing experiment are shown. The *Bam*HI end of the 0.9 *Bam*HI-*Eco*RI (MA-2) fragment contains a sequence highly homologous to the central region of cytochrome oxidase subunit I (Co I) from cow and *Drosophila*. It makes possible to establish the location and orientation of this gene in the *Artemia* mitochondrial genome from the *Bam*H_1 site towards the MA-2 region.

This result indicates that the Co I gene is oriented in the opposite direction to that of the 16s rRNA, similarly to that found in *Drosophila* and different to the well-conserved gene order present in mammals mt-DNAs.

The Southern (1975) blot analysis of *Artemia* mt-DNA expanding this region is also consistent with these results. The H clone from *Drosophila melanogaster* contains a series of genes (including Co I) in a well-conserved order in most of the animal mt-DNAs sequenced so far. Using the nick translated DNA as probe, we detected positive signals in the fragments 0.9 kb *Bam*HI-*Eco*RI (MA-2), 1.5 kb *Eco*RI (MA-2), and 1.0 kb *Eco*RI (MA-2) (Fig. 5). In addition

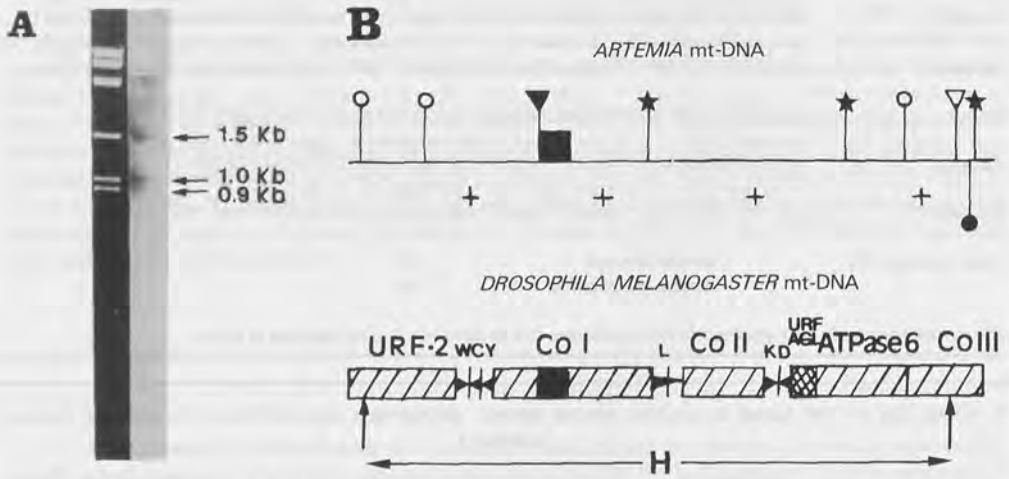

FIG. 5. Southern (1975) blot of MA-2 with the *Drosophila* cytochrome oxidase I and II-ATPase 6 probe. The *Drosophila melanogaster* H clone contains the genes depicted in panel B. Panel A represents a Southern blot containing the *Eco*RI fragments of the MA-2 clone which was hybridized with the nick translated H DNA. The fragments of 0.9 kb, 1 kb and 1.5 kb show positive signal (arrows). The upper region of the gel was excised in order to avoid the strong hybridization produced by the 4 kb pBR322 fragment generated in the MA-2 digestion. (⌊) *Hin*dIII ; (⍑) *Eco*RI ; (⍑) *Bam*HI ; (⍒) *Pst*I ; (⍑) *Ava*I. In panel B it is shown that the region of *Artemia* mitochondrial DNA is homologous to the H clone as deduced by Southern blot analysis.

the 1.0 kb *Bam*HI-*Hin*dIII (MA-3) fragment also hybridizes strongly with the H clone (data not shown).

These results are summarized in Fig. 5 and suggest that in *Artemia* mt-DNA the gene order Co I - Co II - URFA6L - ATPase 6 is also conserved. A complete analysis of this region is in progress in our laboratory and will be published elsewhere.

Discussion

The genome organization of mitochondrial DNA changes between different animals maintaining the coded information. In order to acquire a more complete picture of the evolution of mitochondrial DNA sequences, much more information needs to be obtained from organisms representing different phyla and classes.

In this paper, we have initiated the characterization of *Artemia* mitochondrial DNA, to our knowledge the first crustacean mt-DNA characterized at this level. We report here the cloning of the entire molecule, a restriction map for different enzymes : *Pvu*II, *Bam*HI, *Pst*I, *Eco*RI, *Hin*dIII, and *Ava*I, and the identification of some genes in the molecule.

We have mapped 27 sites uniformly distributed along the entire molecule.

The distance between two adjacent sites does not exceed 2.0 kb pairs. The strategy used to draw the map was based mainly on combined digestions with two enzymes. With this approach it is possible that some sites were lost if they are close enough to yield fragments shorter than 50 base pairs. On the other hand, the accuracy of the site position is of the order of 50 to 150 bp because of the rough estimation of the size of relatively high molecular weight fragments (3 to 6 kb).

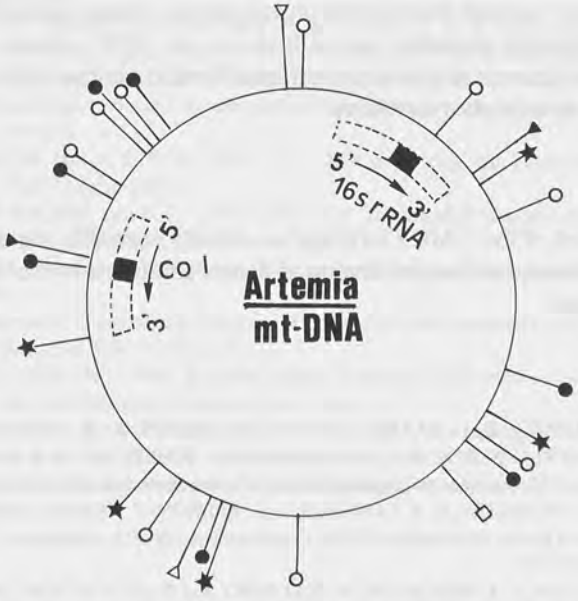

FIG. 6. Restriction map of *Artemia* mitochondrial DNA. The figure summarizes the results presented in this paper. Broken lines indicate the presumptive limits for 16 s rRNA and Co I genes. Dark boxes indicate the sequenced regions. (▽) *Hin*dIII ; (▼) *Eco*RI ; (▼) *Ava*I ; (▽) *Pvu*II ; (▽) *Pst*I ; (▽) *Bam*HI.

With these limitations in mind, the availability of recombinant plasmids containing a detailed physical map opens the possibility of continuing the sequence determination and provides specific probes for future studies. For example, it allows to explore the presence of mitochondrial sequences in *Artemia* genomic DNA, as has been shown in other species (Gellisen *et al.*, 1983 ; Jacobs *et al.*, 1983).

In addition, we have mapped some genes in the *Artemia* mitochondrial genome, as summarized in Fig. 6. The 16s rRNA and Co I genes have been precisely localized, their orientation deduced, and their preliminary limits established. The major conclusion has been the detection in *Artemia* mt-DNA of a similar organization to the *Drosophila* mitochondrial genome, and different of the gene order present in mammals (Bibb *et al.*, 1981) and sea urchin (Roberts *et al.*, 1983). In the former, the Co I is transcribed in the same direction that 16s rRNA, and in seah urchin the Co I gene is directly adjacent to the 16s rRNA. Neither of these gene organizations is present in *Artemia*.

Furthermore, the limited sequence data obtained from *Artemia* mt-DNA indicate a similar variability at the nucleotide level when compared to *Drosophila* and bovine mt-DNA (29 % and 30.7 % respectively). This variability is considerably decreased at the amino acid level, particularly when compared with *Drosophila* (*Drosophila* 6.5 % and bovine 17 %). The data also suggest that in *Artemia* in addition to common mitochondrial codon variations, like TGA for tryptophan and ATA for methionine, instead of termination and isoleucine respectively, a codon usage similar to *Drosophila* is found, namely AGA codes for a serine instead of termination (de Bruijn, 1983).

In the course of this work we have isolated mitochondrial DNA from other *Artemia* sources. Surprisingly, their restriction map has shown an amazing degree of divergency evoking the interesting possibility of an even faster evolution rate in the *Artemia* mitochondrial sequences, compared to the previously described ones (Brown *et al.*, 1979 ; Brown, 1980). Thus the physical map described here is valid for *Artemia* from a North American origin. A detailed report of these findings will be published elsewhere.

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The expression of a gene for eukaryotic elongation factor Tu in *Artemia*

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Abstract

Total *in vivo* proteins from *Artemia* embryos at different developmental stages were examined by 2-D gel electrophoresis. A variety of peptides change during development, with one of them, the eukaryotic elongation factor Tu (eEF-Tu), presenting a dramatic increase from dormant embryos to nauplii. When poly(A)$^+$ RNA is translated *in vitro*, the same relative increase is seen for eEF-Tu during development. Based on the published amino acid sequence for *Artemia* eEF-Tu, a synthetic oligodeoxynucleotide was prepared and used to prime the synthesis of cDNA with poly(A)$^+$ RNA from 12-h developing embryos as template. Direct sequence analysis of the 900-base primary cDNA product shows it to be specific for the 5′ end of *Artemia* eEF-Tu mRNA. Hybridization of a "Northern" blot of denatured poly(A)$^+$ RNA with this cDNA reveals a major band migrating at about 1 800 bases. Quantitation of eEF-Tu mRNA during development shows that it parallels the *in vitro* translation results. When poly(A)$^+$ RNA is separated on a non-denaturing gel, blotted to poly(U)-paper, and hybridized with the eEF-Tu cDNA, a single band is observed migrating faster than 18S. Elution and *in vitro* translation of this band results in a major product migrating with eEF-Tu in a dodecylsulfate polyacrylamide gel. Immunoprecipitation of the *in vitro* translation products with eEF-Tu-specific antibodies identifies the 50 kDa protein as eEF-Tu.

Since the amino acid sequence of the region of eEF-Tu from which the oligomer sequence was derived, is conserved in *Escherichia coli* and *Artemia*, we isolated poly(A)$^+$ RNA from a variety of species to investigate possible conservation of sequences across other taxonomic boundaries. In the species surveyed the eEF-Tu oligomer hybridized to mRNA of about the same size. When the various RNAs were used for reverse transcription primed by the same oligomer, the primary products were the same size on polyacrylamide/urea gels.

Abbreviations used

eEF-Tu : eukaryotic protein synthesis elongation factor Tu, the high M_r subunit of EF-1 ;
EF-Tu : prokaryotic protein synthesis elongation factor Tu.

Introduction

Embryogenesis in *Artemia* is characterized by several unique and interesting features (Lochhead, 1961 ; Olson and Clegg, 1978). During late gastrulation and under the appropriate conditions, the embryo may encyst and enter a dormant state. Released into the environment, the cyst becomes desiccated and remains metabolically inactive. Dormant *Artemia* embryos are characterized by the accumulation of 80S ribosomes (Clegg and Cavagnaro, 1976) and a pool of mRNA which is found in the cytoplasm associated with proteins in the form of ribonu-

cleoprotein particles, or mRNPs (Hultin and Morris, 1968 ; Moens and Kondo, 1976 ; Amaldi *et al.*, 1977 ; Grosfeld and Littauer, 1976 ; Slegers and Kondo, 1977). Resumption of development, which is triggered by hydration (Clegg, 1976) and subsequent development of the freshly hydrated embryos is relatively synchronous. RNA and protein synthesis reinitiate during the first 12 h following hydration, in the absence of both DNA synthesis and cell division (Golub and Clegg, 1968 ; Clegg and Cavagnaro, 1976).

One of the major proteins synthesized by *Artemia in vivo* is a 50 000 M_r polypeptide which has been identified as the eukaryotic elongation factor Tu (eEF-Tu). This factor, which is responsible for binding aminoacyl-tRNA to the ribosome, has been shown to be present in abundance in the cytoplasm of both prokaryotic and eukaryotic cells (Slobin and Möller, 1976). The importance of eEF-Tu in protein synthesis makes the regulation of its synthesis a central problem in the study of development. However, no one has as yet studied changes in eEF-Tu during *Artemia* embryogenesis.

In this work we examined protein synthesis directed by mRNA isolated from both dormant and developing *Artemia* embryos. Among the many proteins produced *in vitro* by mRNA from different stages, eEF-Tu is clearly one of the major products at every stage. We also followed the expression of the eEF-Tu gene during development. By using an eEF-Tu-specific cDNA probe, we identified the mRNA for eEF-Tu, and quantitated the changes in its levels at all stages of development.

We also began an investigation into the interspecies homology of eEF-Tu. The amino acid sequence of eEF-Tu from *E. coli* (Arai *et al.*, 1980), *Euglena* chloroplasts (Montandon and Stutz, 1983), and yeast mitochondria (Nagata *et al.*, 1983) has been determined, as well as that for eEF-Tu from yeast cytoplasm (Cottrelle *et al.*, 1985) and *Artemia* (Amons *et al.*, 1983). Several regions of homology have been identified which may reflect functional domains. To extend this work to other species, we began by identifying the species-specific mRNA for eEF-Tu, and performed eEF-Tu-specific primer extensions on mRNA from several species. In all species examined both the sizes of the various mRNAs and the sizes of the primary primer extension products gave identical results.

Materials and methods

Preparation of *Artemia* embryos

Cysts were obtained from San Francisco Bay Brand (Newark, CA) and were washed, cultured, and harvested as previously described (MacRae *et al.*, 1979 ; Warner *et al.*, 1979). Embryos were frozen in liquid nitrogen immediately after collection and were stored at -70 °C.

Analysis of proteins extracted from *Artemia* embryos

Embryos were ground at 90-100 °C in 2.5 volumes (w/v) of 10 % trichloroacetic acid, with a Potter-Elvehjem homogenizer. Following lyophilization the proteins were analyzed by two-dimensional gel electrophoresis as described for *in vitro* proteins.

RNA isolation

RNA was extracted by grinding embryos at 4 °C in 30 mM Tris-Cl, pH 7.4, 5 mM magnesium acetate, 0.5 % Triton X-100, and 10 mM vanadyl ribonucleoside complexes (Berger and

Birkenmeier, 1979) with a Polytron Homogenizer (Brinkman, Westberry, New York). Protein was removed by phenol extraction (Perry *et al.*, 1972) and glycogen by the method of Bellamy and Ralph (1968). Intact RNA was also prepared by the guanidinium isothiocynanate/cesium chloride gradient method (Chirgwin *et al.*, 1979 ; Maniatis *et al.*, 1982). Poly(A)$^+$ RNA was isolated by two passages through oligo d(T)-cellulose (Aviv and Leder, 1972).

In vitro translation of poly(A)$^+$ RNA

Cell-free protein synthesis was performed in 10 µl reaction mixtures containing 7 µl of nuclease-treated rabbit reticulocyte lysate (Promega Biotec) as described by Woodley *et al.* (1981). All reactions were performed in triplicate.

Gel electrophoresis of *in vitro* translation products

Two-dimensional gel electrophoresis was performed by a modification of the procedure of O'Farrell (1975). RNase-digested *in vitro* translation products were made 9 M with urea and diluted with five volumes of 9 M urea, 2 % ampholytes (Servalyt pH 2-11), 2 % Triton X-100, and 5 % 2-mercaptoethanol.

For discrete visualization of eEF-Tu, nonequilibrium pH gradient gel electrophoresis (NEPHGE) was employed (O'Farrell *et al.*, 1977). The second dimension was run according to Laemmli (1970).

Proteins were stained by one of two procedures. The procedure of Oakley *et al.* (1980) was used for silver staining. For visualization prior to autoradiography, the gels were stained with Coomassie Brilliant Blue R-250 and impregnated with 0.25 M sodium salicylate (Chamberlain, 1979). Fluorography was performed at -70 °C with Kodak XAR-5 film.

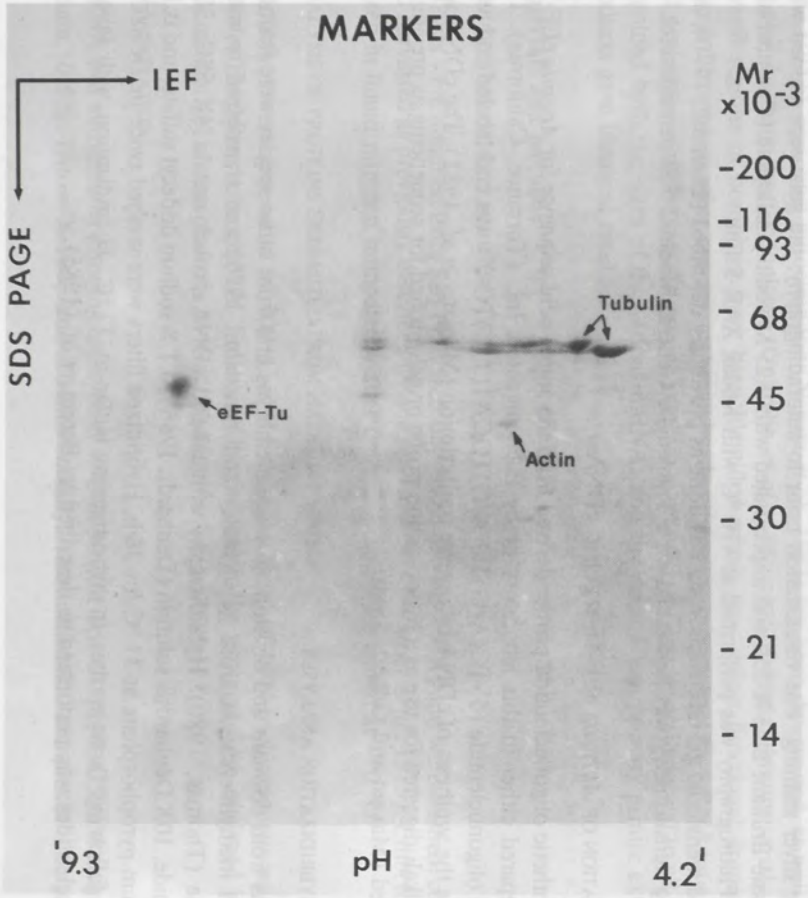
One-dimensional gel electrophoresis was done as previously described (Laemmli, 1970), and scanning densitometry was performed on a Transidyne General Model 2 500 densitometer.

Preparation of *Artemia* eEF-Tu-specific cDNA

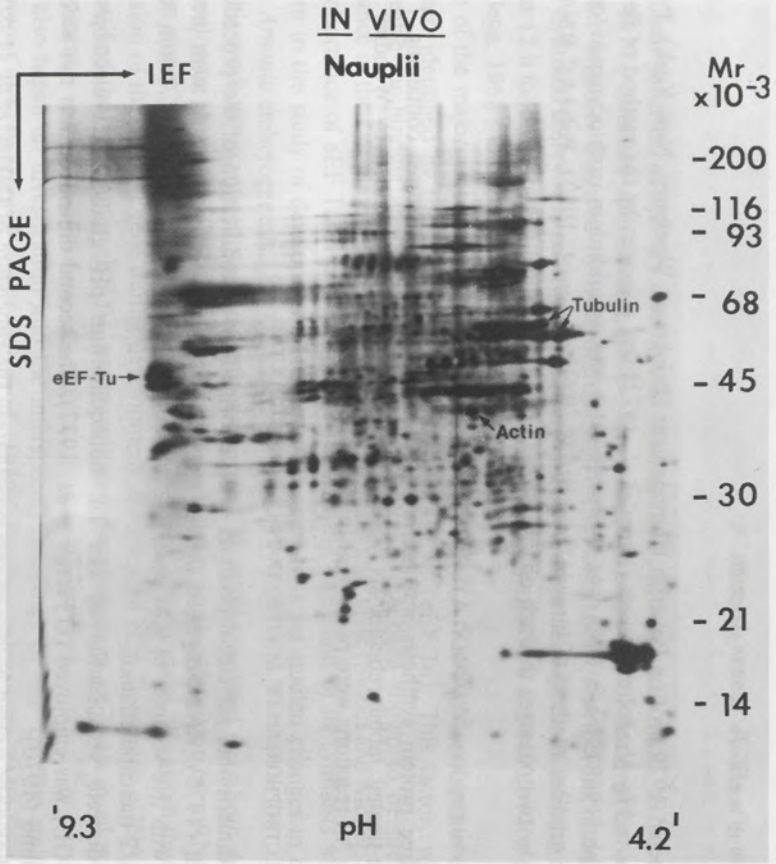
A synthetic oligonucleotide primer derived from the amino acid sequence for *Artemia* eEF-Tu was prepared either in this laboratory or by Bachemgentec, Inc. (Torrance, California). The purified oligonucleotide (5'-TC(A/G)TG(A/G)TGCAT(T/C)TC-3') was end-labeled and used to prime the synthesis of cDNA by reverse transcription (Maniatis *et al.*, 1982). The cDNA was either alkali-digested for use as a hybridization probe or gel-purified for sequencing as previously described (Maxam and Gilbert, 1980).

RNA hybridization analyses

RNAs from dormant and developing *Artemia* embryos and from other species were fractionated on methylmercury/agarose gels (Bailey and Davidson, 1976) and transferred to nitrocellulose (Thomas, 1980). Hybridization with labeled cDNA probes was in 4X SSC, 30 % formamide, 10X Denhardt's solution (Denhardt, 1966), 0.1 % sodium dodecyl sulfate, and 0.1 % potassium pyrophosphate at 37 °C for 36 h. Hybridized filters were washed twice in 1X SSC at 65 °C, following three washes in hybridization buffer at 37 °C. Hybridization with labeled oligonucleotides was performed as described by Berent *et al.* (1985).


MARKERS
IEF
SDS PAGE
Mr x10-3
-200
-116
- 93
- 68
- 45
- 30
- 21
- 14
Tubulin
eEF-Tu
Actin
'9.3
pH
4.2'

1a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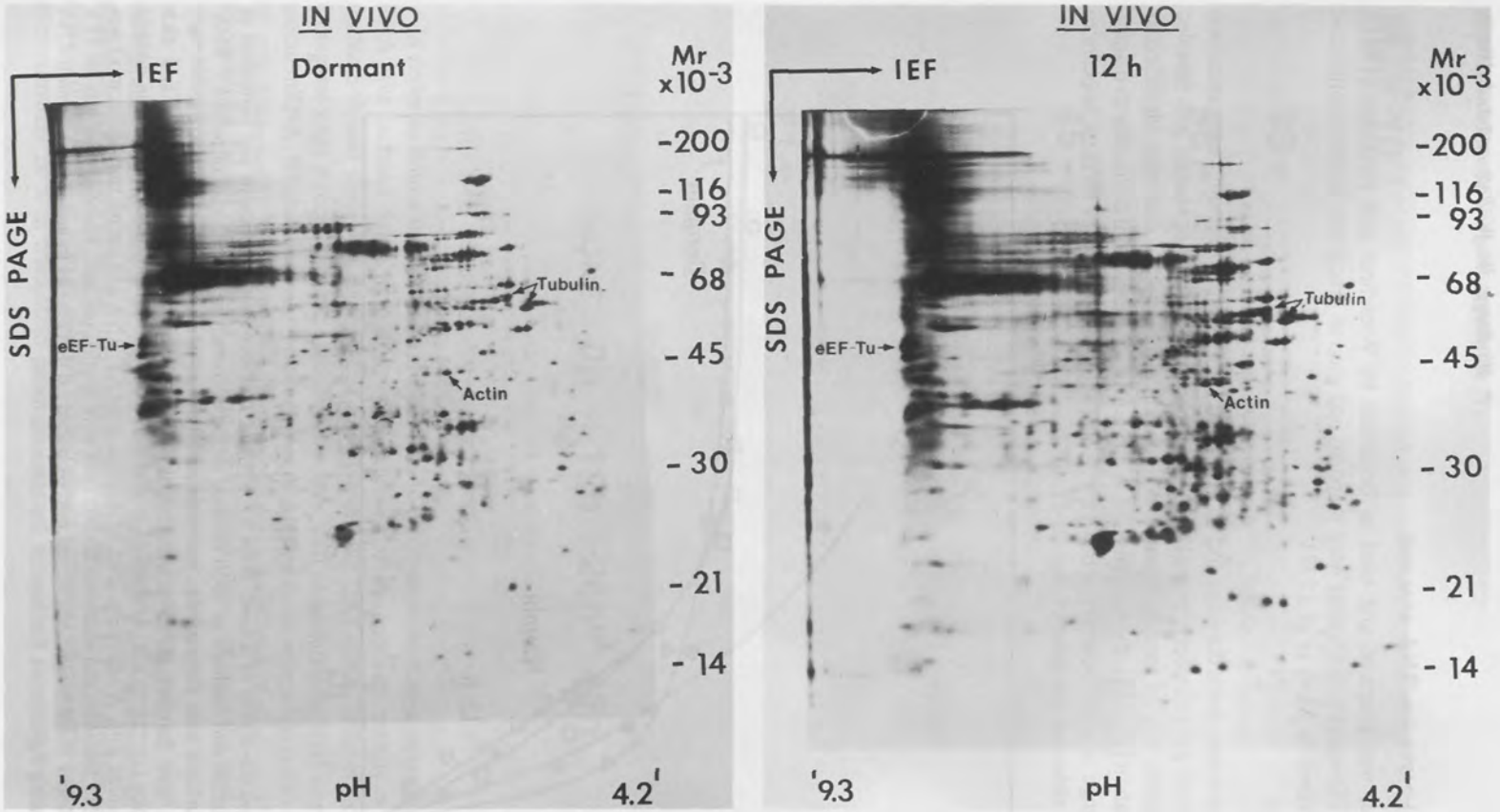
1b



1c 1d

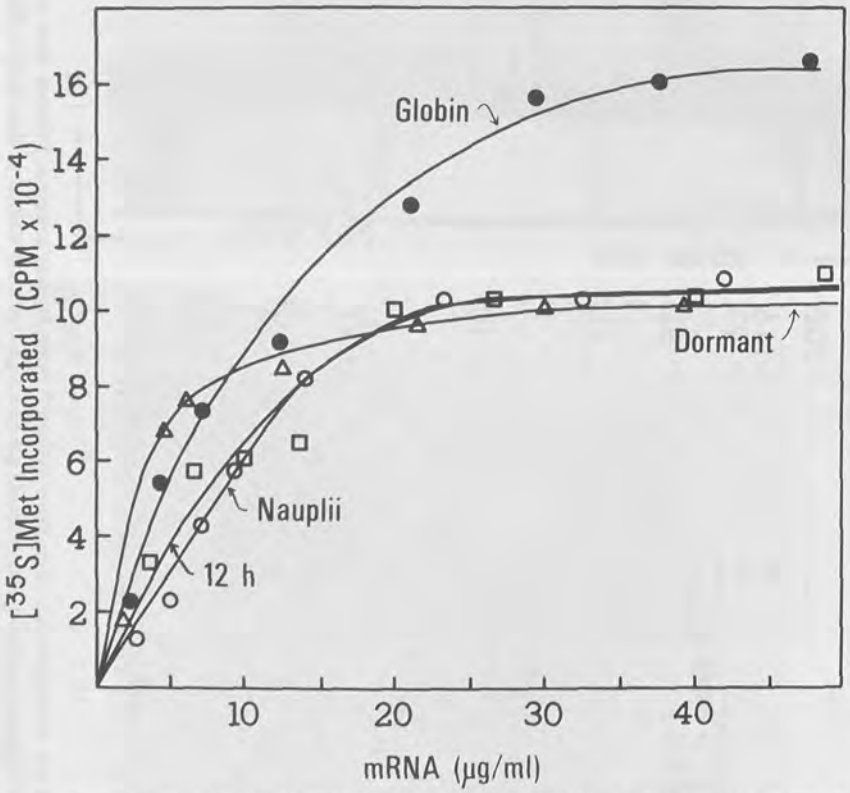
FIG. 1. Two-dimensional gel electrophoresis of proteins extracted from *Artemia* embryos. Proteins were extracted from *Artemia* embryos at various stages of development, separated by IEF/dodecyl sulfate-polyacrylamide gel electrophoresis and stained as described in Materials and methods. An aliquot, 15 µg, was applied to the first dimension (IEF) gels. a. markers : *Artemia* eEF-Tu, *Artemia* tubulin, and chicken muscle actin ; b. dormant *Artemia* embryos ; c. 12 h developing embryos ; d. 48 h developing embryos (nauplii).

USE OF POLY(U)-PAPER FOR RNA ANALYSIS

Poly(U)-paper was prepared and used as described by Wreschner and Herzberg (1984). Rabbit anti-eEF-Tu-specific antibodies were prepared and used to precipitate *in vitro*-synthesized eEF-Tu as described by Mori *et al.* (1981).

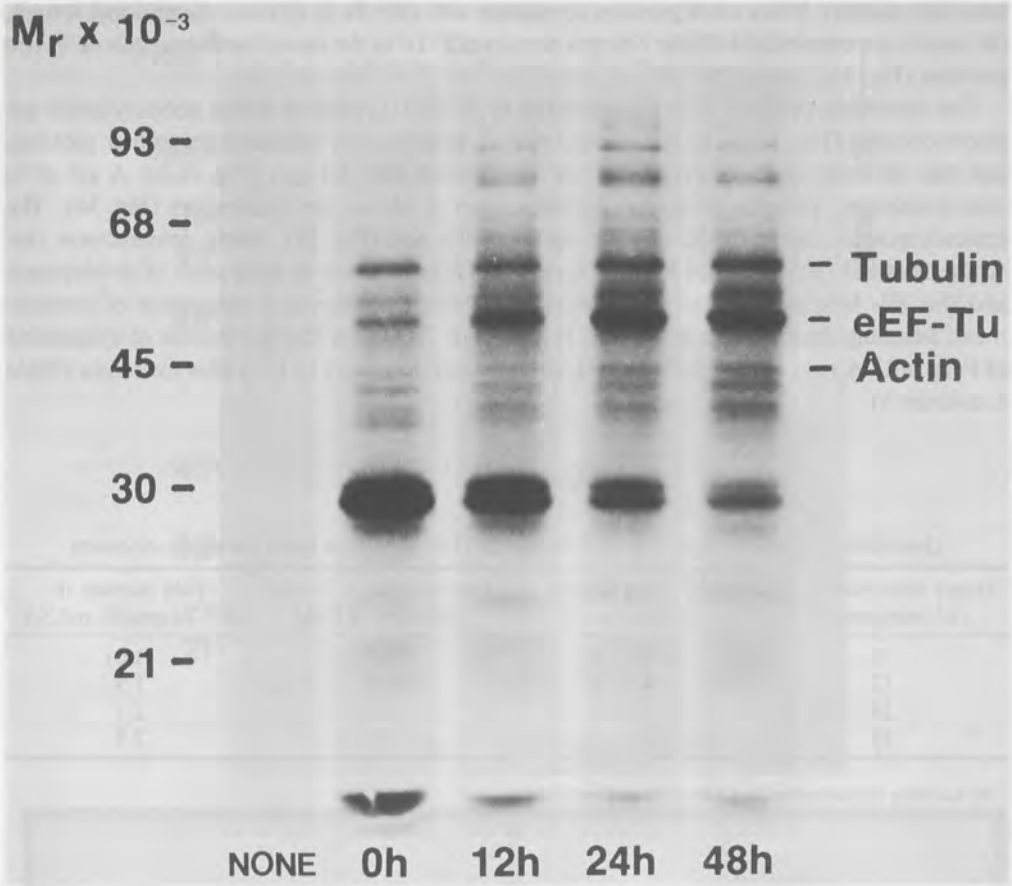
Results

Total *in vitro* proteins from dormant and 12-h embryos, and from 48-h nauplii were examined by two-dimensional gel electrophoresis (Fig. 1a-d). Analyses of the silver-stained gels revealed that several proteins vary in amount during development. Among those that were identified by marker co-migration (Fig. 1a), the tubulins and actin show an increase from dormancy to nauplii. A dramatic change is seen, however, when eEF-Tu is followed ; its rate of increase appears to be greater than either of the others (Fig. 1b-d).



2a

FIG. 2. *In vitro* translation of *Artemia* poly(A)$^+$ RNA. a. Cell-free protein synthesis was performed as described in Materials and methods in 10 µl reaction mixtures containing 0.05-0.5 µg of poly(A)$^+$ RNA. [^{35}S]Met incorporation into polypeptides was measured in 1 µl aliquots. Globin mRNA (●——●), *Artemia* mRNA from dormant embryos (Δ——Δ), 12 h developing embryos (□——□), and 48 h developing embryos (○——○). b. Analysis of *in vitro* translation products by electrophoresis in dodecyl sulfate-polyacrylamide gels. A saturating level of *Artemia* poly(A)$^+$ RNA (40 µg/ml translation mix) was used as described in Materials and methods. 80 000 cpm were applied per lane. Fluorography was for 12 h at -70 °C with Kodak XAR-5 film. *NONE*, no mRNA added ; 0 h, 12 h, 24 h, and 48 h *Artemia* mRNA isolated from developing embryos harvested at the indicated times.



2b

In order to follow the synthesis of eEF-Tu and other proteins during development, poly(A)$^+$ RNA was isolated from various *Artemia* stages, and its template activity was tested in a nuclease-treated rabbit reticulocyte cell-free system (Fig. 2A). RNA isolated from every developmental stage directed protein synthesis at a very high and equivalent level when compared to globin mRNA. When the translation products of these reactions were subjected to electrophoresis in one-dimensional SDS polyacrylamide gels, the overall distribution of [^{35}S]Met-labeled polypeptides did not vary with the different RNAs. Most of the polypeptides seen in a given stage were also present in the other stages (Fig. 2b). However, prominent quantitative changes were seen in the synthesis of a polypeptide(s) of approximate M_r 28 000, as well as for proteins co-migrating with tubulin, actin, and eEF-Tu. For example, scanning densitometry followed by peak integration shows that the band migrating at the M_r of eEF-Tu comprises only 7 % of total protein synthesized *in vitro* by mRNA from dormant embryos, whereas this percentages rises to approximately 20 % in translation products directed by mRNA from 24-h embryos (Fig. 2b ;

scans not shown). While other proteins co-migrate with eEF-Tu in this one-dimensional system, the results are consistent with the changes seen in eEF-Tu in the two-dimensional gels of *in vivo* proteins (Fig. 1).

The translation products were also analyzed by NEPHGE/dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 3a-b). In this system, eEF-Tu is completely separated from other proteins, and may be more easily analyzed than in conventional IEF/2D gels (Fig. 1a-b). A gel of *in vitro*-synthesized proteins from the dormant stage is shown for illustration (Fig. 3a). The autoradiographic image of the eEF-Tu region of the gels (Fig. 3b) clearly demonstrates that synthesis of eEF-Tu is directed by mRNA extracted from embryos at every stage of development and that this synthesis increases dramatically following hydration and resumption of development. Scanning densitometry of the eEF-Tu area (Fig. 3b) shows that the amount of translatable eEF-Tu mRNA increases by more than three-fold from dormancy to 12-h after hydration (Table I, column 3).

TABLE I

Quantitation of *in vitro* translated eEF-Tu and of eEF-Tu mRNA levels during development

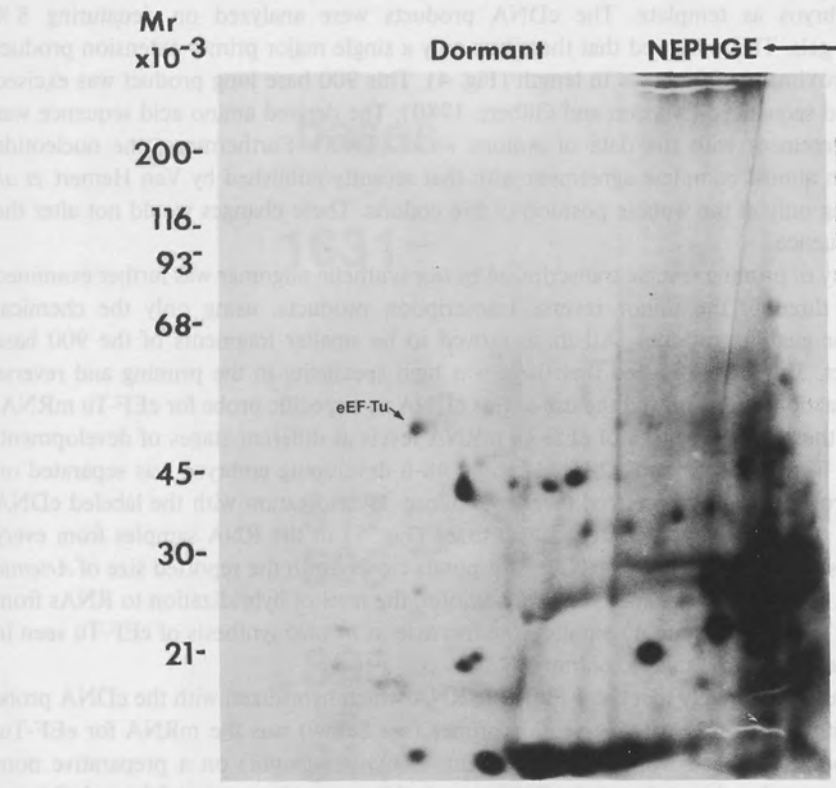
Hours after resumption of development	mg Total RNA/g hydrated cysts	Fold increase in *in vitro* translated eEF-Tu[1]	Fold increase in eEF-Tu-specific mRNA[1]
0	1.2 - 1.4	(1.0)	(1.0)
12	1.4 - 1.6	3.7	1.9
24	1.2 - 1.7	3.7	2.1
48	1.3 - 1.6	2.9	2.8

[1] By scanning densitometry ; see Materials and methods.

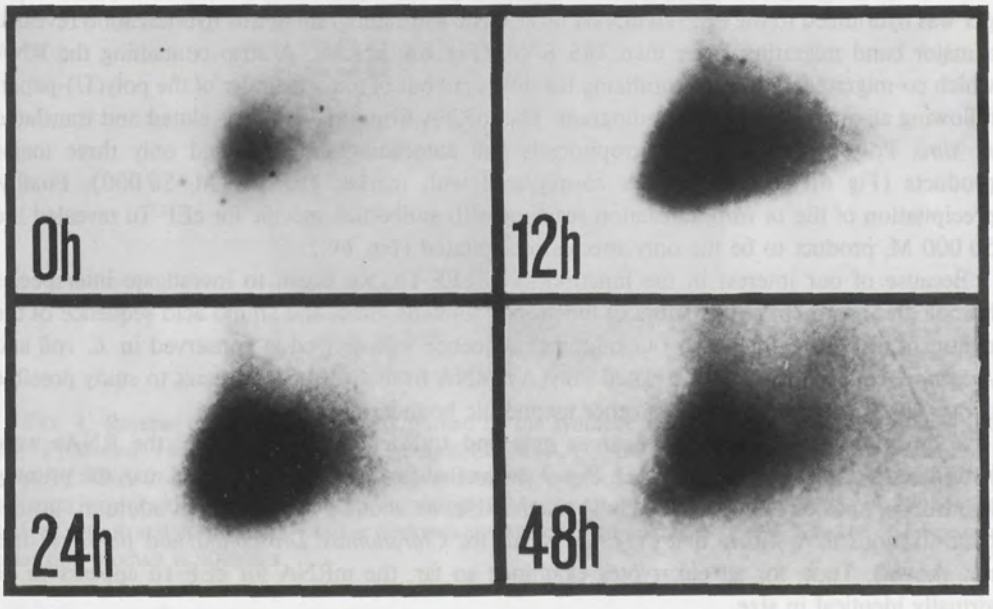
The second stage at which to analyze gene expression is at the level of messenger RNA. Potential changes in the appearance of eEF-Tu mRNA at different stages of development were measured by hybridization to a specific cDNA probe. As primer for the production of eEF-Tu cDNA, a mixed sequence tetradecanucleotide oligomer was prepared, based on amino acids numbers 259 through 263 of the sequence of Amons *et al.* (1983). Following labeling with ^{32}P at the 5'-end, this oligomer was used to synthesize cDNA with poly(A)$^+$ RNA from 12-h

→

FIG. 3. Identification of *in vitro*-synthesized eEF-Tu. NEPHGE/dodecyl sulfate-polyacrylamide gel electrophoresis of *Artemia* poly(A)$^+$ RNA-directed *in vitro* translation products was performed as described in Materials and methods. Prior to electrophoresis 5 µg of purified *Artemia* eEF-Tu was added as unlabeled marker to 180 000 cpm of translation products ; less than 1 % of the applied protein failed to enter the second dimension gel. a. Fluorograph of a single stage (dormant) is shown for illustration. Arrow indicates eEF-Tu. b. Fluorographs of *in vitro* synthesized eEF-Tu with *Artemia* poly(A)$^+$ RNA from different stages of development. Fluorography of all gels was for 10 days at -70 °C with Kodak XAR-5 film.


Mr
x10-3
Dormant
NEPHGE
SDS PAGE
200-
116-
93-
68-
eEF-Tu
45-
30-
21-

3a


0h
12h
24h
48h

3b

developing embryos as template. The cDNA products were analyzed on denaturing 8 % polyacrylamide gels. These showed that there was only a single major primer extension product which was approximately 900 bases in length (Fig. 4). This 900 base long product was excised from the gel and sequenced (Maxam and Gilbert, 1980). The derived amino acid sequence was in complete agreement with the data of Amons *et al.* (1983). Furthermore, the nucleotide sequence was in almost complete agreement with that recently published by Van Hemert *et al.* (1984), differing only at the wobble position of five codons. These changes would not alter the amino acid sequence.

The specificity of priming reverse transcription by our synthetic oligomer was further examined by sequencing three of the minor reverse transcription products, using only the chemical modification for guanine residues. All three proved to be smaller fragments of the 900 base primary product. This demonstrated that there was high specificity in the priming and reverse transcription reactions, and allowed the use of this cDNA as a specific probe for eEF-Tu mRNA.

To examine the relative changes of eEF-Tu mRNA levels at different stages of development, poly(A)$^+$ RNA from dormant and 12-h, 24-h, and 48-h developing embryos was separated on denaturing agarose gels and transferred to nitrocellulose. Hybridization with the labeled cDNA probe revealed a band at approximately 1 800 bases (Fig. 5) in the RNA samples from every developmental stage. The size of this RNA corresponds closely with the reported size of *Artemia* eEF-Tu mRNA (Van Hemert *et al.*, 1984). In addition, the level of hybridization to RNAs from different stages (Table I, column 4) parallels the increase in *in vitro* synthesis of eEF-Tu seen in Fig. 3b and quantitated in Table I, column 3.

To establish unquestionably that the 1 800 base RNA which hybridized with the cDNA probe (Fig. 5) and with the 14-base oligonucleotide primer (see below) was the mRNA for eEF-Tu, we separated poly(A)$^+$ RNA from 48-h developing embryos (nauplii) on a preparative non-denaturing agarose gel and transferred the RNA to poly(U)-paper. A portion of the poly(U)-paper was hybridized to the eEF-Tu cDNA probe. The autoradiogram of this hybridization revealed a major band migrating faster than 18S RNA (Fig. 6A, arrow). A strip containing the RNA which co-migrated with this hybridizing band was cut out of the remainder of the poly(U)-paper, following alignment of the autoradiogram. The mRNA from this strip was eluted and translated *in vitro*. Polyacrylamide gel electrophoresis and autoradiography revealed only three major products (Fig. 6B), one of which co-migrated with marker eEF-Tu (M_r=50 000). Finally, precipitation of the *in vitro* translation reaction with antibodies specific for eEF-Tu revealed the 50 000 M_r product to be the only species precipitated (Fig. 6C).

Because of our interest in the function(s) of eEF-Tu, we began to investigate interspecies homologies as potential indicators of functional domains. Since the amino acid sequence of the region of eEF-Tu from which our oligomer sequence was derived is conserved in *E. coli* and *Artemia* (see Discussion), we isolated poly(A)$^+$ RNA from a variety of species to study possible conservation of sequences across other taxonomic boundaries.

Following electrophoresis in agarose gels and transfer to nitrocellulose, the RNAs were hybridized with the labeled oligomer. Fig. 7 shows that for *Artemia*, mouse, and man the primary hybridizing species is approximately the same size, or about 1 800 bases. In addition, further hybridizations have shown that the same is true for *Chironomus*, *Drosophila*, and *Bombyx* (data not shown). Thus, for all eukaryotes examined so far, the mRNA for eEF-Tu appears to be virtually identical in size.

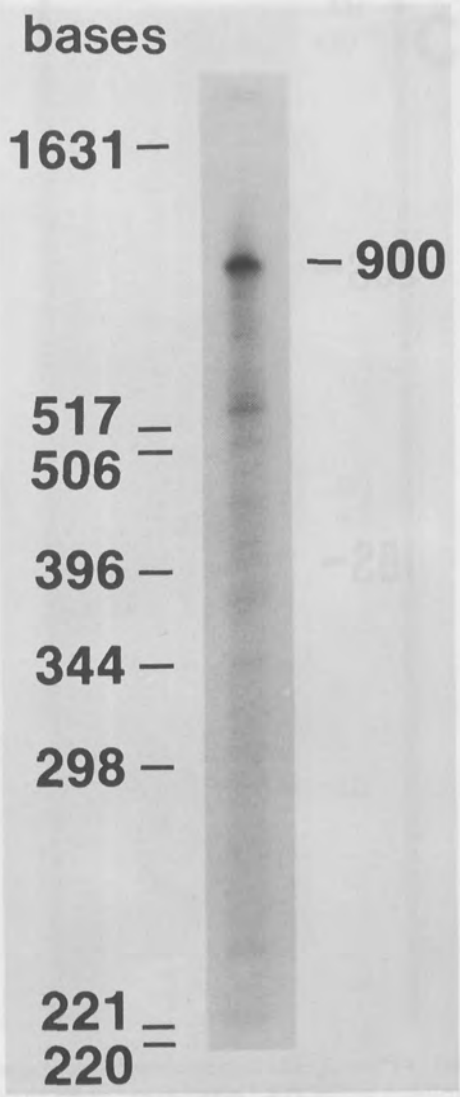


FIG. 4. Reverse transcription products primed by the synthetic eEF-Tu-specific oligonucleotide. The 5′-[^{32}P]-labeled 14-base oligonucleotide primer (5′-TC(A/G)TGCAT(T/C)CC-3′) was hybridized to poly(A)$^+$ RNA from *Artemia* and reverse transcribed as described in Materials and methods. The cDNA products were fractionated in an 8 % polyacrylamide/8M urea gel, and exposed for autoradiography for 2 h at 4 °C with Kodak XAR-5 film. Marker positions are labeled HinfI fragments of pBR322. The major primer extension product is 900bases.

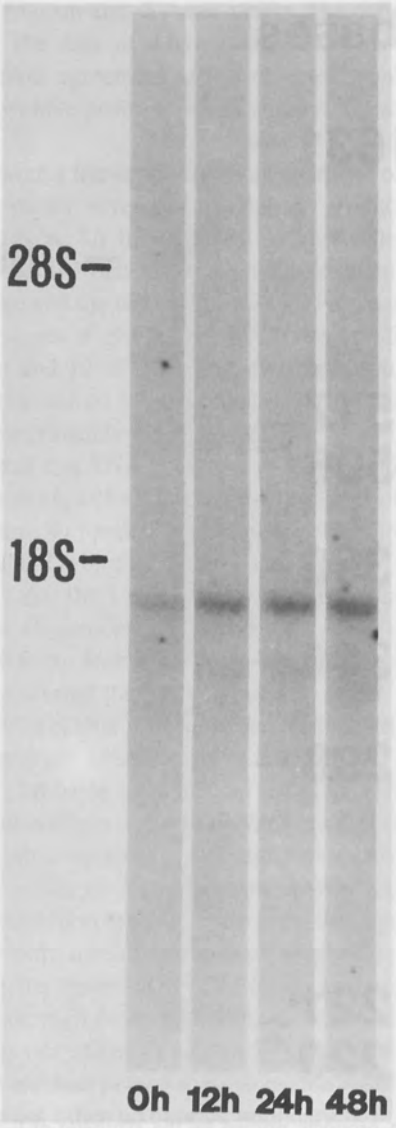


FIG. 5. Hybridization of eEF-Tu cDNA to *Artemia* poly(A)$^+$ RNA. Poly(A)$^+$ RNA (4 μg) from *Artemia* embryos at different stages of development was denatured with 10 mM CH_3HgOH and separated on a 10 mM CH_3HgOH/agarose gel. The RNA was transferred to nitrocellulose and hybridized with 2.0×10^6 cpm of the eEF-Tu-specific cDNA. Exposure for autoradiography was for 72 h at −70 °C.

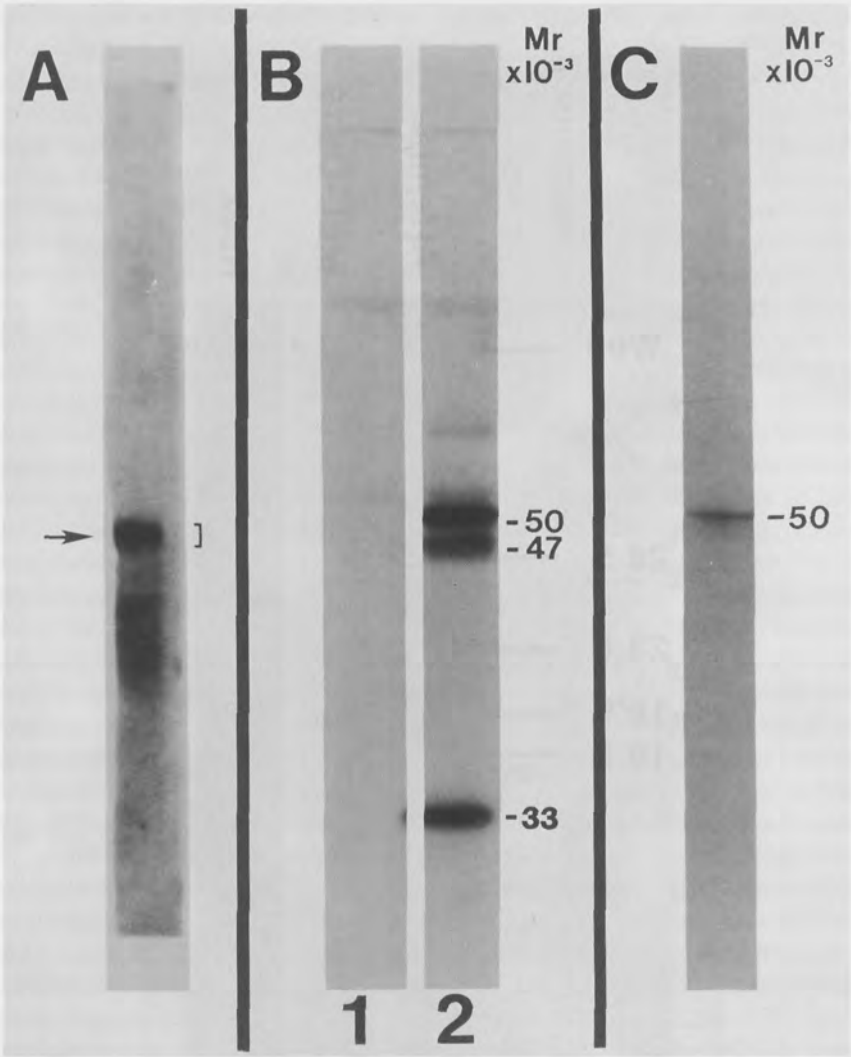


FIG. 6. Poly(U)-paper selection and translation of eEF-Tu mRNA. Poly(A)$^+$ RNA from 48 h developing embryos was electrophoresed on a preparative 1.5 % agarose non-denaturing gel. Following transfer to poly(U)-paper, a strip along the edge was cut off and hybridized with 1.0×10^6 cpm/ml of eEF-Tu cDNA. A. Autoradiography of the hybridized strip, exposed for 16 h at -70 °C. The autoradiograph was aligned with the remainder of the poly(U)-paper, the area (bracket) co-migrating with the hybridizing band (arrow) cut out, and the RNA eluted. B. Dodecyl sulfate-polyacrylamide gel electrophoresis of the *in vitro* translation products. The 50 000 M_r product co-migrates with *Artemia* eEF-Tu ; 1, no RNA added ; 2, poly(U)-paper selected RNA. C. Dodecyl sulfate-polyacrylamide gel electrophoresis of the eEF-Tu antibody precipitate from *in vitro* translation products.

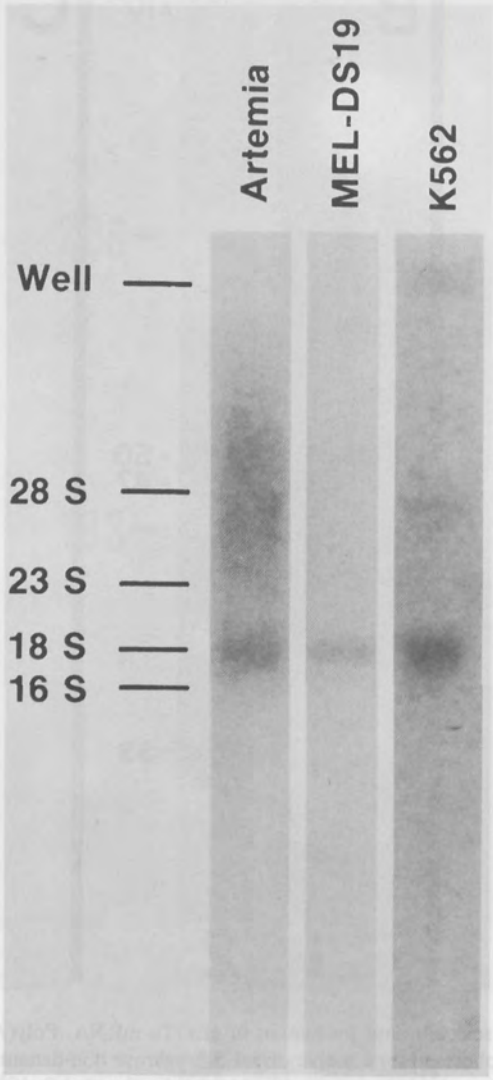


FIG. 7. Hybridization of the oligonucleotide primer to poly(A)-containing RNA from various species. RNA (4 μg) from *Artemia*, K562 cells (human) and MEL-DS19 cells (mouse) was fractionated on denaturing agarose gels and transferred to nitrocellulose. Exposure for autoradiography was for 24 h at -70 °C.

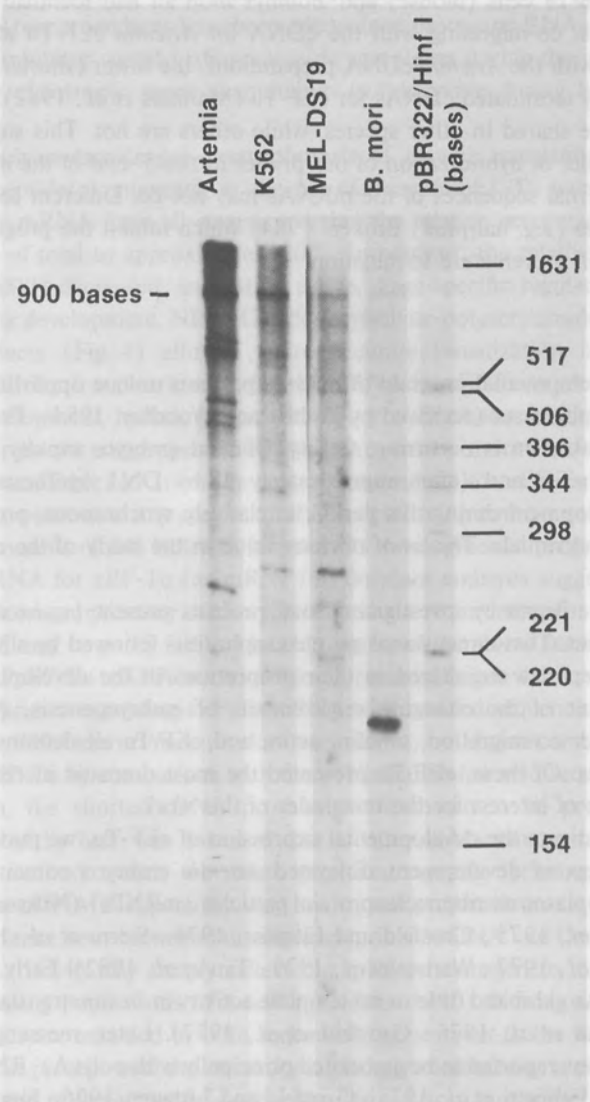


FIG. 8. Primer extension of mRNA from various species. The mixed-sequence 14-base oligonucleotide was end-labeled and used to prime reverse transcription of 10 μg of poly(A)$^+$ RNA. The cDNA products were fractionated on 8 % polyacrylamide/8 M urea gels. Marker positions are HinfI-digested and labeled pBR322.

We next performed primer extensions of the poly(A)-containing RNAs from different species. The 14-base oligonucleotide was utilized as the eEF-Tu-specific primer for reverse transcription. The results, shown in Fig. 8, revealed that the fully extended products from *Artemia*, K562 cells (human), MEL DS-19 cells (mouse) and *Bombyx mori* all had identical sizes on denaturing polyacrylamide gels, co-migrating with the cDNA for *Artemia* eEF-Tu at approximately 900 bases. By analogy with the *Artemia* cDNA preparations, the lower (shorter) extension products may be prematurely terminated cDNAs for eEF-Tu (Maniatis *et al.*, 1982). Interestingly, bands in some species are shared in other species, while others are not. This suggests that while the distance from the site of hybridization of our primer to the 5′-end of the molecule is extremely conserved, the internal sequences of the mRNAs may not be. Different sequences may lead to secondary structures (*e.g.* hairpins ; Broker, 1984) which inhibit the progress of reverse transcriptase, resulting in a premature termination.

Discussion

The unusual developmental program of *Artemia* presents unique opportunities for elucidating cellular regulatory processes (reviewed by Wahba and Woodley, 1984). From a stage in which apparently no metabolism is occurring, freshly-hydrated embryos rapidly initiate protein synthesis, RNA synthesis, and (after approximately 18 h) DNA synthesis and cell division. Furthermore, development during this period is relatively synchronous, providing a population of closely-staged individuals. This is of obvious value in the study of the molecular aspects of development.

In this report we began by investigating total proteins present *in vivo* at different stages of *Artemia* development. Two-dimensional gel electrophoresis followed by silver staining revealed that a variety of peptides are altered in their proportions in the developing embryo (Fig. 1), probably as a result of the changing requirements of embryogenesis. Among the proteins identified by marker co-migration, tubulin, actin, and eEF-Tu all demonstrated stage-specific quantitative changes. Of these, eEF-Tu presented the most dramatic increase (Fig. 1b-d), and became the protein of interest for the remainder of this work.

In order to investigate the developmental expression of eEF-Tu, we purified *Artemia* mRNA from different stages of development. Encysted *Artemia* embryos contain a pool of mRNA present in the cytoplasm as ribonucleoprotein particles (mRNPs) (Nilsson and Hultin, 1972, 1974 ; Felicetti *et al.*, 1975 ; Grosfeld and Littauer, 1976 ; Sierra *et al.*, 1976 ; Amaldi *et al.*, 1977 ; Grosfeld *et al.*, 1977 ; Warner *et al.*, 1979 ; Tan *et al.*, 1982). Early reports claimed that these stored mRNAs exhibited little or no template activity in *in vitro* translation assays (Felicetti *et al.*, 1975 ; Sierra *et al.*, 1976 ; Grosfeld *et al.*, 1977). Later, messenger RNA activity in dormant embryos was reported to be associated principally with poly(A)$^+$ RNA, and no poly(A)$^+$ RNA was present (Felicetti *et al.*, 1975 ; Grosfeld and Littauer, 1976 ; Sierra *et al.*, 1976). The results reported here do not support the previous observations.

When we analyzed the translation products of poly(A)$^+$ RNA in dodecylsulfate-polyacrylamide gels (Fig. 3), the overall distribution of [^{35}S]Met-labeled polypeptides did not vary greatly with mRNAs extracted from embryos at different stages of development. Most of the proteins seen in a given stage are present in the other stages. However, quantitative differences are readily apparent in the products, reflecting the differences seen in Fig. 1. For example, bands which co-migrate with tubulin, eEF-Tu, and actin are seen to increase, just as in the *in vivo* gels. We

believe, therefore, that previous reports of no template activity, or of activity associated only with poly(A)$^-$ RNA in dormant embryos may have been the result of the action of RNases in the cysts. We have found that consistent mRNA template activity with the poly(A)$^+$ fractions was observed only when sufficient precautions were taken to eliminate RNase activity in the preparation of RNA. Thus far, only two procedures have been adequate at protecting RNA : either the inclusion of the ribonuclease inhibitor vanadyl ribonucleoside complexes during the isolation step, or the use of the extreme chaotropic agent guanidinium isothiocyante during homogenization and purification.

Dodecylsulfate polyacrylamide gel electrophoresis of *in vitro* translation products (Fig. 3) revealed that polypeptide(s) migrating at the M_r of *Artemia* eEF-Tu were among the major proteins directed by mRNA from all stages, and that the relative percentage of those proteins increased from 7 % of total to approximately 20 %. In contrast, the relative amount of another protein(s) (M_r 28 000) decreased, suggesting active, stage-specific regulation of the levels of some mRNAs during development. NEPHGE/dodecylsulfate-polyacrylamide gel electrophoresis of the same products (Fig. 4) allowed more accurate visualization and quantitation of [^{35}S]Met-labeled eEF-Tu. It is clearly seen that the mRNA for eEF-Tu is present and translatable a every stage of development, including dormant embryos. Furthermore, the amount of eEF-Tu produced *in vitro* from the stage-specific RNAs increased by more than three-fold following hydration of the cysts (Table I). Given the central requirement for eEF-Tu in protein synthesis and other regulatory processes, it is consistent that a developing embryo such as *Artemia* would require increased amounts of this factor to maintain its developmental program. In addition, the presence of the mRNA for eEF-Tu (as mRNP) in dormant embryos suggests that resumption of development following hydration carries the immediate requirement for more of this factor, a requirement which may be partly fulfilled by recruitment of the stored message into polysomes.

The expression of eEF-Tu mRNA at various developmental stages was examined with a cDNA probe, prepared by primer-extending eEF-Tu mRNA with a mixed-sequence 14-base oligodeoxyribunocleotide. The 900-base primary product was shown, by Maxam and Gilbert sequencing, to be specific for eEF-Tu by comparison with the published amino acid sequence (Amons *et al.*, 1983). In addition, the shorter cDNA products were shown to be premature termination products, as indicated by both our data and that of others (Maniatis *et al.*, 1982). Thus, the primer extension method which we used produced specific priming and the level of non-eEF-Tu-specific cDNA is apparently very low in our preparations.

The five-base variance seen between our oligomer-primed cDNA and that reported elsewhere (Van Hemert *et al.*, 1984) may simply reflect the species heterogeneity that apparently exists between closely existing populations of *Artemia* (Collins, 1980). It is significant, however, that the same amino acids are encoded by both sequences, a testament to the functional importance of that region of the protein and the necessity of strictly maintaining a particular amino acid chain. The comparison of the amino acid sequence derived from our primer-extended cDNA for *Artemia* eEF-Tu to the published sequence for the same region of *E. coli* EF-Tu (Arain *et al.*, 1980), yeast mitochondrial EF-Tu (Nagata *et al.*, 1983), *Euglena* chloroplast EF-Tu (Montandon and Stutz, 1983) and yeast cytoplasmic eEF-Tu (Cottrelle *et al.*, 1985) is shown in Table II. The yeast cytoplasmic sequence is 92 % homologous with the *Artemia* sequence over this region, while the others range between 40-50 % homology to *Artemia* eEF-Tu. Of interest in this region are two sites (Gly-Thr-Val and Gly-Arg-Val-Glu) which are absolutely conserved. These two regions lie just amino-terminal from an absolutely conserved lysine. Recent work from Bosch's

laboratory (Van Noort *et al.*, 1982) has shown that when *E. coli* forms a ternary complex (EF-Tu·GTP·aminoacyl tRNA), the tRNA occupies a specific site on the protein. A second tRNA binding site on the elongation factor is created when the ternary complex associates with the ribosome/mRNA complex or when EF-Tu·GDP or EF-Tu·GTP interacts with the antibiotic kirromycin. Another recent report demonstrates that periodate-oxidized tRNA residing in the ribosomal A site crosslinks exclusively to Lys-237 and EF-Tu·GTP (Van Noort *et al.*, 1984). The completely conserved lysine in Table II is Lys-237 (asterisk). The adjacent, absolutely conserved regions undoubtedly play a significant role in the function of eEF-Tu, which may include aminoacyl tRNA recognition, binding, and/or orientation on the ribosome. In any case, the sequence homology data, combined with the crosslinking experiments, prove that the region around Lys-237 is highly conserved and strongly suggests that it constitutes a functional domain of both EF-Tu and eEF-Tu. Further investigation of eEF-Tu sequence homology in this region among several species is currently underway in our laboratory (see below).

Hybridization of electrophoretically separated RNA from different stages of *Artemia* development with the eEF-Tu cDNA revealed a single band migrating at approximately 1 800 bases. This is the reported size for eEF-Tu mRNA (Amons *et al.*, 1983). The same hybridizing species was found only in poly(A)$^+$ RNA (hybridization of poly(A)$^-$ RNA not shown). Furthermore, the relative intensity of hybridization to the RNA from each stage (Table I, column 4) parallels the *in vitro* translation results seen in Fig. 4 and quantitated in Table I, column 3. This suggests that the primary regulatory control on the amount of eEF-Tu in the cell following hydration may be an increase in the steady-state level of eEF-Tu mRNA.

To confirm that the RNA species hybridizing to our probe was indeed eEF-Tu mRNA, we separated poly(A)$^+$ RNA from 48-h embryos on a non-denaturing preparative agarose gel and transferred the RNA to poly(U)-paper. A single major band was seen after hybridization with the cDNA probe. When the co-migrating, non-hybridized RNA was eluted and translated, three major bands were seen (Fig. 6), one of which migrated at the M_r of marker eEF-Tu. Finally, antibody precipitation of the translation reaction identified the 50 kDa protein as authentic eEF-Tu, and established the validity of our primer extension-produced cDNA as a probe for a specific message.

In order to investigate interspecies homology, we began by searching for eEF-Tu mRNA among other eukaryotes. We found that in each case the mRNA for mouse, human, *Drosophila*, *Bombyx*, and *Chironomus* eEF-Tu co-migrates with *Artemia* eEF-Tu mRNA, at approximately 1 800 bases. Furthermore, when the 14-mer was used for primer extension with mouse, human, and *Bombyx* mRNA, the fully extended products were identical in size, at 900 bases, to the *Artemia* extension product. These results suggest that the mRNA for eEF-Tu is highly conserved in size. When considered together with the known eukaryotic sequence homology data (Table II, rows 1 and 2), eEF-Tu emerges as an amazingly conserved molecule across wide taxonomic boundaries.

We have continued to study interspecies sequence homology, both by sequencing the primer extension products and by selecting eEF-Tu genes from a variety of genomic libraries. Using the known *Artemia* sequence and employing a labeled oligonucleotide as probe, we have already isolated the gene for mouse eEF-Tu and begun its characterization. To our knowledge, this is the first example of an oligomer, produced complementary to the mRNA from one species, being successfully used to screen a genomic library from another species.

TABLE II

Interspecies amino acid sequence homology of eEF-Tu

Artemia[1]	R L P L Q D V Y K I G G I G T V P V G R V E T G I I K* P G M I V T F A P A N I T T . E V K S . . V E M H H
Yeast[2]	– V – – – – – V – – – – – – G V – – . – – – – . . – – – – –
E. coli[3]	L – – I E – – F S – S O R – – – V T – – – – R – – – – V – E E – E I V G I K E – Q . K S T C T G – – – F R
Yeast mitochondria[4]	L M – V E – I F S – S – R – – – V T – – – – R – N L – K – E E L E I V G H – S P L K T T V T G I – – F R
Euglena chloroplasts[5]	L M A I E D – L S – T – R – – – A T – – – – R – T – – V – E T – E L V G L K D – R . K S T I T G L – – F Q

– Indicates homology with *Artemia* eEF-Tu.
. Indicates an adjustment for sequence alignment.
* Indicates Lys 237 (see Discussion).

[1] Daum *et al.*, 1985.
[2] Cottrelle *et al.*, 1985.
[3] Arai *et al.*, 1980.
[4] Nagata *et al.*, 1983.
[5] Montandon and Stutz, 1983.

By analyzing both primer extension products and the genes which we select from genomic libraries, we hope to develop a definitive picture of the interspecies homology of eEF-Tu. This analysis should point to additional functional domains which may be investigated by *in vitro* mutagenesis and functional studies.

Summary

1. We have examined by 2-D gel electrophoresis total *in vivo* proteins extracted from *Artemia* embryos at different developmental stages. Several polypeptides increased dramatically during development, including one which comigrates with eEF-Tu.
2. When poly(A)$^+$ RNA isolated from *Artemia* embryos was translated *in vitro*, the same relative increase was seen for eEF-Tu during development.
3. A synthetic oligodeoxynucleotide was used to prime the synthesis of cDNA from *Artemia* poly(A)$^+$ RNA. The primer extension products were shown by sequence analysis to be specific for eEF-Tu mRNA.
4. We used the major cDNA primer extension product as a hybridization probe in order to determine more accurately the level of eEF-Tu mRNA in *Artemia* embryos. Northern hybridization of poly(A)$^+$ RNA with our cDNA probe showed that the eEF-Tu mRNA is about 1 800 bases in length. The level of eEF-Tu message increases approximately 3-fold during the course of development.
5. *Artemia* poly(A)$^+$ was separated under non-denaturing conditions and blotted to poly(U)-paper. As a further demonstration that the cDNA hybridizes with eEF-Tu mRNA, the ^{32}P-labeled cDNA was used to identify the position of the RNA on a portion of the paper. RNA was then eluted from the poly(U)-paper and translated *in vitro*. A 50 000 M_r translation product was identified by immunoprecipitation as eEF-Tu.
6. We have also investigated interspecies homologies of eEF-Tu. We have seen that the synthetic oligodeoxynucleotide hybridizes to *Artemia* RNA and to RNA from mammals and other invertebrates. In all species examined the hybridizing band was about 1 800 bases. RNA preparations from various species were used as templates for primer extension. In all cases the major product was approximately 900 base pairs long, although shorter reverse transcription products were different for each species examined.

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Organization and sequence of 5S rRNA genes in *Artemia*

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Abstract

In *Artemia* 5S rRNA genes are linked to histone genes, as reported by Bagshaw *et al.* (1984). We have studied the genomic structure of this 5S-histone repeat. There are several types of repeat, according to their restriction maps. We have been able to identify at least three different types of repeats. From our results, it seems that all 5S genes are linked to histone genes. We have, however, isolated a clone that contains a 5S gene not linked to the histone ones. We have sequenced the 5S region of the main type-gene and the orphon 5S gene ; the coding sequence in both of them seems to be functional. The two sequences are homologous only in the coding region and around 140 bp upstream from the initiation site.

Introduction

In eukaryotes 5S rRNA genes are repeated genes that usually are not linked to the large rRNA repeat (Long and Dawid, 1980), being transcribed by RNA polymerase III instead of RNA polymerase I. In almost all organisms studied, 5S rDNA is organized in tandem repeats of variable length. The most studied system is *Xenopus* (Long and Dawid, 1980). The exception to this organization is *Neurospora*, in which 5S rRNA genes are scattered all along the genome (Selker *et al.*, 1981). During our studies on the large rRNA genes, we isolated a recombinant plasmid that contained a 5S rRNA gene (Díaz-Guerra *et al.*, 1982). This clone (pMD59) was 5.5 kb long and contained a single 5S gene. Later on, Bagshaw *et al.* (1984) reported that 5S genes were linked to the histone genes in the *Artemia* genome.

We present in this communication our results on the genomic organization of this 5S-histone repeat, its heterogeneity and the sequence around the 5S rRNA gene.

Materials and methods

Purification of DNA

Artemia larvae (San Francisco Bay Brand, batch 1808) were grown as described by Osuna and Sebastián (1980). DNA was purified by a modification of the procedure of Cruces *et al.* (1981).

Plasmids

pMD59 was obtained by cloning *Pst*I-digested *Artemia* DNA into the *Pst*I site of pBR322. pJC700 is the *Hin*dIII-*Hin*cII fragment of pMD59 that contains the 5S rRNA gene, cloned into the same sites of pUC9 (Vieira and Messing, 1982).

pJC9.0 and pJC6.5 were obtained as follows : *Artemia* DNA was centrifuged to equilibrium in CsCl-bisbenzimide H33258 density gradients. In these conditions a light satellite band is obtained (Cruces *et al.*, 1987) and, also, bands denser than the main band. The densest band was obtained, cleaved with *Sal*I (Gallego *et al.*, 1987) and cloned in λEMBL-4 (Frischauf *et al.*, 1983). The phages were transferred to nitrocellulose membranes (Benton and Davis, 1977) and hybridized with [^{32}P]-labelled 5S rRNA. Two positive phages were isolated and purified. The inserts of these two phages were then subsequently cloned into the *Sal*I site of pUC9.

cDm500 (Lifton *et al.*, 1978) was a generous gift of Dr. D. Hogness, from Stanford University.

Other methods

Other methods were essentially as described in the manual of Maniatis *et al.* (1982). Sequencing of DNA was done by the chemical degradation method of Maxam and Gilbert (1980).

Results and discussion

Different clones containing 5S rRNA genes

Fig. 1 shows three different clones that contain a 5S rRNA gene. pMD59 was the first we isolated (Diaz-Guerra *et al.*, 1982), pJC9.0 and pJC6.5 were obtained later, as described in the Material and methods section. From the restriction maps shown, it is evident that the three isolates represent different organizations of the 5S rRNA genes. In all three cases, there is only one 5S gene per repeat.

When Bagshaw *et al.* (1984) reported that the histone repeat of 8.5-9.0 kb contained the 5S genes, we investigated the presence of histone genes in pMD59, using as probe the 4.8 kb repeat unit of *Drosophila melanogaster* histone genes present in cDm500. The 5′ upstream region (with respect to the 5S rRNA gene) hybridizes with cDm500, up to the *Hin*dIII sites (Fig. 1). pMD59, thus, seems to be one class of 5S-histone genes, although it has an extra *Pst*I site in the 8.5-9.0 kb repeat. pJC9.0 also contains histone sequences, but does not have *Pst*I sites, and therefore corresponds to another class of 5S-histone gene repeat. As could be expected, the region of pMD59 and pJC9.0 that contains the histone genes have similarities. This is clearly seen in the distribution of the restriction sites for *Xba*I, *Pvu*II, *Bgl*I, and *Bst*EII.

pJC6.5 does not hybridize with histone sequences, and raises other questions : are there more 5S genes besides those linked with histone genes, or does pJC6.5 represent an orphon, as described by Childs *et al.* (1981)?

Genomic organization of 5S rRNA genes

To address the questions raised in the previous section, namely, how many types of 5S-histone genes are in the *Artemia* genome, and if there are more 5S genes which are not linked to histone genes, we made Southern genomic blots and hybridized them with different probes. The probes

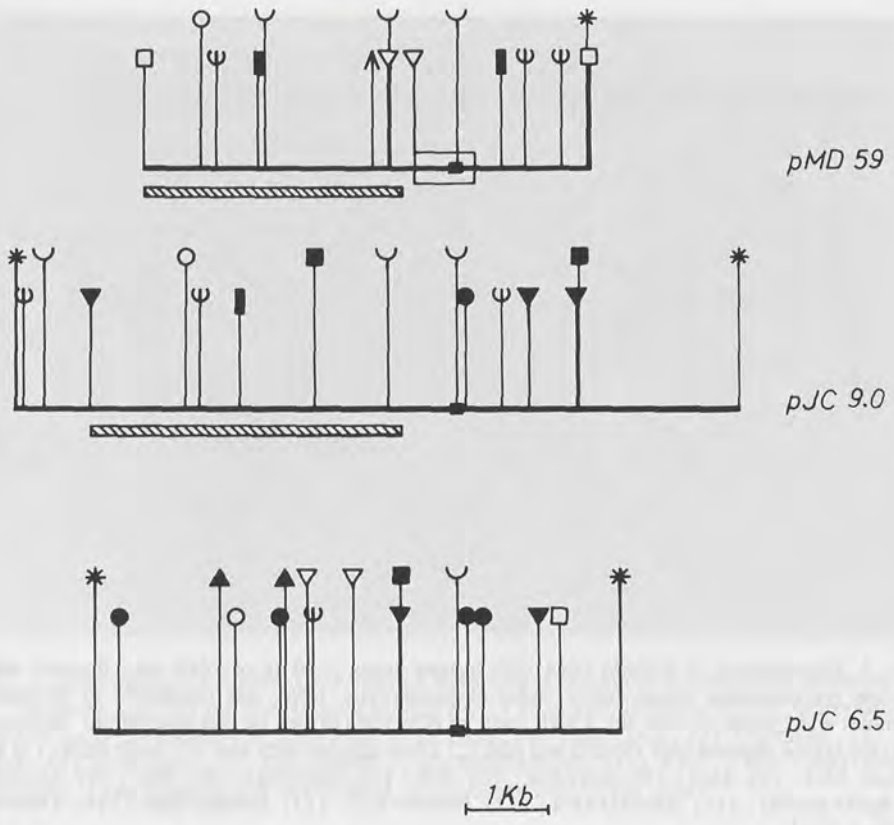

FIG. 1. Restriction maps of different clones that contain a 5S RNA gene. The small black box in the maps represents the 5S RNA coding sequence. The white box in pMD59 is the *Hin*dIII-*Hinc*II fragment subcloned in pJC700. Stripped bars in pMD59 and pJC9.0 represent the regions that hybridize with the 4.8 kb repeat of *D. melanogaster* histone genes. Restriction endonucleases are : (▽) *Pst*I ; (▽) *Xba*I ; (▽) *Pvu*II ; (▽) *Bge*I ; (▽) *Bst*EII ; (▽) *Bam*HI ; (▽) *Hin*dIII ; (▽) *Sal*I ; (▽) *Hpa*I ; (▽) *Sma*I ; (▽) *Eco*RI ; (▽) *Xho*I. Besides the enzymes shown, the following have been tested and do not cleave these plasmids : pMD59 (*Eco*RI, *Sma*I, *Hpa*I, *Bgl*II, *Xho*I, and *Kpn*I) ; pJC9.0 (*Bam*HI, *Pst*I, *Bgl*II, *Xho*I, and *Kpn*I. *Hin*dIII cuts pJC9.0 giving 9 fragments, that have not been mapped) ; pJC6.5 (*Bam*HI, *Bgl*I, *Bgl*II, and *Kpn*I).

used were : a) cDm500 (to identify histone gene bands) ; b) the *Hae*III inner band of the coding sequence of the 5S rRNA gene (to identify 5S rRNA sequences) ; c) pJC9.0 (to identify bands corresponding to the 5S-histone gene repeat) ; and d) pJC6.5 (to identify bands containing this type of 5S gene, not linked to histones).

Some representative autoradiograms are shown in Fig. 2, 3, 4, and 5 respectively.

The conclusions that can be brought from these experiments are the following :

1. The 5S-histone gene repeat is of 8.5-9 kb, defined by digestions with a wide variety of enzymes, and as reported previously by Bagshaw *et al.* (1984).

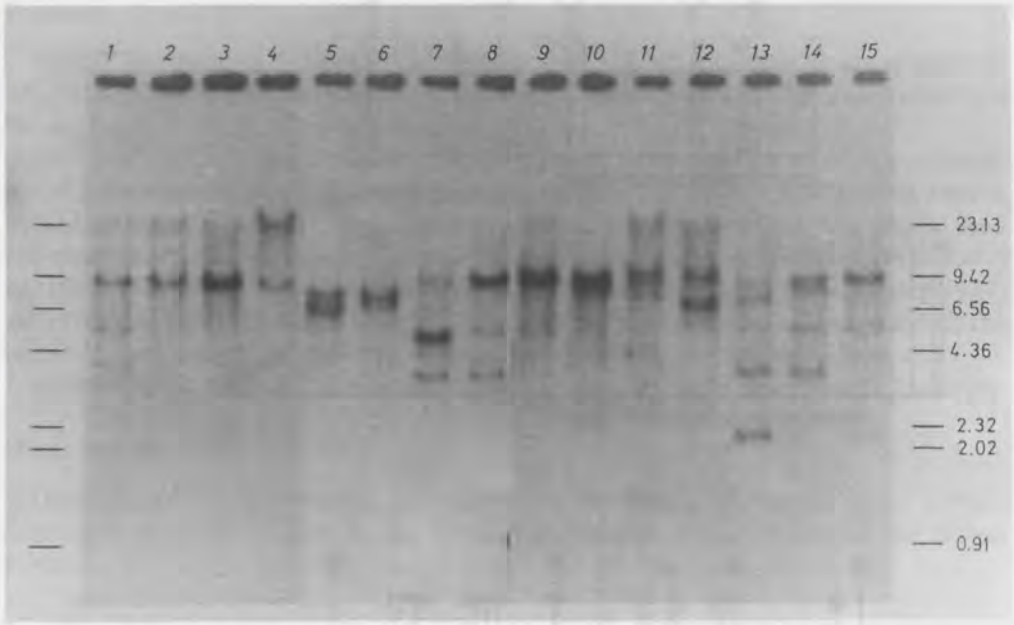

FIG. 2. Hybridization of *Artemia* DNA with histone genes. 5-10 µg of DNA were digested with the restriction endonucleases shown below. After electrophoresis, DNA was transferred to nitrocellulose membranes and hybridized with the 4.8 kb insert of cDm500 (labeled by nick translation). Size markers (in kb) are λDNA digested with *Hin*dIII and pBR322 DNA digested with *Alu*I. (1) *Sal*I+*Bgl*II ; (2) *Bgl*II ; (3) *Sal*I+*Xho*I ; (4) *Xho*I ; (5) *Sal*I+*Bcl*I ; (6) *Bcl*I ; (7) *Sal*I+*Xba*I ; (8) *Xba*I ; (9) *Xho*I+*Bgl*II ; (10) *Bgl*II+*Bam*HI ; (11) *Bam*HI+*Xho*I ; (12) *Bam*HI+*Sal*I ; (13) *Bam*HI+*Xba*I ; (14) *Xba*I+*Bgl*II ; (15) *Xba*I+*Xho*I.

2. As already suggested by the different clones obtained, the 5S-histone gene repeat is heterogeneous. Several restriction endonucleases give the 9 kb band plus larger, non-defined, bands. Other enzymes give, besides, some smaller bands. As the main 5S-histone repeat is of 9 kb, all smaller bands must represent the different classes of repeats, *i.e.* heterogeneous repeats that have more than one site for a given restriction endonuclease.
3. Some classes are more abundant than others. When the hybridization patterns with different probes are compared, it is clear that the different types of repeats are not equally represented. One typical case is that of *Pst*I. As shown in the previous section, there must be, at least, three different repeats, one with no *Pst*I sites, another with one site per repeat, and another with an extra site (represented by pMD59). Hybridization with cDm500 and pMD59 shows that around 50 % of the repeats must have the extra site (giving the 5.5 kb band cloned in pMD59).
4. There are no 5S rRNA genes not linked with histones. We hybridized genomic blots with an inner band of the coding sequence. As shown in Fig. 3, the hybridization signals are accounted by the 5S histone repeat. We can not rule out, however, the presence of some copies of 5S genes scattered along the genome ; as the 5S histone unit is repeated around 100 times (Bagshaw *et al.*, 1984), the hybridization signal of the unique genes would always be

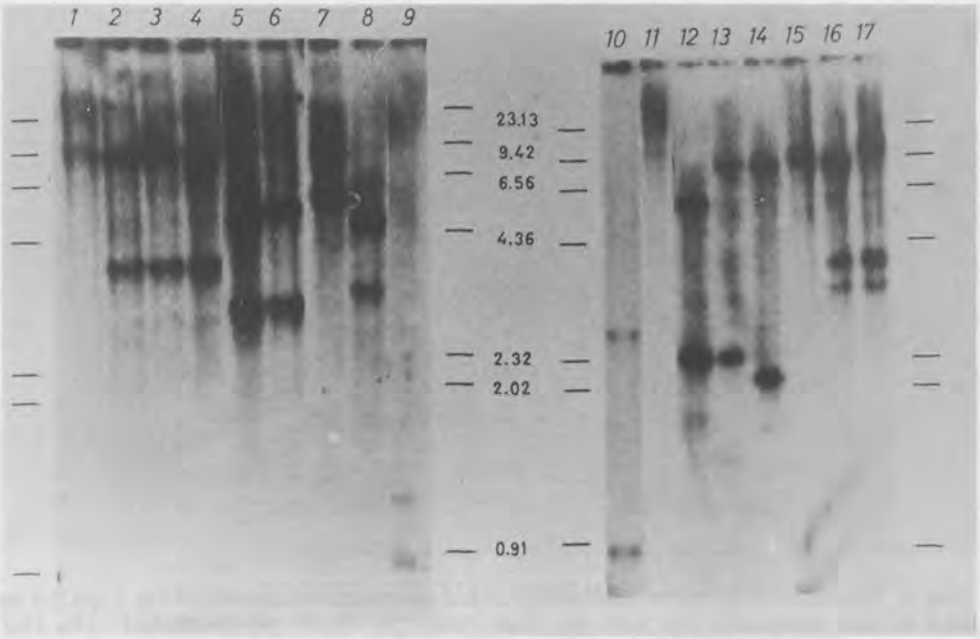

FIG. 3. Hybridization of *Artemia* DNA with 5S RNA coding sequences. Experimental details are as in Fig. 2. The probe used was the *Hae*III inner band of the 5S RNA gene labeled with polynucleotide kinase and (γ-[^{32}P])ATP. (1) *Sal*I ; (2) *Sal*I+*Pvu*II ; (3) *Pvu*II ; (4) *Pvu*II+*Xho*I ; (5) *Xho*I+*Bgl*I ; (6) *Bgl*I ; (7) *Pst*I ; (8) *Pst*I+*Xba*I ; (9) *Xba*I+*Bst*EII ; (10) *Bst*EII+*Bgl*II ; (11) *Kpn*I ; (12) *Eco*RI+*Hin*dIII ; (13) *Hin*dIII ; (14) *Hin*dIII+*Sal*I ; (15) *Hpa*I ; (16) *Hpa*I+*Sma*I ; (17) *Sma*I.

much weaker, and therefore fall below the limits of detection. Moreover, pJC6.5 gives hybridization bands different from those of pJC9.0, pJC9.0 and pMD59 and from the 5S gene. We think, therefore, that pJC6.5 probably represents an orphon.

Our conclusions about the different classes of 5S histone genes are as follows : the class represented by pMD59 is the major one, probably accounting for approximately 50 % of the total repeats. The other 50 % is also heterogeneous, and probably the clones reported by Bagshaw *et al.* (1984) belong to these other classes. When the genomic blots are hybridized with pJC9.0, the detected bands are not present in the plasmid, so the 5S-histone repeat representend by pJC9.0 must be in very low proportion.

SEQUENCE OF 5S rRNA GENES

It could be possible that pJC6.5 represents a pseudogen. To check this possibility, we sequenced the 5S genes (and flanking regions) from pJC700 and pJC6.5. As stated in Materials and methods, pJC700 consists in the *Hin*dIII-*Hin*cII fragment of pMD59 that contains the 5S gene.

Fig. 6 shows both sequences. The 5S gene from pMD59 is exactly the same as described by Diels *et al.* (1981). The gene from pJC6.5 contains five changes, as shown. These changes do not seem to disrupt the secondary structure proposed for 5S rRNA (Diels *et al.*, 1981). The

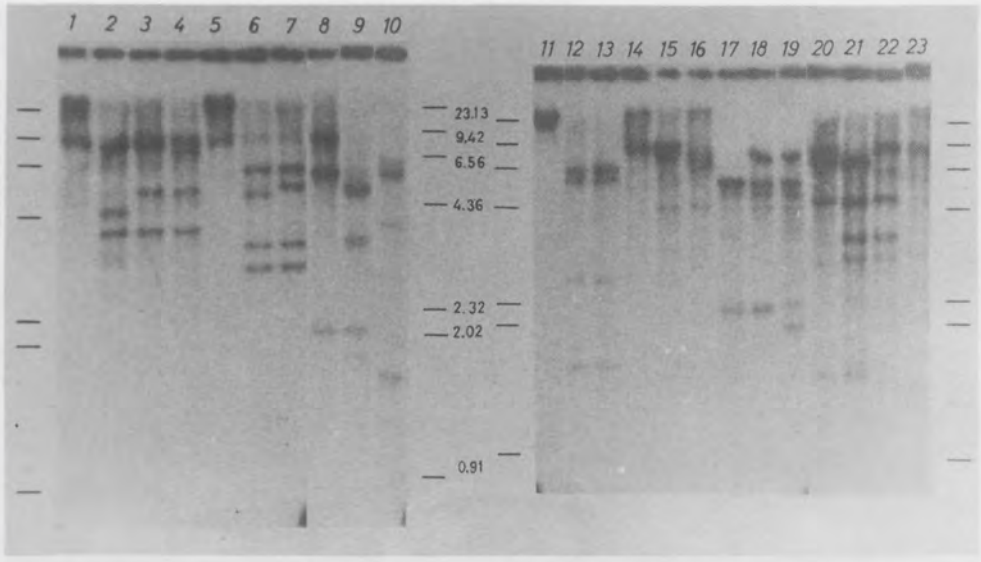

FIG. 4. Hybridization of *Artemia* DNA with pJC9.0. Experimental details are as in Fig. 2. pJC9.0 was labeled by nick translation. (1) *Sal*I ; (2) *Sal*I+*Pvu*II ; (3) *Pvu*II ; (4) *Pvu*II+*Xho*I ; (5) *Xho*I ; (6) *Xho*I+*Bgl*I ; (7) *Bgl*I ; (8) *Pst*I ; (9) *Pst*I+*Xba*I ; (10) *Xba*I+*Bst*EII ; (11) *Kpn*I ; (12) *Bst*EII ; (13) *Bst*EII+*Bgl*II ; (14) *Bgl*II ; (15) *Bgl*II+*Eco*RI ; (16) *Eco*RI ; (17) *Eco*RI+*Hin*dIII ; (18) *Hin*dIII ; (19) *Hin*dIII+*Sal*I ; (20) *Hpa*I ; (21) *Hpa*I+*Sma*I ; (22) *Sma*I ; (23) *Kpn*I+*Sal*I.

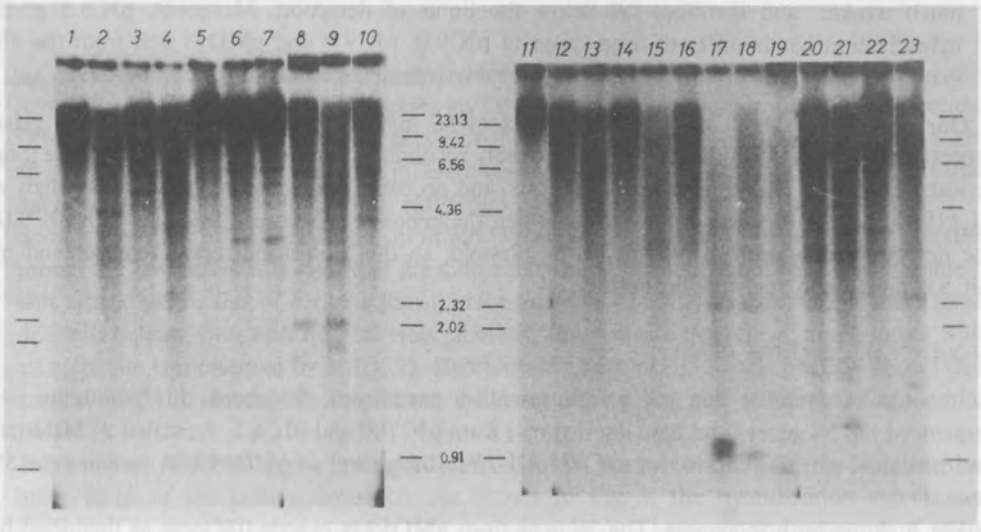

FIG. 5. Hybridization of *Artemia* DNA with pJC6.5. Experimental details and digestions are as in Fig. 4, except that pJC6.5 was used instead of pJC9.0.

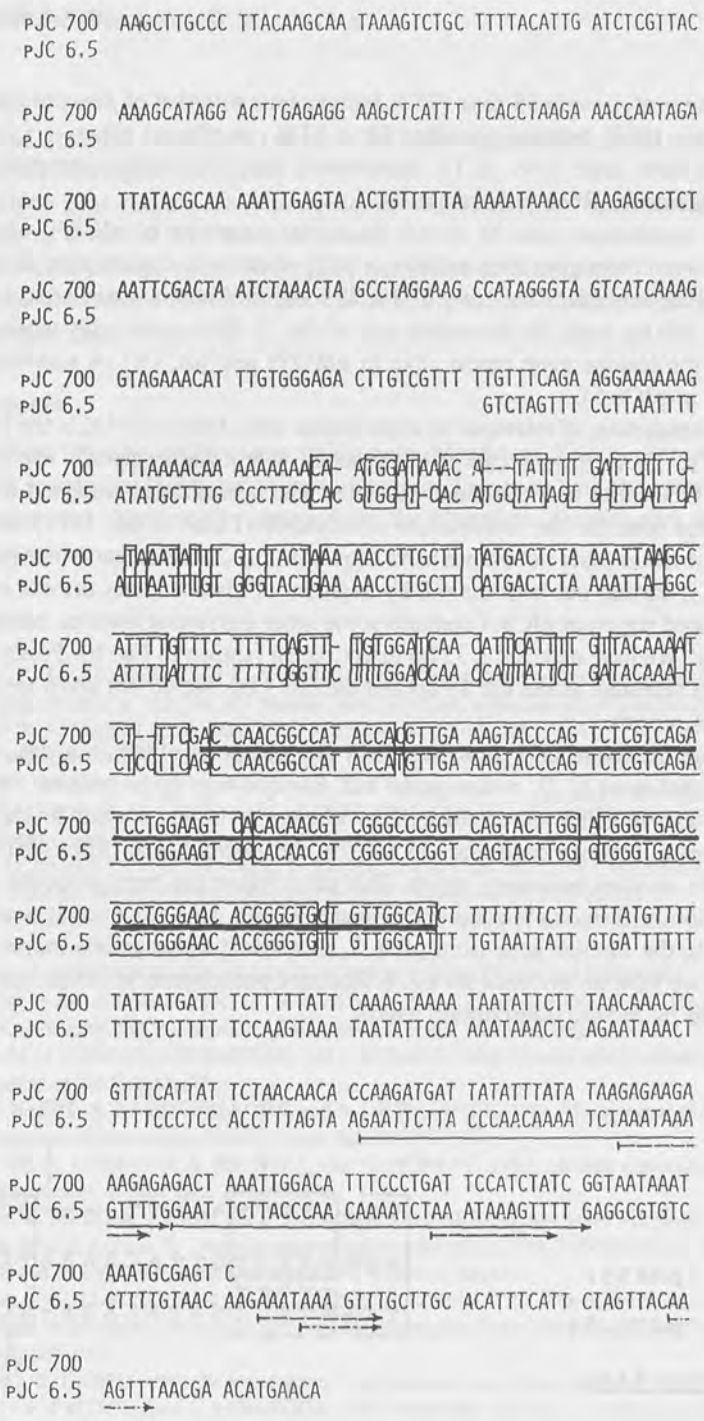

FIG. 6. Sequence of 5S rDNA and flanking regions from pJC700 and pJC6.5. pJC700 was sequenced from the *Bst*EII site outwards and from the *Hin*dIII and *Hin*cII sites. pJC6.5 was sequenced from the *Bst*EII site outwards. The coding sequence is represented by a line in between the two sequences. Boxes represent homology regions between pJC700 and pJC6.5. The different direct repeats of pJC6.5 are also shown below the sequence : (———) the 35 bp repeat ; (– – –) the 11 bp repeat ; (—·—) the 7 bp repeat.

internal promoter of *Artemia* 5S gene (78 % homologous with that of *Xenopus* oocyte 5S rRNA) (Ciliberto *et al.*, 1983) between positions 50 to 83 is not affected either.

Both genes have large rows of T's immediately after the coding sequence, known to be termination signals for RNA polymerase III.

Two more conclusions can be drawn from the sequences of pJC700 and pJC6.5. The homology between them goes from position -140 (+1 is the first nucleotide of 5S RNA) to the end of the coding sequence. The rows of T's, although not homologous, seem to expand about 40 bp. These 300 bp could be the repeat unit of the 5S RNA gene ; this repeat unit would be inserted into the histone gene repeat (like in pMD59 and pJC9.0) or somewhere else in the genome (like in pJC6.5).

The other conclusion, of relevance in experiments with *Artemia* DNA, is the large proportion of A's and T's. These regions can give artefactual hybridization signals. We have hybridized *Artemia* total DNA with nick translated cDm500. The hybridization observed was a smear, due to the A-T tails used for the construction of cDm500 (Lifton *et al.*, 1978) and the A-T rich sequences in *Artemia* (data not shown). As shown in Fig. 2, this smear is not present when the hybridization is carried out with the 4.8 kb fragment of histone genes present in cDm500.

The sequenced region of pJC6.5 contains some other interesting features, probably not related to the 5S gene. We have detected a 35 bp direct repeat (shown in Fig. 6). Portions of this direct repeat are also repeated, as the last 11 bp and the last 7 bp. We do not know yet the significance of these direct repeats.

Fig. 7 shows the sequence 5′ upstream from the start point of the 5S genes. As can be seen, *Artemia* is homologous to *D. melanogaster* and *Bombyx mori* up to position -9 ; this region is homologous also in other lower eukaryotes, whereas it is not present in higher eukaryotes. Further upstream, between positions -19 to -26, the *Artemia* gene (from pMD59) and the *B. mori* gene have another homology region. This latter region has been proposed to be important for transcription in *B. mori* (Morton and Sprague, 1984). This sequence at position -20 is less homologous in the *Artemia* gene from pJC6.5, although the change is a transition.

Therefore, we have no evidence for pJC6.5 being a pseudogene, although this question could only be settled by *in vitro* transcription assays.

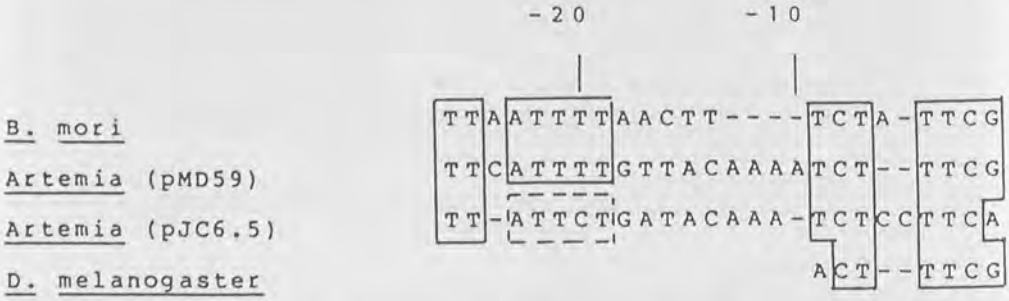

FIG. 7. 5′ upstream sequences from 5S RNA genes from *Artemia* and other Arthropoda. The 5′ flanking sequence (from position -1) is shown for *Artemia* 5S RNA genes from pMD59 and pJC6.5, *B. mori* (Morton and Sprague, 1982), and *D. melanogaster* (Tschudi *et al.*, 1982). Boxes represent homologous regions.

Conclusions

1. The 5S rRNA genes in *Artemia* are linked to the histone genes, in a repeat unit of 9 kb. There is only one 5S RNA gene per repeat.
2. The 5S histone gene repeats are heterogeneous with respect to their restriction maps. One class represents about 50 % of the total genes.
3. We have detected an orphon 5S RNA gene not linked to histones.
4. The sequence of a histone-linked 5S RNA and the orphon have been determined. Both genes seem to be functional.

Acknowledgements

We thank Elvira Dominguez for the technical help. Magdalena Zalacain introduced us in the Maxam-Gilbert sequencing technique. Alberto Domingo helped us with the computer programs. This investigation was supported by grants from the Comisión Asesora para la Investigación Científica y Técnica.

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Identification of a satellite in the *Artemia* genome

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Abstract

When *Artemia* DNA is digested with a large variety of restriction endonucleases, a resistant fraction of around 23 kb is obtained. This fraction (1-2 % of the genome) is only cleaved by *Mbo*II, *Taq*I, and *Alu*I, confirming its low complexity. The same satellite has been obtained by equilibrium density centrifugation in CsCl-bisbenzimide H33258 gradients. The repeat unit of this satellite has been cloned and sequenced.

Introduction

Highly repetitive DNA is present in the genome of all eukaryotic organisms. These satellite DNAs (Bouchard, 1982 ; Singer, 1982) have been classified in several types according to their distribution in the genome, and some of them seem to be transcribed (Jelinek and Schmid, 1982). Except for some types, their function remains unknown.

During our studies on the genome organization in *Artemia*, we noticed that a wide variety of restriction endonucleases leave an undigested fraction, of high molecular weight, also resistant to double digestions. We show in this paper that this fraction is a satellite with very low complexity, and also present the sequence of the basic unit (113 bp).

Materials and methods

Organism and growth conditions

Artemia cysts came from San Francisco Bay Brand. Newly-hatched nauplii were grown as described by Osuna and Sebastián (1980).

Purification of DNA

DNA of *Artemia* larvae was obtained by a modification of the procedure described by Cruces *et al.* (1981).

Plasmids

Artemia DNA was cleaved with *Alu*I and ligated to M13mp8 (Messing and Vieira, 1982) or pUC9 (Vieira and Messing, 1982) linearized with *Sma*I. Transformation and screening of recombinants was done by conventional techniques (Maniatis *et al.*, 1982).

Other methods

Gel-electrophoresis, labeling of DNA probes and other techniques were essentially done as described by Maniatis *et al.* (1982). DNA sequencing was done both by the chemical degradation method of Maxam and Gilbert (1980) and by the dideoxy procedure of Sanger *et al.* (1977). Hydrolysis of DNA was done according to the method of Bendich (1956) and the separation of bases as described by Junowicz and Spencer (1969).

Results and discussion

General characteristics of *Artemia* DNA

Molecular biology of *Artemia* has been done several years without a clear knowledge of its DNA basic properties. The published G+C content of *Artemia* DNA varies from 41 % (Vaughn, 1977) down to 33.4 % (Warner and Bagshaw, 1984). We have calculated the G+C content by three different methods : buoyant density, melting point (both in SSC and in 0.1×SSC), and chemical determination (Cruces, 1982). The results are shown in Table I. We found a value of 32.4 % which is lower than previously described. In Table I the content of DNA per haploid genome, measured by chemical determination with diaminobenzoic acid (Pestaña *et al.*, 1978) is shown. In our experiments the value found is slightly higher than that determined by Roberts and Vaughn (1982). This difference could be due to the existence of polyploid cells in *Artemia* cysts.

The high proportion of A+T in *Artemia* DNA is of relevance when hybridization experiments are performed (Cruces *et al.*, 1987).

Table I

Properties of *Artemia* DNA

		% (G+C)
Content per haploid genome	1.65×10^9 bp (1.8 pg)	
Buoyant density	1.692 g/cm^3	32.6
Tm (0.1 × SSC)	67.1 ± 1.2 °C	32.3 ±2.3
Tm (1 × SSC)	82.5 ± 1.4 °C	32.2 ± 2.5
% (G+C) by chemical method		32.5 ± 1.5
	Mean value	32.4 ± 2.8

Restriction analysis of *Artemia* DNA

When *Artemia* DNA is digested with restriction endonucleases, a band of around 23 kb is clearly seen (Fig. 1). This band accounts for 1-2 % of the genome. In some digestions – with restriction endonucleases that poorly cut *Artemia* DNA – the resistant fraction is not evident, due to the masking by other large DNA fragments. Double digestions do not degrade this fraction (Fig. 2). These experiments demonstrate the low complexity of this fraction. The only endonucleases that cleave these fractions are *Mbo*II, *Taq*I and *Alu*I. In Fig. 3, digestions with *Alu*I and *Mbo*II are shown. Digestion with both enzymes gives a prominent band of 110-120 bp. The observed ladder suggests that the 23 kb-resistant fraction is organized in tandem repeats of this basic unit.

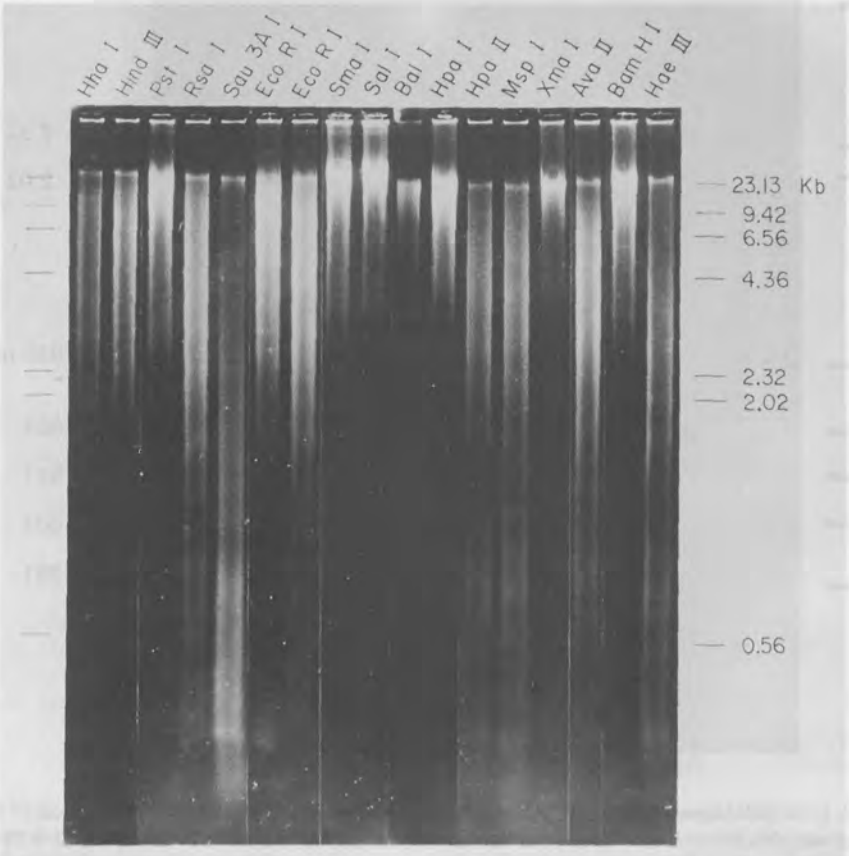

Fig. 1. Restriction analysis of *Artemia* DNA. A total of 2 μg of *Artemia* DNA were digested with the restriction endonucleases shown and electrophoresis applied in a 0.8 % agarose gel at 1 V/cm. Size markers (in kb) are λ DNA digested with *Hin*dIII.

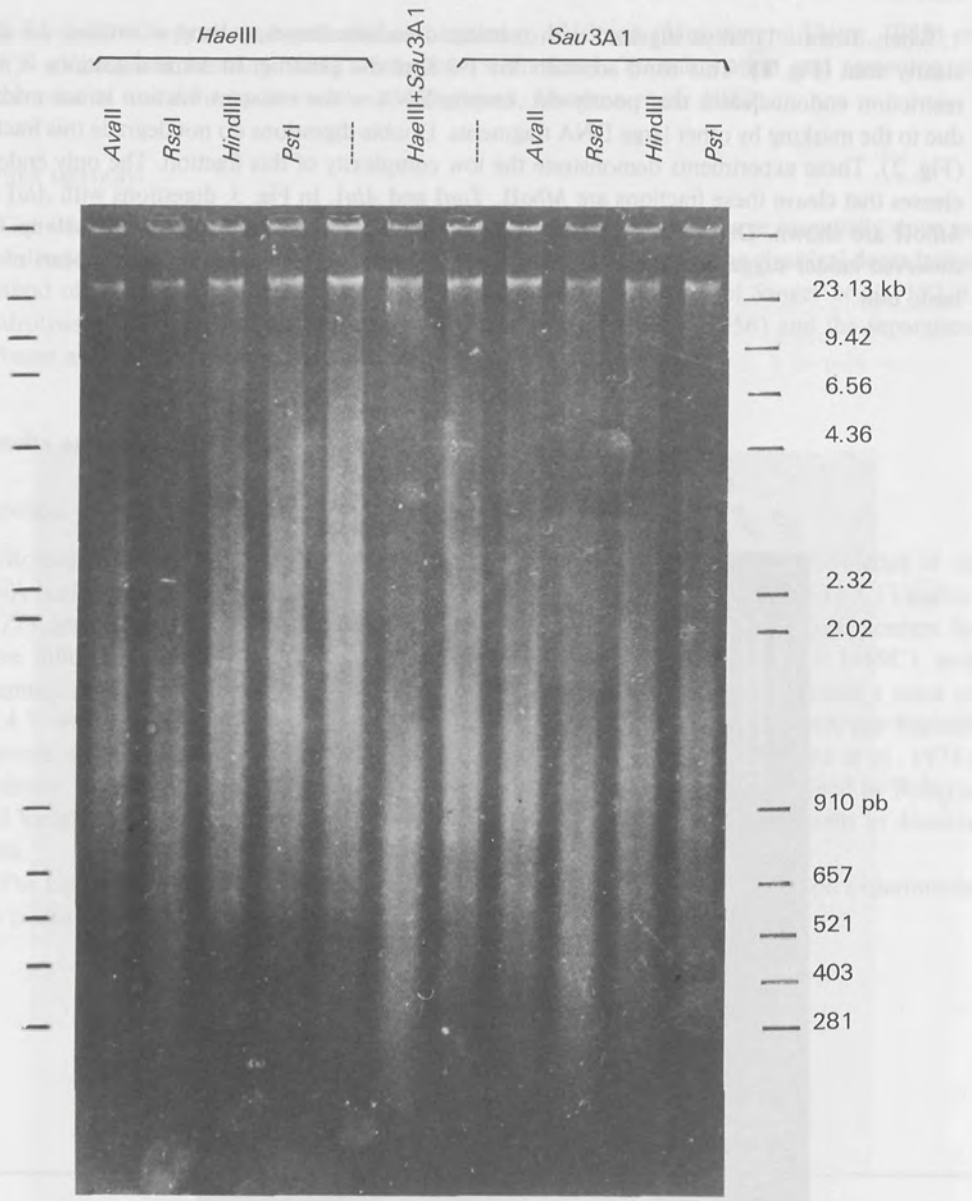

FIG. 2. Double digestion of *Artemia* DNA with restriction endonucleases. A total of 1 μg of DNA was digested with *Hae*III or *Sau*3A plus the restriction endonucleases indicated in the figure, to demostrate the low complexity of the 23 kb resistant fraction. Electrophoresis conditions were the same as in Fig. 1.

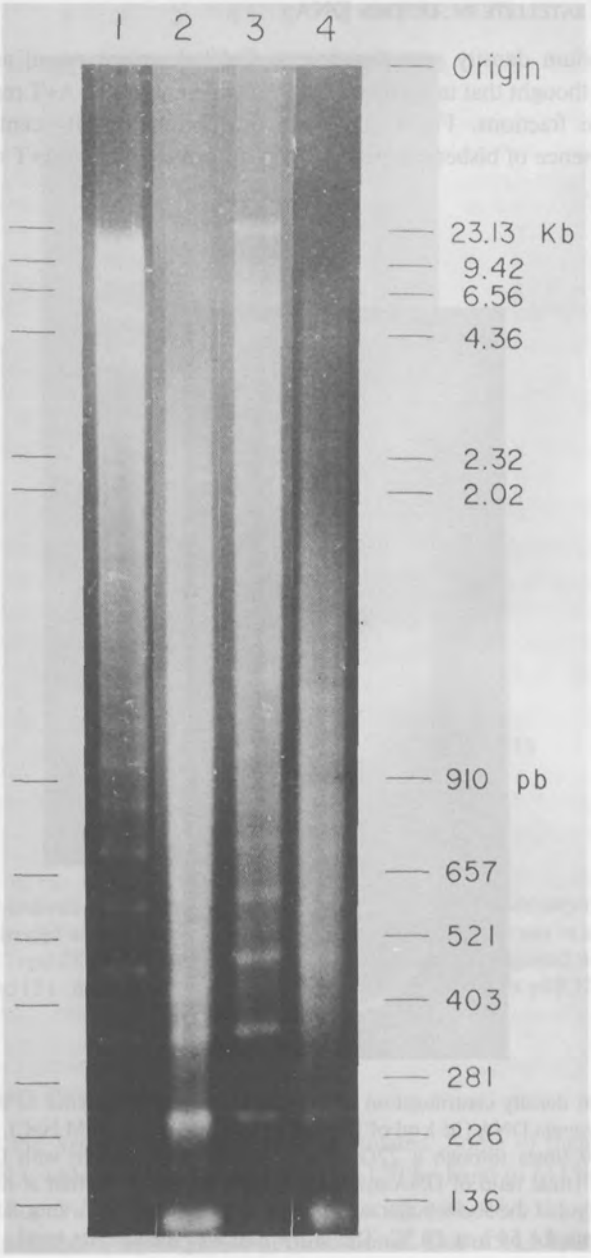

FIG. 3. *Artemia* DNA digested with *Alu*I and *Mbo*II. A total of 10 μg of *Artemia* DNA were digested with *Alu*I (0.8 units) of *Mbo*II (41 units) for different times. In each case the amount of enzyme was such as to give partial digestions in the times used. Lane 1, digestion with *Alu*I for 25 min. Lane 2, digestion with *Alu*I for 7 h. Lane 3, digestion with *Mbo*II for 10 min. Lane 4, digestion with *Mbo*II for 12 h. Aliquots of 2 μg were submitted to electrophoresis in 1 % agarose gels. Size markers were λ DNA digested with *Hin*dIII and pBR322 DNA digested with *Alu*I.

VISUALIZATION OF A SATELLITE IN *ARTEMIA* DNA

Although equilibrium density centrifugation in CsCl does not reveal any satellite fraction (Cruces, 1982), we thought that using dyes that bind preferentially to A+T regions could perhaps reveal such satellite fractions. Fig. 4 shows an equilibrium density centrifugation in CsCl gradients in the presence of bisbenzimide H33258, known to bind to A+T regions of the DNA (Manuelidis, 1977).

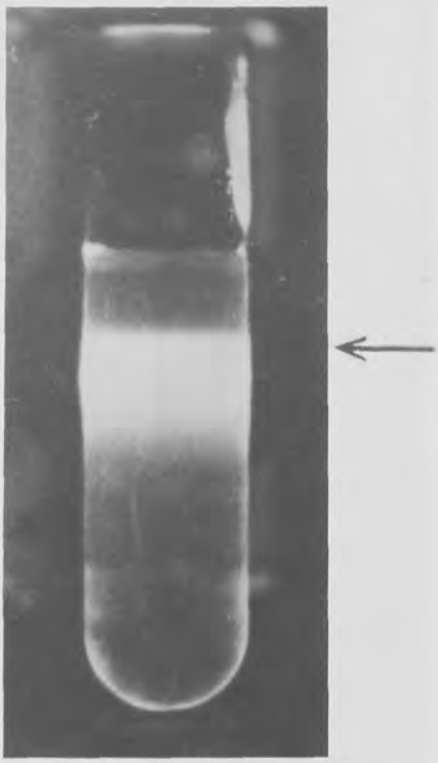

FIG. 4. Equilibrium density centrifugation of *Artemia* DNA in the presence of bisbenzimide H33258. A total of 100 μg of *Artemia* DNA (in 1 ml of 10 mM Tris-Cl, pH 8.0, 10 mM NaCl, 0.5 mM EDTA) were sheared by passing 10 times through a 22G needle. It was slowly mixed with 0.49 ml of 200 μg/ml bisbenzimide H33258 (final ratio of DNA:dye was 1:1) and incubated 10 min at room temperature. The mix was diluted with 5 ml of the above buffer and brought to ρ=1.638 g/cm^3 with solid CsCl. Centrifugation was done at 35 000 rpm for 64 h at 20 °C. The arrow indicates the satellite band.

This satellite fraction was isolated and digested with the restriction endonucleases previously shown to digest the 23 kb-resistant fraction. Fig. 5 shows that the satellite obtained by equilibrium centrifugation is also cleaved, giving the 110-120 bp band. Therefore we conclude that both types of satellite, obtained by two different methods, are the same.

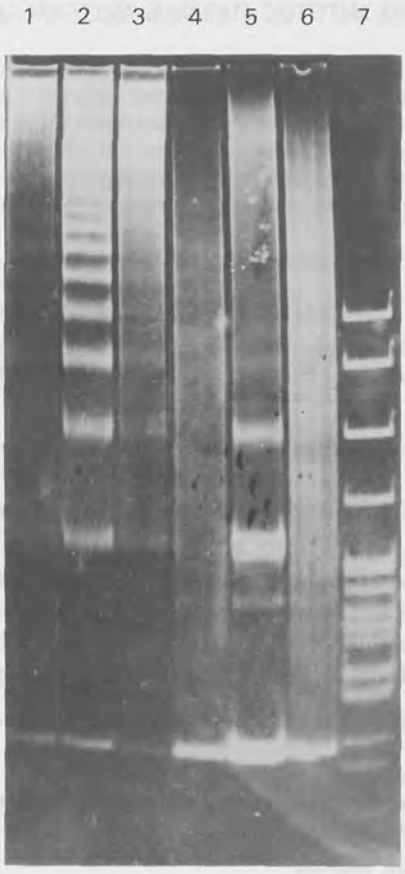

FIG. 5. Restriction analysis of the satellite band obtained by equilibrium density centrifugation. A total of 3 μg of DNA was digested as described below and subjected to electrophoresis in a 10 % polyacrylamide gel, at 100 V for 11 h. Total DNA (1) ; satellite band (2) ; main band (all digested with *Mbo*II) (3) ; total DNA (4) ; satellite band (5) ; main band (all digested with *Alu*I) (6) ; markers pBR322 DNA digested with *Hpa*II (7).

SEQUENCE OF SATELLITE FROM *ARTEMIA*

Several clones of the *Artemia* satellite have been isolated. Some of them were obtained by digestion of total DNA with *Alu*I and cloning the 110-120 bp band in M13mp8 (pMW002 and 003). pMW103, 115 and 116 were obtained by digesting with *Alu*I the gel-purified resistant fraction of 23 kb. Fig. 6 shows the sequence of these clones. Out of 113 bp, there are only seven changes, always in the same position in the five clones.

Other recombinant obtained from the digestion of total DNA (pMW001) did contain the three restriction sites characteristics of the satellite (*Alu*I, *Mbo*II and *Taq*I), but its sequence was completely different. pMW001 did show very high secondary structure (data not shown). We think that there are more satellites in the *Artemia* genome, the more abundant is the one we have sequenced.

```
pMW 002   CTATTAGCCT CGAAAACTAA AACTTTTGAC ATAGGAAAAG AGCCTTTAAT CACATTCTTT ACCATTTGCA
pMW 003         G                                                                   G
pMW 103         C                                                                   G
pMW 115         T                                                                   G
pMW 116         T                                                                   T

pMW 002   ATCATAAAAT AGTCTAATAT CTTCATTTTT TCAACATACG TAG
pMW 003             T                     A   T  G T
pMW 103             T                     C   G  A A
pMW 115             T                     C   G  G A
pMW 116             A                     A   T  G A
```

FIG. 6. Sequence of different satellite clones. The sequence is given for plasmid pMW002. The other four plasmids have the same sequence except for the changes in positions 7, 66, 81, 103, 107, 119, and 111, that are indicated.

The G+C content of the satellite is 30 %. As the G+C content of total DNA is 32.4 %, it is not surprising that conventional CsCl density centrifugation failed to detect it. Even in the presence of bisbenzimide H33258 (Fig. 4), the separation between the main and the satellite bands is poor.

According to our data, the satellite would be repeated between 700 and 1 400 copies per haploid genome, each copy consisting of around 200 repeats of 113 bp.

Conclusions

1. *Artemia* DNA has 32.4 % (G+C). This value has been obtained by three different methods.
2. The presence of a satellite in *Artemia* DNA was identified, both by equilibrium density centrifugation and by restriction analysis.
3. Five clones corresponding to this satellite have been sequenced. The basic repeat is 113 bp long.

Note

When this paper was being written, it came to our attention that Barigozzi's group (Barigozzi *et al.*, 1984) has identified the presence of a satellite in the heterochromatin of some *Artemia* species. This satellite is clearly the same one we have studied and sequenced. In view of their results, it would be interesting to look for the 23 kb band in other *Artemia* species besides the one we examined.

Acknowledgements

We thank Elvira Dominguez for technical help and Juan Ortin for valuable discussions and suggestions. Dr. R. Garesse assisted with the dideoxy sequencing method and Alberto Domingo with the computer analysis of the sequences. This investigation was supported by grants from the Comisión Asesora para la Investigación Cientifica y Técnica.

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Size and unfolding of an eukaryotic ribosomal RNA free in solution

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Abstract

The size and the unfolding behaviour have been studied of the large RNA of the large subunit and of the RNA of the small subunit of eukaryotic ribosomes isolated from *Artemia*.

The influence of Mg^{2+} on the size of the rRNAs was studied. At low Mg^{2+} concentrations (<3 mM) the RNA of the small subunit was much more expanded than the RNA of the large subunit in the same conditions. At high Mg^{2+} concentration (20 mM) a similar behaviour of both RNAs was observed.

In order to obtain quantitative information about the compactness and the size of the RNAs, which are reflected by the frictional coefficient ratio and the hydrodynamic radius, the density increment or the partial specific volume was measured ($\frac{\delta\rho}{\delta c} = 0.50 \pm 0.01$; $\bar{v}^o = (0.49 \pm 0.01)$ cm^3/g).

The size of the RNAs is compared with the size of the corresponding subunit : the size of the large RNA is still larger than the corresponding subunit, the size of the RNA of the small subunit resembles more the size of the complete small subunit itself. Photon correlation spectroscopy of laser light scattered from rRNA solutions, has yielded the diffusion coefficient. The hydrodynamical size, calculated directly only from the diffusion coefficient, is in good agreement with the size derived from the sedimentation coefficient.

The molar mass, M, results by combining s, D and $\frac{\delta\rho}{\delta c}$ in the Svedberg relation. The close agreement between our values for M and values obtained by completely different techniques (Nieuwenhuysen and Clauwaert, 1981 ; Cruces *et al.*, 1982 ; Nelles *et al.*, 1984) confirms our experimental results, especially the rather high value of $\frac{\delta\rho}{\delta c}$.

Introduction

The conformation of RNA free in solution should be known in order to understand the assembly of the RNA into biological structures such as viruses and ribosomes. The conformational information, required for the assembly of the ribosomal subunits, is contained in the structure of the ribosomal proteins and/or RNA (*e.g.* Tam *et al.*, 1981). Magnesium ions have shown to be essential cations for maintaining the structural integrity and functioning of ribosomes and rRNA (Cammack *et al.*, 1970 ; Weiss and Morris, 1973 ; Schulte *et al.*, 1974 ; Nisbet and Slayter, 1975 ; Allen and Wong, 1978 ; Tam *et al.*, 1981 ; Yi and Wong, 1982).

In this paper a comparative study is reported of the hydrodynamical behaviour of the RNA of the small ribosomal subunit and the large RNA isolated from the large ribosomal subunit of

the eukaryote *Artemia*. The known size of both RNAs allows a comparison with the corresponding total ribosomal subunit. The conformation of the rRNA, free in solution, is mainly determined by the ionic strength and the amount of Mg^{2+} in solution and plays an important role in the assembly of the subunit from the RNA and the proteins.

Our results indicate that the hydrodynamical behaviour of the RNA of the small and the large subunit is different : at low ionic strength and without Mg^{2+}, the RNA of the small subunit is much more unfolded than the large rRNA of the large subunit. This behaviour can be related to the secondary structure of the RNAs. At higher ionic strength and with 20 mM Mg^{2+}, both RNAs show a more similar behaviour.

Our attention has been focused on the ribosomal RNA of the brine shrimp *Artemia*, allowing us to widen the knowledge on the physical properties of the complete ribosomes and the ribosomal subunits of *Artemia* (Nieuwenhuysen and Clauwaert, 1980, 1981).

Materials and methods

Preparation of rRNA

Ribosomal subunits were isolated from the cryptobiotic embryos of *Artemia* from San Francisco Bay (Nieuwenhuysen and Clauwaert, 1981). They were concentrated by centrifugation and their RNA was extracted by the SDS-phenol-chloroform method or the SDS-method (Donceel *et al.*, 1982) with only one modification : the RNA was dissolved in a Tris-buffer without EDTA (20 mM Tris/HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 M KCl), which resulted in quite homogeneous samples, specially for rRNA of the small subunit. The RNA was purified by rate zonal centrifugation in a linear gradient of 10-30 % (w/v) sucrose in the same buffer in a Beckman SW 40-rotor at 4 °C and at 145 000 g_{av} for 18 h for the RNA of the small ribosomal subunit. When large amounts of rRNA were needed (*e.g.* for determining the density increment) the RNA was extracted from the whole ribosomes with the SDS-phenol-chloroform method. The RNA was separated and purified by rate zonal centrifugation in a 15-50 % (w/v) gradient of sucrose in the Tris-buffer as mentioned above in a Ti 14 zonal rotor at 4 °C and at 92 000 g_{av} for 26 h. Heparin was added to the RNA before dialysing to avoid RNase degradation (0.1 mg heparin for 1 ml solution). The rRNA fractions were finally dialysed against the appropriate buffers as specified below.

UV-spectrophotometry

UW absorbance of the RNA solutions was measured with a Zeiss PMQ 3 spectrophotometer.

Determination of density increment

Density increments

A resonant oscillator densimeter was used (DMA 02C, Anton Paar, Graz, Austria) (Kratky *et al.*, 1973). The temperature of the oscillator was kept constant within 0.005 °C at 25.0 °C. Dry air and twice-destilled water, which were made dust-free by filtration through a 0.1 µm Millipore filter, were used for calibration. Before measuring the density of the RNA solution, it was brought to thermodynamic equilibrium of diffusible components by extensive dialysis against the following buffer : 20 mM Tris/HCl, pH 7.5, 1 mM $MgCl_2$ and 100 mM KCl.

Concentration measurements

A measure of the concentration of the RNA, present in the solution is given by the UV-absorbance at 260 nm. To determine the concentration in g/ml, a relation is needed between the UV-absorbance at 260 nm and the mass of the dissolved RNA. This relation is derived from phosphorus determinations (Nieuwenhuysen *et al.*, 1978). The phosphorus content of the rRNAs was calculated from its base composition (Lava-Sanchez *et al.*, 1972).

Calculation of the density increment

The density increment at vanishing RNA concentration $\left(\frac{\delta\rho}{\delta c}\right)_{\mu}^{\circ}$ was calculated from the density and the concentration measurement by a linear least-square program. The value $\bar{v}^{\circ}$ was calculated from the equation :

$$\left(\frac{\delta\rho}{\delta c}\right)_{\mu}^{\circ} = 1-\bar{v}^{\circ} \cdot \rho_o$$

were ρ_o is the density of the buffer.

ANALYTICAL BOUNDARY SEDIMENTAION

The sedimentation coefficient and degree of aggregation of the RNA in various buffers were measured by analytical boundary sedimentation. Centrifugation runs were performed in a MSE-analytical ultracentrifuge with a six-hole rotor. Double sector cells were used, with UV-absorption optics coupled to a photoelectric scanner. RNA concentrations were smaller than 45 µg/ml, so that concentration effects were considered negligible. The temperature was kept at 4.0 °C±0.1 °C. Temperature and solvent differences were taken into account to reduce the apparent sedimentation coefficient to $s_{20,w}$.

PHOTON CORRELATION SPECTROSCOPY OF SCATTERED LASER LIGHT

The translation diffusion coefficient, D, of the rRNA in Brownian motion was determined by analysing the intensity fluctuations of their laser-light scattering, by using single-clipped photon-count autocorrelation spectroscopy (*e.g.* Koppel, 1974 ; Nieuwenhuysen, 1978 ; Nieuwenhuysen and Clauwaert, 1978). The measured correlation function of a monodisperse solution decays exponentially with a decay time proportional to D :

$$G(it) = A + B \exp(-2\ D\ K^2\ i\ T)$$

where i is the channel number, T the chosen sample time and K the known modulus of the scattering vector ; A and B are constants.

The apparent diffusion coefficient was corrected for temperature and solvent differences to give $D_{20,w}$. The diffusion coefficient at infinite dilution, $D^{\circ}_{20,w}$ was obtained after extrapolation to zero concentration of various values for $D_{20,w}$ measured at known concentrations.

Results and discussion

The low value for the partial specific volume of the rRNA

In the literature, values in the wide range of 0.49 cm^3/g and 0.58 cm^3/g are mentioned for the partial specific volume of RNA (cf. references in Nieuwenhuysen and Clauwaert, 1980). This large spread is not surprising in view of the differences in the origin, degree of purity of the sample, and in the method of measurement. But even small variations of the partial specific volume cause large changes in the calculation of the hydrodynamical properties. Therefore we have determined the density increments of the large RNA of the large subunit of *Artemia* ribosomes.

From phosphorus determinations we found that 1 A_{260} unit corresponds to (44.0±0.5) µg rRNA, or an absorbance $A_{260\ nm,\ 1\ cm}$ of 22.7±0.3. This value is smaller than for the RNA incorporated in the ribosomes (Nieuwenhuysen and Clauwaert, 1981). The density of rRNA samples in a concentration range between 1.5 mg/ml to 20 mg/ml were measured (Fig. 1). From these data we calculated $\left(\frac{\delta\rho}{\delta c}\right)^{\circ}_{\mu}$ = (0.50±0.01). This is a rather large value compared with those published earlier (cf. references in Nieuwenhuysen and Clauwaert, 1980). A close value has been obtained for the density increment of native MS2 RNA (Slegers *et al.*, 1973).

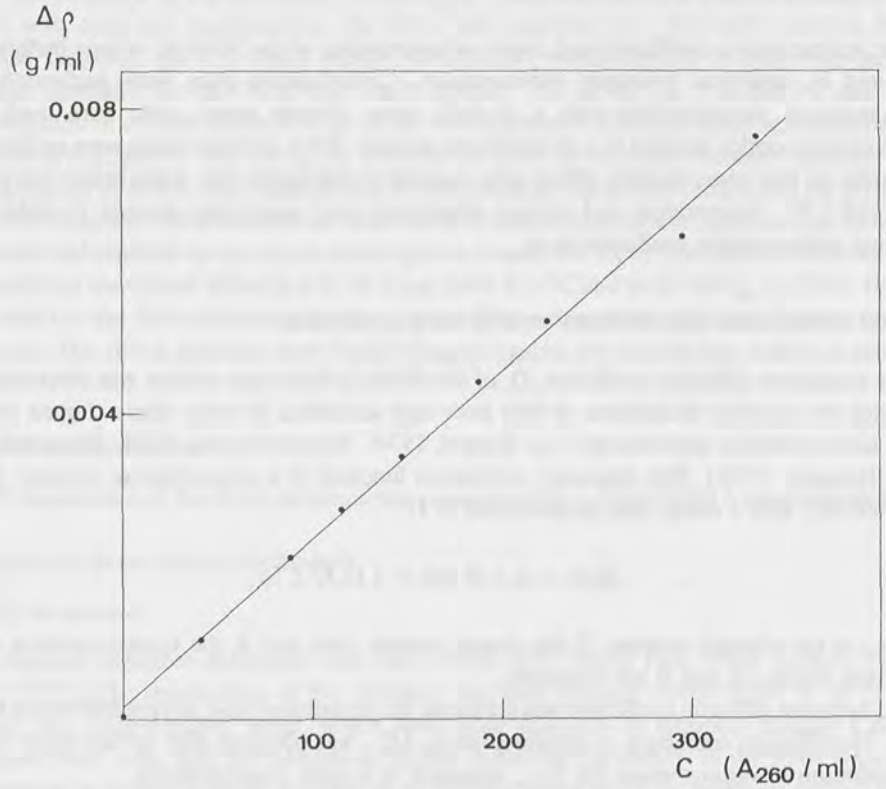

Fig. 1. Determination of the density increment of the large RNA of the large subunit.

In the subsequent calculations the same density increment is assumed for the rRNA of the small subunit in similar conditions. A correct value of the density increment is especially important for the interpretation of neutron scattering studies of structures containing RNA (Moore *et al.*, 1974 ; Chrichton *et al.*, 1977 ; Serdyuk *et al.*, 1979, 1983).

Difference in the unfolding of the rRNAs of the two subunits

The sedimentation coefficient of the large rRNA of the large subunit and of the rRNA of the small subunit were measured in similar conditions in order to compare their hydrodynamical behaviour. We have preferred a Tris/HCl buffer (pH 7.5) containing 0.1 M KCl and various amounts of $MgCl_2$ as indicated ; higher concentrations of KCl, such as 0.36 M KCl used in an earlier study of the large rRNA of the large subunit (Donceel *et al.*, 1982), induces aggregation of the rRNA of the small subunit. On the other hand, at lower concentration of KCl and in the absence of $MgCl_2$, the measurements were hampered by degradation of the rRNA of the large and small subunit. To avoid RNase hydrolysis, heparin was added to the rRNA solutions before dialysing. The rRNA of the small subunit is always less homogeneous than the rRNA of the large subunit ; this may be related to the lower stability of the small ribosomal subunit in comparison with the large subunit, noted earlier (Nieuwenhuysen and Clauwaert, 1981). A plot of the $s^\circ_{20,w}$-values versus the Mg^{2+} concentration, is shown in Fig. 2. A striking difference between the behaviour of the two RNAs is the large change in sedimentation coefficient of the rRNA of the small subunit at low $MgCl_2$ concentration, which does not occur for the large rRNA of the large subunit. The sedimentation coefficient of the rRNA of the small subunit in a buffer lacking Mg^{2+} is relative small, indicating that the rRNA is very expanded. Such a 'jump' in $s^\circ_{20,w}$ value does not show up for the rRNA of the large subunit and suggests that this rRNA is not as highly expanded. This supports the model in which both ends of the 5.8S rRNA are joined by H-binding to different regions of the RNA chain of the large subunit (Peters *et al.*, 1982). So the RNA is hampered to unfold completely, even at low $MgCl_2$ concentrations, as long as the interaction with the 5.8S rRNA prevails.

Diffusion coefficient of both rRNAs

The diffusion coefficient of both rRNAs has been measured in a solvent suited to minimize degradation and aggregation : 0.02 M Tris/HCl buffer, (pH 7.5), containing 3 mM $MgCl_2$ and 0.1 M KCl. The highest concentration of the rRNA of the small subunit was 0.7 mg/ml and that of the large rRNA of the large subunit was 0.8 mg/ml. After extrapolation to zero concentration, the diffusion coefficient reduces to $D^\circ_{20,w}$. A value of $(1.17 \pm 0.08)\ 10^{-7}$ cm^2/s can be assigned to the $D^\circ_{20,w}$ of the large rRNA of the large subunit and a value of $(1.70 \pm 0.08)\ 10^{-7}$ cm^2/s to the $D^\circ_{20,w}$ of the rRNA of the small subunit.

Molar mass of both rRNAs and consistency of measured values

The Svedberg relation allows to calculate the molar mass, M, from the $s^\circ_{20,w}$, $D^\circ_{20,w}$ and $\left(\frac{\delta\rho}{\delta c}\right)^\circ_\mu$ values we have determined experimentally :

$$M = \frac{s^\circ_{20,w} \cdot RT}{D^\circ_{20,w} \cdot \left(\frac{\delta\rho}{\delta c}\right)^\circ_\mu}$$

where R is the gas constant.

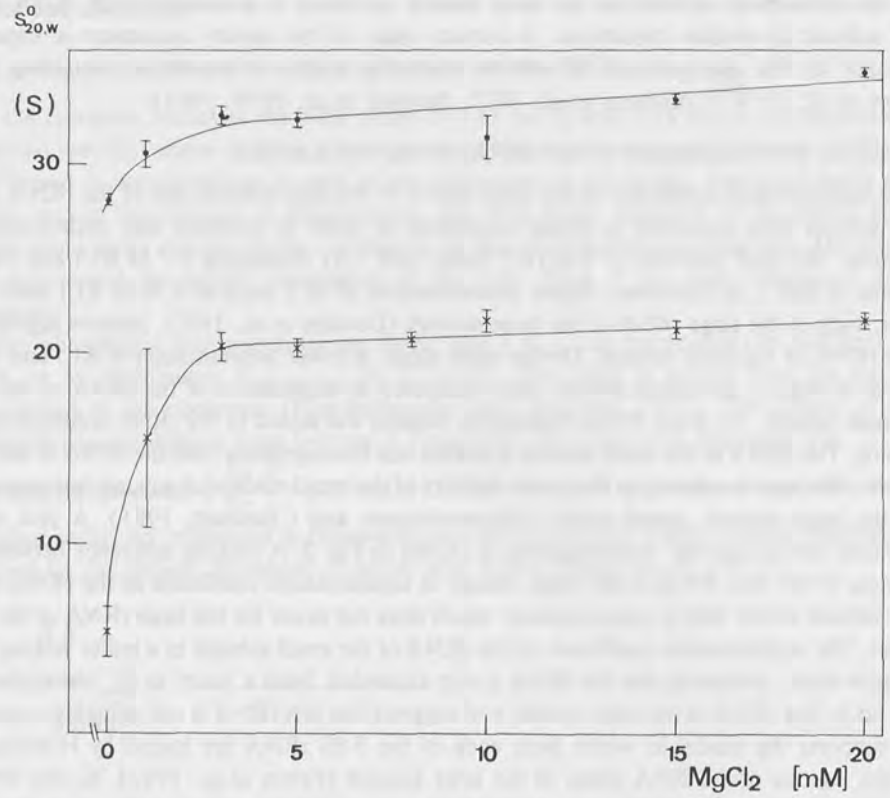

FIG. 2. The sedimentation coefficient, $s^{\circ}_{20,w}$ of the large RNA of the large subunit (●) and of the RNA of the small subunit (x), as a function of Mg^{2+} concentration at a constant K^+ concentration of 100 mM. The error bars indicate the variation in $s^{\circ}_{20,w}$ of different measurements of different samples.

For the molar mass (g/mol) of the large RNA of the large subunit we calculated a value of (1.24 ± 0.02) 10^6, using $s^{\circ}_{20,w}$ = (30.5 ± 0.05) S, $D^{\circ}_{20,w}$ = (1.17 ± 0.08) 10^{-7} cm^2/s and $\left(\frac{\delta\rho}{\delta c}\right)^{\circ}_{\mu}$ = (0.50 ± 0.01) ; for the rRNA of the small subunit we found a value of (0.60 ± 0.02) 10^6 using $s^{\circ}_{20,w}$ = (21.5 ± 0.4) S, $D^{\circ}_{20,w}$ = (1.70 ± 0.08) 10^{-7} cm^2/s and $\left(\frac{\delta\rho}{\delta c}\right)^{\circ}_{\mu}$ = (0.50 ± 0.01) cm^3/g. These values for the molar mass are very close to the values (1.23 ± 0.08) 10^6 and (0.62 ± 0.04) 10^6, calculated by combining the total molar mass of the ribosomal subunits with its RNA content (Nieuwenhuysen and Clauwaert, 1981), and by taking into account the contribution of the 5.8S RNA for the large RNA of the large subunit. The values obtained by our experiments agree also with those determined by gel electrophoresis in denaturing conditions (Cammarano *et al.*, 1975 ; Cruces *et al.*, 1982), where the migration rate of *Artemia* rRNA is compared with that of *E. coli* rRNA species, provided the values for the molar mass of the *E. coli* are used which can be calculated from the reported nucleotide sequence (Brosius *et al.*, 1978,

1980). Furthermore the molar mass of the rRNA of the small subunit can be derived from the sequence, which gives a number of 1 810 nucleotides (Nelles *et al.*, 1984). This corresponds to a molar mass of 0.60×10^6, which agrees with the value determined in this study. This gives us confidence in the correctness of our experimental results, specially the $\left(\frac{\delta\rho}{\delta c}\right)^{\circ}_{\mu}$ of 0.50.

Compactness and size of the RNAs

The degree of compactness of the rRNA is given quantitatively by the frictional coefficient ratio f/f_{min} (*e.g.* Tanford, 1961 ; Cantor and Schimmel, 1980) ; this can be calculated from their $s^{\circ}_{20,w}$, $\bar{v}^{\circ}$ and molar mass M :

$$f/f_{min} = \frac{\left(\frac{4}{3}\right)^{1/3} (1-\bar{v}^{\circ}\ \rho_{20,w})\ M^{2/3}}{6\ \eta_{20,w}\ (\pi\ N_A)^{2/3}\ .\ (\bar{v}^{\circ})^{1/3}\ s^{\circ}_{20,w}}$$

where $\eta_{20,w}$ is the viscosity and $\rho_{20,w}$ is the density of water at 20 °C, N_A is Avogadro's number and M the molar mass we calculated.

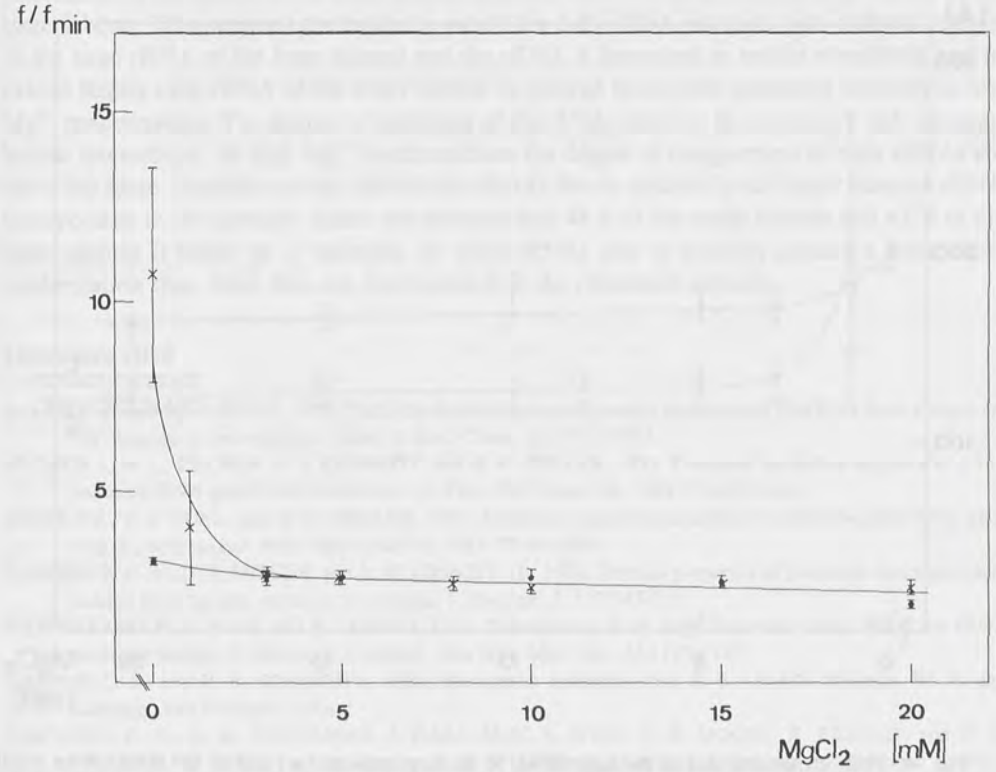

FIG. 3. The degree of unfolding as shown by the frictional ratio f/f_{min} of the large RNA of the large subunit (●) and of the RNA of the small subunit (x) as a function of the Mg^{2+} concentration, at a constant K^+ concentration of 100 mM. The error bars are derived from the uncertainty of the M, $s^{\circ}_{20,w}$ and $\bar{v}^{\circ}$.

A plot of f/f_{min} of both rRNAs versus the Mg^{2+} concentration is shown in Fig. 3. At higher Mg^{2+} concentration both RNAs have the same f/f_{min} within experimental error : the rRNA of the small subunit is as compact as the rRNA of the large subunit. At lower Mg^{2+} concentration, the f/f_{min} of the rRNA of the small subunit is much larger than the f/f_{min} of the rRNA of the large subunit, which indicates that the rRNA of the small subunit is more unfolded. The hydrodynamic radius, R_h, of the rRNA in solution is calculated using the equation :

$$R_h = \frac{M\ (1-\bar{v}^\circ\ .\ \rho_{20,w})}{6\ \pi\ \eta_{20,w}\ N_A\ .\ s^\circ_{20,w}}$$

and plotted in Fig. 4. This allows comparison with the R_h values of the complete subunits (Nieuwenhuysen and Clauwaert, 1981). We notice that the compact large rRNA of the large subunit, at high Mg^{2+} concentration, has still a larger size than the complete subunit (Donceel *et al.*, 1982). The size of the rRNA of the small subunit resembles more the size of the complete small subunit itself.

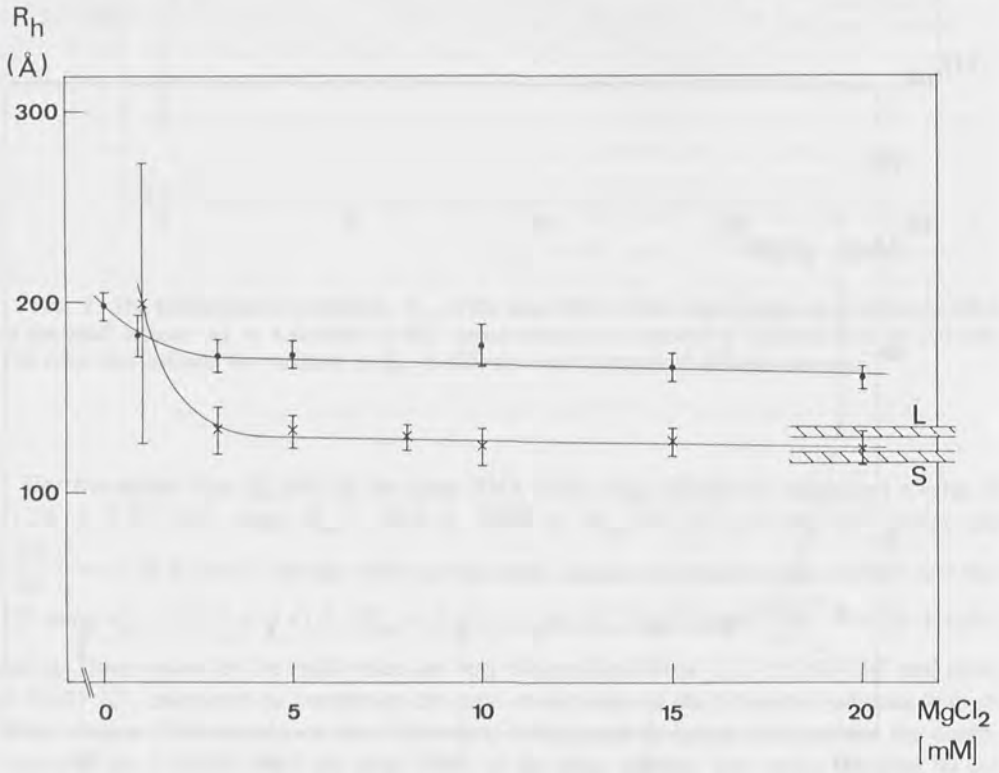

FIG. 4. Hydrodynamical size of the large RNA of the large subunit (●) and of the RNA of the small subunit (x), as a function of the Mg^{2+} concentration. The error bars are derived from the uncertainty of the M, $s^\circ_{20,w}$ and $\bar{v}^\circ$. The hydrodynamical radius of the large subunit (L) and of the small subunit (S) are indicated by the shaded regions.

f/f_{min} and R_h can also be calculated using the diffusion coefficient, independently of the sedimentation coefficient :

$$f/f_{min} = \frac{\left(\frac{4 \pi N_A}{3}\right)^{1/3} k T}{6 \eta_{20,w} \cdot (\pi)^{2/3} (\bar{v}^o M)^{1/3} D^o_{20,w}}$$

$$R_h = \frac{k T}{6 \pi \eta_{20,w} D^o_{20,w}}$$

where T is the temperature of 293 K and k is the Boltzman's constant.

Conclusion

Using the sedimentation coefficient, the density increment, and the diffusion coefficient we have derived values for f/f_{min} and R_h of the rRNAs of the small and large subunit in function of the Mg^{2+}-concentration. These values reflect the degree of unfolding and the size of the rRNA free in solution. Comparing the behaviour of both rRNAs, we conclude that at low Mg^{2+} concentration the rRNA of the small subunit is much more unfolded than the large rRNA of the large subunit. This supports the model in which the 5.8S rRNA interacts with different regions of the large rRNA of the large subunit and the rRNA is hampered to unfold completely and to extend largely ; the rRNA of the small subunit is allowed to an open expanded structure at low Mg^{2+} concentration. The degree of unfolding of the RNA seems to be correlated with intramolecular interactions. At high Mg^{2+}-concentrations the degree of compactness of both rRNAs are about the same. Nevertheless the size of both rRNAs free in solution is still larger than the rRNA incorporated in the subunits, taken into account that 48 % of the small subunit and 42 % of the large subunit is made up of proteins. So both rRNAs free in solution assume a more loose conformation than when they are incorporated in the ribosomal subunits.

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Ribosomal RNA gene types in *Artemia*

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Abstract

We have analyzed the ribosomal RNA genes in different populations of *Artemia*. We have previously described a repeat of 16.5 kb (Cruces *et al.*, 1981) for the San Francisco Bay strain. In some other batches we have found a repeat of 12.2 kb, and in others, a mixture of both types of repeat. We have also analyzed different, geographically isolated, populations of *Artemia*. Our results are compatible with a geographical distribution of rRNA gene types: North American populations are 16.5 kb and/or 12.2 kb. Caribbean populations are 16.5 kb and 13.5 kb in length, although different in organization from the North-American ones. Eurasian populations (mainly for the Iberian peninsula) have genes of 12.2 kb or 13.5 kb. The number of genes in the different populations studied have also been determined.

We have cloned and studied in further detail the North-American 16.5 and 12.2 kb repeat. In this paper we present the results about the organization of the external spacer and the site of initiation of the pre-rRNA.

Introduction

Ribosomal RNA genes (rDNA) in eukaryotes are well described (Long and Dawid, 1980). They consist in tandem repetitions of a basic unit composed (5′ to 3′) of an external non-transcribed spacer, the external transcribed spacer, the 17S coding region, the internal transcribed spacer (with the 5.8S coding sequence) and the 28S coding sequence. We have previously described in *Artemia* a repeat unit of 16.5 kb (Cruces *et al.*, 1981). We have found that other batches of commercially available cysts contain other type of repeat, of 12.2 kb. Other types of rDNA have also been described for *Artemia*. Vaughn *et al.* (1982) have described a recombinant with a 13.9 kb repeat, whereas Piot *et al.* (1981) reported (from genomic studies) a repeat of 11.5 kb. The existence of these different types of repeat moved us to investigate other commercial batches and other populations of *Artemia*.

Regulatory elements for rDNA transcription have not yet been established in great detail (Sommerville, 1984). The same is true for the structure of the external spacer and its relevance in transcription regulation. In *Xenopus* these regions have been extensively studied (Reeder *et al.*, 1983; Labhart and Reeder, 1984). The external spacer is composed of repeated sequences that have been postulated to act like enhancer elements (Reeder, 1984).

To address these questions in *Artemia*, we have cloned the 16.5 and 12.2 kb repeats and investigated the structure of the external spacers, as well as the initiation region of the pre-rRNA.

Materials and methods

Purification of DNA

High molecular weight DNA was obtained by a modification of the procedure of Cruces *et al.* (1981).

Isolation and labeling of RNAs

Artemia nauplii were grown as described by Osuna and Sebastián (1980). rRNA was obtained as described by Cruces *et al.* (1982) and labeled with [^{125}I] (Cory and Adams, 1977) or [^{32}P] (Cruces *et al.*, 1981). Total RNA from *Artemia* larvae was obtained by the method of Adams *et al.* (1977).

Cloning of rDNA

To enrich the DNA preparation for rDNA, purified DNA was fractionated in CsCl gradients in the presence of bisbenzimide H 33258 (Cruces *et al.*, 1987). The band with the highest density was freed of dye, dialized extensively to eliminate the CsCl and digested with *Sal*I. Bacteriophage λEMBL-4 (Frischauf *et al.*, 1983) DNA was also digested with *Sal*I. λEMBL-4 DNA (1.8 μg) were ligated with 1.5 μg of *Artemia* DNA in a final volume of 20 μl, under the conditions described by Maniatis *et al.* (1982) with 400 units of T4 DNA ligase (New England Biolabs). The ligation mixture was extracted twice with phenol:chloroform:Isoamyl alcohol (50:49:1), extracted with ether, ethanol precipitated, resuspended in TE buffer, and packaged *in vitro* as described by Hohn (1979) *In vitro* packaged phages were plated on Q359 cells. Recombinant phages were transferred to nitrocellulose membranes according to the method of Benton and Davis (1977). Filters were hybridized with [^{125}I]-rRNA and positive phages selected. Minipreparations of phage DNAs were made as described by Leder *et al.* (1977).

Two positive recombinant bacteriophages were chosen for further studies : λArt16 (which contains the complete 16.5 repeat) and λArt12 (which contains the complete 12.2 repeat).

Plasmids

The plasmid pArt115, previously obtained in our laboratory (Diaz-Guerra *et al.*, 1983) is the 3.2 kb *Bam*HI fragment that contains the first 600 bp of the 17S coding region and part of the external spacer (Fig. 1) cloned in pBR322.

pArt3000 is the 3kb *Sal*I-*Bam*HI fragment of λArt12, containing the 3′ region of 25S rRNA and part of the external spacer cloned into pUC9 (Vieira and Messing, 1982).

Other methods

S1 mapping was essentially done as described by Berk and Sharp (1977). The other methods used have been reported by Maniatis *et al.* (1982).

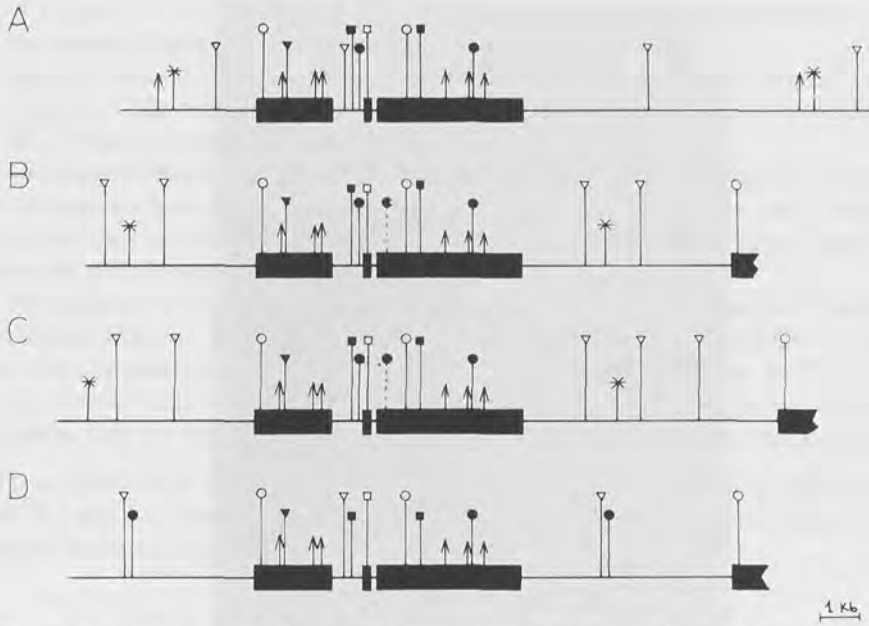

FIG. 1. Restriction maps of different rDNA repeats found in *Artemia*. (A) 16.5 kb repeat (Cruces *et al.*, 1981) ; (B) 12.2 kb repeat ; (C) 13.5 kb repeat ; (D) 12.2 kb repeat from *Artemia* grown in Tarragona (Spain). The restriction endonucleases are : (↑) *Bam*HI ; (↑) *Sal*I ; (↑) *Hin*dIII ; (↑) *Xba*I ; (↑) *Hpa*I ; (↑) *Sma*I ; (↑) *Eco*RI ; (↑) *Pst*I. Black boxes represent (from left to right) 17S, 5.8S, and 25S coding sequences respectively.

Results and discussion

rDNAs present in different batches of cysts

Genomic blots of different commercially available *Artemia* show that there are two different organizations of rDNA. Fig. 2 shows the restriction pattern with *Hin*dIII, and *Eco*RI for representative batches. One of them corresponds to the previously described by our laboratory (Cruces *et al.*, 1981). The other one has a repeat length of 12.2 kb ; both restriction maps are shown in Fig. 1A and B.

Table I shows that batches from the Salt Lake (Utah) have only the 16.5 kb repeat, whereas San Francisco Bay batches have 16.5 and 12.2 kb repeats alone or mixtures of both. The existence in one batch of two different rDNA repeats rises the questions whether these two types are present in the same organism or whether it is due to a mixed population of cysts.

In cysts from batch Newark 00, the 12.2 kb repeat accounts for 80 % (Fig. 2). In Newark nauplii, the 12.2 kb are only 5 %, and this percentage can be changed if nauplii are grown in media with different salinity. When we look at rDNA from different individuals from a batch which showed the two repeats (80 % of 12.2 in cysts), 19 out of 20 gave the 16.5 repeat and only one the 12.2.

These results strongly suggest that rDNA types are unique for a given population and that mixed repeats are due to a mix of cysts from different populations.

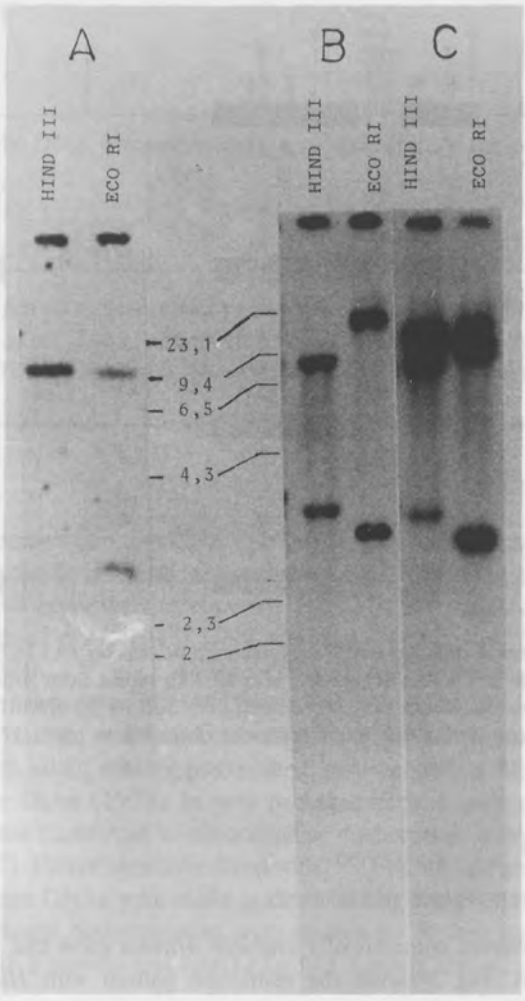

FIG. 2. Southern blot analysis of different commercial batches of *Artemia* cysts. A total of 5-10 µg of DNA were digested with the restriction endonucleases *Eco*RI and *Hin*dIII. After electrophoresis, DNA was transferred to nitrocellulose membranes and hybridized with [^{32}P]-labeled rRNA. Size markers (in kb) are λDNA digested with *Hin*dIII.

rDNA IN VARIOUS *ARTEMIA* POPULATIONS

As shown in Table I, we have also studied various geographically isolated *Artemia* populations. Representative restriction patterns (with *Eco*RI) are shown in Fig. 3. These results can be summarized as follows :

a) Some populations (Sanlúcar de Barrameda, Ayamonte, and S. Fernando, southern Spain) have only the 12.2 repeat already reported. Isolates from Tarragona (Spain) have a repeat

of 12.2 kb, but the *Eco*RI site in the internal spacer is missing and another site appears in the external spacer. This repeat has not the *Sal*I site (Fig. 1D).

b) Saelices, Delta del Ebro, and Bonmati (Spain) have a novel type of repeat, *i.e.* 13.5 kb (Fig. 1C). This repeat is very similar to the 12.2 repeat, except for a *Hind III* insertion in the 5′ region of the external spacer.

c) Alcochete (Portugal) and Tianjin (China) have a mixture of the 12.2 kb and 13.5 kb repeats. Although we have not investigated these two populations further, the results obtained with the San Francisco Bay batches and the fact that these DNAs were from cysts suggest that these isolates are not pure.

d) Two populations from the Caribbean Sea have been tested, from Bocachica (Venezuela) and Yucatán (México). Bocachica presents a 13.5 kb repeat which is different from the one found in Eurasian populations. Yucatán have a 16.5 kb repeat that differs from the North American 16.5 kb one. Both Caribbean populations, however, present a close restriction map. Macau isolates have the typical 16.5 kb repeat, in accordance with their San Francisco Bay origin.

These results show a striking geographical distribution of rDNA : North American *Artemia* have 16.5 and 12.2 repeat ; Caribbean *Artemia* have a repeat different to all others studied, and Eurasian populations have 12.2 and 13.5 kb repeats.

TABLE I

rDNA in various *Artemia* populations

Geographical distribution	Populations and batches	rDNA unit repeat length (kb)
North America	San Francisco (Newark-3462)	12.2
	San Francisco (Newark-1808)	16.5
	Utah (Longlife Aquarium Products)	16.5
	Utah (Sandtech. Co)	16.5
	San Francisco (Newark-3520)	12.2 + 16.5
	San Francisco (Newark-00)	12.2 + 16.5
	San Francisco (Newark-SN)	12.2 + 16.5
	Macau	16.5
Eurasia	Cádiz (S. Fernando) Spain	12.2
	Cádiz (Sanlúcar de Barrameda) Spain	12.2
	Huelva (Ayamonte) Spain	12.2
	Tarragona, Spain	12.2
	Tarragona (Delta del Ebro) Spain	13.5
	Guadalajara (Saelices) Spain	13.5
	Alicante (Bonmati) Spain	13.5
	Alcochete, Portugal	12.2 + 13.5
	Tianjin, China	12.2 + 13.5
Caribbean Sea	Bocachica, Venezuela	13.5
	Yucatán México	16.5

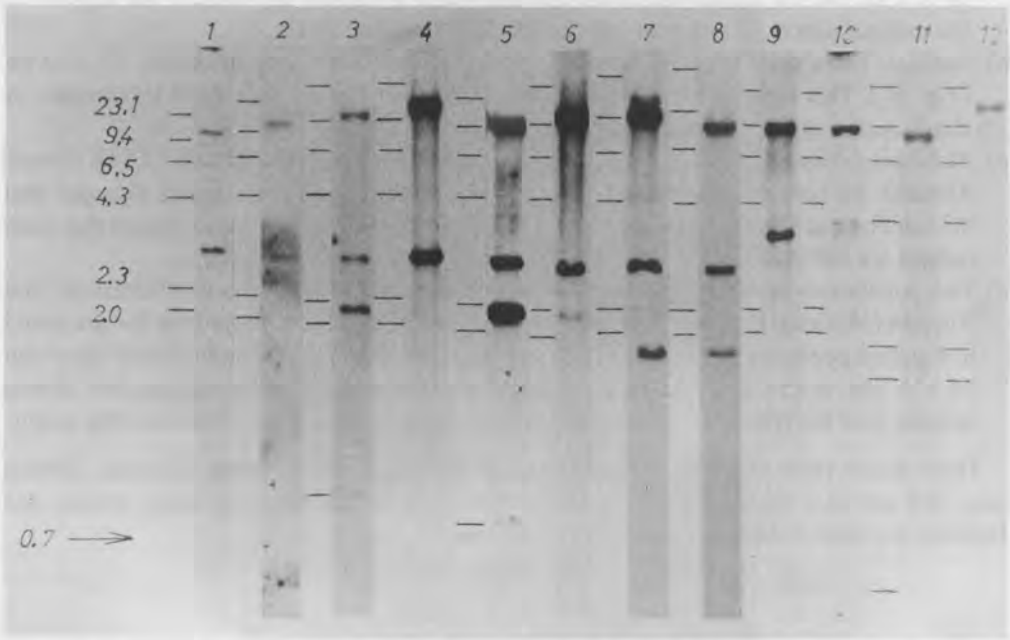

FIG. 3. Southern blot analysis of *Eco*RI digested DNAs from different populations of *Artemia*. Experimental details are as in Fig. 2. (1) Guadalajara, Saelices (Spain) ; (2) Tianjin (China) ; (3) Cádiz, S. Fernando (Spain) ; (4) Delta del Ebro (Spain) ; (5) Cádiz, Sanlúcar de Barrameda (Spain) ; (6) Alcochete (Portugal) ; (7) Bocachica (Venezuela) ; (8) Yucatán (México) ; (9) Tarragona (Spain) ; (10) Alicante, Bonmati (Spain) ; (11) Huelva, Ayamonte (Spain) ; (12) Macau (Brazil).

NUMBER OF rDNA REPEATS IN VARIOUS POPULATIONS

We have studied the number of rDNA repeats in various *Artemia* populations having the 16.5 and 12.2 kb repeat unit.

The different rDNAs were digested with *Sal*I and hybridized with the related clones of 16.5 and 12.2 kb, labeled with [^{32}P] by nick translation. As standard we used different concentrations of 16.5 and 12.2 kb clones (Simpson *et al.*, 1984). *Artemia* genome size was taken as 1.65×10^6 kb (Cruces, 1982).

The hybridization results are shown in Fig. 4 and the calculated gene number in Table II. The values found are between two and three times those previously reported by Roberts and Vaughn (1982), except for a Salt Lake population, that is ten times higher. We think of two possible explanations for this discrepancy : 1) we have consistently observed that a given amount of DNA gives different concentration values when analyzed by different methods [absorbance at 260 nm, fluorescence with ethidium bromide, fluorescence with bisbenzimide H 33258 (Labarca and Paigen, 1980), and deoxyribose determination with diaminobenzoic acid (Pestaña *et al.*, 1978)] ; 2) the size of the *Artemia* genome used for the calculations.

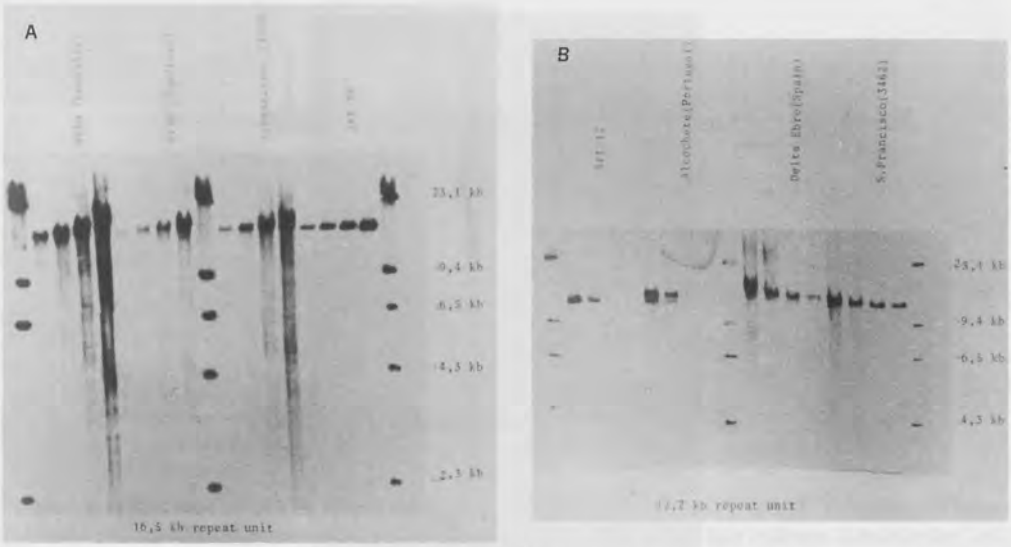

FIG. 4. Copy number of *Artemia* 16.5 and 12.2 rDNA repeats. Southern blots of *Sal*I-digested *Artemia* DNA were hybridized with [^{32}P]-labeled λArt 16 (A) and λArt12 (B). The concentrations of DNA used were : A. San Francisco (Newark-1808) and Utah (Sandtech), 0.4, 0.8, 1.6, and 3.2 µg ; Utah (Longlife), 0.5, 1, 2, and 4 µg ; λArt16, 0.8, 1.6, 3.2, and 6.4 ng. B. Alcochete, 0.25, 0.5, 1, and 2 µg ; Delta del Ebro, 0.35, 0.7, 1.4, and 2.8 µg ; San Francisco (Newark-3462), 0.28, 0.56, 1.12, and 2.24 µg ; λArt 12, 2.5, 5, 10, and 20 ng. DNA concentrations were determined by the diaminobenzoic acid assay.

TABLE II

Number of rDNAs genes in various *Artemia* populations

Cysts origin and batches	Gene number and % per haploid genome	Gene size (kb)
Alcochete, Portugal	1 900 (1.36 %)	12.2 + 13.5
Delta Ebro, Spain	2 222 (1.6 %)	13.5
San Francisco (Newark-3462)	1 950 (1.4 %)	12.2
San Francisco (Newark-1808)	1 855 (1.8 %)	16.5
Utah (Sandtech)	1 443 (1.4 %)	16.5
Utah (Longlife)	5 360 (5.2 %)	16.5

INITIATION SITE OF PRE-rRNA

Pre-rRNA has not been detected in *Artemia* because of the difficulty of labeling RNA to high specific activity. To circumvent this problem, we used the S1 mapping method. pArt115 was labeled in the 5′ ends and hybridized with total RNA obtained as described in Materials and methods. As shown in Fig. 5, a resistant band of 1.5 kb is observed, besides the 600 bp band corresponding to the 17S region contained in pArt115.

FIG. 5. Identification of the pre-rRNA initiation point. A total of 20 ng of 5′-labeled pArt115 were hybridized with 50 μg of total RNA. Hybrids were digested with different concentrations of S1 nuclease at 42 °C and electrophorized in agarose gels. A. Hybridization at 60 °C. B. Hybridization at 52 °C. The concentrations of S1 nuclease were : (1) 1 000 units ; (2) 400 units ; (3) 100 units ; (4) 40 units.

Assuming that, like in other organisms, pre-rRNA ends just after 25S rRNA, *Artemia* pre-rRNA must have a size of 7.6 kb. This figure is in agreement with northern gel-analysis of this total preparation, and also with the size of pre-rRNA from related species, as *D. melanogaster* (Lewin, 1980).

ORGANIZATION OF THE EXTERNAL SPACER

To obtain some information on the organization of the external spacer in *Artemia* rDNA, we hybridized both λArt16 and λArt12 with pArt3000 and the *Sal*I-*Bam*HI fragment of pArt115. pArt115 contains the 5′ region of the external spacer from the 16.5 repeat, whereas pArt3000 contains the 3′ region of the external spacer from the 12.2 kb repeat.

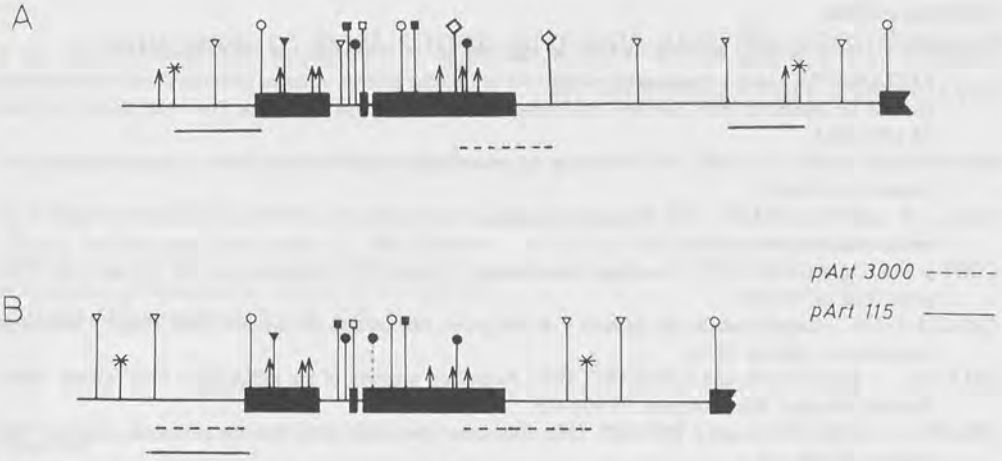

FIG. 6. Hybridization of λArt16 (A) and λArt12 (B) with pArt3000 and pArt115. The solid line below the maps indicates the fragments that hybridize with pArt115. The dashed line indicates hybridization with pArt3000. The symbols used for the restriction endonucleases are those of Fig. 1 and ?, *Bgl*II.

The results are summarized in Fig. 6. pArt115 hybridizes with both 5′ and 3′ regions of the 16.5 repeat and only with the 5′ region of the 12.2 repeat. pArt3000 hybridizes with the 3′ region of the 16.5 kb and with the 5′ and 3′ regions of the 12.2 repeat. As pArt115 and pArt3000 do not hybridize with each other, we conclude that the hybridization of pArt3000 with the 3′ region of 16.5 repeat is only due to the common 25S sequences, and that the hybridization of pArt115 with the 5′ region of the 12.2 kb repeat is due to the common 17S and pre-rRNA sequences.

The hybridization of pArt115 with the 3′ region of the 16.5 repeat, and that of pArt3000 with the 5′ region of the 12.2 repeat must therefore be due to sequences common to these regions, and this repetition has to be different in the 16.5 and 12.2 repeats.

Conclusions

1. North American *Artemia* have rDNAs of 16.5 and 12.2 kb, whereas Eurasian populations have 12.2 and 13.5 kb repeats. Caribbean populations have repeats with the same sizes (16.5 and 13.5 kb) but with different restriction maps. The difference between these types of repeat are only in the spacers, and within each population rDNA is homogeneous.
2. Pre-rRNA from *Artemia* has a size of 7.6 kb ; the initiation site is 900 bp 5′ from 17S coding region.
3. The external spacers have common sequences in the 5′ and 3′ regions but there are no common external spacer sequences between the 16.5 and 12.2 repeats.

Acknowledgements

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Synthesis of heat shock proteins and thermotolerance in *Artemia* cysts and larvae

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Abstract

A preliminary investigation into the heat shock response in *Artemia* has been carried out with a view to understanding the resistance of this animal to environmental stress. Nauplius larvae which have been hatched at 28 °C respond to a rapid, temporary increase in ambient temperature (heat shock) in a manner similar to many other organisms. There is a 50 % survival after a 1 h heat shock at 40 °C while a further one degree increase in temperature reduces survival practically to zero. When returned to 28 °C a high degree of thermotolerance develops during the 4 h following the initial 40 °C heat shock, such that 95 % of the larvae survive a second heat treatment for 30 min at 42 °C. Normally such a combination of time and temperature would be lethal.

By labelling larvae with $NaH^{14}CO_3$, the enhancement of synthesis of an 89 kD protein (HSP89) can be seen even at 32 °C. As the temperature increases a 68 kD protein (HSP68) also increases such that at 39 °C HSP68 and HSP89 are the major products of protein synthesis. At 40 °C normal protein synthesis is strongly suppressed and HSP68 and HSP89 are virtually the only proteins synthesised. No low molecular weight heat shock proteins were seen.

Hydrated pre-emergent cysts display a natural thermotolerance. Neither protein synthesis nor viability is affected by prolonged (17 h) incubation at 40 °C. Cysts of 2 h constitutively synthesise HSP68 and HSP89 with synthesis being further enhanced by heat shock at 40 °C or 45 °C. The level of synthesis of these proteins is reduced in 12 h cysts. Immuno-blotting with a cross-reacting anti-chick HSP70 antibody shows that cysts contain a relatively high constitutive level of a 68 kD protein which appears identical to the inducible HSP68. The amount of this protein also decreases during development but can be elevated again by heat shock. HSP68 and HSP89 may therefore be involved in the production of thermotolerance and possibly other forms of stress resistance including dehydration stress in cysts and larvae.

Small but significant increases in the levels of the bis(5′-nucleosidyl) oligophosphates Ap_3A and Ap_4A were also observed upon heat shock. A role for these nucleotides in the control of the heat shock response in prokaryotes has been postulated. Their involvement in the stress response in *Artemia* remains to be investigated.

Introduction

Since the initial demonstration of the heat-induced puffing of polytene chromosomes in *Drosophila* (Ritossa, 1962), the response of living systems to a sub-lethal heat shock has been shown to be a universal phenomenon with one of the most profound metabolic changes being the suppression of normal protein synthesis and the expression of a set of 'new' proteins

commonly referred to as heat shock proteins (HSPs) (Schlesinger *et al.*, 1982ab ; Alahiotis, 1983 ; Marx, 1983 ; Tanguay, 1983).

HSPs fall roughly into three size classes as determined by electrophoresis in denaturing polyacrylamide gels : high (80-100 kD), intermediate (60-70 kD), and low (12-30 kD) molecular weight (Gutmann *et al.*, 1980 ; Loomis and Wheeler, 1980 ; Schlesinger *et al.*, 1982a ; Stephanou *et al.*, 1983). With the use of high resolution two-dimensional techniques, many more presumptive HSPs have now been detected, for example in primary rat thymocytes (Maytin *et al.*, 1985).

Regulation of the expression of the major HSPs appears to be through the rapid induction and preferential translation of new mRNA species (Ashburner and Bonner, 1979 ; Bonner and Kerby, 1982). However, in HeLa cells the expression of a set of rapidly appearing HSPs involves the translational regulation of a pre-existing pool of HSP mRNAs (Reiter and Penman, 1983). There is also evidence for both types of regulation in rat thymocytes (Maytin *et al.*, 1985) and *Xenopus* embryos (Bienz and Gurdon, 1982). Thus the synthesis of HSPs may be under both transcriptional and translational control.

A close degree of homology has been shown to exist between the equivalent HSPs of species ranging from vertebrates to lower eukaryotes and even prokaryotes (Kelley and Schlesinger, 1982 ; Moran *et al.*, 1983 ; Voellmy *et al.*, 1983). The fact that an HSP70-like protein is present in *E. coli, dna* K protein (Neidhardt *et al.*, 1982), suggests an ancient origin and indeed a fundamental requirement for this protein in all living systems, however the precise functions of the HSPs are still poorly understood.

Physiological changes associated with heat shock include the disruption of cytoplasmic polysomes (Duncan and Hershey, 1984), actin stress fibre bundles and microtubules (Coss *et al.*, 1982 ; Glass *et al.*, 1985) and alterations in nucleolar morphology (Pelham, 1984), and DNA synthesis (Piperakis and McLennan, 1984). It seems likely that a major role of HSPs is in the repair of such damage and in the protection of such macromolecular structures from further disruption. Indeed thermotolerance, that is the enhanced protection against subsequent thermal damage afforded by an initial sub-lethal heat shock, is another generally occurring phenomenon (McAllister and Finkelstein, 1980 ; Subjeck and Sciandra, 1982 ; Taylor and Holliday, 1984). In many systems, some or all of the same sets of HSPs can be induced by a wide variety of agents other than heat, *e.g.* ethanol, heavy metals, amino acid analogues or by recovery from anoxia (Gutmann *et al.*, 1980 ; Tanguay, 1983).

The dry, cryptobiotic cyst of *Artemia* is well known for its high resistance to chemical and physical insults (Clegg and Conte, 1980). Even hydrated cysts are remarkably tolerant to stress. For example, they can be held at 40 °C for several days with little effect on ultimate viability (Sorgeloos *et al.*, 1975), while during the early period of pre-emergence development they can be subjected to multiple cycles of dehydration and rehydration with no damage to macromolecular complexes (De Chaffoy and Kondo, 1976 ; Clegg and Conte, 1980). The factors responsible for dehydration resistance include glycerol and possibly trehalose (Clegg and Conte, 1980) ; however, a role for some of the ubiquitous stress proteins also seems likely since dehydration stress is known to induce HSP70 in plants (Heikkila *et al.*, 1984).

In the hope that *Artemia* may prove to be a useful system in which to study the induction and role of these proteins in stress tolerance, we have initiated a study of the effect of heat shock on protein synthesis in cysts and larvae.

Materials and methods

Reagents

Phenylmethanesulphonyl fluoride (PMSF) and soybean trypsin inhibitor (STI) were from Sigma. $NaH^{14}CO_3$ (50-60 mCi/mmol) and Amplify fluorographic reagent were from Amersham and the GAR-HRP Immuno-blot assay kit was purchased from Bio-Rad. Molecular weight standards for denaturing polyacrylamide gel electrophoresis were : phosphorylase b, 92 500 ; bovine serum albumin, 62 200 ; ovalbumin, 45 000 ; carbonic anhydrase, 31 000, and soybean trypsin inhibitor, 21 500. A polyclonal antibody raised against purified chick fibroblast HSP70 was the generous gift of Prof. M. Schlesinger, Washington University School of Medicine, St. Louis, USA.

Cysts, larvae, and heat shock

Great Salt Lake cysts were obtained from the Sanders Brine Shrimp Co., Ogden, UT, USA, in 1980. These were prepared, decapsulated, and incubated as previously described (McLennan and Prescott, 1984). The decapsulated cysts were stored in saturated NaCl solution for up to 8 days before use. Larvae were separated from unhatched cysts by transferring the incubate to a 100 ml measuring cylinder, allowing the cysts to settle and carefully aspirating the swimming larvae. In the following text 24 h larvae, for example, refers to all larvae which have hatched up to 24 h after rehydration.

Heat shocks were delivered by immersion of aliquots of cysts or larvae contained in 3 ml polycarbonate tubes in a water bath at the appropriate temperature. The percentages of viable, swimming larvae after heat shock were determined in replicate tubes by the method of Warner *et al.* (1979).

Protein labelling and analysis

Unless otherwise stated, 25 mg wet weight samples of cysts or larvae were harvested after heat shock by filtration on 2.5 cm paper discs and transferred to 1 ml thoroughly degassed seawater containing 50 µCi $NaH^{14}CO_3$ in a 3 ml polycarbonate tube. The tubes were flushed with O_2, sealed and incubated at 28 °C for 3 h. Organisms were reharvested and washed with ice-cold 20 mM potassium phosphate buffer pH 7.2, 1 mM EDTA. Samples were then homogenised in a Kontes glass homogeniser in 250 µl 10 mM potassium phosphate buffer pH 7.2, 0.3 M KCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM $NaHSO_3$, 2.5 mM PMSF, 100 µg/ml STI. The homogenates were centrifuged (16 000 g, 10 min, 4 °C) and the supernatants dialysed extensively against 62.5 mM Tris-HCl pH 6.8, 1 mM EDTA and then frozen at -70 °C. The pellets were washed three times in the same buffer, then resuspended in 25 µl 62.5 mM Tris-HCl pH 6.8, 2 % SDS, 5 mM 2-mercaptoethanol, 10 % glycerol and boiled for 5 min. After centrifugation as above, the supernatants were stored at -70 °C. Protein concentrations were estimated by the method of Bradford (1976).

Samples of the dialysed soluble extracts corresponding to the same numbers of organisms in each case (50-100 µg protein, 1 500-7 000 cpm) were boiled for 5 min in sample buffer and subjected to denaturing polyacrylamide gel electrophoresis (Laemmli, 1970). Since the protein concentrations of the pellet extracts solubilised directly in SDS sample buffer could not be

measured accurately, qualitative estimates based on gel profiles stained with Coomassie blue were used. In general, 10 µl samples of pellet extracts were loaded beside the soluble samples. Polyacrylamide gels of 10 % were run either at 25 mA constant current with cooling or at 10 mA overnight without cooling. Incorporation of ^{14}C label was visualised by impregnating the gel with Amplify fluorographic reagent and exposing the dried gel to a pre-flashed sheet of Fuji RX film at -70 °C for between 4 days and 3 weeks depending on the degree of incorporation of label.

Immunological visualisation of *Artemia* HSP68 was achieved by protein blotting (Towbin *et al.*, 1979). Proteins were electrophoretically transferred overnight from the gel to nitrocellulose at 30 V, 150 mA in 25 mM Tris, 192 mM glycine pH 8.3, 20 % methanol, 0.1 % SDS. After blocking in 3 % gelatin, the blot was incubated for 24 h at room temperature with 35 µg/ml anti-chick HSP70 IgG in 20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 % gelatin, 0.01 % thimerosal and the bands visualised using the GAR-HRP Immuno-blot kit. The second antibody, peroxidase-conjugated goat anti-rabbit IgG, was used at a dilution of 1:2 000.

Measurement of Ap_3A and Ap_4A

Nucleotides were separated from TCA extracts of heat-shocked cysts and larvae by reversed phase ion-pair h.p.l.c. on a Waters RP-18 Radial-Pak cartridge as previously described (McLennan and Prescott, 1984). Pooled fractions containing Ap_3A and Ap_4A were assayed using the coupled luciferase procedures (Ogilvie and Jakob, 1983 ; McLennan and Prescott, 1984).

Results

Effect of heat shock on viability of larvae

When 24 h larvae which had been hatched at 28 °C were incubated for 1 h at various temperatures and their viability determined after a 1 h recovery period at 28 °C, 50 % survival resulted at 40 °C (Fig. 1). A further one degree increase in temperature was sufficient to reduce survival practically to zero. Longer periods of recovery did not significantly alter the observed effect which is in contrast to the behaviour of hydrated cysts which will tolerate a temperature of 40 °C for up to 3 days (Sorgeloos *et al.*, 1975 ; McLennan, unpubl.). Nevertheless a significant degree of thermotolerance is induced in the larvae by a 1 h heat shock at 40 °C. When the survivors of this treatment were harvested and given a second heat shock at 42 °C for 30 min, normally a lethal combination, after increasing times of recovery at 28 °C, a maximum survival rate of 95 % was attained after a recovery period of 4 h (Fig. 2). Thermotolerance was temporary, only 40 % surviving the second heat treatment after 19 h recovery at 28 °C.

Effect of heat shock on protein synthesis

In order to determine whether alterations in the pattern of protein synthesis accompanied the reduction in survival at high temperatures, 24 h larvae were labelled with $NaH^{14}CO_3$ after a 1 h heat shock at different temperatures and the labelled KCl-soluble proteins analysed on a denaturing polyacrylamide gel. Two responses were apparent. The first was the gradual enhancement of the synthesis of two proteins of 68 kD and 89 kD (HSP68 and HSP89) ; at 39 °C these two proteins were very prominent (Fig. 3a). The second was the sudden suppression of 'normal' protein synthesis which occurred at 40 °C due to the disaggregation of existing polysomes

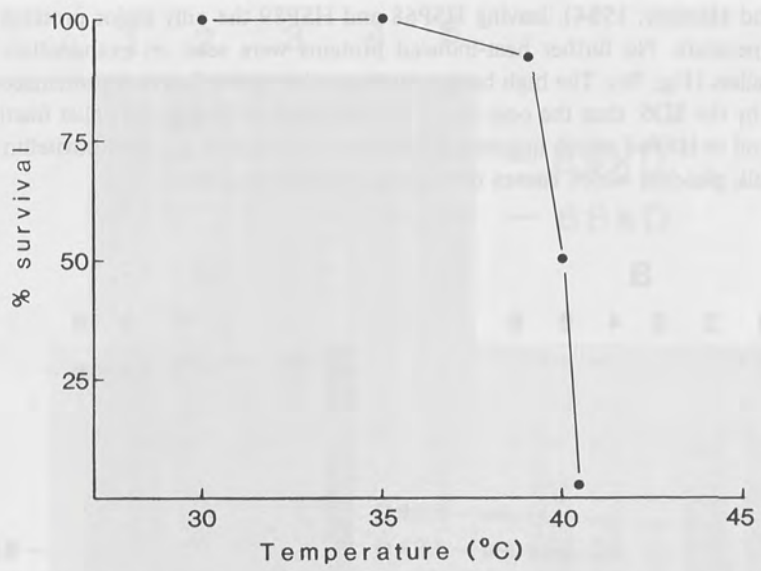


FIG. 1. Viability of 24 h larvae after heat shock at different temperatures. Larvae were harvested and given a 1 h heat shock at different temperatures as described in "Materials and methods." The % survival was determined after a 1 h recovery period at 28 °C. Points represent the average from several experiments.

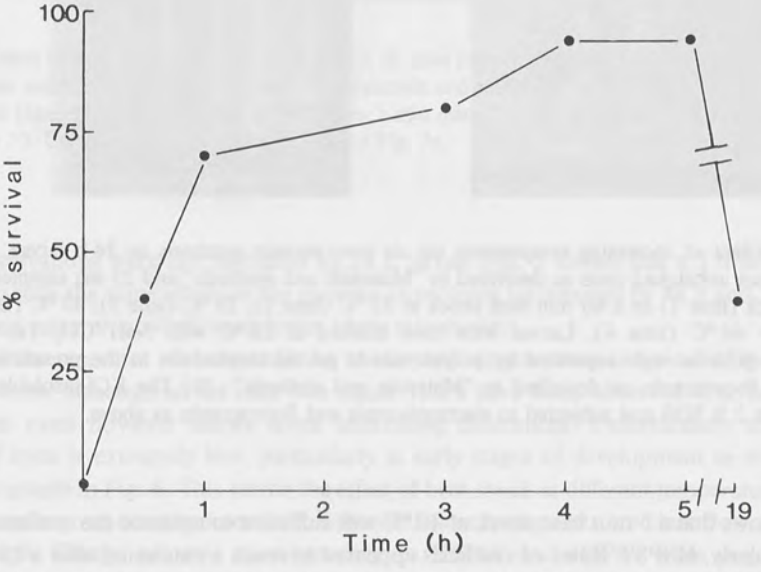


FIG. 2. Induction of thermotolerance in heat-shocked 24 h larvae. Larvae which survived a 1 h heat shock at 40 °C were harvested and allowed to recover at 28 °C for various times before the application of a second heat shock at 42 °C for 30 min. Survivors were determined as before.

(Duncan and Hershey, 1984), leaving HSP68 and HSP89 the only major proteins synthesised at this temperature. No further heat-induced proteins were seen on examination of the KCl insoluble pellets (Fig. 3b). The high backgrounds are due to labelled non-proteinaceous material solubilised by the SDS, thus the only major heat-induced protein in the pellet fractions appears to correspond to HSP68 which migrates just behind the major 68 kD apolipovitellin polypeptide from the yolk platelets which causes quenching of the fluorogram.

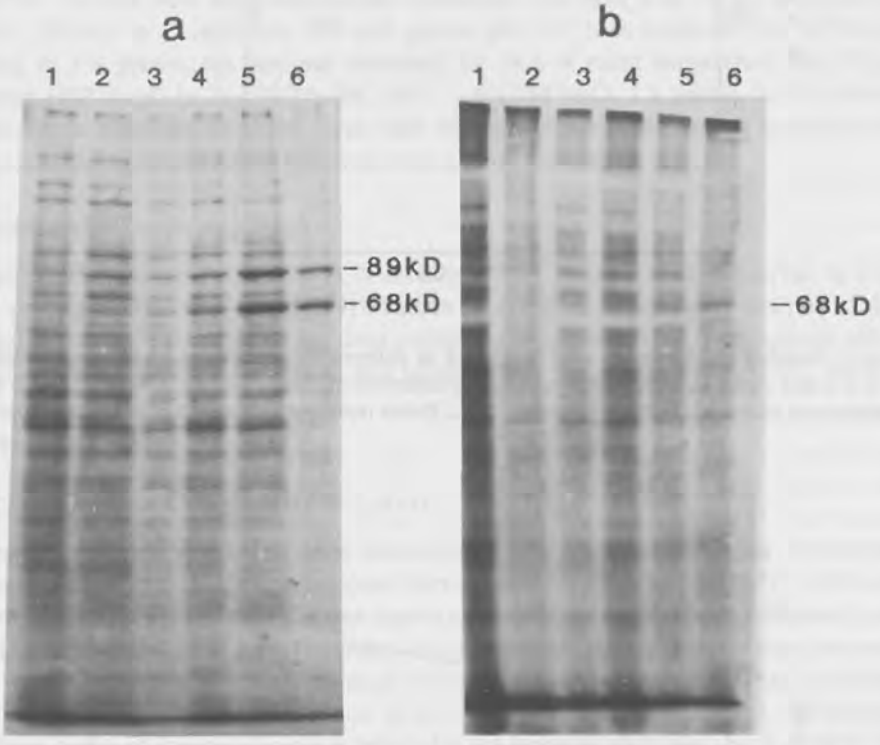


FIG. 3. Effect of increasing temperature on *de novo* protein synthesis in 24 h larvae. Larvae were separated from unhatched cysts as described in "Materials and methods" and 25 mg samples given either no heat shock (lane 1) or a 60 min heat shock at 32 °C (lane 2), 35 °C (lane 3), 37 °C (lane 4), 39 °C (lane 5), or 40 °C (lane 6). Larvae were then labelled at 28 °C with $NaH^{14}CO_3$. (a) The labelled KCl-soluble proteins were separated by polyacryamide gel electrophoresis in the presence of SDS and analysed by fluorography as described in "Materials and methods"; (b) The KCl-insoluble pellets were solubilised in 2 % SDS and subjected to electrophoresis and fluorography as above.

Fig. 4 shows that a 5 min heat shock at 40 °C was sufficient to enhance the synthesis of HSP68 and, particularly, HSP89. Rates of synthesis appeared to reach a maximum after a 15 min shock, however longer periods at 40 °C were required to cause suppression of normal protein synthesis, so the two responses appear to be under different controls. After 60 min even the synthesis of the HSPs was reduced.

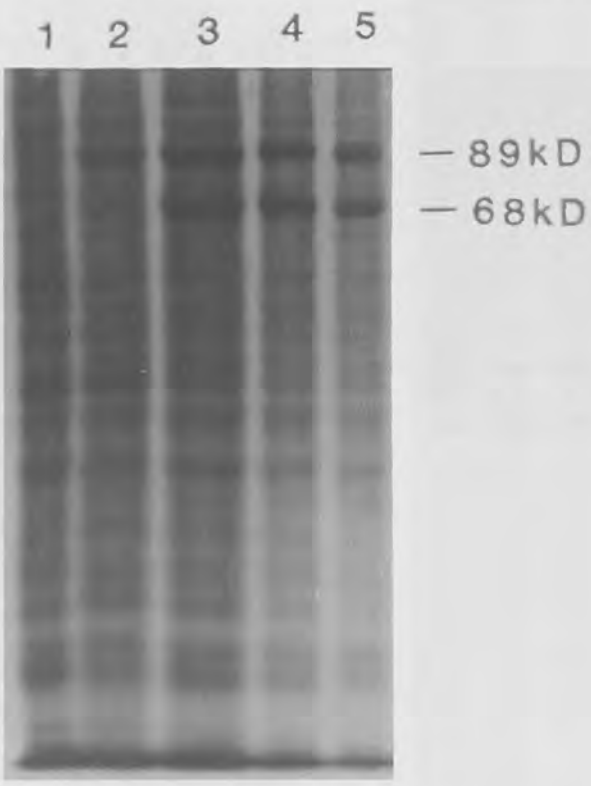
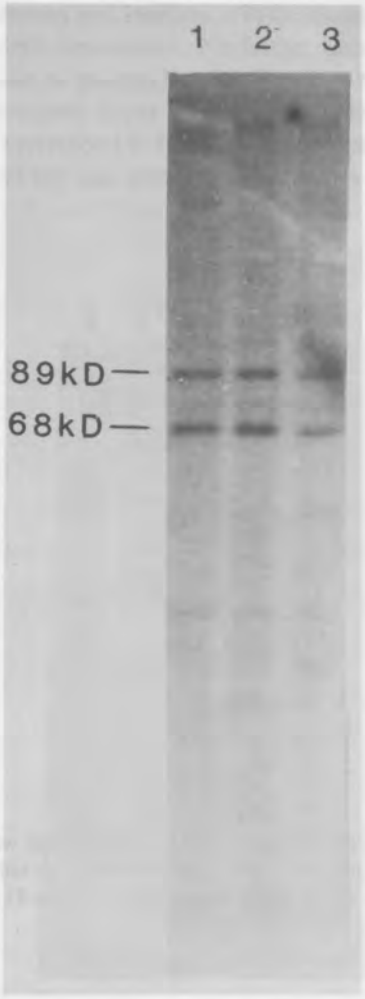


FIG. 4. Effect of increasing duration at 40 °C on *de novo* protein synthesis in 24 h larvae. Larvae were separated from unhatched cysts as described in "Materials and methods" and 25 mg samples given either no heat shock (lane 1) or a heat shock at 40 °C for 5 min (lane 2), 15 min (lane 3), 30 min (lane 4), or 45 min (lane 5). Larvae were then processed as for Fig. 3a.

These observations were not restricted to 24 h larvae. Fig. 5 shows that a 1 h heat shock at 40 °C had exactly the same effect on the patterns of proteins synthesised by 48 h and 72 h larvae, although their rates of survival were lower (data not shown).

In general the response of *Artemia* larvae to elevated temperatures is typical of that observed in other systems, although so far only two major HSPs have been observed. The behaviour of pre-emergent cysts however shows some interesting differences. Unfortunately the labelling efficiency of cysts is extremely low, particularly at early stages of development as evidenced by the autoradiograph in Fig. 6. This shows the effect of heat shock at different temperatures on cysts which have undergone development for 2 h. Nevertheless, two points are clear from the original autoradiograph. Firstly, proteins corresponding to HSP68 and HSP89 were very prominent amongst those synthesised before heat shock, much more so than in larvae (lane 1). The synthesis of both proteins was further enhanced by a 1 h heat shock at either 40 °C (lane 2) or 45 °C (lane 3). Secondly, it is evident that a 1 h heat shock at 40 °C did not suppress 'normal'



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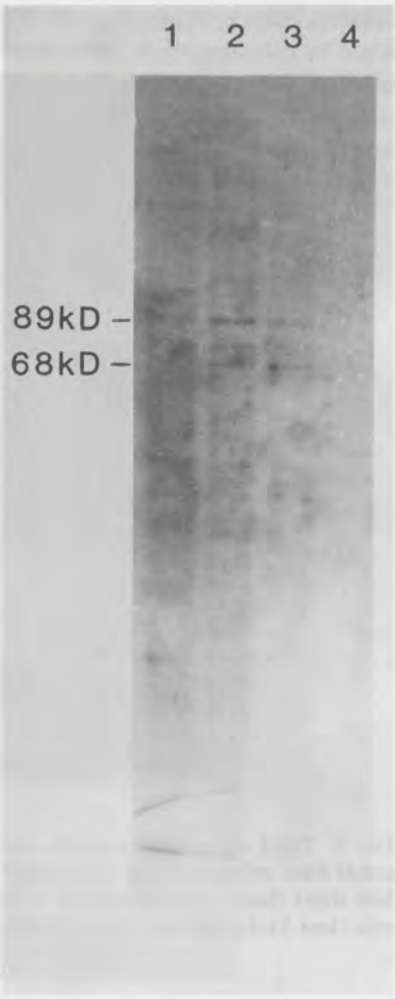

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FIG. 5. Expression of heat shock proteins in larvae at different developmental stages. 24 h (lane 1), 48 h (lane 2), and 72 h (lane 3) larvae were given a 1 h heat shock at 40 °C. Further processing as for Fig. 3a.

FIG. 6. Effect of heat shock on *de novo* protein synthesis in 2 h cysts. Decapsulated cysts were incubated at 28 °C for 2 h and 50 mg samples given either no heat shock (lane 1) or a 60 min heat shock at 40 °C (lane 2), 45 °C (lane 3), or 50 °C (lane 4). Further processing as for Fig. 3a. Because of the faint image, photography was with reflected light, rather than the usual transmitted light.

protein synthesis at all (lane 2). Even after heating at 45 °C some residual synthesis of 'normal' proteins was apparent (lane 3). A heat shock for 1 h at 50 °C resulted in the complete inhibition of all protein synthesis (lane 4).

Protein synthesis in 12 h cysts was also found to be resistant to suppression by a 1 h heat shock at 40 °C (Fig. 7, lanes 1 and 2). The effect of such a treatment on 24 h larvae is shown again for comparison (lanes 3 and 4). However, the amounts of HSP68 and HSP89 synthesised by

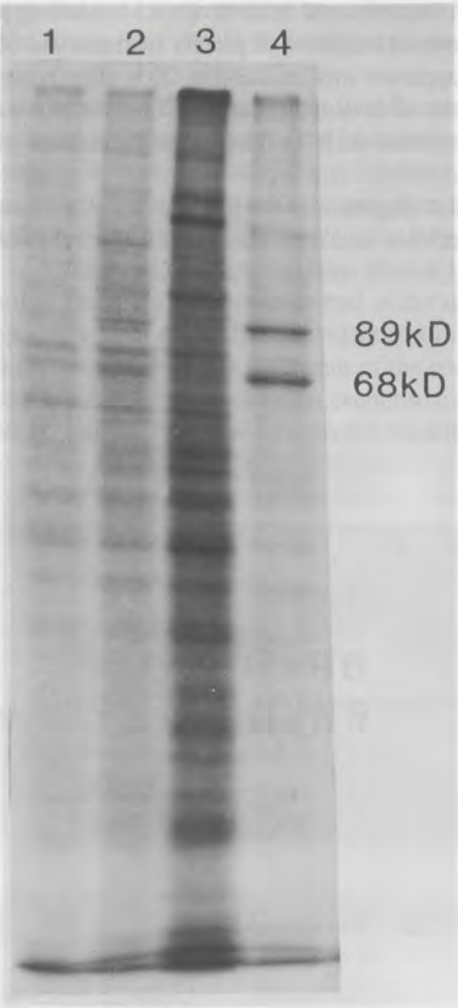

FIG. 7. Effect of heat shock on *de novo* protein synthesis in 12 h cysts. Decapsulated cysts were incubated at 28 °C for 12 h and 50 mg samples given either no heat shock (lane 1) or a 60 min heat shock at 40 °C (lane 2). Control lanes are 24 h larvae before (lane 3) and after (lane 4) a 1 h heat shock at 40 °C.

12 h cysts before heat shock appeared to be a much smaller percentage of the total protein synthesised than was the case with 2 h cysts. In this respect 12 h cysts appear more similar to 24 h larvae. Fig. 7 also shows an apparent increase in the synthesis of proteins of approximately 135 kD, 85 kD, and 50 kD after heat shock (lanes 1 and 2) but the possible significance of this remains to be established.

What is most striking about these results is the natural thermotolerance of the developing cysts. A 45 °C shock for 1 min is sufficient to kill larvae and abolish all labelling of proteins. This thermotolerance extends to prolonged periods at high temperatures. For example, when 8 h cysts

were held at 40 °C for 17 h then returned to 28 °C for a 5 h labelling period, HSP68 and HSP89 were evident but again no overall inhibition of protein synthesis was observed (Fig. 8). The cysts began to hatch with no apparent loss in viability 20 h after being returned to 28 °C. This complete and reversible arrest of development at 40 °C and the increase in accumulated hatching time at 28 °C from the normal 16 h to 28 h is in agreement with previous observations (Sorgeloos *et al.*, 1975).

The results with cysts also suggest that the mRNAs for HSP68 and HSP89 may be present in the dry gastrulae and activated and translated shortly after rehydration. The content of these

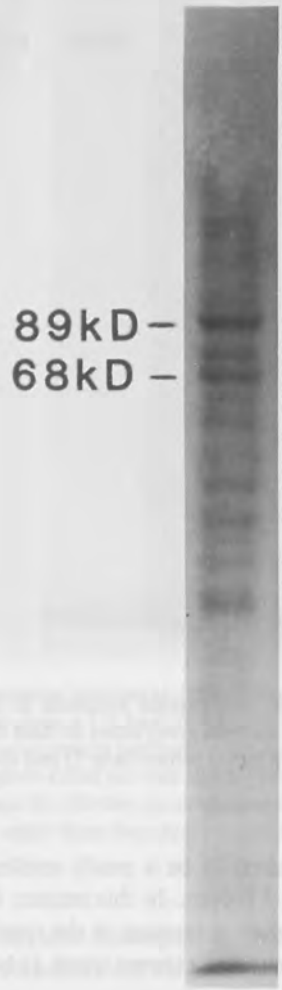

FIG. 8. Effect of prolonged hyperthermia on *de novo* protein synthesis in cysts. Decapsulated cysts were incubated as follows: 8 h at 28 °C, then 17 h at 40 °C, then 5 h at 28 °C in the presence of 50 μCi $NaH^{14}CO_3$. Further processing as for Fig. 3a.

mRNAs seems to decrease during pre-emergence and larval development, though they retain the ability to reappear upon heat shock. This should be readily confirmed by translation of cyst and larval mRNA *in vitro*.

If indeed HSP mRNAs are present in the dry gastrulae then the corresponding proteins might also be expected to be constitutive components. A semi-quantitative method for the detection of certain HSPs involves immunological visualisation with a heterologous antibody. Anti-chick HSP70 cross-reacts with proteins of similar molecular weight from a wide variety of organisms (Kelley and Schlesinger, 1982). When a nitrocellulose blot of soluble proteins from 0 h and 7 h cysts and 24 h, 48 h, and 72 h larvae, all of which had been given a 1 h heat shock at 40 °C and allowed to recover for various times at 28 °C, was probed with this antibody as described in "Materials and methods", a single band at 68 kD was evident in all cases (Fig. 9). This shows : 1) that the *Artemia* 68 kD protein is indeed the equivalent of the corresponding HSPs in other systems ; 2) that it is present at all developmental stages examined even before heat shock ; and 3) that the HSP68 induced by heat shock is the same as the constitutive protein rather than a

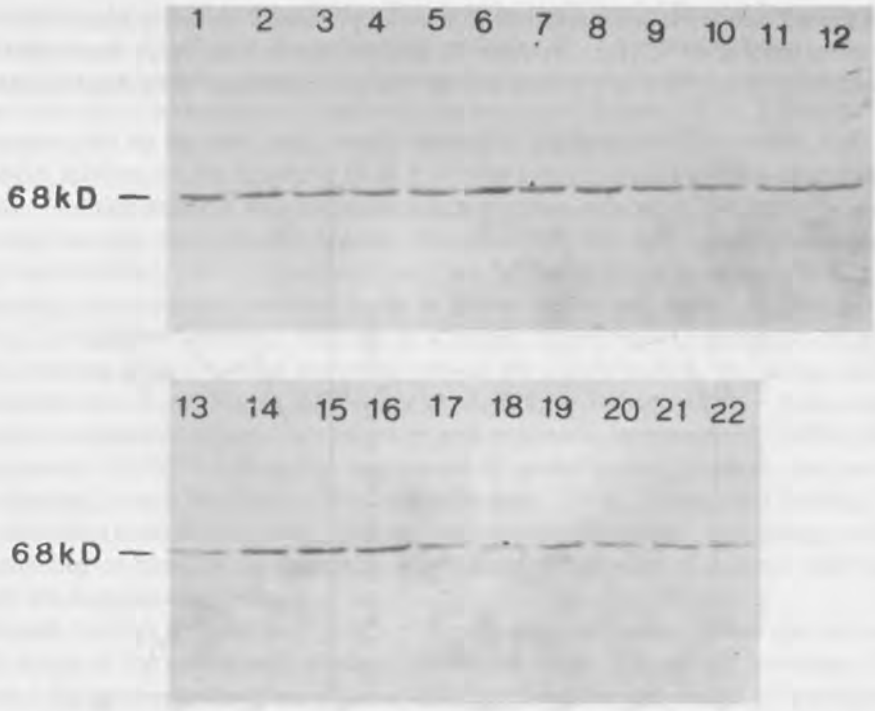

FIG. 9. Immunological detection of HSP68 at different developmental stages on nitrocellulose transfers. Cysts and larvae at 0 h (lanes 1-4), 7 h (lanes 5-8), 24 h (lanes 9-12), 48 h (lanes 13-17), and 72 h (lanes 18-22) of development were given either no heat shock (lanes 1, 5, 9, 13, 18) or a 1 h heat shock at 40 °C followed by a recovery period at 28 °C of 0 h (lanes 2, 6, 10, 14, 19), 1 h (lanes 15, 20), 3 h (lanes 3, 7, 11, 16, 21), or 5 h (lanes 4, 8, 12, 17, 22). KCl-soluble proteins were prepared, separated by polyacrylamide gel electrophoresis in the presence of SDS and nitrocellulose transfers of the gels probed with anti-chick HSP70 as described in "Materials and methods."

related member of the same family as in L-cells (Lowe and Moran, 1984). A visible increase in its content only occurs upon heating 48 h (lanes 13-17) and particularly 72 h (lanes 18-22) larvae. Although its synthesis can be detected at early developmental stages under these conditions, the increase in amount at early times is not so great compared to the existing content of control 0 h (lanes 1-4) and 7 h (lanes 5-8) cysts and 24 h larvae (lanes 9-12) as it is at later stages. Thus it appears that HSP68 may be a major constitutive protein in the dormant cyst and that it decreases in content during development although it can still be induced to higher levels at later times by heat shock. HSP89 has yet to be examined in this way.

Regarding the intracellular signal responsible for the induction of these proteins, Lee *et al.* (1983) have recently shown that all those agents which turn on the heat shock response in *E. coli* and *S. typhimurium* lead to the prior accumulation of adenine-containing bis(5′-nucleosidyl) oligophosphates including Ap_3A and Ap_4A and have suggested that these molecules may be responsible for mediating the heat shock response. Ap_3A also accumulates in yeast cells after heat shock (Denisenko, 1984).

We have previously determined the levels of Ap_3A and Ap_4A during pre-emergence and early larval development of *Artemia* (McLennan and Prescott, 1984 ; McLennan, unpubl.). Here we have examined their levels in 2 h cysts and 48 h larvae, prepared from freshly decapsulated cysts, after various times at 40 °C (Fig. 10). After 20 min, the optimal time, Ap_3A was found to have increased by 76 % and 80 % in 2 h cysts and 48 h larvae respectively, while Ap_4A increased by

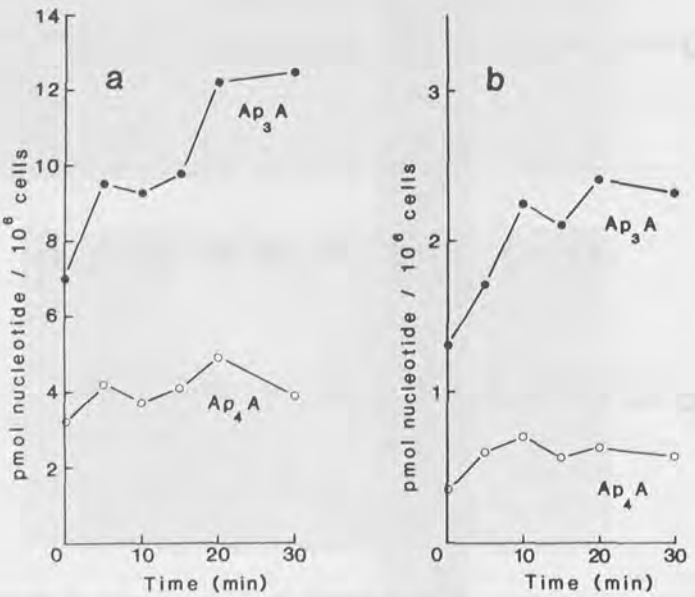

FIG. 10. Levels of Ap_3A and Ap_4A in heat-shocked cysts and larvae. The experimental procedure for the measurement of these nucleotides has been described in detail (McLennan and Prescott, 1984). Briefly 1 g samples of (a) 2 h cysts and (b) 48 h larvae were incubated at 40 °C in 50 ml seawater for different times then rapidly homogenised in TCA. After neutralisation, samples were chromatographed on a RP-18 hplc column to separate Ap_3A and Ap_4A from each other and from nucleotides which would interfere with the highly sensitive luciferase assays employed.

53 % and 65 % respectively. These changes are to be contrasted with the expected 5 % increase in 2 h cysts due to normal development for 20 min at 28 °C and the expected negligible change in 48 h larvae at 28 °C. The slightly greater increases in Ap_3A compared to Ap_4A probably reflect corresponding changes in the ADP and ATP pools, the respective substrates, after heat shock (data not shown). Hence small but significant increases in both Ap_3A and Ap_4A levels are produced in cysts and larvae by a heat shock at 40 °C with no preferential change in either nucleotide. These changes appear lower than the increases from undetectable levels observed in *E. coli, S. typhimurium* and yeast cells, and it is not yet possible to say whether they simply reflect fortuitous, temperature-dependent changes in the relative rates of synthesis and degradation or whether they are specific, heat-triggered components of the heat-shock response.

Discussion

The magnitude of temperature shift from the normal or optimal growth temperature of a cell to that required for the induction of HSPs and thermotolerance and for cell killing varies widely from cell type to cell type. It probably reflects the temperature range which an organism is likely to encounter in its natural environment and tends to be much greater for poikilotherms than for homoiotherms. In this respect, the temperature response of 24 h *Artemia* larvae is very similar to that of *Drosophila* where a jump of 14 °C is required to produce maximal expression of HSP70 and suppression of normal protein synthesis (Ashburner and Bonner, 1979 ; Lindquist, 1980). Mammalian cells on the other hand usually respond to much smaller temperature shifts.

In other respects too the behaviour of 24 h *Artemia* larvae is typical of many other biological systems : 1) a high degree of thermotolerance such as can be induced in 24 h larvae by an initial heat shock has also been observed in yeast (McAllister and Finkelstein, 1980) and *U. maydis* (Taylor and Holliday, 1984) ; 2) virtually every species examined so far reponds to heat shock by inducing the synthesis of proteins similar in size to HSP68 and HSP89 (Schlesinger *et al.*, 1982a) ; 3) HSP89 is generally obtained in a readily soluble form indicating a cytoplasmic location whereas HSP70 is found distributed between the soluble fraction, the nucleus, and other sedimentable structures (Fig. 3 ; Schlesinger *et al.*, 1982a ; Pelham, 1984) ; 4) the time and temperature-dependent sequence of changes in gene expression (appearance of HSP89 followed by appearance of HSP70 followed by suppression of normal protein synthesis) has previously been observed in chick fibroblasts (Kelley and Schlesinger, 1978), *Drosophila* (Lindquist, 1980) and HeLa cells (Burdon *et al.*, 1982). We have yet to determine whether these changes are under transcriptional or translational control in *Artemia* and experiments to measure HSP mRNA content are currently under way.

Although the data obtained with cysts is still preliminary, it is quite evident that they exhibit a high degree of thermotolerance relative to unadapted larvae, that optimal induction of HSPs requires a higher temperature than in larvae and that, at least at early stages of redevelopment, HSPs are synthesised in the absence of heat shock to a much greater degree than in larvae. It will be of interest to determine whether cysts are maximally thermotolerant in their normal state or if a level of super-thermotolerance can be attained by some form of heat treatment.

Although more direct quantitation is required by immunoprecipitation or from 2-D gels, it appears that HSP68 at least is a prominent constitutive component of cysts. A major polypeptide of 68 kD is also consistently seen on poyacrylamide gels stained with Coomassie blue. There are several possible reasons for its presence : 1) members of the HSP70 family of proteins are normal

components of all cells and may increase in content as part of the normal developmental programme (Bienz, 1985) ; 2) HSP70 is induced to high levels in cells entering the quiescent G_0 phase of the cell cycle and may be involved in the maintenance of G_0 (Iida and Yahara, 1984) ; the dormancy of *Artemia* cysts may in some respects be analogous to G_0 and therefore require the presence of HSP68 ; 3) HSP68 may accumulate prior to encystment to afford protection against thermal and desiccation damage to the cyst in its natural environment where it may be subjected to repeated cycles of hydration to levels which are high enough to allow molecular damage but too low to permit transcription and translation *de novo*.

Precisely what form of protection HSP70-like proteins give to the cell is not certain although Pelham (1984) has shown that it accelerates the recovery of nucleolar morphology after heat shock and may catalyse the reassembly of thermally disrupted pre-ribosomes and other RNP particles. Such an activity could be of particular importance to an organism like *Artemia* which contains a large store of latent mRNP particles (Lake, 1984). The apparent reduction in the constitutive level of HSP68 during development would be expected from any of the above possibilities.

So far we have been unable to detect the synthesis of any HSPs of low molecular weight in either cysts or larvae. Such a situation has also been noted with *Xenopus* oocytes (Bienz, 1985), chick myotubes (Atkinson, 1981) and reticulocytes (Morimoto and Fodor, 1984), and some human cell lines (Voellmy *et al.*, 1983) and so is not without precedent. On the other hand our failure to see such proteins may be due to their induction at low levels at temperatures where normal protein synthesis is unaffected or to the quenching of the autoradiograms by the unusually large quantities of proteins in the 20-30 kD range which are present in *Artemia*. Further work with 2-D gels should resolve this problem.

One of these low molecular weight *Artemia* proteins deserves particular mention in the context of stress tolerance. This protein has been studied extensively by De Herdt *et al.* (1980) and by Slobin (1980) who has termed it 'artemin'. Artemin is a 27 kD RNA-binding protein which aggregates to form the abundant 19S complexes which are found in the cyst cytoplasm. It is cyst-specific, forming 13 % of the total protein of post-ribosomal supernatants from cysts but disappearing rapidly after hatching. For this reason it has been assigned a tentative role in the maintenance of the integrity of the dry cyst, possibly as a cytoskeletal component (De Herdt *et al.*, 1980). Some of the low molecular weight HSPs, like chick HSP24, may also be cytoskeletal components (Kelley and Schlesinger, 1982), therefore the possibility exists that artemin may belong to the class of small HSPs, accumulating during encystment to protect the infrastructure of the cells but not synthesised thereafter. A role for the small HSPs in conferring thermotolerance has been proposed (Berger and Woodward, 1983). These proteins share considerable sequence homology with lens α-crystallins (Ingolia and Craig, 1982 ; Wistow, 1985). Like artemin, both types of protein have the capacity to form aggregates of high molecular weight, particularly, as is the case with α-crystallins, in a relatively dehydrated environment. The abundance of artemin should allow its amino acid and gene sequence to be determined without difficulty and so allow this possible relationship to the small HSPs to be investigated.

The possible role of adenylated bis(5′-nucleosidyl) oligophosphates in modulating stress protection deserves further investigation. Ap_4A, Ap_3A, Ap_3G, Ap_4G, and Ap_3Gp_2 have all been shown to respond rapidly to those agents which elicit the heat shock response in bacteria (Lee *et al.*, 1983). In addition to containing Ap_3A and Ap_4A, *Artemia* is virtually unique in its high content of guanine-containing bis(5′-nucleosidyl) oligophosphates for which various roles have

been suggested (Warner, 1980). A more detailed investigation of the relationship between the high stress tolerance of *Artemia* cysts and the possession of an unusually wide range of these nucleotides should prove fruitful in the future.

Conclusions

Growing interest in the molecular biology of the stress response in a wide variety of organisms has given *Artemia* a renewed position of importance both as a model system in which to study the functions of the 'classical' heat shock proteins and as a unique system in its own right. Factors identified as being involved in the protection of the cyst from its environment now include polyols, an abundant low molecular weight protein and two high molecular weight heat-inducible proteins. Further studies will undoubtedly add to this list and reveal the precise functions of these factors.

Acknowledgements

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Studies on elongation factor 1 and ribosomes of *Artemia*

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Abstract

The protein and gene structure of elongation factor 1 (EF-1), a regulatory protein in protein synthesis, is discussed for *Artemia* and attention is paid to its role during development.

There are stage-specific changes in the aggregation behaviour of this factor during the transition from cyst to nauplius. Deaggregation seems a prerequisite for the revival of the protein synthetic apparatus out of the dormant stage in the cyst.

The two parts of elongation factor 1, EF-1α and EF-1βγ have been thoroughly purified and characterized with respect to individual properties of aminoacyl tRNA binding to the ribosome and capacity to exchange guanine nucleotides. EF-1α binds aminoacyl tRNA to the ribosome. EF-1β catalyzes the actual exchange of GDP for GTP on EF-1α while EF-1γ possesses a strong tendency to aggregate specificially with EF-1β and in isolation to associate with itself especially in non-detergent solvents. The function of EF-1γ probably is connected to this tendency to react with detergents and other proteins.

mRNA levels of EF-1α and ribosomal proteins eL12 and eL12' increase coordinately alredy after 4 h of development of the cyst whereas transcription of histone genes (Bagshaw and Pacey, 1979) and hemoglobin (Moens, 1987) takes place much later. This behaviour suggests that the transcription of factors and ribosomal proteins is coordinated via a mechanism involving recognition of common elements within this group of genes.

Sequence studies on EF-1α show a large homology between EF-1α's from different species and a clear similarity to the sequence of bacterial EF-Tu. Comparison with the deduced sequence of bacterial EF-Ts with that of EF-1β sofar does not reveal convincing similarities, consistent with a less demanding cellular function of EF-1β or EF-Ts compared to EF-1α or EF-Tu.

Introduction

For most „Artemiologists" studies on elongation factor 1 (EF-1) may sound fairly specialistic. It is spectacular that after a number of hours in seawater, the shell of the encysted embryo bursts and a pear-shaped embryo becomes visible ; shortly thereafter it crawls out of the membrane and starts life as a free-swimming nauplius (Sorgeloos, 1980). However, it is during this period of morphological changes, that the embryo resumes protein synthesis without any need for cell division or DNA synthesis (Clegg and Conte, 1980 ; Hultin and Nilsson, 1980). Elongation factor 1 is an important protein in this process because it attaches aminoacyl tRNA to the ribosomes (Miller and Weissbach, 1977) and in this sense participates in the control of protein

synthesis. In particular there is a remarkable change in the size of active EF-1 during embryonic development (Slobin and Möller, 1975). This change occurs during a period of redistribution of mono- and dinucleoside compounds (Finamore and Clegg, 1969 ; Warner, 1980) which may effect the rate of guanine nucleotide binding and recycling of EF-1. The stage-specific change in EF-1 on resumption of protein synthesis thus offers a good opportunity to study a regulatory process of chain elongation.

This review summarizes the present knowledge on the structure and function of elongation factor 1 of *Artemia* and its enzymatic activity during early development. Secondly, a study is made of the structure of a gene of EF-1α, the actual tRNA binding protein, and the way its transcription is regulated, during different stages of development. The problem is discussed as to how RNA- sequences coding for EF-1α and certain components of the protein synthetic machinery are turned on after a period of deep dormancy in the dehydrated cysts. Since recombinant DNA technology permitted us to identify and characterize several genes for *Artemia* EF-1α (Van Hemert *et al.*, 1984 ; Lenstra *et al.*, 1986) together with that for two other ribosomal proteins, eL12 and eL12' (Maassen *et al.*, 1985), we have explored possible signals for the coordinate expression of genes for EF-1α and these ribosomal proteins during embryogenesis of *Artemia*. It is pertinent to mention here that stage-specific gene expression of histones and hemoglobin during early development of *Artemia* has been reported (Bagshaw and Acey, 1979).

Excellent reviews and papers on the molecular biology of *Artemia* with emphasis on messenger RNA expression and ribosome function during development can be found in the first comprehensive treatise on *Artemia* (Persoone *et al.*, 1980).

Irrespective of the limitations of *Artemia* for in vivo studies of precursors of protein and nucleic acids due to the impermeability of the cyst towards radioactive compounds, *Artemia* embryos offer great potential as an organism to study the gene regulation of components involved in ribosome structure and function. The molecular genetics of *Artemia* are less well-defined compared to unicellular organisms such as yeast ; genetic polymorphism may be another disadvantage. Compared to other invertebrates, however, the relative ease with which large amounts of pure proteins can be obtained, makes *Artemia* an attractive research object for everyone interested in the basic mechanisms of protein synthesis and development.

Change in elongation factor form during the development of *Artemia*

Two forms of elongation factor 1 can be distinguished in *Artemia* : a heavy heterogeneous form EF-1_H (M_r about 250 000 and 500 000) and a light form, EF-1_L. EF-1_H is composed of three different peptides, α, β, and γ (M_r 51 000, 26 000, and 46 000) while EF-1_L is formed by only one, which corresponds to peptide α of EF-1_H. EF-1_H occurs as oligomers of a 1:1:1 complex of EF-1α, EF-1β, and EF-1γ or EF-1αβγ. As indicated, on development the relative amount of the heavy form of EF-1 with respect to EF-1_L decreases and in the free-swimming nauplii the light form EF-1_L (or EF-1α) has become dominant (Slobin and Möller, 1975 ; Slobin 1980). *Artemia* EF-1α binds aminoacyl tRNA to the ribosome under hydrolysis of GTP, while EF-1βγ is a guanine nucleotide exchange enzyme, acting on EF-1α. GDP under formation of EF-1α.GTP from free GTP. As in pig liver EF-1βγ, the actual nucleotide exchange capacity of *Artemia* EF-1βγ resides in the β subunit, the function of EF-1γ being unknown (Iwasaki *et al.*, 1976 ; Slobin and Möller, 1976). Characterization of the other elongation factor in protein synthesis, EF-2 in dormant cysts and developing embryos of *Artemia*, shows that during early

development the amount of EF-2 in the particulate fraction decreases markedly concomitantly with an increase in the amount of soluble EF-2. In the case of EF-2 proteases have been postulated to be important (Yablonka-Reuveni and Warner, 1979). In our opinion, changes in guanine nucleotide levels and preferential synthesis or breakdown of one of the components of EF-1 may be also important during this period. Perhaps most intriguing is that the observed changes in the form of EF-1 occur during a period in which protein synthesis starts again. A good correlation between the level of protein synthesis and the degree of EF-1_H to EF-1_L conversion has been reported in the development of wheat-seeds (Sacchi *et al.*, 1984). Specific changes in the amounts of the two forms of EF-1 during the recovery from the dormant state to an active state of protein synthesis, are therefore indicated in two different cryptobiotic systems. This strengthens the view that the change in the observed size distribution of at least EF-1 is related to the resumption of protein synthesis (Fig. 1). Furthermore, the striking correspondence in subunit M_r's between *Artemia* EF-1_H and wheat embryo EF-1_H (Bollini *et al.*, 1974) – both contain three different polypeptides – support our suggestion that EF-1_H would be a storage form of EF-1α with EF-1βγ helping to stabilize EF-1_L against gradual inactivation during periods of biological dormancy (Slobin and Möller, 1975). To what extent the observed excess of EF-1_L to EF-1_H in prenauplii is due to differential EF-1α synthesis, EF-1_H dissociation or other factors

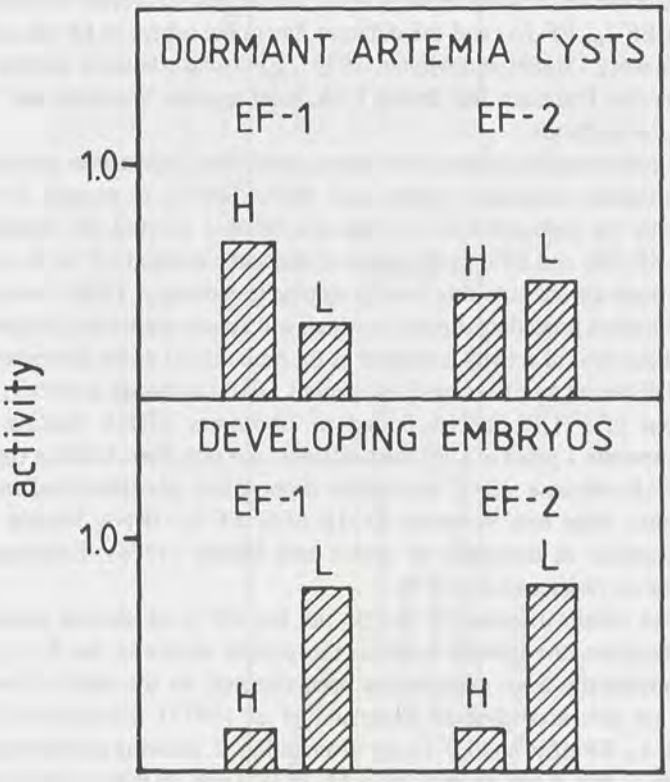

FIG. 1. Influence of development on the apparent amount of heavy and light form of elongation factor 1 and 2 espectively (Slobin and Möller, 1975 ; Yablonka-Reuveni and Warner, 1979).

will be discussed in a separate paper (Maessen *et al.*, in prep.). Because in pig-liver a M_r 30 000 protein possesses structural and functional properties strongly resembling bacterial EF-Ts (Iwasaki *et al.*, 1976 ; Nagata *et al.*, 1976) it was tried whether heat-treatment of EF-1_H may inactivate EF-1α and release selectively the guanine nucleotide exchange activity assigned to EF-1β of M_r 26 000 (Slobin and Möller, 1977). The results showed that *Artemia* EF-1_H indeed consists of an EF-1α. EF-1$\beta\gamma$ complex which is functionally analogous to bacterial EF-Tu. EF-Ts and thus protects the labile EF-1α molecule against inactivation in the cyst. We think that the heat-induced release of EF-1β under conditions known to inactivate EF-1α from *Artemia* EF-1_H was significant for understanding the function of EF-1_H (Slobin and Möller, 1977). It also may explain part of the inability of several laboratories to detect in the beginning EF-1β activity in their EF-1_H preparations (Legocki *et al.*, 1974 ; Grasmuk *et al.*, 1976 ; Kemper *et al.*, 1976) ; recently the important role of temperature in the dissociation of EF-1_H into subunits in the presence of GTP has been documented more fully for pig-liver (Hattori and Iwasaki, 1983) and *Artemia* (Janssen and Möller, 1988a).

Properties and Purification of elongation factor 1 of *Artemia*

Here we confine ourselves to some remarks about the method to prepare *Artemia* EF-1$\beta\gamma$. For the preparation of EF-1_H, EF-1α, and the different functional assays of EF-1α and EF-1$\beta\gamma$ we refer to our earlier work (Slobin and Möller, 1979). EF-1 and ribosome studies used *Artemia* cysts, Metaframe or San Francisco Bay Brand, USA, local supplier Voorwald and Van Nikkelen, Kuyper, Haarlem, Netherlands.

The last 5 years considerable advances have been made in the purification and characterization of EF-1$\beta\gamma$. The original procedure (Slobin and Möller, 1978) to prepare EF-1β has been improved leading to the development of a fast and reliable method for obtaining milligram quantities of pure EF-1$\beta\gamma$ and EF-1β. By means of the same method EF-1γ is also obtained in pure form, but in aqueous solvents this protein aggregates strongly. Table I contains a scheme of the present purification procedure, details of which will appear elsewhere (Janssen and Möller, 1988b). In Table I one unit of activity is defined as the amount : a) which promotes the exchange of 1 pmol [^{3}H]GDP bound to EF-1α with exogenous GDP (exchange activity) ; b) required to double the amount of [^{3}H]Phe-tRNA bound to ribosomes (tRNA binding stimulation) ; c) required to incorporate 1 pmol of [^{3}H]phenylalanine into polyphenylalanine (polyphe-synthesis). After the DEAE-cellulose step 2, nucleoside diphosphate phosphotransferase activity was no longer detectable. Step four separates EF-1β from EF-1γ. tRNA binding and polyphe-synthesis were measured as described by Slobin and Möller (1976). Exchange activity was measured according to Nagata *et al.*, 1976.

Since methods to obtain *Artemia* EF-1$\beta\gamma$ do not use EF-1_H as starting material but rather 100 000 × *g* supernatants, the identity between the protein chains in the EF-1_H complex and those prepared individually from extracts has been checked on the basis of one-dimensional peptide mapping on gels according to Cleveland *et al.* (1977). Examples of partially pure preparations of EF-1_H, EF-1$\beta\gamma$, and EF-1α are given in Fig. 2, showing artemin as a contaminant of EF-1_H. In a similar way it was shown that a M_r 28 000 and 30 000 doublet seen sometimes in eEF-Ts type preparations originates from proteins structurally unrelated to EF-1β (Roobol *et al.*, 1980). Peptide mapping according to Cleveland *et al.* (1977) appeared highly useful because

TABLE I

Purification of EF-1βγ from *Artemia* cysts (850 g)

Step	Volume (ml)	Total protein (mg)	Specific activity (units/μg)		
			Exchange	tRNA binding	Polyphe-synthesis
1. 35-50 % ammonium sulfate saturation	500	6800	1.7	nd[1]	nd
2. DEAE-cellulose	88	55	26	12	32
3. Phenyl-Sepharose	41	2.9	49	18	57
4. DEAE-cellulose (6M ureum)	39	0.59	120	61	150

[1] nd : not determined.

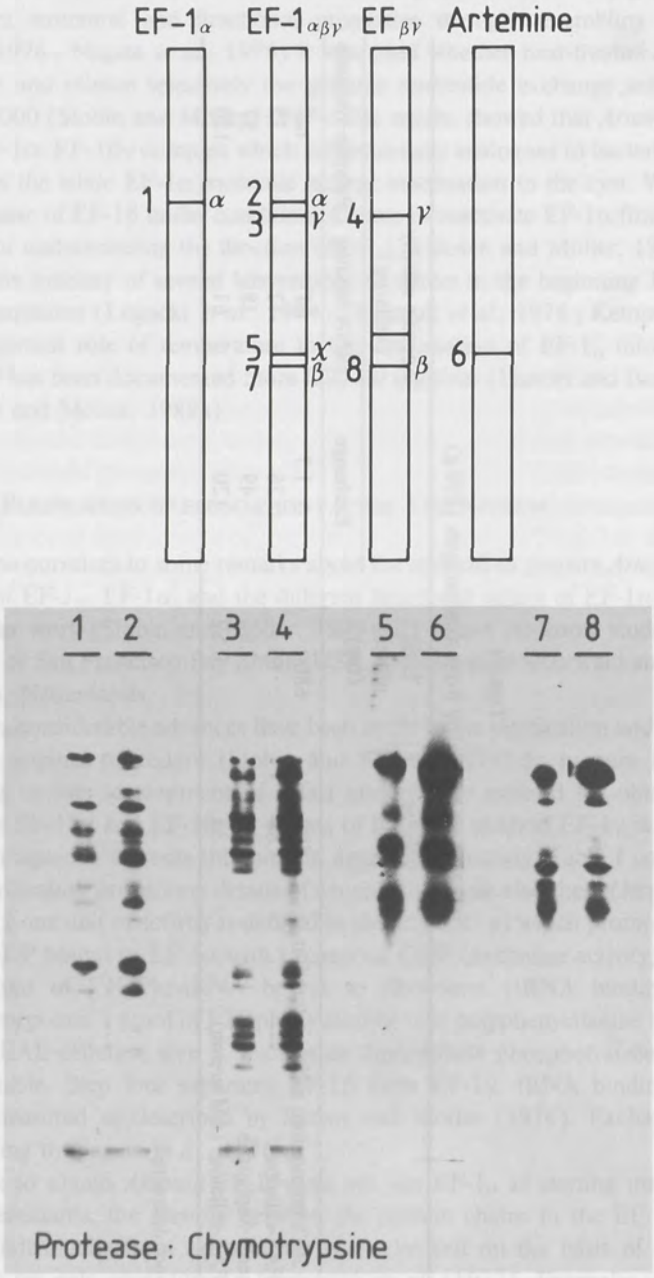

FIG. 2. Peptide maps of isolated EF-1 proteins. Individual proteins were isolated on a SDS-polyacrylamide gel (top). Peptide maps were produced from individual bands using chymotrypsin as described by Cleveland *et al.* (1977) (bottom).

contaminants like artemin, tubulin, and nucleoside diphosphate (NDP) kinase hindered previous efforts to routinely obtain a high degree of purity of EF-1βγ.

Table II summarizes several structural properties of EF-1 among which the molecular weight of the subunits and the aggregation behaviour of the protein.

TABLE II

Elongation factor 1 from *Artemia*

Property	EF-1αβγ	EF-1α	EF-1βγ	EF-1β	EF-1γ
Molecular weight of subunits	α 51 000 β 26 000 γ 46 000	α 51 000	β 26 000 γ 46 000	β 26 000	γ 46 000
Aggregate Molecular weight	500 000		250 000 (in 1 % cholate)		Aggregate
EF-Tu like activity	+	+	-	-	-
EF-Ts like activity	+ (heat induced)	-	+	+	-
GTP binding	+	+	-	-	-
Isolation[1]	Hydroxy-apatite	CM-sephadex	DEAE-cellulose	β-γ Separation on DEAE cellulose + 6 M urea	

[1] See Slobin and Möller (1979).

On Sephacryl S300 an aggregate of EF-1αβγ elutes just behind artemin, having a M_r of 600 000 in the analytical ultracentrifuge. Artemin is a major protein in the cyst (Slobin, 1980) and a frequent contaminant of EF-1_H. The subunit M_r of artemin is 27 000 and contrary to earlier suggestions (Slobin, 1980) there is no reason to invoke an immunological relationship between EF-1β and artemin. Unrelatedness of the 19S crypto-specific M_r 27 000 protein of *Artemia* to EF-1β (De Herdt *et al.*, 1980) has also been found by Kondo *et al.* (pers. commun.). This 19S protein is identical to artemin according to S-value, M_r, aminoacid composition, immunological cross-reaction, and regular electron microscopic structure (De Herdt *et al.*, 1080 ; and our own unpublished work).

NUCLEOTIDE EXCHANGE PROPERTIES OF EF-1βγ

The enzymatic interaction of elongation factor EF-1α.GDP and elongation factor EF-1βγ from *Artemia* has been investigated kinetically according to Chau *et al.* 1981. Without EF-1βγ, the release reaction of [^{3}H]GDP bound to EF-1α can be described by a single exponential decay of radioactivity of EF-1α.[^{3}H]GDP left on the filter. When the amount of unlabeled GDP is in large excess, a release constant of 2×10^{-3}/s is found at 21 °C. In the presence of enzymatic amounts of EF-1βγ the loss of [^{3}H]GDP from *Artemia* EF-1α[^{3}H]GDP proceeds at a higher rate suggesting an increased dissociation of the labeled GDP due to the formation of a transient ternary complex of EF-1α.[^{3}H]GDP.EF-1βγ. For instance, at an initial ratio of 1:100 of EF-1βγ and EF-1α.[^{3}H]GDP, the rate of release of [^{3}H]GDP can be described by a release constant $k = 2 \times 10^{-2}$, the decay being exponential in the beginning. This constant is ten times larger than the release rate constant without EF-1βγ. Although it appears that EF-Ts from *E. coli* enhances

the catalytic process of [^{3}H]GDP release from EF-Tu.[^{3}H]GDP more effectively than EF-1βγ does in the presence of EF-1α.[^{3}H]GDP, the results leave no doubt that EF-1βγ is enzymatically active in releasing bound GDP from EF-1α (see Table III). Furthermore, *Artemia* and pig-liver EF-1βγ catalyze the release of [^{3}H]GDP from *Artemia* and pig-liver EF-1α at almost the same rate under comparable conditions. Rate constants of pig-liver EF-1βγ were calculated from Fig. 1 given by Nagata *et al.*, 1976. A summary of the different release rate constants of *Artemia* and pig liver EF-1βγ and the comparison with the *E. coli* system are given in Table III. Conditions for *Artemia*: 0.02 M Tris HCl pH 7.5 ; 0.01 M magnesium acetate ; 0.05 m NH_4Cl ; 10 % v/v glycerol ; 140 μM GDP ; 0.3 μM EF-1α [^{3}H]GDP and 0.03 μM EF-1βγ. For pig-liver : 0.02 M Tris HCl pH 7.5 ; 0.01 M magnesium acetate ; 0.05 M NH_4Cl ; 10 % v/v glycerol ; 150 μM GTP ; 0.4 μM EF-1α [^{3}H]GDP and 0.008 μM EF-1βγ. For *E. coli*: 0.02 M Potassium phosphate pH 6.8 0.1 M NH_4Cl ; 0.01 M $MgCl_2$; 0.001 M dithiothreitol ; 50 μM GDP ; 0.01 μM EF-Tu.[^{3}H].GDP ; 0.0005 μM EF-Ts.

TABLE III

Rate constants k for enzymatic loss of [^{3}H]GDP from EF-1a [^{3}H]GDP under influence of EF-1βγ

Condition	k		
	Artemia	*E. coli* [1]	Pig-liver [2]
No EF-1βγ	2×10^{-3}/s (21 °C)	2×10^{-3}/s (21 °C)	2×10^{-3}/s (0 °C)
EF-1βγ : EF-1α = 1 : 100	2×10^{-2}/s (21 °C)	1×10^{-1}/s (21 °C)	5×10^{-3}/s (0 °C)

[1] Chau *et al.*, (1981).
[2] Nagata *et al.*, (1976).

A full description of the kinetic properties of EF-1βγ based on a procedure first used by Chau *et al.* (1981) for bacterial EF-Ts is in press (Janssen and Möller, 1988a). The results show that EF-1βγ catalyzes the guanine nucleotide exchange on EF-1α so that the recycling rate of the latter becomes consistent with the known elongation rates *in vivo*.

The conservation of the nucleotide exchange-properties of EF-1βγ in pig-liver (Nagata *et al.*, 1976), *Artemia* (Slobin and Möller, 1976), reticulocytes (Slobin, 1979), and chick brain (Murakami and Miyamoto, 1983) give additional weight to the catalytic function of EF-1βγ in the recycling reaction of eukaryotic tRNA binding enzymes.

Previously it was found that certain eukaryotic EF-Ts like preparations from *Artemia* contain a nucleoside diphosphate phosphotransferase (NDP-kinase) activity which could transfer the γ-phosphate of GTP to [^{3}H]GDP (Roobol and Möller, 1981). The suggestion was made that eukaryotic EF-Ts possesses a NDP-kinase activity which may be an essential aspect of both the structure and function of the protein. However, this kinase activity has a high enzymatic rate and is ubiquitous so that it is frequentlty encountered as a contaminant in the purification of nucleotide metabolizing enzymes (Parks and Agarwal, 1973). Therefore a study has been made to determine whether the kinase-like activity results from either trace contamination, a protein with chemical and physical properties very similar to EF-1βγ or a protein which interacts with eukaryotic eEF-Ts (EF-1βγ). The final results showed that on DEAE-Sephadex chromatography

the NDP-kinase activity can be fully resolved from the nucleotide exchange activity and from the stimulatory activity of EF-1α dependent Phe-tRNA binding (Janssen and Möller, 1988b). Furthermore, both the two latter activities reside in an EF-1βγ complex, having on its own no detectable NDP-kinase activity like pure EF-1_H. This refutes the idea of EF-1βγ having intrinsic NDP-kinase activity and agrees with the results of pig-liver EF-1βγ being solely a nucleotide exchange enzyme (Iwasaki *et al.*, 1976). A similar conclusion has been drawn recently for the bacterial elongation factor Ts and a nucleoside diphosphate kinase activity (Eccleston, 1984). The possibility that NDP-kinase activity associates specifically with EF-1βγ cannot be excluded at the present time. It may explain the observation that NDP-kinase activity from *Artemia* appears in an EF-1_H form upon addition of partially purified EF-1βγ preparations to EF-1α (Roobol and Möller, 1981). In this respect the situation looks analogously to microtubules containing a NDP kinase, which is associated to tubulin. Here, however, the NDP kinase is believed to act directly on tubulin GDP (Jacobs and Huitorel, 1979). Interestingly EF-1βγ preparations often display a protein kinase activity with EF-1β being phosphorylated at a specific serine residue. The possible significance of this phosphorylation for the function of EF-1β is under study.

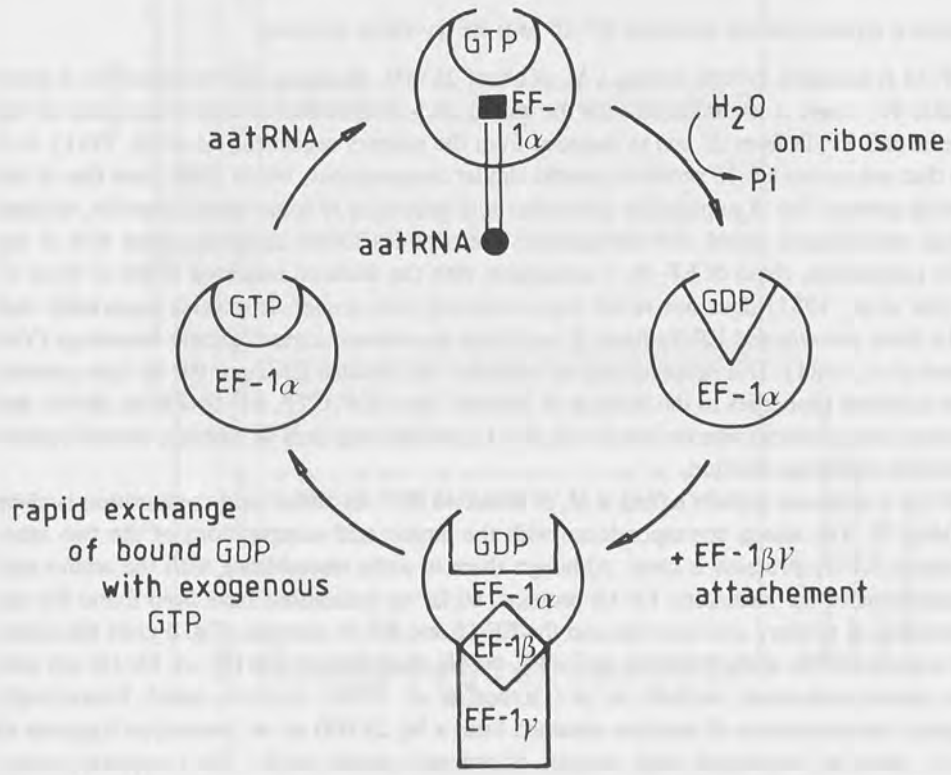

FIG. 3. Recycling mechanism of EF-1.

EF-1γ RATHER THAN EF-1β POSSESSES AGGREGATING PROPERTIES

Elongation factor 1 occurs as a high molecular weight aggregate ranging in molecular weight of about 50 000 to 500 000. A heavy form of EF-1 composed of three non-identical subunits EF-1αβγ has been isolated from wheat germ (Bollini *et al.*, 1974), *Artemia* (Slobin and Möller, 1976), pig-liver (Hattori and Iwasaki, 1983), silk worm (Ejiri *et al.*, 1977), and rabbit reticulocytes (Moretti *et al.*, 1979). *Artemia* EF1α, being functionally equivalent to EF-Tu, does not aggregate in solution whereas EF-1βγ does. In fact, even in 1% cholate solutions, EF-1βγ elutes from Sephacryl S300 columns as an aggregate having an apparent M_r of about 250 000. This aggregation behaviour seems to be due to the γ-chain as judged from the fact that isolated EF-1γ in contrast to EF-1β, aggregates strongly and partitions into the detergent phase in Triton X-114 solutions (Janssen and Möller, 1988b). Presently the hydrophobic properties of EF-1γ are studied in relation to its possible function and sequence, which shows an unusual high frequency of aromatic aminoacids (see section sequence studies on the proteins EF-1β and EF-1γ from *Artemia*). It has been found that EF-1γ interacts specifically with tubulin thereby supplying a possible anchor of parts of the protein synthetic machinery to the cytoskeleton and other structures of the cell (Janssen and Möller, 1988b).

SEQUENCE STUDIES ON THE PROTEINS EF-1β AND EF-1γ FROM *ARTEMIA*

EF-1β is an acidic protein having a M_r of about 26 000. Its amino acid composition is given in Table IV, where it is compared with the amino acid composition of other eukaryotic EF-1β proteins and EF-Ts from *E. coli* as deduced from the primary sequence (An *et al.*, 1981). It is seen that eukaryotic EF-1β proteins possess similar compositions, which differ from that of the bacterial protein. Fig. 4 summarizes the amino acid sequences of some tryptic peptides, isolated by high performance liquid chromatography. The peptides shown comprise about 40% of the whole polypeptide chain of EF-1β. Comparison with the deduced sequence of EF-Ts from *E. coli* (An *et al.*, 1981) does not reveal any convincing homologies. It remains remarkable that EF-1α from *Artemia* and EF-Tu from *E. coli* show on average a much greater homology (Van Hemert *et al.*, 1984). This property may be related to the fact that EF-Tu or EF-1α type proteins share common properties in the binding of 'ligands' like GDP/GTP, EF-1β/EF-Ts, tRNA, and ribosomal components, whereas the EF-1β/EF-Ts proteins may only be mutually related by their nucleotide exchange reaction.

EF-1γ is a neutral protein having a M_r of about 46 000. Its amino acid composition is given in Table IV. The strong correspondence with the amino acid compositions of the two other eukaryotic EF-1γ proteins is clear. Although there is some resemblance with the amino acid compositions of the eukaryotic EF-1β proteins, so far no indications have been found for any relationship in primary structure between the EF-1β and EF-1γ proteins. Fig. 4 gives the amino acid sequence of the amino terminal part of EF-1γ. No resemblance with EF-1α, EF-1β, nor with other protein sequences, available to us (Dayhoff *et al.*, 1978), could be noted. Interestingly, sequence determinations of peptides obtained from a M_r 24 000 m. w. proteolytic fragment of EF-1γ, show an impressive high content of aromatic amino acids. The complete primary structure of both EF-1β and EF-1γ has been published recently (Maessen *et al.*, 1986, 1987).

TABLE IV

Amino acid compositions of the proteins EF-1β/EF-Ts and EF-1γ

Amino acid	EF-Ts/EF-1β					EF-1γ		
	E. coli [1]	Chick brain [2]	Pig liver [3]	Silk gland [4]	*Artemia*	Chick brain [2]	Pig liver [3]	*Artemia*
AsX	7.8	12.1	12.6	13.6	13.8	9.5	9.4	10.7
Thr	3.9	4.7	2.7	4.8	2.2	4.4	5.0	2.7
Ser	3.6	6.5	6.2	6.2	5.8	7.8	7.0	8.2
Glx	14.9	18.1	15.7	15.4	15.7	15.2	16.1	12.4
Pro	1.8	3.6	4.9	5.3	4.5	5.0	5.0	2.5
Gly	8.5	6.5	7.2	6.1	4.8	7.8	7.1	8.7
Ala	13.8	8.4	9.0	9.9	9.7	8.5	9.3	8.8
Cys	0.7	n.d.	0.9	trace	n.d.	n.d.	1.2	n.d.
Val	10.3	5.9	7.4	7.4	6.7	6.0	6.3	5.3
Met	3.2	1.8	1.4	1.1	2.8	1.5	1.3	3.1
Ile	6.7	3.3	3.7	4.1	5.8	3.4	2.8	5.0
Leu	5.7	8.7	7.6	8.3	7.6	8.9	7.1	6.7
Tyr	1.1	3.1	4.0	3.1	2.5	2.8	2.6	4.6
Phe	3.5	3.4	2.3	2.3	3.9	5.7	6.0	5.7
Lys	9.9	9.0	9.0	8.8	11.5	6.7	6.6	9.7
His	1.4	1.1	1.0	0.6	0.9	2.1	1.6	0.6
Arg	3.2	3.8	2.8	3.3	1.9	4.6	4.1	4.4
Trp	0	nd	1.6	nd	nd	nd	1.5	nd

[1] An *et al.* (1981).
[2] Murakami and Miyamoto (1983).
[3] Motoyoshi and Iwasaki (1977).
[4] Ejiri *et al.* (1977).
[5] nd = not determined.

Tryptic peptides from elongation factor EF-1β from *Artemia*

Ser-Tyr-Leu-Gln-Gly-Tyr-Glu-Pro-Ser-Gln-Glu-Asp-
Val-Ala-Ala-Phe-Asn-Gln-Leu-Asn-Lys

Ala-Pro-Ser-Asp-Lys-Phe-Pro-Tyr-

Gly-Gln-Glu-Gln-Leu-Asn-Glu-Leu-Leu-Ala-Lys

Val-Gln-Met-Asp-Gly-Leu-Val-Trp-Gly-Ala-Ala-Lys

Ile-Ser-Glu-Phe-Glu-Asp-Phe-Val-Gln-Ser-Val-Asp-
Ile-Ala-Ala-Phe-Glu-Lys

Ser-Ser-Met-Ile-Leu-Asp-Ile-Lys-Pro-Trp-Asp-Asp-
Glu-Thr-Asp-Met-Ala-Glu-Met-Glu-Lys

Gly-Phe-Pro-Gly-Ile-Pro-Thr-Asn-Ala-Ala- x -Glu-

Aminoterminal sequence of elongation factor EF-1γ from *Artemia*

Val-Ala-Gly-Lys-Leu-Tyr-Thr-Tyr-Pro-Glu-Asn-Phe-
Ala-Ala-Phe-Ala-Ala-Leu-Ile-Ala-Ala-Glx-Tyr-Gly-
Gly-Ala

FIG. 4. Partial sequences of EF-1β and EF-1γ from *Artemia*.

mRNA LEVELS OF EF-1α AND RIBOSOMAL PROTEIN eL12 DURING EMBRYOGENESIS

As mentioned in the introduction, the brine shrimp *Artemia* shows the unique property of a development within 24 h from a dormant gastrula (cyst) to a free-swimming nauplius when incubated under the appropriate conditions. In addition, during the first 16 h of development the cells differentiate without appreciable cell division. These properties make *Artemia* well suited for a study of genes related to the ribosomal machinery.

We have chosen to study the genes for the α subunit of elongation factor 1 (EF-1α) and two ribosomal proteins from the large subunit, eL12 and eL12'. The function of EF-1α has been described in the previous section ; eL12 and eL12' are acidic phosphoproteins from the large ribosomal subunit and are structurally related to L7/L12 from *E.coli* (Amons and Möller, 1980). The amino acid sequences of eL12 and eL12' have been determined and the data show that both proteins have an identical carboxy terminus and a completely different amino terminus. Therefore the proteins are considered to form a gene-family (Amons *et al.*, 1982). The proteins are involved in elongation-factor-dependent processes during protein biosynthesis and, because of their phosphorylation, may constitute a regulatory element in the rate of the elongation cycle. We have analyzed the relative levels of the mRNA's of these proteins during embryogenesis of *Artemia* by means of Northern blot analysis. For this purpose, RNA from *Artemia* in different states of

development was isolated and used for preparation of Northern blots. These blots were successively hybridized with labeled cDNA probes for eL12, eL12', and EF-1α. The isolation of these cDNA probes has been published (Van Hemert *et al.*, 1984 ; Maassen *et al.*, 1985). The autoradiographs and the quantitated data are presented in Fig. 5. Autoradiographs (top) of RNA blots after successive hybridization of the filters with [^{32}P]cDNA probes for eL12, eL12', and EF-1. *Artemia* cysts were developed for the time indicated and total RNA was isolated, electrophoresed, and blotted. 100 µg total RNA was applied in each lane. It should be noted that the order of time points on the blot is inverted at 12 h and 16 h. B. Quantified data (bottom) on the relative levels of mRNAs for eL12, eL12', and EF-1 during the development of *Artemia* were obtained by microdensitometric scanning of the autoradiographs shown at the top.

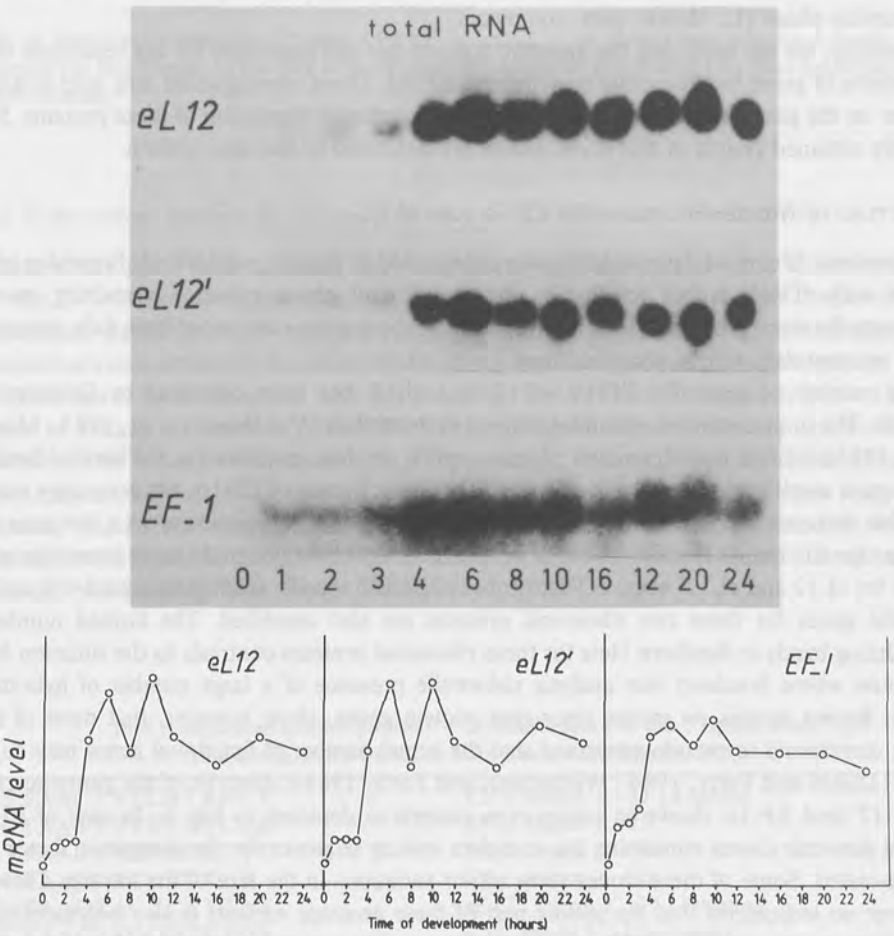

FIG. 5. Relative levels of each mRNA coding for eL12, eL12', and EF-1.

The data show that the dormant cyst contains already mRNA for these proteins, though in low concentrations. After 3 h of development a steep increase takes place in the relative levels of these mRNA's, after which the steep increase levels off. It is remarkable that all three mRNA's show similar variations in their concentrations throughout the developmental process.

In general, the synthesis of ribosomal proteins is coordinated in eukaryotes. Our data show that in *Artemia* also that the gene for EF-1α is expressed in coordination with ribosomal protein genes. Though the possibility exists that the coordinate expression of these genes in *Artemia* reflects a more general resumption of transcriptional activity in the developing cyst, there are two findings that argue against this possibility.

1) The level of polyA$^+$RNA in total cellular RNA during development increases very little from 3.5 % in the dormant cyst to 4.9 % after 24 h of development.

2) The expression of histone genes has been studied during development of *Artemia* and these data show that expression of these genes takes place only in the nauplius stage after 24 h of development (Bagshaw and Acey, 1979); transcription of hemoglobin starts around the prenauplius phase (L. Moens, pers. commun.).

Presently, we are analyzing the genomic regions that are important for the regulation of the expression of genes for ribosomal proteins and EF-1α. These investigations may help to give an answer on the genomic elements that regulate the coordinate expression of these proteins. Some recently obtained results of this investigation are described in the next section.

Structure of the genes coding for EF-1α and eL12'

A genomic library of *Artemia* DNA was constructed in the phage EMBL-3. Screening of this library with cDNA probes resulted in the isolation of phage particles containing genomic fragments for these proteins. Though the analysis of these genes has not yet been fully completed, some relevant data will be discussed here.

The number of genes for EF-1α, eL12, and eL12' has been estimated by Southern blot analysis. The limited number of bands obtained in these blots (Van Hemert *et al.*, 1983; Maassen *et al.*, 1985) indicate a small number of genes coding for these proteins. On the basis of Southern blots, gene amplification may be overlooked. Therefore, in case of EF-1α, the gene copy number was also determined by gene titration. The results from this experiment support a low gene copy number for EF-1α. As the EF-1α bands in Southern blots have about the same intensities as the bands for eL12 and eL12' when cDNA probes of similar specific activities are used it is unlikely that the genes for these two ribosomal proteins are also amplified. The limited number of hybridizing bands in Southern blots for these ribosomal proteins contrasts to the situation found in mouse where Southern blot analysis shows the presence of a large number of hybridizing bands. Recent studies on mouse ribosomal protein genes, show however, that most of these bands correspond to pseudo-genes and that the actual number of functional genes may be very small (Dudov and Perry, 1984; Wiedemann and Perry, 1984). Analysis of the genomic clones for eL12' and EF-1α shows an intron-exon pattern as depicted in Fig. 6. In case of EF-1α, several genomic clones containing the complete coding sequence for the elongation factor have been isolated. Some of these clones show minor variations in the size of the introns. There are however no indications that the coding part of these genomic variants is also polymorphic. In case of the gene coding for eL12', only part of the coding sequences have been found sofar. The genomic clones for this protein analyzed contain two exons coding for amino acid 1 to 81. The

sequence of eL12' contains 110 amino acids. The DNA region coding for the carboxy terminal part of the protein is absent in these genomic clones. As EF-1α show a coordinated expression during the development of *Artemia*, we have characterized these genes in part by DNA sequencing. The sequence data obtained so far show several interesting features.

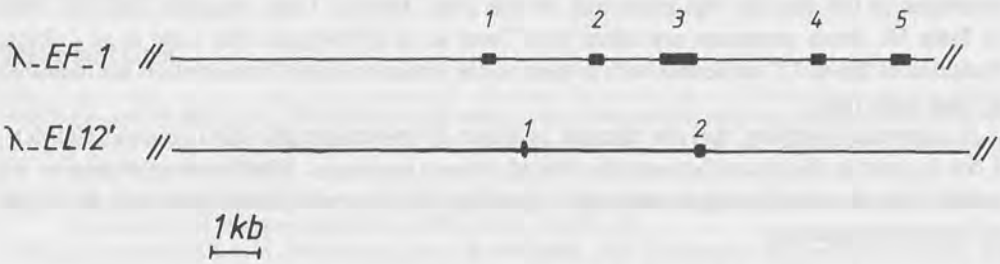

FIG. 6. Intron-exon structure of the genes for EF-1α and eL12' as derived from genomic λ-clones. EF-1α comprises the entire gene. In the case of eL12', only the exons for amino acid 1-81 have been detected yet in the λ clones.

Most of the introns contain the sequence pyrimidineTAACPyrimidine (PyTAACPy)

Table V lists intron sequences near the 3'-end splice junctions in the genomic clones for EF-1α and eL12'. It is seen that the sequence PyTAACPy is present with a high frequency at an approximate position of 20 base-pairs upstream from the splice site. Interestingly, these sequences are also present in the introns of the genes for two ribosomal proteins in mouse. There, each gene has at least one intron with this sequence. The PyTAACPy sequence is not a general feature of eukaryotic introns. A search among published intron sequences shows that the presence of this sequence element around 20 bp upstream from the 3'-splice site has a frequency of occurrence of about 10 %. It would be interesting to see whether this sequence represents a regulatory sequence involved in coordinate processing of primary gene transcripts for these proteins. In this context it is remarkable that the PyTAACPy sequence is found in a region where the actual process of splicing occurs (Ruskin *et al.*, 1984).

TABLE V

Intron sequences around position − 20 with respect to the 3'-splice sites.
Mouse sequences are taken from Dudov and Perry (1984)

GGTTAACTTTGATAGCT	EF-1, Intron 1, -31 (*Artemia*)
TACTAATTTTCCATCAA	EF-1, Intron 2, -17
TTAACCATTACATTT	EF-1, Intron 3, -19
TTCTAACCTCCTCTGT	EF-1, Intron 4, -16
CTCTAACTACTCTTTAT	eL12'. Intron 1, -22 (*Artemia*)
TACTAACTTGGGTCTGA	L32, Intron 1, -22 (mouse)
GACTAACTTGCCTGTGT	L32, Intron 2, -18

Upstream sequences in the Artemia eL12'-gene are also present in yeast ribosomal protein genes

Analysis of yeast ribosomal protein genes has revealed the presence of consensus-like sequences upstream from the transcription initiation site. These sequences are considered to be important for the regulation of gene expression (Teem *et al.*, 1984 ; Van Leer *et al.*, 1985). We have also analyzed this region in eL12' from *Artemia* and the sequence data show similar sequences in the gene for this ribosomal protein from *Artemia.* These sequence data are listed in Table VI. Yeast sequences are taken from Teem *et al.* (1984) and Van Leer *et al.* (1985). Positions of the eL12' sequences with respect to the initiation site of transcription are indicated in base pairs (bp).

It is especially striking that the distance of about 20 bp between the RPG box and HOMOL 1 box in yeast is also found between the related *Artemia* sequences. Whether these sequences are indeed important for the regulation of the expression of ribosomal protein genes will be a topic of future investigations.

TABLE VI

Compilation of upstream "HOMOL" and "RPG" sequences in yeast ribosomal protein genes and the possible counterpart-sequences in *Artemia* eL12' (Maassen *et al.*, 1986)

AT(T/A)TTNCA	HOMOL 4, yeast
ATTTCCA	eL12', -258
GC(TG)CTTC(T/C)T	HOMOL 3, yeast
CTTCC	eL12', -38
CTTCTT	eL12', -245
(T/A)CATCTNTA	HOMOL 2, yeast
TGATCTTTA	eL12', -283
GCATCTCTG	eL12', -47
AACATC(TA/CG)T(G/A)CA	HOMOL 1, yeast ... ← 20bp ... ACCCATACAT RPG, yeast
AACAT	eL12' .... 22bp ... ACGAATACAT eL12', -181

Conclusions and summary

1. There is a stage-specific change in the form of elongation factor 1 during development : in the cyst most of the tRNA binding activity to ribosomes is found as a high molecular weight form of EF-1, whereas in the nauplii mainly a low molecular weight form of EF-1 can be distinguished.

2. The high molecular weight form of EF-1 is composed of three different subunits. EF-1α binds aminoacyl tRNA to the ribosome under hydrolysis of GTP, while EF-1$\beta\gamma$ exchanges guanine nucleotides bound to EF-1α with exogenous guanine nucleotides. The exchange activity resides in the β-chain (M_r 26 000), the function of the γ-chain (M_r 46 000) being unknown.

3. EF-1γ rather than EF-1α or EF-1β, aggregates strongly in aqueous solvents and distributes preferentially in lipophylic solvents.

4. Gene transcription of EF-1α and acidic 60S ribosomal proteins is closely coordinated during development ; both types of proteins contain the intron sequence PyTAACPy near the splice junction.

5. The *Artemia* genome contains a small number of gene-copies for EF-1α. Characterization of an EF-1α gene shows four introns ; different EF-1α clones exhibit some gene variance in the non-coding parts.

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The regulation of cytoplasmic polyadenylation in *Artemia* sp. cryptobiotic gastrulae

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Abstract

A manganese dependent poly(A) polymerase is purified from the postmitochondrial supernatant of *Artemia* sp. cryptobiotic gastrulae as described previously (Roggen and Slegers, 1985). Under not yet defined conditions poly(A) polymerase applied to a poly(A)-Sepharose 4B column is separated into two enzyme fractions. Approximately 40 % of the poly(A) polymerase activity is recovered in the unbound fraction while 60 % is eluted with 750 mM KCl. Both enzyme preparations show identical physical and enzymatic properties if purified to near homogeneity.

Poly(A) polymerase from *Artemia* sp. cryptobiotic embryos is a glycoprotein composed of several isoforms ranging from pI 6.6 to 7.6. In the presence of divalent cations the enzyme is hydrolyzed in two fragments with an approximately equal molecular weight of 35 000 daltons. This hydrolysis results in a complete loss of the enzyme activity and is not observed in the presence of ATP. The kinetics of polyadenylation are studied and the effect of several factors competing with the enzyme for substrate or primer. Our results indicate that the initiation step of polyadenylation is a very important level of regulation. Enzyme phosphorylation and poly(A)-binding proteins are shown to be involved in the regulation of polyadenylation. A complete lack of primer specificity is observed when RNAs are used as primers. If the efficiency of polyadenylation is compared for a variety of native ribonucleoproteins, AMP incorporation catalyzed by poly(A)-Sepharose 4B bound poly(A) polymerase is highly specific towards non-polysomal poly(A)-containing 17S mRNP. The latter RNP is the main RNP component of cryptobiotic gastrulae of *Artemia* sp. The latter specificity is due to the presence of a small ribonucleoprotein that can be separated from poly(A) polymerase by gel filtration in alkaline conditions. The M_r 58 000 RNP is composed of an M_r 29 000 $\pm$ 1 000 ribonucleic acid and an M_r 38 000 protein. Preliminary results indicate that this protein is the main poly(A)-binding protein present in cryptobiotic gastrulae of *Artemia* sp.

Introduction

It is generally accepted that both hnRNA and mRNA are complexed with proteins and that the main protein-binding site within these ribonucleoprotein particles is the 3'-OH terminal poly(A) sequence (Preobrazhensky and Spirin, 1978 ; Brawerman, 1981 ; Standart *et al.*, 1981 ; Ilyin and Georgiev, 1982 ; Van Eekelen *et al.*, 1982). In nuclei the majority of hnRNP is polyadenylated *de novo* after cleavage of the RNA polymerase II primary DNA transcript approximately 20 nucleotides downstream from the Proudfoot-Brownlee sequence (Proudfoot and Brownlee, 1976 ; Tosi *et al.*, 1981 ; Nevins, 1983 ; Frayne, 1984 ; Manley *et al.*, 1985). During transport of functional mRNP to the cytoplasm the poly(A) tail is hydrolyzed to a more heterogenous but more stable steady-state length, while the transfer of mRNA into polysomes is preceded by polyadenylation (Abraham *et al.*, 1980 ; Saunders *et al.*, 1980 ; Adams *et al.*,

1981). From the universal distribution of poly(A) sequences and the high amount of energy that is spent by the cell to synthesize and maintain this sequence a significant cellular function is to be expected. However, after nearly two decades of intensive research the function of polyadenylation remains unknown. The poly(A)-protein complex may be involved in selection of the 3'-terminal region during mRNA biogenesis (Manley *et al.*, 1979 ; Tosi *et al.*, 1981 ; Nussinov, 1982 ; Amara *et al.*, 1984 ; Nishikura and Vuocolo, 1984 ; Boardman *et al.*, 1985). It also may be involved in mRNP transport to the cytoplasm (Moffett and Webb, 1981 ; Nussinov, 1982) stabilization of mRNA if the remaining poly(A) fragment is longer than 21 nucleotides (Carlin, 1978 ; Müller *et al.*, 1983) and regulation of protein synthesis (Brawerman, 1981).

Beside structural and functional research these hypotheses have stimulated the study of enzymes that metabolize 3'-OH terminal poly(A)-sequences. To date three such enzymes have been characterized *e.g.* poly(A) polymerase, poly(A) endoribonuclease IV and 2', 3'-exoribonuclease (Edmonds and Winters, 1976 ; Jacob and Rose, 1978, Müller *et al.*, 1983). Poly(A) polymerase activities have been localized in nuclei, mitochondria, microsomes, ribosomes and cytosol (Edmonds and Winters, 1976 ; Jacob and Rose, 1978). *In vitro* a common observation with poly(A) polymerase is the lack of primer specificity of the enzymes purified from different origins towards hnRNA or mRNA. Oligo- as well as polyribonucleotides are used as primers. The preference seems to be correlated with the divalent cofactor that stimulates the enzyme activity. The optimal rate of polyadenylation is observed with oligo(A) primers longer than three nucleotides (Winters and Edmonds, 1973 ; Tsiapalis *et al.*, 1975 ; Nevins and Joklik, 1977).

Recently polyadenylation in whole cell lysate has indicated the requirement of one or more factors for efficient and specific elongation (Manley, 1983 ; Moore and Sharp, 1984). This observation and the relation that seems to exist between the protein composition of mRNP particles and their functional state (Bergmann *et al.*, 1982) suggest that mRNA associated proteins may be involved in the regulation of polyadenylation. To date only a few attempts to polyadenylate RNP have been reported. Although difficult to interpret due to the lack of analysis of the reaction products the latter reports suggest that non-polysomal mRNP is more efficiently polyadenylated than its extracted RNA or polysomal mRNP (Dennis and Kisilevsky, 1980). Recently several factors that may participate in the regulation of poly(A) metabolism in eukaryotic cells have been identified *e.g.* poly(A)-binding proteins, protein kinase activities and the cytoskeletal proteins tubulin and actin (Corti *et al.*, 1976 ; Rose and Jacob, 1980 ; Brawerman, 1981 ; De Herdt *et al.*, 1982 ; Müller *et al.*, 1983).

Artemia sp. cryptobiotic gastrulae are very useful for the study of cytoplasmic poly(A)-containing mRNP and the mechanisms involved in cell reactivation. Shortly after reactivation stored non-translatable mRNP is activated and incorporated into polysomes while no transcription of new poly(A)-containing mRNA occurs in nuclei (Felicetti *et al.*, 1975 ; Susheela and Jayaraman, 1976). As the cell differentiates, selection of specific mRNP by the translational machinery is responsible for morphogenesis during the early stages of development (Finamore and Clegg, 1966). This uncoupling of cell differentiation and DNA synthesis requires the existence of a cytoplasmic selection mechanism that may be triggered by polyadenylation of stored poly(A)-containing mRNP as suggested by Brawerman (1981).

Several reports concerning the purification and characterization of poly(A) polymerase from cryptobiotic embryos of *Artemia* sp. have been published (Jeyaraj *et al.*, 1980 ; Sastre and Sebastian, 1981 ; Roggen and Slegers, 1985). The purified enzyme activities are Mn^{2+} dependent and have all the characteristics of poly(A) polymerase as reviewed by Jacob and Rose (1978)

and Edmonds (1982). Measurement of the enzyme activity in different cellular fractions indicates that in *Artemia* sp. poly(A) polymerase is mainly localized in the cytoplasm (Sastre and Sebastian, 1981 ; Roggen and Slegers, 1985). The very low amount of nuclear poly(A) polymerase may be a consequence of cryptobiosis as it has been demonstrated that during the course of embryogenesis the enzyme activity is shifted towards the nucleus (Jeyaraj *et al.*, 1980 ; Sastre and Sebastian, 1981). A number of RNAs have been compared for their capacity to function as primers. The results confirm the absence of any pronounced primer specificity towards a specific RNA (Sastre and Sebastian, 1983 ; Roggen and Slegers, 1985). Sastre and Sebastian (1982) however have published data that suggest a specificity towards non-polysomal mRNP.

In this communication we report the purification of cytoplasmic poly(A) polymerase of *Artemia* sp. cryptobiotic embryos specific in the *in vitro* polyadenylation of 17S non-polysomal poly(A)-containing mRNP. Our results suggest the existence of a factor which determines the *in vivo* primer specificity of poly(A) polymerase. The involvement of enzyme modifications and the poly(A)-binding protein in the initiation of polyadenylation is discussed.

Materials and methods

Materials

Artemia sp. cryptobiotic gastrulae were obtained from the Artemia Reference Center, University of Ghent (Belgium) ; GF/C glass fiber filters from Whatman Biochem. Ltd (Maidstone, Kent, UK) ; CL-Sepharose 4B, CNBr-activated Sepharose 4B, marker proteins from Pharmacia Fine Chemicals (Uppsala, Sweden) ; calf liver tRNA from Boehringer (Mannheim, FRG) ; ATP, CTP, GTP, UTP, calf thymus DNA, poly(A) from Sigma Chemical Co. (St. Louis, MO, USA) ; (^{3}H)-ATP, (^{3}H)-poly(U), (^{32}P)-ATP from Amersham (Buckinghamshire, UK) ; $(A)_4$, $(A)_6$, $(A)_8$, $(A)_{10}$ from P.L. Biochemicals GmbH (St. Goar, FRG) ; $(A)_{54}$ from Miles Lab. Inc. (Kankakee, IL, USA) ; hydroxyapatite and Bio-gel P2 from Bio-Rad Labs (Richmond, CA, USA).

Buffers

Buffer A : 50 mM Tris-HCl pH 8.6, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 5 % (v/v) glycerol.

Buffer B : 20 mM potassium phosphate pH 5.8, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 10 % (v/v) glycerol.

Buffer C : 10 mM Hepes pH 7.5, 10 mM 2-mercaptoethanol, 50 mM KCl, 20 % (v/v) glycerol.

Buffer D : 50 mM Tris-HCl pH 6.8, 100 mM NH_4Cl, 20 mM 2-mercaptoethanol, 50 % (v/v) glycerol.

Buffer E : 10 mM Hepes pH 7.2, 100 mM KCl, 0.1 mM dithiothreitol.

Buffer F : 200 mM Tris-HCl pH 8.6, 40 mM NH_4Cl, 10 mM 2-mercaptoethanol, 20 % (v/v) glycerol).

Purification of cytoplasmic poly(A) polymerase

Poly(A) polymerase is purified from the postmitochondrial supernatant of *Artemia* sp. as previously described (Roggen and Slegers, 1985). The supernatant from 600 g (dry weight) cryptobiotic gastrulae is prepared in buffer A, dialyzed and applied to a DEAE-cellulose column. Bound material is eluted with a linear 0-200 mM NH_4Cl gradient. The poly(A) polymerase containing fractions are pooled, diluted to a final volume of 1 l and rebound to DEAE-CL-Sepharose 6B. After a pH gradient from pH 8.6 in buffer A to pH 5.8 in buffer B the enzyme activity is eluted with 100 mM potassium phosphate pH 5.8. Pooled fractions are concentrated by dialysis against 95 % (v/v) glycerol and applied to a CL-Sepharose 6B column equilibrated with the latter buffer and 200 mM KCl. Poly(A) polymerase containing fractions are chromatographed on phosphocellulose P11 in the same buffer conditions. The flow contains all the poly(A) polymerase activity and is diluted 4 times and reapplied to phosphocellulose P11. The enzyme activity eluted at 400 mM KCl and is further purified by affinity chromatography on poly(A)-Sepharose 4B (0.65 mg poly(A)/ml swollen gel) equilibrated with buffer C. The bound poly(A) polumerase fraction is eluted with 750 mM KCl. The final enzyme preparations are dialyzed against buffer D. After addition of ATP to a final concentration of 0.6 mM and incubation at 30°C for 30 min the enzyme can be stored at - 70 °C for several months without loss of activity.

Preparations of RNP and RNA

Cytoplasmic RNPs are separated from the postmitochondrial supernatant of 400 g (dry weight) cryptobiotic embryos of *Artemia* as previously described (Nieuwenhuysen and Slegers, 1978 ; De Herdt *et al.*, 1979 ; Slegers *et al.*, 1981). Non-polysomal poly(A)-containing mRNP, 22S poly(A)-lacking mRNP, ribosomes and ribosomal subunits are concentrated by ultrafiltration on a PM30 membrane and dialyzed against buffer E. RNA is extracted by phenolyzation and dissolved in buffer E.

Assays

a) poly(A) polymerase assays are in 200 mM Tris, pH 8.4, 0.9 mM (^{3}H)-ATP (specific activity 15 cpm/pmol ATP), 20 mM NH_4Cl or KCl, 0.9 mM $MnCl_2$, appropriate concentration of primer RNA or RNP and 1.25 µg protein/100 µl assay of the active enzyme. Incubation is at 30 °C for an appropriate time ; b) poly(A)-sequences are localized by hybridization with (^{3}H)-poly(U) (Slegers *et al.*, 1981) ; c) the phosphorylation assay of poly(A) polymerase by non-polysomal poly(A)-containing mRNP associated protein kinase is described previously (Thoen *et al.*, 1985).

Protein determination

The protein concentration is measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis is as described by Weber and Osborn (1969). Two-dimensional gel electrophoresis is performed according to the procedure of O'Farrell (1975).

Specific staining of glycoproteins using fluorochrome-labelled lectins

Electrophoretically separated glycoproteins are electroblotted to nitrocellulose and stained using fluorochrome-labelled concanavalin A, as described by Peeters *et al.* (1985).

Purification of the Mr 38 000 poly(A)-binding protein

The 38 000 Mr poly(A)-binding protein of non-polysomal mRNP is prepared from 400 g (dry weight) cryptobiotic gastrulae of *Artemia* sp. as described previously (De Herdt *et al.*, 1984).

Purification of protein kinase associated with non-polysomal poly(A)-containing mRNP

The protein kinase activity is isolated from 400 g (dry weight) cryptobiotic embryos as described by Thoen *et al.* (1985).

Results

Binding of poly(A) polymerase to poly(A)-Sepharose 4B

The binding of poly(A) polymerase activity on poly(A)-Seharose 4B is not evident from the results presented in Table I. 500 µl aliquots obtained in the different purification steps are brought to the appropriate buffer conditions by gel filtration on Bio-gel P2 columns (5 ml) and are applied to poly(A)-Sepharose 4B columns (2 ml) equilibrated with the respective buffers. Subsequently bound material is eluted discontinuously with 400 and 800 mM KCl respectively. The enzyme activity is measured in standard conditions with 8 µM $(A)_4$ primer. Incubation is for 1 h at 30 °C. The total enzyme activity is calculated from the slope of a protein concentration variation curve. In Table I the relative amount of enzyme is measured in the poly(A) Sepharose bound and unbound fraction. The results suggest that the binding of poly(A) polymerase to poly(A)-Sepharose 4B is optimal at physiological pH. At more acidic conditions the binding is progressively reduced while a sharp decrease is observed in more alkaline conditions. Furthermore the binding seems to be correlated with the degree of purification. Acidification of the poly(A) polymerase preparation seems to be crucial as a pH gradient is the only difference between DEAE-cellulose and DEAE-Sepharose CL 6B ion exchange chromatography. Analysis by sodium dodecylsulfate polyacrylamide gel electrophoresis of the proteins that are eluted by this gradient reveals than an M_r 38 000 protein is one of the major components (data not shown).

After purification by gel filtration on CL-Sepharose 6B in buffer B and 200 KCl the enzyme becomes very unstable at acidic pH. This inactivation is due to the dilution which occurs after gel filtration on the Bio-gel P2 column and is not observed if fractions are dialyzed against acidic buffers. However no binding on poly(A)-Sepharose 4B is observed in these conditions (data not shown). The presence of detectable enzyme activity after chromatography on phosphocellulose P11 may be explained by the high protein concentrations used (Roggen and Slegers, 1985).

Specificity of *in vitro* polyadenylation

A number of RNAs has been compared for their capacity to function as primers for purified poly(A) polymerase (Table II). The poly(A)-Sepharose 4B bound poly(A) polymerase shows a preference for oligo(A) if adenylic acids are used as substrates. Approximately eight nucleotides seem to be the optimal primer length. Of the ribonucleic acids only 9S poly(A)-lacking mRNA

TABLE I

Binding of poly(A) polymerase to poly(A)-Sepharose 4B

Enzyme fractions	Relative poly(A) polymerase distribution (%)											
	pH 8.6			pH 7.5			pH 6.5			PH 5.8		
	F	400	800	F	400	800	F	400	800	F	400	800
Postmitochondrial supernatant	> 99.9	< 0.1	< 0.1	> 99.9	< 0.1	< 0.1	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
DEAE-cellulose	> 99.9	< 0.1	< 0.1	> 99.9	< 0.1	< 0.1	n.m.	n.m.	n.m.	9.7.	54.0	36.3
DEAE-cellulose CL 6B	> 99.9	< 0.1	< 0.1	1.6	11.5	86.9	2.3.	20.4	77.3	0.9	52.5	46.6
CL-Sepharose 6B	> 99.9	< 0.1	< 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phosphocellulose a	> 99.9	< 0.1	< 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phosphocellulose b	> 99.9	< 0.1	< 0.1	22.5	2.5	75.0	13.5	14.1	72.4	< 0.1	> 99.9	< 0.1

n.m. : not measured.
n.d. : not detectable.

Poly(A) polymerase has been purified as described in Materials and methods. From the enzyme preparation obtained in each purification step 2 ml is taken and divided in 500 µl aliquots. These aliquots are applied on Bio-gel P2 columns of 5 ml equilibrated with buffer A, buffer B at pH 6.5 and 5.8 respectively, and buffer C. This gel filtration step results in a 5-fold dilution to a final volume of 2.5 ml. Subsequently the enzyme fractions are applied on a 2 ml poly(A)-Sepharose 4B column (0.65 mg poly(A)/ml swollen gel). Bound material is eluted discontinuously with 400 mM and 800 mM KCl respectively. The enzyme activity is assayed in the presence of 8 µM $(A)_4$ at 30 °C. Incubation is for 1 h. From the slope of the protein concentration variation curve the total enzyme activity in each fraction is calculated. The enzyme distribution on poly(A)-Sepharose 4B is expressed relative to the total poly(A) polymerase activity recovered.

TABLE II

Comparison of the efficiency of primer polyadenylation

RNA primers	Polyadenylation		RNP primers	Polyadenylation	
	pmol AMP.pmol primer^{-1} . µg protein^{-1}	%		pmol AMP.pmol primer^{-1} . µg protein^{-1}	%
$(Ap)_3A$	28.0	100.0	80S ribosomes	n.d.	n.d.
$(Ap)_5A$	34.0	121.4	40S ribosomal subunits	n.d.	n.d.
$(Ap)_7A$	35.6	127.0	60S ribosomal subunits	n.d.	n.d.
$(Ap)_9A$	34.2	122.0	22S poly(A)$^-$ mRNP	n.d.	n.d.
$(Ap)_{53}A$	21.4	76.4	7S poly(A)$^+$ RNP	n.d.	n.d.
poly(A)	n.d.	n.d.	17S poly(A)$^+$ RNP	n.d.	n.d.
tRNA	7.0	25.0	17S poly(A)$^+$ mRNP	140.0	500.0
28S rRNA	11.2	40.0			
18S rRNA	12.3	44.0			
5S rRNA	0.6	2.1			
13S poly(A)$^+$ mRNA	14.7	52.5			
9S poly(A)$^-$ RNA	n.d.	n.d.			

n.d. : not detectable.

Cytoplasmic RNPs are isolated from the postmitochondrial supernatant of 400 g (dry weight) cryptobiotic gastrulae of *Artemia* sp. 17S poly(A)-containing mRNP, 22S poly(A)-lacking mRNP and ribosomes are purified as described previously (Nieuwenhuysen and Slegers, 1978 ; De Herdt *et al.*, 1979 ; Slegers *et al.*, 1981). The respective RNP fractions are concentrated by ultrafiltration on a PM 30 membrane, dialyzed against buffer E and stored at - 20 °C. 9S poly(A)-lacking mRNA, 13S poly(A)-containing mRNA and rRNAs are extracted from 22S poly(A)-lacking mRNP, 17S poly(A)-containing mRNP, and 80S ribosome respectively. Extraction is with phenol/chloroform/isoamyl alcohol in a final concentration of 25 %/ 24 %/ 1 % (v/v). RNA is precipitated overnight with 2 volumes of ethanol at - 20 °C. The precipitate is washed with ether, dried and dissolved buffer E. 9S poly(A)-lacking mRNA, 13S poly(A)-containing RNA and 28S, 18S, and 5S rRNA are purified from the respective RNA preparations by centrifugation on a 10-30 % (w/v) sucrose density gradient. Centrifugation is in a SW40 Beckman rotor at 160 000 × *g* for 18 h at 4 °C. The other primers are commercial preparations. The efficiencies are calculated from the slope of a primer concentration variation curve and are corrected for polyadenylated degradation material. Correction factors are calculated from analysis of reaction mixtures by 10-30 % (w/v) sucrose density gradient centrifugation at 160 000 × *g* for 18 h at 4 °C in a SW40 Beckman rotor. The enzyme (1.25 µg enzyme/100 µl) assays are incubated at 30 °C for 3 h.

is not polyadenylated by the enzyme. 5S rRNA, tRNA, 13S poly(A)-containing mRNA, 18S and 28S rRNA incorporate AMP although less efficiently as compared to the oligo(A) primers.

In Table II the efficiency of polyadenylation of ribonucleoproteins is compared with the primer efficiency of their extracted RNAs. Clearly efficient and specific polyadenylation is only obtained with ribonucleoproteins. Indeed 22S poly(A)-lacking mRNP, ribosomes and ribosomal subunits which are not polyadenylated *in vivo* are also not polyadenylated *in vitro* although extracted rRNAs can be polyadenylated *in vitro*. Furthermore the purified poly(A) polymerase only polyadenylates the non-polysomal poly(A)-containing 17S mRNP and no elongation of the degraded 7S poly(A) protein complex is observed in the same conditions. The ribonucleoprotein used as substrate by the enzyme is characterized by gel filtration of poly(A)-containing ribonucleoproteins on a CL-Sepharose 4B column and assay of the fractions for polyadenylation by the purified poly(A) polymerase (Fig. 1). A single activity peak is detected at the position of the M_r

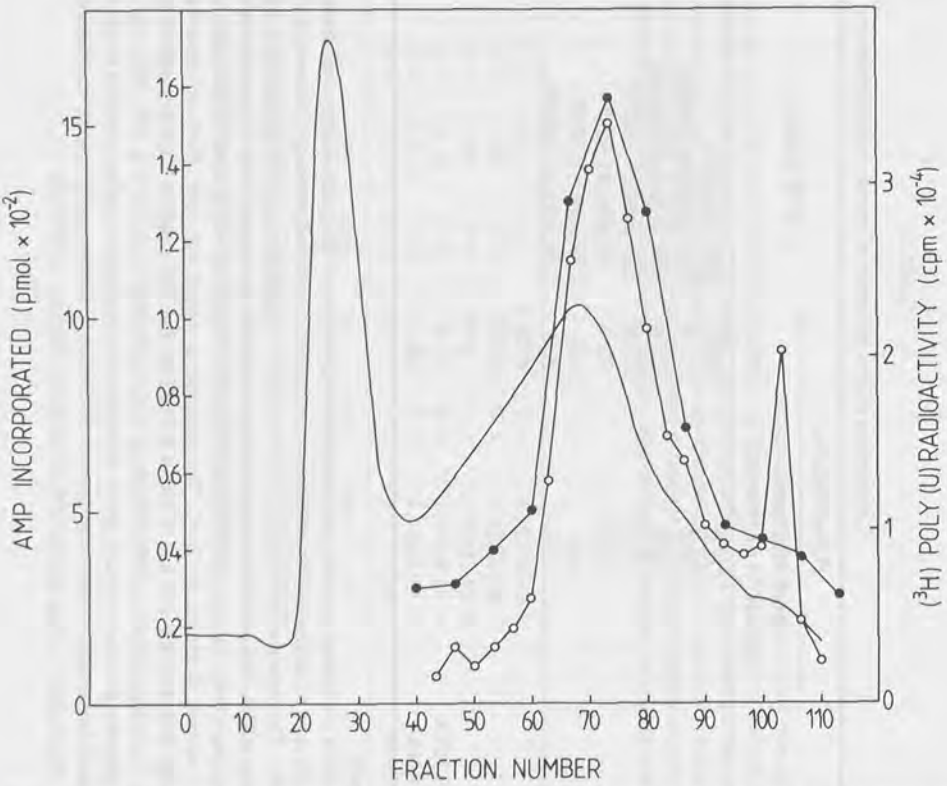

FIG. 1. Analysis of poly(A)-containing RNP by gel filtration on CL-Sepharose 4B. Non-polysomal poly(A)-containing RNP is isolated from the postmitochondral supernatant of 400 g (dry weight) cryptobiotic gastrulae of *Artemia* sp. by affinity chromatography on oligo (dT)-cellulose (Slegers *et al.*, 1981). The bound ribonucleoprotein is chromatographed on a CL-Sepharose 4B column (1.4 × 100 cm) equilibrated with buffer D and fractions of 1.8 ml are collected. Poly(A) sequences are detected by hybridization with (^{3}H)poly(U) (○—○). Poly(A) polymerase assays are performed as described in the legend of Table I. Assay mixtures of 100 µl contain 35 µl of the indicated fractions. Incubation is at 30 °C for 3 h (●—●). Absorbance is measured at 280 nm (—).

500 000 ± 60 000 17S ribonucleoprotein particle. Degradation material is not elongated and no endogenous poly(A) polymerase activity can be measured. Polyadenylation is coincident with the presence of poly(A) sequences as demonstrated by hybridization with (^{3}H)-poly(U). Sucrose density gradient centrifugation demonstrates that this particle has the same size as the well-characterized non-polysomal 17S poly(A)-containing mRNP (Fig. 2). This identity is further confirmed by SDS polyacrylamide gel electrophoresis. Both particles show the same protein composition (data not shown). From a primer concentration variation curve it is calculated that the poly(A)-containing mRNP is extended with approximately 1 400 AMP residues (Roggen *et al.*, 1985). However analysis of the reaction products by sucrose density gradient centrifugation demonstrates that 50 % of the precipitable radioactivity is incorporated in small RNA molecules (Fig. 2). As no polyadenylation of degraded material is observed (Fig. 1) the latter elongated small RNA is generated by degradation of poly(A) sequences synthesized on RNP during the polyadenylation reaction. After correction for degradation the 17S poly(A)-containing mRNP is elongated with approximately 700 nucleotides and is ten times more efficiently polyadenylated as compared to its extracted 13S poly(A)-containing mRNA and five times as compared to $(A)_4$ (table II).

If poly(A)-Sepharose bound poly(A) polymerase is purified by gel filtration on Sephadex G-100 in buffer A containing 200 mM NH_4Cl the primer specificity towards 17S poly(A)-containing mRNP is abolished. As observed for the unbound enzyme fraction only oligo(A) primers are efficiently polyadenylated (Roggen *et al.*, 1985).

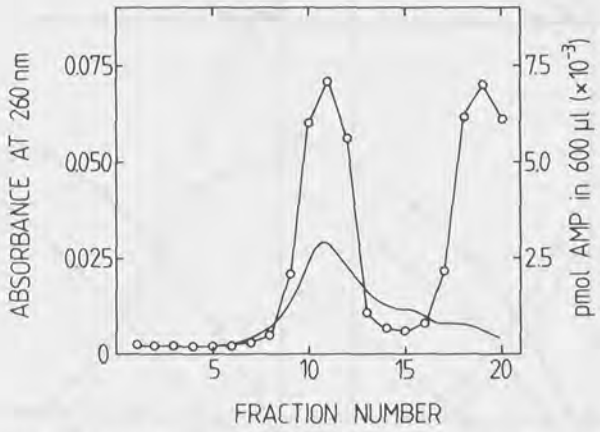

FIG. 2. Analysis of the 17S poly(A)-containing mRNP polyadenylated with purified poly(A) polymerase. Assay mixtures of 400 µl containing 5 µg enzyme and 20 nM ribonucleoprotein are incubated for 3 h at 30 °C. The assay is analyzed on a 10-30 % (w/v) sucrose density gradient in buffer D. Centrifugation is in a SW40 Beckman rotor for 18 h at 160 000 × *g* and at 4 °C. The gradient is fractionated in 600 µl fractions and the distribution of the radioactivity is measured (○—○). Absorbance at 260 nm (—).

KINETICS OF POLYADENYLATION CATALYZED BY POLY(A) POLYMERASE

The kinetics of polyadenylation are studied as a function of time. Polyadenylation by the purified enzyme is preceded by a lag phase which is independent of a pre-incubation in the

absence of oligo(A) primer. This lag phase is primer length dependent and disappears if ATP, oligo(A) or enzyme concentration is increased. Of the oligo(A) primers used an oligo(A) with eight adenosine residues is the shortest primer resulting in a lag phase (Fig. 3). Several factors competing with the enzyme for the primer RNA or the substrate have been added to the reaction mixture. CTP, GTP, and UTP are not incorporated but inhibit polyadenylation at concentrations equivalent to ATP (data not shown).

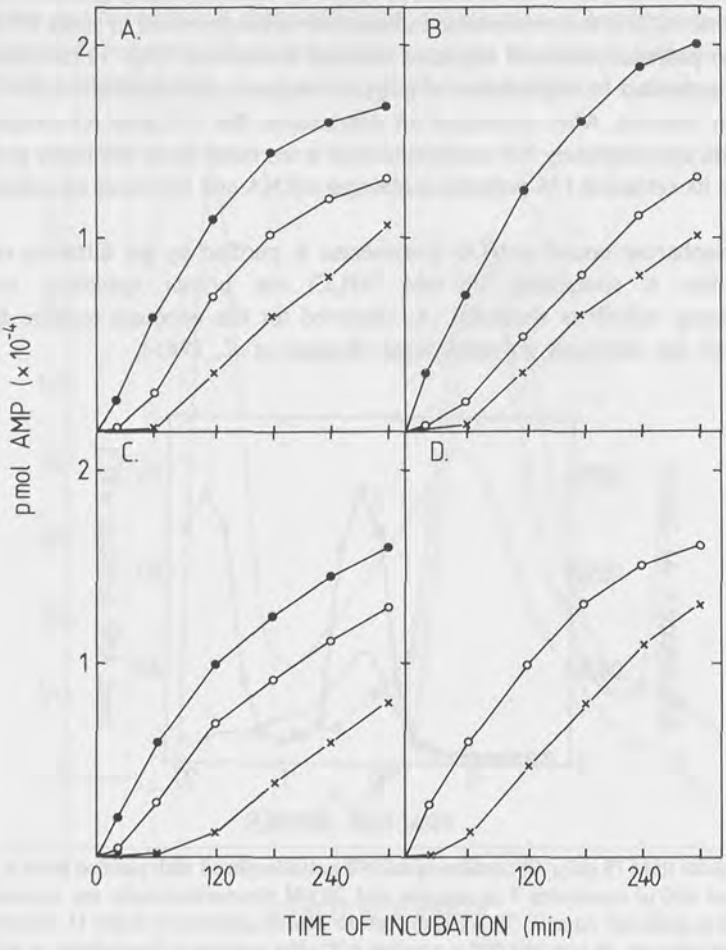

FIG. 3. Polyadenylation as a function of time. Poly(A) polymerase purified to near homogeneity is incubated in standard conditions and the AMP incorporation is determined. (A) with 0.2 mM (×—×), 0.4 mM (○—○), and 0.9 mM (●—●) ATP, 8 μM of $(A)_4$ primer and 9.2 μg of protein ; (B) with 4 μM (×—×), 8 μM (○—○), and 20 μM (●—●) $(A)_4$ primer, in the presence of 0.4 mM ATP and 9.2 μg protein ; (C) with 3.6 μg (×—×), 9.2 μg (○—○), and 18.4 μg (●—●) protein, 0.4 mM ATP and 8 μM $(A)_4$ primer ; (D) with 8 μM $(A)_4$ (×—×), and $(A)_{10}$ (○—○) respectively), 9.2 μg protein and 0.4 mM ATP.

Addition of the M_r 38 000 poly(A)-binding protein (P38) isolated from non-polysomal cytoplasmic poly(A)-containing mRNP has a dual effect on polyadenylation of $(A)_4$. In the absence of P38 the rate of the AMP incorporation is decreased at an oligo(A) length of 10 ± 2 nucleotides but is increased to the initial rate at a length of 13 ± 2 nucleotides. When P38 is added to the reaction mixture at concentrations equimolar to the primer it competes with poly(A) polymerase for oligo(A) as indicated by the increase in the lag phase. Once polyadenylation is initiated the poly(A)-binding protein changes the elongation rate into a linear AMP incorporation (Fig. 4A). Pre-incubation of purified poly(A) polymerase with 1 μM AMP, 6 mM $MgCl_2$, and purified non-polysomal poly(A)-containing mRNP associated protein kinase results in a decrease of the lag phase but does not alter the elongation rate of polyadenylation of $(A)_4$. The rate of polyadenylation still decreases at an oligo(A) length of 10 ± 2 nucleotides (Fig. 4B).

Similar studies have been performed with 17S poly(A)-containing mRNP as primer. 6×10^{-3} A_{260} units of the RNP preparation equivalent to 2 pmol poly(A)-containing mRNA are added to the reaction mixture and are incubated with 5 μg of the poly(A)-Sepharose bound enzyme fraction. As a function of time a very broad lag phase of nearly 2 h is observed. Based on the determined AMP incorporation P38 is added up to a final P38:AMP ratio of 1:10. This ratio is the *in vivo* stoichiometry of the protein in mRNP (De Herdt *et al.*, 1984). As shown in Fig. 5 no AMP incorporation is observed.

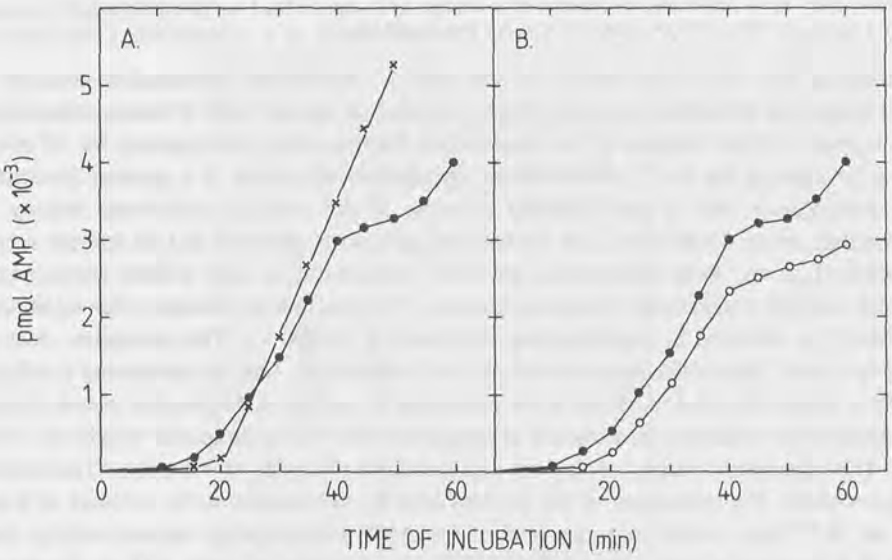

FIG. 4. Effect of factors on the lag phase of polyadenylation. Poly(A) polymerase purified to near homogeneity is assayed as described in Materials and methods with $(A)_4$ as primer. (A) The M_r 38 000 poly(A)-binding protein is added to the reaction mixture at a concentration equimolar to the primer concentration. The AMP incorporation is measured as a function of time with (×—×) and without (●—●) protein. (B) Poly(A) polymerase is phosphorylated as described. 200 μl enzyme fraction is added to an equal volume of protein kinase assay mixture and incubated for 60 min. Subsequently 20 μl of this mixture is assayed for poly(A) polymerase activity. The AMP incorporation is determined as a function of time (●—●). Assay in the absence of protein kinase (○—○).

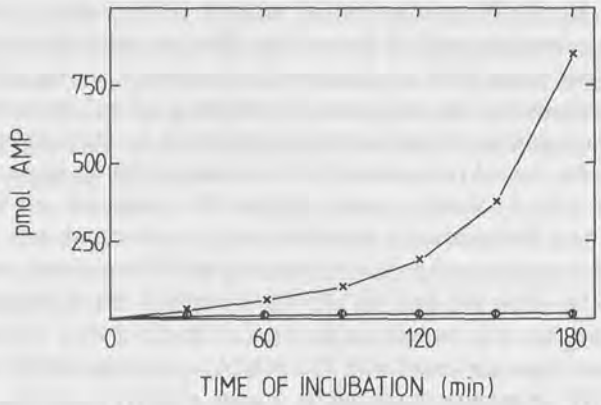

FIG. 5. Effect of the poly(A)-binding protein on the polyadenylation of the 17S poly(A)-containing mRNP. Poly(A) polymerase bound on poly(A)-Sepharose 4B is assayed with 17S poly(A)-containing mRNP as primer. 5 µg of enzyme is incubated with 6×10^{-3} A_{260} RNP (2 pmol poly(A)-containing mRNA) in standard conditions (×—×). The poly(A)-binding M_r 38 000 protein is added until a P38:AMP ratio of 1:10 (about 7.5 pmol) and the incubation is repeated (○—○). Poly(A)-containing mRNP and P38 are purified as described in Materials and methods.

MODIFICATIONS OF THE CYTOPLASMIC POLY(A) POLYMERASE

Addition at 4 °C of 1 mM $MnCl_2$ to the poly(A) polymerase preparation obtained by poly(A)-Sepharose 4B affinity chromatography and dialysis against buffer F causes precipitation and an increase of the viscosity of the supernatant fraction after centrifugation for 10 min at 10 000 g. Increasing the Mn^{2+} concentration discontinuously results in a gradual increase of protein precipitation and a concommitant decrease of the poly(A) polymerase activity. At concentrations above 4 mM $MnCl_2$ no further precipitation is observed and no enzyme activity is detected (Fig. 6). Both phenomena are Mn^{2+} concentration and protein concentration dependent but pH independent (data not shown). The precipitate obtained after addition of 4 mM $MnCl_2$ is collected by centrifugation for 10 min at 10 000 × *g*. This precipitate does not dissolve in a Mn^{2+} free buffer regardless of pH and temperature. Only in denaturing conditions the pellet is slowly dissolved. Analysis of the precipitate by sodium dodecylsulfate polyacrylamide gel electrophoresis indicates the presence of a single protein with a molecular weight of 35 000 daltons. The supernatant fraction reveals the disappearance of the M_r 70 000 poly(A) polymerase (data not shown). Pre-incubation of the purified poly(A) polymerase in the presence of 6 mM $MgCl_2$ at 30 °C also results in a decrease of the AMP incorporating enzyme activity when assayed as a function of time between 0 and 180 min. Addition of 1 µM ATP to the enzyme mixture prevents this loss of enzyme activity. Pre-incubation of the poly(A) polymerase in the absence of divalent cation has no effect on the AMP incorporation (Fig. 7). Analysis by sodium dodecylsulfate polyacrylamide gel electrophoresis of the Mg^{2+}-treated enzyme fractions reveals the appearance of an M_r 35 000 protein concomitant with a decrease of the M_r 70 000 poly(A) polymerase (data not shown). A decrease in molecular weight of poly(A) polymerase is also observed in the enzyme fractions stored in buffer D at - 70 °C. Analysis by sodium dodecylsulfate polyacrylamide gel electrophoresis demonstrates that in the thawed preparations the M_r 70 000

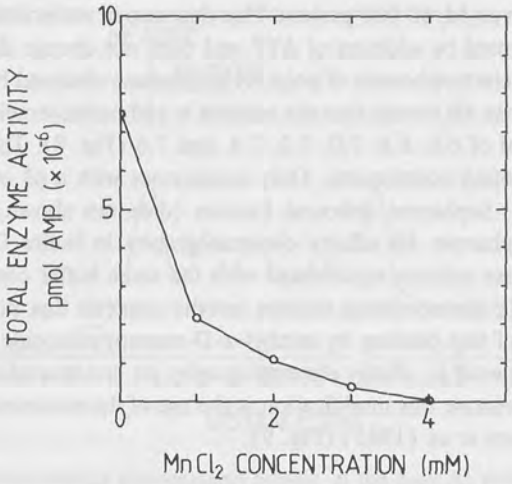

FIG. 6. Inactivation of poly(A) polymerase by Mn^{2+}-ions. Poly(A)-polymerase obtained by affinity chromatography on poly(A) Sëharose 4B in buffer C is incubated at 4 °C for 10 min in the presence of 1 mM $MnCl_2$. The precipitate is collected by centrifugation for 10 min at 10 000 × g_{max}. The supernatant is removed and placed for another 10 min at 0 °C. No further precipitation is observed. The enzyme activity is assayed with 800 pmol $(A)_4$ as primer. Incubation at 30 °C is for 180 min. The $MnCl_2$ concentration is increased discontinuously in 1 mM steps. The sample is processed as described. At a final concentration of 4 mM $MnCl_2$ precipitation is no longer observed.

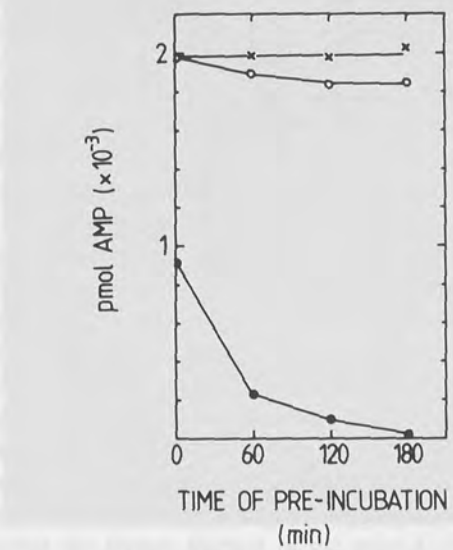

FIG. 7. Inactivation of the enzyme by pre-incubation in 6 mM $MgCl_2$. Poly(A) polymerase is pre-incubated for 180 min in the absence (○—○) and presence (●—●) of 6 mM $MgCl_2$. Pre-incubation with 6 mM $MgCl_2$ is also performed in the presence of 1 µM ATP (×—×). At different times 20 µl fractions are incubated for 180 min in the poly(A) polymerase assay mixture.

protein has changed into an M_r 60 000 protein. This decrease in molecular weight of the poly(A) polymerase is not prevented by addition of ATP and does not change the enzyme activity.

Two dimensional gel electrophoresis of poly(A) polymerase obtained by affinity chromatography on poly(A)-Sepharose 4B reveals that the enzyme is a phosphoprotein. It consists of at least six isoenzymes with a pI of 6.6, 6.8, 7.0, 7.2, 7.4, and 7.6 (Fig. 8). The fractions having a pI of 7.0 and 7.4 are the main components. Only isoenzymes with a pI of 6.8, 7.0, and 7.2 are detected in the poly(A)-Sepharose unbound fraction (date not shown). Poly(A) polymerase purified by poly(A)-Sepharose 4B affinity chromatography in buffer C is bound on a 5 ml concanavalin A-Sepharose column equilibrated with the same buffer containing 150 mM KCl. The binding of the AMP incorporating enzyme activity suggests that poly(A) polymerase is a glycoprotein. Reversal of this binding by methyl-α-D-mannopyranoside has been unsuccessful but this is often encountered in affinity chromatography on concanavalin A-Sepharose 4B. An alternative way to demonstrate this modification is the use of fluorochrome labeled concanavalin A as described by Peeters *et al.* (1985) (Fig. 9).

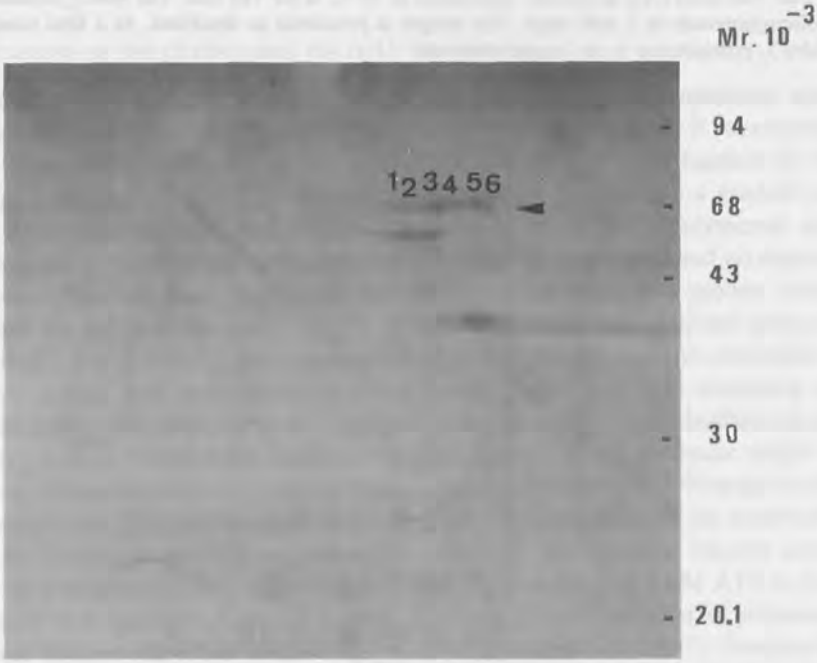

FIG. 8. Two-dimensional gel electrophoresis of purified poly(A) polymerase. Two-dimensional gel electrophoresis of poly(A) polymerase is performed according to the procedure described by O'Farrell (1975). Marker proteins are phosphorylase b (M_r 94 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100). Respective pI ; 1 : 6.6 ; 2 : 6.8 ; 3 : 7.0 ; 4 : 7.2 ; 5 : 7.4 ; 6 : 7.6.

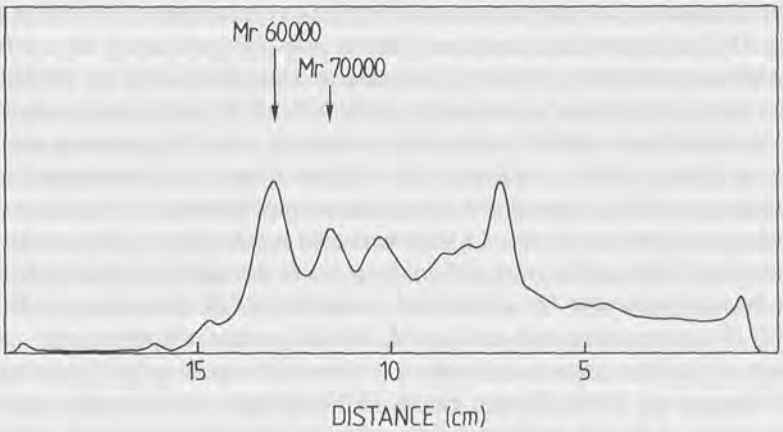

FIG. 9. Staining of electroblotted glycoproteins present in the poly(A) polymerase fraction by fluorochrome-labeled concanavalin A. The poly(A) polymerase fraction obtained by affinity chromatography on poly(A)-Sepharose 4B is analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis. The proteins are fluorescein labelled, electroblotted onto nitrocellulose and stained with Concanavalin A and processed as described by Peeters *et al.* (1985).

Discussion

In agreement with the results of others (Winters and Edmonds, 1973 ; Tsiapalis *et al.*, 1975 ; Edmonds and Winters, 1976 ; Nevins and Joklik, 1977 ; Jacob and Rose, 1978 ; Sastre and Sebastian, 1983) cytoplasmic poly(A) polymerase purified to near homogeneity from the postmitochondrial supernatant of *Artemia* sp. cryptobiotic embryos does not show any pronounced primer specificity (Roggen and Slegers, 1985 ; Roggen *et al.*, 1985). Evidence that at least one additional factor is involved in specific polyadenylation is obtained by the binding of the enzyme to poly(A)-Sepharose 4B. To date only Nevins and Joklik (1977) have reported the binding of poly(A) polymerase to poly(A)-Sepharose. We observed that binding occurs in very restricted conditions which are not yet completely understood. Optimal binding seems to occur at pH 7.5 if the enzyme has been subjected to acidic conditions previously what renders the enzyme much more labile at low protein concentration. Analysis of the protein fractions that elute from DEAE-Sepharose CL-6B by a pH gradient shows that an M_r 38 000 protein is the main component which is removed from the poly(A) polymerase containing fractions. This protein has the same molecular weight as the main poly(A)-binding protein of stored poly(A)-containing mRNP of cryptobiotic gastrulae of *Artemia* sp. In spite of those data the binding of poly(A) polymerase to poly(A)-Sepharose 4B remains unknown although it is very reproducible in our hands. We have been able to bind approximately 75 % of the applied enzyme activity to poly(A)-Sepharose. Although bound and unbound activities have identical physical and enzymatic properties the former fraction of the poly(A)-Sepharose 4B column shows a striking specificity towards the non-polysomal poly(A)-containing mRNP. Gel filtration demonstrates that polyadenylation is confined to a single mRNP particle with a molecular weight of 500 000 ± 60 000 daltons. Protein analysis by sodium dodecylsulfate polyacrylamide gel electrophoresis and sucrose gradient centrifugation show that the polyadenylated RNP particle is

similar if not identical to the non-polysomal 17S poly(A) containing mRNP which is the main cytoplasmic RNP of cryptobiotic gastrulae of *Artemia* sp. (Slegers *et al.*, 1981). The enzyme catalyzed AMP incorporation is primer concentration dependent and is not limited in length. After correction for degradation an elongation of the 17S mRNP with approximately 700 AMP residues is calculated from a primer concentration variation curve. In agreement with the results of Moore and Sharp (1984) obtained with cell-free lysates, polyadenylation of the 17S poly(A)-containing mRNP is preceded by a lag phase of approximately 1-2 h. The loss of primer specificity after purification of the poly(A)-Sepharose 4B bound poly(A) polymerase fraction by gel filtration suggests the presence of a factor responsible for specific polyadenylation. Indeed recently we have demonstrated the presence of a small M_r 58 000 ribonucleoprotein composed of an M_r 29 000 ribonucleic acid and an M_r 38 000 protein (Roggen *et al.*, 1985). The characteristics of the latter protein are identical to those of the main poly(A)-binding protein of mRNP (De Herdt *et al.*, 1984). To date this M_r 38 000 protein is the only factor able to change the elongation rate of *in vitro* polyadenylation into a linear AMP incorporation. Elongation of $(A)_4$ primer by poly(A) polymerase is not linear and a rate decrease is observed at an oligo(A) length of 10 ± 2 nucleotides. A rate decrease at approximately 8 ± 2 nucleotides is also observed for the polyadenylation by calf thymus poly(A) polymerase (Ackerman *et al.*, 1979). In the initiation of polyadenylation a competition between the poly(A)-binding protein and poly(A) polymerase is suggested by the increase of the lag phase. In the presence of this protein elongation occurs at a constant rate probably by inducing a configurational change in the poly(A) sequences (Nowak *et al.*, 1980). The latter effect of the poly(A)-binding protein is not observed when 17S poly(A)-containing mRNP is used as primer. Addition of the poly(A)-binding protein abolished AMP incorporation completelty. This may be explained by the presence of the M_r 38 000 protein on the poly(A) sequence of mRNP presenting an already unstacked sequence to the enzyme at the beginning of the reaction.

A function of mRNP proteins in polyadenylation is also suggested by the differences in the efficiency of elongation of RNP and its extracted RNA. These proteins may be involved in the induction of structural alterations of the primer but may also function at the level of initiation or elongation of polyadenylation. Several enzyme activities which are involved in polyadenylation have been demonstrated to exist in the protein moiety of poly(A) containing mRNP. A poly(A) degrading endonuclease activity is associated with the non-polysomal 17S mRNP (our unpublished results) and recently the presence of a protein kinase has been demonstrated (Thoen *et al.*, 1985). As phosphorylation/dephosphorylation is often proposed as a regulatory mechanism involved in protein synthesis we have studied the effect of phosphorylation on polyadenylation. Phosphorylation of purified poly(A) polymerase by mRNP associated protein kinase results in a decrease of the lag phase of polyadenylation. As the latter phase is a measure of the time necessary to form an active initiation complex (Sano and Feix, 1976) phosphorylation may increase the affinity of the enzyme for the RNA primer as observed for other RNA-binding proteins (Beyer *et al.*, 1977 ; McDonald and Agutter, 1980). Poly(A) polymerase is found to be a modified enzyme. At least six isoenzymes are detected with isoelectric points ranging from 6.6 to 7.6. Several of these isoenzymes are more acidic than the pI of 7.4 reported for calf thymus polymerase (Tsiapalis *et al.*, 175) but are more alkaline than the pI of 6.0 measured by Sastre and Sebastian (1981) for poly(A) polymerase of *Artemia* sp. cryptobiotic gastrulae. Using a recently described procedure (Peeters *et al.*, 1985) we have been able to reveal the presence of saccharide residues covalently bound to poly(A) polymerase. The function of this modification

is still unknown. It is known that glycosylation influences the protein conformation and protects the protein against protease degradation. It also may be a signal for transport and secretion (Horowitz, 1980). As cytoplasmic poly(A) polymerase has to be stored in an active state during cryptobiosis glycosylation may be functional in the protection ageinst proteolytic degradation. This modification also may account for the change of the M_r 70 000 enzyme into an M_r 60 000 one as proteins containing O-acyl functions may hydrolyze spontaneously above pH 7. This explanation is supported by the shift observed after hydroxyapatite column chromatography. The glycosylated form of the enzyme being negatively charged is only eluted by phosphate while the more positively charged deglycosylate form, probably formed spontaneously after the prolonged storage at pH 8.6, is eluted from hydroxyapatite with Na^+- or K^+-ions (our unpublished results). These results indicate that glycosylation as well as phosphorylation may account for the existence of the isoenzymes. The hypothesis that poly(A) polymerase is stabilized by glycosylation seems to be in disagreement with the Mg^{2+}/Mn^{2+} dependent degradation of the enzyme. To date no such degradation has been reported. Even more surprising is the appearance of two equal sized fragments with a molecular weight of 35 000 daltons. The mechanism and the function of this symmetrical proteolysis remains to be elucidated.

In spite of the amount of data that are available now the regulation mechanism for polyadenylation remains unresolved. Our results suggest that the M_r 38 000 poly(A)-binding protein has an important function. Removal of this protein seems to be necessary for the binding of poly(A) polymerase to poly(A). Furthermore it is a component of the small RNP which determines the primer specificity of the enzyme towards non-polysomal 17S poly(A)-containing mRNP of cryptobiotic gastrulae of *Artemia* sp. At least it has a dual effect on polyadenylation and influences both initiation and elongation. The latter protein is confined to the cryptobiotic state of *Artemia* sp. (L. Van Hove, pers. commun.). After binding of poly(A) polymerase to the 17S mRNP activation of the initiation of polyadenylation seems to be regulated by the mRNP itself. An mRNP bound protein kinase is able to phosphorylate the enzyme resulting in an increase of the affinity towards the RNA primers. Further work is in progress to resolve the regulation and specificity of polyadenylation of mRNP.

Conclusions

A cytoplasmic poly(A) polymerase has been purified which shows a high *in vitro* specificity towards native non-polysomal poly(A)-containing mRNP in the presence of a small ribonucleoprotein. It is suggested that the latter particle takes part in the primer recognition mechanism. Kinetic studies show that the initiation of polyadenylation is influenced by a variety of factors. It has to be noted however that the latter factors affect the lag phase but have no effect on the kinetics of elongation except for the M_r 38 000 poly(A)-binding protein. This protein seems to be functional in the regulation mechanism of polyadenylation.

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Structure of ribosomal RNA constituents of *Artemia* and comparison with other species

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Abstract

Fast growing sequence information derived from ribosomal RNAs allows the derivation, on a comparative basis, of universal secondary structure models for these molecules and the construction of evolutionary trees. Especially the last aspect causes some problems : the determined 5S rRNA sequence of *Artemia* proved to occupy an unusual position relative to other arthropods. 5S rRNA from other related organisms and 5.8S and 18S rRNA from *Artemia* were sequences in order to test possible causes for these aberrant findings.

Introduction

The flow of sequence information derived from ribosomal RNAs is growing at a high rate. In a recent review (Erdmann *et al.*, 1985), covering data up to the end of 1984, some 238 5S rRNA sequences and 29 5.8S rRNAs are compiled. The number of known 5S rRNA sequences is expected to exceed 350 by the end of 1985. A majority of about 60% accounts for sequences from eukaryotic species, 30 % comes from eubacteria and the remainder originates from archaebacteria but also from chloroplasts and plant mitochondria possessing organelle-specific 5S rRNA.

Although until recently poorly documented, the number of large ribosomal RNA sequences is constantly growing : 30 small subunit RNAs and 17 large subunit RNAs being reported in the latest surveys (Nelles *et al.*, 1984a ; Noller, 1984).

The large flux of information and the universal occurrence of the rRNA molecules, makes them useful for reconstructing evolutionary relationships between all living species. The fact that sequences are available for different species allows derivation of secondary structure characteristics on a comparative basis. We determined the sequences of 5S, 5.8S, and 18S rRNA from *Artemia* and compared them with other sequences.

Materials and methods

Isolation of rRNA

5S rRNA from *Artemia* was obtained by isolating ribosomes (Zasloff and Ochoa, 1971) followed by phenol treatment, a gel filtration step (Monier, 1971), and purification on a preparative 10% polyacrylamide gel in the presence of 7 M urea.

For the isolation of 5.8S rRNA and 18S rRNA, dissociated subunits were prepared (Zasloff and Ochoa, 1971), treated with phenol and the RNA precipitated with ethanol. In order to dissociate 5.8S from 28S rRNA, a solution containing 8 M urea was heated for 10 min at 80 °C and further purified on a preparative polyacrylamide gel (Ursi *et al.*, 1982).

Isolation of rDNA

DNA was isolated from newborn *Artemia* larvae. A *Hin*dIII fragment containing the complete 18S rRNA gene (Cruces *et al.*, 1981) was cloned in pBR327 (Nelles *et al.*, 1984a) and identified using a[^{125}I]-labelled (Commerford, 1971) 18S rRNA probe. Large quantities of the *Hin*dIII fragment were made by fractionation of the digested plasmid on low melting agarose and extraction (Wieslander, 1979).

Sequencing 5S and 5.8S rRNA

The major part of both nucleotide sequences was obtained by the chemical degradation gel reading method (Peattie, 1979). Often additional evidence was obtained by gel electrophoresis of enzymatically degraded RNA (Dams *et al.*, 1983) and by a two-dimensional combination of gel and thin layer electrophoresis of randomly degraded RNA (Tanaka *et al.*, 1980). Determination of the terminal nucleotides and of occasionally occurring modified nucleotides was performed as described (Diels *et al.*, 1981 ; Ursi *et al.*, 1982).

Sequencing of the 18S rRNA gene

Subfragments of the *Hin*dIII fragment were obtained by digestion with a series of restriction enzymes and subcloned in M13mp8. Sequencing was according to Sanger *et al.* (1980) and sequencing gels were prepared according to Garoff and Ansorge (1981) and run either at room temperature or at elevated temperature using a special electrophoresis setup (Macrophor, LKBn, Bromma, Sweden).

Construction of evolutionary trees

Trees were constructed by weighted pairwise clustering (Sokal and Michener, 1958) starting from a dissimilarity matrix corrected for double and back mutations (Kimura and Ohta, 1972). Preceding positions of the sequences into an alignment was based on the occurrence of conserved sequence elements and secondary structure features (De Wachter *et al.*, 1982).

Results and discussion

Structure of 5S rRNA

The 5S rRNA primary structure of *Artemia* was determined, aligned with other 5S sequences and found to fit a general eukaryotic 5S secondary structure model (Fig. 1a). This model is also applicable to eubacterial 5S RNAs with minor changes (Fig. 1b,c). Archaebacterial molecules show more heterogeneity, but share some secondary structure features with eukaryotes and eubacteria. The general topology of 5S RNA secondary structure, with five helix segment separated by loops as shown in Fig. 1, is universal. The length of certain loops as hairpin loop H_1, and the degree of symmetry of the internal loops, is characteristic for the eukaryotic, eubacterial, or archaebacterial origin of the considered species.

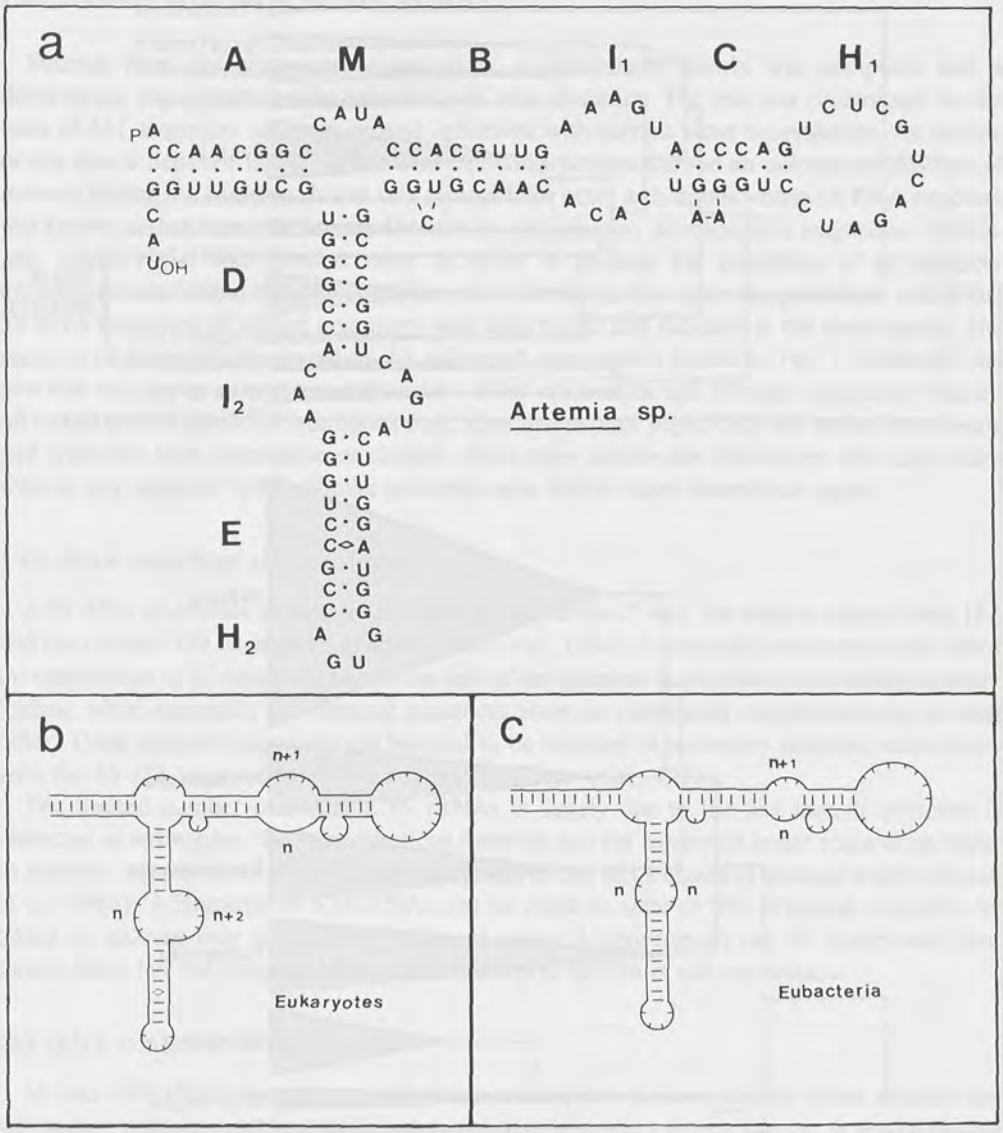

FIG. 1. Secondary structure of 5S rRNA of *Artemia* and derived generalized structures. The sequence of *Artemia* is shown folded in the proposed secondary structure model (a) containing helices (A-E) interrupted by loops (M, I_1, I_2) and ending in hairpin loops (H_1, H_2). Characteristic bulges are present on the helices. Base pairing is indicated by dots and G · U is considered as a base pair even at the end of a helix. An "odd" base pair occurs in helix E and is indicated by a losenge. The secondary structure of *Artemia* was generalized for eukaryotes (b) and eubacteria (c) as outlined. Characteristic symmetry or asymmetry of internal loops is indicated by the number defining the relative length of the two strands.

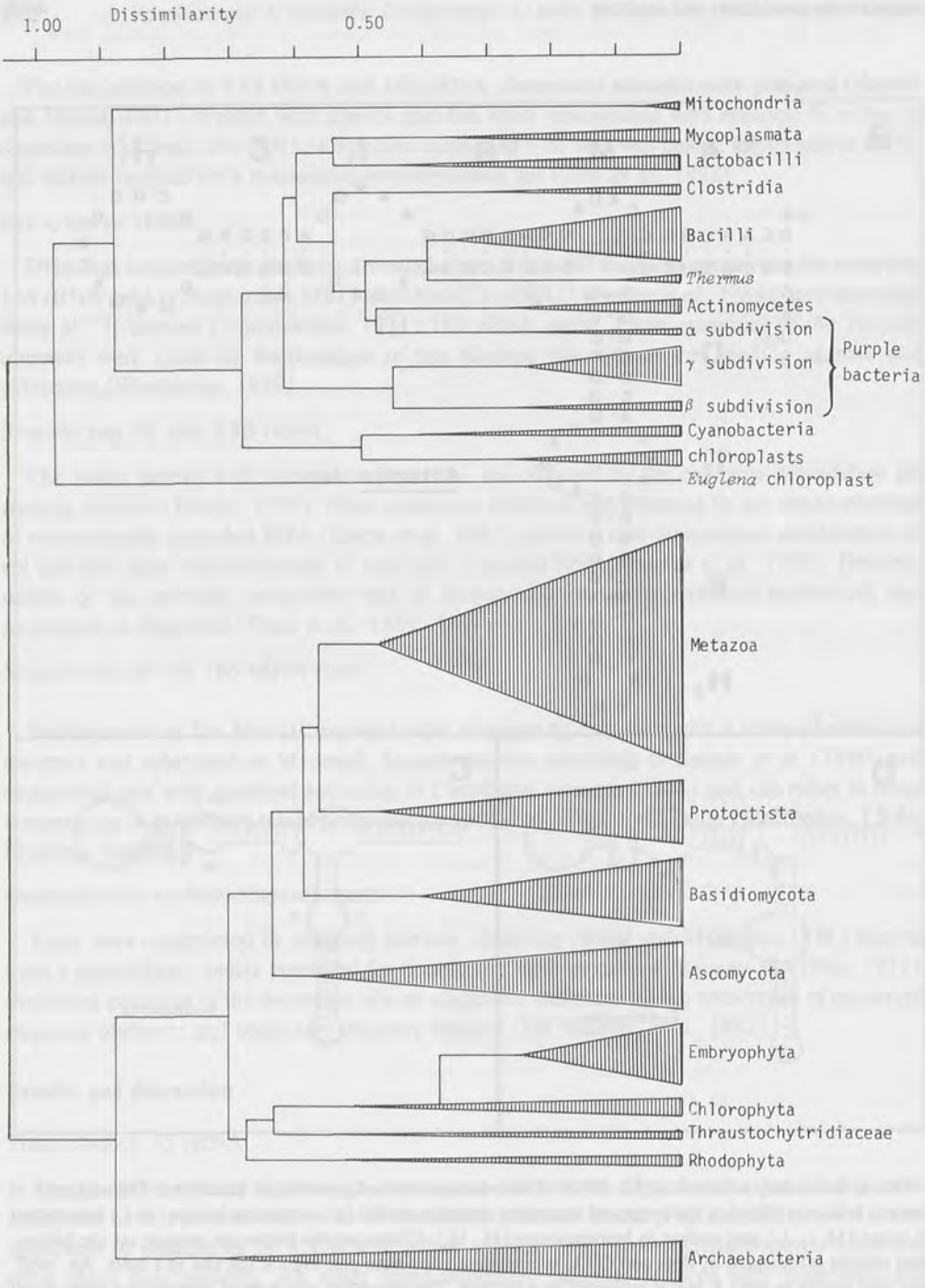

FIG. 2. Outline of a phylogenetic tree constructed from 251 sequences of 5S rRNA. Sequences are grouped into major taxa, which are represented by triangles. The base of these triangles is proportional to the number of incorporated sequences. The top corresponds to the earliest point of divergence within the taxon.

Evolutionary tree on the basis of 5S rRNA

Starting from the alignment of sequences, a dissimilarity matrix was computed and a dendrogram was constructed by weighted pair wise clustering. The tree was constructed on the basis of 251 sequences and is in general agreement with current views on evolution. An outline of this tree is depicted in Fig. 2. Previous tree constructions showed an unexpected position of *Artemia* among the Metazoa. It was very remote from other arthropods whose 5S RNA structure was known at that time : the insects *Drosophila melanogaster, Acyrthosiphon magnoliae, Philosamia cynthia-ricini,* and *Bombyx mori.* In order to exclude the possibility of an apparent evolutionary gap within the *Arthropoda* due to an artefact in the clustering procedure, additional 5S RNA sequences of related organisms were determined and included in the dendrogram. The result, a phylogenetic tree based on 15 arthropod sequences is shown in Fig. 3. However, this new tree still shows several inconsistencies : lower crustaceans and *Limulus polyphemus* branch off before the divergence of arthropods from other invertebrate phyla. Only the higher crustaceans and arachnids form homogeneous clusters, while other species are interwoven with each other without any apparent order. Similar problems arise within other invertebrate phyla.

5.8S rRNA structure and evolution

5.8S RNA of *Artemia* showed length heterogeneity at the 5'-end, the longest variant being 162 and the shortest 159 nucleotides in length (Ursi *et al.*, 1982). A secondary structure model based on comparison of 12 sequences shows that part of the molecule is involved in secondary structure folding, while essentially the terminal sequences show no convincing complementarity to each other. These terminal sequences are believed to be involved in secondary structure interactions with the 26-28S large subunit RNA component (Ursi *et al.*, 1983).

The limited number of available 5S rRNAs is largely due to the fact that its presence is restricted to eukaryotes, the more laborious isolation and the somewhat larger sequencing work. In addition, some insect 5.8S RNAs are composed of two RNA chains of unequal length (Jordan *et al.*, 1980). Alignments of 5.8S RNAs can be made in spite of this structural variability by taking in account only homologous sequence areas. A dendrogram can be constructed (not shown here) but the coupling between the arthropod species is still too distant.

18S rRNA structure and evolution

5S and 5.8S rRNA are rather small molecules and the question can be raised whether the information content is not too small to reconstruct relationships among species. A way to resolve this question is to look at larger molecules and for this reason, the nucleotide sequence of 18S rRNA was determined using the DNA cloning and sequencing approach (Nelles *et al.*, 1984a). Previously attempts to elucidate directly the RNA sequence were hindered by the presence of a "hidden break" in the 28S rRNA component (Nelles *et al.*, 1984b). When RNA was prepared from total ribosomes, 18S was mixed up with two fragments of about equal length derived from 28S rRNA. The 3'-terminal labelling resulted in incorporation of radioactivity in the fragments and sequencing revealed – rather by chance – the sequence preceding the knick in 28S rRNA. Alignment with the yeast 26S rRNA sequence showed the knick to correspond with position 1 565 in the latter rRNA.

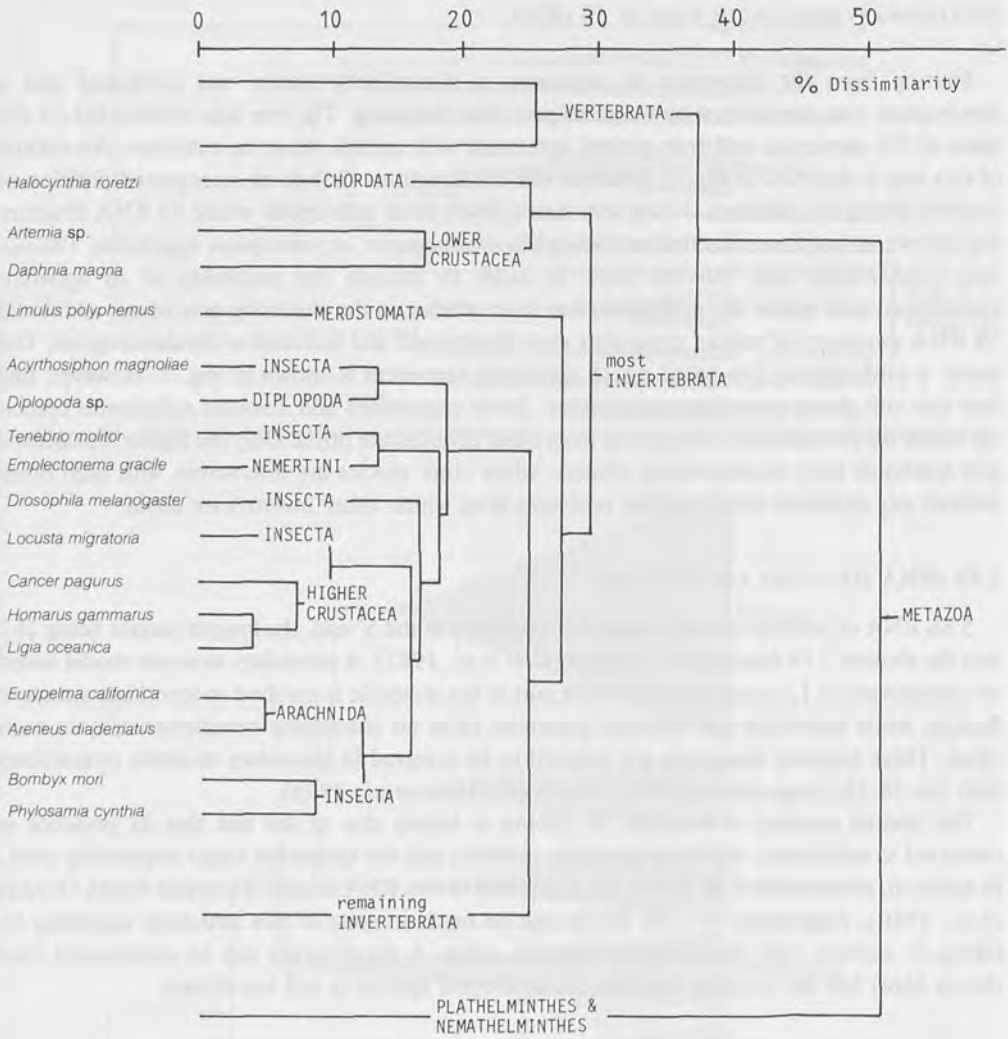

FIG. 3. Simplified version of a tree based on 75 metazoan sequences. In this tree, 15 arthropod sequences are incorporated. *Halocynthia roretzi* and *Emplectonema gracile* do not belong to this phylum.

The sequence of 18S rRNA of *Artemia* was aligned with other small subunit rRNAs and a detailed secondary structure model was presented (Nelles *et al.*, 1984a). The alignment construction is laborious due to the large variation in length especially when species from mitochondrial origin are included : *e.g.* mammalian mitochondrial small subunit RNA is almost half the size of the 1 810 nucleotides long *Artemia* sequence. The deletions in one taxon (*e.g.* animal mitochondria) with respect to others (*e.g.* eukaryotes) are localized in certain regions of high sequence variability. A preliminary tree was constructed excluding the mammalian mitochondrial sequences and is depicted in Fig. 4.

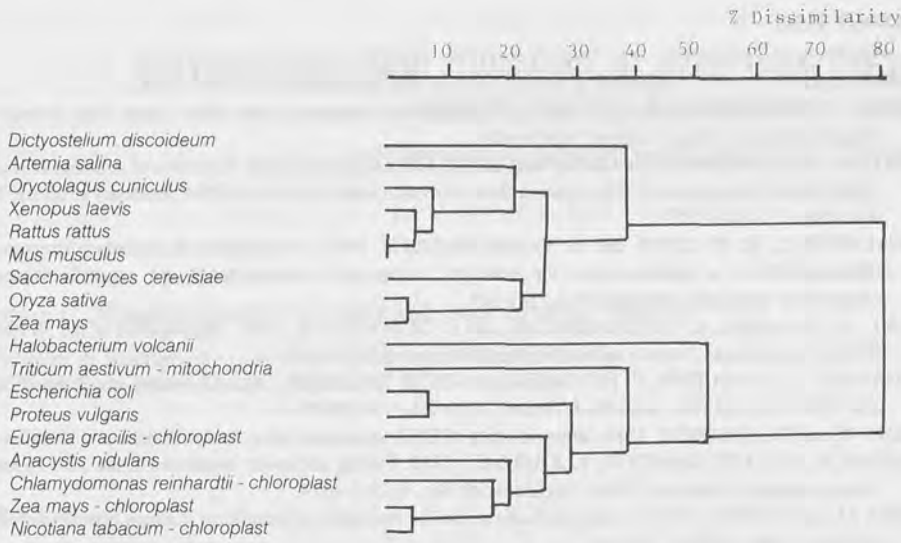

FIG. 4. Phylogenetic tree constructed for 18 small subunit rRNAs. Mammalian mitochondrial sequences were not incorporated and insertions were counted as one difference position, regardless the length of the insert.

The number of small subunit ribosomal RNA sequences is presently rather limited and the derived tree is yet too poorly documented to draw firm conclusions. However, a flow of new sequence information can be expected from several laboratories in the near future. Meanwhile we started to further document the collection by sequencing the 18S rRNA of the spider *Eurypelma californica.*

In the mean time, methods to correct for differences in evolutionary rate in the tree construction are studied in order to come to a better clustering of arthropod species.

Conclusions

The sequences of *Artemia* 5S, 5.8S, and 18S rRNA were established and incorporated in respective alignments of sequences of other species. These allowed the construction of secondary structure models and computer-aided building of phylogenetic trees. In contrast with 5.8S rRNA, a large number of 5S rRNAs is known. It was found difficult to derive the correct phylogenetic position of *Artemia* among the arthropods on the basis of 5S RNA sequences. To further document the evolutionary background, other 5S rRNA sequences from related organisms were established but did not provide a completely satisfactory clustering. Larger ribosomal RNA molecules are now being compared and improved tree construction methods are studied.

Acknowledgements

At the time of the experiments, L.H., L.D., L.N. and D.U. received IWONL fellowships and E.H. was a research assistant from the FKFO. Our research was supported in part by a grant of the NFWO to A.V. and R.D.

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Polypeptide chain initiation in *Artemia* embryos : functional and structural studies on chain initiation factor in *Artemia*

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Abstract

Significant differences exist between eIF-2 preparations from *Artemia* embryos and rabbit reticulocytes. Under conditions where Mg^{2+} inhibits ternary (eIF-2·Met-tRNA$_f$·GTP) and binary (eIF-2·GDP) complex formation with reticulocyte eIF-2, Mg^{2+} stimulates these reactions with *Artemia* eIF-2. The reticulocyte eIF-2·GDP binary complex is stable in the presence of Mg^{2+} and GTP and requires another factor (GEF, guanine nucleotide exchange factor) for the exchange of bound GDP for free GTP. *Artemia* eIF-2 readily exchanges bound GDP for GTP and this reaction is not significantly enhanced by GEF. Phosphorylation of the α-subunit of reticulocyte eIF-2 by the heme-controlled repressor (HCR) results in the inhibition of protein synthesis. In contrast, *Artemia* eIF-2 remains active in protein synthesis after phosphorylation by HCR. Inhibition of protein synthesis in reticulocyte lysates is due to the inability of GTP to displace GDP from the eIF-2(α-P)·GDP binary complex and may be relieved by the addition of eIF-2 or GEF, or by high levels of GTP (1 mM). Phosphorylation of the *Artemia* eIF-2 reduces the rate of nucleotide exchange by less than 50 % even at low GTP concentrations (0.2 mM).

We examined some of the structural features of *Artemia* and reticulocyte eIF-2 in order to have a better understanding of their functional properties. The tryptic phospho- and iodopeptides of the isolated subunits of *Artemia* and reticulocyte eIF-2 were compared. Partial trypsin digestion of *Artemia* eIF-2(α-P) after phosphorylation by HCR generates a small phosphopeptide (M_r 4 000) as reported for reticulocyte eIF-2 (Zardeneta *et al.*, 1982). Upon extensive trypsin digestion, the two-dimensional phosphopeptide maps of the α-subunit are identical for the *Artemia* and reticulocyte factors, whereas the ^{125}I-maps of the isolated subunits do not appear to be similar. Antibodies prepared against the individual subunits of *Artemia* eIF-2 do not react efficiently with any of the subunits of the reticulocyte factor.

We isolated a casein kinase from the ribosomal high salt washes and the post-ribosomal supernatants of *Artemia* embryos that phosphorylates the α-subunit of *Artemia*, yeast and wheat germ eIF-2. This enzyme, however, phosphorylates the β-subunit of reticulocyte eIF-2. Two-dimensional mapping of the tryptic phosphopeptides of *Artemia* eIF-2(α) demonstrates that HCR and the *Artemia* kinase phosphorylate different sites. Phosphorylation by this kinase does not appear to alter the ability of *Artemia* eIF-2 to undergo GDP/GTP exchange or bind Met-tRNA$_f$. The purified kinase appears to have a native M_r of 150 000 to 180 000 M_r, and phosphorylates α-, β-, and γ-casein, and phosvitin, but not histones or ribosomal proteins. The kinase specifically requires Mg^{2+}, utilizes ATP or GTP as the phosphoryl donor, and is not influenced by the addition of cyclic AMP, cyclic GMP, EGTA, or phospholipid.

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Abbreviations used

eIF-2 : the eukaryotic initiation factor 2 ;
eIF-2(α-P) : eIF-2 in which the α subunit is phosphorylated ;
GEF : the guanine nucleotide exchange factor ;
HCR : the heme-controlled repressor ;
CK II : casein kinase II ;
DPCC-trypsin : diphenyl carbamyl chloride-treated trypsin.

Introduction

Polypeptide chain initiation factor 2 is a central component in the regulation of protein synthesis (Safer *et al.*, 1982 ; Voorma and Amesz, 1982). Much of the data collected on the regulation of eIF-2 activity is derived from initiation factor preparations from rabbit reticulocytes. Because of the differences in properties in certain functional assays between *Artemia* and reticulocyte eIF-2 preparations (Mehta *et al.*, 1983 ; Wahba and Woodley, 1984), it is possible that the *Artemia* factor may not be regulated in the same manner as eIF-2 derived from higher eukaryotes such as rabbit reticulocytes and human tissue culture cells (Mariano *et al.*, 1985).

Mechanisms for regulating eIF-2 activity in higher eukaryotes may involve : 1) the energy charge (the ratio of GTP to GDP) ; 2) a heme-controlled repressor (HCR) that phosphorylates the α-subunit of eIF-2 ; and 3) the activity of a multisubunit factor required for nucleotide exchange after the release of eIF-2 from the ribosomal initiation complex. A scheme for the function of eIF-2 in polypeptide chain initiation is illustrated in Fig. 1.

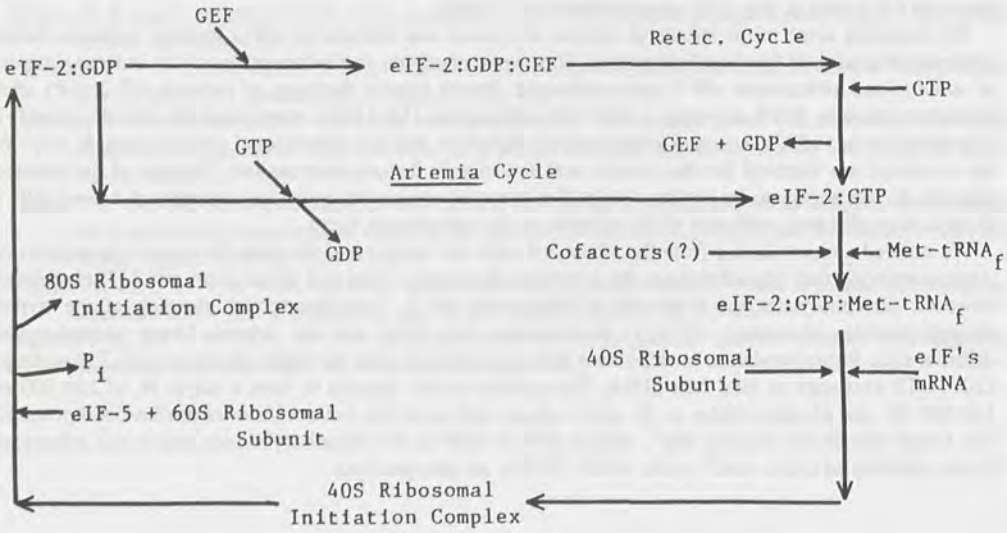
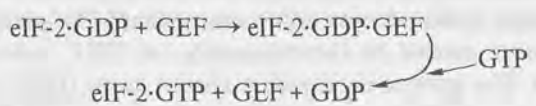


FIG. 1. Scheme for the eIF-2 cycle in polypeptide chain initiation.

GDP binds to reticulocyte eIF-2 with a very high affinity (K_D = 30 nM) (Walton and Gill, 1975), and is released very slowly from an eIF-2·GDP binary complex in the presence of physiological levels of Mg^{2+} and GTP (Panniers and Henshaw, 1983). Since eIF-2 is presumably released from the ribosomal initiation complex as a eIF-2·GDP binary complex (Trachsel and Staehelin, 1978), the GDP must be removed before the eIF-2 can enter the next cycle of polypeptide chain initiation (Clemens *et al.*, 1982 ; Salimans *et al.*, 1984). A guanine nucleotide exchange factor (GEF) is required for the GTP-dependent release of GDP from this binary complex (Goss *et al.*, 1984) :



The relative concentrations of GTP and GDP may play a role in regulating the level of active eIF-2 available for the formation of an eIF-2·GTP·Met-$tRNA_f$ ternary complex. The points at which these nucleotides may exert their effects include : 1) competition between GDP and GTP for binding to eIF-2 (Walton and Gill, 1975 ; Safer *et al.*, 1979) ; 2) the GTP requirement for displacement of GDP from an eIF-2·GDP·GEF ternary complex (Safer *et al.*, 1982 ; and 3) regulation of the phosphorylation/dephosphorylation of eIF-2(α) (Ranu and London, 1979 ; Petryshyn *et al.*, 1982 ; Ranu, 1982).

The absence of heme (Legon *et al.*, 1973 ; Farrell *et al.*, 1977) or the addition of ds-RNA (Levin and London, 1978 ; Levin *et al.*, 1980 ; Ranu, 1980) to reticulocyte lysates activates a cAMP-independent protein kinase that phosphorylates the α-subunit of eIF-2 (eIF-2(α-P)). Phosphorylation of eIF-2 by these kinases is associated with the cessation of protein synthesis in reticulocyte lysates and is due to the inability of GEF to catalyze the exchange of GTP for GDP (Siekierka *et al.*, 1982 ; Matts *et al.*, 1983 ; Goss *et al.*, 1984).

The affinity of GEF for eIF-2(α-P)·GDP is less than for the non-phosphorylated factor (Goss *et al.*, 1984). However, at physiological concentrations of eIF-2 and GEF most of the GEF will be in a eIF-2(α-P)·GDP·GEF complex (Konieczny and Safer, 1983). The net effect is to trap GEF as a eIF-2(α-P)·GDP·GEF complex, rendering it unavailable to catalyze nucleotide exchange with nonphosphorylated eIF-2·GDP binary complexes.

There are significant differences between rabbit reticulocyte eIF-2 and the *Artemia* factor. Although both eIF-2 preparations consist of three nonidentical subunits, the M_r of these subunits differ markedly (MacRae *et al.*, 1979). Furthermore, upon phosphorylation of *Artemia* eIF-2 by reticulocyte HCR, it remains active in protein synthesis in reticulocyte lysates (Woodley *et al.*, 1981). In an attempt to understand these differences, we compared the properties of both factors under various assay conditions, and examined some of the structural features of these preparations. These two eIF-2 preparations were compared in the following ways : 1) the effect of Mg^{2+} on binary (eIF-2·GDP) and ternary (eIF-2·GTP·Met-$tRNA_f$) complex formation ; 2) ability to exchange GDP for GTP ; 3) one- and two-dimensional peptide mapping after partial and extensive trypsin digestion ; 4) the immunological reactivity of reticulocyte subunits with antibodies raised against the individual (α, β and γ) subunits of *Artemia* eIF-2 ; and 5) specificity of reticulocyte HCR and casein kinases isolated from rabbit muscle and *Artemia* embryos in phosphorylating specific subunits of eIF-2.

Materials and methods

Preparations

Polypeptide chain initiation factors

Desiccated *Artemia* embryos (San Francisco Bay Brand, Newark, CA) and rabbit reticulocyte lysates (Green Hectares, Oregon, WI, USA) were used as the source of initiation factors. Both *Artemia* and reticulocyte eIF-2 were prepared by a modification of the procedure described by MacRae *et al.* (1979). Neither factor was chromatographed on hydroxylapatite and the heparin-Sepharose step in the preparation of reticulocyte eIF-2 was omitted. The phosphocellulose fraction of each factor was further purified by chromatography on FPLC cation and anion exchange columns (Pharmacia). The guanine nucleotide exchange factor (GEF) was purified from the post-ribosomal supernatant of rabbit reticulocyte lysates by chromatography on DEAE- and phosphocellulose. This fraction was further purified by chromatography on FPLC cation and anion exchange columns (Pharmacia). The wheat germ embryo and yeast eIF-2 preparations were kindly provided by Joanne M. Ravel (Department of Chemistry, University of Texas, Austin) and Naba K. Gupta (Department of Chemistry, University of Nebraska, Lincoln), respectively.

eIF-2-specific protein kinases

The rabbit reticulocyte heme-controlled repressor (HCR) was prepared as previously described (Kramer *et al.*, 1976). Casein kinase II from rabbit muscle was a gift from Erwin Reimann (Department of Biochemistry, Medical College of Ohio, Toledo). The *Artemia* kinase was isolated from dormant *Artemia* embryos and appears to be similar to casein kinase II. The purification of the enzyme is outlined as follows : The *Artemia* post-ribosomal supernatant and ribosomal salt wash fractions were prepared from hydrated embryos as described earlier (MacRae *et al.*, 1979). The 25-60 % $(NH_4)_2SO_4$ fraction of the 0.5 M NH_4Cl wash from the ribosomes contained the protein kinase activity. The ammonium sulfate fraction was applied to a DEAE-cellulose column previously equilibrated with buffer containing 100 mM KCl, 20 mM Tris-Cl (pH 7.5), 1 mM dithiothreitol, 50 mM EDTA, 10 % glycerol and the protein kinase activity eluted with buffer containing 300 mM KCl. The protein was then applied to a phosphocellulose column previously equilibrated with buffer containing 300 mM KCl. The column was thorougly washed with this buffer, and the kinase was eluted with a linear gradient (300-1 000 mM KCl). These fractions were dialyzed and chromatographed on a second phosphocellulose column as before. The active fractions from the second phosphocellulose column were combined and concentrated by dialysis for 4 h against buffer containing 300 mM KCl and 20 % polyethylene glycol (M_r 20 000). This fraction was chromatographed on an Ultrogel AcA 34 column equilibrated with buffer containing 300 mM KCl. Proteins used as molecular weight standards were catalase (M_r 230 000), bovine γ-globulin (M_r 160 000) and bovine hemoglobin (M_r 64 000). On the basis of its elution from Ultrogel AcA 34, it was estimated to have a native M_r of 150-180 000. The fractions from Ultrogel AcA 34 having protein kinase activity were concentrated by dialysis against buffer containing 20 % (w/v) polyethylene glycol (M_r 20 000) and were then applied to a heparin-Sepharose 4B column previously equilibrated with buffer containing 100 mM KCl. The column was thoroughly washed with buffer and the bound protein successively eluted with buffer containing 300 and 700 mM KCl. Fractions containing protein

kinase activity were eluted with buffer containing 700 mM KCl and were dialyzed overnight against buffer containing 100 mM KCl and 50 % (v/v) glycerol. The enzyme activity is retained for at least 6 months when stored at - 20 °C. A preliminary description of this kinase was reported (Dholakia *et al.*, 1984).

Methods

eIF-2 activity assays

The factor was assayed in ternary (eIF-2·GTP·Met-tRNA$_f$) and binary (eIF-2·GDP) complex formation and nucleotide exchange as previously described (MacRae *et al.*, 1979 ; Mehta *et al.*, 1983).

Kinase activity assays

Phosphorylation of eIF-2 by HCR was performed in the presence of [γ-^{32}P]ATP (1 000 cpm/pmol), 2 mM Mg^{2+} and 100 mM KCl and was re-isolated by chromatography on phosphocellulose (Woodley *et al.*, 1981). Conditions for the phosphorylation of eIF-2 by the *Artemia* casein kinase were similar to those for phosphorylation by HCR, except 10 mM Mg^{2+} and 200 mM KCl were used. The specificity of phosphorylation was determined by electrophoresis of the reaction products in dodecylsulfate/polyacrylamide gels (Laemmli, 1970), followed by autoradiography of the gel (Woodley *et al.*, 1981). The activity of the *Artemia* and rabbit muscle casein kinases were quantitated by precipitation of the reaction products in 10 % trichloroacetic acid containing 5 mM KH_2PO_4. The precipitates were filtered through nitrocellulose membranes (0.45 µ), dried, and counted for radioactivity in a liquid scintillation counter.

Peptide mapping

eIF-2 was phosphorylated with [γ-^{32}P]ATP using either HCR or the *Artemia* kinase as described above. For [^{125}I]-labeling, the subunits of eIF-2 were separated by electrophoresis in dodecylsulfate/polyacrylamide gels and detected by brief staining. Excised gel slices containing the proteins were washed with 25 % isopropanol and then with 10 % methanol. The proteins in the dried gel slices were radioiodinated by the sequential addition of 10 µl containing 0.5 mCi of Na^{125}I, 25 µl of 0.5 M phosphate buffer, pH 7.5 and 5 µl of chloramine-T (5 mg/ml). The reaction was stopped after 30 min at room temperature by the addition of 1.0 ml sodium metabisulfite (1 mg/ml) and noncovalently bound ^{125}I was removed by washing the gel slices in 10 % methanol (Montelaro *et al.*, 1984).

Limited (up to 30 min) trypsin digestion of *Artemia* eIF-2(α-^{32}P) was as described by Zardeneta *et al.* (1982). Aliquots of the partial eIF-2 digests were removed at different time intervals, mixed with 1 µg soybean trypsin inhibitor and analyzed either by electrophoresis in dodecylsulfate/polyacrylamide gels or by two-dimensional peptide mapping (Elder *et al.*, 1977a, b ; Mehta *et al.*, 1983 ; Montelaro *et al.*, 1984). For extensive digestion subunits were initially digested for 24 h at 37 °C with DPCC-treated trypsin (60 µg/ml). Samples digested for longer than 24 h received 60 µg/ml of additional trypsin after each 24 h interval (Gross *et al.*, 1981).

Immunological methods

Purified *Artemia* eIF-2 was subjected to dodecylsulfate/polyacrylamide gel electrophoresis. After Coomassie Brilliant Blue R-250 staining, the gel pieces containing the individual eIF-2

subunits were washed and homogenized in phosphate-saline buffer (sodium phosphate, 10 mM, pH 7.2 ; NaCl, 150 mM), mixed with equal volume of Freund's complete adjuvant and injected into rabbits intradermally. The initial injection of each subunit (500 μg) was followed by boosters of 300 μg at 30 and 40 days after the initial injection. Serum was collected by centrifugation and solid $(NH_4)_2SO_4$ was added to 50 % saturation. The precipitates were dissolved in phosphate saline buffer, dialyzed and stored at - 80 °C.

For Western blot analysis, proteins in the eIF-2 preparations were separated by electrophoresis in dodecylsulfate/polycrylamide gels and electroblotted onto a nitrocellulose sheet for 12-15 h to achieve quantitative transfer of the proteins (Towbin *et al.*, 1979) and the blot was processed according to the method of Johnson *et al.* (1984). The immunoblot was dried and exposed to Kodak XAR-5 film.

Results

Functional properties of *Artemia* and reticulocyte eIF-2

There are both similarities as well as differences between eIF-2 preparations from *Artemia* embryos and rabbit reticulocytes. Both factors have three subunits, and in each case the lowest M_r subunit (α-subunit) is phosphorylated by HCR. However, upon phosphorylation by HCR, the reticulocyte factor unlike *Artemia* eIF-2, is no longer catalytically functional in polypeptide chain initiation. This observation led us to investigate further the functional differences between these initiation factor preparations.

Effect of Mg^{2+} on formation of eIF-2· nucleotide binary complexes

Artemia or reticulocyte eIF-2 forms a binary complex with GDP or GTP (Walton and Gill, 1975 ; MacRae *et al.*, 1979). With *Artemia* eIF-2, the K_D for GDP is 28 nM and that for GTP about 10 μM (MacRae *et al.*, 1979). Similar values were obtained for reticulocyte eIF-2 (Walton and Gill, 1975). GDP binding is stimulated by the addition of Mg^{2+} to binary complex assays with *Artemia* eIF-2, whereas with the reticulocyte factor Mg^{2+} reduces the extent of GDP binding approximately 3-fold (Table I and Fig. 2).

The addition of Mg^{2+} to reticulocyte eIF-2 stabilizes the binding of GDP which is present in the factor preparation. Consequently, further binding of exogenous GDP is reduced (Bagchi *et al.*, 1982 ; Siekierka *et al.*, 1982). We, therefore, compared the ability of *Artemia* and reticulocyte eIF-2 to form stable eIF-2·GDP binary complexes. Each eIF-2 preparation was incubated with [^{3}H]GDP at 30 °C in the absence of Mg^{2+}, chilled to 0 °C, and the concentration of Mg^{2+} adjusted to 1 mM. The binary complex was then isolated by chromatography on phosphocellulose (Mehta *et al.*, 1983). The isolated eIF-2·GDP complex thus formed with eIF-2 from either *Artemia* or rabbit reticulocytes is stable at 30 °C in the absence of added nucleotide (Fig. 3). However, the *Artemia* binary complex readily exchanges bound GDP for free GTP (Fig. 3, panel A). This is in contrast to the reticulocyte eIF-2·GDP complex, with is stable in the presence of added GTP (Fig. 3, panel B).

Formation of eIF-2· GDP· Met-tRNA ternary complexes

The effect of Mg^{2+} on ternary complex formation with *Artemia* eIF-2 was also examined (Fig. 2). Ternary complex formation is stimulated 35-40 % by the addition of up to

TABLE I

Formation of binary (eIF-2·GDP) and ternary (eIF-2·GTP·Met-tRNA$_f$) complexes with phosphorylated [eIF-2(α - P)] and nonphosphorylated eIF-2 from rabbit reticulocytes and *Artemia*

Additions	[^{3}H]GDP bound		[^{35}S]Met-tRNA$_f$ bound	
	- Mg^{2+}	+ 1 mM Mg^{2+}	- Mg^{2+}	+ 1 mM Mg^{2+}
	(cpm)		(cpm)	
Reticulocyte				
eIF-2	11 200	3 300	8 700	1 700
eIF-2 + GEF	10 300	11 300	9 900	7 700
eIF-2(α - P)	9 500	1 300	6 900	1 600
eIF-2(α - P) + GEF	9 000	1 400	8 800	1 600
Artemia				
eIF-2	5 300	8 800	4 200	6 200
eIF-2 + GEF	8 200	8 500	7 500	8 400
eIF-2(α - P)	4 900	8 200	4 800	6 400
eIF-2(α - P) + GEF	6 600	8 500	6 300	7 100

Reaction mixtures contained, where indicated, 0.5 μg (3.8 pmol) of reticulocyte eIF-2 or eIF-2(α - P); 1 μg (7.1 pmol) of *Artemia* eIF-2 or eIF-2(α - P); nucleotide exchange factor, 0.2 μg (approximately 0.4 pmol assuming 70 % purity); and 0.2 mM GTP. Filtration backgrounds for [^{3}H]GDP (14 000 cpm/pmol), [^{35}S]Met-tRNA$_f$ (10 000 cpm/pmol), and ^{32}P-labeled eIF-2 have been subtracted.

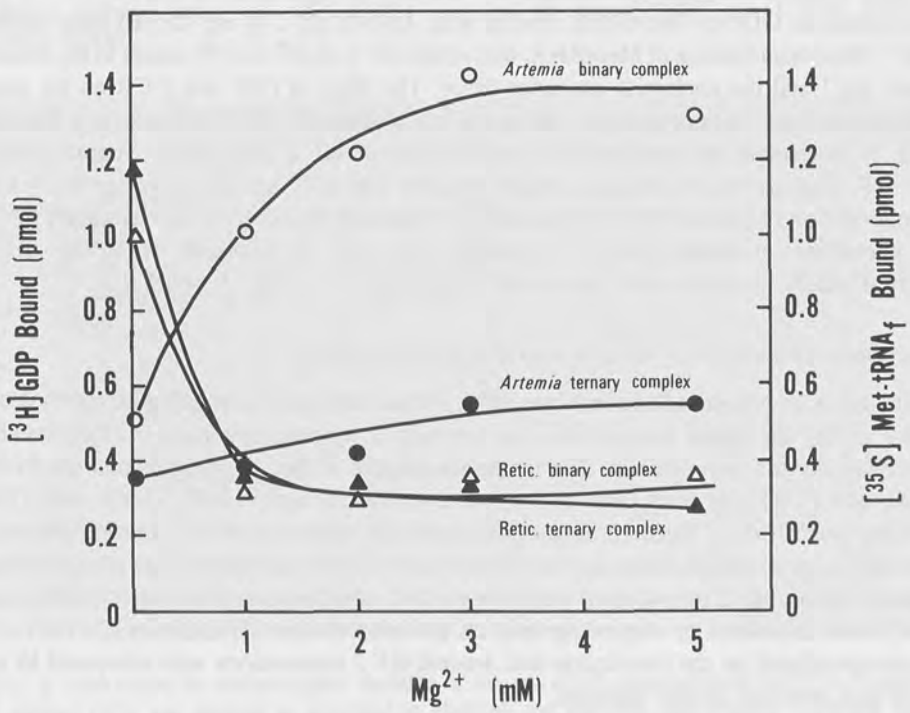


FIG. 2. Effect of Mg^{2+} on binary and ternary complex formation. For ternary complex formation each 75 μl reaction contained 0.8 μg of *Artemia* or 0.57 μg of reticulocyte eIF-2, 0.2 mM GTP and 3 pmol of Met-tRNA$_f$ (14 500 cpm/pmol). Assay of binary complex formation was similar, except that no Met-tRNA$_f$ was present and unlabeled GTP was replaced by 0.53 μM [^{3}H]GDP (5 000-6 000 cpm/pmol). From Mehta *et al.* (1983).

4-5 mM Mg^{2+}. In contrast, the addition of 1 mM Mg^{2+} to ternary complex assays with reticulocyte eIF-2 causes a 3-fold reduction in Met-$tRNA_f$ binding. This inhibition parallels the effect of Mg^{2+} on binary complex formation (Table I, Fig. 2).

Effect of GEF and phosphorylation on nucleotide exchange and ternary complex formation

A multisubunit factor, GEF, isolated from postribosomal supernatants and ribosomal high salt washes of rabbit reticulocytes, is required for GTP-dependent release of GDP from reticulocyte eIF-2 (Konieczny and Safer, 1983 ; Panniers *et al.*, 1983 ; Siekierka *et al.*, 1983). Nucleotide exchange is inhibited if the α-subunit of reticulocyte eIF-2 is phosphorylated by the heme controlled repressor (HCR) (Siekierka *et al.*, 1982 ; Pain and Clemens, 1983 ; Konieczny and Safer, 1983). However, the requirement for a nucleotide exchange factor with eIF-2 preparations from lower eukaryotic systems has not yet been demonstrated. The effect of the nucleotide exchange factor and Mg^{2+} on GDP binding and ternary complex formation with phosphorylated eIF-2 from *Artemia* and rabbit reticulocytes was therefore examined. In the absence of Mg^{2+} phosphorylated and nonphosphorylated eIF-2 from either source forms stable binary or ternary complexes (Table I). However, Mg^{2+} inhibits both binary and ternary complex formation with reticulocyte eIF-2 and eIF-2(α-P), but not with either *Artemia* eIF-2 or eIF-2(α-P). Addition of the nucleotide exchange factor to binary or ternary complex reactions readily reverses the Mg^{2+} inhibition with reticulocyte eIF-2 , but not with eIF-2(α-P). In contrast to this observation there is an increase in GDP or Met-$tRNA_f$ binding with *Artemia* eIF-2 or eIF-2(α-P) upon addition of Mg^{2+}. Maximum binding of Met-$tRNA_f$ with either eIF-2 or eIF-2(α-P) occurs in the presence of both Mg^{2+} and the nucleotide exchange factor. The effect of GEF and HCR on the rate of nucleotide exchange with preformed *Artemia* and reticulocyte eIF-2·GDP complexes is illustrated in Fig. 3. Nucleotide exchange with the isolated *Artemia* eIF-2·GDP binary complex requires only GTP, whereas the reticulocyte complex requires both GTP and the exchange factor GEF. Phosphorylation of reticulocyte eIF-2(α) almost completely blocks [^{3}H]GDP binding (Table I) and nucleotide exchange (Fig. 3), whereas the rate of exchange with the *Artemia* eIF-2(α-P)·GDP binary complex is reduced by less than 50 % (Fig. 3, panel B).

Structural comparison of *Artemia* and reticulocyte eIF-2

Although both *Artemia* and reticulocyte eIF-2 preparations have three subunits, the molecular weights of the individual polypeptides, as determined by electrophoresis in dodecylsulfate/polyacrylamide gels, are different. The molecular weights of the *Artemia* subunits are 52 000, 43 000, and 41 000, whereas those of the reticulocyte factor are 55 000, 52 000, and 37 000 (Woodley *et al.*, 1981). Since HCR phosphorylates the α-subunit of both *Artemia* and reticulocyte eIF-2, we wished to determine whether the same site on each factor was phosphorylated. For this purpose, eIF-2 preparations from *Artemia* and rabbit reticulocytes were phosphorylated by HCR and re-isolated by chromatography on phosphocellulose (Woodley *et al.*, 1981). The site phosphorylated on the reticulocyte and *Artemia* eIF-2 preparations were compared by both limited and extensive trypsin digestion.

Limited digestion of eIF-2

The tryptic digestion was terminated at different time intervals by the addition of soybean trypsin inhibitor. The results in Fig. 4 show that as the trypsin digestion proceeds the α-subunit

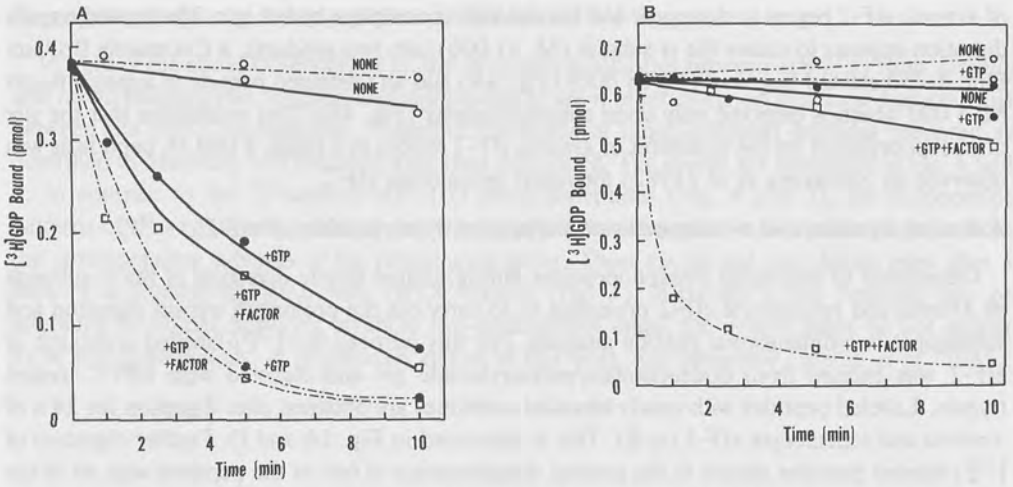


FIG. 3. Kinetics of GDP release from eIF-2·GDP or eIF-2(α - P)·GDP. The assay mixture in 75 µl contained 100 mM KCl, 20 mM Tris-Cl (pH 7.8), 1 mM Mg^{2+}, 0.2 mM GTP and, as indicated, 42 ng of nucleotide exchange factor, 0.6 pmol of reticulocyte eIF-2·GDP or eIF-2(α - P)·GDP (panel A), or 0.4 pmol *Artemia* eIF-2·GDP or eIF-2(α - P)·GDP (panel B). No addition (○); GTP (●); GTP and nucleotide exchange factor (□); eIF-2·GDP (-·-·-) and eIF-2(α - P)·GDP (——). From Wahba and Woodley (1984).

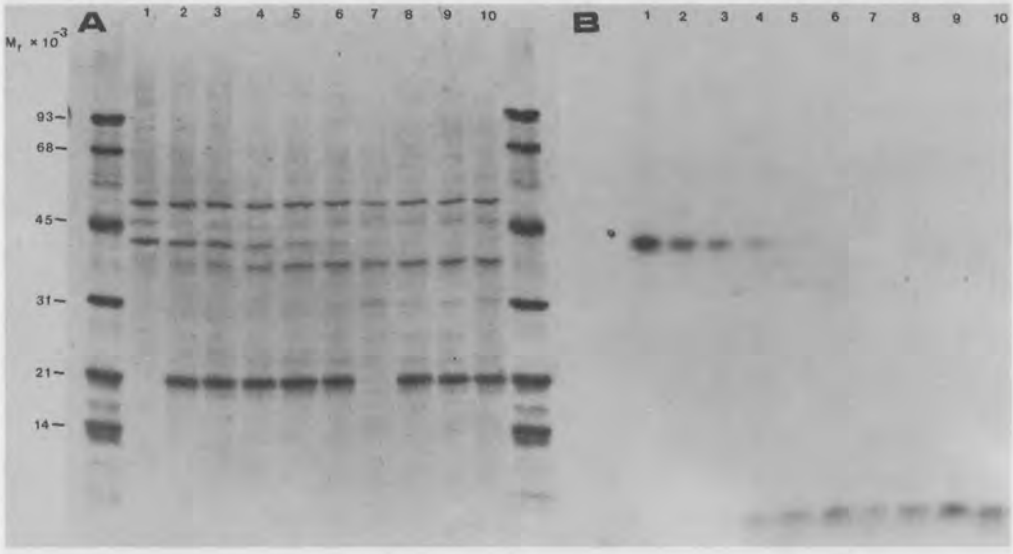


FIG. 4. Time course for limited trypsin digestion of Artemia eIF-2 phosphorylated by HCR. Phosphorylated *Artemia* eIF-2 was digested as described in Materials and methods, and aliquots containing approximately 4 µg eIF-2 were removed at the indicated times and fractionated by electrophoresis in dodecylsulfate/polyacrylamide (12.5 %) gels. Panel A, Coomassie Brilliant Blue R-250 stained gel; panel B, the autoradiogram of the gel. Lanes 1-10 represent aliquots taken at 0, 0.5, 1, 3, 6, 9, 12, 16, 20, and 30 min, respectively.

of *Artemia* eIF-2 begins to disappear and the cleavage is complete by 6-9 min. The limited trypsin digestion appears to cleave the α-subunit (M_r 41 000) into two products, a Coomassie Brilliant Blue R-250 stained fragment (M_r 38 000) (Fig. 4A) and an unstained peptide of approximately M_r 4 000 which is detected only upon autoradiography (Fig. 4B). This establishes that the site of phosphorylation on the α-subunit of *Artemia* eIF-2 resides in a small, 4 000 M_r peptide as was observed by Zardeneta *et al.* (1982) for rabbit reticulocyte eIF-2.

Extensive digestion and two-dimensional mapping of tryptic peptides of eIF-2(α - ^{32}P)

Generation of analogous phosphopeptides during limited tryptic digestion of the α-subunits of *Artemia* and reticulocyte eIF-2 prompted us to carry out the prolonged trypsin digestion and subsequent two-dimensional peptide analyses. For this purpose the [^{32}P]-labelled α-subunit of eIF-2 was excised from dodecylsulfate/polyacrylamide gel and digested with DPCC-treated trypsin. Labeled peptides with nearly identical mobilities are obtained after digestion for 24 h of *Artemia* and reticulocyte eIF-2 (α-P). This is illustrated in Fig. 5A and D. Further digestion of [^{32}P]-labeled peptides results in the gradual disappearance of two of the peptides with all of the label remaining in a single phosphopeptide (Fig. 5, Panels C, F). Results are identical for both *Artemia* and reticulocyte preparations when phosphorylated with HCR and are similar to the observation of Gross *et al.* (1981) for reticulocyte eIF-2.

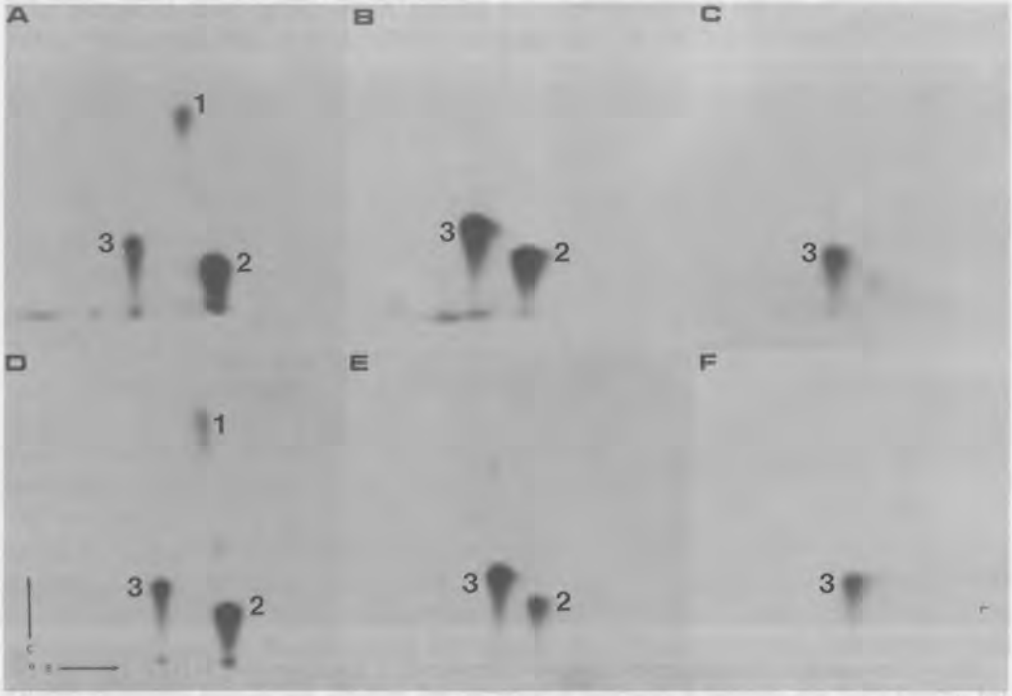


FIG. 5. Extensive trypsin digestion of *Artemia* and reticulocyte eIF-2 phosphorylated by HCR. Autoradiograms of two-dimensional thin-layer chromatograms of ^{32}P-labeled *Artemia* (panels A-C) and reticulocyte eIF-2 (panel D-F) after trypsin digestion for 1, 3, and 5 days. Trypsin digestion and electrophoresis were as described in Materials and methods.

Fingerprint analysis of tryptic peptides of [^{125}I]-labeled eIF-2

In light of the above observations on the structural similarities between eIF-2(α) from *Artemia* and rabbit reticulocytes, we extended our studies to see if other common structural features exist between the eIF-2 preparations. Fingerprint analyses of radio-iodinated peptides prepared as described in Materials and methods were then performed. The results are presented in Fig. 6 and 7. In contrast to the ^{32}P-labeled eIF-2(α) phosphopeptides (Fig. 4 and 5), the iodopeptide patterns of the α-, β- and γ-subunits of *Artemia* eIF-2 do not appear to be similar to those from the corresponding subunits of the reticulocyte factor. These results did not change even after 5 days of continued digestion with DPCC-trypsin (Fig. 7). Although a few peptides may be migrating to similar positions on these two-dimensional maps, the overall maps do not appear to be highly analogous. A preliminary report of this work was presented (Mehta *et al.*, 1985).

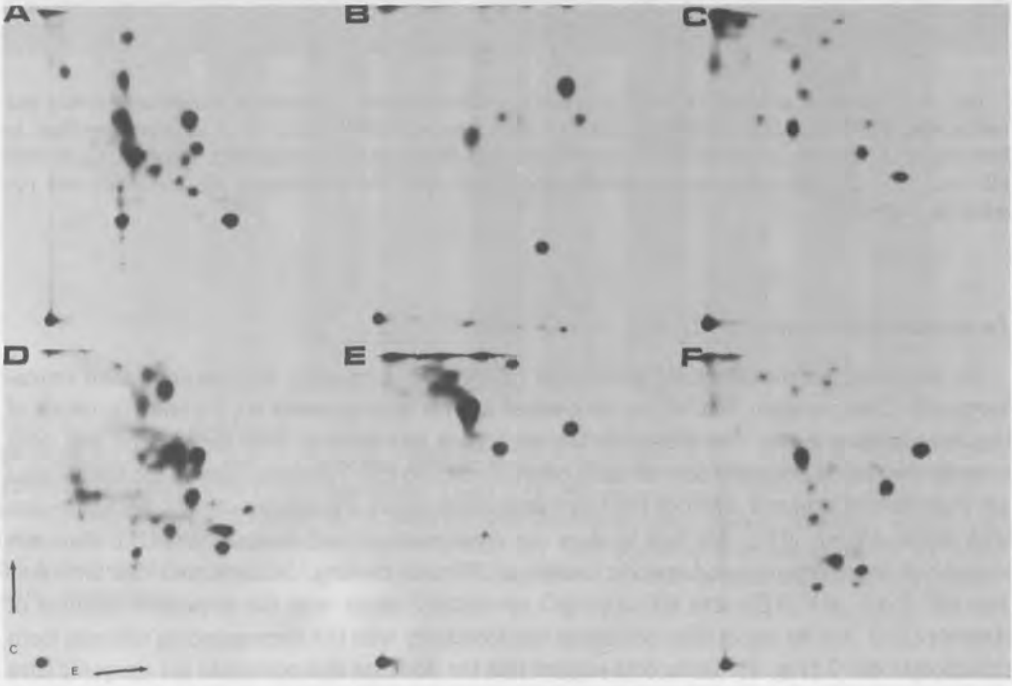


FIG. 6. Fingerprint analyses of 14 h digested ^{125}I-labeled tryptic peptides of individual *Artemia* and reticulocyte eIF-2 subunits. Individual subunits were digested with trypsin for 1 day as described in Materials and methods, and analyzed by two-dimensional thin-layer chromatography. Panels A-C, *Artemia* eIF-2(α), (β) and (γ) subunits, respectively ; panels D-F, the reticulocyte eIF-2(α), (β) and (γ) subunits, respectively. Iodination and fingerprint analyses were as described in Materials and methods.

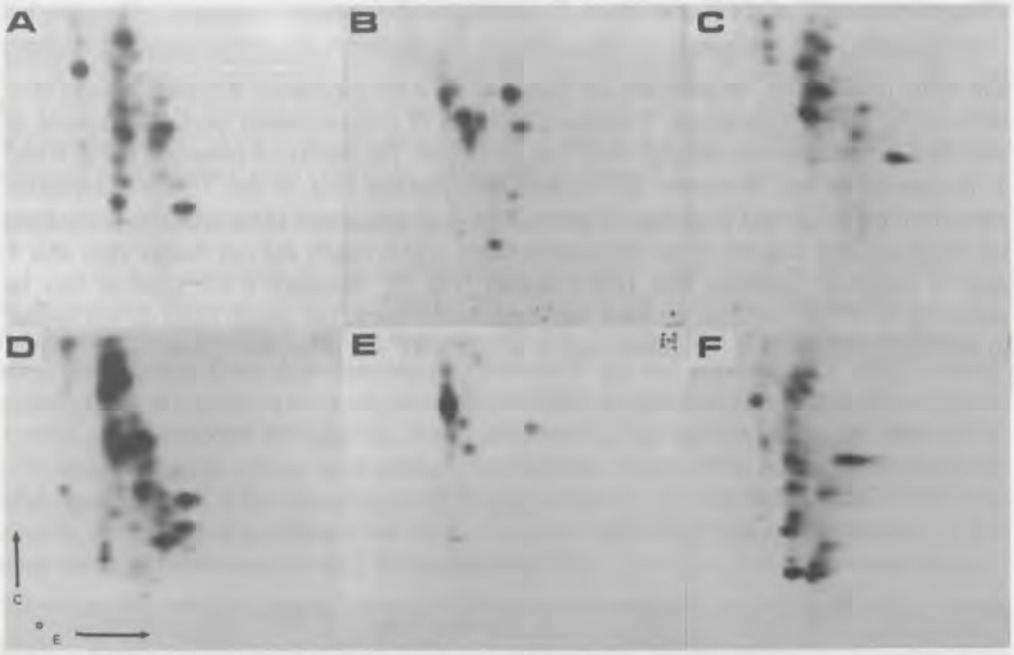


FIG. 7. Fingerprint analyses of 5-day digested ^{125}I-labeled tryptic peptides of individual *Artemia* and reticulocyte eIF-2 subunits. Individual subunits were digested with trypsin for 5 days as described in Materials and methods, and analyzed by two-dimensional thin-layer chromatography. Panels A-C, *Artemia* eIF-2(α), (β) and (γ) subunits, respectively, and panels D-F, the reticulocyte eIF-2(α), (β) and (γ) subunits, respectively.

Immunochemical studies of eIF-2

To probe further the structural similarities or differences between *Artemia* and rabbit reticulocyte eIF-2 preparations, antibodies were raised against homogeneous α-, β-, and γ-subunits of the brine shrimp factor. The DEAE-cellulose purified IgG fraction from each serum was used to study the shared antigenic determinants between the two eIF-2 preparations. In the simple agar gel immunodiffusion test, each of the three antibodies shows a single prominent precipitin line with native *Artemia* eIF-2, but fails to show any crossreactivity with reticulocyte eIF-2 (data not shown). A more sensitive and specific technique, Western blotting, demonstrates that anti-*Artemia* eIF-2(α)-, eIF-2(β)- and eIF-2(γ)-IgG specifically reacts with the respective subunits of *Artemia* eIF-2, but do not exhibit consistent crossreactivity with the corresponding subunits from reticulocyte eIF-2 (Fig. 8). These data suggest that the domains that constitute the antigenic sites on the *Artemia* eIF-2 subunits differ from those of the reticulocyte factor.

We also compared the immunological crossreactivity of a partial trypsin digestion of both *Artemia* and reticulocyte eIF-2, since trypsin digestion of both eIF-2 preparations demonstrated that HCR phosphorylates a homologous 4 000 M_r peptide (Fig. 4 and 5). The protocol for the experiment in Fig. 4 was followed but with only anti-*Artemia* eIF-2(α) IgG being used.

Antibodies to the α-subunit of *Artemia* eIF-2 reacted with the 38 000 M_r polypeptide but not with the 4 000 M_r fragment. No significant crossreactivity is observed with the intact reticulocyte α-subunit or with any of the trypsin digestion products of that subunit (Fig. 9).

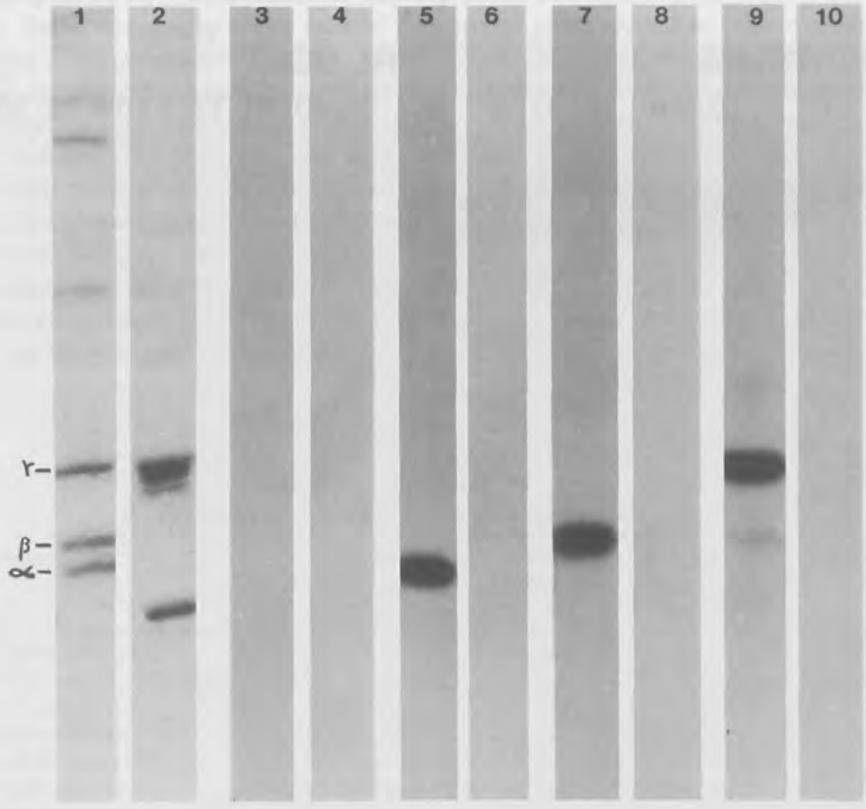


FIG. 8. Immunological reactivity of *Artemia* and reticulocyte eIF-2 to antibodies raised against *Artemia* eIF-2 subunits. Lanes 1 and 2 : Coomassie Brilliant Blue R-250 stained dodecylsulfate/polyacrylamide gel of *Artemia* and reticulocyte eIF-2, respectively. Lanes 3-10 : the autoradiogram of ^{125}I-labeled immunoblot showing *Artemia* and reticulocyte eIF-2 reactivity with antibodies raised against individual subunits of *Artemia* eIF-2. *Artemia* and reticulocyte eIF-2 reactivity with pre-immune serum (lanes 3, 4), with anti-*Artemia* eIF-2(α) (lanes 5, 6), anti-*Artemia* eIF-2(β) (lanes 7, 8) and with anti-*Artemia* eIF-2(γ) (lanes 9, 10), respectively.

ISOLATION AND CHARACTERIZATION OF A CASEIN KINASE FROM *ARTEMIA* EMBRYOS THAT PHOSPHORYLATES *ARTEMIA* eIF-2(α)

The specificity of reticulocyte HCR for phosphorylating the α-subunit and casein kinase for phosphorylating the β-subunit of eIF-2 preparations has been used as a means to identify the respective subunits (Kramer *et al.*, 1976 ; de Haro *et al.*, 1978 ; de Haro and Ochoa, 1979 ;

Woodley *et al.*, 1981 ; Crouch and Safer, 1984). We examined *Artemia* extracts for a protein kinase that would phosphorylate the α-subunit of eIF-2. Reticulocyte HCR was the only known enzyme to phosphorylate the α-subunit of both *Artemia* and reticulocyte eIF-2. As discussed below, the surprising result was that the enzyme isolated was a casein kinase that phosphorylates the α- rather than the β-subunit of *Artemia* eIF-2.

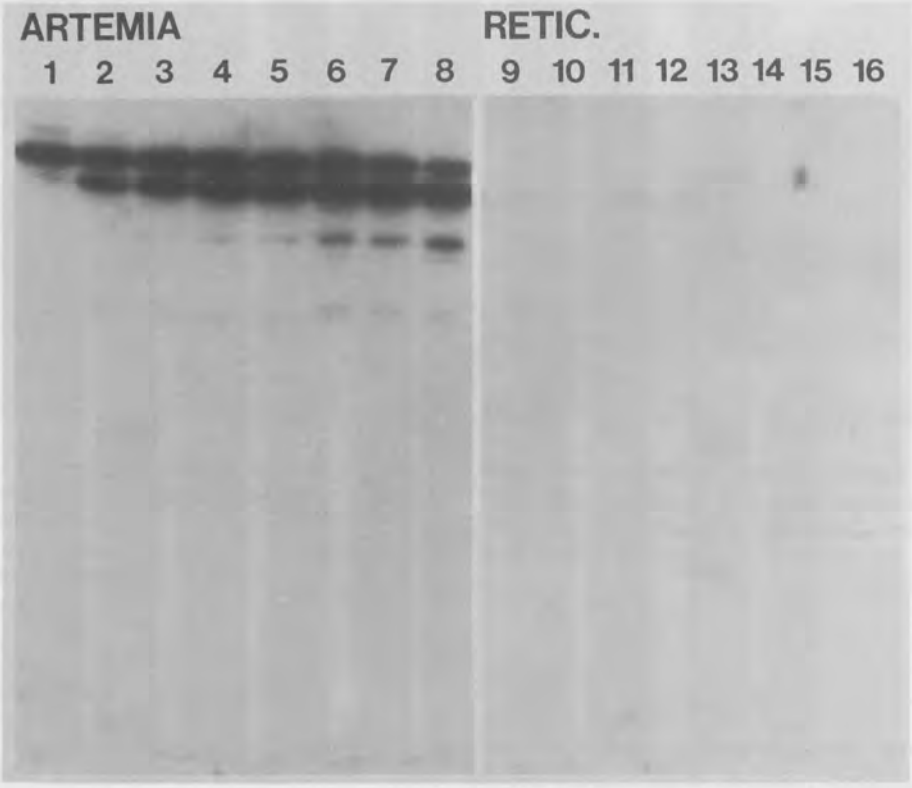


FIG. 9. Immunological reactivity of anti-*Artemia* eIF-2(α) IgG with tryptic peptides of *Artemia* and reticulocyte eIF-2(α - P). *Artemia* and reticulocyte eIF-2 preparations were digested with trypsin as in Fig. 4, electrophoresed in dodecylsulfate/polyacrylamide gels, and subjected to Western blotting as in Fig. 8 using antibodies raised against *Artemia* eIF-2(α). Lanes 1-8, autoradiogram of immunoblot with *Artemia* eIF-2 digested for 0, 1, 3, 6, 9, 12, 16, and 20 min, respectively. Lanes 9-16, same as 1-8, except the tryptic digest of reticulocyte eIF-2.

Purification of the casein kinase

A summary of the procedure for purification of the kinase from ribosomal high salt washes of dormant embryos is given in Table II. Kinase activity is found in the 25-60 % $(NH_4)_2SO_4$ fraction of the ribosomal wash and this step is used to remove glycoprotein and lipids which interfere with the subsequent column chromatography steps. Approximately 3 % of kinase activity

specific for casein but not for eIF-2 is found in the fraction not adsorbed to DEAE-cellulose at 100 mM KCl. The initial fractions containing kinase activity also contain significant amounts of eIF-2. About 75 % of eIF-2 elutes from the DEAE-cellulose column at 100 mM KCl and the remainder is separated from the kinase by chromatography on phosphocellulose and heparin-Sepharose. The kinase activity elutes from DEAE-cellulose at 300 mM KCl after which it is chromatographed on phosphocellulose. This step results in a 20-fold purification of the kinase activity. Rechromatography of this material on a second phosphocellulose column results in an additional 2-fold purification. This step removes a 35 000 M_r protein which becomes phosphorylated in the absence of added casein or eIF-2. The kinase may be further purified by filtration on Ultrogel AcA 34, followed by chromatography on heparin-Sepharose. Each step results in a 2-fold increase in the specific activity of the enzyme. The final preparation is about 580-fold purified with respect to the ribosomal salt wash fraction and with the exception of the ammonium sulfate fractionation, approximately 60 % of the kinase activity is recovered at each stage of purification, giving an overall yield of 5 % of the initial activity. Chromatography on either casein-Sepharose, hydroxylapatite or ATP-Sepharose does not separate the casein kinase activity from the eIF-2 kinase activity (data not presented). The general characteristics of this casein kinase are summarized in Table III.

TABLE II

Purification of a protein kinase from dormant embryos of *Artemia*

Purification step	Total protein [1] (mg)	Specific activity (units/mg)	Total activity [2] (units × 10^{-4})	Yield	
				%	-fold
1. Ribosomal salt wash	2 533	540	137	100	1
2. 25-60 M% $(NH_4)_2SO_4$ fraction	2 300	565	129	94	1.0
3. DEAE-cellulose	330	2 575	85	62	4.8
4. Phosphocellulose	10	55 000	55	40	100
5. Phosphocellulose	1.4	78 333	11	8	145
6. Ultrogel AcA 34	0.45	156 666	7	5	290
7. Heparin-Sepharose	0.17	313 330	5.3	4	580

[1] The protein kinase phosphorylating eIF-2 and casein was purified from 6 000 g wet weight of dormant *Artemia* embryos as described in Materials and methods.

[2] One unit equals 1 pmol of [^{32}P] incorporated per minute into casein under standard assay conditions.

This enzyme was also purified from the postribosomal supernatant by the same procedure as described here, and has similar characteristics as that purified from the ribosomal high salt wash (data not presented). The total units of eIF-2 kinase activity are 2-fold higher in the postribosomal supernatant as compared to that of the ribosomal salt wash. However, the specific activity of the eIF-2 kinase is 3-fold higher in ribosomal salt wash fractions. The final specific activity of this kinase for phosphorylated eIF-2(α) is 454 pmol eIF-2 phosphorylated/mg kinase/min.

TABLE III

Comparison of protein kinases

Characteristics	Casein kinase I [1]	Casein kinase II [2]	Reticulocyte ds-RNA induced eIF-2α kinase [3]	Reticulocyte HCR [4]	*Artemia* eIF-2α-kinase
M_r	37 000	130 000	67 000	90 000	150 000-180 000
Subunits	1	4	1	1	
Phosphate donor	ATP only	ATP (10 μM) GTP (40 μM)	ATP only	ATP	ATP (13 μM) GTP (10 μM)
Elution from DEAE-cellulose	50 mM KCl	175-220 mM KCl	50 mM KCl	100-250 mM KCl	100-250 mM KCl
Elution from phosphocellulose	500 mM KCl	700 mM KCl	300 mM KCl	100 mM KCl	700 mM KCl
pH optimum	–	8.8	7.2	7.2	7.5
Mg^{2+} optimum	2-5 mM	10 mM	6 mM	2 mM	10 mM
KCl optimum	200 mM	200 mM	60 mM	100 mM	200 mM
Substrates :					
histones	No	No	Yes	Poor	No
casein	Yes	Yes	No	Poor	Yes
phosvitin	–	–	No	No	Yes
ribosomal proteins	–	–	No	No	No

[1] Hathaway (*et al.*) (1983).
[2] Hathaway and Traugh (1983).
[3] Levin *et al.* (1980).
[4] Farrell *et al.* (1977).

Reaction characteristics of the protein kinase

In order to determine the optimal conditions for enzymatic activity, the effect of pH and Mg^{2+} and KCl concentrations on the phosphorylation of eIF-2(α) and casein was studied. The enzyme is optimally active between pH 7.2-7.5. KCl stimulated both eIF-2 and casein kinase activities and maximal enzyme activity is at 200 mM KCl, and the optimum concentration of Mg^{2+} is 10 mM. Ca^{2+}, Mn^{2} +, Mn^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} would not substitute for Mg^{2+} in the kinase reaction. The purified protein kinase has a significantly greater affinity for eIF-2 (K_M = 2 μM) than for casein (K_M = 68 μM) (Table IV). The protein kinase could phosphorylate eIF-2 and casein with either ATP or GTP as the phosphoryl donor, and the respective K_M values for these nucleotides are presented in Table IV. The K_M for GTP is lower than that for ATP, but the rate of phosphorylation is 3.8-fold greater with ATP than with GTP as the phosphoryl donor.

TABLE IV

Specificity of the *Artemia* protein kinase

Substrate	Protein	ATP	GTP
		Km (μM)	
Casein	69	13	10
eIF-2	2	7	2

Effect of various compounds on protein kinase activity

The enzyme was further characterized by examining the effect of a variety of compounds on its activity (Table V). The protein kinase activity is not influenced by the addition of cyclic AMP, cyclic GMP and phosphatidyl serine, and is not affected by the presence of EGTA, N-ethyl-maleimide or 40 μM hemin. Therefore, this enzyme is different from the calcium and phospholipid-dependent protein kinases (Le Peuch *et al.*, 1983). Unlike the cyclic nucleotide-independent protein kinases which were reported to be stimulated by basic proteins and polyamines (Yamamoto *et al.*, 1979 ; Hathaway and Traugh, 1984), the *Artemia* kinase under the assay conditions is not stimulated by histones or spermine. Sodium fluoride and spermine inhibited kinase activity only at high concentrations (10 mM). GDP, diguanosine triphosphate, diguanosine tetraphosphate and diguanosine pentaphosphates, which are present in abundance in dormant *Artemia* embryos (Warner and Finamore, 1967), inhibit enzyme activity. The respective concentrations required for 50 % inhibition are presented in Table VI.

Substrate specificity of the Artemia *casein kinase*

The *Artemia* casein kinase was evaluated for its ability to phosphorylate different substrates. The kinase phosphorylates partially hydrolyzed and dephosphorylated casein, purified α-, β-, and γ-casein and phosvitin. It does not phosphorylate histones or *Artemia* ribosomal proteins (Fig. 10).

TABLE V

Effect of various compounds on protein kinase activity

Addition	Concentration	Kinase activity (% control)	
		eIF-2	Casein
Cyclic-AMP	10 μM	100	100
	20 μM	99	99
Cyclic-GMP	20 μM	100	99
	40 μM	99	99
EGTA	1 mM	100	100
	2 MM	101	100
Phosphatidyl serine	60 μg/ml	100	100
$CaCl_2$, phosphatidyl serine	1 mM, 60 μg/ml	100	100
N-ethylmaleimide	10 mM	100	100
Histones	170 μg/ml	100	100
Hemin	20 μM	91	94
	40 μM	82	89
Sodium fluoride	10 mM	69	71
	20 mM	40	45
Spermine	5 mM	62	65
	10 mM	32	35

The agents to be tested were added to the standard assay for protein kinase (0.2 μg) as described in Materials and methods. Results are the average of triplicate samples, expressed as percent of control ; 100 % was equivalent to 2 and 1 525 units of the kinase activity for eIF-2 and casein phosphorylation, respectively.

TABLE VI

Effect of various inhibitors on casein kinase activity

Inhibitor	Protein kinase I_{50} (μM)[1]
Guanosine diphosphate	37
Diguanosine triphosphate	200
Diguanosine tetraphosphate	140
Diguanosine pentaphosphate	65
Heparin	0.03

The protein kinase was assayed as described in Materials and methods with various concentrations of the inhibitor under standard assay conditions.

[1] I_{50} is defined as the concentration of inhibitor required to give 50 % inhibition of the casein kinase activity.

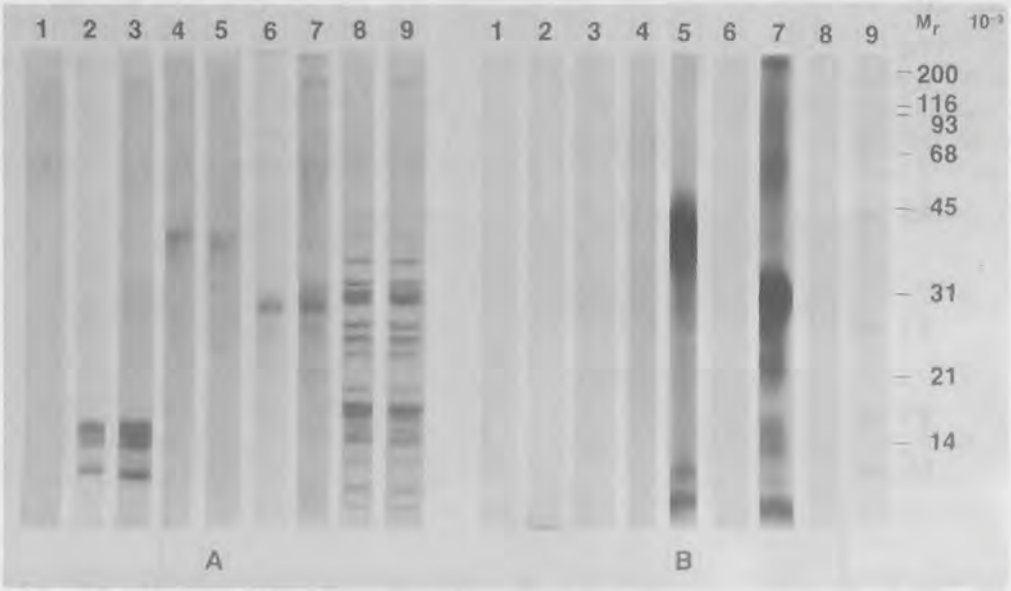


FIG. 10. Substrate specificity of the *Artemia* kinase. Phosphorylation was carried out with [γ-^{32}P]ATP as described in Materials and methods, and the products were electrophoresed in 12.5 % dodecylsulfate/polyacrylamide gels, stained with Coomassie Brilliant Blue R-250 (A) and autoradiographed (B). Where indicated 2 µg of the *Artemia* kinase were added to the incubation reaction. Lane 1, *Artemia* kinase ; lane 2, 5 µg calf thymus histones ; lane 3, calf thymus histones plus kinase ; lane 4, 5 µg partially hydrolyzed and dephosphorylated casein ; lane 5, casein plus kinase ; lane 6, 5 µg phosvitin ; lane 7, phosvitin plus kinase ; lane 8, 0.5 A_{260} unit *Artemia* 40S ribosomal subunits ; and lane 9, *Artemia* 40S ribosomal subunits plus kinase.

The properties of the *Artemia* casein kinase are compared with those of several other protein kinases in Table III. The molecular weight of the enzyme, the use of either ATP or GTP as the phosphoryl donor, the elution from DEAE-cellulose and phosphocellulose, as well as the Mg^{2+} and KCl optima, suggest that the *Artemia* enzyme is a type II casein kinase.

Since the *Artemia* casein kinase phosphorylates the α-subunit of *Artemia* eIF-2 we evaluated its ability to phosphorylate eIF-2 preparations from wheat germ embryos, yeast, and rabbit reticulocytes. For comparison, these different eIF-2 preparations were phosphorylated with reticulocyte HCR. The reticulocyte HCR efficiently phosphorylates the α-subunit of eIF-2 from all the sources tested (Fig. 11). However, with the *Artemia* casein kinase the result obtained were unexpected. This enzyme phosphorylates the β-subunit of reticulocyte eIF-2, as was reported earlier for casein kinase II (de Haro and Ochoa, 1979 ; Crouch and Safer, 1984). With the other eIF-2 preparations, either the *Artemia* or rabbit muscle casein kinase II phosphorylates the α-subunit, the subunit recognized by reticulocyte HCR. The phosphorylation of *Artemia* eIF-2 by casein kinase II does not affect its ability to form binary (eIF-2·GDP) or ternary (eIF-2·GTP·Met-tRNA$_f$) complexes, or to exchange GDP for GTP (data not shown).

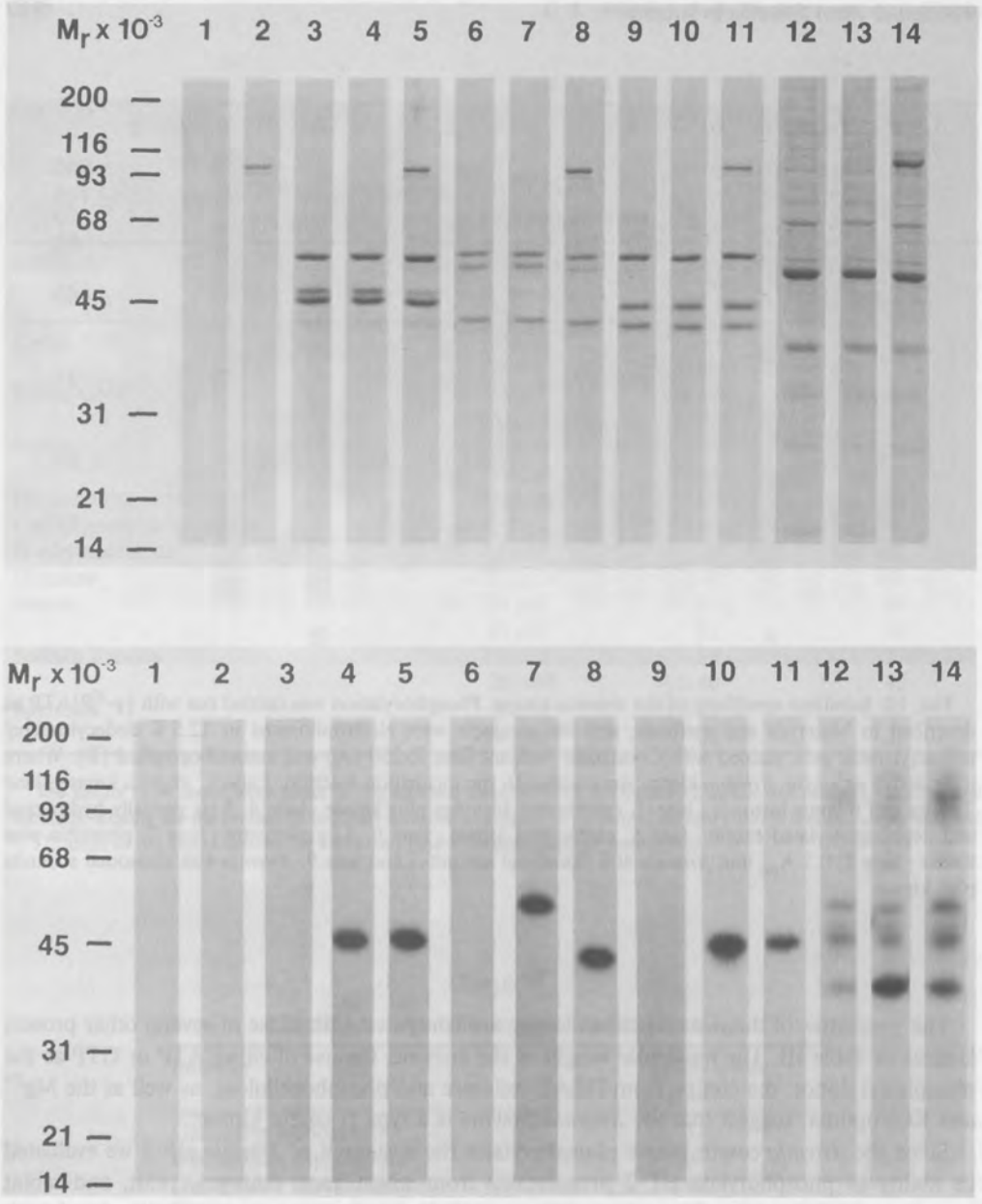


FIG. 11. Phosphorylation of eIF-2 from different sources by casein kinase II and HCR. Phosphorylation was carried out with [γ-^{32}P]ATP as described in Materials and methods, and the products electrophoresed in dodecylsulfate/polyacrylamide (10 %) gels and (A) stained with Coomassie Brilliant Blue R-250 and (B) autoradiographed. Two µg of either *Artemia* (lanes 3 to 5), rabbit reticulocyte (lanes 6 to 8), wheat germ embryos (lanes 9 to 11), or yeast (lanes 12 to 14) eIF-2 were used. Lanes 1, 4, 7, 10, and 13 also included 0.2 µg of the *Artemia* kinase, and reaction mixtures for lanes 2, 5, 8, 11, and 14 contained 1 µg of HCR. The phosphorylation was terminated with an equal volume of sodium dodecylsulfate-buffer. The molecular weight of each standard protein is indicated at the right. (Not shown is the phosphorylation of the different eIF-2 preparations by 0.2 µg of the muscle casein kinase II. This reaction yielded identical results as the *Artemia* kinase (lanes 1, 4, 7, 10, and 13).

Sites on Artemia *eIF-2 phosphorylated by the* Artemia *kinase and reticulocyte HCR*

As shown above, the *Artemia* kinase and reticulocyte HCR phosphorylate the α-subunit of *Artemia* eIF-2. In order to check the phosphorylation site specificity of the two kinases, *Artemia* eIF-2 was phosphorylated with each kinase. After extensive trypsin digestion of the phosphorylated α-subunits, two-dimensional peptide mapping demonstrates that different sites are phosphorylated by the two kinase preparations (Fig. 12). The amino acid phosphorylated by these

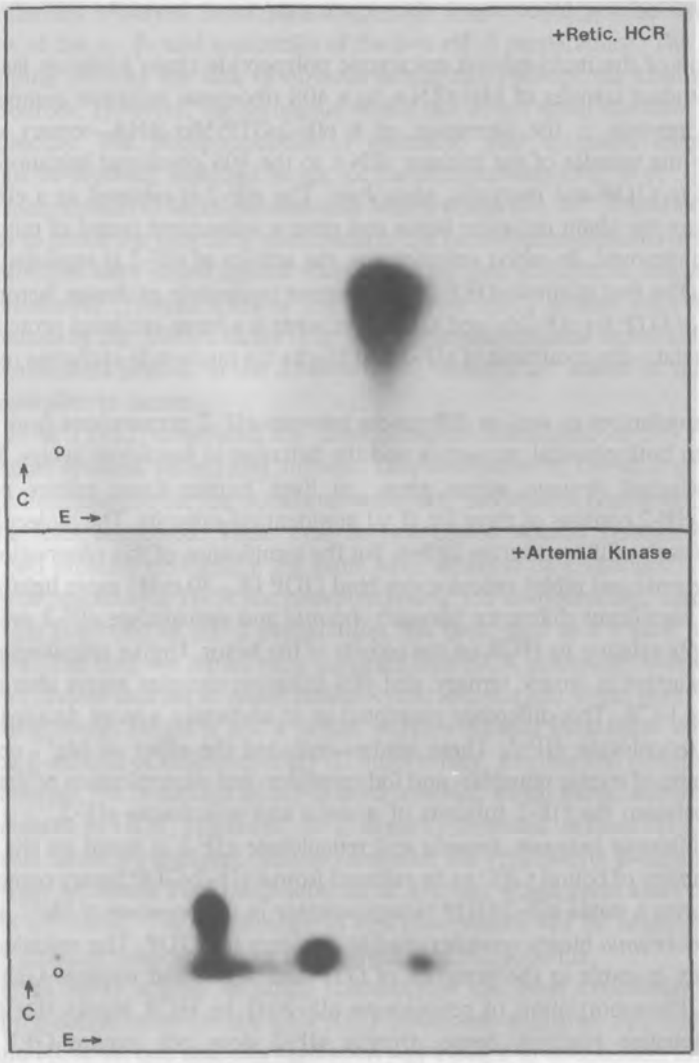


FIG. 12. Two-dimensional tryptic maps of phosphopeptides derived from *Artemia* eIF-2 phosphorylated by reticulocyte HCR or *Artemia* kinase. The *Artemia* eIF-2 was phosphorylated by either reticulocyte HCR (upper panel) or by the *Artemia* kinase (lower panel), followed by trypsin digestion and two-dimensional mapping as described in Materials and methods. The (o) indicates the origin, (C) the direction of the first dimension, and (E) the electrophoresis in the second dimension.

two kinases was determined by chromatography on thin-layer cellulose plates of the acid hydrolysate of ^{32}P-labeled eIF-2(α). In each case the results indicate that serine is the phosphate acceptor (data not shown).

Under optimum conditions of the assay, the stoichiometry of eIF-2 phosphorylation by the *Artemia* kinase is 0.92 pmol phosphate/pmol of eIF-2, and with HCR, 0.9 pmol phosphate/pmol eIF-2.

Discussion

The function of the multi-subunit eukaryotic polypeptide chain initiation factor 2 (eIF-2) is the GTP-dependent transfer of Met-tRNA$_f$ to a 40S ribosomal initiation complex. An intermediate in this process is the formation of a eIF-2·GTP·Met-RNA$_f$ ternary complex. Upon completion of the transfer of the initiator tRNA to the 40S ribosomal initiation complex, GTP is hydrolyzed to GDP and inorganic phosphate. The eIF-2 is released as a eIF-2·GDP binary complex. Before the chain initiation factor can enter a subsequent round of initiation the bound GDP must be removed. In rabbit reticulocytes, the activity of eIF-2 is regulated at this point by two proteins. The first of these, GEF or the guanine nucleotide exchange factor, is required for the exchange of GTP for eIF-2-bound GDP. The other is a heme-inhibited protein kinase (HCR) that phosphorylates the α-subunit of eIF-2 and blocks the nucleotide exchange reaction catalyzed by GEF.

There are similarities as well as differences between eIF-2 preparations from various sources with respect to both physical properties and the behavior in functional assays. In most systems examined, including *Artemia*, wheat germ, rat liver, human tissue culture cells, and rabbit reticulocytes, eIF-2 consists of three (α, β, γ) nonidentical subunits. The molecular weight of the subunits from each of these sources differs, but the significance of this observation is not known. eIF-2 from *Artemia* and rabbit reticulocytes bind GDP (K_D 30 mM) more tightly than GTP (K_D 1-10 μM). A significant difference between *Artemia* and reticulocyte eIF-2 preparations is the effect of phosphorylation by HCR on the activity of the factor. Unlike reticulocyte eIF-2, *Artemia* eIF-2 remains active in binary, ternary, and 40S initiation complex assays after phosphorylation by reticulocyte HCR. This difference prompted us to undertake a more detailed examination of *Artemia* and reticulocyte eIF-2. These studies included the effect of Mg^{2+} on *Artemia* eIF-2 activity, mapping of tryptic phospho- and iodopeptides, and determination of the immunological relationship between the eIF-2 subunits of *Artemia* and reticulocyte eIF-2.

A major difference between *Artemia* and reticulocyte eIF-2 is based on the effect of 1 mM Mg^{2+} on the ability of bound GDP to be released from a eIF-2·GDP binary complex. Each eIF-2 preparation forms a stable eIF-2·GDP binary complex in the presence of Mg^{2+}, and the addition of GTP to the *Artemia* binary complex readily displaces the GDP. The reticulocyte eIF-2·GDP binary complex is stable in the presence of GTP and Mg^{2+}, and requires GEF to remove the bound GDP. Phosphorylation of reticulocyte eIF-2(α) by HCR blocks this GEF-dependent nucleotide exchange reaction. Since *Artemia* eIF-2 does not require GEF for nucleotide exchange, phosphorylation of *Artemia* eIF-2(α) by HCR reduces the rate of nucleotide exchange by approximately 50 %.

The results of our limited and extensive trypsin digestion of *Artemia* and reticulocyte eIF-2 after phosphorylation with HCR demonstrate that the structure near the phosphorylation site is highly conserved and is near either the C- or N-terminal end of eIF-2(α). Our observations are

consistent with those of Zardeneta *et al.* (1982) and Gross *et al.* (1981) for reticulocyte eIF-2. It was interesting to note that the phosphopeptides 1, 2, and 3 seem to be related by a precursor/product relationship, the sequence apparently being 1 to 2 to 3 (Fig. 5). The complete digestion to generate peptide 3 required unusually large amounts of trypsin and long periods of digestion (up to 5 days). This may be due to either Arg-Arg, Arg-Lys or Lys-Lys sequences in the peptide which may produce random and incomplete trypsin cleavage at these sites, or to the presence of trace amounts of another protease in the trypsin.

Structural similarities observed from phosphopeptide maps could not be extrapolated to iodopeptide maps of the α-, β- and γ-subunits of the two eIF-2 preparations. The results of the iodopeptide mapping indicate the lack of obvious similarities between the analogous subunits from these two sources. However, the technique would not detect small stretches of conserved amino acid sequences. The phosphopeptide(s) generated after extensive trypsin digestion (Fig. 5) could not be precisely matched to any of the iodopeptides (Fig. 6 or 7), presumably because the phosphopeptide(s) lacks tyrosine and hence would not be ^{125}I-labeled.

In our attempts to probe the structural relatedness of the homologous subunits of the two eIF-2 preparations, antibodies were raised against each of the purified subunits of *Artemia* eIF-2. We observed no reproducible crossreactivity of any reticulocyte eIF-2 subunit with the antibodies raised against subunits of the *Artemia* factor (Fig. 9). This immunological technique revealed that the antigenic determinants present in the *Artemia* eIF-2 subunits are absent or modified in the subunits of the reticulocyte factor.

Brown-Luedi *et al.* (1982) compared the immunological relatedness of eIF-2 preparations from two mammalian systems, rabbits and humans. They concluded on the basis of immunoblots that the antigenic determinants for the α-subunit were only moderately conserved, and those of the β-subunit the least conserved. Thus, even in two closely related systems, the antigenic determinants of two proteins performing the same basic function have changed.

The specificity of reticulocyte HCR for phosphorylating the α-subunit and casein kinase for phosphorylating the β-subunit of eIF-2 preparations has been used as a means to identify the respective subunits. The data we report here confirms that HCR phosphorylates the α-subunit of eIF-2 from such diverse sources as rabbit reticulocytes, *Artemia* and wheat germ embryos, and yeast. Rabbit muscle casein kinase II and a similar enzyme isolated from brine shrimp embryos phosphorylate the β-subunit of reticulocyte eIF-2. Surprisingly, *Artemia* and rabbit muscle casein kinase II phosphorylate the α-subunit of eIF-2 from *Artemia*, wheat germ, and yeast, the same subunit phosphorylated by HCR. Moreover, the sites phosphorylated on these eIF-2 preparations by HCR and casein kinse are different. The sequence on the reticulocyte β-subunit recognized by casein kinase may be absent from the β-subunits of *Artemia*, wheat germ and yeast eIF-2, but is present on the α-subunit. The significance of this observation will be better understood by further sequence and functional studies of the individual eIF-2 subunits.

The regulation of eIF-2 activity in rabbit reticulocytes has been extensively studied. The primary point of regulation occurs at the GDP/GTP nucleotide exchange step and involves two proteins, GEF and HCR. At one time this regulation was thought to be common to all eukaryotic systems. Since neither GEF nor HCR have as pronounced an influence on the activity of *Artemia* eIF-2 as on the reticulocyte factor in *in vitro* assays, the role of these factors in the regulation of eIF-2 activity in *Artemia* and other systems remains to be established. The possibility that *Artemia* embryos may contain such factors should be investigated. Other important questions

dealing with the regulation of eIF-2 in *Artemia* embryos include the possible role of other protein kinases and phosphatases as well as the control of eIF-2 synthesis during embryogenesis.

Acknowledgements

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Summary

1. Both *Artemia* and reticulocyte eIF-2 preparations form stable eIF-2·GDP binary complexes in the presence of Mg^{2+}. When GTP is added to this binary complex the *Artemia* factor readily exchanges GTP for the bound GDP, whereas the reticulocyte eIF-2 requires the guanine nucleotide exchange factor for this exchange reaction to occur.
2. The heme controlled repressor, isolated from reticulocytes phosphorylates the α-subunit of *Artemia*, wheat germ, yeast and reticulocyte eIF-2. After phosphorylation nucleotide exchange with the reticulocyte factor was completely inhibited, whereas the nucleotide exchange rate with the *Artemia* binary complex is reduced by approximately 50 %.
3. A casein kinase isolated from *Artemia* embryos phosphorylates the α-subunit of eIF-2 preparations from *Artemia* and wheat germ embryos and yeast. This phosphorylation has no effect on binary or ternary complex formation or nucleotide exchange with *Artemia* eIF-2. The β-subunit and not the α-subunit of reticulocyte eIF-2 is phosphorylated by this *Artemia* kinase.
4. Reticulocyte HCR phosphorylates a homologous 4 000 M_r tryptic peptide on *Artemia* and reticulocyte eIF-2 preparations.
5. Different sites on *Artemia* eIF-2(α) are phosphorylated by the *Artemia* kinase and reticulocyte HCR.
6. Iodopeptide mapping of *Artemia* and reticulocyte eIF-2 revealed that there was little homology between the α-, β- and γ-subunits of *Artemia* eIF-2. Furthermore, there was no significant homology of the *Artemia* subunits with the corresponding reticulocyte eIF-2 subunits.
7. Antibodies prepared against the individual subunits of *Artemia* eIF-2 demonstrated that there was little immunological identity between the subunits of *Artemia* and rabbit reticulocyte eIF-2.

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Concluding remarks for Symposium Session II : Physiology, Biochemistry, Molecular biology

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In Session II, "**Physiology, Biochemistry and Molecular biology of *Artemia***", 42 communications were presented.

A first session "**Metabolic enzymes**" mainly focused on metabolism of purine nucleotides. Despite much effort it is still doubtful whether *Artemia* is able to synthesize purines *de novo*. The major source of purine nucleotides is diguanosine tetraphosphate. For its synthesis the enzyme GTP-GTP guanyltransferase is essential. Diguanosine tetraphosphate is stored in high quantities in cysts and it disappears after about 5 days of development. For this degradation the major pathways of classical purine interconversion are used.

In a second session on "**Metabolism and development**" several important topics were discussed.

Proteolytic enzymes

At least five different groups of proteases were characterized and probably there are many more. Several of them may be masked enzymes which are activated by different triggers. However, too little information is available about the localization of the enzymes. Are they from lysosomal origin or do they come from the digestive track ? The elucidation of their structural-functional relationship, as was worked out for the alkaline phosphatases, will be very welcome.

Chitin degrading enzymes

For the first time chitin degrading enzymes were characterized in *Artemia* as well as the change in their activity during development. It is, however, still uncertain whether they play a role in hatching. Maybe, these enzymes will offer us a tool to make cysts more permeable. In the same context the quantitation of moulting hormones and their function will become more and more important.

Pigments

The structure of the fascinating molecule, artemocyanin is now determined but its functional significance has to be determined. The same is true for the carotenoproteins with their extraordinary cis-trans ratio of canthaxanthin.

A third session on "Enzymes related to DNA and RNA metabolism" mainly focused on the DNA- and RNA polymerases. Both groups of enzymes were quite well characterized. The DNA-polymerase seems to lack the β form and is not able to bind Ap_4A as reparted for other systems.

The last session on "Genome structure and expression" was probably the most promising one.

Mechanism of protein synthesis

A considerable progress was made on the elucidation of the structure and function of the initiation – and elongation factors. Unraveling the composition of the mRNP particle and the significance of phosphorylation/dephosphorylation will probably help to understand the activation of the stored mRNP in the cryptobiotic gastrulae. The establishment of specific and inducible heat choc proteins as well in cysts as in nauplii, may become a link between environmental factors and protein synthesis.

The *Artemia* genome

Presently and in the near future, the main progress will be made in the characterization of the *Artemia* nuclear- and mitochondrial genetical information. For the first time polymorfism in *Artemia* was established by molecular biological techniques. The comparison of gene structure by restriction analysis will become more and more important.

Contents of Volume 1

Strain characterization

Contents of Volume 2

Enzymes related to nucleotide and nucleic acid metabolism

Contents of Volume 3

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