Heart Transaminase in the Rock Crab, *Cancer irroratus*, Exposed to Cadmium Salts

by EDITH GOULD, RIES S. COLLIER, JOHN J. KAROLUS, and SHARON GIVENS
National Marine Fisheries Service
Middle Atlantic Coastal Fisheries Center
Milford Laboratory
Milford, Conn. 06460

Enzymes of amino acid metabolism are an important link between carbohydrate and protein metabolism and have been strongly implicated in the production of animal energy. Demands upon an animal's energy reserves are invariably accompanied by an increase in nitrogen metabolism, as in starvation or exposure to cold, or in diseased states like alloxan diabetes, or with toxic diets; other triggers of gluconeogenesis, such as glucagon or sex hormones, or a high-protein diet, cause increased transaminase activity (KNOX and GREENGARD, 1965; CAMPBELL, 1973). BUTTERY and ROWSELL (1974), in their study of bodysize-dependent enzyme activities in mammalian liver, speculated that transamination may largely promote the regulatory mechanism of basal metabolism. The breakdown of amino acids in small mammals rousing from hibernation provides oxidative substrates for heat production and intermediates for the maintenance of blood glucose, replenishment of tissue glycogen, and oxidation of fatty acids (WHITTEN et al., 1974). Protein catabolism is unquestionably accelerated in man and animals by stress, with transaminase activity directly related to changes in the size of the amino acid pool in liver (NICHOL and ROSEN, 1963).

Of the two major transaminases, both widely distributed in the animal kingdom, aspartate aminotransferase (EC 2.6.1.1, AAT) has the higher capacity for substrate turnover (NICHOL and ROSEN, 1963) and appears to be the more essential: in the liver and muscle of the African lungfish (Protopterus), AAT activity remained unchanged during starvation and aestivation, while the alanine enzyme (EC 2.6.1.2, AlAT) decreased tenfold (JANSSENS, 1964). Clinical use of elevated serum AAT for indicating heart and liver damage, a well-established diagnostic tool for mammals, has been applied to teleosts exposed to toxic hydrocarbons (BELL, 1968), to copper (MCKIM et al., 1970), and to pesticides (LANE and SCURA, 1970).

Important as transamination is in vertebrates, it is perhaps even more so in invertebrates, where protein is a major source of energy. In insects, a direct relation has been established between amino acid metabolism and energy production (MCALLAN and CHEFURKA, 1961; BURSELL, 1966). For marine crustaceans, the few available data are not so clear-cut, and some controversy still exists over the relative importance of protein and carbohydrate in energy production (SCHEER et al., 1952; NEEDHAM, 1957;

Bulletin of Environmental Contamination & Toxicology, Vol. 15, No. 6 © 1976 by Springer-Verlag New York Inc.

WOLVEKAMP and WATERMAN, 1960; VONK, 1960). At present, however, the generally accepted view is that amino acids figure more largely in these animals for the storing and release of energy than either glucose or lipides, and are important as well in osmoregulation (e.g. FLORKIN, 1960; VINCENT-MARIQUE and GILLES, 1970; CAMPBELL, 1973; GILLES, 1973). Glucose is apparently more prominently involved in chitin formation (SCHEER and SCHEER, 1952; HOHNKE and SCHEER, 1970); the Krebs cycle operates only slowly in decapods, and a considerable body of evidence suggests a major metabolic pathway that gives rise to glutamic and aspartic acids (SCHOFFENIELS and GILLES, 1970). In the study of marine crustaceans exposed to heavy metals, therefore, it seemed advisable to investigate first those systems largely involved in the mobilization of energy, the transaminases.

In mammalian tissues, AAT is most highly concentrated in heart muscle (VELICK and VAVRA, 1962; BRAUNSTEIN, 1973). ZANDEE et al. (1958) reported that there was no AAT in normal invertebrate blood, and CHAPLIN et al. (1967) studied the distribution of AAT in Carcinus maenas tissues, but the heart was not a part of either of these studies. Preliminary work here established that there is relatively little AlAT in normal crab hearts (Carcinus and Callinectes), but very large amounts of the aspartate enzyme, AAT.

Because the crustacean heart is a discrete organ easily dissected from surrounding tissues, because it is not so subject to diet-induced fluctuations in enzyme concentrations as are organs like the hepatopancreas, and because it contains high concentrations of the major transaminase, we chose to investigate heart AAT in our study of cadmium-exposed rock crabs, Cancer irroratus, reported here.

Cadmium was selected as the challenge metal because it is a widespread trace pollutant of high toxicity not only to warm-blooded vertebrates but also to aquatic animals (e.g. SHUSTER and PRINGLE, 1969; GARDNER and YEVICH, 1970; EISLER, 1971; CALABRESE et al., 1973; THURBERG et al., 1973). The chloride salt was used because of its ready solubility and because its anion is the most abundant in seawater. Additionally, anticipating future work with silver (which is commonly added as the nitrate), we exposed crabs to cadmium nitrate, also a readily soluble salt, both for a more logical basis of future comparison and for information on the relative toxicities of cadmium salts. Finally, because cadmium has been reported to alter the metabolism of copper (EVANS et al., 1970), the crab serum was monitored for any change in the electrophoretic patterns of total protein and of hemocyanin.

MATERIALS AND METHODS

Animal Exposure: The crabs were collected in eel traps and lobster traps within 2 Km of Milford Harbor, Connecticut, and

held in tanks of flowing harbor water, salinity 25 ± 2 ppt and temperature 20° ± 2°C. To acclimate the animals to the test medium, they were transferred to tanks of flowing ozonized harbor water at least 1 week before each test exposure. The ozonizing treatment produced an initial 0.5 mg/l residual ozone concentration, which was removed by filtration through coconut charcoal (BLOGOSLAWSKI et al., 1975). During acclimation the crabs were fed minced hardshell clams, Mercenaria mercenaria, daily; they were unfed for 2 days prior to and during the test exposures.

Crabs were exposed individually for 96 hrs in 3.7-liter jars filled to 3 liters with test medium. The medium was prepared by filtering ozonized harbor water to 1 µm with spun polypropylene cartridge filters, which removed excessive organic and particulate matter. The water was aerated throughout the exposure period.

Cadmium background of the medium was less than 1 ppb. Cadmium salts used in exposing the animals were $CdCl_2.2\text{-}1/2\ H_2O$ and $Cd(NO_3)_2.4H_2O$, and were added as a calculated 1 ppm Cd.

Tissue Preparation: At the end of each exposure period, hemolymph samples were taken from the crabs individually by puncture at the base of the walking legs and clarified by centrifugation at 1720 g, 6°C. The sera were stored frozen (-16°C) until electrophoresis.

The hearts were pooled (3 crabs/pool) in small plastic pouches, as air-free as possible, and frozen-stored (-29°C) until testing. From each heart pool a 5% homogenate was made, w/v in cold glass-distilled H_2O, in a small glass homogenizer containing 25-µ glass powder to facilitate grinding. Centrifugation was at 15,000 g, 4°C, for 45 min. The supernates, diluted 1:1 with cold H_2O, served as the enzyme preparation, E^{10x}. Aliquots of 1.00 ml were used for protein biuret determinations (GORNAL et al., 1949) and the remainder was halved; one portion was refrigerated (6°C) and the balance frozen-stored (-16°C) for in vitro electrolyte stress, EX^{10x}.

Enzyme Assay: The AAT assay was based on the standard coupled reaction as described by BERGMEYER and BERNT (1963), except for the proportions and concentrations of some reagent solutions. No malic dehydrogenase was added. Water used in preparing all solutions was doubly glass-distilled; solutions of reduced nicotinamide adenine dinucleotide (NADH) were prepared fresh daily; α-ketoglutarate (KG) was prepared in 200-ml volumes and frozen-stored in small amounts (ca. 5-7 ml). A double-beam ratio-recording spectrophotometer, chamber temperature 25°C, and linear-log recorder were used to follow reaction rates.

Protocol - 2.60 ml buffer-amino acid solution (0.1 M phosphate buffer, pH 7.5, containing 0.25 M L-aspartic acid, neutralized with KOH before addition); 0.10 ml NADH (9 mg/ml H_2O);

0.05 ml E^{10x} (or 0.10 ml, according to preparation activity; the same volume was used for each set of preparations); and, to start the reaction, 0.20 ml KG (0.1 M, neutralized with KOH).

Except KG, the solutions were pipetted into an optical cuvette, 1-cm pathlength, and allowed to stand for 10 min at RT. Absorbance was read at 340 nm against a reference cuvette (H_2O) adjusted with small amounts of NADH to a differential absorbance of 0.800-1.000. When the absorbance was stable and no further oxidation of NADH could be detected, the reaction was started by the addition of KG. The log mode of the recorder was used with a linear chart and the slope was drawn from the fastest linear section, some 40-90 sec after the reaction's start. Unit of activity was the change in absorbance at 340 nm at the rate of 0.001/min/mg protein.

Electrophoresis: Crab serum was subjected to electrophoresis on polyacrylamide gel columns, 3 µl/column. Electrophoresis was performed at 4°C on 7% acrylamide, pH 9.1, with photopolymerized stacker and sample gels of 3% acrylamide, pH 5.2. Electrode buffers were Tris-glycine, pH 8.3. Both gel formularies and electrophoretic procedure are based on the work of DAVIS (1964) and have been fully described elsewhere (GOULD and MEDLER, 1970).

Stains - Subsequent to electrophoresis, gel columns were stained in test tubes for either total protein, or copper, or peroxidatic activity. The stain used for total protein was Amido Schwartz 10B (Buffalo Black), 1% in 7.5% acetic acid. Gels were destained by passive diffusion in several changes of methanol-glacial acetic acid-H_2O (5:1:5) for a total of about 20 hrs. Hemocyanin sites were marked by an aqueous tetrazolium-cyanide stain for copper (GOULD and KAROLUS, 1975) and by a stain for peroxidase (PANDEY, 1967), on replicate gels.

RESULTS AND DISCUSSION

When rock crabs, Cancer irroratus, were exposed for 4 days to 1 ppm cadmium as the chloride salt, aspartate aminotransferase activity in heart muscle preparations increased significantly over heart AAT in the control crabs (Table 1). When crabs were exposed to 1 ppm cadmium as the nitrate salt in analogous experiments, however, the heart AAT was slightly depressed, but not to a significant degree:

TABLE 1

AAT activity in heart muscle of Cancer irroratus exposed to cadmium salts for 4 days

Experimental conditions	Number sample pools[a]	Range[b]	Mean[b] ± S.E.	Level of significance	
Controls	26	631-3690	1784 ± 204		
1 ppm Cd as $CdCl_2$ .2-1/2 H_2O	9	1715-5850	2963 ± 422	$P < .01$	
1 ppm Cd as $Cd(NO_3)_2.4H_2O$	14	494-2740	1510 ± 274	N.S.	$P < .001$

a) 3 hearts/sample pool.

b) AAT unit of activity = change in absorbance of 0.001/min/mg protein under assay conditions.

Serum magnesium was elevated in crabs exposed to cadmium chloride, an observation true of both sexes, but not in crabs exposed to cadmium nitrate (NELSON and WENZLOFF, 1974). Moreover, more mortalities occurred in crab groups exposed to cadmium as chloride than occurred either in those exposed to cadmium as nitrate or in the control groups.

In a similar study, THURBERG et al. (1973) found that freshly-excised gills of rock crabs exposed to cadmium chloride had depressed rates of oxygen consumption, a clear indication of metabolic distress. BROWN and NEWELL, looking at the effect of copper and zinc on Mytilus edulis metabolism (1972), suggested that suppression of metabolism is very probably "... due to the inhibition of an energy-consuming process ... rather than a direct effect of the metal on respiratory enzymes." In the work reported here, we ascribe the elevated AAT values to a compensatory mechanism attempting to provide energy to drive an impaired metabolism.

That the nitrate salt of cadmium did not produce the same effect as the chloride salt underscores the importance of the form in which a metal occurs in the environment. Metal ions bind to various ligands, metabolic and otherwise, to an extent largely dependent upon the nature of their immediate chemical association (e.g. LEWIS et al., 1972). The results reported here clearly indicate that cadmium is more toxic as the chloride salt than as the nitrate.

Important as it is to determine fluctuations in the amount of enzyme activities, perhaps equally as important is the measure of an enzyme's capacity to function under stress in vitro. The aqueous extracts of crab hearts, frozen at -16°C for 3 days and then thawed at 6°C, may reasonably be considered to have undergone electrolyte stress. During the formation of ice, small pockets of unfrozen solution remain that contain soluble protein and relatively high concentrations of salts in close association. Less of the original AAT activity remained in preparations from the control crabs than in those from crabs exposed to cadmium chloride (Table 2). Again, the nitrate salt did not differ significantly from the controls:

TABLE 2

Residual AAT activity in frozen and thawed crab heart preparations (% of original activity)

Experimental conditions	Number sample pools	Range (%)	Mean±S.E. (%)	Level of significance	
Controls	12	21-52	31.1 ± 3.2		
1 ppm Cd as $CdCl_2$ .2-1/2H_2O	7	51-99	64.3 ± 7.9	P<.001	
1 ppm Cd as $Cd(NO_3)_2.4H_2O$	5	10-42	19.0 ± 13.3	N.S.	P<.001

We construe these data to mean that some enzymes in metal-exposed animals may develop a degree of tolerance to higher-than-normal concentrations of cations, a tolerance that may lessen the necessary sensitivity to biochemical modulators, such as magnesium. We favor this interpretation in the light of observations that high assay concentrations of magnesium, for instance, reveal perturbed enzymatic function in the skeletal muscle of aging haddock, Melanogrammus aeglefinus (GOULD, 1969), and in the liver of cadmium-exposed cunner, Tautogolabrus adspersus (GOULD and KAROLUS, 1974); and more recently, evidence supporting this view has been found in the tissues of flounder, Pseudopleuronectes americanus (GOULD, in press), and lobster, Homarus americanus (THURBERG et al., in press).

Electrophoretic analysis of the hemolymph showed no significant difference between either group of the cadmium-exposed crabs and control crabs, either in total-protein patterns or in hemocyanin structure and peroxidatic activity.

REFERENCES

BELL, G. R.: J. Fish. Res. Bd. Can. 25, 1247 (1968).

BERGMEYER, H.-U., and E. BERNT: in Methods of Enzymatic Analysis. Editor BERGMEYER, H.-U., New York, Academic Press, 1963, p. 839.

BLOGOSLAWSKI, W. J., C. BROWN, E. W. RHODES, and M. BROADHURST: in Proceedings, 1st International Symposium on Ozone for Water and Wastewater. Editors BROWNING, M., and R. RICE, Waterbury, Conn., International Ozone Inst., 1975, p. 674.

BRAUNSTEIN, A. E.: in The Enzymes, 3rd edition. Editor BOYER, P. D., New York, Academic Press, 1973, p. 379.

BROWN, B. E., and R. C. NEWELL: Mar. Biol. 16, 108 (1972).

BURSELL, E.: Comp. Biochem. Physiol. 19, 809 (1966).

BUTTERY, P. J., and E. V. ROWSELL: Comp. Biochem. Physiol. 47B, 473 (1974).

CALABRESE, A., R. S. COLLIER, D. A. NELSON, and J. R. MACINNES: Mar. Biol. 18, 162 (1973).

CAMPBELL, J.: in Comparative Animal Physiology, 3rd edition. Editor PROSSER, C. L., Philadelphia, W. B. Saunders Co., 1973, p. 279.

CHAPLIN, A. E., A. K. HUGGINS, and K. A. MUNDAY: Comp. Biochem. Physiol. 20, 195 (1967).

DAVIS, B. J.: Ann. N. Y. Acad. Med. 121, 404 (1964).

EISLER, R.: J. Fish. Res. Bd. Can. 28, 1225 (1971).

EVANS, G. W., P. F. MAJORS, and W. E. CORNATZER: Biochem. Biophys. Res. Comm. 40, 1142 (1970).

FLORKIN, M.: in The Physiology of Crustacea, Vol. 1. Editor WATERMAN, T. H., New York, Academic Press, 1960, p. 395.

GARDNER, G. R., and P. P. YEVICH: J. Fish. Res. Bd. Can. 27, 2185 (1970).

GILLES, R.: Netherlands J. Sea Res. 7, 280 (1973).

GORNAL, A. G., C. S. BARDAWILL, and M. M. DAVID: J. Biol. Chem. 177, 751 (1949).

GOULD, E.: J. Fish. Res. Bd. Can. 26, 3175 (1969).

GOULD, E.: in Physiological Responses of Marine Organisms to Pollutants. Editors, VERNBERG, F. J., A. CALABRESE, F. P. THURBERG, and W. B. VERNBERG, New York, Academic Press, in press (1976).

GOULD, E., and J. J. KAROLUS: in Physiological Response of the Cunner, Tautogolabrus adspersus, to Cadmium. NOAA Tech. Rept. NMFS SSRF-681. 1974, p. 21.

GOULD, E., and J. J. KAROLUS: Anal. Biochem., 67, 515 (1975).

GOULD, E., and M. J. MEDLER: J. Assoc. Offic. Anal. Chemists 53, 1237 (1970).

HOHNKE, L., and B. T. SCHEER: in Chemical Zoology, Vol. 5. Editors FLORKIN, M., and B. T. SCHEER, New York, Academic Press, 1970, p. 147.

JANSSENS, P. A.: Comp. Biochem. Physiol. 11, 105 (1964).

KNOX, W. E., and O. GREENGARD: in Advances in Enzyme Regulation, Vol. 3. Editor WEBER, G., London, Pergamon Press, 1965, p. 247.

LANE, C. E., and E. D. SCURA: J. Fish. Res. Bd. Can. 27, 1869 (1970).

LEWIS, A. G., P. H. WHITFIELD, and A. RAMNARINE: Mar. Biol. 17, 215 (1972).

MCALLAN, J. W., and W. CHEFURKA: Comp. Biochem. Physiol. 2, 290 (1961).

MCKIM, J. M., G. M. CHRISTENSEN, and E. P. HUNT: J. Fish. Res. Bd. Can. 27, 1883 (1970).

NEEDHAM, A. E.: Physiol. Comparata et Oecol. 4, 209 (1957).

NELSON, B., and D. R. WENZLOFF: NOAA, Natl. Mar. Fish. Serv., Milford, Conn. 06460, personal communication (1974).

NICHOL, C. A., and F. ROSEN: in Advances in Enzyme Regulation, Vol. 1. Editor WEBER, G., Oxford, Pergamon Press, 1963, p. 341.

PANDEY, K. K.: Nature 213, 669 (1967).

SCHEER, B. T., and M. A. R. SCHEER: Physiol. Comparata et Oecol. 2, 198 (1952).

SCHEER, B. T., C. W. SCHWABE, and M. A. R. SCHEER: Physiol. Comparata et Oecol. 2, 327 (1952).

SCHOFFENIELS, E., and R. GILLES: in Chemical Zoology, Vol. 5. Editors FLORKIN, M., and B. T. SCHEER, New York, Academic Press, 1970, p. 199.

SHUSTER, C. N., JR., and B. H. PRINGLE: Proc. Natl. Shellfish. Assoc. 59, 91 (1969).

THURBERG, F. P., M. A. DAWSON, and R. S. COLLIER: Mar. Biol. 23, 171 (1973).

THURBERG, F. P., A. CALABRESE, E. GOULD, R. GREIG, M. A. DAWSON, and R. K. TUCKER: in Physiological Responses of Marine Organisms to Pollutants. Editors VERNBERG, F. J., A. CALABRESE, F. P. THURBERG, and W. B. VERNBERG, New York, Academic Press, in press (1976).

VELICK, S. F., and J. VAVRA: in The Enzymes, 2nd edition, Vol. 6. Editors BOYER, P. D., H. LARDY, and K. MYRBÄCK, New York, Academic Press, 1962, p. 219.

VINCENT-MARIQUE, C., and R. GILLES: Comp. Biochem. Physiol. 35, 479 (1970).

VONK, H. J.: in The Physiology of Crustacea, Vol. 1. Editor WATERMAN, T. H., New York, Academic Press, 1960, p. 291.

WHITTEN, B. K., R. F. BURLINGTON, and M. A. POSIVIATA: Comp. Biochem. Physiol. 47A, 541 (1974).

WOLVEKAMP, H. P., and T. H. WATERMAN: in The Physiology of Crustacea, Vol. 1. Editor WATERMAN, T. H., New York, Academic Press, 1960, p. 35.

ZANDEE, D. I., H. J. NIJKAMP, I. ROOSHEROE, J. DE WAART, J. W. SEDEE, and H. J. VONK: Arch. Internat. de Physiol. et de Biochim. 66, 220 (1958).