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5 November 1981

Transport of Dissolved Amino Acids by the Mussel, *Mytilus edulis*: Demonstration of Net Uptake from Natural Seawater

Abstract. *High-performance liquid chromatography provides direct evidence for substantial removal of naturally occurring specific free amino acids during a single passage of water through the mantle cavity of mussels. This occurs during the few seconds required for passage of the water across the gill, and removal proceeds unabated at ambient substrate concentrations as low as 38 nanomoles per liter.*

The total mass of organic material in solution in the oceans is vast, outweighing the total biomass on the earth (*1*). Marine biologists have speculated for more than a century that this very large potential resource may be available to marine organisms as a nutritional supplement (*2*). Demonstration of rapid influx of specific radiolabeled substrates into marine invertebrates (*3*) has kindled interest in this possibility and stimulated work in the field. Studies in the past two decades strongly support the widespread occurrence of uptake and utilization by marine organisms of elements of the dissolved organic pool (*4*). However, evidence for this conclusion is less direct than is desirable, and considerable controversy still exists about the role of dissolved organic material in invertebrate nutrition (*5*). Since concentrations of specific organic compounds in the natural pool are extremely low, there is no direct analytical evidence for net entry of organic substrates at the submicromolar levels characteristic of natural waters. Furthermore, it has been impossible to exclude completely a possible role of heterotrophic microorganisms, which have been present in the experimental systems studied to date (*6*). We report here that net entry of specific organic solutes from natural waters has been established by direct chemical analysis. A significant contribution to the process by contaminant microorganisms was ruled out by the very rapid changes in concentration, which occurred within seconds.

We prepared fluorescent derivatives of amino acids and other amines in solution with *o*-phthaldialdehyde (OPA). Derivatives were separated by high-performance liquid chromatography (HPLC). Peaks were monitored with a fluorometer, identified by elution time, and quantified by peak area (*7, 8*). The technique has a sensitivity in the picomole range. With a sample of 200 μl of seawater, a quantitative measure of specific amino acids present at concentrations as low as 10 n*M* can be obtained. The linearity of the relation between peak area and concentration was established by chromatography of standards ranging in concentration from 83.5 to 1000 n*M*. Coefficients of determination (r^2) for the three amino acids used were aspartic acid (Asp), .99 (N = 19); serine (Ser), .99 (N = 21); and glycine (Gly), .98 (N = 21).

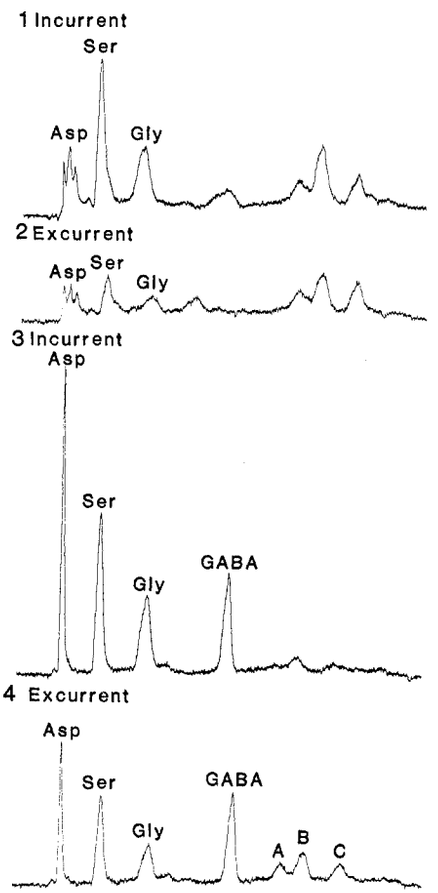

Fig. 1. HPLC chromatograms of natural seawater and an artificial seawater solution. Tracing 1 is natural seawater collected from the immediate habitat of *Mytilus edulis*. Tracing 2 is the same water after passage through the mantle cavity of the animal. Tracing 3 is an artificial seawater solution containing 0.25 μmole per liter of Asp, Ser, Gly, and GABA. Tracing 4 is the artificial solution after passage through the mantle cavity of the animal. Peaks of the four amino acids are labeled. Peaks A, B, and C are material added by the animal during passage of water through the mantle cavity.

Specimens of the mussel *Mytilus edulis* were collected locally from Newport Bay in southern California, cleaned with a wire brush, and adapted to room temperature for 24 to 48 hours before use. An animal was placed in 400 ml of medium and allowed to open and begin pumping. The medium was withdrawn and replaced; normally, pumping resumed promptly. Samples were then collected directly from the excurrent siphon of *Mytilus* with a fine plastic cannula positioned with a micromanipulator. Such samples were compared with water samples from the incurrent margin of the animal. Since the animal was unrestrained and pumping normally, differences between incurrent and excurrent samples represented changes that occurred during a single passage of water through the mantle cavity. This procedure has been described in detail (*9*).

The first two tracings in Fig. 1 are chromatograms obtained from 200-μl samples of natural seawater taken respectively from the incurrent margin and the excurrent siphon of an animal as soon as possible after the onset of pumping. All chromatograms are photographs of original records. Fluorescent derivatives of aspartate, serine, and glycine are labeled. The water was collected from the immediate habitat of the animal, brought to the laboratory, and passed through a 0.2-μm Nucleopore filter to remove particulate matter and microorganisms. Analysis of a small sample of water filtered immediately in the field indicated that the brief period between collection of the water sample and filtration in the laboratory did not result in noticeable changes in amino acid composition. In the case presented in Fig. 1, 63, 84, and 72 percent of aspartate, serine, and glycine were removed by the animal during passage of the water through the mantle cavity.

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For further observations, a solution of these three amino acids and γ-aminobutyric acid (GABA) was prepared in artificial seawater. This was done to reduce the complexity of the initial medium and to avoid any ambiguity in identification of peaks. Each substrate was present in this artificial medium at an initial concentration of 0.25 μ*M*. The GABA was included as a control of the cannulation procedure since preliminary observations indicated that its rate of entry into the animal was considerably lower than that of the other amino acids and its OPA derivative was clearly separated from the other test substrates. Use of the artificial medium also facilitated detection and characterization of any OPA-reactive compounds liberated into the medium by the mussels. Previous work showed that influx of free amino acids is accompanied by liberation of fluorescamine-positive material interpreted as unknown primary amines (*10*).

Tracings 3 and 4 in Fig.1 are chromatograms of samples of the artificial medium, taken respectively from the incurrent margin and excurrent siphon of an actively pumping animal. This procedure was repeated several times. Percentages of the substrates removed during passage of water through the mantle cavity of four animals are shown in Table 1. In another experiment, concentrations of the substrates were compared in ex-

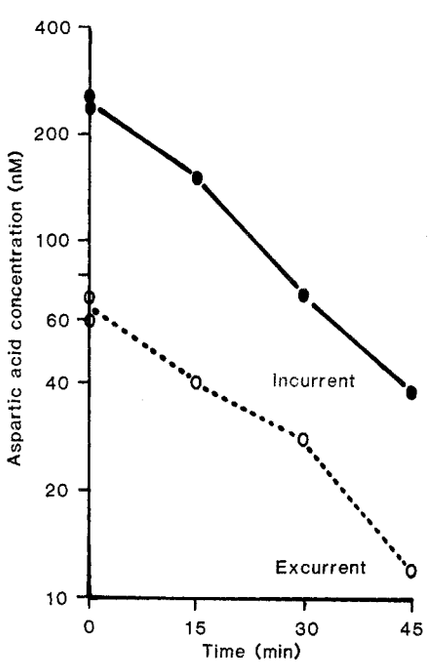

Fig. 2. Removal of aspartate from an artificial seawater solution containing Asp, Ser, Gly, and GABA at an initial concentration of 0.25 μ*M* for each substrate. (●) Samples of the medium taken from the incurrent margin of the animal; (○) samples taken with a fine plastic cannula from the excurrent siphon.

Table 1. Percentage of substrate removed during passage of seawater through the mantle cavity of four specimens of *M. edulis*. Initial concentration of each substrate is 0.25 μ*M*.

Animal	Percent removed			
	Asp	Ser	Gly	GABA
1	73	74	76	26
2	57	52	58	34
3	69	71	94	42
4	36	48	64	0

current and incurrent samples taken at 15-minute intervals after the onset of pumping. Figure 2 presents the data for aspartate. Notice that passage through the mantle cavity at 45 minutes after observations were initiated reduced the aspartate concentration from 38 to 12 n*M*. Thus 68 percent of the substrate was removed even at this very low ambient concentration. There was no discernible decrease in efficiency of removal of aspartate as ambient concentration decreased from 250 n*M* down to the limit of resolution of the analytical procedure.

The peaks A, B, and C in the fourth tracing of Fig. 1 were present in all samples taken from the excurrent siphon of animals. They represent material released by the animal during passage of water through its mantle cavity. As expected, these peaks appear in the bulk medium in which the animal is placed and increase in height with the passage of time. *Mytilus* was placed in seawater that contained no organic substrate. Tracing 5 in Fig. 3 is a chromatogram of a sample taken 45 minutes after the animal began pumping. Appearance of these peaks occurs independent of substrate entry; tracing 6 in Fig. 3 is a chromatogram of the artificial medium 45 minutes after exposure to an animal. Peaks A, B, and C increase in size for the 5 hours during which observations were made; no other peaks appear during this time.

Chromatogram 7 in Fig. 3 was obtained by injecting a solution containing 4 nmole of NH_4Cl in 200 μl of artificial seawater into the HPLC. The presence of three peaks whose elution time corresponds to that of peaks A, B, and C strongly suggests that ammonia is the principal excretory product being added to the medium during passage through the animal (*11*). The possibility that some other material that coelutes with one of the ammonia peaks is being excreted is not excluded, but there is no evidence to support this.

The rapid removal of specific amino acid substrates observed in this work is consistent with reports of influx of ^{14}C-labeled amino acids and disappearance of fluorescamine-positive amines in mussels (*10*). Ammonia is a major excretory product of bivalves (*12*) and reacts to form fluorescent derivatives with both OPA and fluorescamine (*13*). Its appearance in the medium is consistent with the slow excretion of "unknown amines" previously reported. Rapid removal of amino acids from the medium is observed only if the animal is actively pumping. Close examination of scanning and transmission electron micrographs of *Mytilus* gill provides no morphological evidence of any bacterial association; autoradiograms of gills exposed for very brief periods to radiolabeled amino acids show intense labeling within the cells of the gill epithelium (*14*). Since dramatic decreases in concentration occur in a period of seconds during passage of water across the gill, no significant role can reasonably be assigned to heterotrophic microorganisms in removal of substrate in these observations.

Rapid removal of naturally occurring amino acids from seawater is not confined to *Mytilus*, nor is it limited to bivalve mollusks. Preliminary observations indicate that the ascidian *Styela montereyensis* removes half to two-thirds of specific amino acids from natural seawater during passage of the water through the branchial basket. The work reported here strongly supports inferences of a nutritional role of dissolved organic material in marine organisms based on less direct observations (*2*, *4*). The use of HPLC separation combined with fluorescent detection of substrates

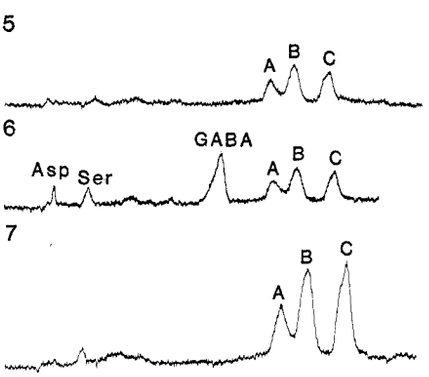

Fig. 3. HPLC chromatograms 5 and 6 are samples of the medium taken 45 minutes after the onset of pumping by an animal. Tracing 5 represents a medium of artificial seawater to which no organic substrate was added initially. Tracing 6 is artificial seawater containing Asp, Ser, Gly, and GABA (initial concentrations, 0.25 μ*M*). The same animal was used to obtain chromatograms 5 and 6. Tracing 7 is the chromatogram obtained when 4 nmole of NH_4Cl in 200 μl of artificial seawater was derivatized and injected into the HPLC.

allows quantitative assessment of the input of specific solutes from the natural pool of dissolved organic material and should greatly facilitate assessment of the importance of this transport pathway in energy flow in marine communities.

DONAL T. MANAHAN
Department of Developmental and Cell Biology, University of California, Irvine 92717

STEPHEN H. WRIGHT
Department of Physiology, School of Medicine, University of California, Los Angeles 90024

GROVER C. STEPHENS
MICHAEL A. RICE
Department of Developmental and Cell Biology, University of California, Irvine

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10 August 1981; revised 2 October 1981

Hydrolysis of Nerve Gas by Squid-Type Diisopropyl Phosphorofluoridate Hydrolyzing Enzyme on Agarose Resin

Abstract. *An enzyme purified from squid nerve that hydrolyzes the cholinesterase inhibitor diisopropyl phosphorofluoridate (DFP) has now been coupled to agarose beads. A column of this agarose-DFPase hydrolyzes the nerve gas 1,2,2-trimethylpropyl methylphosphonofluoridate (Soman). Although the more inhibitory of the four diastereoisomers of Soman are hydrolyzed least rapidly, a column of sufficient length will accomplish 95 percent hydrolysis whether measured by fluoride release or loss of cholinesterase-inhibiting power. The results suggest a means for detoxifying unwanted chemical warfare agents.*

From a perusal of scientific commentary and informed popular writing it appears that the powerful cholinesterase inhibitor 1,2,2-trimethylpropyl methylphosphonofluoridate (Soman) is a potential chemical warfare agent (*1*). A similar compound, diisopropylphosphorofluoridate (DFP), is widely used for research and has even been used for clinical purposes (*2*). The discovery of an enzyme or enzymes capable of hydrolyzing DFP (*3*), the seeming absence of a natural substrate or physiological role for the enzyme, and the discovery of a particular variety of the enzyme in the squid giant axon (*4*) has led us to use the term "squid-type DFPase" (*5*). This enzyme will also hydrolyze Soman (*6*). In contrast to DFP, Soman has a property that makes it especially insidious as a nerve gas. Its powerful inhibition of acetylcholinesterase (AChE) is irreversible by any known means because of the very rapid loss of the alkoxy side chain after the initial phosphonylation of AChE (*7*). Data showing that Soman may act on components of the cholinergic system other than AChE (*8*) have renewed our interest in Soman hydrolysis by DFPase. Since one of the two phosphorus-centered stereoisomers (there are a total of four diastereoisomers) is enzymatically hydrolyzed more rapidly than the other (*9*), the use of squid-type DFPase for ascribing these other actions to one or another isomer seemed an attractive possibility. Although this has not yet been realized, another accomplishment of some possible practical significance is presented here. This is the covalent linking of squid-type DFPase to a resin, and the use of this immobilized enzyme for the detoxication of Soman.

Prior to the immobilization of the enzyme, we determined the molecular weight of 2000-fold purified squid-type DFPase (*5*) by gel filtration on Sephadex G-100 resin (*10*). The elution volume of the DFPase, determined with DFP and a fluoride electrode (*11*), was compared to the elution volumes of cytochrome *c*, myoglobin, α-chymotrypsinogen, ovalbumin, and bovine serum albumin, determined by their absorbancies at 280 nm. From these determinations the molecular weight of squid-type DFPase was estimated to be 26,600 ± 1,000 (± standard deviation).

The coupling of this relatively low molecular weight enzyme to an agarose resin has been carried out three times by the method of Cuatrecasas and associates (*12*) with consistent results. In a typical preparation, 2 g of Sepharose-6B resin was activated with cyanogen bromide and was then added to squid-type DFPase (approximately 2 mg of protein capable of hydrolyzing 12 μmole of DFP per minute; the DFPase of highest purity has not been used for coupling) contained in 2 ml of 0.2*M* sodium bicarbonate, *p*H 7.5, and the suspension was shaken overnight in the cold. The resulting agarose-DFPase was collected on a coarse sintered glass filter and washed with 40 ml of a solution made 0.5*M* in sodium chloride, 0.1*M* in tris(hydroxymethyl)aminomethane, *p*H 7.5, all by gravity filtration over about 30 minutes. Finally, the agarose-DFPase was water-washed repeatedly by low-speed centrifugation. Approximately 80 percent of the enzyme was bound to the resin, and about 20 percent was accounted for in the filtrate. With either DFP or Soman as substrates, the Michaelis constants (K_M) and relative maximum velocities (V_{max}) of a dilute slurry of agarose-DFPase are essentially the same as those reported by us for the soluble enzyme (*6*).

We refer to the immobilized enzyme as agarose-DFPase; our interest is predominantly in its Soman-hydrolyzing properties. Two measures of this have been used: the release of fluoride ion measured by a fluoride-sensitive electrode (*11*) and the loss of AChE-inhibiting power in which AChE activity is measured by the method of Ellman *et al.* (*13*).

Figure 1 shows that when $10^{-4}M$ Soman is passed through a 5 by 50 mm agarose-DFPase column at a flow rate of 0.1 ml/min, fluoride ion appears in the column effluent at almost the rate of fluoride appearance if $10^{-4}M$ fluoride is passed through the column. By contrast, $10^{-4}M$ Soman passed through a simple agarose column of the same dimensions releases fluoride ion at a rate compatible

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