The close agreement between CO_2 invasion measurements based on naturally occurring and bomb-produced ^{14}C methods and those obtained from radon measurements allows us to place limits on the role of catalysis in the oceans (*17*). These results support our overall conclusion that enzymatic catalysis, even if it does occur, would have little effect on the mass transport of CO_2 into the oceans, given the degree of wind-induced turbulence present.

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9. With the valve closed to the liquid phase, air in the gas phase was first circulated through a CO_2 trap consisting of Ascarite (sodium hydroxide–coated asbestos) to remove CO_2 and establish a baseline. The trap was then bypassed and sufficient 100 percent CO_2 gas was injected through a septum on the gas injection bulb to raise the CO_2 partial pressure in the gas phase to the desired concentration.
10. The rate coefficient K (min^{-1}) was calculated from the equation $K = \Delta t^{-1} \ln (C_0 C_t^{-1})$, in which C_0 was the initial CO_2 concentration in the gas phase and C_t was the concentration after the time interval Δt. The liquid phase had a surface area of 18.1 cm^2 and a volume of 450 cm^3.
11. The concentration of CO_2 in our artificial seawater varied from ~ 40 nmole at *p*H 7.8 to ~ 0.5 nmole at *p*H 9.2. The time interval 30 to 45 minutes was chosen to ensure that decreases in *p*H never exceeded 0.1 *p*H unit and that equilibrium between CO_2 in the gas and liquid phases was never attained. We expressed the rate coefficient in units of reciprocal minutes rather than in the more conventional CO_2 exchange units of meters per year or moles per square meter per year used by oceanographers because the geometry of our system was so unlike that of the ocean.
12. The estimated film thickness (μm) was calculated from the expression DA/KV, in which D is the diffusion coefficient of CO_2 in seawater (taken to be 2×10^{-5} cm^2/sec), A is the surface area, and V is the volume.
13. Quinn and Otto (*5*) estimated that chemical enhancement of CO_2 transport into seawater becomes important only for surface film thicknesses ≥ 400 μm, which is consistent with our findings. In contrast, Broecker and Peng (*4*) estimated a conservative surface film thickness of the oceans to be < 60 μm, far less than is necessary for chemical enhancement to be effective.
14. Over the range of *p*H in the natural seawater samples (7.96 to 8.40) the concentration of total inorganic carbon varied between 25.5 and 27.1 mg/liter, whereas the concentration of total inorganic carbon in the artificial seawater decreased from 27.5 mg/liter at *p*H 7.96 to 25.6 mg/liter at *p*H 8.40.
15. Carbonic anhydrase is an essential enzyme in the regulation of CO_2 excretion in marine fish and has been found in numerous shellfish species, even though its function in marine invertebrates is not well understood [T. H. Maren, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **26**, 1097 (1967); H. F. Bundy, *Comp. Biochem. Physiol. B* **57**, 1 (1977); J. N. Cameron, *Mar. Biol. Lett.* **1**, 3 (1979)].
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17. The best estimate for CO_2 invasion rates in the ocean, based on radon measurements, is 16 mole/m^2-year, whereas estimates determined from ^{14}C methods are 19 to 22 mole/m^2-year. Hence, enzymatic catalysis of CO_2 transport could at best account for a 40 percent enhancement effect, assuming the different measurements were essentially error-free [W. S. Broecker, T.-H. Peng, G. Mathieu, R. Hesslein, T. Torgensen, *Radiocarbon* **22**, 676 (1980)].
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Fibronectin Binds to Some Bacteria but Does Not Promote Their Uptake by Phagocytic Cells

Abstract. *The involvement of plasma fibronectin in phagocytosis of bacteria was investigated by testing the binding of fibronectin to several species of bacteria and by evaluating the ability of fibronectin to promote binding and endocytosis of two species of these bacteria by phagocytic cells. Fibronectin binds non-covalently to Gram-positive and Gram-negative bacteria and to yeast but did not appear to be necessary or sufficient for uptake of* Staphylococcus aureus *and* Salmonella typhimurium *by several different phagocytic cell types.*

Experiments showing a correlation between the levels of plasma fibronectin and the clearance of gelatin-coated particles suggest that fibronectin may act as an opsonin to augment the clearance of various particulate materials from the circulation (*1*). Fibronectin contains several binding sites, including one for gelatin (*2*), and stimulates the endocytosis of gelatin-conjugated particles by peritone-

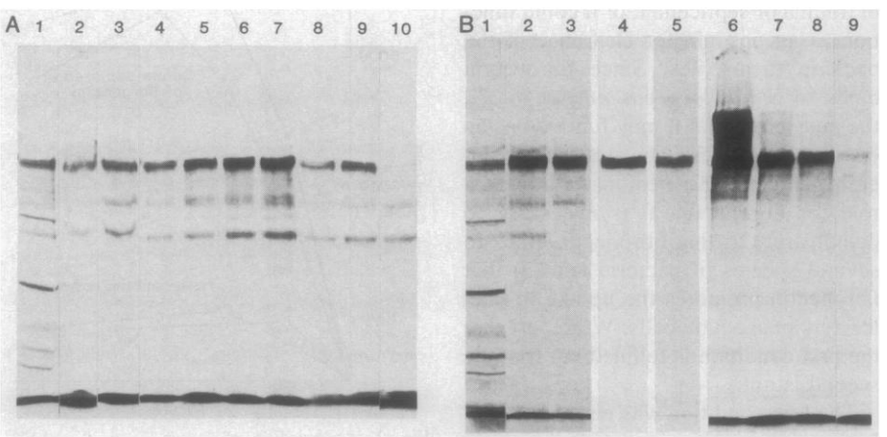

Fig. 1. Fluorograph of gels showing fibronectin binding to different microorganisms. (A) Portions (0.5 ml) of [^{35}S]methionine-labeled culture medium prepared from NIL8 hamster cells and containing 5 percent fetal calf serum (lane 1) (3.4×10^6 cpm/ml) were incubated for 30 minutes at room temperature with 0.1 ml (lanes 2, 4, 6, 8, and 10) or 0.2 ml (lanes 3, 5, 7, and 9) of a 10 percent suspension of each microorganism (*14*). The microorganisms tested were *Salmonella typhimurium* (lanes 2 and 3), *Bacillus subtilis* (lanes 4 and 5), *Staphylococcus aureus* (lanes 6 and 7), *Escherichia coli* (lanes 8 and 9), and *Saccharomyces cerevisiae* (lane 10). Bound proteins were released by boiling in buffer containing 2 percent SDS and 0.1*M* dithiothreitol and were analyzed on a 5 percent SDS gel. Arrowheads mark proteins at 230,000 (fibronectin), 185,000 (pro-C3), 180,000 (procollagen), and 130,000 daltons (C3α). (B) In similar experiments, [^{35}S]methionine-labeled conditioned medium (lane 1) was incubated with *S. aureus* after incubation without (lane 2) or with (lane 3) purified collagenase. Fibronectin was bound whether or not the collagen band was present. Furthermore, fibronectin purified by gelatin-affinity and gel filtration chromatography (*15*) (lane 4) was also bound by *S. aureus* (lane 5). *Staphylococcus aureus* prepared untreated (lane 6), fixed (lane 7), or heated and fixed (lane 8) were each incubated in medium conditioned with ^{35}S; all samples bound fibronectin. Bound proteins were analyzed by SDS electrophoresis on a 5 percent gel as above. When bacteria were omitted, very little fibronectin was sedimentable (lane 9). Furthermore incubation with Sepharose beads did not lead to binding of fibronectin (not shown). Slowly migrating radioactivity (lane 6) was seen whenever unfixed bacteria were used, presumably because lysis of the bacteria led to trapping of radioactive proteins at the top of the gel.

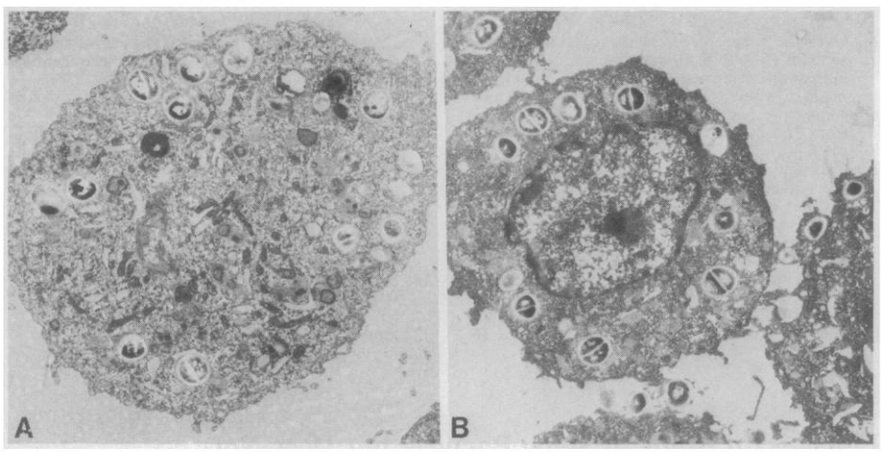

Fig. 2. Fibronectin-independent endocytosis of *S. aureus* by rabbit alveolar macrophages. Samples of human serum (A) containing or (B) depleted of fibronectin (to less than 0.1 percent) (*4*) were mixed with an equal volume of *S. aureus* (10 percent by volume; 10^{10} organisms per milliliter) (*14*), incubated for 15 minutes at 37°C, washed in phosphate-buffered saline (PBS), mixed with 2×10^6 rabbit alveolar macrophages in a total volume of 0.9 ml of PBS, and incubated for 10 minutes at 37°C. Washed cell pellets were fixed and prepared for electron microscopy (Phillips EM 201) (*4*). Bacteria were endocytosed whether or not plasma fibronectin was present.

al macrophages (*3*) and macrophage-like cell lines (*4*). Furthermore, fibronectin appears to reverse some of the clinical complications of septicemia in injured patients (*5*). Fibronectin could ameliorate the clinical symptoms in these patients by aiding in the clearance of nonbacterial debris arising as a consequence of trauma or septicemia, or it could function as an opsonin for clearance of the bacteria themselves. Since fibronectin binds to *Staphylococcus aureus* (*6, 7*), the possibility that it may function as an opsonin for bacteria has aroused considerable interest. To demonstrate such a role for fibronectin, it is necessary to demonstrate (i) that fibronectin binds to several species of bacteria and (ii) that fibronectin promotes the uptake of bacteria by phagocytic cells. We report that the first condition is fulfilled but that the second is not.

The first condition was tested by incubating [^{35}S]methionine-labeled culture medium prepared from NIL8 hamster cells with various microorganisms. This medium contains several secreted proteins, including cellular fibronectin, some type of collagen, and the third component of complement (C3) (*8*). Bound proteins were eluted with sodium dodecyl sulfate (SDS) and analyzed on gels (Fig. 1A). Binding of proteins at 230,000, 200,000, and 180,000 daltons was reproducibly observed with Gram-positive and Gram-negative bacteria and with yeast. In some experiments, another minor band at 300,000 to 400,000 daltons was also bound (Fig. 1B). The 230,000-dalton band is fibronectin, and the 180,000-dalton band is collagenous [(*8*), and see also below]. The identities of the 200,000-dalton band and the minor band at 300,000 to 400,000 daltons are not known. Although C3 was present in this culture medium and would bind to erythrocytes coated with complement components C1, C4, and C2 (*8*), it did not bind to the fixed bacteria used here (Fig. 1A).

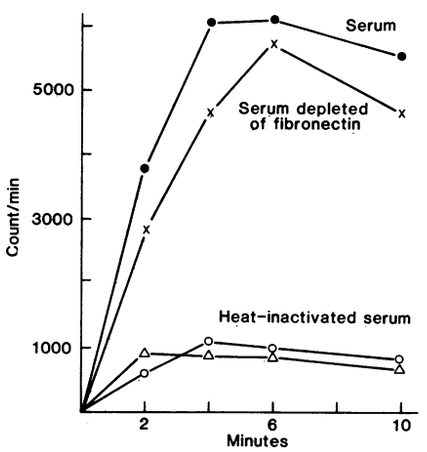

Fig. 3. Association of *S. aureus* with rabbit alveolar macrophages. Fixed *S. aureus* (0.1 ml; 10 percent by volume; 10^{10} organisms per milliliter), labeled with [^{3}H]thymidine, were incubated with 0.1 ml of human serum (with or without heat inactivation at 56°C for 30 minutes, and with or without depletion of fibronectin by gelatin-affinity chromatography). After being washed with PBS, the bacteria were incubated with 1×10^7 rabbit alveolar macrophages in a final volume of 0.9 ml PBS containing divalent cations. At intervals, 0.2 ml was removed to ice-cold PBS with *N*-ethylmaleimide (2 m*M*) to block further endocytosis, and the cells were washed (at about 1000*g* for 10 seconds). Heat-inactivated serum, with (○) or without (△) fibronectin, is inactive as an opsonin. Controls in serum-free medium (not shown) were similar to those in heat-inactivated serum. Results of similar experiments under different conditions and with other cell types are shown in Table 2.

This experiment shows that fibronectin binds non-covalently to the bacteria, since it can be eluted by SDS. An earlier report described covalent cross-linking of fibronectin to *S. aureus* catalyzed by factor XIIIa transamidase (*7*). In our experiments, this presumably did not occur since the culture medium probably contained little active XIIIa. In any event, our experiments deal with non-covalently bound fibronectin.

The nature of the binding of fibronectin to the bacteria was further investigated by competition experiments (Table 1). Neither EDTA nor sulfhydryl-blocking reagents inhibited the binding of fibronectin to *S. aureus*. High concentrations of several sugars did not inhibit binding. However, glucuronic acid did inhibit binding, and this inhibition was slightly potentiated by *N*-acetylglucosamine, which was itself inactive. There was partial inhibition of binding by heparin (0.2 mg/ml) but none by similar concentrations of heparan sulfate, dermatan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, or hyaluronic acid. Gelatin slightly inhibited binding, and arginine, lysine, and glutamine were inhibitory at high concentrations.

The observed inhibition, on the one hand, by heparin and glucuronic acid and, on the other, by some amino acids (arginine, lysine, glutamine) is consistent with interaction through the basic NH_2-terminal region of fibronectin (*7*). This region also appears to bind to heparin (*9*); therefore heparin and related haptenic sugars might be expected to inhibit the binding of fibronectin to bacteria.

We next conducted experiments to determine whether endocytosis of bacteria is promoted by fibronectin. Alveolar macrophages endocytosed *S. aureus* whether or not exogenous fibronectin was present (Fig. 2). Labeled bacteria were used with phagocytic cells in a suspension assay that enabled us to test a large number of variables (Fig. 3 and Table 1). With rabbit alveolar macrophages, uptake of *S. aureus* occurred within 10 minutes at 37°C in serum either containing or depleted of fibronectin; heat inactivation of the serum blocked uptake (Fig. 3). Neither heat-inactivated serum nor buffer alone stimulated significant binding or uptake of bacteria (Fig. 3). Therefore, over the time course of this experiment, endogenous synthesis of fibronectin by alveolar macrophages was not sufficient to promote the uptake of *S. aureus*.

This centrifugation assay was used to test other bacteria-cell pairs for dependence on serum, fibronectin, or heparin. In all cases there was a strong dependence on serum (experiment 1 in Table 2). Heat inactivation of the serum greatly reduced the association of *S. aureus* or *Salmonella typhimurium* with several of the cells tested (Fig. 3 and experiment 2 in Table 2). No dependence on fibronectin was observed when complete serum was compared with serum depleted of fibronectin (Fig. 3 and experiment 3 in Table 2), or after reconstitution with purified fibronectin (experiment 4 in Table 2). Sera prepared from human, mouse, or rabbit blood were all competent to promote binding unless they were inactivated by heating. Human platelet-poor plasma gave similar results. Although a strict dependence on fibronectin and heparin was observed in parallel experiments on the uptake of gelatin-latex by mouse cell lines P388D_1 and RAW 309Cr.1, mouse peritoneal exudate cells, or human neutrophils (*4*), added heparin had no stimulatory effect on the binding of bacteria and, in fact, slightly inhibited binding in some experiments (experiment 5 in Table 2). In one set of experiments, the treatment of the *S. aureus* was modified (experiment 6 in Table 2), but this did not result in significant fibronectin-dependent uptake by P388D_1 cells. Therefore, for the bacteria-cell pairs we tested, exogenous fibronectin is not an opsonin under the conditions tested.

These results demonstrate that plasma and cellular fibronectin, which bind to several Gram-positive and Gram-negative bacteria, do not promote the binding and endocytosis of *S. aureus* and *S. typhimurium* by several types of phagocytic cells. Binding and uptake of bacteria by these cells does depend on a heat-labile opsonin present in serum, but the presence or absence of plasma fibronectin appears to be unimportant. Some of the cells we tested (peritoneal exudate cells, RAW 309Cr.1, alveolar macrophages) do synthesize and secrete fibronectin (*4, 10*). If this endogenous fibronectin can act as an opsonin, it is apparently not sufficient to do so by itself under our experimental conditions (Fig. 3 and Table 2). Perhaps most clearly, the macrophage-like cell line P388D_1, which does not synthesize fibronectin and is dependent on exogenous fibronectin for uptake of gelatin-conjugated latex particles (*4*), did not use plasma fibronectin as an effective opsonin for bacteria. It is possible that other microorganisms, other cell types, or different assay conditions will show an involvement of fibronectin in endocytosis of bacteria.

Table 1. Inhibition of binding of fibronectin to *S. aureus*. Results were obtained from assays such as shown in Fig 1. Bound fibronectin was quantified from scans of fluorographs of gels. The concentrations shown are the maximum concentrations tested.

Inhibitor	Maximum inhibition of binding
EDTA (10 m*M*)	0
Iodoacetic acid (10 m*M*)	0
N-Ethylmaleimide (10 m*M*)	0
Glucose (0.5*M*)	0
Galactose (0.5*M*)	0
α-Methyl mannoside (0.5*M*)	0
Glucosamine (0.2*M*)	0
N-Acetylglucosamine (0.2*M*)	0
Sialic acid (0.2*M*)	0
Glucuronic acid (0.2*M*)	80
Glucuronic (0.2*M*) + *N*-acetylglucosamine (0.2*M*)	90
Arginine (0.2*M*)	90
Lysine (0.2*M*)	80
Glutamine (0.5*M*)	30
Heparin (0.2 mg/ml)	60
Gelatin (0.2 mg/ml)	20

Lanser and Saba (*11*) reported that although fibronectin alone does not promote binding of *S. aureus* by neutrophils, complete serum is 1.5 to 2 times as effective as fibronectin-depleted serum in functioning as an opsonin. They suggest that fibronectin may act as a cofactor for phagocytosis in this system. We did not observe this result in any of the bacteria-cell pairs we tested, including the pair (*S. aureus* and neutrophils) examined by Lanser and Saba (*11*). Proctor *et al.* and Verbrugh *et al.* (*12*) reported that fibronectin binds to *S. aureus* but does not promote phagocytosis.

If fibronectin does not act as a true opsonin for phagocytosis of bacteria, other interpretations for the reported effects of fibronectin on the clinical symptoms of patients with septic injuries (*5*) must be considered. It is established that fibronectin binds to a wide variety of materials (*2*), that levels of fibronectin are correlated with the clearance of particulates in vivo and that fibronectin promotes the endocytosis of gelatin-coated particles by phagocytic cells (*3, 4*). It is therefore possible that fibronectin plays a role in the clearance of debris such as fragments of extracellular matrix or blood clots arising as a consequence of trauma and sepsis. Since fibronectin also binds to the C1q component of complement (*13*), it could promote the clearance of complement-bound materials.

In conclusion, there is currently no convincing evidence that fibronectin promotes endocytosis of bacteria, although it apparently can act as an opso-

Table 2. Uptake of bacteria in suspension assay (see Fig. 3). Bacteria labeled with [^{3}H]thymidine (New England Nuclear; 5 μCi/ml, 23 Ci/mmole) were prepared by the method of Kessler (*14*), except in experiment 6. Uptake of phagocytes is the ratio (expressed as percentage) of labeled bacteria associated with cells in treated serum to cell-associated bacteria in untreated serum; incubation with phagocytes for 20 minutes at 37°C. In experiment 3, gelatin-Sepharose chromatography was used to deplete serum of fibronectin (*15*). The purified fibronectin used in experiment 4 was prepared by elution from gelatin-Sepharose with either urea or NaBr; both plasma and cellular fibronectins were tested at various doses in the presence and absence of heat-inactivated, fibronectin-depleted serum. No increase above background levels was seen in either case. PEC, peritoneal exudate cells

Experiment	Variable tested	^{3}H-labeled microorganism	Phagocytes	Uptake (%)
1	Absence of serum	*S. aureus*	Rabbit alveolar	5
2	Heat inactivation of serum	*S. aureus*	Rabbit alveolar	5
		S. aureus	P388D_1, RAW 309Cr.1, mouse PEC, human neutrophils	20 to 50
		S. typhimurium	RAW 309Cr.1	30 to 50
3	Depletion of fibronectin	*S. aureus*	P388D_1, RAW 309Cr.1, mouse PEC, human neutrophils, rabbit alveolar	100
		S. typhimurium	P388D_1	100
4	Reconstitution of heat-inactivated, fibronectin-depleted serum with purified fibronectin	*S. aureus*	P388D_1, RAW 309Cr.1, human neutrophils, mouse PEC, rabbit alveolar	5
		S. typhimurium	P388D_1	5
5	Addition of heparin (10 U/ml)	*S. aureus*	P388D_1	100
		S. aureus	Rabbit alveolar	35 to 50
		S. typhimurium	RAW 309Cr.1	50
6	Bacteria preparation			
	Live	*S. aureus*	P388D_1	170
	Fixed	*S. aureus*	P388D_1	130
	Heat-fixed	*S. aureus*	P388D_1	100

nin for other materials. In the treatment of patients with septic injuries (*5*), fibronectin might perform other functions of clinical relevance, such as augmenting the clearance of tissue debris or denatured proteins; these other roles need further analysis in both experimental and clinical studies.

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Bacteria-Free Sea Urchin Larvae: Selective Uptake of Neutral Amino Acids from Seawater

Abstract. *Bacteria-free suspensions of larvae of* Strongylocentrotus purpuratus *(Stimpson) were prepared without the use of antibiotics. Net rates of removal of 18 amino acids, each supplied at 125 nanomoles per liter, and the appearance of ammonia were measured by high-performance liquid chromatography. Taurine and acidic and basic amino acids were not taken up. Removal of neutral amino acids from the medium occurred at rates adequate to contribute to the carbon and nitrogen balance of the larvae at ecologically relevant substrate concentrations.*

Strong antibiotic solutions have been used in the past to produce bacteria-free soft-bodied marine invertebrates, but the process is lengthy and success is not assured (*1*). Antibiotics may kill only certain groups of bacteria and may actually increase total bacterial numbers by removing interspecies competition (*2*). Even if successful, the intensive use of antibiotics may inhibit animal growth, and the toxic effects can persist for several weeks after the initial exposure (*3*). A reliable technique has been introduced whereby axenic marine invertebrate larvae can be obtained by taking advantage of the fact that gametes in the gonad are free of bacteria (*4*).

We have studied axenic larvae of the sea urchin, *Strongylocentrotus purpuratus*, obtained without the use of antibiotics. Adult animals were collected from the Newport Bay area in southern California. When brought to the laboratory, they were injected with several milliliters of 0.5*M* KCl to induce spawning. After this treatment, animals continued to release gametes for at least 30 minutes. The surface spines and tissues on the aboral surface of spawning animals were removed, and the cleaned area was sprayed with 95 percent ethanol. All reagents and equipment were sterilized by autoclaving and aseptic procedures were followed. The animals were handheld over a 10-ml beaker of MBL-seawater (Marine Biological Laboratory) (*5*) in a transfer hood with the gonopores just touching the surface of the water. The first gametes shed were discarded. Subsequent eggs and sperm obtained in this way were sterile (*6*).

The possibility that marine invertebrates may obtain an important nutritional supplement by absorbing dissolved organic material (DOM) directly from seawater has long been of interest to marine biologists (*7*). Previous studies were done in the presence of heterotrophic microorganisms, and therefore bacteria may have contributed to the

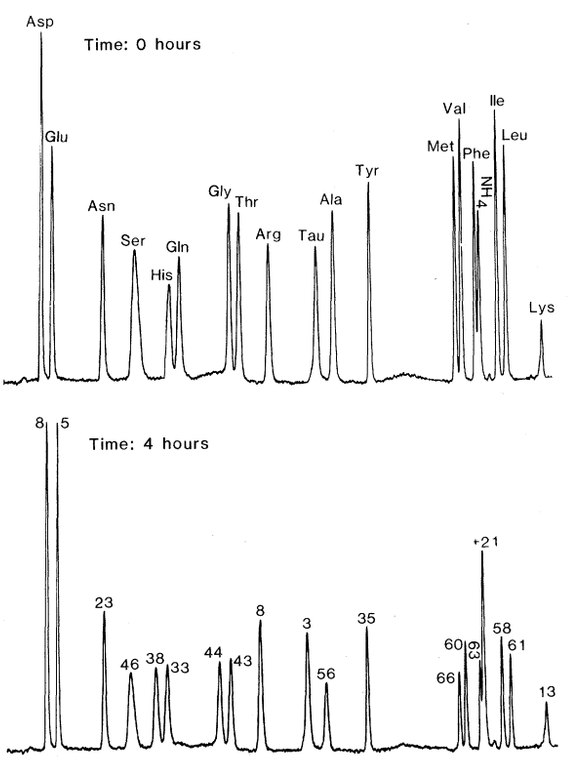

Fig. 1. Chromatograms (HPLC) of the uptake of 18 amino acids and the release of ammonia by axenic sea urchin larvae (*S. purpuratus*). The top tracing (at start) shows the amount of amino acid present in a 400-μl sample. Each amino acid is present at 125 n*M*. The bottom tracing shows the amino acid concentrations after a 4-hour exposure to larvae (102 per milliliter). The numbers above the peaks give the percent of each amino acid removed and the amount of ammonia released. Although the glutamic acid peak has increased in height, the total area has decreased by 5 percent. Concentration is related to area, not peak height (*9*). Amino acid abbreviations are: Asp, aspartic acid; Glu, glutamic acid; Asn, asparagine; Ser, serine; His, histidine; Gln, glutamine; Gly, glycine; Thr, threonine; Arg, arginine; Tau, taurine; Ala, alanine; Tyr, tyrosine; Met, methionine; Val, valine; Phe, phenylalanine; Ile, isoleucine; Leu, leucine; Lys, lysine.