Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 7430–7434, August 1995
Physiology

Free amino acids exhibit anthozoan "host factor" activity: They induce the release of photosynthate from symbiotic dinoflagellates *in vitro*

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Communicated by Andrew A. Benson, University of California, San Diego, La Jolla, CA, April 24, 1995

ABSTRACT **Reef-building corals and other tropical anthozoans harbor endosymbiotic dinoflagellates. It is now recognized that the dinoflagellates are fundamental to the biology of their hosts, and their carbon and nitrogen metabolisms are linked in important ways. Unlike free living species, growth of symbiotic dinoflagellates is unbalanced and a substantial fraction of the carbon fixed daily by symbiont photosynthesis is released and used by the host for respiration and growth. Release of fixed carbon as low molecular weight compounds by freshly isolated symbiotic dinoflagellates is evoked by a factor (i.e., a chemical agent) present in a homogenate of host tissue. We have identified this "host factor" in the Hawaiian coral *Pocillopora damicornis* as a set of free amino acids. Synthetic amino acid mixtures, based on the measured free amino acid pools of *P. damicornis* tissues, not only elicit the selective release of ^{14}C-labeled photosynthetic products from isolated symbiotic dinoflagellates but also enhance total $^{14}CO_2$ fixation.**

Dinoflagellates freshly isolated from the Hawaiian coral *Pocillopora damicornis* and the Pacific giant clam *Tridacna crocea* and incubated in sea water with a crude homogenate of their own host tissue fix $^{14}CO_2$ in the light and release a substantial fraction of the fixed carbon to the incubation medium, principally as [^{14}C]glycerol, [^{14}C]alanine, and [^{14}C]glucose. In contrast, release of labeled organic carbon compounds by freshly isolated dinoflagellates incubated in sea water alone is always significantly less. Moreover, as a crude homogenate of animal tissue from the one host species also induces dinoflagellates from another host species to selectively release fixed carbon, the inducing factor would appear to be nonspecific (1).

Release of fixed carbon by symbiotic dinoflagellates has been demonstrated in several cnidarian symbioses. Although a host factor (HF) has never been identified, its properties, depending on the host, have been described as heat labile (1–3) or heat stable (1, 4), enhancing carbon fixation (2) or not enhancing carbon fixation (3), absent from aposymbiotic hosts but inducible by reinfection with dinoflagellates, constitutive in aposymbiotic hosts (5), evoking release of fixed carbon from cultured symbiotic dinoflagellates and free-living nonsymbiotic dinoflagellates, not evoking the release of fixed carbon from cultured symbiotic dinoflagellates and free-living nonsymbiotic dinoflagellates (5, 6), and behaving like a substance with a molecular mass of <10 kDa (3) or ≈1 kDa (5). Here we identify HF in the Hawaiian reef coral *P. damicornis* as a set of free amino acids (FAAs) that not only evoke the selective release of photosynthate from symbiotic dinoflagellates *in vitro* but also enhance dinoflagellate carbon fixation.

P. damicornis and the tropical sea anemone *Aiptasia pulchella* were collected from Checker Reef adjacent to the Hawaiian Institute of Marine Biology, Oahu, Hawaii, and the animals were transported by air to the University of California, Los Angeles. The corals were sacrificed within a week of collection, and the anemones were maintained in marine aquaria until used (26°C, 12-h photoperiod, 40 μmol of quanta per m^2 per s).

A crude aqueous extract exhibiting HF activity was obtained from *P. damicornis* by scrubbing several individual branches of coral with a toothbrush in a small volume (<3 ml) of filtered sea water (FSW; Millipore; 0.45 μm, pore size) containing the protease inhibitors (all from Sigma) aprotinin (1 μg/ml), pepstatin (1 μg/ml), leupeptin (1 μg/ml), and 1 mM phenylmethylsulfonyl fluoride. The dinoflagellates were removed from the resulting homogenate by centrifugation (15,000 × *g*, 15 min at 4°C). The resulting supernatant was passed through glass wool and kept frozen at −20°C.

HF was isolated from the crude aqueous *P. damicornis* extract by size-exclusion chromatography and HPLC. HF activity was bioassayed at each step of the isolation procedure by incubating symbiotic dinoflagellates freshly isolated from either *A. pulchella* or *P. damicornis* with the host-derived fraction in the presence of $^{14}CO_2$ in the light. Specifically, the dinoflagellates were freshly isolated from *A. pulchella* by homogenization in FSW (0.22-μm pore size) and from *P. damicornis* by crushing the coral with a hammer and vigorously shaking the crushed fragments in a small volume of FSW. The animal fraction was removed from the mixture by repeated (three times) centrifugation (1000 × *g* for 5 min) and discarded. The isolated dinoflagellates were resuspended in FSW, their concentration was adjusted to 4 × 10^6 cells per ml of FSW, and 250 μl was placed in a 1.5-ml microfuge tube (several tubes were used). The substance to be tested for HF activity (250 μl) and NaH$^{14}CO_3$ (1 μCi in 10 μl; 1 Ci = 37 GBq) were added simultaneously to the dinoflagellates, and the suspension was incubated for 60 min in the light (300 μmol of quanta per m^2 per s) at 26°C. FSW alone (pH 8.3) replaced the HF in controls. After the incubation, each tube was mixed thoroughly to resuspend the dinoflagellates and two 50-μl samples were removed to assess the total amount of ^{14}C fixed. The dinoflagellates were separated from the medium by centrifugation (5000 × *g* for 3 min) and two 50-μl samples were removed to assess the total organic ^{14}C released. All samples were acidified with 0.1 M HCl and purged for 60 min to remove unincorporated [^{14}C]bicarbonate. The amount of radioactivity in each sample was determined as dpm by liquid scintillation counting (LKB model 1214). HF activity has customarily been expressed as the percentage of the total ^{14}C fixed by the dinoflagellates that is released to the medium (1–5). As

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Abbreviations: HF, host factor; FAA, free amino acid; FSW, filtered sea water; MAA, mycosporine-like amino acid.

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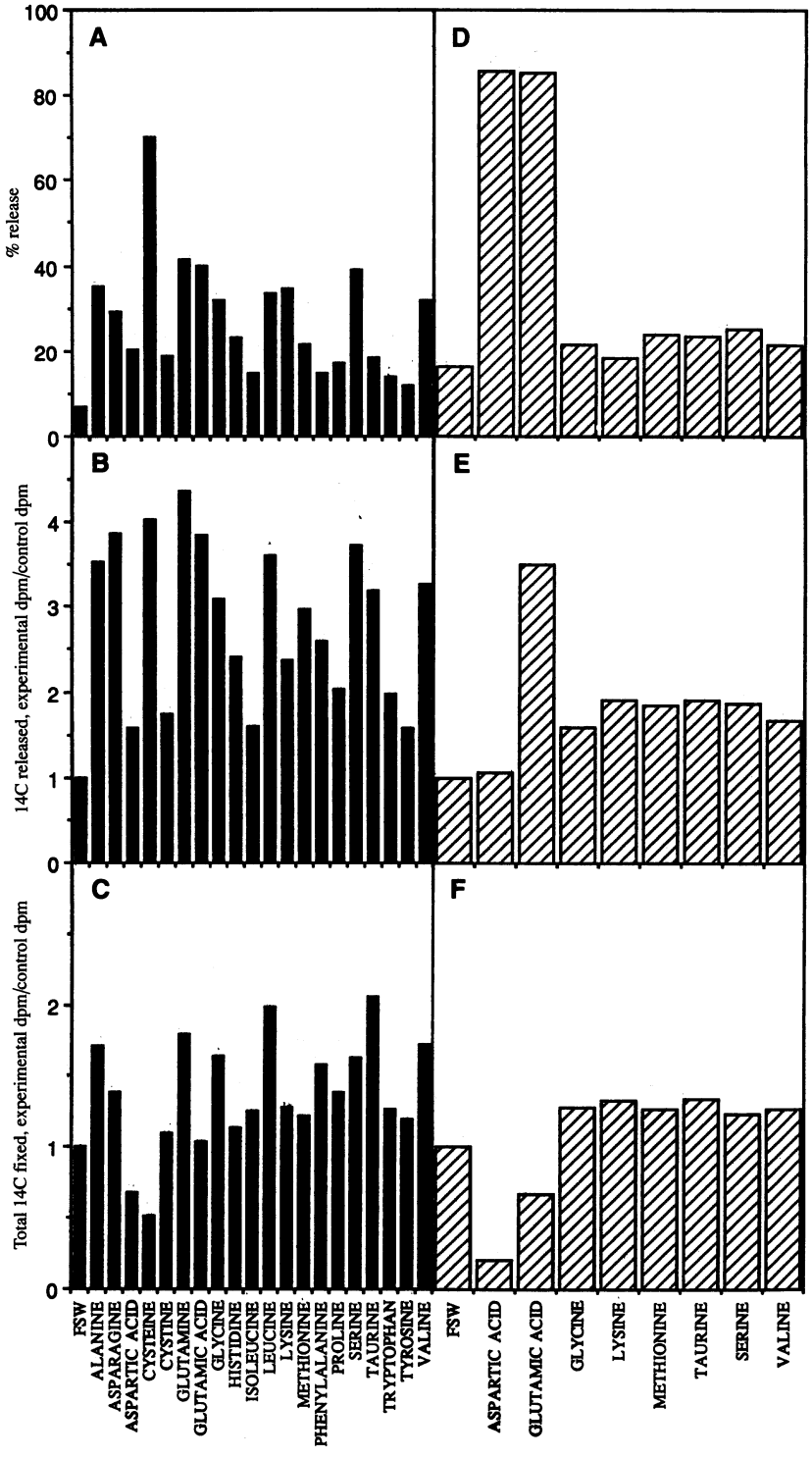

FIG. 1. Fixation and release of acid-stable ^{14}C by isolated symbiotic dinoflagellates incubated with individual amino acids (50 mM in distilled H_2O, salinity was adjusted to 33 parts per thousand, and pH was adjusted to 8.3) and in FSW alone. (*A–C*) Data collected using dinoflagellates freshly isolated from *A. pulchella*. (*D–F*) Data collected for dinoflagellates isolated from *P. damicornis*. (*A* and *D*) Percent of the total ^{14}C dpm fixed and subsequently released to the incubation medium. (*B* and *E*) Ratio of ^{14}C dpm released to the medium in experimental (amino acid in FSW) vs. control (FSW alone) samples. (*C* and *F*) Ratio of the total ^{14}C dpm fixed in experimental vs. control samples.

dinoflagellates incubated with crude aqueous *P. damicornis* HF also exhibited enhanced carbon fixation compared to the controls in FSW, HF activity was also expressed as the ratio of the ^{14}C fixed in experimental (with an HF active fraction) vs. control (with FSW alone) incubations and as the ratio of ^{14}C released to the incubation medium in experimental vs. control incubations.

For size-exclusion analysis, crude aqueous *P. damicornis* HF (1 ml) was loaded onto a 60 × 1 cm glass column containing Sephadex G-25 (Pharmacia) and the material was eluted with distilled water at a flow rate of 0.7 ml/min. The absorption spectrum of the 25 3-ml fractions collected was monitored between 200 and 450 nm. Each fraction was lyophilized and subsequently bioassayed for HF activity. The HF active frac-

Table 1. Free amino acid pools of two colonies of *P. damicornis*

Amino acid	Concentration, mM	
	Colony I	Colony II
Aspartic acid	1.80	0.68
Glutamic acid	21.80	8.50
β-Glutamic acid	1.20	2.41
Serine	1.96	1.65
Histidine	1.84	0.00
Glycine	11.27	2.98
Arginine	2.25	1.96
Taurine	3.85	2.41
Alanine	12.89	7.93
Tyrosine	1.32	0.61
Methionine	3.08	0.37
Valine	4.73	7.12
Phenylalanine	3.17	1.47
Isoleucine	2.26	3.56
Leucine	2.64	2.22
Asparagine	0.00	1.28
Cysteine	ND	ND
Proline	ND	ND
Tryptophan	ND	ND
Total	76.06	45.15

ND, not determined.

tion was not eluted in the void volume of the column, suggesting that HF had a molecular mass of <4 kDa. In addition to absorbance features typical of proteins and nucleic acids, the HF active fraction exhibited a pronounced absorbance peak at 320 nm, a wavelength characteristic of the hydrophilic mycosporine-like amino acids (MAAs) abundant in marine cnidarians (7, 8). The correlation between HF activity and absorbance at 320 nm raised the possibility that HF might be one or more MAAs. Indeed, partially purified MAAs (HPLC standards, gifts from B. E. Chalker, Australian Institute of Marine Science, and D. F. Gleason, University of Houston) from a symbiotic zoanthid (*Palythoa tuberculosa*), a red alga (*Porphyra sp.*), and the coral trout (*Plectropomus leopardus*) evoked a 22.73 ± 1.77% (mean ± SD) release of photosynthate fixed by dinoflagellates isolated from the sea anemone *A. pulchella* compared to 6.8 ± 0.9% released by controls in sea water. MAAs did not enhance carbon fixation in these bioassays; however, the relative concentrations of partially purified MAAs in these standards (estimated from absorbance at 320 nm) were an order of magnitude lower than those present in the crude aqueous *P. damicornis* extract used as an HF activity control in these experiments.

To further investigate the role of MAAs as HF, we isolated individual MAAs from lyophilized methanolic extracts of *P. damicornis* tissues by using analytical hydrophilic interaction HPLC (polyhydroxyethyl A column 200 × 4.6 mm; 5 μm, pore size; PolyLC). Samples were passed through the column at 1 ml/min by using an isocratic gradient for 15 min in 20% Nanopure filtered water containing 50 mM hexafluoroisopropanol (solvent A) to 80% (vol/vol) acetonitrile containing 50 mM hexafluoroisopropanol (solvent B) followed by a 30-min linear gradient of 20–100% solvent A and 80–0% solvent B. Samples were monitored at a detection wavelength of 320 nm. Six major absorbance peaks were resolved, each of which was collected and purified by repeated passage through the hydrophilic interaction column. The six peaks represented individual MAAs, as judged by their specific absorbance maxima between 310 and 360 nm (9, 10), but all were present in low concentration. Again, each of the MAAs evoked the selective release of fixed carbon from symbiotic algae but did not enhance carbon fixation. To determine the exact composition of these partially active HF samples, each MAA peak was subjected to amino acid analysis before and after alkaline hydrolysis at the UCLA microsequencing facility. The prehydrolysis analyses revealed that each of the six peaks also contained FAAs in addition to an individual MAA. To determine whether they were HF-active, known FAAs were bioassayed in the millimolar concentration range, based on the published FAA data for marine cnidarians (11, 12). All FAAs tested induced the selective release of photosynthate from symbiotic dinoflagellates isolated from the tropical sea anemone *A. pulchella* (Fig. 1 *A* and *B*) and from *P. damicornis* (Fig. 1 *D* and *E*). The majority of FAAs also enhanced total carbon fixation (Fig. 1 *C* and *F*).

To investigate further the possible role of FAAs as HF, we prepared synthetic FAA mixtures based on the composition of measured FAA pools of *P. damicornis* colonies (Table 1). The tissue volume was estimated from fluid displacement volume of the live coral colonies minus fluid displacement volume of tissue-free skeleton. The FAAs were extracted with 70% ethanol for 24 h at 4°C. The amino acids were derivatized with phenyl isothiocyanate and their composition was assessed by reverse-phase HPLC (courtesy of D. Manahan and J. Welborn, University of Southern California). FAA concentrations were calculated based on tissue volume. Synthetic FAA mixtures based on the measured FAA pools for colonies I and II were made by dissolving each amino acid (Sigma) in distilled water. The pH of the mixtures was adjusted to 8.3 and the salinity was brought to 33 parts per thousand by the addition of NaCl. After filtration (0.22-μm pore size, Millipore), the synthetic mixtures were stored at 4°C to prevent the formation of a precipitate, which occurred upon freezing. Although the pools from two colonies varied qualitatively and quantitatively, glutamic acid, glycine, alanine, and valine were among the most abundant FAAs (Table 1), and the total amino acid concentration was in the millimolar range. The synthetic FAA mixtures, like crude HF, evoked the release of carbon by dinoflagellates isolated from *A. pulchella* and enhanced their rate of carbon fixation (Table 2).

The ^{14}C-labeled photosynthetic products released and fixed by dinoflagellates isolated from *P. damicornis* and incubated with a synthetic FAA pool were identified by chromatography carried out using Whatman no. 4 paper (19 × 19 cm) with

Table 2. Effect of two synthetic FAA mixtures (prepared as in Table 1), crude aqueous *P. damicornis* extract (crude HF), and FSW on $^{14}CO_2$ fixation and release of acid-stable ^{14}C by the symbiotic dinoflagellates *Symbiodinium pulchrorum* isolated from *A. pulchella*

	Synthetic FAA mixture			
	Colony I	Colony II	Crude HF	FSW
^{14}C released, %	22.19 (7.0)	18.57 (5.5)	43.12 (00.0)	6.74 (3.0)
^{14}C released, experimental dpm/control dpm	7.48 (0.64)	7.48 (1.72)	12.01 (6.21)	1.00 (0.09)
^{14}C fixed, experimental dpm/control dpm	2.47 (0.25)	2.62 (0.59)	1.97 (0.58)	1.00 (0.09)

Data are the mean ± SD (in parentheses); $n = 16$. The effect of the synthetic FAA mixtures appears to be synergistic, in that they evoke significantly greater dinoflagellate $^{14}CO_2$ fixation and ^{14}C release ratios than any individual FAA at a similar concentration (compare with Fig. 1 *B* and *C*).

phenol/water, 72:28 (wt/wt), in the first dimension (horizontal) and butanol/propionic acid/water, 1246:620:874 (vol/vol), in the second dimension (vertical). Chromatograms were exposed to Kodak X-Omat AR film for 2 weeks. Under the influence of synthetic FAA mixtures, the release of ^{14}C-labeled products was selective and not the result of dinoflagellate lysis, as judged by chromatographic comparison of the ^{14}C-labeled products in an extract of symbiotic dinoflagellates with those in the incubation medium (Fig. 2 *A* and *B*). Moreover, the released products were qualitatively identical to those released by dinoflagellates incubated in crude aqueous extracts of their own host (Fig. 2 *B* and *C*). The products released by dinoflagellates freshly isolated from *P. damicornis* differed from those released by dinoflagellates freshly isolated from *A. pulchella* (Fig. 2 *C* and *D*). We speculate that these differences may be a reflection of the diversity of dinoflagellate species found in symbiosis with cnidarians (15–17), rather than HF specificity.

To determine whether the FAAs were interacting with a residual component of the host adhering to the surface of freshly isolated symbiotic dinoflagellates, we assessed the impact of HF on the nonsymbiotic green alga *Chlamydomonas reinhardtii*. Cultured algae in stationary growth phase (high salt medium, HSM) were incubated in synthetic HF reconstituted in HSM or in HSM alone with $^{14}CO_2$ in the light for 1 h. In synthetic HF, *C. reinhardtii* released fixed carbon to the surrounding incubation medium (23.2% of the total carbon

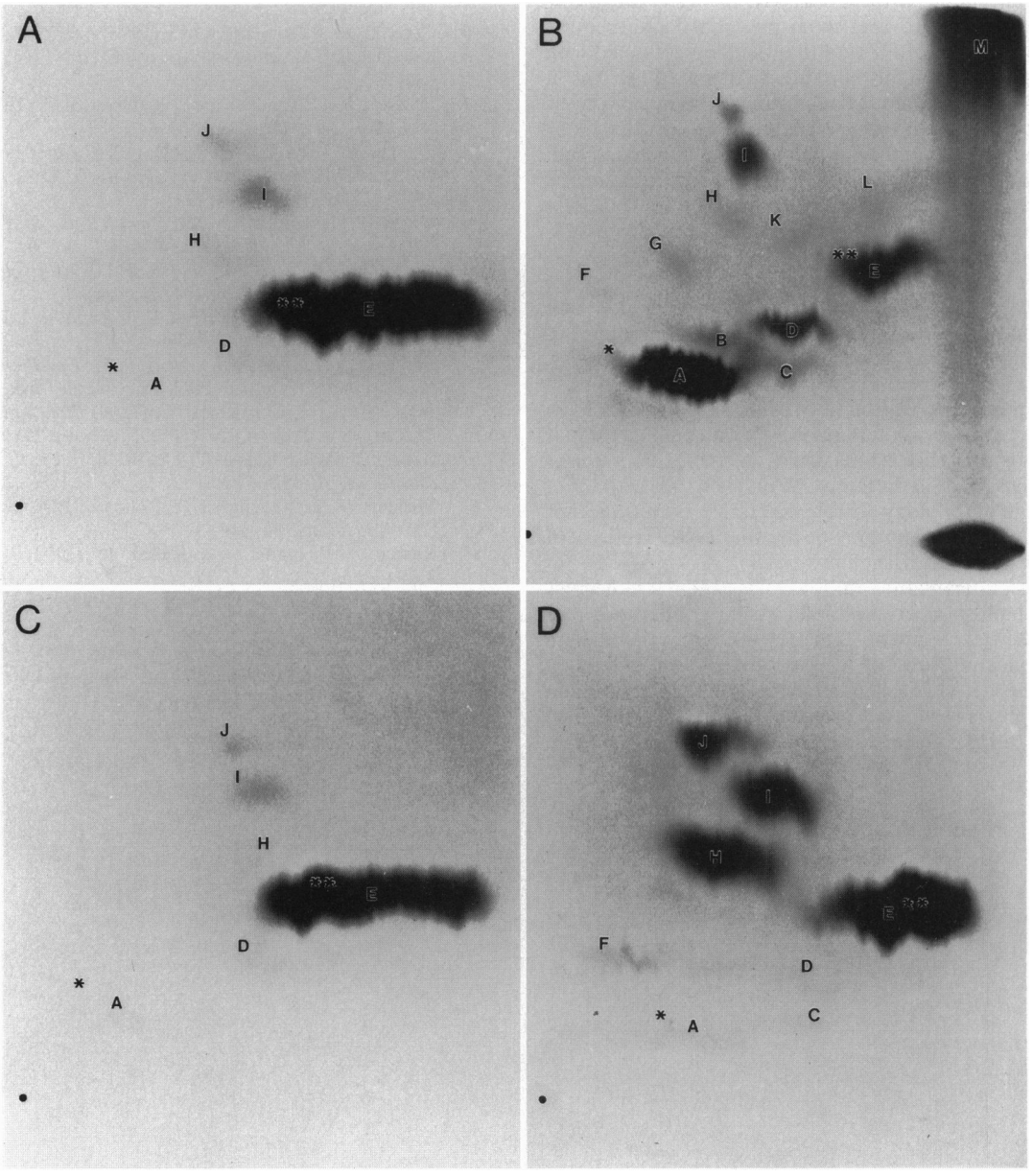

FIG. 2. Radiochromatograms showing the ^{14}C-labeled photosynthetic products released (*A*) and fixed (*B*) by dinoflagellates isolated from *P. damicornis* and incubated with a synthetic FAA pool (see Table 1). (*C*) ^{14}C-labeled products released by dinoflagellates isolated from *P. damicornis* and incubated with crude aqueous *P. damicornis* extract. (*D*) ^{14}C-labeled products released by dinoflagellates isolated from *A. pulchella* incubated with a synthetic FAA mixture. The radiochromatograms represent ^{14}C-labeled products resulting from 60-min incubations. Glucose, alanine, and glycerol were provisionally identified by cochromatography with authentic compounds in three solvents. All other compounds were tentatively identified by R_f with published chromatographic maps for the solvents employed (13, 14). Spots: A, glucose; B, glycine and serine; C, glutamine; D, alanine; E, glycerol; F, citrate; G, glycerate; H, glycolate; I, lactate; J, succinate; K, valine; L, phenylalanine and leucine; M, lipid; ●, origin; *, internal marker dye Ponceau 3R; **, internal marker dye *p*-(2-hydroxy-L-naphthylazo)benzenesulfonic acid, sodium salt. Labeled spots corresponding to glucose in *A*; 3-phosphoglyceric acid, sugar phosphates, aspartate, and glutamate in *B*; and glycolate in *C* were detectable on the x-ray films but were too faint for photographic reproduction.

fixed; 4.03:1 experimental/control ratio dpm released) and exhibited enhanced carbon fixation compared with algae in HSM alone (1.27:1 experimental/control ratio of dpm fixed). These cultured algae appeared to respond to HF in a manner identical to that of freshly isolated symbiotic dinoflagellates.

The crude HFs used in this and previous studies represent complex mixtures of metabolically active substances and may be contaminated with microorganisms. As such, the overall activity of HF may be adversely affected by its mode of preparation, chemical changes, interactions between other components of the mixture, and substances that might have a negative impact on algal photosynthesis. Such complications are confounded by the extended maintenance of crude HF once prepared and the temperature at which the preparation is maintained and utilized for a bioassay. In this context, it is not surprising that there are numerous conflicting reports regarding the behavior of HF and that the data collected with these preparations are highly variable. FAAs are heat-stable, and indeed the crude aqueous *P. damicornis* HF and synthetic FAA mixtures used in this study were not adversely affected by boiling. In contrast, a number of studies have concluded that crude HF is heat labile (1–3), yet these results could also be interpreted as representing an inhibition of dinoflagellate photosynthesis resulting from heat-induced breakdown of a constituent other than HF.

As synthetic FAA mixtures can duplicate the full effect of crude aqueous extracts of *P. damicornis*, we conclude that FAAs function as HF *in vitro*. As MAAs are a component of the FAA pool, they may contribute additional HF activity. However, their activity remains to be demonstrated with pure synthetic compounds. Unfortunately, MAAs have not yet been synthesized. The observation that FAAs exhibit HF activity is at variance with an earlier estimation of the molecular mass of HF as >10 kDa (3). The latter could either be an artifact of size-exclusion methodology or representative of yet another class of molecules from another species that evokes release of photosynthate from dinoflagellates *in vitro*.

FAAs play significant and multiple roles as signal molecules in regulatory biology as exemplified by their cooperative role in *Myxococcus* development (18, 19). In cnidarians, high exogenous concentrations of FAAs evoke specific feeding responses and other changes in behavior, and they function in both neurotransmission and osmoregulation (20–22). The identification of FAAs as factors that also elicit photosynthate release and enhanced carbon fixation in symbiotic dinoflagellates *in vitro* opens the way for detailed investigations into their role and relevance as HF *in hospite* (in the host).

We thank Drs. R. S. Alberte, R. K. Trench, and P. Edmunds for reviewing the manuscript; A. Alpert (PolyLC), J. Welborn, and D. Manahan for technical support; and J. Erickson for the *C. reinhardtii* cultures. This work was supported by grants from the Office of Naval Research to L.M. and M.M.-N.

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