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ALANINE UPTAKE BY ISOLATED ZOOXANTHELLAE OF THE MANGROVE JELLYFISH, *CASSIOPEA XAMACHANA*. II. INHIBITION BY HOST HOMOGENATE FRACTION

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ABSTRACT

A low molecular weight fraction (Inhibitory Fraction, IF) was isolated from whole animal homogenates of the symbiotic jellyfish, *Cassiopea xamachana*. This fraction (2–10 kDa) strongly inhibited the uptake of L-(U-^{14}C)alanine by isolated zooxanthellae at concentrations as low as 5 μg protein ml^{-1}. The magnitude of suppression was unaffected by the length of exposure time to IF prior to monitoring uptake. Uptake rates were linear for control and experimental cell suspensions, suggesting that algal metabolic integrity was maintained in the presence of IF. Zooxanthellae incubated in IF, and then washed free of this fraction, continued to show suppressed alanine uptake with only partial recovery. These cells, when placed in culture media, exhibited normal morphology, continued to grow and divide, and developed flagellated stages. The possible regulation of nutrient exchange between symbionts by symbiosis-specific host factors is discussed.

INTRODUCTION

Algal-invertebrate associations provide unique opportunities for investigating the regulation of the metabolic interactions necessary for the maintenance of this symbiosis in two such diverse organisms. While it has been recognized that endozoic algae translocate photosynthate to their animal hosts (for reviews, see Muscatine, 1974, 1980), it has only recently been appreciated that they also may compete for the available pool of nutrients obtained via host feeding (Cook, 1971; Thorington and Margulis, 1981). Since the activity of various metabolic pathways is directed by the availability of substrate, the stability and widespread occurrence of algal-invertebrate symbioses may result from an ability of the symbionts to regulate reciprocal transfers. Few studies, however, have focused on this regulation and the mechanisms involved.

An intriguing possibility is that this might be accomplished, in part, through the induction of certain regulatory molecules or factors specific to the symbiotic state. Factors that increase the rate of photosynthate liberation by isolated zooxanthellae have been reported in various cnidarians (Trench, 1971; Muscatine *et al.*, 1972). In addition, these factors appear to lack species specificity (Muscatine, 1967). The present investigation shows that a homogenate fraction of the jellyfish *Cassiopea xamachana* can significantly inhibit the back-transfer of nutrients such as alanine to zooxanthellae. Should such factors be common to algal-cnidarian associations, their differential production could conceivably regulate the levels of organic compounds available to each partner. The regulation of nutrient availability to zooxanthellae, or the production of appropriate host factors, could also serve to regulate algal populations within cells.

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Steele and Goreau (1977) have reported that a protein obtained from a distilled water extract of the anemone *Phyllactis* reduced the concentration of zooxanthellae suspensions from this species, as well as *Aiptasia* and *Zoanthus,* although *Aiptasia* lacked this protein.

Materials and Methods

Cassiopea xamachana were obtained from commercial sources and maintained in the laboratory as previously described (Carroll and Blanquet, 1984). Whole animal homogenates, prepared via mechanical disruption in an Osterizer blender, were subsequently centrifuged to remove the zooxanthellae and other particulate material. A low molecular weight fraction of the resulting supernatant was obtained by vacuum filtration using Millipore immersible CX-10 Ultra-filtration units having a molecular weight cut-off of 10 kDa. Extensive dialysis of the filtrate in benzoylated tubing (Sigma, St. Louis, Missouri) against filtered sea water (FSW) removed all compounds less than 2 kDa. This fraction (2–10 kDa), referred to as Inhibitory Fraction (IF), significantly suppressed the uptake of exogenous alanine by zooxanthellae. Concentration of IF was determined by protein measurement according to the methods of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Effect of IF on alanine uptake by zooxanthellae

Cassiopea zooxanthellae were freshly isolated for all experiments according to the methods of Blanquet *et al.* (1979). Cell suspensions were adjusted to standard densities of $3 \cdot 10^6$ cells ml^{-1}. Total chlorophyll (chl a and c_2) was determined by the methods of Jeffrey and Haxo (1968). Cell suspensions, in the presence or absence of IF, were allowed to preincubate for one hour with gentle agitation under standard conditions of light (45 ± 3 μEinsteins m^{-2} s^{-1}) and temperature (23 ± 2°C) prior to the addition of L-(U-^{14}C) alanine (144 mCi $mmole^{-1}$) to obtain a final concentration of 1 μCi ml^{-1}. Radiolabeled alanine was obtained from ICN Biochemicals (Irvine, California).

The effects of IF (1 to 100 μg protein ml^{-1}) on the uptake of alanine were monitored after 3-hour incubation periods. In all experiments, triplicate samples were prepared as previously reported (Carroll and Blanquet, 1984) and counted in a Beckman (Model LS7500) programmable liquid scintillation counter.

To determine whether the length of time of exposure of isolated zooxanthellae to IF affected subsequent uptake of L-(U-^{14}C) alanine, standard algal suspensions were divided into two fractions. Fraction I (control) contained algae suspended in FSW, while algae in fraction II were exposed to IF (50 μg protein ml^{-1}) for periods of time ranging from 9 to 180 minutes. The latter were subsequently washed free of the inhibitor prior to the addition of alanine to both fractions. In these experiments the one-hour preincubation step was omitted.

Effects of inhibitory fraction previously used in inhibition of alanine uptake

Assays were performed to determine whether IF retained maximal activity throughout the course of uptake experiments. In these assays, freshly isolated zooxanthellae were divided into two fractions, each of which were incubated under standard experimental conditions for one hour. Fraction I (control) contained standard concentrations of suspended algae in sea water without IF, while Fraction II contained sea water with IF (50 μg protein ml^{-1}). At time zero, aliquots (20 ml) of each fraction were put into separate beakers and L-(U-^{14}C)alanine (1 μCi ml^{-1}) was added to each.

Uptake was monitored at intervals throughout the experiment. At 2.3 hours, ^{14}C-alanine was added to an additional aliquot (20 ml) taken from Fraction I, and uptake again monitored. In addition, zooxanthellae from Fraction I were washed in fresh sea water and resuspended to standard densities either in a supernatant from Fraction II, termed "used IF," or in IF not previously exposed to zooxanthellae termed "fresh IF." Radiolabeled alanine was added and uptake again monitored.

Recovery of alanine uptake after prior exposure to inhibitory fraction

The reversibility of IF inhibition of alanine uptake in zooxanthellae was experimentally determined by dividing cell suspensions into two fractions. Fraction I (control) contained no IF, while Fraction II contained IF at a concentration of 50 μg protein ml^{-1}. L-(U-^{14}C) alanine was added at time zero. After 2 hours, two measured aliquots were removed from Fraction II. One of the aliquots was washed three times in FSW containing the experimental concentration of IF; the other aliquot was similarly washed, using FSW to remove the IF previously present. L-(U-^{14}C) alanine was added to these aliquots at the initial experimental concentration, and uptake was again monitored.

RESULTS

Low molecular weight fractions (2–10 kDa), prepared from *Cassiopea* homogenates, were found to suppress significantly the uptake of exogenous alanine by isolated zooxanthellae at concentrations as low as 5 μg protein ml^{-1} (Fig. 1). Concentrations above 25 μg protein ml^{-1} showed near maximum effectiveness, and 50 μg protein ml^{-1} was chosen as the standard concentration for subsequent experiments. The magnitude of uptake suppression appeared to be unaffected by the length of exposure time (9–180 minutes) to this fraction (Table I). Comparison of 3-hour uptake values of paired samples of pre-dialysis (0–10 kDa) and post-dialysis (2–10 kDa) fractions

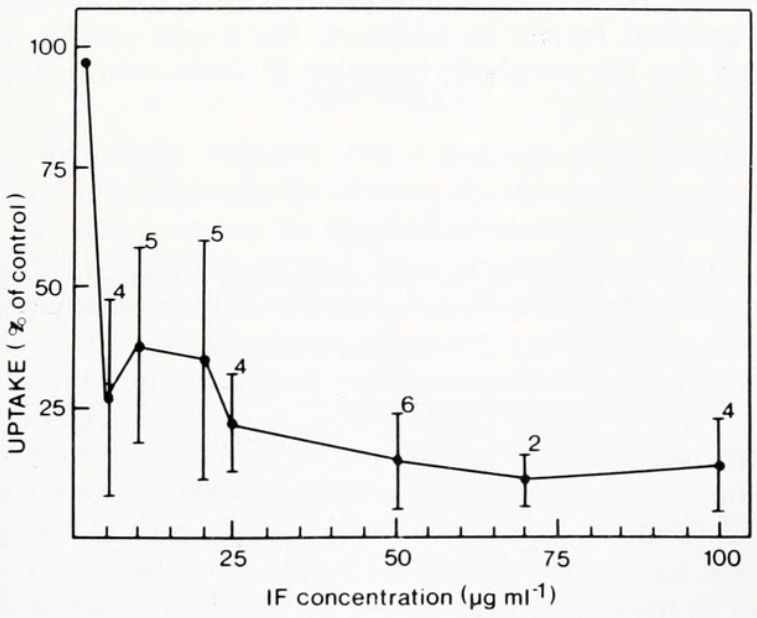

FIGURE 1. Uptake of L-(U-^{14}C) alanine by isolated zooxanthellae in the presence of different concentrations of Inhibitory Fraction (IF). Data points are mean values ± S.D. as percentage of control. Data point at 1 μg protein ml^{-1} is the mean of two experiments. The number of experiments for other concentrations are given above the bars.

TABLE I

Effect of length of exposure to Inhibitory Fraction (IF) on subsequent uptake of L-(U-^{14}C) alanine by isolated zooxanthellae

Experiment	Control slope	Experimental slope		
1	461	205 (9)	214 (25)	168 (60)
2	511	264 (10)	284 (80)	293 (180)
3	802		397 (60)	405 (120)
4	428		126 (60)	108 (80)
5	503		143 (25)	134 (40)

Uptake rates (slope) are expressed as DPM · μg chl^{-1} ml^{-1} h^{-1}. Exposure times (in minutes) are given in parentheses.

of individual homogenates (Table II) showed no significant difference ($P > 0.10$), indicating that there are no active molecules smaller than 2 kDa.

As shown here and in previous experiments (Carroll and Blanquet, 1984), the rate of alanine uptake by isolated zooxanthellae was highly linear. This was also true for algae either continuously exposed to IF or after previous exposure to this fraction (Figs. 2, 3). Correlation coefficients for the slopes of all experiments were greater than 0.97. As shown in Figure 2, IF retained its effect over the course of a 3-hour incubation with zooxanthellae, and could subsequently inhibit fresh algal suspensions to the same extent.

Zooxanthellae which were washed free of IF after a 2-hour exposure period continued to demonstrate suppressed uptake, with partial recovery, when resuspended in FSW at initial alanine concentrations (Fig. 3). Algal cells washed in IF and resuspended in IF, showed uptake rates similar to those prior to washing.

Viability of zooxanthellae exposed to IF for 2–4 hours was monitored by inoculating washed cells into FSW containing Carolina Biological Alga-Gro Concentrate (20 ml liter^{-1}). Aliquots from all cultures, examined by phase contrast microscopy after 2–5 days, contained cells of normal morphology and size, actively dividing stages, and numerous flagellated forms. In addition, the linear uptake rates noted in all experiments suggest that the metabolic integrity of the zooxanthellae remained intact during exposure to IF.

TABLE II

Inhibition of uptake of L-(U-^{14}C) alanine by Pre- and Post-dialysis Inhibitory Fraction

Experiment	Control	Pre-dialysis IF	Post-dialysis IF
1	1162.7	516.3 (44.4%)	575.8 (49.5%)
2		376.5 (32.4%)	423.7 (36.4%)
3	2009.2	344.7 (18.2%)	470.1 (23.4%)
4		351.5 (17.5%)	347.6 (17.3%)
Mean ± S.D.		397.3 ± 80.5	454.3 ± 95.5

Uptake values are expressed as DPM · μg chl^{-1} ml^{-1} for 3-hour samples. Percentage of control is given in parentheses.

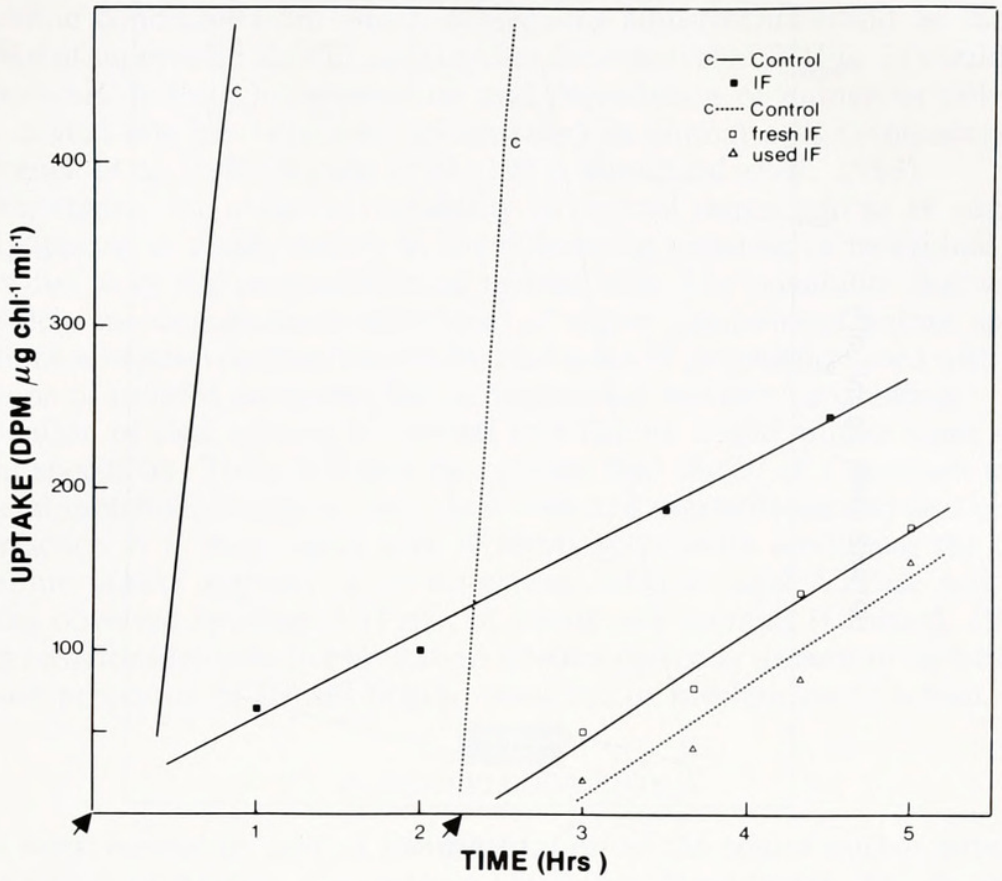

FIGURE 2. Isolated zooxanthellae were divided into two fractions, one of which contained no added IF (Fraction I), the other (Fraction II) contained IF at a concentration of 50 μg protein ml^{-1}. At time zero (first arrow), ^{14}C-alanine was added to 20 ml aliquots from Fraction I (C —— and Fraction II (■ IF) and uptake monitored. Again at 2.3 hours (second arrow) ^{14}C-alanine was added to an additional aliquot from fraction I (C - - -) and uptake rate determined. In addition, algae from fraction I were washed in FSW and resuspended either in a supernatant from Fraction II, termed "used IF" (△), or in IF not previously exposed to zooxanthellae, "fresh IF" (□), to which radiolabeled alanine was added. Each regression line was drawn to the calculated slope obtained from 5 data points, although not all points are shown on each line. Regression analyses were carried out according to Snedecor and Cochran (1971).

DISCUSSION

The results of this study indicate that a fraction from *Cassiopea xamachana* homogenates (IF) is able to suppress significantly the uptake of alanine by zooxanthellae *in vitro* and thus provide a means by which back-transfer of nutrients *in vivo* may be controlled. While IF inhibits amino acid uptake, it has little or no effect on photosynthesis (Blanquet, unpub.), indicating that IF is not acting as a general metabolic inhibitor. Since aposymbiotic *Cassiopea* were not available for this investigation, it was not possible to determine whether production of IF is symbiosis-specific, as has been shown for host factors in other cnidarians (Trench, 1971).

The possibility that the measured suppression of ^{14}C-alanine uptake in the presence of IF was due to a reduction in the specific activity of exogenous, radiolabeled alanine through the enhanced release of this amino acid from zooxanthellae by some component of IF does not appear to be tenable. Trench (1974) and Muscatine and Cernichiari (1969) have shown that alanine is a minor component of the photosynthate released from zooxanthellae in the presence of cnidarian host homogenates. The latter investigators demonstrated that, in the coral *Agaricia,* only 7.2% of the fixed ^{14}C released in one hour by host homogenate was in the form of alanine. Though this

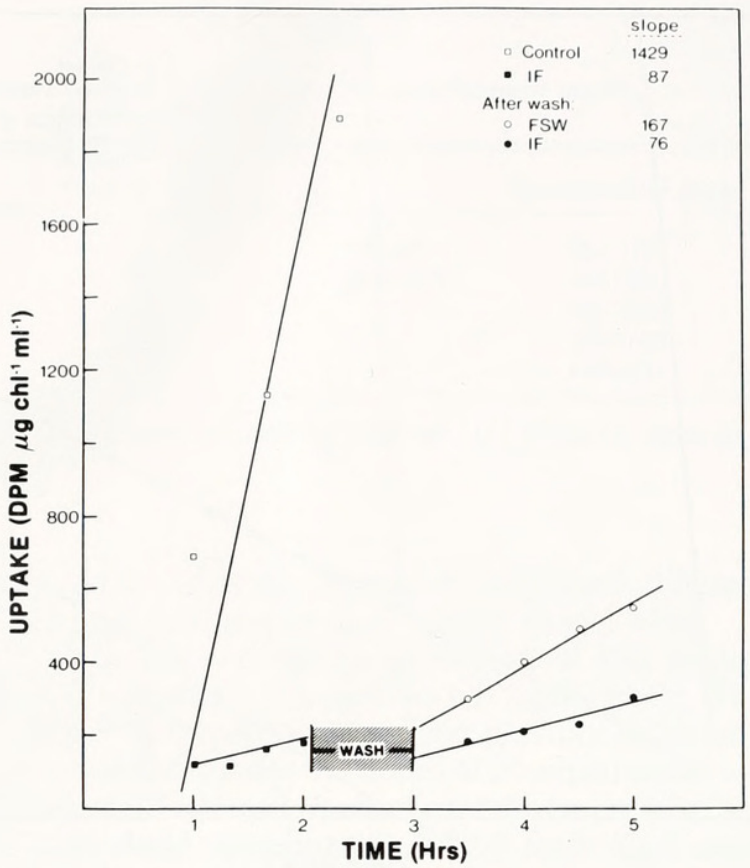

FIGURE 3. Uptake of L-(U-^{14}C) alanine by isolated zooxanthellae in medium without IF (□) or with IF (■). At 2 hours, cells were washed, either in the experimental concentration of IF (●) or in FSW (○). Cells were then resuspended in the appropriate medium to which ^{14}C-alanine was added and uptake again monitored.

percentage increased with time, the apparently small amounts of alanine released from these algae, together with the magnitude of suppression and linearity of alanine uptake by *Cassiopea* over three hours in the presence of IF, would argue against the above interpretation. In addition, alanine uptake remains suppressed after IF has been removed (Fig. 3).

Inhibitory fraction was Lowry-positive, with a molecular weight range of 2–10 kDa, and thus it is probable that the active factor(s) may be peptides or low molecular weight proteins. This fraction was shown to be effective at concentrations as low as 5 μg protein ml^{-1}. Since it is reasonable to assume that IF consists of several components, not all of which may be active in amino acid uptake regulation, the active component(s) would be present at even lower concentrations. Therefore, to inhibit effectively back-transfer of amino acids, animal cells *in vivo* may need to synthesize only small amounts of the inhibitory component(s) of IF. Alternatively, host cells might produce and sequester greater amounts of IF, which could then be released as needed to regulate amino acid levels. Such a strategy, which is characteristic for various neurosecretory peptides and endocrines, would enable the host cells to have a faster response time than if *de novo* synthesis of the inhibitor were required. On the other hand, decreased production or release of IF would allow increased uptake during periods of excess amino acid availability, thereby enhancing the recycling of nutrients.

Internalization or down-regulation of putative IF-alanine receptor complexes by zooxanthellae could also supply a means for IF degradation, thereby allowing a return

to a baseline condition from which subsequent adjustments could be made. The persistence of suppressed alanine uptake after the removal of IF (Fig. 3) would support this hypothesis. Indeed, internalization and degradation of numerous polypeptides through coated pits has been well documented in animal cells (Schlessinger *et al.*, 1978; Branca *et al.*, 1982; Krupp *et al.*, 1982; Blanchard *et al.*, 1983).

In this regard, the observed variability in control slopes and in IF suppression (Table I) appears to reside mainly in the differential response of individual cell suspensions due to *in situ* preconditioning in host cells. The possibility that variability resides solely in concentration differences of active components within specific IF preparations is negated by observations that the same IF preparation used with different suspensions of isolated zooxanthellae demonstrated variable suppression.

Regulation of algal activity by animal host factors would require some degree of symbiont specificity. Thus, it would be unlikely that the IF of *Cassiopea* would act as a general metabolic inhibitor, since host cells and organelles would also be affected by its presence. It is more likely that IF either specifically modulates the action of algal alanine uptake systems or, in some way, inhibits algal ATPase activity. The differences observed between ATPases of plants and animals (Leonard, 1982) may provide a basis for such selective inhibition. Studies currently underway on the chemical nature and properties of IF will help to elucidate its mechanism of action.

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