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AMINO ACID UTILIZATION BY *GYMNODINIUM BREVE*

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Key Word Index—*Gymnodinium breve*; Florida Red Tide; dinoflagellate; amino acid utilization; protein synthesis; extracellular protein.

Abstract—The marine dinoflagellate *Gymnodinium breve* utilizes exogenous amino acids for the synthesis of proteins in the light. During logarithmic growth, L-valine and L-methionine are incorporated into proteinaceous material which is retained by the cell. Glycine is also incorporated, but the glycine-containing proteins are extruded. When cells are no longer growing exponentially, all proteins that incorporated these supplied amino acids are extruded. The pronase-susceptible extruded material has a MW in excess of 300000. When chloramphenicol is used to inhibit protein synthesis, glycine is not taken up. L-Methionine is rapidly metabolized intracellularly and is used in the synthesis of other macromolecules. L-Valine accumulates intracellularly and remains unaltered. Glycine and L-methionine appear to be transported via facilitated diffusion systems, while L-valine uptake appears to be active.

INTRODUCTION

Most marine phytoplankton are capable of utilizing any one of a number of nitrogen sources including ammonium, urea, nitrate, nitrite, free amino acids, and peptides. The environmental concentration of any one is typically less than 50 μM [1].

Any species able to utilize more than one nitrogen source probably has a distinct advantage over species not possessing such an ability. Utilization of amino acids provides a further advantage in that they may be used intact for the synthesis of protein, or they may be metabolized, providing an ancillary source of carbon, in addition to nitrogen.

Transport of amino acids has been demonstrated in a variety of marine phytoflagellates [2]. Rapid metabolism of the amino acid, retention of the amino nitrogen, and extrusion of a variety of non-nitrogen-containing compounds frequently operate to keep the effective intracellular concentration low, and thus present a favorable osmotic gradient for continued uptake by facilitated diffusion [3]. Although amino acid utilization in the open ocean may be low, phytoplankton exposed to the nutrient-rich organic medium present in restricted bays may utilize amino acids significantly. Florida's red tide organism, the marine dinoflagellate *Gymnodinium breve*, is photoauxotrophic since it requires growth factors (i.e. vitamins) [4]. Based largely on the work of Aldrich [5] who observed no stimulation of growth in the light or dark on a variety of substrates, *G. breve* is presumed to be incapable of heterotrophic existence. This paper reports the uptake and metabolism of three amino acids by *G. breve*.

RESULTS

Separatory funnel settling of viable *G. breve* cells, and the isolation of whole cells using 6% sucrose NH-15 solution are methods we designed to minimize cellular destruction during concentration, as laboratory cultures of *G. breve* are very fragile. These procedures have been published previously [6].

The rate of incorporation of each amino acid into TCA insoluble material increased linearly with respect to increasing substrate concentration. The maximum rates attained were 4.5×10^{-8} mol of glycine incorporated/mg cellular protein/hr (K_m = 110 μM), 1.4×10^{-8} mol of valine incorporated/mg cellular protein/hr (K_m = 150 μM), and 4.5×10^{-9} mol of methionine incorporated/mg cellular protein/hr (K_m = 125 μM) (see Fig. 1).

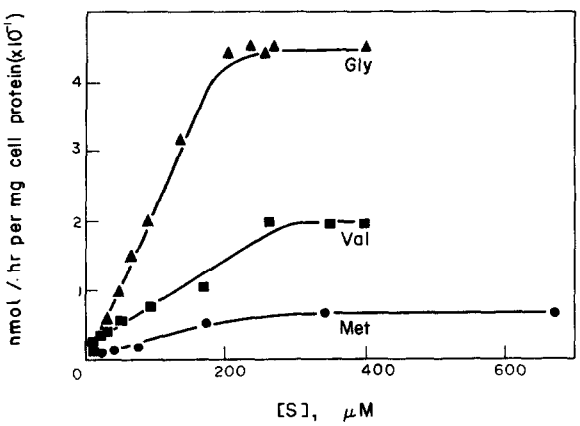

Fig. 1. Uptake and incorporation of amino acids into TCA insoluble material. Each point is the result of a 6 hr incubation. The rate of uptake was determined as radioactivity of glycine (▲), valine (■), or methionine (●), incorporated into TCA insoluble material. ^{14}C substrate concentrations ranged from 0–0.4 mM (glycine and valine), and 0–0.6 mM (methionine). The rate of incorporation of ^{14}C amino acid into TCA insoluble material was linear with respect to time at each substrate concentration.

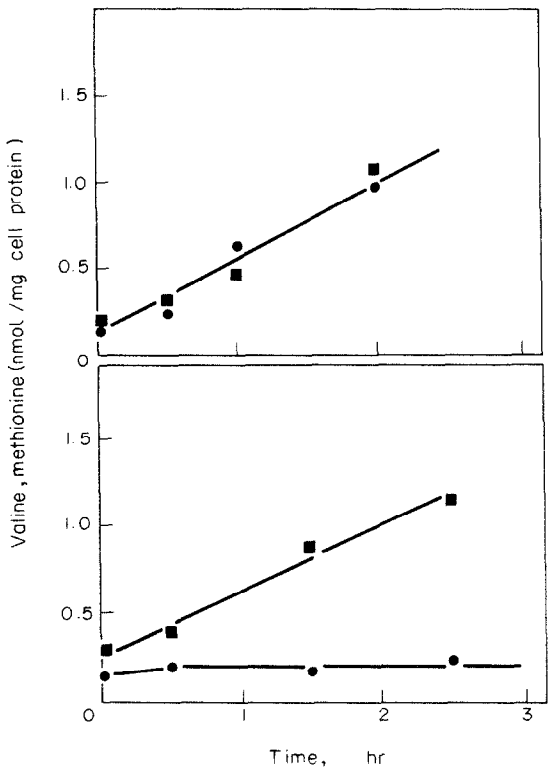

Fig. 2. Incorporation of L-valine and L-methionine into whole cells and TCA insoluble materials. Substrate concentration was 20 μM. Comparison of L-Valine or L-methionine present in whole cells (●) and TCA insoluble materials (■) from whole cultures. Both uptake and incorporation were graphed as nmol substrate taken up/mg cellular protein/hr. The upper panel is *G. breve* in logarithmic growth and the lower panel represents amino acid utilization in stationary phase cells.

The fate of the labeled macromolecular material synthesized from valine and methionine is different if cells are in logarithmic growth phase or in stationary phase. Methionine and valine uptake (Fig. 2) were graphed together, as at 20 μM substrate concentration, little difference was noted in their respective uptake and incorporation rates. When ^{14}C valine or methionine is supplied to logarithmic cells (Fig. 2, top panel), the radioactivity associated with whole cells and TCA precipitates of whole cells in the experimental medium are equivalent, suggesting that no detectable intracellular pools of free valine or methionine are exchangeable with these exogenous amino acids. It also suggests that transport is the rate-limiting step in valine or methionine utilization. TCA insoluble counts do not accumulate extracellularly in log-phase cells.

When cells have ceased exponential growth (Fig. 2, lower panel), no counts remain with the cell pellet, but TCA precipitable counts increase linearly in the medium, suggesting extrusion of the proteinaceous material following intracellular synthesis. The rate of synthesis of the macromolecular material is the same regardless of cell growth pattern. Only the ultimate location of the macromolecule is different.

Glycine utilization (Fig. 3) shows a different pattern in that, regardless of cell growth phase, the ^{14}C radioactivity associated with whole cells is always lower than the ^{14}C radioactivity associated with macromolecular material. Again, the rate of incorporation of glycine into TCA insoluble material is the same regardless of growth phase.

At standard culture (no separatory funnel settling) concentrations of bacteria (3×10^6–3×10^7 cells/ml) and *G. breve* (1×10^4 cells/ml), the bacterial contribution to extracellular TCA insoluble counts is 5–7 %. Following separatory funnel settling, bacterial concentrations are reduced 1000-fold. TCA insoluble counts arising from bacteria are likewise reduced.

If *G. breve* is removed by low speed centrifugation (70 *g*) from a control culture supplied with any of the 3 amino acids in either log or stationary phase, and the supernatants are incubated further, TCA insoluble counts cease to accumulate at the time *G. breve* are removed. As such isolation techniques do not remove bacteria which may be present, the absence of further accumulation indicates *G. breve* to be the organism responsible for the synthesis of the material.

The labeled macromolecular material synthesized from ^{14}C amino acids, when hydrolysed and chromatographed on silica gel TLC plates using butanol–acetic acid–water (4:1:5) and compared with the migration of the supplied ^{14}C amino acid, the radioactivity in each case co-migrates. The growth phase of the culture makes

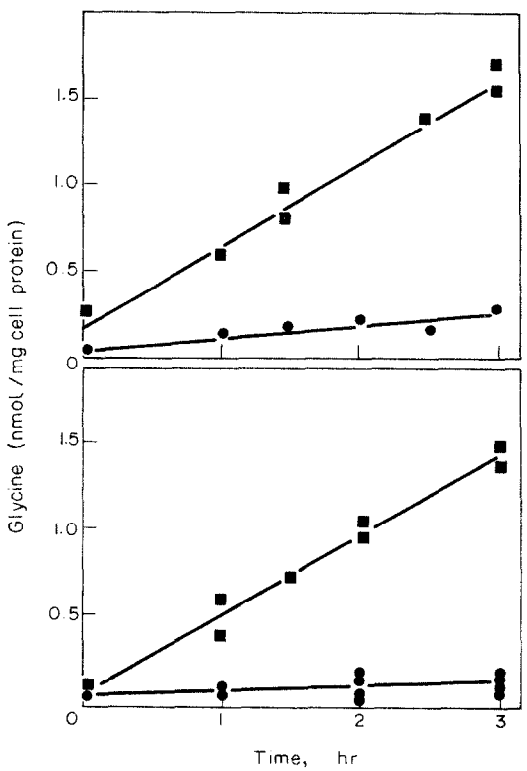

Fig. 3. Incorporation of glycine into whole cells and TCA insoluble materials. Glycine concentration was 20 μM. Comparison of glycine present in whole cells (●) and TCA insoluble materials (■) from whole cultures. Both uptake and incorporation were graphed as nmol glycine taken up/mg cellular protein/hr. The upper panel represents amino acid utilization in logarithmic cells and the lower panel represents utilization in stationary phase cells.

no difference in the labeling pattern—the amino acids are utilized intact for the synthesis of covalently-bound material.

The majority of the extracellular material synthesized from glycine-[^{14}C], following dialysis and gel chromatography, was found to elute at the void volume of a Sephadex G-200 column, corresponding to a MW in excess of 300000. The labeled macromolecular material was susceptible to digestion by pronase, indicating that it might be proteinaceous. The native glycine-[^{14}C]-labeled material consisted of *ca* 71% material of MW greater than 300000 and 29% of material *ca* 20000. After a 1 hr pronase digestion, 20% of the higher MW fraction had been degraded. Further incubation for 24 hr, degraded another 15%. There was no change in the MW of the control incubations in the absence of enzyme.

Protein synthesis was inhibited in *G. breve* by only 2 of the 9 antibiotics examined. One of these, cycloheximide, inhibited only to the extent of 5%. The other, chloramphenicol, inhibited protein synthesis up to 90%. Concentrations as low as 10 μg/ml inhibited protein synthesis over 80%. The resulting inhibition was similar when glycine-[^{14}C] or methionine-[^{14}C] were used as tracers of protein synthesis, except the degree of inhibition was less in methionine-supplied cultures.

Utilizing chloramphenicol at 20 μg/ml, it was possible to examine the kinetics of amino acid uptake in the absence of appreciable protein synthesis. Under these conditions, cells supplied with glycine-[^{14}C] failed to show any accumulation. Cells supplied with methionine-[^{14}C] or valine, accumulated the labeled amino acids several fold over the external concentration. Lysis of the cells following accumulation and chromatography gave the following results. Methionine-[^{14}C] was extensively metabolized in the absence of protein synthesis. Most of the radioactivity remained at the origin (R_f = 0–0.13) and few counts co-chromatographed with the radioactive standard (R_f = 0.8). Valine-[^{14}C] remained unaltered inside the cells and most of the radioactivity co-chromatographed with the valine standard (R_f = 0.71). The uptake of valine is linear with respect to time. In 3 hr, valine is concentrated 5.8-fold over the external concentration.

DISCUSSION

The 3 amino acids chosen for these studies have been shown to be transported, in most organisms, by separate amino acid transport systems [7]. Transport appears to be the rate-limiting step in the utilization of any of the 3 amino acids, as no free amino acid is accumulated at any substrate concentration examined.

It is interesting to note that inhibitors of eucaryotic protein synthesis have little effect on protein synthesis in *G. breve*. Conversely, chloramphenicol, an inhibitor of procaryotic protein synthesis and chloroplast protein synthesis [8], inhibits *G. breve* at low concentration. Therefore, in our opinion, the major part of protein synthesis takes place in the chloroplast.

The finding that *G. breve* utilizes intact amino acids for the synthesis of protein is also surprizing. Many phytoplankton metabolize transported amino acids, retaining the amino nitrogen and extruding the carbon skeleton. This type of metabolism is generally observed under artificially-induced conditions of nitrogen starvation [3]. The *G. breve* cultures used in our experiments are not nitrogen-starved and so imply that this organism will utilize amino acids when ample nitrogen is present. Because amino acids are used when nitrogen is available in other forms (NO_3^-,NH_4^+), *G. breve* appears able to utilize such pre-formed nitrogen-containing compounds for other than nitrogen metabolism. Although amino acid concentrations in the ocean are low [9], the amounts can become substantial in areas of low water turnover and high productivity [10], which coincidently are the sites of many red tide outbreaks.

Glycine and methionine appear to be assimilated via facilitated diffusion systems. The evidence supporting this hypothesis include: (1) saturation with increasing substrate concentration, (2) lack of an intracellular pool of free amino acid at any substrate concentration examined and (3) for glycine, lack of uptake in the absence of protein synthesis. It appears that the incorporation of glycine into protein and the extrusion of the latter back into the medium is the driving force for glycine transport, for, in the absence of a mechanism for glycine 'trapping', no uptake is seen.

Under conditions of chloramphenicol inhibition, no detectable pool of free methionine-[^{14}C] was evident. Methionine was still transported, but was now extensively metabolized, thereby exercising a degree of control over methionine transport. Small amounts of free methionine may be present in whole cell pellets. The equilibrium concentration of methionine (based on calculations and measurement of intracellular water volume) in whole cells isolated from 100 ml of culture medium approximates 1350–1500 cpm. TLC analysis showed that no more than 150 cpm or 10% could possibly be free methionine. The rate of uptake in the absence of protein synthesis was somewhat lower, further suggesting the existence of a facilitated diffusion system for the uptake of methionine.

The third amino acid, valine, is not extensively metabolized by *G. breve* under any of the conditions studied, but is instead utilized intact for the synthesis of protein—or, in the absence of protein synthesis, remains unaltered and accumulates. The rate of uptake is independent of protein synthesis. L-Valine uptake is still saturable at high substrate concentration. An active uptake of L-valine is implied.

The extruded material, of MW greater than 300000 is much more than can be accounted for by cell lysis. If cellular lysis were responsible for the observed location of the macromolecular material in logarithmic cultures, one would expect a similar observation in experiments utilizing any of the three amino acids. This is not the case. Why cells, which have incorporated glycine-[^{14}C] into proteinaceous material, extrude it under all growth conditions is not fully understood. A primary consideration, from a cellular viewpoint, may be the ease with which each is autotrophically synthesized, glycine (and serine) being the most easily produced. Methionine and valine on the other hand, have a complex biosynthetic pathway and, in the case of methionine, an ample source of sulfur is also necessary. Another plausible explanation is the degree of accessibility of exogenous amino acids to the intracellular pools of amino acids.

The finding that all examined exogenous amino acids are extruded in proteinaceous material when cells are stationary may be significant. This release, again, is not due to cellular lysis, as whole cell pellets do not change

volume or size, as compared to log phase pellets. Stationary cells appear no more fragile than logarithmic cells. It is not known at this time if the extruded protein(s) have any enzymatic activity analogous to the extruded phosphatases produced by *Ochromonas* under conditions of phosphorus depletion [11]. The fact that cultures are stationary is an indication that some factor or nutrient is limiting. Perhaps, in an effort to scavenge the nutrient, *G. breve* extrudes molecules of similar function. The extruded proteins may also be a substrate for the maintenance of a 'phycosphere' as proposed by Bell and Lang [12], as it is known, that when uni-algal cultures reach stationary phase, the number of contaminating bacteria increases [13]. As *G. breve* has been implicated as a faculative phagotroph (pers. commun. D. L. Taylor, K. A. Steidinger), the production of a nutrient-rich layer for the cultivation of micro-organisms as a source of food (or an essential vitamin such as B_{12}) could present a great ecological advantage for *G. breve*.

EXPERIMENTAL

Standard culture conditions. Uni-algal cultures of *G. breve* were grown in the laboratory in 3 l. cotton-plugged conical flasks containing 2.5 l. of medium (cell generation time = 2.5–3 days). The cultures were maintained as previously described [6]. *G. breve* cells were counted using either a Coulter counter Model B (100 μm aperture) or by direct observation of 0.1 ml of culture medium under low power magnification. Bacterial counts were obtained microscopically in a Petroff-Hausser counting chamber.

Preparation of experimental cultures. Cultures in early log-phase or late stationary phase of growth were used in all studies. In log-phase standard cultures, *G. breve* cells numbered 5000/ml and bacteria numbered 3×10^6 cells/ml. In stationary standard cultures, *G. breve* numbered 2×10^4 cells/ml and bacteria numbered 3×10^7 cells/ml. *G. breve* was separated from bacteria using our separatory funnel settling technique [6]. Following separatory funnel settling, appropriate dilutions were made to yield a final *G. breve* concn of $1–2 \times 10^4$ cells/ml and a final bacterial concn calculated to be, for log phase cultures *ca* 6×10^3 cells/ml, and for stationary cultures less than 1.4×10^4 cells/ml. Bacterial counts were obtained using Difco Bacto-nutrient agar plates as previously described [6]. Before and after each expt, cultures were examined under high power magnification to determine if the cells had retained normal size, shape, and motility. Cultures less than 90% motile were discarded.

Isolation of whole cells. Hopkins vaccine tubes were filled to 1 ml with 6% sucrose dissolved in the culture medium NH-15. Aliquots (10 ml) of the experimental culture were carefully layered on top of the sucrose soln and the tubes centrifuged at 70 *g* for 4 min. The culture supernatant was removed by pipette and saved, and the sucrose soln removed. The whole cell pellet in 10 μl of sucrose soln, was removed with the aid of a microsyringe. The latter was rinsed with 2×0.1 ml vol. of H_2O following removal and expulsion of the pellet.

Isolation of TCA insoluble material. TCA (10 M) was added to disrupted (by freeze-thawing or sonication) cell suspensions or supernatants following whole cell isolation, to give a final TCA concn of 10%, and the suspensions were cooled at 0°. They were then rapidly filtered on Gelman Type A-E glass fiber filters and washed with 10 ml cold 5% TCA. They were finally washed with 10 ml cold MeOH and assayed for radioactivity as outlined below.

Determination of radioactivity. All radioactive pellets and their appropriate syringe washes were suspended in 10 ml Aquasol (New England Nuclear, Inc.) liquid scintillation fluid in Beckman Poly Q vials. Gelman filters were treated similarly. TLC plates, once chromatographed, were scraped in 0.5 cm fractions from origin to solvent front. The fractions were suspended in 10 ml scintillation fluid for counting. Radioactivity was assessed using a liquid scintillation counter with an efficiency for ^{14}C of 60%. In all cases, appropriate controls such as unlabeled cells, liquids and plates were assayed for background radiation. In all expts, methionine-[U-^{14}C] and valine-[U-^{14}C] (sp. act. 50 mCi/mmol) and glycine-[2-^{14}C] (50 mCi/mmol) were used.

Experimental incubation procedure. The rate of amino acid uptake was examined during 6 hour incubations at standard culture conditions and with substrate concns from 1–600 μM in 100 ml vol. Whole cell and TCA insoluble samples were prepared at 0, 0.5, 1, 2, 4 and 6 hr. The rate of uptake and the rate of incorporation into TCA insoluble material was determined in each case. Cellular protein was determined according to ref. [14].

Hydrolysis and chromatography of labeled macromolecular material. Following 6 hr incubations with glycine-[^{14}C], methionine-[^{14}C], or valine-[^{14}C], 100 ml cultures were flash-evaporated, resuspended in a small amount of H_2O and transferred to dialysis tubing (MW exclusion 16–18000). Following exhaustive dialysis against H_2O, the dialysates were flash-evaporated and hydrolysed in 6N HCl/0.1% PhOH for 18 hr at 105°. The hydrolysates were flash-evaporated, applied to a Si gel TLC plate and chromatographed in *n*BuOH–HOAc–H_2O (6:7:1). They were compared to the migration of the supplied amino acid.

Pronase digestion of labeled macromolecular material. Macromolecular material synthesized from glycine [^{14}C] was dialysed and reduced in vol. The material from a 100 ml culture was divided into 5 equal parts. Each was resuspended in 1 ml of 3.5% NaCl and one sample was frozen immediately. Pronase (40 PUK units) was added to 2 of the samples. The 2 enzyme mixtures and 2 samples containing no enzyme were incubated 24 hr at 37°. Samples of enzyme mixtures and controls were taken at 1 hr and 24 hr and were frozen.

Column chromatography of extruded material and pronase digest. A 1.2(ID) × 22 cm Sephadex G-200 gel filtration column was equilibrated in 3.5% NaCl. The radioactive macromolecular materials were applied to the column in 0.1 ml 3.5% NaCl. The flow rate was adjusted to 25 ml/hr and 0.5 ml fractions collected for radioactive measurement.

Inhibition of protein synthesis. Duplicate 10 ml cultures containing 8 μM valine-[^{14}C] or glycine-[^{14}C] were supplemented with inhibitors of protein synthesis as listed below: chloramphenicol (0.02–0.3 mM), cycloheximide (0.01–1 mM), NaF (0.05–10 mM), and neomycin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline (0.05–0.3 mM). Cells were incubated 3 hr under standard conditions. Following incubation, TCA was added to the cultures to yield a final TCA concn of 5%. The ppts were isolated and analysed as previously described. Inhibition was determined by comparison with control cultures incubated in the absence of inhibitor. Cultures were also examined microscopically for physical anomalies which might result from exposure to such inhibitors.

Uptake of amino acids in the absence of protein synthesis. Duplicate cultures containing valine-[^{14}C], glycine-[^{14}C], or methionine-[^{14}C] at 8.6 μM and chloramphenicol at 20 μg/ml were incubated 3 hr under standard contions. Whole cell and TCA insoluble samples were prepared as before. At the end of 3 hr, whole cell pellets from 10 ml aliquots of valine-[^{14}C] and methionine-[^{14}C] cultures were expelled into 0.5 ml H_2O and frozen in a MeOH dry ice bath to lyse the cells. The samples were thawed and applied to a Si gel TLC plate, dried under a stream of N_2 and chromatographed as before. Samples from the plate were removed and assayed.

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