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NITROGEN ASSIMILATION IN THE SYMBIOTIC MARINE ALGA *GYMNODINIUM MICROADRIATICUM*: DIRECT ANALYSIS OF ^{15}N INCORPORATION BY GC–MS METHODS

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Key Word Index—*Gymnodinium microadriaticum*; zooxanthellae; nitrogen assimilation; stable isotopes; gas chromatography–mass spectrometry.

Abstract—^{15}N-Labelled ammonium chloride, sodium nitrate and urea were supplied *in vitro* to freshly isolated *Gymnodinium microadriaticum*, the algal symbiont (zooxanthellae) of the clam *Hippopus hippopus*. Subsequent extraction of the resulting labelled amino acids and determination of the distribution of ^{15}N in glutamine, glutamic acid and aspartic acid, using gas chromatography–mass spectrometry revealed a light dependent assimilation from all substrates, primarily via the GS–GOGAT pathway.

INTRODUCTION

Mutualistic symbioses between living plant cells and animal tissues are particularly prevalent in the tropical marine environment where their high incidence has been correlated with extremely low levels of dissolved inorganic nutrients [1–3]. This correlation implies that the algae play a critical role in host nutrition and vice versa although the extent to, and mode by, which host and symbiont derive their respective nutritional benefits, is still poorly understood. Alternative possibilities for the heterotrophic host include digestion of algae, autolytic degradation of senescent algae or continuous release of some proportion of algal photosynthate. One way the autotrophic symbiont presumably benefits is by access to a continuous supply of nitrogenous substrates, the end-products of host metabolism [1–4].

Gymnodinium microadriaticum (Dinophyceae) is a ubiquitous symbiont with extensive geographical distribution and range of associations, the most prominent of which are the hematypic corals, and the large molluscs of the family Tridacnidae.

Several aspects of N-metabolism in symbiotic associations have recently been investigated. Webb and Wiebe [5] observed the removal from sea water by coral of dissolved-N in the form of NO_3^-. Utilization of N in the form of NH_3 and urea has also been observed [2, 6]. The effects of NH_3, NO_3^- and urea on some aspects of the photosynthesis and release of radio-labelled substrates from *G. microadriaticum in vitro* and *in vivo*, as well as calcification rate of an intact coral were also recently studied [7]. Ammonia and urea at concentrations up to 80 μM were found to stimulate the *in vivo* photosynthetic rate, the release of algal photosynthate and the calcification rate of *Acropora cervicornis*, with NH_3 having the greatest effect. Nitrate was not found to stimulate any of the above functions although axenic culture of alga appeared equally successful with each substrate as the sole N-source [7].

In this paper, we present the results of our experiments using the stable isotope ^{15}N in which we directly determined: (a) the incorporation of N from NH_3, NO_3^- and urea into the amino acid pool of *G. microadriaticum* and the nature of its light dependence and (b) the enzymic pathway of this incorporation.

RESULTS AND DISCUSSION

Isolation and mass spectrometric analysis of algal amino acids

Specific N-enrichments for each amino acid were determined by GC–MS of their trimethylsilyl derivatives. This derivative was selected because its formation requires a one step reaction, which when properly controlled gives a single, homogeneous and relatively stable derivative of glutamine [8]. Of the alternative methods of amino acid derivatization, the most common involve the formation of *N*-acyl-alkyl esters and these are not suitable for determination of glutamine since the acid catalysts used in the esterification reaction cause its conversion to the corresponding glutamate ester. In addition, glutamic and aspartic acids were separated from the neutral amino acid fraction containing glutamine [9] prior to derivatization to further preclude the possibility of cross contamination during GC–MS analysis. Following an analysis of the total nitrogen enrichment of glutamine from the neutral amino acid fraction an aliquot was subjected to acid hydrolysis to glutamic acid for separate determination of the amino nitrogen enrichment. Glutamine amide nitrogen enrichment could then be estimated by difference.

Isotope enrichments in each individual amino acid were calculated from the intensities of the isotope clusters of selected ions in complete EI MS scans (m/z 600 to m/z 140) obtained on a double focussing sector instrument, a technique previously used successfully in our laboratory to measure ^{18}O-enrichment patterns [10–12]. Alternative

Table 1. Results of 1 hour feedings of ^{15}N-labelled substrates to *G. microadriaticum*

Substrate and treatment	^{15}N-enrichment of individual amino acids as % molecules labelled (±s.e.m.)*							^{15}N-enrichment of glutamine nitrogens	
	Glutamic acid		Aspartic acid		Glutamine			Amino-N (atom % Found)	Amido-N (atom % Calc.)
	R	R1	R	R1	R	R1	R2		
Control	99.5 (1.3)	0.3 (1.8)	98.6 (2.8)	0.1 (2.4)	99.8 (0.9)	0.2 (0.9)	0.8 (0.2)	0.1 (0.6)	0
$^{15}NH_4^+$, light	47.5 (0.2)	52.7 (0.4)	48.4 (1.3)	50.9 (2.4)	11.2 (0.7)	40.9 (2.3)	45.6 (2.1)	46.0 (4.3)	86
$^{15}NH_4^+$, dark	79.9 (0.8)	20.1 (0.9)	80.9 (0.7)	18.6 (0.7)	41.0 (2.2)	47.4 (2.9)	9.2 (1.8)	12.4 (2.9)	53
$^{15}NO_3^-$, light	92.8 (1.7)	6.5 (1.8)	93.6 (2.1)	5.4 (2.2)	64.8 (3.1)	32.2 (4.3)	5.3 (1.6)	4.6 (1.8)	37
$^{15}NO_3^-$, dark	97.0 (0.6)	2.8 (0.7)	97.3 (1.2)	2.7 (1.1)	76.0 (2.3)	18.4 (3.7)	2.4 (2.7)	1.7 (2.5)	21
$(^{15}NH_2)_2CO$, light	93.6 (0.9)	5.6 (0.7)	94.3 (2.6)	4.9 (1.6)	59.5 (5.4)	18.4 (3.8)	10.7 (3.9)	No result	—
$(^{15}NH_2)_2CO$, dark	96.5 (1.9)	3.2 (1.5)	95.6 (1.8)	3.3 (1.7)	73.8 (5.9)	15.2 (3.8)	4.1 (2.7)	3.0 (1.0)	19

* R denotes the number of unlabelled molecules, R1 the number of singly labelled molecules and R2 the number of doubly labelled molecules. For glutamic and aspartic acids, R1 equates with atom % amino-N enrichment. Specific enrichment of glutamine amino-N was determined by hydrolysis and analysis of resulting glutamate. Enrichment of glutamine amido-N was determined by difference.

methods based on CI–SIM–GC–MS [13, 14] have also been used successfully for this purpose and can reliably detect as little as 0.08 atom% excess isotope. These methods have not, however, been used for the determination of individual nitrogen enrichments in glutamine.

The MS fragment selected for measurement of ^{15}N-enrichment was the ion formed by α-cleavance loss of ·COOTMS from the molecular ion ($M^{+} - 117$) in every case, as this ion is uncomplicated by alternative fragmentation pathways and for the compounds measured had relative intensities varying between 20 and 40% of the base peaks. At least 6 sets of data from triplicate injections were then compared with the patterns expected for the constituent elements at their natural abundances (Table 2) using the method described by Biemann [15]. The standard deviations varied with intensity of the spectra and were least (1–5%) when measuring relatively large enrichments. As expected, larger deviations were encountered when attempting to detect lower enrichments, particularly in the case of glutamine from the urea feedings where recovery was low and consequently subject to larger interferences from other substances. Bearing in mind these limitations the results are sufficiently reliable for several valid biochemical deductions to be made.

Isotope feeding experiments

The results presented in Table 1 show the enrichments (atom%) for individual N-atoms of the amino acids glutamine, glutamic acid and aspartic acid following a 1 hr feeding of each of the substrates $^{15}NH_4Cl$, $Na^{15}NO_3$ and $(^{15}NH_2)_2CO$ (all at 100 μM) to *G. microadriaticum*. Each labelled feeding experiment was carried out at half full sunlight with a dark control, *in vitro*, on algal cells immediately following their isolation from a freshly collected *Hippopus hippopus* clam.

For all three substrates assimilation was clearly light dependent and was most rapid in the case of NH_3, with the enrichment about 5 to 10-fold higher after 1 hr than for NO_3^- and urea. Since assimilation from the latter substrates is dependent on inducible enzymes (nitrate reductase and urease) which may have significant induction periods, and perhaps also upon slower transport processes, this trend is not unexpected. Nevertheless, the magnitude of the preference for NH_3 is striking and accords with Taylor's observation [7] of marked *in vitro* stimulation of photosynthetic activity and excretion rates for *G. microadriaticum* by high NH_3 concentrations (up to 80 μM).

For all three substrates the distribution of label is consistent with the assimilation occurring primarily via the enzymes glutamine synthetase (GS; EC.6.3.1.2) and glutamate synthetase (GOGAT; EC.2.6.1.53) rather than via the alternative glutamate dehydrogenase (GDH; EC.1.4.1.3) pathway [16]. Following all feedings the highest relative enrichment was found in the amido-N of glutamine. Hydrolysis of glutamine and comparison of the relative enrichments of all three amino-N groups in glutamine, glutamic acid and aspartic acid revealed an approximate equilibrium between them at an enrichment 2 to 8 times lower than that of the glutamine amido-N. This is consistent with dilution of amido-N from glutamine in a larger pool of glutamate but complicated by the possible existence of multiple compartments of glutamine synthesis [18]. Amino acid analysis of an alga sample, unperturbed by feeding, showed that each 0.5 g (dry weight) used per incubation contained approximately 790 nmol free glutamate and 160 nmol free glutamine, that is, the ratio of respective pool sizes is approximately 5:1. This ratio, however, may be shifted in favour of glutamine by high NH_3 concentrations [17], a phenomenon which could account for the lower than expected dilution (approx. 2:1) of label between glutamine amido-N and glutamate amino-N in the NH_3 (light) incubation.

Because the relative enrichments of the amino-N of glutamine and of glutamate are similar in most experiments, and correspond roughly to the % of doubly labelled glutamine, we suggest there is only a small proportion of glutamine singly labelled in the amino-N position and therefore most of the amino labelled glutamine is in fact doubly labelled material which has cycled at least once through a larger and poorly enriched pool(s) of glutamate. However, data from kinetic experiments are needed before we can precisely attribute this pattern of labelling to be a result of simple dilution or the existence of multiple glutamine/glutamate pools.

The small but consistent difference in the relative enrichment of glutamine amino-N and glutamate amino-N could reflect the contribution that GDH makes to the total amount of NH_3 assimilated. The higher relative enrichment of glutamate-N compared to glutamine amino-N is mainly evident in the dark experiments and particularly the dark ammonia feeding where glutamate-N is 20.1%, aspartate-N is 18.6% and glutamine amino-N is only 12.4%. In this experiment the number of doubly labelled glutamines is only 9.2% and hence there is a proportion, albeit small, of glutamine molecules singly labelled in the amino nitrogen. Since this glutamine must

Table 2. Natural abundance isotope patterns of TMS-amino acid fragments

Amino acid	M^{+} (*m/z*)	α-Cleavage fragment (*m/z*)	(Composition)	Isotope peak ratios* R + 1	R + 2	R + 3	R + 4
Asp	349	232	$C_9H_{22}NO_2Si_2$	0.21099	0.08881	0.01234	0.00211
Glu	363	246	$C_{10}H_{24}NO_2Si_2$	0.22212	0.09116	0.01338	0.00226
Gln	362	245	$C_{10}H_{25}N_2OSi_2$	0.21886	0.09246	0.01352	0.00240

* Where R = 1.

be derived from glutamate, the source of its label could be that proportion of the glutamate pool labelled directly from the substrate ($^{15}NH_4^+$) pool via GDH. This is consistent with the observations of Canvin and Atkins [19, 20] who have shown that $^{15}NH_3$ can be assimilated in the dark in barley leaves, possibly via mitrochondrial GDH. Recent experiments with leaves of *Lemna* [18], *Datura* [21] and *Pisum* [22] do not support the view that GDH contributes in a significant way to N-assimilation in the light. Nevertheless, until we have kinetic and other evidence to the contrary we should acknowledge the possibility of an active but minor role in N-assimilation for GDH in the highly specialized organism studied here.

The results of this preliminary investigation suggest merit and practicality in the further use of ^{15}N-labelled substrates and GC–MS techniques to study nitrogen assimilation in intact algal–invertebrate symbioses as well as movement of nitrogenous compounds between respective partners. Such studies could contribute significantly to our understanding of how these systems function so efficiently in a low nutrient environment.

EXPERIMENTAL

Isolation of algal cells. The isolation of algal tissue and incubation with labelled substrate was carried out in the field at Lizard Island Research Station (14° 40′ S; 145° 28′ E) at the northern end of Australia's Great Barrier Reef. Mantle tissue (140 g) from a single, fresh specimen of *H. hippopus* (approx. 2 kg live body wt) was finely dissected and homogenized in fresh sea water. Animal tissue was largely removed by filtration through Miracloth and the algal cells washed (×4) with fresh filtered sea water by centrifugation with subsequent resuspension. The final algal pellet (approx. dry wt 6.1 g) was suspended in 240 ml fresh sea water and aliquots (20 ml) used for each incubation. Aliquots were reserved for total amino acid analysis and work-up controls.

Incubation. Each aliquot of algal cells was made up in 250 ml with fresh filtered sea water and concentrated aliquots of labelled substrates (97 atom % ^{15}N) added so that the final concentration in all incubations was 100 μM. Dark controls were incubated in foil wrapped flasks, all treatments being for 1 hr at 30° with constant agitation. Feedings were terminated by centrifugation of the cells and addition of EtOH (5 ml) and $H{\cdot}CO_2H$ (100 μl) to the resulting pellet. Samples were maintained at −10° prior to amino acid isolation.

Amino acid isolation and GC–MS analysis. The EtOH suspension of algal cells from each inbubation was filtered and the alga extracted twice more with a soln of EtOH–HOAc–H_2O (8:1:2), the combined filtrates evapd to remove EtOH and applied to a column (5 ml) of Dowex 50-X8 (H^+ form) and the column washed with H_2O (25 ml). The ammonia eluate (2N, 20 ml) from the column was dried (<25°), resuspended in H_2O and applied to a column (2.5 ml) of Dowex 1-X8 (acetate form) and washed with H_2O (15 ml). This fraction contained the neutral and basic amino acids including glutamine. A subsequent elution with HOAc (2N, 15 ml) yielded a fraction containing the acidic amino acids. A fraction (20%) of the neutral/basic fraction was heated with HCl (2N, 200 μl) for 1 hr at 100° and then dried under a stream of N_2. Aliquots of these three fractions were dried and treated with acetonitrile (20 μl) and BSTFA–TMCS (99:1) (20 μl) and the mixture heated at 70° for 15 min. Aliquots were subjected to GC–MS: column (2 m × 2 mm i.d.) of 3% Dexsil 300 on 80–100 Gaschrom Q; He 30 ml/min; 100–250° at 10°/min; injector 200°; line 220°; jet separator 220°. Mass spectra were recorded using a Du Pont 21-491 B mass spectrometer (70 eV, source temp 220° scanned at 6 sec/decade) interfaced to a VG 2025 Data System. Raw ion intensity data (for the peak groups summarized in Table 2) were used since no improvement in precision was noted when attempting to correct for background signals. Results were processed as previously described [15, 10] to give the molecule % unlabelled species (R), the molecule % species labelled at one mass higher (R1, one atom ^{15}N) and for glutamine the molecule % species labelled at two a.m.u. higher (R2, containing two atoms ^{15}N). For glutamate and aspartate, R1 corresponds to amino-N atom % enrichment in excess of natural abundance. For glutamine, the atom % amino-N enrichment (x) was determined from the glutamate obtained by hydrolysis, and the atom % amido-N was estimated to be the sum of all labelled molecules (R1 + R2) less % molecules singly labelled at amino-N.

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