Biosynthesis of 'essential' amino acids by scleractinian corals

Lisa M. FITZGERALD* and Alina M. SZMANT†

Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Cswy., Miami, FL 33149, U.S.A.

Animals rely on their diet for amino acids that they are incapable either of synthesizing or of synthesizing in sufficient quantities to meet metabolic needs. These are the so-called 'essential amino acids'. This set of amino acids is similar among the vertebrates and many of the invertebrates. Previously, no information was available for amino acid synthesis by the most primitive invertebrates, the Cnidaria. The purpose of this study was to examine amino acid synthesis by representative cnidarians within the Order Scleractinia. Three species of zooxanthellate reef coral, *Montastraea faveolata*, *Acropora cervicornis* and *Porites divaricata*, and two species of non-zooxanthellate coral, *Tubastrea coccinea* and *Astrangia poculata*, were incubated with ^{14}C-labelled glucose or with the ^{14}C-labelled amino acids glutamic acid, lysine or valine. Radiolabel tracer was followed into protein amino acids. A total of 17 amino acids, including hydroxyproline, were distinguishable by the techniques used. Of these, only threonine was not found radiolabelled in any of the samples. We could not detect tryptophan or cysteine, nor distinguish between the amino acid pairs glutamic acid and glutamine, or aspartic acid and asparagine. Eight amino acids normally considered essential for animals were made by the five corals tested, although some of them were made only in small quantities. These eight amino acids are valine, isoleucine, leucine, tyrosine, phenylalanine histidine, methionine and lysine. The ability of cnidarians to synthesize these amino acids could be yet another indicator of a separate evolutionary history of the cnidarians from the rest of the Metazoa.

INTRODUCTION

Protein amino acids are fundamental components of all life forms. Some extant groups are able to synthesize all of the 20 protein amino acids (plants, and many fungi, bacteria and Protista; Table 1). All animals studied so far, however, lack a number of amino acid synthetic pathways, or the rates of synthesis of these amino acids are insufficient to meet metabolic needs. These amino acids, termed essential, must be obtained by animals from their environment (e.g. food). All vertebrates have the same nine essential amino acids (threonine, valine, methionine, leucine, isoleucine, phenylalanine, histidine, lysine and tryptophan), and some have additional essential amino acids (Table 1). More variability exists within the invertebrates: some (but not all) representatives of the Crustacea, Mollusca and Nematoda have been attributed with the ability to synthesize one or more of the vertebrate essential amino acids (Table 1). One phylum for which capabilities for amino acid synthesis had not yet been studied was the Cnidaria. The purpose of this study was therefore to examine amino acid synthesis within the order Scleractinia.

Overall, available information on amino acid synthesis shows that the pattern of essential compared with non-essential amino acids is similar among most Metazoa. Possible explanations for this similarity are: (1) animals evolved from early protozoan groups that had already lost or never acquired all the amino acid synthetic pathways; (2) the loss of these pathways occurred early in the evolution of the Metazoa; or (3) certain amino acid synthetic pathways may be more prone to loss than others. In general, essential amino acids are those with long, complex synthetic pathways. Random deleterious mutations may be more likely to occur in pathways with many steps than in those with few steps. The cnidarians are among the most primitive extant metazoans in most phylogenetic schema (see, for example, [1–3]). In this regard, their pattern of amino acid synthetic abilities

might be indicative of their divergence from other metazoan groups relative to the loss of amino acid synthetic pathways.

Glucose and amino acids can be precursors for amino acid synthesis. Glucose is rapidly absorbed and used in many metabolic pathways. Hence carbon atoms derived from glucose have a high probability of being incorporated into all newly synthesized amino acids. Glutamic acid is the direct precursor of several other amino acids via the tricarboxylic acid cycle, from where, like glucose, its carbon atoms have a high probability of being incorporated into newly synthesized amino acids. Lysine and valine, on the other hand, are not direct precursors of other amino acids. Although carbon atoms derived from these amino acids can feed into the tricarboxylic acid cycle, their degradation pathways are long and complex.

The approach used in this study was to incubate corals with ^{14}C-radiolabelled D-glucose or one of three ^{14}C-radiolabelled amino acids (glutamic acid, lysine and valine), and follow the radiolabel into coral protein amino acids. Five species of scleractinian coral were used: three that have dinoflagellate symbionts (known as zooxanthellae) living within their gastrodermal cells, and two that do not. The zooxanthellate species were *Montastraea faveolata* (= *M. annularis* [4]), *Acropora cervicornis* and *Porites porites*; the two non-zooxanthellate species were *Tubastrea coccinea* and *Astrangia poculata* (= *A. danae* [5]).

EXPERIMENTAL

Coral collection and maintenance

Pieces of the tropical zooxanthellate corals *M. faveolata*, *Ac. cervicornis* and *P. divaricata* were collected in the Bahamas east of the Joulters Cays. Colonies of the tropical, non-zooxanthellate species *T. coccinea* were collected from a shallow wreck in the Bahamas, and colonies of the non-zooxanthellate species *As. poculata* were collected under a pier in Woods Hole,

* Present address: Myriad Genetics, 390 Wakara Way, Salt Lake City, UT 84108, U.S.A.
† To whom correspondence should be addressed. Present address: The Institute for Genomic Research, 9712 Medical Center Drive, Rockville, MD 20850, U.S.A.

Table 1   Survey of amino acid synthetic capabilities of 12 groups of organisms

Most information is from studies that followed the fate of radiolabelled amino acid precursors into amino acids. Feeding studies are marked with an asterisk. Symbols: +, amino acids that can be synthesized; −, amino acids that cannot be synthesized; ±, amino acids for which synthetic capability varies within the group; no symbol, amino acids that were not analysed for in study/studies.

Organism	Asx	Ser	Glx	Gly	Ala	Pro	Cys	Arg	Tyr	Thr	Val	Met	Leu	Ile	Phe	His	Lys	Trp	References
Human	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	[35]
Rat	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	[48]
Chicken	+	+	+	−	+	+	−	+	+	−	−	−	−	−	−	−	−	−	[48]
Fish	+	+	+	+	+	+	+	−	±	−	−	−	−	−	−	−	−	−	[49*,50*,51]
Insect	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	[52]
Crustacean	+	+	+	+	+	+	+	−	±	±	−	±	−	−	−	±	±	−	[53–58]
Mollusc	+	±	+	+	+	±	+	−	−	−	±	±	±	−	−	−			[13,59–63]
Nematode	+	+	+	+	+	+	+	−	±	±	±	−	±	±	−	±	±	±	[16–18,64–65]
Flatworm	+		+							+									[65]
Cnidarian	+	+	+	+	+	+		+	+	−	+	+	+	+	+	+	+		This study
Protists																			
Phytoflagellates	±	+	±	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	[48,66–67]
Other	+	+	+	±	+	+	+	±	+	±	±	−	±	±	±	±	±	±	[48,66–67]

Massachusetts. For the reef corals, all replicates for each experiment were collected from a single colony. Discs of tissue and skeleton of *M. faveolata* were cut with a hole saw 2.4 cm in diameter and a pneumatic drill driven by a scuba tank. Branch tips of *Ac. cervicornis* and *P. divaricata* approx. 3–4 cm in length were collected with wire cutters. Individual colonies of *T. coccinea* and *As. poculata* were removed from the substrate with a hammer and chisel.

Corals gathered in the Bahamas were held in aquaria with running sea water on board the R/V *Calanus* and used 24–48 h after collection. Colonies of *As. poculata* were maintained in running sea water for several months at the University of Miami before use. During this period they were fed with thawed frozen brine shrimp.

Additional discs of *M. faveolata* were bleached (loss of algal symbionts; see, for example, [6]) by placing them tissue-side down on the bottom of the aquarium. The cores turned snowy white within 48 h. The bleached corals were righted, held under reduced light intensities and fed with brine shrimp for 5 weeks, during which time they did not recover their algal symbionts.

Procedures to minimize bacterial contamination

A major concern was to reduce the numbers of coral-associated bacteria that could take up the radiolabelled precursors and contaminate the coral samples with their metabolites. Two procedures were used to remove externally attached bacteria: first, immediately before incubation with the radiolabelled compounds, each coral was rinsed vigorously in three successive dishes of sea water filtered through a 0.22 μm filter; secondly, before tissue removal was begun (see below), the living surface of each coral was squirted with a stream of cold deionized water until the coral began to release streams of mucus, which was assumed to carry with it surface bacteria. The efficiency of these procedures was assessed twice, first with a set of laboratory-maintained samples of *M. faveolata*, and the second time with freshly collected samples of this species from the Florida Keys. The procedures tested were: (1) 0.22 μm filtration of the sea water, (2) one to five vigorous rinses of the coral in filtered sea water, (3) squirt-bottle rinses of the coral surfaces with chilled deionized water, (4) successive rinses in filtered sea water, followed by a rinse in deionized water, and (5) incubation with either 500 μg/ml gentamicin or 500 μg/ml streptomycin for 30 min. Bacterial numbers in the sea water before and after filtering were assessed by plating 250 μl samples of unfiltered and filtered sea water on agar plates (fortified seawater media: 0.05 g of yeast extract, 0.5 g of tryptone, 0.1 g of sodium glycerophosphate and 12.0 g of noble agar per litre of sea water; [6a]). The effectiveness of each of the various coral rinses was assessed by gently pressing the living surface of each coral on an agar plate. Untreated *M. faveolata* samples served as controls.

In the first test series (laboratory-maintained corals), bacterial counts in the 0.22 μm-filtered sea water were 98 % lower than in unfiltered sea water. Rinsing *M. faveolata* three times with filtered sea water decreased the number of surface bacteria transferred to the plates by 90–96 %, and the deionized-water rinse further decreased the bacteria by a total of 96–99 % compared with controls. The number of bacterial colonies plated from corals incubated with antibiotics were similar to or higher than those from rinsed corals. In the second test series (freshly collected corals), the first seawater rinse decreased the plated colonies by 83 %; subsequent seawater rinses did not significantly decrease bacterial counts further. A single deionized water rinse decreased bacterial counts by 74 %. The greatest decrease in bacterial counts (in both test series) was obtained by the combination of seawater and deionized-water rinses, the treatment used in our experiments.

In addition to these tests, the mixtures of mucus, bacteria and water generated by the deionized-water rinses performed before each recovery of experimental tissue were collected and assayed for radiolabel content. The average amount of radiolabel in these rinses ranged from 5 % to 7 % of the total radiolabel recovered in the crude coral homogenates. Together with the results of the plating experiments, these results suggest that contamination of the coral homogenates with external bacterial contaminants was most probably minimal.

Incubations with radiolabelled glucose

After rinsing in filtered sea water as described above, coral pieces were incubated for 2 or 3 h in 0.22 μm-filtered sea water containing D-[U-^{14}C]glucose (20.94–2.39 μCi/ml, 50–348 mCi/ mmol; purchased from ICN). Each dish was gently aerated and maintained at a temperature within 1 or 2 °C of ambient sea water. Incubations were conducted during the corals' normal

dark period, when their polyps were expanded, so that water circulation through the coelenteron and tissue area exposed to the radiolabel would be maximized. At the end of the incubation period, the corals were rinsed several times in fresh (non-radiolabelled) filtered sea water to remove excess radiolabel and moved to an aquarium with flow-through sea water for various post-chase periods before tissue recovery. *M. faveolata* and *Ac. cervicornis* were maintained for 1, 4, 12 or 24 h after termination of the incubation, those of *T. coccinea* were maintained for 1, 4 or 24 h, *P. divaricata* and *As. poculata* were maintained for 4 or 12 h, and the laboratory-maintained zooxanthellate and bleached *M. faveolata* were maintained for 16 h.

Incubations with radiolabelled amino acids

Preincubation rinses, incubation procedures and post-incubation handling of the corals used in the amino acid experiments were similar to those used in the glucose experiments. The three zooxanthellate species were incubated together in two dishes, one containing [U-^{14}C]glutamic acid (0.37–0.5 μCi/ml, 272.6 mCi/mmol; ICN) and the other containing [U-^{14}C]lysine (0.08–0.2 μCi/ml, 329.4 mCi/mmol; ICN). The bleached pieces of *M. faveolata* and the two non-zooxanthellate species were incubated in separate dishes for each species and each amino acid. The non-zooxanthellate species were incubated with [U-^{14}C]valine (0.08–0.09 μCi/ml, 264.0 mCi/mmol) in addition to glutamic acid and lysine. Field corals were maintained for 24 h, and the laboratory zooxanthellate and bleached *M. faveolata* for 16 h, before processing for tissue removal.

Tissue removal

Coral tissues were removed from their skeletons with a stream of high-pressure air and chilled 0.22 μm-filtered sea water from an artist's airbrush [7]. The tissue slurry was collected in a graduated glass tube on ice. This technique is similar to the Water Pik technique [8], but yields a more concentrated tissue preparation in about the same amount of time. The tissue slurries were homogenized for 30 s with an Ultra-Turrax homogenizer, and subsamples were counted for total tissue radioactivity by liquid-scintillation counting.

Animal tissue/zooxanthellae separation and assessment of procedures

Zooxanthellae were removed from the tissue homogenates of the zooxanthellate species by centrifugation (1625 *g*, 3 min) in an IEC clinical centrifuge at 4 °C. Centrifugation also pellets animal cell debris such as nematocysts and skeletal/tissue fragments. The pellets from the non-zooxanthellate species were larger than those from the zooxanthellate species, attesting to differences in tissue characteristics of the corals. Subsamples of each supernatant were taken for radioactivity counts, and the remains frozen in liquid nitrogen and stored at -70 °C until processed for protein extraction. The zooxanthellae/cell-debris pellets were resuspended in filtered sea water and repelleted three times to decrease animal contamination. The washed pellets were frozen in liquid nitrogen and stored at -70 °C. Pellets of the non-zooxanthellate species were not washed before freezing.

The primary interest of this study was the biosynthetic capabilities of the coral animals, not that of the symbiotic association. Therefore we performed tests to determine that the animal supernatant was not contaminated with zooxanthellae metabolites during tissue processing. Szmant-Froelich [9] found that when freshly isolated *As. poculata* zooxanthellae were incubated with NaH^{14}CO$_3$, added to non-zooxanthellate animal homogenates of the same host species, and separated again by centrifugation, only 3.0 ± 1.3 % of the radiolabel was found in the supernatant. Similar tests were performed with *M. faveolata* colonies and zooxanthellae. Freshly isolated zooxanthellae were incubated with NaH^{14}CO$_3$ and re-pelleted by centrifugation. Half of the pellet was frozen in liquid nitrogen. Either living or frozen zooxanthellae preparations were smeared across the tops of *M. faveolata* colonies; then the coral tissues were removed from the skeletons and processed as described above. Only 1.4 % of the radiolabel from the living zooxanthellae was recovered in the animal supernatant, but 19.3 ± 9 % of the radiolabel from frozen zooxanthellae leaked into the animal supernatant. These results confirm that zooxanthellae do not leak a significant portion of their soluble compounds into the coral homogenate during normal tissue processing of living corals, but that similar processing of frozen corals can result in substantial contamination of the animal supernatant with zooxanthellae components.

Contamination of animal tissues with zooxanthellae-derived amino acids could also occur if the zooxanthellae took up radiolabelled glucose, synthesized amino acids, and translocated them to the animal. Although translocation of radiolabelled amino acids from the algae to the animal was not assessed in these experiments, the magnitude of their potential contribution was estimated from the percentage of the total radiolabel that was in the algal pellet. Debris pelleted from coral homogenates of the three zooxanthellate species initially contained only 7–13 % of the radiolabel taken up by the whole (animal plus algae) coral association. After washing three times with filtered sea water, these pellets contained only 1–9 % of the total radiolabel. Although washing removed most of the animal debris from the pellets, intact nematocysts and larger cell fragments of animal origin were still visible when examined under a microscope. Thus much of the radiolabel remaining in these pellets could be accounted for by animal contaminants. In addition, the percentage of total radiolabel in the animal supernatant remained unchanged over the 24 h sampling period for the three zooxanthellate species. This contrasts with results from studies with H^{14}CO$_3^{2-}$, where the animal fraction increases in activity over this time-frame owing to algal translocation (see, for example, [9–11]). The small amount of radioactivity in the washed zooxanthellae pellets and the stability of the radiolabel content of these pellets, coupled with the fact that the algae are unlikely to take up much glucose, suggests that the zooxanthellae did not contribute much to the radiolabelled amino acids recovered from the zooxanthellate animal hosts.

Isolation of protein amino acids

Protein was precipitated from the animal supernatants by adding concentrated trichloroacetic acid to a final concentration of 8 % (w/v). The mixtures were incubated on ice for 30 min, after which the trichloroacetic acid-insoluble material was pelleted by high-speed centrifugation (12 000 *g*) for 20 min at 4 °C in a Sorvall RC-5B Superspeed centrifuge.

The trichloroacetic acid-precipitated protein pellets were transferred to Microfuge tubes and resuspended (three times) in 1 ml of 8 % (w/v) trichloroacetic acid and re-pelleted; the supernatant was decanted to remove trichloroacetic acid-soluble contaminants such as sugar and lipid moieties, nucleic acids and adherent trichloroacetic acid-soluble compounds. The pellets were then extracted three times with 1 ml of acetone to remove lipids from lipoproteins. The pellets were dried, resuspended in 50 % formic acid and transferred to a glass tube for hydrolysis. The formic acid was evaporated in a Savant Speed Vac and the
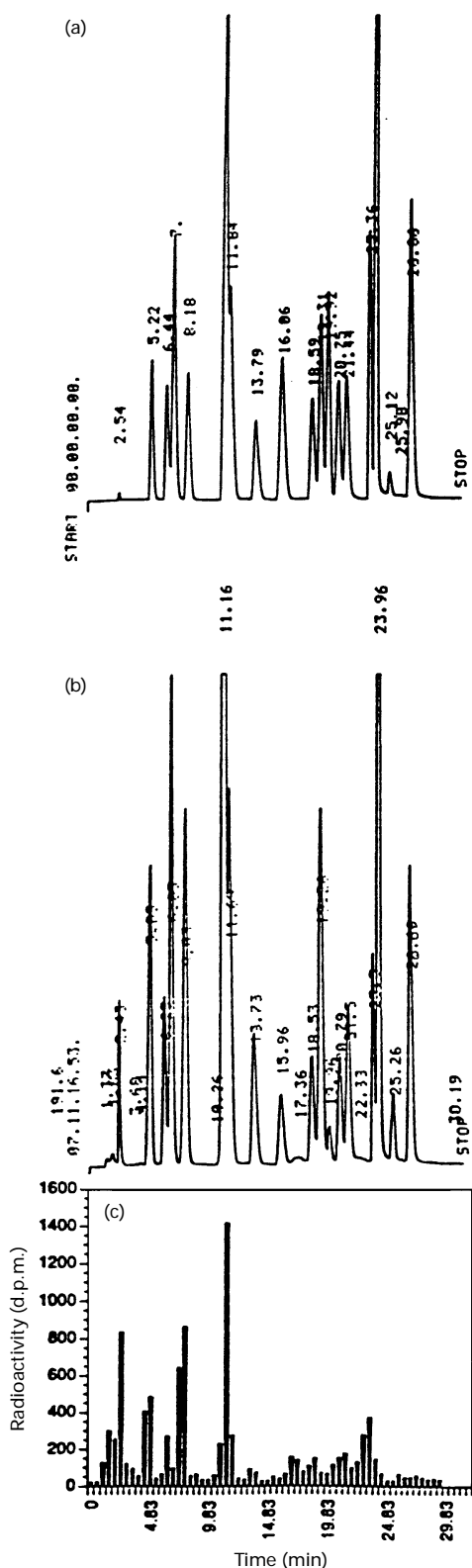
Figure 1   Example HPLC chromatograms and radiolabel profile

(**a**) HPLC profile of an amino acid standard. The amino acids are separated in the following order, left to right: Asx, Thr, Ser, Glx, Gly, Ala, Val, Met, Ile, Leu, norleucine (internal standard), Tyr, Phe, His, Lys, ammonium (not quantitative), Arg. The elution times are shown above each peak. (**b**) Profile of a protein hydrolysate from *M. faveolata*. An unknown, *o*-phthaldialdehyde-positive compound was eluted at 2.43 min, before the first protein amino acid. (**c**) The radiolabel content of the 30 s effluent fractions from the sample shown in (*b*).

residue hydrolysed over 6 M HCl in an evacuated vessel at 110 °C with a Waters PicoTag workstation. Acid hydrolysis converts glutamine into glutamate (the pair is referred to as Glx), asparagine into aspartic acid (the pair is referred to as Asx), and breaks down tryptophan completely, especially in the presence of carbohydrates [12], which were plentiful in these samples. Cysteine is also susceptible to degradation in the presence of carbohydrates [12]. A small percentage of threonine and serine, 3 % and 6 % respectively, can be degraded during hydrolysis [12]. Thus our methods could not differentiate between the synthesis of glutamine and glutamate, or that of asparagine and aspartic acid, could not detect the synthesis of tryptophan or cysteine, and might have underestimated the synthesis of threonine and serine.

The hydrolysates were dissolved in Pierce HPLC citrate sample buffer (pH 2.2) containing 0.1 mM norleucine as an internal standard. Much of the carbohydrate in the pellet caramelized during acid hydrolysis and did not dissolve easily in the citrate buffer. Undissolved particulate material, including caramelized carbohydrates, were removed before HPLC by filtration through a 0.22 μm nylon filter.

Chromatography

HPLC

Samples were loaded on a 0.46 cm × 12 cm HPLC cation-exchange column (sulphonated polystyrene–divinylbenzene copolymer resin, Pierce High Speed AA511) at 65 °C. Amino acids were eluted with a sodium buffer gradient of increasing pH and ionic strength using Pierce Buffelute buffers in accordance with the 30 min protocol recommended by Pierce. Post-column derivatization of primary amines with 2-mercaptoethanol and *o*-phthaldialdehyde provided for fluorimetric detection (excitation at 340 nm, emission at 455 nm) of 15 protein amino acid peaks (Figure 1a). The Glx and Asx pairs are each detected as one peak. Proline and hydroxyproline, which do not have a primary amine, and cysteine, which has a poor response to *o*-phthaldialdehyde, are not detected. The effluent was collected in 30 s fractions and the amount of radiolabel in each aliquot was determined by liquid-scintillation counting with a Beckman model LS180 counter and Ecolume scintillant.

An average of 100 ± 10 % of the radiolabel injected into the HPLC was recovered in the collected effluent, but only 42 ± 10 % and 64 ± 18 % of the radiolabel was attributable to amino acids in the glucose metabolism and amino acid-metabolism experiments respectively. A broad peak of radiolabel was eluted before the first protein amino acid (Figures 1a and 1b), accounting for 30 ± 11 % and 7 ± 7 % of the total radiolabel in the samples from the glucose and amino acid metabolism experiments respectively. A narrow *o*-phthaldialdehyde-positive peak was co-eluted with this broad radiolabel peak (Figure 1b) in all samples except those from *Acropora cervicornis*. Allen and Kilgore [13] also noted a large radiolabel peak that was eluted before the first protein amino acid after incubation of the mollusc *Haliotis rufescence* with radiolabelled glucose. Although we did not identify this peak, we determined experimentally that it was not glucose.

A single 30 s fraction of the HPLC effluent was assigned to each amino acid on the basis of the amino acid elution times of each sample run. Some fractions associated with an amino acid contained a low level of radioactivity (less than 100 d.p.m.). The radioactive counts in these fractions were compared with the mean background obtained for the same amino acid during the separation of an unlabelled amino acid standard (range from 21 ± 3 d.p.m. for arginine to 26 ± 5 d.p.m. for histidine). The background d.p.m. values for each amino acid were also corrected

for chemifluorescence. Fractions were considered to contain a radiolabelled amino acid only in those cases in which sample d.p.m. values exceeded two standard deviations above the mean background value.

The amino acids threonine and serine were eluted within 30 s of each other, as were histidine and lysine. A shorter collection interval (2 s) was used to obtain a more precise determination of the co-occurrence of these amino acids and the radiolabel during the elution of these amino acid pairs for three samples of each species. Glycine and alanine were eluted too closely to be separated clearly with 2 s effluent fractions. However, separation of these amino acids was distinct on the TLC plates.

The integrated peak areas for each amino acid were converted to concentrations by using calibration (F) factors derived from amino acid standards (Pierce amino acid standard H) to which an internal standard, norleucine, was added. F factors were calculated from 36 runs as the ratio of the peak area of each amino acid in the standard to the peak area of the norleucine. The same internal standard was added to each coral sample, and the concentration of each amino acid in the sample was calculated by comparison with the internal norleucine peak and the F factors. Amino acid concentrations in the coral samples were normalized to total amino acid nitrogen recovered in each HPLC run. Specific radioactivities, in μCi/mmol, were calculated from the radiolabel attributed to each amino acid, and the amount of that amino acid in each HPLC sample.

TLC and autoradiography

Three protein-hydrolysate samples per species from corals incubated with radiolabelled glucose and analysed by HPLC were also analysed by two-dimensional TLC. The hydrolysates were desalted with 55 mm × 5.4 mm Dowex W50-8X columns equilibrated in 5 % (v/v) formic acid. Four column volumes of formic acid were used to remove uncharged compounds such as sugars. The amino acids were eluted with 1 M NH₄OH, dried in a Savant Speed-Vac evaporator and redissolved in 500 μl of deionized water; a subsample was counted by liquid-scintillation counting. Subsamples containing approx. 0.2 mg of amino acid (calculated from HPLC analysis of each sample) were loaded on 20 cm × 20 cm × 160 μm Eastman cellulose chromatography sheets. Solvents for the first dimension were n-butanol/acetic acid/distilled water (60:15:25, by vol.) and for the second dimension t-butanol/s-butanol/methyl ethyl ketone/distilled water/diethylamine (40:40:80:50:4, by vol.) (modified from [14,15]). The plates were dried under vacuum overnight, sprayed with 0.2 % ninhydrin in acetone to locate amino acid spots, and autoradiographed with Kodak X-OMAT AR X-ray film for 30–45 days. Spots on the films were mapped over the ninhydrin-positive spots.

Statistics

All statistics were calculated with Systat 5 1990–91 on a Macintosh II computer. Bartlett's Box test was used to test for homogeneity of variance among groups, and a Kolmogorov–Smirnov comparison with a standard normal curve was used to test the distribution of each group. Whenever the sample groups did not have a homogeneous variance and/or did not have a normal distribution, either Mann–Whitney U tests or Kruskal–Wallis tests were performed as indicated, depending on the number of groups in each comparison. Otherwise, analyses of variance and Student's t tests were performed.

RESULTS

Amino acid profile of composite coral protein

The overall distribution of the 15 HPLC-detectable amino acids in the recovered proteins was similar for the five species of scleractinian corals (Figure 2). To test for statistical differences in the patterns of amino acid distribution, the deviation of the percentage composition of each amino acid was standardized by the following protocol: a grand mean and standard deviation was calculated for each amino acid, pooling the percentages for all coral species. The grand mean was then subtracted from each sample value, and the result divided by the grand standard deviation. This procedure results in the number of standard deviations by which each sample falls away from the grand mean. A Kruskal–Wallis two-way analysis of variance demonstrated that the patterns of amino acid distribution in the composite protein samples were not significantly different between the field-collected colonies of the five coral species, nor between the field-collected and laboratory-maintained colonies of *M. faveolata* ($P > 0.05$).

Synthesis of amino acids from glucose

HPLC analyses indicate that after incubation with radiolabelled glucose at least 14 amino acids were radiolabelled in all five species of coral, including the laboratory-maintained zooxanthellate and bleached *M. faveolata* (Asx, Ser, Glx, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, His, Lys and Arg) (Table 2). The 2 s HPLC fractions showed that threonine was not radiolabelled, but that serine, lysine and histidine were. These results, and the attribution of radiolabel to both glycine and alanine, were confirmed with TLC.

In addition, the TLC autoradiographs clearly indicated the presence of radiolabelled proline in all five species and radiolabelled hydroxyproline in four species (the exception being *M. faveolata*.) The presence of hydroxyproline in *M. faveolata* cannot be ruled out, because separation on that part of the TLC plates was poor. The composite results of the HPLC (15 amino acids) and TLC (17 amino acids) analyses indicate the synthesis of 16 amino acids (Table 2).

The percentage of the total radiolabel in each amino acid was similar between species (Table 2). However, the pattern of radiolabel content differed from the amino acid profile of composite coral protein, which indicates a lack of correlation between amino acid synthesis and net assimilation into protein. For example, composite coral protein contained fewer histidine than leucine or lysine residues (Figure 2), yet histidine had a higher radiolabel content than leucine or lysine (Table 2). However, those amino acids in highest abundance (Glx, Asx and Gly) also contained large amounts of radiolabel.

Histidine is an essential amino acid for most animals studied (Table 1), yet on average it was one of the most radiolabelled amino acids in this study (Table 2).

Fate of radiolabelled glutamic acid, lysine and valine

Radiolabel from glutamic acid was incorporated into 14 of the detectable protein amino acids, although the amount of radiolabel in some of the amino acids (Val, Met, Ile, Leu and Tyr) was low or at background in some of the replicates. On average, only 48 % of the total radiolabel in the protein was recovered as glutamic acid.

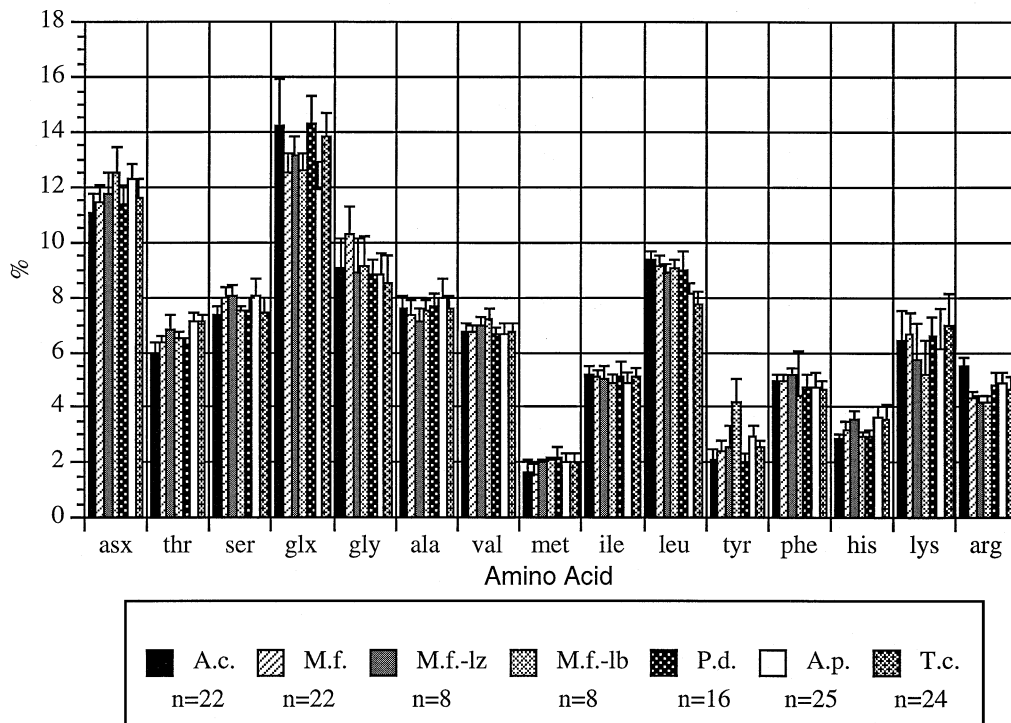Much less radiolabel from lysine and valine appeared in other

Figure 2   Amino acid composition of trichloroacetic acid-precipitable protein from five species of scleractinian coral

Bar heights are the mean molar percentages of each amino acid in the protein samples. Abbreviations: A.c., *Ac. cervicornis*; M.f., *M. faveolata*, field corals; M.f.-lz, *M. faveolata* laboratory zooxanthellate corals; M.f.-lb, *M. faveolata* laboratory bleached corals; P.d., *Porites divaricata*; A.p., *As. poculata*; T.c., *Tubastrea coccinea*; n, number of replicates. Error bars indicate 1 S.D.

Table 2   Radiolabelled amino acids in hydrolysates of proteins extracted from corals incubated with [U-^{14}C]glucose

Symbols in column 2 (TLC): −, no spot on autoradiograph; +, faint spot on autoradiograph; + +, dark spot on autoradiograph; *, proline was detected by TLC in all species except *M. faveolata*. Symbols in column 3 (HPLC): −, no detectable radiolabel in amino acid; +, less than 5% of total radiolabel in amino acid; + +, 5% or more of total radiolabel; no symbol, amino acid not detectable by HPLC. Values in columns 4–8 are percentages of total radiolabel in each amino acid recovered by HPLC.

Amino acid	Presence (all corals)		Radiolabel recovered by HPLC (% of total)				
	TLC	HPLC	*Ac. cervicornis*	*M. faveolata*	*P. divaricata*	*T. coccinea*	*As. poculata*
Asx	+ +	+ +	10	4	5	10	7
Thr	−	−	0	0	0	0	0
Ser	+ +	+ +	26	10	12	5	6
Glx	+ +	+ +	8	5	7	11	12
Pro	+ *						
H-Pro	+						
Gly	+ +	+ +	4	5	8	8	11
Ala	+ +	+ +	22	17	31	25	32
Val	+	+	>1	2	1	>1	3
Met	+	+	4	1	3	>1	2
Isl	+	+	2	3	3	5	3
Leu	+	+	1	1	1	5	2
Tyr	+	+	2	3	8	2	3
Phe	+	+	2	2	4	2	3
His	+	+ +	16	43	14	20	13
Lys	+	+	1	3	2	4	2
Arg	+	+	>1	1	1	>1	>1

amino acids; 80% and 76% respectively remained as the administered amino acid. The protein amino acids derived from lysine were similar to those made from glutamic acid, but in smaller quantities. A greater proportion of the radiolabel from valine appeared in tyrosine, phenylalanine and histidine than appeared in these amino acids from glutamic acid or lysine.

DISCUSSION

The ability to synthesize protein amino acids *de novo* varies only slightly within the animal kingdom. The results of this study suggest that corals might be able to synthesize many protein amino acids that are thought to be essential in most non-cnidarian Metazoa (Table 1). This assertion is not entirely new for the Metazoa. Evidence for the synthesis of amino acids usually considered essential also exists for some representatives of the the the Crustacea, Mollusca and Nematoda (Table 1). Interestingly, patterns of amino acid synthesis seem to vary by species than by phylum, and might indicate biochemical adaptation of individual organisms. However, the variable patterns of amino acid synthetic abilities within and among groups might also be an artifact of experimental design, as illustrated by two different patterns of amino acid synthesis reported for the nematode *Caenorhabditis briggsae*. Dougherty et al. [16] and Nichols et al. [17] reported that none of the essential amino acids could be synthesized by *C. briggsae*, but in a later study Rothstein and Tomlinson [18] reported that in axenic culture this nematode could synthesize Tyr, Thr, Val, Met, Leu, Ile, His and Lys *de novo*. Even Rothstein and Tomlinson [18], however, observed that synthesis of these amino acids by *C. briggsae* was curtailed in the presence of penicillin and streptomycin, and that the nematode was unable to grow or reproduce on anything other than complete media.

Eight amino acids putatively synthesized by these corals (Val, Ile, Leu, Tyr, Phe, His, Met and Lys) cannot be synthesized by most Metazoa (Table 1). This finding might not be as novel as it first seems when recent findings relating to cnidarian phylogeny are considered. Phylogenetic trees based on 18 S rRNA sequences suggest the Cnidaria might be on an early or separate branch from the rest of the metazoans [1–3]. According to these phylogenetic reconstructions, the Cnidaria are more closely related to the plants, fungi and ciliates than to other metazoans. Schlichter et al. [19] also suggested that the Cnidaria are primitive evolutionarily because their amino acid uptake sites are on their external rather than their internal surfaces. A different or early evolutionary origin of the Cnidaria is consistent with our observations and suggests that the Cnidaria might have diverged from other metazoans before amino acid synthesis pathways were lost.

Attribution of amino acid synthesis

The radiolabelled amino acids found in the present experiments with radiolabelled glucose could have been synthesized by (1) the coral animal, (2) the zooxanthellae for the zooxanthellate species only, and/or (3) bacterial symbionts or contaminants. Many dinoflagellates can synthesize all protein amino acids [20,21]. However, synthesis of the recovered amino acids by zooxanthellae can be discounted because the pattern of protein amino acid radiolabelling did not differ between zooxanthellate and non-zooxanthellate species, nor did it differ between zooxanthellate and bleached colonies of *M. faveolata*. Furthermore, other studies have shown only minimal translocation of amino acids from zooxanthellae to their coral hosts [10,22–26]. Only a few non-essential amino acids such as alanine and glutamate have been shown to be excreted from zooxanthellae *in vivo* [26] or in the presence of host tissue extracts [25,27–31].

Synthesis of the radiolabelled amino acids by bacteria is more difficult to dismiss. Undoubtedly some bacteria were present on the surface of the corals in spite of the rinsing techniques, and no effort was made to remove or inactivate those in polyp coelenterons. Furthermore two of the coral species used, *Ac. cervicornis*

and *Porites divaricata*, have colonies of rod-shaped bacteria within their tissues that are probably endosymbionts of some sort ([32], and A. M. Szmant, unpublished work). Such bacterial colonies have not been found, however, in the other three species used in these experiments (A. M. Szmant, unpublished work). Given that neither the amount of radiolabelled-glucose uptake nor the pattern of amino acid radiolabelling differed between the two coral species with bacterial colonies and the three without, it can be reasoned that the bacterial colonies were not responsible for a significant amount of the amino acid synthesis. The same logic cannot be applied to bacteria living on internal or external surfaces of the coral tissues. However, tests of the rinsing techniques suggest that 90 % or more of the bacteria were eliminated from external surfaces, and given the relatively large biomass of coral it is unlikely that bacteria remaining on the exterior coral surfaces, together with coelenteric bacteria, could have been responsible for the rather substantial amount of amino acid synthesis from radiolabelled glucose, even considering that bacteria have higher metabolic rates than corals. Even so, we made a rough calculation of the amount of radiolabelled glucose that could have been taken up by surface bacteria, based on (1) bacterial densities on the surface of scleractinian corals (276 cells/cm²) [33], (2) K_m and V_{max} for the uptake of D-glucose by an oligotrophic marine bacterium [34], and (3) the experimental values of substrate concentration, incubation time and coral surface areas. Our calculated estimate for bacterial glucose uptake is 0.1 % of the administered radiolabel. This value is likely to be an overestimate because we used the minimum K_m and maximum V_{max} from Nissen et al. [34] (adjusted for bacterial cell number), and the maximum substrate concentration for all the incubations. Furthermore we assumed that substrate concentration was constant throughout the incubation period, whereas it decreased by 21–70 %, and we did not account for the removal of surface bacteria by our pre-rinsing techniques. In addition, much of the radiolabelled glucose taken up by bacteria would undoubtedly have been used for bacterial respiration and not for the synthesis of amino acids. Lastly, the amount of radioactivity recovered in the distilled-water rinses (which included coral mucus and associated bacteria) was small, indicating that most of the radiolabelled glucose was taken up directly by the coral.

Attribution to bacterial synthesis of essential amino acids radiolabelled in only trace quantities might be warranted, but not the synthesis of essential and non-essential amino acids found radiolabelled in large quantities, such as alanine, glycine, leucine, isoleucine and especially the essential amino acid histidine (Table 2). While we are confident that these arguments indicate that the contribution of bacteria to the synthesis of radiolabelled amino acids was minimal during the experiments, the doubt about bacterial contributions cannot be eliminated entirely without further testing.

Pathways of amino acid synthesis

Carbon-14 atoms from glucose can be incorporated into amino acids via intermediates in the glycolytic pathway (Ser, Gly, Ala, Val, Leu, Cys, Tyr, Phe and Trp), the pentose phosphate shunt (Tyr, Phe, Trp and His), and the tricarboxylic acid cycle (Asx, Glx, Thr, Pro, Met, Ile, Lys and Arg) [35]. The eight 'essential' amino acids synthesized by these corals are produced from intermediates from all three of these pathways. Valine and isoleucine are synthesized by the same enzymes but from different precursors, and leucine is synthesized by a similar series of reactions. Tyrosine and phenylalanine are synthesized from intermediates in glycolysis and the pentose phosphate shunt, and

therefore radiolabel in these two amino acids could have been derived from either pathway. Histidine, however, is synthesized entirely from an intermediate in the pentose phosphate shunt, which indicates that radiolabelled glucose was moved through this pathway. Glutamic acid can be synthesized from α-oxoglutarate by the addition of ammonia by the action of glutamate dehydrogenase. In animals, however, glutamate dehydrogenase is generally considered to play a larger role in the oxidation of glutamic acid than in its synthesis [36]. Glutamic acid is usually made by the transamination of α-oxoglutarate and other amino acids, and incorporation of ammonia into amino acids is mediated by the action of glutamine synthetase. Recovery of radiolabel in glutamine, arginine and proline, which are synthesized from glutamic acid, as well as in glutamic acid, indicates the action of glutamate dehydrogenase in the direction towards glutamic acid synthesis. Miller and Yellowlees [37] noted a high affinity of coral-tissue glutamate dehydrogenase for ammonium, which is consistent with glutamate dehydrogenase-mediated glutamic acid synthesis. Furthermore, in other experiments both *M. faveolata* and *Ac. cervicornis* assimilated significant amounts of $^{15}NH_4$ into animal tissue after being maintained in total darkness for 48 h, by which time their zooxanthellae no longer took up nutrients ([7], and A. M. Szmant, L. M. Fenner and L. M. FitzGerald, unpublished work).

Fates of radiolabelled amino acids

Carbon-14 atoms from the administered amino acids (Glu, Lys and Val) can be used to synthesize other amino acids directly or indirectly (after entering glycolysis, the tricarboxylic acid cycle, etc.), or they can be released as $^{14}CO_2$ if the amino acid is oxidized for energy [35]. Glutamic acid enters the tricarboxylic acid cycle as α-oxoglutarate after deamination and transamination. The carbon backbone from valine enters the tricarboxylic acid cycle as succinyl-CoA, which is converted in several steps into oxaloacetate. From oxaloacetate, ^{14}C atoms from valine can be diverted to synthesis of amino acids (Asx, Met, Thr, Lys and Ile), used in gluconeogenesis or remain in the tricarboxylic acid cycle and end up in Glx, Arg and Pro from α-oxoglutarate. Carbon atoms that enter the gluconeogenic pathway can be used for amino acid synthesis at a number of intermediary steps (Ser, Gly, Ala, Val, Leu, Cys, Tyr, Phe and Trp), or they can be incorporated into glucose. Glucose can then enter the pentose phosphate shunt. The carbon backbones from lysine can be degraded into acetyl-CoA and then enter the tricarboxylic acid cycle, or be directed toward cholesterol/ketone-body synthesis (but not gluconeogenesis directly, because animals in general cannot convert acetyl-CoA into pyruvate).

The appearance of radiolabel from glutamic acid, lysine and valine in other amino acids indicates that their ^{14}C atoms moved through anabolic as well as catabolic pathways. The activity of gluconeogenic and pentose phosphate pathways is suggested by the relatively high specific radioactivity of tyrosine, phenylalanine and histidine in corals administered valine. The likely pathway for ^{14}C from the radiolabelled lysine recovered in Gly, Ala and Ser, and the essential amino acids Tyr, Phe and His, was through the tricarboxylic acid cycle, followed by gluconeogenic, glycolytic or phentose phosphate pathways.

Rate of amino acid synthesis

Rates of synthesis of the individual amino acids were not measured in this study, and yet some deductions can be made about the relative flux of carbon from glucose into each amino acid. The specific radioactivities of the protein amino acids were the net result of (1) the initial intracellular pool size for each free amino acid, (2) the amount of each amino acid synthesized from the radiolabelled glucose, (3) the number of ^{14}C atoms in each of these amino acids, (4) the amino acid composition of the proteins made during the experimental periods and (5) the amino acid composition of the composite pre-existing protein. The highest specific radioactivities were found for Asx, Ser, Glx, Gly, Ala and the essential amino acid His. These amino acids have fewer carbon atoms (2–5) than the remaining amino acids (5–11), and have been reported in higher concentrations in the free amino acid pools of other cnidarians [38–47]. Except for histidine, these amino acids also have shorter, less complex, synthetic pathways. If all amino acids had been synthesized at the same rate, a large free amino acid pool size and small number of ^{14}C atoms per molecule would have resulted in lower specific radioactivities of these amino acids than of the others. Therefore, assuming that the free amino acid pool composition in the experimental corals was similar to that reported for other cnidarians, the higher specific radioactivities of these six amino acids would indicate that net carbon flux into them was greater than into the other amino acids.

Conclusions

In summary, all five species of scleractinian corals tested could synthesize at least 16 of the 20 protein amino acids, eight of which are essential in other Metazoa examined so far. It remains to be determined whether attached or coelenteric bacteria are responsible for some of the synthesis. Even if bacteria are in fact responsible for the synthesis, however, this finding is significant from an ecological-unit perspective. If coral gut bacteria or bacterial endosymbionts regularly convert sugars (which reef corals receive from their zooxanthellae) into amino acids, and if corals have access to bacterial products (e.g. digestion of bacteria or bacterial excretion of amino acids into the coelenteron), then in a functional sense corals have a previously unaccounted-for source of protein and amino acids. Despite the apparent ability of corals to synthesize some essential amino acids, rates of amino acid synthesis seem to be greater for those amino acids that are typically non-essential, and slower for those amino acids that are typically essential, with the exception of histidine. The role of synthesis in satisfying metabolic requirements for 'essential' amino acids still needs to be determined.

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