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Metabolite comparisons and the identity of nutrients translocated from symbiotic algae to an animal host

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Summary

Dinoflagellate algae of the genus *Symbiodinium* in symbiosis with marine animals release much of their photosynthetic carbon to the animal host. The compounds translocated to the host ('mobile compounds') were investigated by metabolite comparison as follows: a substrate was identified as a candidate mobile compound when comparable profiles of metabolites were generated from host metabolism of this substrate (supplied exogenously) and the endogenous mobile compounds. When the sea anemone *Anemonia viridis* was incubated with $NaH^{14}CO_2$ under photosynthesizing conditions, most of the radioactivity in the animal tissue was recovered from the low-molecular-mass fraction and distributed in the ratio 1:2:1 between the neutral, acidic and basic sub-fractions. Prominent ^{14}C-labelled compounds included glucose, malate and glucose-6-phosphate. When the symbiosis was incubated with ^{14}C-labelled glucose plus succinate or fumarate (but none of eight other substrate combinations tested), the ^{14}C-labelled metabolites closely matched those obtained with $NaH^{14}CO_2$. These data suggest that glucose and succinate/fumarate (or metabolically allied compounds) may be important photosynthetic compounds transferred from the *Symbiodinium* cells to the tissues of *A. viridis*. Metabolite comparisons can be applied to study nutritional interactions in symbioses involving photosynthetic algae and, with appropriate modification, other associations between microorganisms and plants or animals.

Key words: *Anemonia viridis*, Cnidaria, dinoflagellate alga, nutrition, photosynthetic metabolism, *Symbiodinium*, symbiosis, zooxanthella.

Introduction

Many corals, sea anemones and other benthic marine animals bear symbiotic dinoflagellate algae of the genus *Symbiodinium*, also known as zooxanthellae. These symbioses have a nutritional basis, involving the sustained and substantial bidirectional translocation of nutrients between living cells of the algal and animal partners. In particular, the algal cells photosynthesize at high rates and release much of their photosynthetically fixed carbon to the animal tissues, making a substantial contribution to the carbon and energy budgets of the animal partner (e.g. Muscatine et al., 1984; Edmunds and Davies, 1986).

The nutrients translocated between the partners in a symbiosis are referred to here as mobile compounds. This study concerns the identity of the mobile compounds derived from the photosynthetically fixed carbon pools of the algal cells. This topic is technically challenging for two reasons. Firstly, the *Symbiodinium* cells in most associations are intracellular, specifically located in the cytoplasm of animal cells and individually enclosed within an animal membrane termed the symbiosomal membrane (Wakefield et al., 2000). Secondly, it is expected that the mobile photosynthetic compounds are metabolized rapidly on receipt by the animal cytoplasm, thereby limiting their backflow to the algal cells (Douglas, 1994). It is widely accepted that the mobile photosynthetic compounds include low-molecular-mass compounds such as glycerol, glucose and organic acids. The principal evidence is that these are the compounds released into the medium by *Symbiodinium* cells freshly isolated from certain symbioses and incubated with extract of the host animal (Trench, 1971a; Hinde, 1988). Exceptionally, in the tridacnid clam–*Symbiodinium* association, the mobile compounds can be identified relatively easily in the intact symbiosis because the algal cells are extracellular. In this system, the dominant photosynthetic compound released from *Symbiodinium* cells is glucose in the symbiosis but glycerol in isolated cells (Streamer et al., 1988; Rees et al., 1993; Ishikura et al., 1999). This discrepancy suggests that it may not be justified generally to equate the compounds released by isolated algal cells and the mobile compounds in the intact symbiosis. Compounding these difficulties, the *Symbiodinium* cells in some animal hosts, e.g. *Zoanthus robustus* and *Anemonia viridis*, release markedly more photosynthetic compounds to the animal tissues in the intact association than after isolation and incubation with host homogenate (Sutton and Hoegh-Guldberg, 1990; L. Whitehead, J. T. Wang and A. E. Douglas, unpublished).

Various approaches have been adopted to study

photosynthate release from *Symbiodinium* cells in the intact symbiosis, thereby circumventing the limitations of studies with isolated algae. These approaches include identification of photosynthetically fixed ^{14}C- and ^{13}C-labelled compounds in the animal partner (Trench, 1971b; Battey and Patton, 1984; Johnston et al., 1995), measurement of respiratory quotients (Gattuso and Jaubert, 1990) and identification of organism-specific compounds (Harland et al., 1991). Although these studies were not designed specifically to identify the mobile photosynthetic compounds, they collectively suggest that intact lipids, glycerol and fatty acids may be translocated from *Symbiodinium* cells to the animal tissues. These data are partly, but not completely, consistent with the candidate mobile compounds identified from studies with isolated algal cells (see above).

The purpose of the present study was to apply the approach of 'metabolite comparisons', summarized in Fig. 1, to explore the identity of mobile photosynthetic compounds in an intact symbiosis. The two treatments are: (1) the 'control animals' incubated with $NaH^{14}CO_3$ under photosynthesizing conditions and (2) the 'experimental animals' incubated with one of a panel of ^{14}C-labelled organic substrates under non-photosynthesizing conditions. The radioactively labelled compounds in the animal fraction are the products of animal metabolism of the mobile compounds and of the exogenously applied organic compounds for the control and experimental animals, respectively. The exogenous organic substrates that generate the same pattern of labelled metabolites in the animal fraction as that obtained with the $NaH^{14}CO_3$ incubations can, consequently, be interpreted as the mobile photosynthetic compound (or a compound metabolically closely allied to the mobile compound). By this approach, candidate mobile photosynthetic compounds have been identified in the symbiosis between the sea anemone *Anemonia viridis* and algae of the genus *Symbiodinium*. This association has been the subject of a number of physiological and metabolic studies (e.g. Taylor, 1969; Schlichter, 1978; Tytler and Davies, 1984; Stambler and Dubinsky, 1987; Harland et al., 1992; Davy et al., 1997; Furla et al., 1998; Roberts et al., 1999) but the mobile compounds have not been identified.

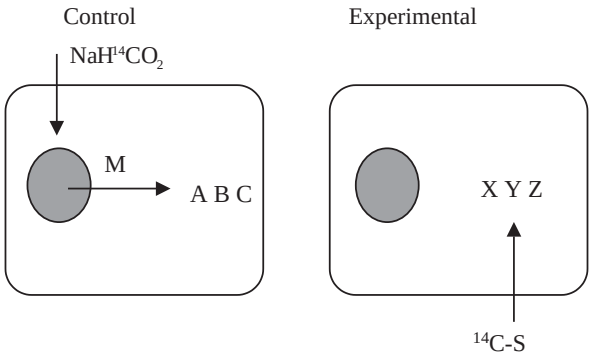

Fig. 1. Identification of candidate mobile photosynthetic compounds by metabolite comparison. The algal cells are shown as the shaded oval within the animal (square). Control symbiosis: $NaH^{14}CO_3$ is supplied to the symbiosis under photosynthesizing conditions; the mobile compound(s), M, labelled with ^{14}C, is translocated from the algal cells to the animal, where it is metabolized to compounds A, B and C. Experimental symbiosis: ^{14}C-labelled organic carbon source (S) supplied to the symbiosis under non-photosynthesizing conditions is metabolized to compounds X, Y and Z in the animal. When compounds X, Y and Z are similar to compounds A, B and C, the carbon source S is interpreted to be the same as, or allied to, the mobile compound M.

Materials and methods

Experimental material

Anemonia viridis (Forskal), green colour morph, were collected from a tide pool at Morfa Nefyn, North Wales, UK (Ordinance Survey grid reference 274, 410) and maintained in aerated artificial seawater (Instant Ocean Salts; Aquarium Systems, Sarrebourg, France) of salinity 35‰ and pH 8.2 at 16°C under a 16 h:8 h light:dark regime at 10–30 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). Experiments were conducted 3–5 days after the animals had received their weekly feed of chopped squid and were performed under the same light and temperature conditions as for anemone culture.

Replicate groups of three freshly excised tentacles of *A. viridis* (taken from three separate individuals) were incubated in 1 ml seawater buffer, comprising 420 mmol l^{-1} NaCl, 26 mmol l^{-1} $MgSO_4$, 23 mmol l^{-1} $MgCl_2$, 9 mmol l^{-1} KCl, 9 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $NaHCO_3$ and 10 mmol l^{-1} Hepes, pH 8.2 (Wang and Douglas, 1997), for 30 min prior to an experiment, by which time the tentacles had relaxed. Where appropriate, algal photosynthesis was inhibited by 5 μmol l^{-1} DCMU (dichlorophenyl dimethyl urea) added 15 min before the experiment. Experiments were initiated by adding radiolabelled substrate (details below) and, unless stated otherwise, comprised a 5-min time course, to minimize the extent of animal metabolism of the radioactive substrate whilst generating sufficient radioactivity for quantification. Samples without added radioactivity were included in all experiments as controls.

$NaH^{14}CO_3$ incubation under photosynthesizing and non-photosynthesizing conditions

After the incubation with 0.37 MBq (10 μCi) $NaH^{14}CO_3$ (ICN Pharmaceuticals, Costa Mesa, CA, USA), the tentacle samples were rinsed in ice-cold 0.5 mol l^{-1} NaCl and homogenized in a hand-held glass homogenizer in 1 ml ice-cold 0.5 mol l^{-1} NaCl. The protein content of each homogenate was quantified using the Bio-Rad protein micro-assay kit (Bio-Rad Laboratories GmbH, Munchen, Germany) according to the manufacturer's instructions, with bovine serum albumin as standard. The homogenate was centrifuged at 1000 ***g*** for 5 min at 4°C, and the pellet was washed twice by centrifugation and resuspension with 1 ml 0.5 mol l^{-1} NaCl. The supernatants were combined to form the animal fraction, and the final

suspension of algal cells was designated the algal fraction. The cell density in the algal fraction was quantified using an improved Neubauer haemocytometer (six replicate counts per sample). To quantify ^{14}C incorporation, 25 μl of each fraction was acidified with 4 mol l^{-1} acetic acid and shaken for 60 min to remove unfixed ^{14}C. Scintillation cocktail (Ultima-Gold™ XR; Packard Bioscience B.V., Gröningen, The Netherlands) was then added, and the radioactivity quantified in a scintillation counter (Packard Tri-Carb Scintillation Analyser) using pre-set ^{14}C windows. The counts in the samples incubated without added radioactivity were subtracted from values for the experimental samples.

Samples of the algal and animal fractions were incubated in 5% trichloroacetic acid (TCA) for 15 min at room temperature and then centrifuged at 10 000 ***g*** for 15 min. The supernatant (TCA-soluble fraction) was extracted three times with ether to remove the TCA, evaporated to dryness in a Speed-Vac (SC110; Savant Instruments, Holbrook, NY, USA) and then resuspended in 80 μl and 200 μl of deionised water for algal cell and animal samples, respectively. (The animal samples required a larger volume to dilute the salt; ethanol extraction was not used to remove the salt because this procedure substantially reduced the yield of radioactivity.) The lipid fraction was extracted from the TCA-insoluble fraction with 1 ml methanol:chloroform (2:1) for 15 min at room temperature followed by centrifugation at 10 000 ***g*** for 15 min. The chloroform layer containing the lipid was separated from the methanol by the addition of 0.4 ml 0.1 mol l^{-1} KCl. The final pellet that contained protein and nucleic acids was solubilized by incubation in 0.2 ml 1 mol l^{-1} NaOH at 100°C for 10 min and then neutralized with 0.2 ml 1 mol l^{-1} HCl. Samples of each fraction were removed for the quantification of radioactivity. The recovery of radioactive compounds after fractionation was, on average, 90% and 65% for the algal and animal samples, respectively. It is unclear why recovery was lower for the animal samples but it seems unlikely to be due to the salt content as similar recovery figures were achieved after salt removal by Streamer et al. (1988).

The TCA-soluble fraction was further separated into neutral, acidic and basic fractions using cation (Dowex 50 × 8 H^+; Sigma-Aldrich, Dorset, UK) and anion (Dowex 1 × 8; Sigma-Aldrich) exchange columns, following the procedure of Quick et al. (1989). To identify individual radiolabelled compounds, samples of the TCA-soluble fraction were separated by thin layer chromatography/thin layer electrophoresis, as in Wang and Douglas (1997), for organic acids, phosphate esters, sugars and sugar alcohols and as in Wang and Douglas (1999) for amino acids. The chromatography/electrophoresis plates were exposed to Kodak X-ray film for 4–6 weeks, and radioactive spots were identified by comparison with authentic compounds. The identification of the sugars, sugar alcohols and amino acids was verified by reverse-phase HPLC using the procedures of Ashford et al. (2000) and Karley et al. (2002).

^{14}C organic substrate incubations

The analyses were performed as for $NaH^{14}CO_2$ incubations with the following modifications. All experiments were performed under non-photosynthesizing conditions (in the presence of 5 μmol l^{-1} DCMU) in autoclaved seawater buffer supplemented with the antibiotics ampicillin (1 mg ml^{-1}) and streptomycin (50 μg ml^{-1}) to inhibit the metabolism of any contaminating bacteria (Wang and Douglas, 1998). Organic substrates {[U-^{14}C]glucose, [U-^{14}C]glycerol, [2,3-^{14}C]succinate, [2,3-^{14}C]fumarate (Sigma-Aldrich), [U-^{14}C]malate and [1,5-^{14}C]citrate (Amersham Pharmacia Biotech UK Ltd, Amersham)} were supplied at a concentration of 1 mmol l^{-1} and 0.11 MBq ml^{-1} for single substrate incubations and 0.5 mmol l^{-1} and 0.06 MBq ml^{-1} for double substrate incubations (i.e. giving a total concentration of 1 mmol l^{-1} and 0.11 MBq ml^{-1}). The chemical purity of radiochemical compounds was >99%, as determined by thin layer chromatography. The experimental design was developed following preliminary experiments on the uptake of

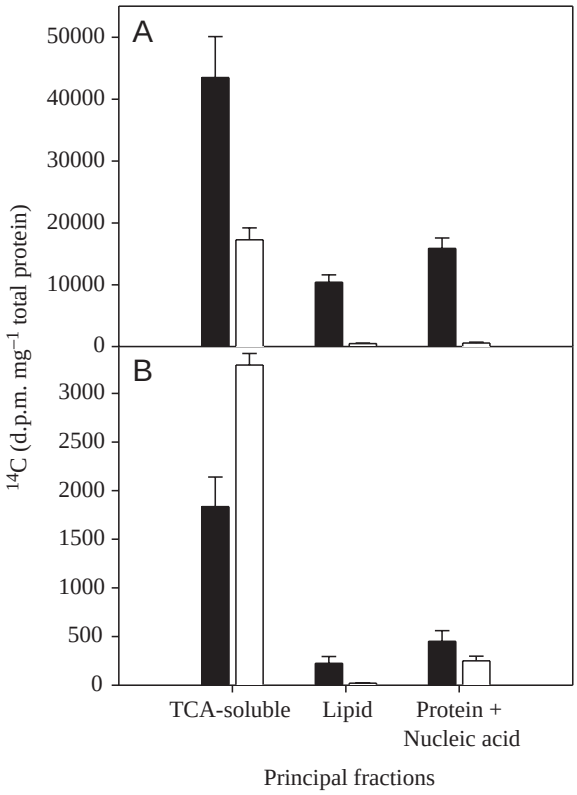

Fig. 2. Incorporation of $NaH^{14}CO_3$ into the principal chemical fractions of the algal cells (black bars) and anemone tissue (white bars) of *A. viridis* under photosynthesizing (A) and non-photosynthesizing (B) conditions. Bars represent means ± S.E.M. of 14 and 11 replicates, respectively. TCA, trichloroacetic acid. Multivariate analysis of variance (MANOVA) for data expressed as d.p.m. mg^{-1} protein after logarithmic transformation: Wilk's λ for algal cells/animal tissue, $F_{3,40}$=72.34, P<0.001; for photosynthesizing/non-photosynthesizing conditions, $F_{3,40}$=103.60, P<0.001; interaction, $F_{3,40}$=20.43, P<0.001. The full data set did not conform to the assumption of normal distribution and homogeneity, but exclusion of the non-conforming data resulted in no change in significance levels.

organic substrates by tentacle samples over time from 5 s to 5 min, which revealed minimal nonspecific binding of ^{14}C with >98% recovery of radioactivity from the anemone fraction, i.e. <2% in the algal cells. Algal cells and animal tissue were therefore not separated in the definitive experiments. The experimental material was homogenized in ice-cold deionised water, and the TCA-soluble fractions, which were essentially salt-free, were resuspended in 80 µl volumes after evaporation.

Results

$NaH^{14}CO_3$ fixation and assimilation

The rate of $NaH^{14}CO_3$ fixation by the excised tentacles of *A. viridis* in the light was $3.7\pm0.29\times10^5$ d.p.m. mg^{-1} total protein h^{-1} (mean ± S.E.M., N=19), equivalent to 0.09 ± 0.008 d.p.m. algal $cell^{-1}$ h^{-1}, and fixation was linear in incubations of 5–60 min duration with 30±1.5% of ^{14}C recovered from the animal tissue. Fixation in the presence of 5 µmol l^{-1} DCMU was, on average, 6% of that in samples without DCMU.

Incorporation of radioactivity into the principal chemical fractions of the algal cells and animal tissue after 5-min incubations is shown in Fig. 2. The distribution of radioactivity (expressed as d.p.m. mg^{-1} protein) across the chemical fractions varied significantly between the algal cells and animal tissues and was significantly altered by the DCMU treatment (statistical analysis shown in legend to Fig. 2). The two principal factors contributing to this variation were: (1) the greater incorporation of radioactivity into the TCA-soluble fraction of animal tissues (94% of the total) compared with algal cells (59%) under photosynthesizing conditions and (2) the disproportionate reduction in ^{14}C incorporation into the lipid fraction in the DCMU-treated samples relative to those under photosynthesizing conditions. The radioactivity in the neutral,

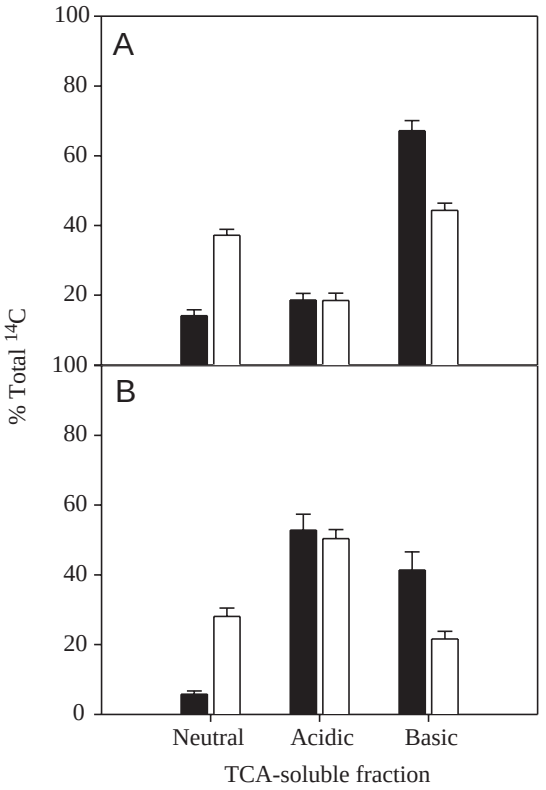

Fig. 3. Incorporation of $NaH^{14}CO_3$ into the trichloroacetic acid (TCA)-soluble fractions of the algal cells (A) and animal tissue (B) of *A. viridis* under non-photosynthesizing (black bars) and photosynthesizing (white bars) conditions, expressed as a percentage of the total radioactivity in the TCA-soluble fraction. Bars represent means ± S.E.M. of 12 and 9 replicates, respectively. Multivariate analysis of variance (MANOVA) for data expressed as d.p.m. mg^{-1} protein after logarithmic transformation: Wilk's λ for algal cells/animal tissue, $F_{3,34}$=53.35, P<0.001; for photosynthesizing/non-photosynthesizing conditions, $F_{3,34}$=169.25, P<0.001; interaction, $F_{3,34}$=19.26, P<0.001. The full data set showed homogeneity and closely approached normality.

Table 1. *Compounds identified in the trichloroacetic acid (TCA)-soluble organic fractions of separated algal cells and animal tissue from* A. viridis *incubated under photosynthesizing conditions with $NaH^{14}CO_3$ for 5 min*

		Intensity of radioactivity*	
Fraction	Compound	Algal cells	Animal tissue
Neutral	Fructose	++	–
	Glucose	+++	++
	Sucrose	++	–
	Glycerol	+	–
Anionic	Glucose 6-phosphate	+++	+++
	Unknown 1†	+++	++
	Unknown 2†	+++	+++
	Citrate	+++	+
	Fumarate	+	+
	Malate	+	+++
	Succinate	+	++
Basic	Arginine	+	–
	Aspartate	+	+
	Asparagine	+++	–
	Glutamate	+++	+
	Glutamine	++	+
	Isoleucine	+	–
	Lysine	+	+
	Serine	+	–
	Proline	–	+
	Threonine	+	++
	Tyrosine	+	–
	Valine	++	–

*Radioactivity in individual compounds was detected using autoradiography with spots identified by comparison with authentic compounds. +++, intense signal; ++, strong signal; +, detectable; –, not detected (these symbols apply relative to other compounds within each fraction and should not be compared between fractions; intensity should also not be compared between columns – algal samples had much more intense labelling overall).

†Unknowns 1 and 2 were compounds that co-eluted with other sugar phosphates but could not be positively identified.

acidic and basic fractions of the TCA-soluble fraction is shown in Fig. 3, with statistical analysis in the legend. For the samples under photosynthesizing conditions, the distribution of radioactivity differed significantly between the algal cells and animal tissues, with recovery from the neutral, acid and basic fractions in approximate ratios of 2:1:2 for algal cells and 1:2:1 for the animal tissues. The distribution of radioactivity was also significantly altered by DCMU treatment.

Various compounds were radioactively labelled in both the algal cells and animal tissue of samples incubated with $NaH^{14}CO_3$ under photosynthesizing conditions (Table 1). Differences between the algal cells and the animal tissue included the detection of ^{14}C-labelled glycerol in the algal cells but not in the animal tissue, and intense labelling of malate in the animal tissue as opposed to citrate in the algal cells. The ^{14}C content of DCMU-incubated samples was so low that identification of individual radiolabelled compounds was not feasible.

Uptake and assimilation of ^{14}C-labelled organic substrates into the animal tissue

Tentacles of *A. viridis* incubated under non-photosynthesizing conditions with each of the six organic substrates (glucose, glycerol, malate, citrate, succinate and fumarate) accumulated radioactivity equivalent to between 2.74 nmol substrate mg^{-1} total protein and 4.20 nmol substrate mg^{-1} total protein over the 5-min incubation period (Table 2). The pattern of incorporation of radioactivity into the principal chemical fractions was very similar for all the organic substrates tested. Radioactivity was recovered predominantly from the TCA-soluble fraction (99%), with a small amount in the protein/nucleic acid fraction (approximately 0.8%) and barely detectable amounts (0.2%) in the lipid fraction. For all the organic substrates, the distribution of radioactivity between the neutral, acidic and basic fractions of the TCA-soluble fraction was different to that recorded from the control experiments where the symbiosis was supplied with $NaH^{14}CO_3$ under photosynthesizing conditions (Fig. 4). For every substrate, radioactivity was detected almost exclusively in just two of the three TCA-soluble fractions: little radioactivity was recovered from the neutral fraction (≤0.7% of total ^{14}C in the TCA-soluble fraction) when an organic acid was supplied or from the basic fraction when glucose (4% of the total) or glycerol (5% of the total) was supplied. For most organic substrates tested, the most strongly radiolabelled fraction corresponded to that of the substrate supplied. Exceptionally, 58% of the total radioactivity derived from exogenous ^{14}C-labelled malate was detected in the basic fraction.

Individual radioactively labelled compounds recovered from tentacles that had been incubated in the various organic

Table 2. *Radioactivity incorporated from exogenous ^{14}C-labelled carbon sources by* A. viridis *over a 5-min period in the presence of 5 µmol l^{-1} DCMU*

Carbon source	Uptake (nmol mg^{-1} total protein)
Single substrate incubations	
Glucose	3.61±0.329
Glycerol	3.52±0.205
Citrate	2.74±0.310
Fumarate	3.74±0.217
Malate	4.20±0.697
Succinate	3.71±0.299
Double substrate incubations	
Glucose + fumarate	4.40±0.342
Glucose + succinate	4.25±0.294
Glycerol + fumarate	5.21±0.378
Glycerol + succinate	5.03±0.368

Values are means ± S.E.M. of 5–9 replicates.

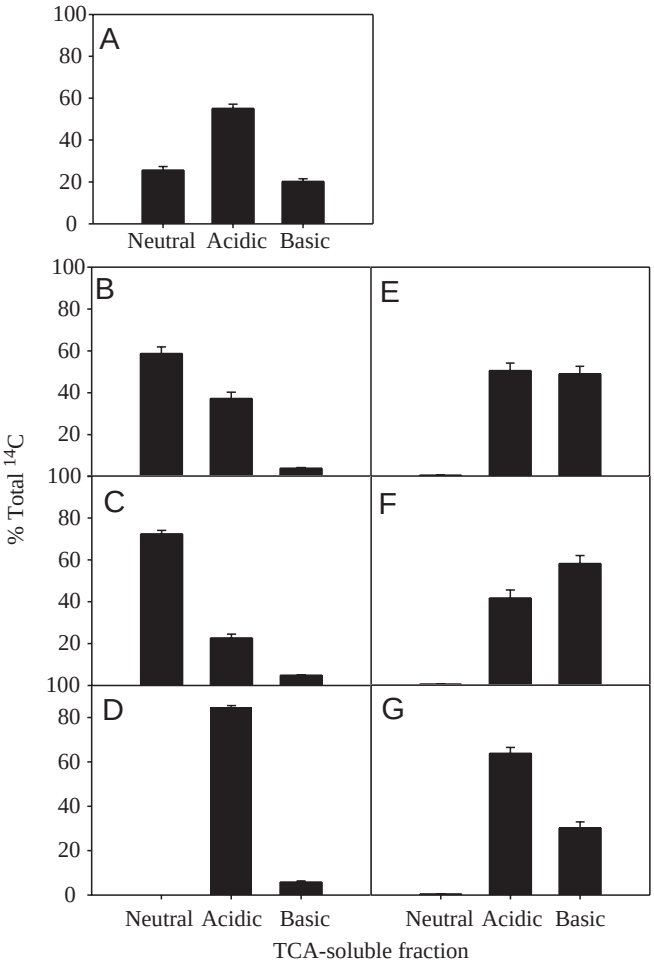

Fig. 4. Incorporation of ^{14}C into the trichloroacetic acid (TCA)-soluble fractions of *A. viridis* after incubation in (A) $NaH^{14}CO_3$, as in Fig. 3B, (B) [^{14}C]glucose, (C) [^{14}C]glycerol, (D) [^{14}C]citrate, (E) [^{14}C]fumarate, (F) [^{14}C]malate and (G) [^{14}C]succinate. Experiments were performed over 5 min and, except in A, in the presence of 5 µmol l^{-1} dichlorophenyl dimethyl urea (DCMU). Data are expressed as a percentage of the total label in the TCA-soluble fraction. Bars (except in A) represent the means ± S.E.M. of 5–9 replicates.

substrates are shown in Table 3, which includes the control data obtained for tentacles incubated with $NaH^{14}CO_3$ (Table 1) for comparison. For all substrates tested, four ^{14}C-labelled compounds – malate and three sugar phosphates – were detected in common with the control. The most strongly labelled compounds were those corresponding to the substrate supplied and to glucose 6-phosphate. In general, when an organic acid was supplied, a greater number of ^{14}C-labelled compounds were detected in common with the control compared with when glucose and glycerol were supplied. However, certain ^{14}C-labelled compounds detected in the control samples (notably proline) were only detected in the experimental samples supplied with ^{14}C-labelled glucose.

When the tentacle samples were incubated with two ^{14}C-labelled substrates simultaneously, the total uptake varied between 4.25 nmol mg^{-1} total protein and 5.21 nmol mg^{-1} total protein (Table 2). Incorporation of radioactivity into the principal fractions was very similar to single substrate incubations, with 99% of the radioactivity recovered from the TCA-soluble fraction. The pattern of incorporation that most closely resembled the control incubations with $NaH^{14}CO_3$ was obtained with glucose and either succinate or fumarate as substrates (Fig. 5).

The profile of labelled compounds in double substrate incubations (Table 4) was identical to the combined profile of compounds labelled in previous experiments using the same substrates applied singly (Table 3). For the incubations with glucose and succinate or glucose and fumarate, the radiolabelled compounds were very similar to those labelled in the control experiments incubated with $NaH^{14}CO_3$. The products of double substrate incubations that included glycerol were also similar to the results from the control experiments, but two of the most strongly labelled compounds, glycerol and an unknown amino acid, in these double substrate incubations were not detected in the control incubations.

In summary, the profile of metabolites in the animal tissues derived from exogenous glucose and succinate/fumarate, but no other exogenous compounds tested, was closely similar to the equivalent profiles derived from photosynthetically fixed compounds translocated from the algal cells. This comparison of metabolite profiles (see Fig. 1) suggests that glucose and a dicarboxylic acid (probably succinate and/or fumarate), or metabolically allied compounds, are important mobile photosynthetic compounds in the *Anemonia viridis–Symbiodinium* symbiosis.

Discussion

A fundamental feature of many symbiotic microorganisms is that they provide the host with nutrients that would otherwise enhance their own growth and proliferation rates. Our understanding of the processes mediating this co-operative trait of nutrient release is limited by uncertainty about the chemical identity of the mobile compounds. The metabolite comparison

Table 3. *Compounds identified in the trichloroacetic acid (TCA)-soluble organic fraction of the anemone tissue from* A. viridis *after single compound incubation*

		Intensity of radioactivity*						
Fraction	Compound	$NaH^{14}CO_3$	Malate	Citrate	Succinate	Fumarate	Glucose	Glycerol
Neutral	Fructose	–	–	–	–	–	+	–
	Glucose	++	–	–	–	–	+++	+
	Sucrose	–	–	–	–	–	+	+
	Glycerol	–	–	–	–	–	–	+++
Acidic	Glucose-6-phosphate	+++	+++	+	+++	+++	+++	++
	Unknown 1†	++	+	+	+	+	++	+
	Unknown 2†	+++	+	+	+	+	++	+
	Citrate	+	+	+++	+	+	–	–
	Fumarate	+	–	–	–	+	–	–
	Malate	+++	+++	+	++	+++	+	+
	Succinate	++	+	+	+++	+	–	–
Basic	Aspartate	+	++	–	++	++	–	–
	Glutamate	+	+	–	+	+	–	–
	Glutamine	+	+	+	+	+	–	–
	Lysine	+	–	–	–	–	++	+
	Proline	+	–	–	–	–	++	–
	Threonine	++	–	–	+++	+	–	+
	Valine	–	–	–	–	+	–	+
	Unknown 3†	–	–	–	–	–	–	+++

*Experiments were performed in the presence of 5 μmol l^{-1} dichlorophenyl dimethyl urea (DCMU). Compounds identified after incubation in $NaH^{14}CO_3$ (data from Table 1) included for comparison. Symbols as in Table 1.

†Unknowns 1 and 2 as in Table 1. Unknown 3 co-eluted with other amino acids but no positive identification could be made.

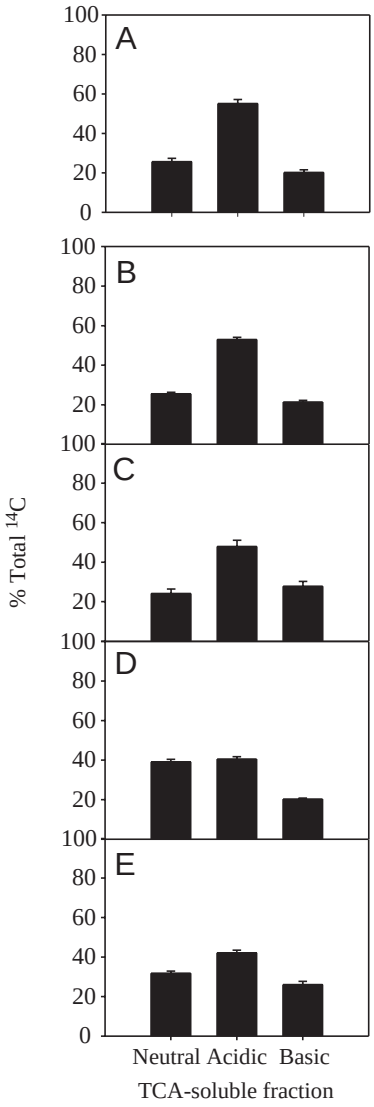

Fig. 5. Incorporation of ^{14}C into the trichloroacetic acid (TCA)-soluble fractions of *A. viridis* after incubation in (A) $NaH^{14}CO_3$, as in Fig. 3B, (B) [^{14}C]glucose + [^{14}C]succinate, (C) [^{14}C]glucose + [^{14}C]fumarate, (D) [^{14}C]glycerol + [^{14}C]succinate and (E) [^{14}C]glycerol + [^{14}C]fumarate. Experiments were performed over 5 min and, except in A, in the presence of 5 μmol l^{-1} dichlorophenyl dimethyl urea (DCMU). Data are expressed as a percentage of the total label in the TCA-soluble fraction. Bars (except in A) represent the means ± S.E.M. of 5–6 replicates.

method outlined in Fig. 1 contributes to the resolution of this limitation by identifying candidate mobile compounds on the basis of their pattern of metabolism by the host. The method assumes that animal metabolism of exogenous compounds taken up into ectodermal cells is equivalent to animal metabolism of photosynthetic compounds translocated across the symbiosomal membrane into endodermal cells. Consistent with this assumption, Trench (1971b) has shown that the pattern of metabolism of photosynthetic compounds is similar in the ectodermal and endodermal cell layers of the sea anemone *Anthopleura elegantissima*. The present study has identified glucose and succinate/fumarate as candidate mobile compounds in *Anemonia viridis* using short duration experiments. This probably does not represent the total complement of mobile photosynthetic compounds because other compounds, possibly including lipid and amino acids, may be translocated many minutes to hours after fixation (e.g. Battey and Patton, 1984; Wang and Douglas, 1999).

To our knowledge, the photosynthetic mobile compounds in the *A. viridis* symbiosis have not previously been identified. This may be because although a number of studies have demonstrated carbon translocation in the intact symbiosis (Stambler and Dubinsky, 1987; Davy et al., 1997), the algal cells isolated from *A. viridis* do not release photosynthetically fixed carbon, even in the presence of host homogenate (L. F. Whitehead and A. E. Douglas, unpublished results). The conclusion that glucose and succinate/fumarate are the likely photosynthetic mobile compounds in *A. viridis* is partly consistent with previous reports, in that these compounds have been cited as mobile compounds in a range of symbioses involving *Symbiodinium* (e.g. Trench, 1971a; Rees et al., 1993; Wang and Douglas, 1997). However, glycerol, which is accepted widely as a dominant mobile photosynthetic compound in many symbioses involving *Symbiodinium*, is apparently not translocated in the *A. viridis* system. If glycerol were mobile in *A. viridis*, it would be expected that ^{14}C-labelled glycerol would be detected in the animal fraction after incubation in $NaH^{14}CO_3$, because exogenous glycerol was not metabolized rapidly in the animal tissues [also noted by Ishikura et al. (1999) in tridacnid clams]. Contrary to this expectation, glycerol was not recovered as a radioactively labelled metabolite in the animal tissues in any of the many $NaH^{14}CO_3$ incubation experiments conducted here. This result is open to at least three alternative explanations. First, as suggested by the study of Ishikura et al. (1999), glycerol may not generally be an important mobile compound in symbiosis. Its dominance among the photosynthetic compounds released from isolated algal cells incubated with host homogenate may reflect a general shift in metabolism associated with the physiological responses either to isolation from the symbiosis or to the host homogenate (Goiran et al., 1997). Second, the symbiosis in *A. viridis* may be metabolically different from many symbioses, linked to its exclusively high latitude distribution (Manuel, 1988) or its genetically distinctive symbiotic algal partner (Savage et al., 2002). Finally, the identity of the mobile photosynthetic compounds may vary with environmental conditions. For example, Gattuso et al. (1993) concluded that glycerol is released from the algal cells to the animal tissues of the coral *Stylophora pistillata* only under high light conditions, whereas the experiments described here on *A. viridis* were conducted under relatively low light conditions. Further research is required to discriminate between these possibilities. For the present, we note that although glycerol is incorporated preferentially into lipid in other symbioses (Trench, 1971b; Schmitz and Kremer, 1977), we found no evidence for this in *A. viridis* and that the

Table 4. *Compounds identified in the trichloroacetic acid (TCA)-soluble organic fraction of the anemone tissue from* A. viridis *after double compound incubation*

		Intensity of radioactivity*				
Fraction	Compound	$NaH^{14}CO_3$	Succinate + glucose	Fumarate + glucose	Succinate + glycerol	Fumarate + glycerol
Neutral	Fructose	–	+	+	–	–
	Glucose	++	+++	+++	+	+
	Sucrose	–	+	+	+	+
	Glycerol	–	–	–	+++	+++
Acidic	Glucose 6-phosphate	+++	+++	+++	+++	+++
	Unknown 1†	++	+	+	+	+
	Unknown 2†	+++	++	++	+	+
	Citrate	+	+	+	+++	+
	Fumarate	+	–	+	–	–
	Malate	+++	++	+++	+	+++
	Succinate	++	+++	–	+++	+
Basic	Alanine	–	–	–	+	+
	Aspartate	+	++	++	++	++
	Glutamate	+	+	+	+	+
	Glutamine	+	+	+	+	+
	Lysine	+	++	++	–	–
	Proline	+	++	++	–	–
	Threonine	++	++	+	++	+
	Unknown 3†	–	–	–	+++	+++

*Experiments were performed in the presence of 5 μmol l^{-1} dichlorophenyl dimethyl urea (DCMU). Compounds identified after incubation in $NaH^{14}CO_3$ (data from Table 1) included for comparison. Symbols as in Table 1.

†Unknowns 1 and 2 as in Table 1. Unknown 3 co-eluted with other amino acids but no positive identification could be made.

generally lower lipid content of temperate symbioses, including *A. viridis*, compared with tropical species (Harland et al., 1991) is consistent with glycerol not being an important mobile photosynthetic compound in the *A. viridis* symbiosis.

Other data obtained in this study support the possibility that the fate of photosynthetic carbon in the *A. viridis* symbiosis may differ from that in low-latitude symbioses. In particular, photosynthetic carbon is preferentially incorporated into acidic compounds in the TCA-soluble fraction of the animal tissue in *A. viridis* (Fig. 3B), but very little radioactivity is recovered from this fraction in low-latitude symbioses, including *Zoanthus flos marinus* and *Condylactis gigantea* (von Holt and von Holt, 1968). A second distinctive feature of the data obtained here is the low incorporation of photosynthetically fixed carbon into the lipid fraction of both the algal cells (16%) and the animal tissue (3%), relative to other symbioses (35–90% and 20–60%, respectively; von Holt and von Holt, 1968; Trench, 1971b; Gattuso et al., 1993). This may reflect the low total lipid content of *A. viridis* compared with tropical anemones and corals (11% and 50% of dry mass, respectively; Harland et al., 1992), which has been hypothesised to result from the lower light and lower temperature in temperate environments (Harland et al., 1992; Muller-Parker and Davy, 2001). However, further research on the impact of season, light and temperature on the incorporation patterns of photosynthetic carbon is required to resolve this issue.

In conclusion, the metabolite comparison method developed here to study the identity of mobile photosynthetic compounds in symbioses involving *Symbiodinium* has potential value to investigate a range of issues that are currently not readily tractable to study. These include comparisons among symbioses from different habitats (e.g. temperate *versus* tropical) involving different algal genotypes and maintained under various environmental conditions, e.g. irradiance and temperature. With appropriate modifications, the approach may also contribute to the study of nutritional interactions in other symbioses between microorganisms and plants or animals, especially where, as with the *Symbiodinium* systems, alternative methods to study nutrient translocation in the intact association are not fully developed.

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