Carbon metabolism in the *Anemonia viridis* symbiosis

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Note

The work described in chapters 2 and 3 has been published in two papers in the journal "Marine Biology". These papers are bound in the back of the thesis

Abstract

This study was undertaken to investigate some aspects of carbon metabolism in the temperate symbiotic sea anemone *Anemonia viridis* (Forskäl). In particular, questions were asked about the nutritional interactions between the algal symbionts and host, and how these affect the lipid stores. Further questions addressed the quantitative importance of the carbon which is fixed in photosynthesis and translocated to the host. Are anemones potentially autotrophic with respect to carbon?

Anemonia viridis contained about 11% lipid on a dry weight basis when maintained at light levels of about 10μE m^{-2} s^{-1} and a temperature of 10°C. Aposymbiotic forms of the anemone had similar lipid levels. These values are very low when compared with tropical symbiotic anthozoa in which lipid levels are up to 50% of dry weight. In symbiotic *A. viridis*, less than 6% of total lipid consisted of the storage lipids, wax esters and triglycerides. Most of the triglyceride was stored in the animal tissues rather than the zooxanthellae. Zooxanthellae contained only small amounts of wax esters. An analysis was made of the triglyceride, wax ester and fatty acid composition of symbiotic anemones, isolated zooxanthellae and aposymbiotic anemones. Wax ester composition was similar in symbiotic and aposymbiotic forms. However, triglyceride composition differed. In particular trimyristin (C42) was found only within the symbiotic association. Fatty acids showed a high degree of unsaturation, and acids with both odd and even numbers of carbon atoms were found. The most abundant fatty acid was 16:0 in all samples except for the total lipids from zooxanthellae in which the major fatty acid was *trans* 18:1.

In order to examine the effect of light level on the total and storage lipids, anemones were exposed to three experimental light regimes of 10, 100 and 300 μE m^{-2} s^{-1}. Anemones were fed once a week. After 30 days there were no significant differences in the total lipid levels between anemones at any of the light intensities. However, after 60 days lipids had increased in proportion to light level in both the zooxanthellae and animal tissue compartments. The higher levels of total lipid were in part due to increases in storage lipid (wax esters and triglycerides). Wax ester levels increased in the animal tissues but remained constant in the zooxanthellae whereas triglycerides increased in both compartments. In contrast to fed anemones, starved anemones which were maintained at 300 μE m^{-2} s^{-1} for 30 or 60 days did not show a statistically significant change in lipid levels at 60 days. However, there was a significant increase in the storage lipids which suggested that the non-storage phospholipids and structural lipids had declined as a result of cellular catabolism. The composition of the wax esters and triglycerides of both fed and starved anemones was analysed and compositional changes were observed at higher light intensities.

Incorporation of ^{14}C by *Anemonia viridis* was measured. Anemones were adapted to 10 μE m^{-2} s^{-1} for two months and then incubated with $NaH^{14}CO_3$. In the TCA-soluble fraction (probably containing water-soluble compounds such as glycerol and glucose) and the whole-anemone compartment there was a significant increase in ^{14}C incorporation over time in both the light and the following dark period. This dark fixation was 25 times higher than that recorded during the dark equilibration period and was also higher than levels of dark fixation previously recorded for other anthozoans. *A. viridis* may have the capacity to fix $^{14}CO_2$ in the dark at the same rate as in the light, possibly by using pathways similar to those described for crassulacean acid metabolism. The total lipid fraction incorporated less ^{14}C than the TCA-soluble fraction and increases in total lipid ^{14}C over time were only observed in the light. Incorporation of fixed ^{14}C into the lipids of the zooxanthellae and host-tissue compartments was also measured and a significant time-dependent increase was observed in the light. In the following dark period, the level of ^{14}C in the zooxanthellae lipids remained constant while there was a significant decline in the labelled lipid in the host-tissues. These data suggested that either the recently fixed host ^{14}C was immediately metabolised in the dark or that there was back-flux of fixed ^{14}C from the host-tissues to the zooxanthellae during the dark period. The incorporation of ^{14}C into the lipid classes of whole-anemone tissue was measured and on average about 10% of the ^{14}C in the total lipid was found in the wax esters and about 36% in the triglycerides. The ^{14}C content of most lipid classes increased significantly in the light over time. No further increases were observed in the dark period. The release of fixed ^{14}C into the incubation medium was measured. On average, less than 1% of the total ^{14}C fixed was lost. It is suggested that as anemones would have contained low levels of lipid at the start of the experiment, any carbon fixed in excess of respiratory and growth requirements, would have been retained for the biosynthesis of both storage and other lipids.

About 60% of anemone total lipid was present in the body of the polyp (oral disc, body wall and mesenteries) and 40% in the tentacles. Storage lipids were found almost exclusively in the gastrodermal cell layer of the polyp and no storage lipid was found in the tentacles. Zooxanthellae were present in the gastrodermis of the tentacles, oral disc and body wall but were absent from mesenteries. There was evidence from macro-autoradiography that carbon was fixed by the zooxanthellae of the tentacles and translocated to the mesenteries. It was speculated that carbon compounds, such as lipid, could also be translocated to these tissues where excess lipid could be released from the polyp. "Mucus" samples were analysed for lipid content and were found to contain wax esters, triglycerides and structural lipid. Mucous gland cells were present throughout the epidermis but were less numerous in the mesenteries. However, as lipid was not stored in the epidermal cell layer, mucus-lipid may have been released from the mesenteries to become "bound" to the mucus secreted into the coelenteron. The mucus and mucus-lipid

would leave the polyp via the mouth.

A 24 h carbon budget was determined for *Anemonia viridis* under experimental conditions of 12 h light and 12 h dark. In the budget, it was assumed that the net amount of carbon fixed by the zooxanthellae during the 12 h light period would represent the carbon available to satisfy the respiratory demand during darkness and any carbon losses over 24 h. Net photosynthesis (Pnet) was estimated by measuring oxygen evolution in the light. Respiration (R) was estimated from measurements of oxygen consumption during darkness and losses of carbon were measured directly. It was originally intended to compare budgets for anemones adapted to 10 μE m^{-2} s^{-1} (low levels of stored lipid) and 300μE m^{-2} s^{-1} (high levels of stored lipid). Unfortunately, data for carbon losses in the low-light adapted anemones were lost and budgets were presented for high-light adapted anemones only. Rates of respiration were similar in both high- and low-light adapted anemones. However, low-light adapted anemones had significantly higher rates of Pnet than high-light adapted anemones when this was measured at either 10 or 300 μE m^{-2} s^{-1}. These differences suggested that low-light adapted anemones were photoadapted. Loss of organic carbon was measured in particulate and soluble fractions in the high-light adapted anemones. Particulate carbon was released continuously in the light and dark but loss of dissolved carbon appeared to be light-dependent. The carbon budget for the high-light adapted anemones demonstrated that at an irradiance of 300 μE m^{-2} s^{-1}, zooxanthellae could supply enough carbon for respiration and losses. However, when the same anemones were exposed to 10 μE m^{-2} s^{-1}, the respiratory demand for carbon and for losses exceeded the levels of carbon fixed resulting in a budget deficit. Potential sources of carbon to meet such a deficit are heterotrophic feeding and drawing upon lipid stores.

Chapter 1

The nutrition of symbiotic anemones

The temperate sea anemone *Anemonia viridis* (Forskäl) lives in association with the symbiotic dinoflagellate *Symbiodinium microadriaticum*. These dinoflagellates are unicellular algae and are commonly called zooxanthellae. Zooxanthellae can be found in all three classes of Cnidaria including many anemones and the tropical reef building corals. In anthozoans, they are intracellular and located in the gastrodermal cell layer of the host whilst in scyphozoans they may be located in the mesoglea (Russell 1970). Until recently, it was assumed that the single species, *S. microadriaticum,* was found in symbiotic association with all cnidarians but this has since been questioned by several authors, and it has been demonstrated that different strains or species of algae may be associated with different species of host (Schoenberg and Trench, 1980 a,b,c, Blank and Trench 1985).

By definition, mutualistic symbiosis requires that both partners in a symbiotic relationship derive some advantage from the association (see Smith and Douglas 1987). For symbiotic anthozoans, the host appears to benefit nutritionally from translocation of the products of algal photosynthesis (Muscatine and Cernichiari 1969). Conversely, the zooxanthellae benefit from the assimilation of host-derived organic compounds (Steen 1987). However, apart from algal photosynthesis, the nutritional requirements of symbiotic anemones may be satisfied in other ways. Predation, suspension feeding and the absorption of dissolved organic material may contribute to nutrition. These methods of feeding have been reviewed extensively by Van-Praet (1985) and Steen (1988) and will only be briefly mentioned.

Anemones are effective predators and their feeding behaviour has been described by Van-Praet (1985). Prey is detected by chemoreception and this initiates a pre-feeding response of oral disc expansion, tentacle waving and movement of the polyp column. These behaviours increase the likelihood of prey capture. When the prey touches the tentacles, nematocysts are discharged and the tentacles holding the prey are moved towards the mouth which opens. Ciliary currents in the pharynx are reversed in the direction of the coelenteron and the prey is ingested.

In suspension feeding, the ciliated ectodermal surfaces of the oral disc and pharynx generate currents which can direct particulate matter towards the mouth. Anemones often have cilia in other regions of the body such as the tentacles and body wall and these may also assist with the suspension feeding process (Van-Praet 1985).

Anemones can absorb dissolved organic material. Taylor (1974) showed that under experimental conditions, an aposymbiotic clone of *Aptaisia pallida* could absorb a range of organic compounds. *Anemonia sulcata* (= *viridis*) absorbed amino acids and glucose from

comparatively low ambient sea-water concentrations indicating active transport of these compounds (Schlichter 1975). It has also been shown that there may be separate transport mechanisms for different amino acids (Schlichter 1978). However, the importance of dissolved organic compounds to the nutritional requirements of symbiotic anemones is at present unresolved. For *Anemonia sulcata*, Schlichter (1975) estimated that anemones could obtain more than 50% of respiratory requirements from dissolved glucose in sea-water at a concentration of 90 $\mu g\ l^{-1}$. Schlichter (1982) measured the uptake by the soft coral *Heteroxenia fuscescens* of 12 amino acids and glucose which were dissolved in sea water at natural substrate concentrations and predicted that this could supply 80% of the coral's energy demand. However, it has been suggested that since these experiments were not carried out under axenic conditions, the uptake of dissolved organic material could have been by bacteria associated with surface mucus (Steen 1988).

Translocation of algal photosynthate

Direct evidence that symbiotic algae contribute to the nutrition of their coelenterate host was first provided by Muscatine and Hand (1958). Using the technique of autoradiography, these authors showed that photosynthetically fixed ^{14}C was translocated from the zooxanthellae to the host tissues of the anemone *Anthopleura elegantissima*. Similar results were observed by Taylor (1969) for *Anemonia sulcata*.

Biochemical form of translocated carbon

To identify the photosynthetic products which were translocated by the zooxanthellae, Muscatine (1967) isolated the algal cells from the clam *Tridacna crocea* and the reef coral *Pocillopora damicornis*. Zooxanthellae were suspended in homogenised host tissues and incubated in the light with ^{14}C bicarbonate. Analysis of the medium by two-dimensional thin-layer chromatography and autoradiography showed that most of the labelled carbon was in the glycerol fraction with a small amount in glucose.

Von Holt and Von Holt (1968) examined ^{14}C uptake in whole symbiotic anemones, corals and zoanthids. After exposure to labelled sea water in the light, specimens were homogenised and divided into zooxanthellae and host tissue fractions. The biochemical composition of both fractions was determined and compounds were analysed for incorporation of radioactive label. In both zooxanthellae and host compartments, the highest levels of ^{14}C were found in lipid although other compounds such as amino acids and carbohydrates were also labelled.

Muscatine and Cernichiari (1969) examined the the uptake of ^{14}C by the reef coral *Pocillopora damicornis* to determine the nature of the compounds translocated from the

zooxanthellae to host. Using an experimental protocol similar to that of Von Holt and Von Holt (1968), intact corals or freshly isolated zooxanthellae *in vitro* were exposed to labelled bicarbonate. Qualitative analysis of the biochemical fractions revealed a large labelled lipid component and because Muscatine (1967) had shown that glycerol was released from zooxanthellae *in vitro*, the host lipid fraction was deacylated to isolate the lipid glycerol component. Label was found exclusively in this fraction and it was suggested that this was a good indication that glycerol or its derivatives were released by the algae and then converted to lipid or other compounds in the host. Release and incorporation of glycerol into host lipid were also observed when freshly isolated zooxanthellae were incubated with host homogenate *in vitro*.

Trench (1971 a), used the same separation procedures as Muscatine and Cernichiari (1969) to examine ^{14}C translocation in the anemone *Anthopleura elegantissima* and the zoanthid *Palythoa townsleyi*. It was found that when the host lipids were deacylated and the products separated by TLC, the only radioactive saponification product was glycerol. Two related studies also showed that a range of low molecular weight water soluble compounds, including glycerol and glucose, were released by isolated zooxanthellae *in vitro* (Trench 1971 b, c) again suggesting that glycerol was probably the main translocated product of photosynthesis.

Using an "inhibition" technique to determine the nature of the translocated material, Lewis and Smith (1971) observed that glycerol, glucose and alanine were released by zooxanthellae *in vivo* and *in vitro* in a range of symbiotic coelenterates. These authors incubated their preparations in labelled bicarbonate in the presence of non-radioactive glycerol (or glucose or alanine) in order to saturate the metabolic machinery with these compounds. Labelled compounds translocated from the zooxanthellae then exchanged with the unlabelled form and accumulated in the incubation medium. If the assumption is correct that the substrate does overload the system, then this study provides good evidence that glycerol, glucose and alanine are translocated.

Patton et al. (1977) examined the rates of lipid biosynthesis from ^{14}C-labelled acetate in the coral *Pocillopora capitata*. It was found that biosynthesis in whole coral tissue was three times higher in the light than in the dark and it was suggested that this indicated that the majority of lipid was produced by the zooxanthellae. However, this result could also be explained if carbon was fixed by the zooxanthellae and translocated to the host for lipid biosynthesis. It was shown that host tissue and zooxanthellae synthesised similar triglyceride fatty acids from ^{14}C-labelled acetate and it was suggested that the unlabelled fatty acids found by Muscatine and Cernichiari (1969) and Trench (1971 a) could be explained by acetate recycling if the fatty acid precursors had come from non-photosynthetically fixed (and therefore unlabelled) carbon in the host tissues. It was suggested that zooxanthellae would take up acetate derived from host metabolism and

convert it to fatty acids which would then be translocated to the host and incorporated into wax esters and triglycerides.

The theory of acetate recycling and lipid translocation gained support from Blanquet et al. (1979) who examined the uptake of labelled acetate by *Anthopleura elegantissima*. In the light, label was incorporated into the fatty acids of both the host and zooxanthellae and it was suggested that fatty acids were translocated. Lipid translocation was also proposed by Crossland et al. (1980 b). These authors observed droplets, which were thought to contain lipid, in electron micrographs of zooxanthellae from *Acropora acuminata*. It was suggested that these droplets could be transported to the host tissues.

Patton et al. (1983), attempted to resolve the question of whether glycerol or lipid was translocated by comparing the metabolism of both labelled acetate and bicarbonate in the coral *Stylophora pistillata*. Patterns of incorporation were similar for both precursors. When lipid from whole coral tissue was deacylated, the majority of fixed ^{14}C was recovered in fatty acids and fatty alcohols. It was suggested that fixed carbon was immediately converted into lipid in the zooxanthellae before translocation to the host. Furthermore, as labelled bicarbonate could be incorporated into fatty acids it was suggested that photosynthetically fixed CO_2 would be the main source of host lipid carbon and that it was unneccessary to propose the acetate-recycling scheme (Patton et al. 1977).

The conclusions reached by Muscatine and Cernichiari (1969) and Trench (1971 a) were criticised by Patton et al. (1983) who suggested that these authors had misinterpreted their data. Muscatine and Cernichiari (1969) used TLC to separate the deacylated lipid samples. Only two radioactive components were detected on the TLC plate. One was "judged" to be a portion of the original lipid which had not been deacylated, the other was confirmed as glycerol. Patton et al. (1983) suggested that the original lipid spot was actually labelled fatty acids or fatty alcohols and other authors have subsequently agreed with this interpretation (Kellog and Patton 1983). It was therefore likely that both the glycerol and fatty acids in the host lipid had been labelled with ^{14}C.

Indirect evidence for the translocation of lipid has also been provided by Patton and Burris (1983). Droplets surrounding freshly isolated zooxanthellae *in vitro* from *Stylophora pistillata* were analysed and found to contain wax esters and triglycerides. Kellog and Patton (1983) isolated lipid droplets from *Condylactis gigantea* by homogenising anemone tissues and collecting the droplets from the surface of the homogenate. It was suggested that the droplets had originated from the zooxanthellae and when analysed, they were found to contain mainly wax ester. After exposing *C. gigantea* to ^{14}C-labelled acetate in the light and then isolating the zooxanthellae and their associated lipid droplets, the specific activity was measured in different lipid classes. It was found that the free fatty acids (which comprised only 4% of the total lipid) of the zooxanthellae had the highest specific activity after 1 h. With 2 h incubation periods, the triglyceride specific activity increased. A

similar labelling pattern was observed in the extra-algal droplets and it was suggested that the zooxanthellae fatty acids were forming a pool of lipid precursors. It was also shown that in both the zooxanthellae and the lipid droplets, over 80% of the ^{14}C was in the fatty acids of triglyceride molecules. Based upon these observations, it was suggested that either triglycerides or unesterified fatty acids may have been secreted by the zooxanthellae. However, as isolated lipid droplets were also observed to incorporate ^{14}C-acetate from the incubation medium in the dark and because exocytosis of lipid droplets was not observed, it was impossible to state with any certainty that the lipid in the droplets had originated from the zooxanthellae.

In direct contrast to the results of experiments carried out by Patton et al. (1983), Schlichter et al. (1983), working with the soft coral *Heteroxenia fuscescens*, showed that most of the ^{14}C from labelled bicarbonate was found in the glycerol component of host lipids. It was suggested that glycerol was the main product transferred and this was then rapidly converted into lipid. However, when Schlichter et al. (1984) used labelled acetate to test the hypothesis that zooxanthellae utilise host derived carbon for lipid synthesis (Patton et al. 1977), it was shown that upon deacylation of host lipid, both fatty acids and glycerol were labelled with the majority of the label in the lipid.

In order to finally resolve the nature of the translocated photosynthate, Battey and Patton (1984) carried out experiments using intact tentacles which had been excised from *Condylactis gigantea* and incubated with three different ^{14}C-labelled precursors. Labelled bicarbonate was used because the original glycerol translocation hypothesis was formulated in studies using this tracer, labelled acetate because this was used in experiments in which it was proposed that zooxanthellae translocated lipid, and labelled glycerol was used to investigate the metabolism of this compound in zooxanthellae and host tissues. After exposure to labelled bicarbonate or acetate in the light for 90 minutes, 80% of the ^{14}C was found in host and zooxanthellae triglyceride fatty acids. However, ^{14}C from labelled glycerol was not incorporated into the fatty acids of zooxanthellae triglycerides in the dark and only 37% of the label appeared in these fatty acids after 90 minutes in the light. Very little glycerol was incorporated into the lipid fraction and less than 5% of the triglyceride fatty acids were labelled. It was suggested that neither the host nor the zooxanthellae could rapidly convert glycerol to fatty acid. However, the host wax esters only accumulated label from exogenously supplied labelled glycerol in the light (wax esters do not contain esterified glycerol and therefore could only have incorporated glycerol carbon atoms as fatty acids or fatty alcohols). To explain the increases in ^{14}C in the zooxanthellae triglyceride fatty acids and host wax esters in the light, it was proposed that the glycerol had first been oxidised by the host and that labelled CO_2 had been re-fixed in the zooxanthellae which then transported this carbon back to the host. It was also suggested that a similar situation might exist under natural conditions if glycerol was translocated

from the zooxanthellae to the host where it would be used for respiration with the respiratory CO_2 being refixed by the zooxanthellae. Battey and Patton (1984) cited Lewis and Smith (1971) and Taylor (1974) to support this hypothesis as these authors had observed rapid oxidation of exogenously supplied glycerol to CO_2 in aposymbiotic anemones and corals in the dark.

Battey and Patton (1987) investigated the hypothesis that both glycerol and lipid are transferred from zooxanthellae to host tissues. Tentacles of *Condylactis gigantea* were incubated with ^{14}C bicarbonate in the presence of sodium cyanide (NaCN) to inhibit respiration but only partially inhibit photosynthesis. NaCN at a concentration of 10^{-5} M resulted in a decrease of 34% in respiration but only a 10% decrease in net photosynthesis, therefore potentially increasing both carbon translocation from the zooxanthellae and accumulation of carbon by the host. In controls without cyanide, 2% of the total fixed carbon was recovered in the glycerol of intact tentacles after 1 h incubation with labelled bicarbonate. With the addition of 10^{-5} M NaCN, this figure increased to about 25% suggesting that under normal circumstances glycerol was catabolised to CO_2. However, there may be an alternative explanation for the observed increase in glycerol. Cyanide inhibits respiration by binding to the terminal cytochrome in the electron transport system, blocking the donation of electrons to oxygen and rendering the organism "functionally" anaerobic. Glucose would then be converted to lactate by anaerobic glycolysis (Fruton and Simmonds 1958), but there are other possible pathways of anaerobic degradation of glucose among which is the conversion of glucose to glycerol and pyruvate. This requires the presence of glycerol phosphate dehydrogenase and a specific or non-specific glycerol phosphate phosphatase and these enzyme activities do exist in animal cells (Fruton and Simmonds 1958). Nevertheless, from their own interpretation of the results of these experiments, Battey and Patton (1987) proposed a scheme by which translocated glycerol is used as a substrate for host respiration while any excess glycerol and translocated lipid would be accumulated in host lipid stores.

Although it appears that both glycerol and lipids may be translocated by zooxanthellae, the problems of positively identifying the translocated carbon compounds in the intact symbiotic association remain and may only be resolved with the application of new experimental approaches and analytical techniques.

Contribution of zooxanthellae to host nutrition

Symbiotic algae can translocate a substantial proportion of the carbon they fix in photosynthesis to the host. The quantitative importance of this carbon to both host and algae has been assessed and carbon budgets for symbiotic anemones and corals constructed.

In earlier studies, anthozoans were incubated in ^{14}C for short periods and the relative

amount of label in the zooxanthellae and host tissues determined. It was shown that up to 60% of fixed carbon may be translocated to host tisssues (Muscatine 1967, Muscatine and Cernichiari 1969, Taylor 1969, Trench 1971 a). However, it is not clear whether ^{14}C fixation by zooxanthellae approximates net or gross photosynthesis since translocated ^{14}C may be respired to $^{14}CO_2$ and then refixed by the zooxanthellae. Furthermore, any ^{14}C which has passed from the tissues into the incubation medium would not have been measured when analysing the ^{14}C content of host tissue (Crossland et al. 1980 a, b). Muscatine et al. (1981) advised caution when interpreting data from tracer experiments because the uptake of labelled and stable carbon may not be equal and also because unlabelled organic compounds may be translocated from zooxanthellae during the incubation period.

A second method for estimating the amount of carbon which the zooxanthellae supply to the symbiotic association is to measure the rates of photosynthesis as oxygen evolution and respiration as oxygen consumption. Gross photosynthesis (production) (Pgross) and respiration (R) can be expressed as a ratio and Pgross/R $>$ 1.0 has been interpreted to mean that more carbon is fixed in photosynthesis than is required for the basic metabolism of the association (McCloskey et al. 1978). Davies (1977), from measurements of photosynthesis and respiration, predicted that in shallow-water corals, zooxanthellae could fix enough carbon to meet the entire carbon demand of the colonies over a 24 h period. These corals would therefore be autotrophic with respect to carbon. In this study, the assumption was made that all fixed carbon that was not required by the zooxanthellae was transferred to the host.

Carbon budgets

Muscatine et al. (1981) developed equations to determine the contribution of zooxanthellae to animal respiration (CZAR) which could be applied to any algal-coelenterate symbiosis. In the corals *Pocillopora damicornis* and *Fungia scutaria*, gross photosynthesis and respiration were measured, the rates of carbon release from freshly isolated zooxanthellae measured *in vitro* and an estimate (based upon whole-animal respiration and the biomass ratios of algae and animal tissues) made of zooxanthellae respiration. It was assumed that *in vitro* carbon translocation was the same as translocation *in vivo* and also that algal and animal respiration rates were similar. CZAR for the two species of coral was estimated to be between 63 and 69% with the remainder of the respiratory carbon requirement presumably coming from heterotrophic sources. These results contradicted the work of Davies (1977) in which it had been shown that symbiotic corals were capable of complete carbon autotrophy.

Davies (1984) constructed an energy budget for the coral *Pocillopora eydouxi* and the

principles which were established in this study apply equally well to a total carbon budget. The energy budget was partitioned between zooxanthellae and host. In the zooxanthellae, carbon input from photosynthesis was used for respiration, growth and algal cell losses with the excess being translocated to the animal tissues. This translocated carbon, along with carbon from exogenous sources, supplied animal respiration, growth and losses. It was estimated that more than 90% of the energy from photosynthesis was translocated from the zooxanthellae. Although the value for losses was not measured but determined by subtraction, Davies (1984) predicted that for corals living at 5 m depth on a cloudless day and over a 24 h period, 51% of photosynthetically fixed energy was used in respiration, 0.9% in growth and 48% was unaccounted for and presumed lost from the colony. When this method was applied to the reef coral *Porites porites*, similar results were obtained (Edmunds and Davies 1986).

Muscatine et al. (1984) developed methods for quantifying the daily flux of carbon using CZAR and four different procedures for estimating ^{14}C translocation. Firstly, translocation of carbon was measured in freshly isolated zooxanthellae *in vitro*. Algae were labelled and translocation expressed as the % of the total fixed ^{14}C recovered in the incubation medium after 1 h in the light. Secondly, the intact association was incubated with ^{14}C and the amount of fixed carbon determined in the algal and host compartments. The third procedure was based upon estimating the amount of ^{14}C remaining in the algae after 24 h, rather than measuring the amount of label appearing in the incubation medium or host tissues. The final procedure was based upon estimating growth rates of the algal population. This method required direct measurement of the standing stock of zooxanthellae carbon and the amount of carbon added each day from photosynthesis. Algal cell division rates were used to estimate the total net daily carbon increment to the zooxanthellae population. The proportion of fixed carbon retained by the zooxanthellae for new cells was therefore obtained and by subtraction, the amount of carbon translocated. Although this method does not account for zooxanthellae respiration it was favoured by Muscatine et al. (1984) as the preferable procedure for estimating carbon translocation because the use of radioactive tracers is not required. It was shown that in the coral *Stylophora pistillata*, the amount of carbon needed for algal growth was extremely small and a prediction was made that 97% of fixed carbon could be translocated.

High rates of carbon translocation have also been predicted for symbiotic anemones. From measurements of photosynthesis and respiration, Tytler and Davies (1984) estimated that under high light, *Anemonia sulcata* could supply all the energy requirements of the symbiotic association from zooxanthellae photosynthesis. Stambler and Dubinsky (1987) adopted the methods of Muscatine et al. (1981) and the *in vivo* technique of Muscatine et al. (1984) to calculate carbon translocation in *A. viridis* and it was estimated that up to 70% of the carbon fixed by zooxanthellae may be translocated.

Carbon may be translocated in excess of the requirements of the symbiotic association (Davies 1984, Muscatine et al. 1984, Porter et al. 1984) and it has been predicted that excess carbon will be excreted. Cooksey and Cooksey (1972) measured the turnover of labelled carbon in the corals *Siderastrea siderea* and *Montastrea annularis*. It was observed that there was a 50% loss of ^{14}C over about 20 h. Similarly, Crossland et al. (1980 a) showed that a substantial fraction of fixed carbon was rapidly lost from tissues of *Acropora formosa*. Corals were incubated in labelled bicarbonate for 2 h periods and then branches were removed and analysed for ^{14}C content over 5 days. Up to 60% of the ^{14}C fixed in photosynthesis was lost during the first 40 h. This estimate was similar to that of Davies (1984) for *Pocillopora eydouxi* and of Muscatine et al. (1984) for shade adapted *Stylophora pistilla*. However, for light adapted *S. pistillata*, it was predicted that only 6% of fixed carbon would be lost.

Carbon may be lost from the symbiotic association as mucus and lipid (Crossland et al. 1980 a, b, Crossland 1987)[1]. Crossland et al. (1980) examined the uptake and excretion of ^{14}C in the coral *Acropora acuminata*. Carbon was excreted as both mucus and mucus-lipid. There was a diurnal pattern of mucus-lipid excretion with the majority being released during the day. Mucus was secreted continuously over the 24 h period. In the excreted mucus-lipid, ^{14}C was detected in different lipid classes with most being present in the wax esters and triglycerides. Crossland (1987) examined mucus and dissolved organic carbon (DOC)-lipid release over 24 h in *Acropora variabilis* and *Stylophora pistillata*. It was shown that most of the mucus and the DOC-lipid was released by corals during the day. In this study, DOC-lipids were mainly composed of wax esters and structural lipids.

Most of the carbon translocated from zooxanthellae to host tissues has been shown to accumulate in the lipid fraction of the host (Muscatine and Cernichiari 1969, Battey and Patton 1984). Symbiotic coelenterates often contain large amounts of lipid (Bergmann et al. 1956, Patton et al. 1977, Blanquet et al. 1979, Kellog and Patton 1983, Stimson 1987) and a high proportion of this may consist of the storage lipids wax esters and triglycerides (Patton et al. 1977). Production of lipid is light-dependent (Patton et al. 1977) and it has been suggested that the storage lipid acts as an energy reserve which can be drawn upon at times when the symbiotic association can not fix enough carbon to satisfy respiration and growth (Davies 1991). Davies (1991) predicted that this situation would occur on heavily overcast days when photosynthesis could not supply the energy demands of corals and that

[1]There are different interpretations of the generic term "mucus". Crossland et al. (1980 b) used *mucus* for secreted mucus-polysaccharide and *mucus-lipid* for the lipid associated with this mucus-polysaccharide. Crossland (1987) defined *mucus* as organic material adsorbed to celite particles and *dissolved organic carbon-lipid* (DOC-lipid) as the lipid associated with the DOC released. The term "*mucus*" is used in the present thesis to describe all particulate and DOC (P/DOC) released from *Anemonia viridis*. The term *mucus* is used for the mucus-polysaccharide component of P/DOC and *mucus-lipid* is the lipid associated with P/DOC.

any deficit would be met by drawing upon lipid stores. It was also suggested that on cloudless days when zooxanthellae fixed more energy than was required for respiration, growth and storage, corals would have to excrete excess energy and that this could be in the form of mucus-lipid.

A theoretical model showing the main pathways for photosynthetically fixed carbon in a symbiotic anthozoan is shown in figure 1.1. Carbon dioxide is fixed by the zooxanthellae and any carbon in excess of zooxanthellae requirements is transferred to the host where it may appear as lipid, glycerol and glucose. This carbon can be used for host respiration, growth, mucus production and storage as lipid. Carbon is excreted as mucus and mucus-lipid.

In the present thesis, the following questions were addressed:

1) Does the lipid composition of the zooxanthellae and host tissues of *Anemonia viridis* provide evidence for the movement of lipid between the symbiotic partners?

2) Is the total lipid content and the level of storage lipids dependent upon the rate of zooxanthellae photosynthesis? If light levels are increased, will the lipid content also increase?

3) What is the pattern of fixed carbon incorporation into the lipids of the zooxanthellae and host tissues in the light? Will this pattern differ in the dark? If comparisons are made between incorporation of fixed carbon into the TCA-soluble (eg. glycerol) and lipid fractions of whole anemone tissue, will this give some indication of the form in which carbon is translocated from the zooxanthellae to the host?

4) Where are the lipid stores located in anemones? Are zooxanthellae located in the same tissues? If not, is carbon or lipid translocated within the anemone polyp? Is lipid released from anemones and what are the possible pathways of excretion?

5) How much carbon is translocated from the zooxanthellae to the host tissues of *Anemonia viridis* and what proportions of this are respired and lost from the symbiotic association?

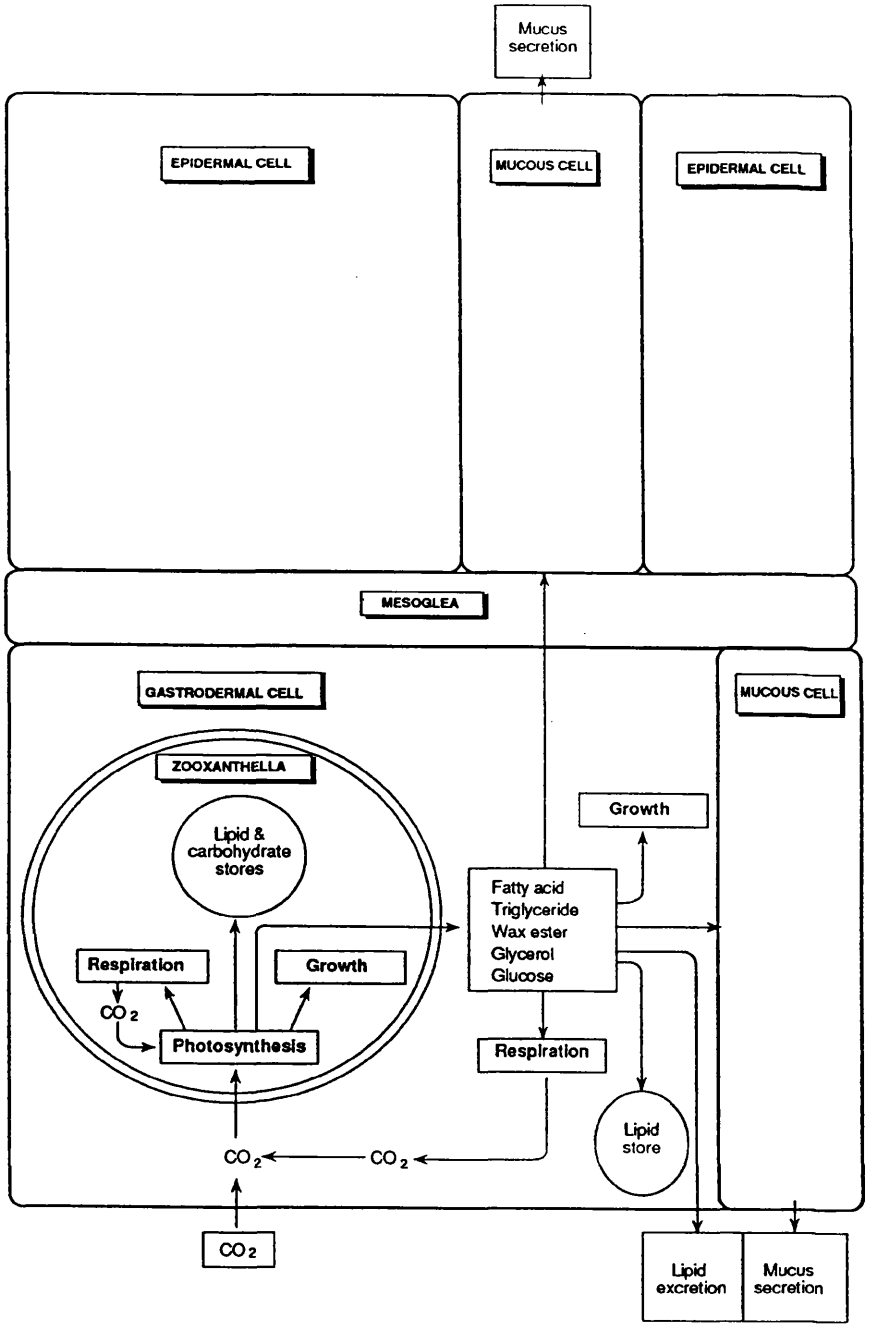


Figure 1.1. A theoretical model showing carbon flux in an anthozoan symbiosis. In this model, carbon dioxide is fixed by the zooxanthellae during photosynthesis. Carbon can then be used for zooxanthellae respiration, growth and the storage of lipid and carbohydrate. Carbon in excess of zooxanthellae requirements is translocated to the host where it appears as lipid, glycerol and glucose. The exact form in which the carbon crosses the algal and anemone vacuolar membranes is not known. However, there is evidence for the translocation of triglycerides, fatty acids, glycerol and other water soluble compounds such as glucose. Host carbon is used in respiration, growth, the provision of mucus and for storage as lipid. Carbon is lost from the symbiotic association as mucus and mucus-lipid which may be excreted from the gastrodermal cell layer. This model is not meant to imply that all routes of carbon flux are known but those shown are consistent with current knowledge.

Chapter 2

Distribution of lipids between the zooxanthellae and animal compartment in the symbiotic sea anemone *Anemonia viridis*: wax esters, triglycerides and fatty acids

Introduction

Symbiotic sea anemones and corals are rich in lipids typically containing large amounts of wax esters and triglycerides (Bergmann et al. 1956, Patton et al. 1977, Blanquet et al. 1979). Many are efficient carnivores and may derive some lipid from the diet. However, the accumulation of large amounts of triglyceride and wax esters probably results mainly from the release of photosynthetically fixed carbon by the algal symbionts (zooxanthellae) into the host tissues. This fixed carbon may be translocated both as glycerol (Muscatine and Cernichiari 1969, Trench 1971a, b, Battey and Patton 1984, 1987) and as lipids (Patton et al. 1977, Kellog and Patton 1983, Patton and Burris 1983, Battey and Patton 1984). The extent to which each of these products contributes to host lipid stores is unclear.

Catabolism of wax esters and triglycerides can provide energy when food intake is reduced. In corals which show little or no evidence of heterotrophic nutrition (Edmunds and Davies 1986) the lipid stores are probably called upon to supply the energy requirements for respiration and growth on cloudy days when light for photosynthesis is limiting (Davies 1991).

In this chapter, the nature of the stored lipids of the temperate symbiotic anemone *Anemonia viridis* is examined and the distribution of lipids between the zooxanthellae and the animal tissues is reported.

Materials and methods

Experimental anemones

Specimens of *Anemonia viridis* were collected from the Isle of Cumbrae, Scotland, in 1989, and were maintained in a recirculating filtered sea-water system at the Zoology Department, University of Glasgow, at about 10°C with a 12h light:12h dark regime. Daylight fluorescent tubes provided a maximum of 10 μE m^{-2} s^{-1} at the water surface measured with a Skye SKP200 quantum light recorder and SKP215 sensor (Skye Instruments Ltd.). Aposymbiont anemones were obtained by treating symbiotic anemones with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU)(Schoenberg and Trench 1980 a).

Anemones were fed weekly with chopped mussels (*Mytilus edulis*) which were stored frozen (-20°C) until used.

Lipids were extracted from the freeze-dried tissues of five symbiotic anemones and their freshly isolated zooxanthellae, five aposymbiotic anemones and the soft tissues of three mussels. Symbiotic anemones were buoyant weighed (Tytler and Davies 1984), cut in half vertically, and each portion re-weighed. One half, which was to be used for the analysis of the lipids of both host and zooxanthellae, was immediately freeze dried. The zooxanthellae from the other half were extracted from anemone tissue by a procedure based upon the homogenisation and washing technique of Davies (1988) (figure 2.1.) before freeze drying. The dry weight of zooxanthellae in whole anemones was calculated from the buoyant weight measurements.

Extraction of lipids

Freeze-dried samples of anemone, zooxanthellae and mussels were weighed and then homogenised with an Ultra-Turrax blender in 5 ml of distilled water. Lipids were extracted as described by Folch et al. (1957). All solvents were Analar grade (BDH Chemicals Ltd.). One hundred ml of chloroform:methanol (2:1, v/v) was added to each homogenate and the extract was filtered through a Whatman number 1 filter paper which was then rinsed with a further 10 ml chloroform:methanol mixture. To the filtered extract, one-fifth the total solvent volume of 0.88% KCl in distilled water was added. The upper aqueous phase was removed by aspiration and the remaining organic phase washed three times with 33 ml methanol:water (1:1, v/v). The organic solvent was evaporated to dryness *in vacuo* under N_2 at 39°C and the lipid re-dissolved in a small amount of chloroform which was then filtered (sintered glass filter, porosity grade 3). Filters were washed five times with about 2 ml chloroform to ensure complete recovery of lipid and the lipid-containing solution was stored at -20°C.

The lipid extracts were evaporated to dryness under N_2 at 39°C, and re-dissolved in 2 ml chloroform. Total lipid was determined gravimetrically using a Sartorius Supermicro balance after drying an aliquot of this solution to constant weight. Wax esters and triglycerides were separated from other components of the remaining lipid extract by preparative thin-layer chromatography (TLC) on silica gel H60 plates using two solvents. Plates were developed first in petroleum spirit (bp = 40 to 60°C):diethyl ether:formic acid (150:50:1 by vol.) until the solvent front had moved half-way up the plate. After drying, the plate was re-developed in toluene until the solvent front reached the top of the plate. Areas containing the lipids were visualised by spraying the plates with 0.1% 2',7'-

dichlorofluorescein (Sigma Chemical Co. Ltd.) in ethanol. The lipids were identified by comparison with the R_f's of standard wax esters and triglycerides. Areas of silica containing the two lipids were transferred seperately to sintered glass filters (porosity grade 3) and the lipids were eluted with chloroform. After evaporation of the solvent under N_2, the purified lipids were dissolved in a small volume of toluene for gas-liquid chromatography (GLC) analysis. Methyl esters of fatty acids were prepared from lipid samples by acid-catalysed transesterification (see Christie 1987).

Analysis of wax esters, triglycerides and fatty acids

Wax Esters

Gas chromatography analysis of wax esters was carried out using a Perkin-Elmer 8420 gas chromatograph fitted with a 12 m x 0.25 mm SGE WCOT column (Scientific Glass Engineering Ltd.) with SGE BP-100 as the stationary phase. The column temperature was programmed at 5C° min^{-1} from 170°C to 300°C and the carrier gas (N_2) flow rate was 1 ml min^{-1}.

Triglycerides

For triglyceride analysis, the gas chromatograph was a Perkin-Elmer 8500 fitted with a Heliflex 4.5 m x 0.25 mm column (Alltech Ltd.) with an RSL-150 stationary phase. The temperature gradient was 240°C to 340°C at 10C° min^{-1} with an isothermal hold at 340°C for 10 min. The carrier gas was helium at a flow rate of 2.5 ml min^{-1}.

Fatty Acids

Fatty acid methyl esters were analysed with the Perkin Elmer 8420 gas chromatograph using a 30 m x 0.25 mm Supelco SPB^{TM}-1 column with SPB-1 stationary phase (Supelco Ltd.), held isothermally at 190°C for 33 min and then increased to 250°C at 15C° min^{-1}, with a final hold of 13 min. The carrier gas (N_2) flow rate was 0.9 ml min^{-1}.

Wax esters, triglycerides and fatty acid methyl esters were identified by comparing retention times to those of standards (Sigma Chemical Co. Ltd., Supelco Ltd.). Identification was confirmed in most cases by gas chromatography-mass spectrometry (GC-MS) with a VG Analytical model 70-250 S and Hewlett Packard model 5890 A gas chromatograph fitted with the Heliflex triglyceride column for triglyceride analysis or a Chrompack CP-SIL 5CB 25 m x 0.32 mm column (Chrompack Ltd.) for the analysis of wax esters and fatty acid methyl esters. The MS source temperature was 240°C and electron ionization potential (EI) 35eV.

Known quantities of hexadecanoyl heptadecanoate (C33:0) and tripentadecanoyl glycerol (C45 - refers to the total number of carbon atoms in the fatty acids) (Sigma Chemical Co. Ltd.) were added to samples prior to TLC separation to facilitate quantitation of the wax esters and triglycerides respectively. These standards were selected since preliminary studies had shown that C33:0 and C45 were not present in lipid samples. Peak areas of individual compounds were measured in relation to the peak area of the internal standards. To control for differences in detector response etc. for the higher molecular weight triglycerides, known quantities of standard triglycerides of C42, C48, C54, C60 and C66 (Sigma Chemical Co. Ltd.) were chromatogrammed and the detector response for each relative to C45 was determined. Statistical procedures were carried out on arcsine transformed data (Sokal and Rohlf 1981).

Results

Examples of TLC separation of lipids are shown in figure 2.2, a GLC trace in figure 2.3. and a GC-MS trace in figure 2.4.

Lipid Content

There was no significant difference in the total lipid content of symbiotic and aposymbiotic anemones (Student's *t*-test, $P>0.05$), both values being approximately 11% of tissue dry weight. However, in the symbiotic anemone this lipid is divided into that within the zooxanthellae and that in the animal tissues. Further comparisons were therefore made between the lipid content of the animal tissues and that in aposymbionts and also between the animal tissues of symbiotic anemones and the zooxanthellae (Table 2.1.).

Symbiotic anemones contained 12.5% ($\pm$3.38 SD) zooxanthellae on a dry weight basis. The zooxanthellae contained approximately 25% of their dry weight as lipid which was about twice that of the animal tissues. The lipid content of the animal tissues at 9.86% of dry weight was similar to the 10.7% of dry weight in the aposymbiont (Student's *t*-test, $P>0.05$).

There was no significant difference in the percentage of triglycerides in the lipids of zooxanthellae and in the animal tissues (Student's *t*-test, $P>0.05$). However, aposymbionts contained significantly less triglyceride than the animal compartment of the symbiotic anemones (*t*-test, $P<0.05$). Wax esters represented 0.33% of zooxanthellae lipids which was significantly different from the wax ester content of the animal tissues which was

2.29% (*t*-test, $P<0.05$). There was no significant difference in the percentage of wax esters of aposymbiotic and symbiotic anemone lipids (*t*-test, $P>0.05$).

Table 2.1. *Anemonia viridis*. Lipid composition of symbiotic and aposymbiotic anemone tissue and freshly isolated zooxanthellae. Values for symbiotic animal tissue were derived by subtraction of zoothanthellae values from those of symbiotic anemones. Standard deviations are shown in parentheses; $n = 5$.

	Symbiotic anemone			Aposymbiotic anemone
	Whole anemone	Isolated zooxanthellae	Animal tissue	
Dry wt. (mg)	324.4 (80.3)	40.6 (10.03)	283.8 (70.23)	87.4 (16.2)
Total lipid (mg)	37.9 (9.13)	9.91 (3.03)	27.9 (10.29)	9.31 (1.83)
% dry wt.	11.7 (1.83)	24.41 (10.73)	9.86 (2.06)	10.7 (0.41)
Triglyceride (mg)	1.54 (0.80)	0.33 (1.16)	1.21 (0.66)	0.07 (0.04)
% total lipid	4.06 (2.08)	3.33 (1.16)	4.32 (2.65)	0.75 (0.44)
Wax ester (mg)	0.67 (0.37)	0.03 (0.02)	0.64 (0.36)	0.11 (0.05)
% total lipid	1.77 (0.91)	0.33 (0.15)	2.29 (1.32)	1.18 (0.81)

Summing the values for the wax esters and triglycerides will give an estimate of the storage lipid content of the anemones. In zooxanthellae 3.6% of lipid was storage lipid whereas in the animal tissues it was 6.6%, and in the aposymbiont it was only 1.9%. The diet mussels contained less lipid than anemones. About 5% of mussel dry weight was lipid and this contained 1.3% triglycerides and 0.05% wax esters.

Wax esters

All wax esters in anemones, zooxanthellae and mussels had an even number of C-atoms (Table 2.2). The wax ester composition of symbiotic and aposymbiotic anemones was very similar with unsaturated wax esters 32:1, 34:2, 34:1 and 36:1 predominating. The major saturated wax ester in both cases was 32:0. In some cases, two different isomers of both 34:1 and 36:1 could be detected. For the purposes of this study such data were combined.

Table 2.2. *Anemonia viridis*. and *Mytilus edulis*. Wax ester composition (percent by weight) of whole-anemone tissue, zooxanthellae isolated from anemone tissue, aposymbiont anemones and whole-mussel tissue. Wax esters were identified by mass spectrometry and by comparison with authentic standards. tr: trace (<0.05%);nd: not detected (estimated to be <0.01%).

Sample No.	Wax ester								
	30:0	32:1	32:0	34:5	34:2	34:1	34:0	36:1	36:0
Anemone									
1	tr	22.2	13.9	3.45	12.4	17.3	tr	29.2	1.55
2	tr	23.5	15.6	tr	9.10	10.2	tr	41.6	tr
3	tr	21.5	14.5	5.18	14.7	23.0	1.44	18.9	0.69
4	tr	21.5	17.1	6.22	11.2	19.6	3.56	19.5	1.31
5	tr	23.8	14.3	5.24	15.0	22.2	tr	18.4	1.04
mean		22.5	15.1	4.02	12.5	18.5	1.00	25.5	0.92
(±SD)		(1.09)	(1.29)	(2.46)	(2.47)	(5.13)	(1.56)	(10.0)	(0.60)
Zooxanthellae									
1	tr	nd	23.4	nd	nd	nd	tr	47.6	29.0
2	20.3	nd	22.8	nd	nd	nd	14.4	36.9	5.60
3	8.43	nd	25.9	nd	nd	nd	6.02	54.8	4.82
4	tr	nd	22.8	nd	nd	nd	7.18	70.0	tr
5	5.17	nd	30.7	nd	nd	nd	6.12	58.0	tr
mean	6.78		25.1				6.74	53.5	7.88
(±SD)	(8.53)		(3.37)				(5.13)	(12.1)	(12.1)
Aposymbiont									
1	nd	18.9	14.0	4.79	18.0	20.0	0.62	20.8	2.86
2	nd	16.1	14.1	tr	12.1	18.1	tr	36.9	2.65
3	nd	13.6	10.9	3.67	10.6	14.3	21.9	25.0	tr
4	nd	17.8	11.7	6.81	14.4	10.3	15.0	19.6	4.43
5	nd	22.4	24.2	6.73	tr	13.9	tr	32.7	tr
mean		17.8	15.0	4.41	11.0	15.3	7.28	27.0	1.99
(±SD)		(3.27)	(5.34)	(2.80)	(6.76)	(3.80)	(9.22)	(7.54)	(1.94)
Mussel									
1	nd	nd	100	nd	nd	nd	nd	nd	nd
2	nd	nd	100	nd	nd	nd	nd	nd	nd
3	nd	nd	100	nd	nd	nd	nd	nd	nd
mean			100						
(±SD)									

Zooxanthellae had a much simpler wax ester composition than anemones and it was dominated by saturated 32:0 and mono-unsaturated 36:1. Zooxanthellae contained a 30:0 wax ester although this was only a major component in one sample. Traces of 30:0 were found in symbiotic anemones and it seems probable that this was due to the 30:0 present in the zooxanthellae. The only mussel wax ester found was 32:0.

Triglycerides

Triglycerides were separated according to molecular weight (Table 2.3.).

Table 2.3. *Anemonia viridis* and *Mytilus edulis*. Triglyceride composition (percent by weight) of whole-anemone tissue, zooxanthellae isolated from anemone tissue, aposymbiotic anemones and whole-mussel tissue. Triglycerides were identified by comparison with authentic standards. tr: trace (<0.05%); nd: not detected (estimated to be <0.01%).

Sample	Triglyceride						
	C42	C44	C46	C48	C50	C52	C54
Anemone							
1	1.25	1.20	tr	19.1	27.7	31.1	19.7
2	7.23	nd	tr	28.5	23.9	32.7	7.59
3	0.91	nd	tr	26.9	41.9	24.8	5.44
4	2.27	nd	tr	25.3	38.2	21.5	12.8
5	1.67	nd	tr	23.5	31.1	28.2	15.6
mean	2.67	0.24		24.7	32.6	27.7	12.2
(±SD)	(2.60)	(0.53)		(3.62)	(7.41)	(4.57)	(5.81)
Zooxanthellae							
1	2.32	nd	17.9	17.8	15.9	26.8	19.4
2	1.85	nd	9.73	18.7	18.0	29.7	22.0
3	2.81	nd	7.50	15.7	16.5	31.4	26.1
4	2.55	nd	7.15	16.8	25.2	24.2	24.1
5	1.75	nd	5.34	20.4	26.9	26.7	18.8
mean	2.26		9.52	17.9	20.5	27.8	22.1
(±SD)	(0.45)		(4.94)	(1.80)	(5.16)	(2.82)	(3.09)
Aposymbiont							
1	nd	nd	nd	18.6	19.6	26.2	35.5
2	nd	nd	nd	18.9	19.3	29.7	32.1
3	nd	nd	nd	21.0	22.4	27.3	29.3
4	nd	nd	nd	11.0	28.5	31.1	30.3
5	nd	nd	nd	10.3	27.6	27.4	34.6
mean				16.0	23.5	28.1	32.4
(±SD)				(4.94)	(4.36)	(1.68)	(2.67)
Mussel							
1	nd	nd	12.0	17.3	18.5	23.7	28.4
2	nd	nd	8.11	16.0	18.3	25.1	32.4
3	nd	nd	6.38	18.3	19.0	27.5	28.8
mean			8.83	17.2	18.6	25.4	29.9
(±SD)			(2.88)	(1.15)	(0.36)	(1.92)	(2.20)

In all samples, trimyristin (C42), tripalmitin (C48) and tristearin (C54) could be detected as single component peaks except in mussels where C54 was a mixture of triglycerides. Other peaks were complex mixtures which could not be separated into molecular species with the GLC system used. Mass spectra fragmentation patterns indicated that the most common

triglyceride fatty acids were palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0) and oleic acid (18:1). However, there was evidence of fatty acids with an odd number of C-atoms in zooxanthellae samples. To obtain more information about triglyceride structure, triglyceride fatty acid composition was determined (see following subsection).

Fatty acids

Twenty one fatty acids were identified in samples of total lipid extract and separated triglycerides (Table 2.4.).

Table 2.4. *Anemonia viridis* and *Mytilus edulis*. Fatty acid composition (percent by weight) of total lipid and triglycerides from one sample each of whole-anemone tissue (Anem.), zooxanthellae isolated from anemone tissue (Zoox.), aposymbiotic anemones (Apo.) and whole-mussel tissue. Fatty acid methyl esters were identified by mass spectrometry and by comparison with authentic standards, except for 11:0 and 12:0 which were identified with authentic standards. IS: internal standard for quantitative TG analysis; a: anteiso; c: cis; tr: trace (<0.5%); nd: not detected (estimated to be <0.01%).

Fatty	Total lipid				Triglyceride			
Acid	Anem.	Zoox.	Apo.	Mussel	Anem.	Zoox.	Apo.	Mussel
11:0	1.96	nd	nd	nd	0.66	0.35	nd	nd
12:0	1.76	1.43	tr	3.36	0.77	1.63	0.82	0.88
13:0	0.50	0.85	2.73	3.34	0.47	1.17	nd	0.60
14:0	1.49	3.33	1.67	3.26	2.29	6.46	3.11	6.35
a15:0	0.98	0.98	0.63	1.42	0.52	4.55	1.24	1.19
15:0	1.14	1.19	1.76	2.91	IS	IS	IS	IS
16:1	1.78	11.1	4.08	2.39	15.6	7.60	6.19	7.83
16:0	27.0	18.3	27.6	45.0	28.7	22.7	26.6	34.7
C17	1.44	1.06	3.68	4.11	0.50	1.41	6.79	3.83
C17	tr	nd	2.89	nd	0.49	0.94	3.83	2.57
C17	1.24	nd	nd	2.64	0.86	3.39	2.34	2.16
17:0	1.49	0.93	3.59	4.78	1.11	15.2	3.78	3.39
c18:1	3.02	1.60	4.42	1.18	11.4	8.58	5.45	4.38
t18:1	1.62	22.9	2.60	1.07	7.02	3.04	3.68	4.71
18:0	15.1	6.47	13.8	13.0	9.11	9.39	12.3	13.0
19:0	7.46	1.43	4.46	0.96	1.91	2.16	11.0	1.02
20:1	13.2	11.4	3.36	2.76	2.60	1.18	5.25	5.23
20:1	2.28	1.69	7.49	2.20	5.44	1.46	7.56	2.72
20:0	14.2	2.44	8.67	3.47	4.34	4.10	nd	3.70
22:5	tr	4.93	nd	nd	3.25	1.06	nd	0.58
22:1	2.43	8.04	6.26	1.73	3.02	3.70	nd	1.16

Fatty acids were highly unsaturated, especially in zooxanthellae total lipid. Even numbered fatty acids were the principle components by mass although fatty acids with an odd number of C-atoms were found in all samples with about 30% in zooxanthellae triglycerides. Any 15:0 fatty acid present in the triglyceride fraction would have been masked due to the

presence of the internal standard tripentadecanoyl glycerol. Three C17 components emerged just before the 17:0 fatty acid. Using a stationary phase of SPB-1, unsaturated components emerge ahead of saturated fatty acids of the same chain length and it is possible that they were different isomers of unsaturated C17 fatty acid. Two 20:1 isomers were resolved although the double bond positions were not ascertained.

In total anemone lipids, 16:0 was the most abundant fatty acid. In mussel total lipids, 16:0 accounted for 45% of total fatty acids. However, the largest component in the zooxanthellae total lipid fraction was *trans*18:1. Analysis of triglyceride fatty acids provided information about the structure of the unidentified triglyceride molecules. The fatty acid profile confirmed earlier predictions based on triglyceride mass fragments with 16:0 as the largest component in all samples. Other major fatty acids were 16:1, *cis*18:1, 18:0 and in zooxanthellae there were appreciable amounts of 17:0.

Other lipids detected

Small amounts of sterol esters were found in all samples of triglyceride, and cholesterol was detected in aposymbionts as a minor component. In samples analysed as wax esters minor components included squalene in zooxanthellae, cholesterol and cholesta-4,6-dien-3-ol in anemones, and a marine sterol (27-norergosta-5,22-dien-3-ol,(3.ß,22Z)) in mussels.

Discussion

Temperate *Anemonia viridis* from this study contained about 11% lipid on a dry weight basis whereas *Anemonia sulcata* (*viridis*) from Brittany, France contained 22.7% (Janssen and Möller 1981) and the American west-coast symbiotic anemone *Anthopleura elegantissima* between 5 and 20% (Blanquet et al. 1979, Jennison 1979). Assuming the dry weight of an anemone to be 10% of wet weight, the lipid content of non-symbiotic temperate anemones was 3 to 13% of dry weight for *Metridium dianthus* (Hooper and Ackman 1971) and 6.7 to 51.5% of dry weight for *Metridium senile* (Mason 1972, Hill-Manning and Blanquet 1979). Ortega and Navarro (1988) found that lipid comprised between 5 and 8.75% of dry weight for non-symbiotic *Actinia equina*. Some of the variability found in lipid levels within and between anthozoan species has been correlated with the stage of the reproductive cycle (Hill-Manning and Blanquet 1979, Jennison 1979, Stimson 1987) and differences in sea-water temperature (Hill-Manning and Blanquet 1979, Glynn et al. 1985).

Tropical symbiotic anemones and corals tend to contain more lipid than temperate anemones. Bergmann et al. (1956) found that lipid comprised 33% of dry weight in the warm water anemone *Condylactis gigantea*, and values of between about 30 and 46% have been reported for reef-building corals (Meyers 1977, Patton et al. 1977, Stimson 1987, Harland et al. 1992). However, the tropical coral *Porites porites* contained only 11% of dry weight as lipid (Harland et al. 1992) and high lipid values have been reported for the temperate anemone *Bolocera tuediae* which contained about one-third of dry weight as lipid (Bergmann et al. 1956).

Zooxanthellae accounted for 12.5% of the dry weight of *Anemonia viridis* and the contribution of algal lipids to whole anemone lipids might be expected to be small. However, about 26% of total lipid was present in the algal fraction. This figure is somewhat higher than the 10% estimated for the reef coral *Pocillopora capitata* (Patton et al. 1977).

In *Anemonia viridis*, less than 1% of the lipid of aposymbionts was accounted for by triglycerides. The animal tissues contained about 4% and the zooxanthellae about 3% of their lipid as triglycerides. These data may suggest synthesis of the triglyceride by the zooxanthellae followed by translocation and storage in the animal compartment. Comparing the triglycerides of *A. viridis* with those of other symbiotic anthozoans showed that levels were much lower than in the tropical coral *Pocillopora capitata* which contained about 38% of lipid as triglyceride in the host tissues and about 5% in the zooxanthellae (Patton et al. 1977). However, levels were similar in the anemone *Anthopleura elegantissima* in which triglycerides were an estimated 3% of the lipid in the animal tissue and 7% of the zooxanthellae lipid (Blanquet et al. 1979).

There was little evidence for the translocation of wax esters from zooxanthellae to the animal compartment. Zooxanthellae contained only 0.33% of lipid as wax ester and the animal tissues and aposymbionts 2.29% and 1.18% respectively. Patton et al. (1977) found similar levels of wax ester in the zooxanthellae of *Pocillopora capitata* although, in contrast to *Anemonia viridis*, lipids of the host tissues contained about 30% wax ester.

In symbiotic anthozoans, there is good evidence for the translocation of photosynthetically-derived lipid from zooxanthellae to host tissues (Patton et al. 1977) and triglyceride is probably the major lipid translocated (Kellog and Patton 1983). Triglycerides and wax esters may be considered as major storage lipids. In *Anemonia viridis*, triglycerides and wax esters together accounted for 5.8% of the total lipid. However, in tropical symbiotic anthozoa, this storage lipid may be as high as 75% (Patton et al. 1977). Since lipid production is light dependent (Patton et al. 1977, Blanquet et al. 1979), this could explain the relatively low levels of storage lipid in *A. viridis* when compared to tropical anthozoa. *Anemonia viridis* was maintained at a light intensity well below the level

of photosynthetic light saturation (Tytler and Davies 1984) and it is conceivable that more triglycerides would be produced under high light conditions.

In this study, both symbiotic anemones and zooxanthellae contained C42 triglyceride which was nearly exclusively composed of glycerol plus three C14:0 fatty acids. As no C42 triglyceride was detected in aposymbiotic anemones or the mussel diet, this may provide indirect evidence for the transfer of triglyceride from zooxanthellae to the anemone tissue. A C46 component was found in the zooxanthellae triglyceride and in trace quantities in symbiotic anemones. No C46 was detected in aposymbiotic anemones and this triglyceride may be exclusive to the algal fraction. Aposymbiotic anemones contained less C50 and more C54 than symbiotic anemones. The major triglyceride in mussels also had a carbon chain length of C54 which suggests a triglyceride compositional shift towards dietary fatty acids in aposymbiotic anemones.

Mytilus edulis wax ester appeared to consist exclusively of C32:0. Wax esters have not been detected in molluscs (for review see Joseph 1982) and it is conceivable that the small amount of wax ester reported in this study may have come from lipid rich algae which probably form the major part of the mussel diet (Volkman et al. 1989). Wax esters from both zooxanthellae and mussels are unlikely to be the only source of this lipid in the host tissues of symbiotic anemones. As the composition of wax esters in symbiotic and aposymbiotic anemones was similar, wax esters are almost certainly produced by the animal compartment. Zooxanthellae wax esters had a simpler composition than those of anemones and were dominated by the C36:1 species. C34 unsaturated wax esters were not detected in zooxanthellae which suggests that they were produced exclusively by the anemones. Some C30:0 was found in the algal fraction with traces of this wax ester in the intact symbiotic association. As C30:0 was absent from aposymbiont anemones, it seems likely that this was synthesised exclusively by the zooxanthellae.

The chromatography column used for the analysis of triglycerides was capable of separating triglycerides by carbon number only and mass-spectrometry showed that most triglyceride components were complex mixtures. However, glycerides with three 14:0, 16:0 or 18:0 fatty acids were positively identified as single-component peaks in all samples. Analysis of the fatty acids enabled predictions to be made about triglyceride composition. Palmitic (16:0) and palmitoleic (16:1) were the most abundant triglyceride fatty acids. Patton et al. (1977) examined the fatty acids of *Pocillopora capitata* and found that both coral and zooxanthellae were dominated by palmitic (16:0), stearic (18:0) and oleic acids (18:1). However, in contrast to *Anemonia viridis*, only a small amount of palmitoleic acid (16:1) was detected in the triglyceride fraction. Palmitic acid appears to be the most common fatty acid in coelenterates although Bergmann et al.(1956) found that myristic acid (C14:0) comprised 51% of the fatty acids of *Condylactis gigantea*. Anemone and

zooxanthellae fatty acid composition showed many similarities with differences being mainly in degree of saturation. It has been postulated that polyunsaturated lipids may be derived from diet and saturated lipids from zooxanthellae (Meyers 1977, Patton et al. 1977). The presence of saturated fatty acids in both aposymbiotic and symbiotic *A. viridis* suggests that they can be synthesised by anemones, independent of algal symbionts. Mussels were not particularly rich in lipid and would not be expected to be a major source of fatty acids. Moreover, mussels contained 45% palmitic acid (16:0) which was far in excess of the percentage found in anemones. If 16:0 is taken up in the diet, it may be transformed into other fatty acids before incorporation into anemone wax esters or triglycerides.

The fatty acids of the total lipid and triglyceride fractions were largely saturated and only one polyunsaturated fatty acid was detected. This observation would appear to be at variance with the view that marine invertebrates contain large amounts of polyunsaturated fatty acids (Bockerhoff et al. 1963). However, symbiotic corals and anemones, in contrast to non-symbiotic species, most often exhibit low or non-detectable levels of polyunsaturated fatty acids (Meyers 1977, Meyers et al. 1978, Patton et al. 1977, Blanquet et al. 1979). Indeed, Patton et al. (1977) proposed that symbiotic coelenterates are characterised by a very saturated fatty acid profile. All lipid and triglyceride samples consisted of fatty acids with even- and odd-numbered C-atom chains. The highest proportion of odd chain length fatty acids was found in zooxanthellae triglyceride with 15% 17:0, a fatty acid also present in the triglycerides of *Metridium senile* (Hooper and Ackman 1971). Of the polyunsaturated fatty acids, the 22:5 acid detected in this study has also been reported for other anemones (Hooper and Ackman 1971, Pollero 1983) but it was a relatively minor component of the total lipid and triglycerides of symbiotic *Anemonia viridis* and its zooxanthellae. Its absence from aposymbiotic anemones may indicate that zooxanthellae are a source of this fatty acid.

Three C17 fatty acids were found in all triglyceride samples although their structure was uncertain. However, Hooper and Ackman (1971) identified iso-17:0, anteiso-17:0, 17:0 and phytanic acid in *Metridium dianthus*. All triglyceride samples from this study contained 17:0 and in symbiotic *Anemonia viridis*, it appears that this fatty acid is found exclusively in the zooxanthellate fraction. Hooper and Ackman (1971) reported *trans*-16:1 for *M. dianthus* and although this fatty acid was not found in *A. viridis*, *trans*-18:1 was identified in all samples. This unusual isomer was a major component of the algal fraction although its presence in aposymbionts suggests that the zooxanthellae may not be the only source in symbiotic anemones.

No quantitative data were obtained for the sterol esters, cholesterol or squalene detected in *Anemonia viridis*. In *Metridium senile*, sterols accounted for 30-35% of neutral lipid, rising to about 60% during gametogenesis (Hill-Manning and Blanquet 1979)

although no sterol esters were present. Relatively high levels of cholesterol have been reported for anemones (Bergmann et al. 1956, Mason 1972) and Mason (1972) proposed that the saturated long-chain fatty acids and cholesterol found in *M. senile* were indicative of a highly-ordered membrane system. Meyers (1977) reported high levels of the hydrocarbon squalene in several species of coral and suggested that this compound was metabolically active. Clearly, there is scope for more work to quantify each of these lipid classes in *A. viridis*.

The results of this study have shown that zooxanthellae did not significantly influence the total level of lipids in symbiotic anemones. However, the relative increase in the levels of triglyceride when compared to aposymbiotic anemones may in part be due to the provision of photosynthetically derived carbon for storage lipids. There may also be evidence for the transfer of particular triglyceride species from the zooxanthellae to the host anemone compartment. Analysis of the diet indicated that mussels were not important in contributing to total anemone lipid nor does mussel fatty acid composition directly influence the fatty acids of anemones.

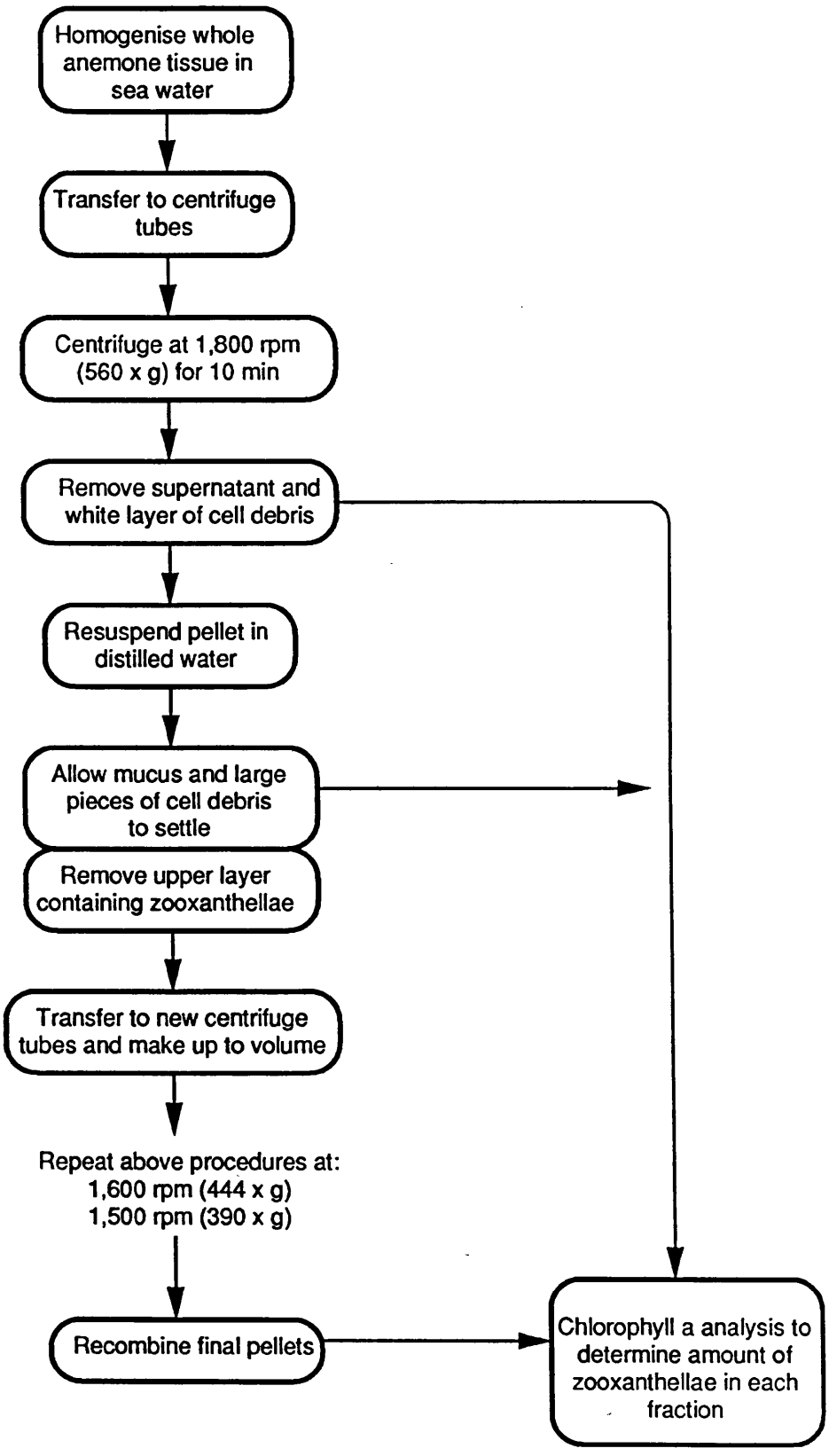


Figure 2.1. *Anemonia viridis*. A method for extracting zooxanthellae from whole-anemone tissue.

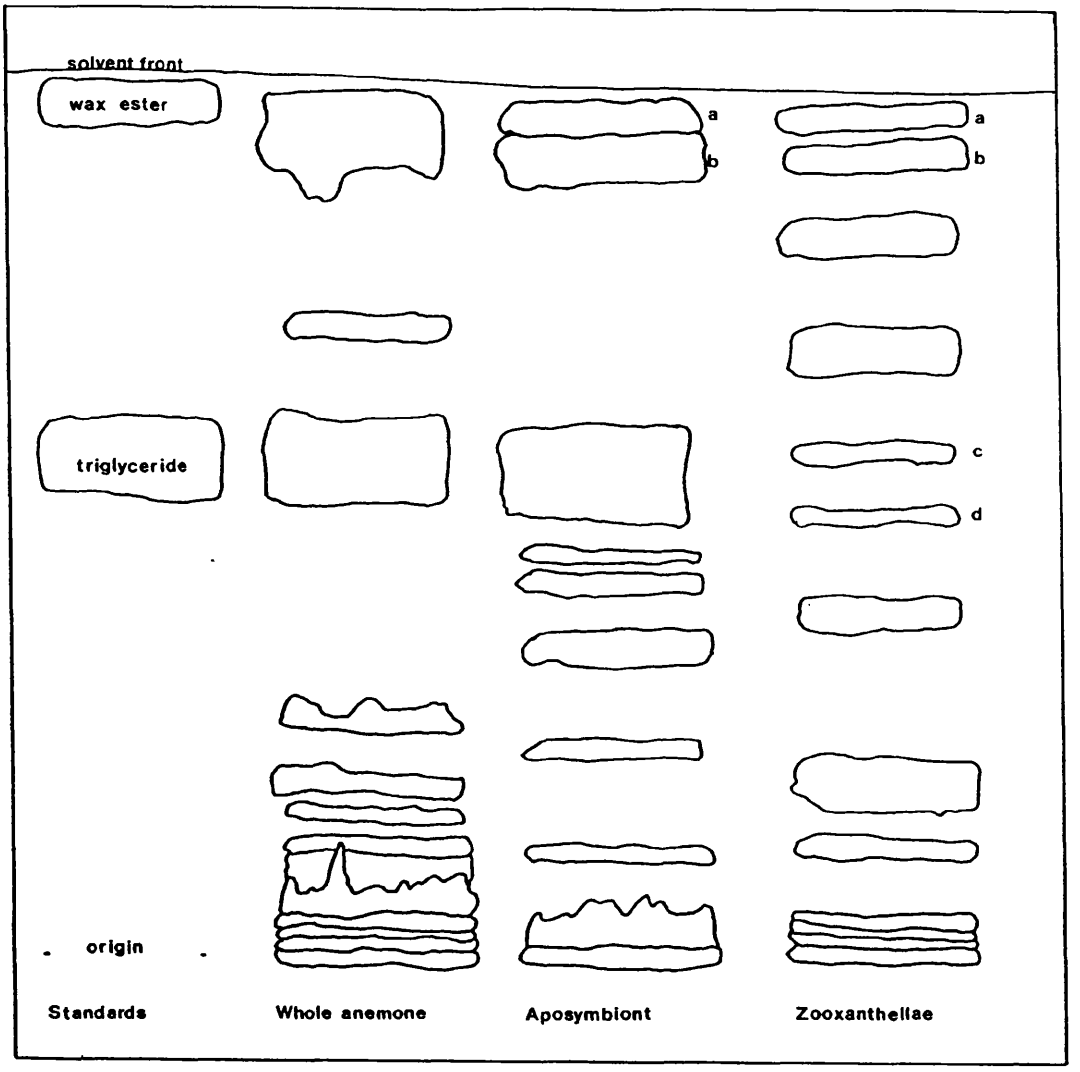


Figure 2.2. *Anemonia viridis*. Separation of whole-anemone, aposymbiont-anemone and zooxanthellae lipid extracts by thin-layer chromatography. Silica gel H60 plates were developed sequentially in two solvents (see materials and methods) and the lipids visualised with 2',7' -dichlorofluorescein. Wax esters and triglycerides were identified by comparison with standards. For aposymbiont and zooxanthellae lipids, two areas of silica (a and b) were removed as the total wax ester and for zooxanthellae lipids, two areas (c and d) were removed as the triglyceride fraction.

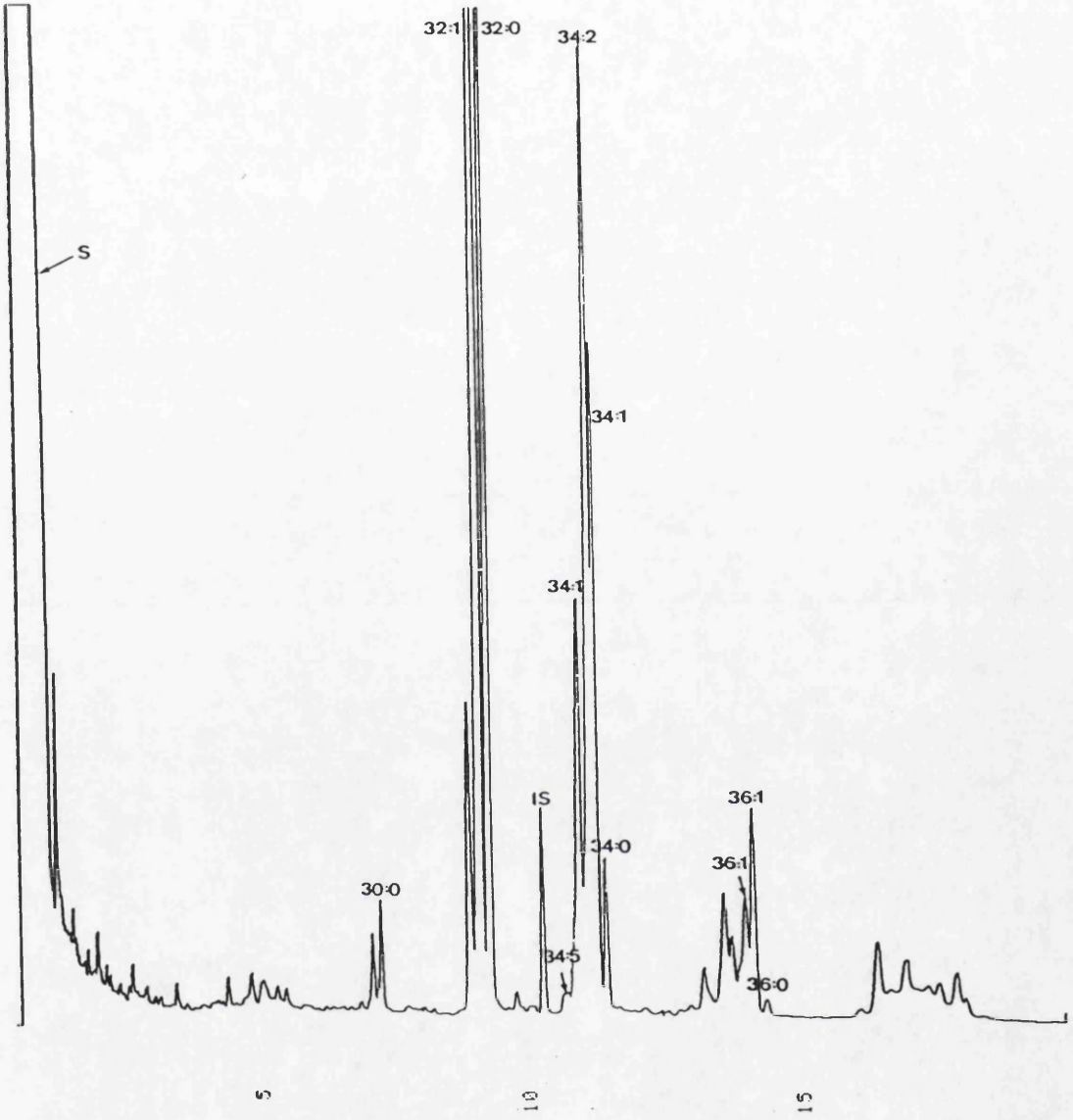


Figure 2.3. *Anemonia viridis*. Separation of whole-anemone wax esters by gas-liquid chromatography with a SGE 12 m x 0.25 mm column. The stationary phase was SGE BP-100, carrier gas N_2 with a flow rate of 1 ml min^{-1}, and the oven temperature programmed from 170°C to 300°C at 5C° min^{-1}. The column gave separations based upon molecular weight and the number of double bonds, with low-molecular weight wax esters emerging first, and unsaturated wax esters emerging before saturated wax esters of the same carbon-chain length. The x-axis is time (min.). The internal standard, C33:0 had a retention time of 10.292 min.

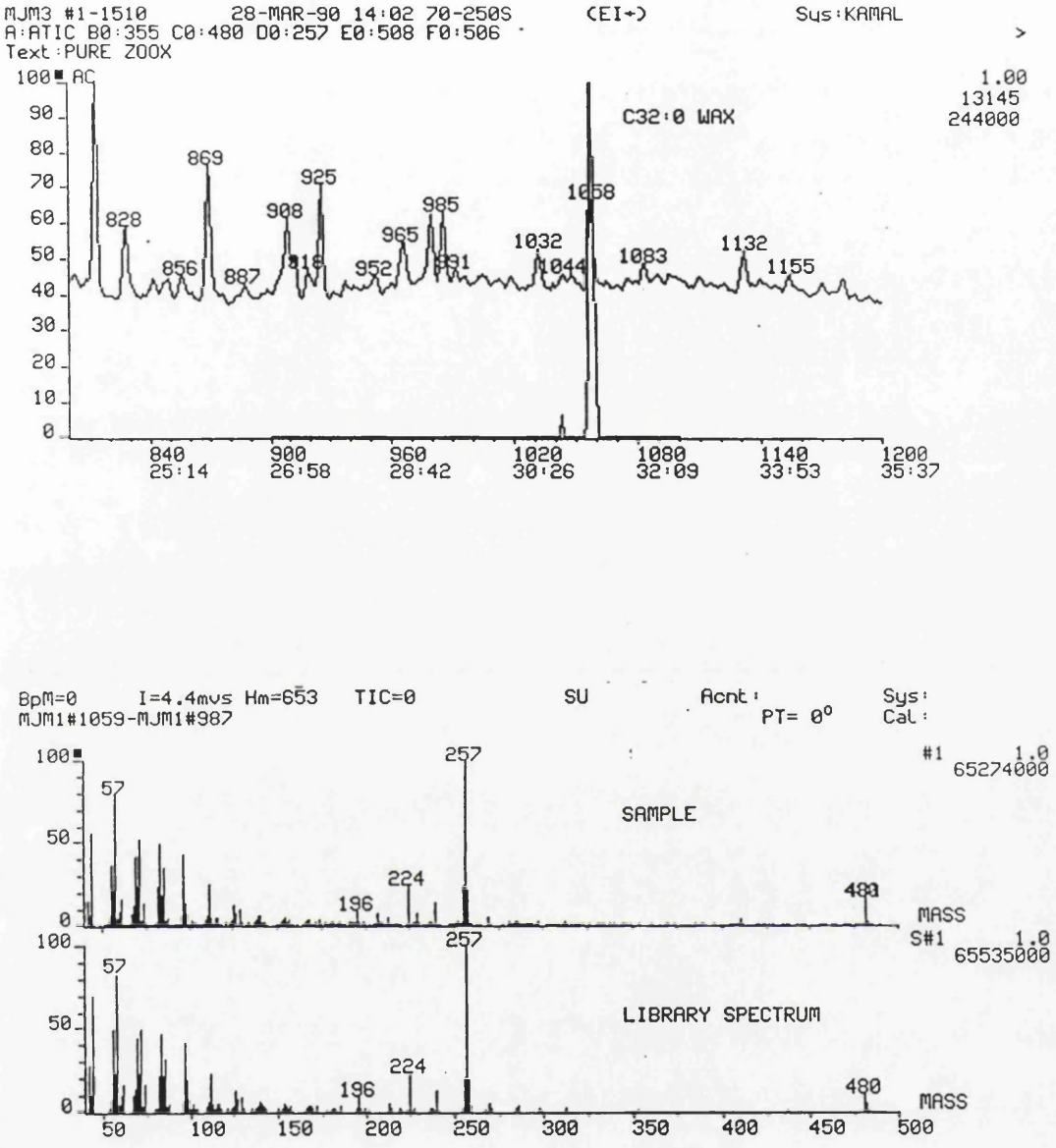


Figure 2.4. *Anemonia viridis*. Identification of the zooxanthellae C32:0 wax ester by gas chromatography-mass spectrometry (GC-MS). The top trace is a section of the gas chromatogram showing peaks numbered 828 to 1155. The x-axis is peak number and time (min.). The mass spectrum of the 1058 peak is shown in the bottom trace. The sample mass spectrum can be compared to a library spectrum for cetyl palmitate (C32:0) in which the x-axis is molecular mass. The parent ion is 480. Other peaks show mass fractions derived from palmitic acid (mass 257) and cetyl alcohol (mass 224) which are the fatty acid and fatty alcohol components of cetyl palmitate.

Chapter 3

Effect of light on the total lipid content and storage lipids of the symbiotic sea anemone *Anemonia viridis*

Introduction

Symbiotic anthozoans contain symbiotic zooxanthellae which export photosynthetically fixed carbon compounds to the host tissue. Carbon may be translocated as intact lipids, fatty acids or glycerol (Muscatine and Cernichiari 1979, Trench 1971 a, Patton et al. 1977, Battey and Patton 1984). Lipid is stored by the host, primarily as triglycerides and wax esters (Patton et al. 1977) and any photosynthetically fixed carbon in excess of requirements for growth or the laying down of lipid stores may be excreted as mucus-lipid (Crossland et al. 1980 b, Crossland 1987). The total amount of lipid appears to vary between temperate and tropical symbiotic Anthozoa. Temperate sea anemones generally contain between 5 and 23% of dry tissue weight as lipid (Blanquet et al. 1979, Jennison 1979, Janssen and Möller 1981, chapter 2) whilst the amount of lipid in tropical anemones and corals is about 30 to 46% (Bergmann et al 1956, Patton et al 1977, Stimson 1987). The lipid levels of corals have been shown to be light-dependent and experimental attenuation of the light available to *Pocillopora damicornis* on a shallow fringing reef resulted in a significant reduction in lipids after 17 d (Stimson 1987).

In the previous chapter, the lipid composition of *Anemonia viridis* which had been maintained in aquaria at 10 μE m^{-2} s^{-1} was described. The total lipid levels under these conditions were about 11% of anemone dry weight. Experiments were therefore carried out to determine if higher storage levels would be found after exposure to higher levels of irradiance. It was anticipated that if at low levels of light (10 μE m^{-2} s^{-1}) the level of excess production of carbon by photosynthesis was only adequate to maintain a small lipid store, at higher levels the store would build up and ultimately become replete. Furthermore, if anemones were not fed and therefore not given access to a nitrogen source, they would not grow. Under these conditions, it would be expected that a relatively larger amount of carbon would be channelled into lipid and the lipid stores would therefore increase to higher levels.

Materials and methods

Experimental design

Anemonia viridis were collected from the Isle of Cumbrae in 1989 and maintained at a light

intensity of 10 μE m^{-2} s^{-1} in the sea water aquarium of the Zoology Department, Glasgow University (see chapter 2). The experimental temperature was about 10°C. Five anemones were removed from the aquaria and the total lipid level of whole anemone tissue was determined. Ten anemones were then placed in each of three glass tanks at light levels of 10, 100 or 300 μE m^{-2} s^{-1} with a 12h light:12h dark photoperiod. Light was provided by a bank of fluorescent tubes. Anemones were fed weekly on chopped mussel. After 30 and 60 d, 5 anemones were removed from each tank. One half of each anemone was analysed for whole anemone lipid while zooxanthellae were extracted from the other half for analysis of the algal lipids (chapter 2). Zooxanthellae were isolated from symbiotic anemone tissue by repeated washing and centrifugation (figure 1.2). A consequence of using this separation technique was that not all zooxanthellae were recovered. To estimate losses, chlorophyll *a* levels were measured (Parsons et al. 1984) in both pelleted algae and discarded supernatants and from these data the total amount of zooxanthellae was calculated. All samples were freeze dried before lipid extraction.

To examine the effect of starvation at saturating light intensity on the lipid content of *Anemonia viridis*, the above procedures were repeated at 300 μE m^{-2} s^{-1} using anemones which were starved for one week before and for the duration of the experiment. Fifteen anemones were buoyant weighed (Davies 1990), 5 of which were sacrificed immediately and freeze dried. The remaining 10 anemones were placed in the experimental tanks and 5 removed at 30 d and 5 at 60 d when they were re-weighed before freeze drying. A linear regression analysis of buoyant weight and dry weight enabled a prediction to be made of the dry weight of the 30- and 60-d anemones at the start of the experiment.

Lipid analysis

Lipids were extracted from freeze dried samples of whole anemone tissue and zooxanthellae as described by Folch et al. (1957). Wax esters and triglycerides were separated by thin-layer chromatography and analysed by gas chromatography. Identification of lipids was confirmed by mass spectrometry. All solvents (Analar, BDH Chemicals Ltd.) were re-distilled before use. Full details of lipid analysis are given in chapter 2.

Statistical procedures

Statistical analyses were carried out on arcsine-transformed data using either analysis of variance or two-way analysis of variance (Sokal and Rohlf 1981). Multiple comparisons of means were done with a Student-Newman-Keuls test (SNK) (Zar 1974).

Results

Lipid content

The total lipid levels in anemones maintained in the aquaria at 10 μE m^{-2} s^{-1} was 9.26% of whole anemone dry weight. For fed anemones which were exposed to 10, 100 or 300 μE m^{-2} s^{-1} for 30 d, there was no significant differences in total lipid between light levels ($P>0.05$, ANOVA). However, at 60 d there was a significant increase with each increase in light level, the percentage lipid in whole anemone dry weight changing from 8.87 at 10 μE m^{-2} s^{-1} to 16.0 at 300 μE m^{-2} s^{-1} ($P<0.05$, ANOVA) (Table 3.1). The increase in lipid was apparent in both the animal tissues and the zooxanthellae compartment, the proportion in each compartment remaining approximately the same between 10 and 300 μE m^{-2} s^{-1}.

Table 3.1. *Anemonia viridis*. Total lipid composition of symbiotic anemones and freshly isolated zooxanthellae. Anemones were fed on chopped mussel (*Mytilus edulis*) and maintained for 60 d at a light intensity of either 10, 100 or 300 μE m^{-2} s^{-1}. Data are expressed as % lipid in the dry weight of whole-anemone tissue. Values for animal tissue were derived by subtraction of zooxanthellae values from those of whole anemones. For symbiotic anemones, $n = 5$, except for 100 μE (*), where $n = 4$. Standard deviations are shown in parentheses. ND: no data.

Light Intensity (μE m^{-2} s^{-1})	Whole anemone (%)	Animal tissue (%)	Isolated zooxanthellae (%)
0 d			
10	9.26 (0.90)	ND	ND
60 d			
10	8.87 (1.70)	5.69 (2.21)	3.18 (0.79)
100*	12.20 (1.53)	8.67 (1.14)	3.56 (0.97)
300	16.0 (2.0)	10.4 (2.12)	5.63 (1.55)

In order to determine whether these lipid increases were due to changes in the lipid stores, the values for wax esters and triglycerides were summed to give a measure of the total storage lipid. In both the animal tissue and the zooxanthellae, there were large increases in the storage lipid with increases in light intensity from 10 to 300 μE m^{-2} s^{-1} ($P<0.05$, ANOVA) (Table 3.2). In the zooxanthellae, wax esters remained almost unchanged at 30 and 60 d at a mean overall level of 0.025% of anemone dry weight ($P>0.05$, two-way analysis of variance). Almost all of the changes in zooxanthellae storage lipid were accounted for by triglycerides which increased by a factor of 10 after 60 d from 0.047 to 0.46%.

In the animal tissue, by contrast, there was a significant increase in both wax esters and triglycerides. At 60 d, the wax esters had increased by a factor of 20 from 0.04% at 10 μE m^{-2} s^{-1} to 0.83% at 300 μE m^{-2} s^{-1} whilst the triglycerides increased by a factor of 25, from 0.04 to 1.0% of anemone dry tissue weight.

In the starved anemones, it was anticipated that since the photosynthetically fixed carbon would not be channelled into growth, it would be incorporated into the lipid stores which would increase. It was found that after 60 d at 300 μE m^{-2} s^{-1} there was only a small increase in the total lipid from 7.6% of dry weight (at an initial light intensity of 10 μE m^{-2} s^{-1}) to 9.3%, the difference being not significant ($P>0.05$, ANOVA) (Table 3.3).

Table 3.2. *Anemonia viridis*. Storage lipid (wax ester and triglyceride) content of animal tissue (anim) and freshly isolated zooxanthellae (zoox). Anemones were fed on mussel and maintained for 30 or 60 d at light intensities of 10, 100 or 300 μE m^{-2} s^{-1}. Where superscripts differ within a time period, lipid class and tissue compartment, data are statistically different (SNK, $P<0.05$). Further details as in legend to table 3.1.

Light Intensity (μE m^{-2} s^{-1})	Storage lipid (%)					
	Wax ester (WE)		Triglyceride (TG)		Total (WE+ TG)	
	anim	zoox	anim	zoox	anim	zoox
30 d						
10	0.014^{a}	0.027^{a}	0.041^{a}	0.034^{a}	0.055^{a}	0.061^{a}
	(0.006)	(0.012)	(0.04)	(0.025)	(0.037)	(0.025)
100	0.31^{a}	0.038^{a}	0.43^{b}	0.32^{b}	0.74^{b}	0.36^{b}
	(0.09)	(0.025)	(0.36)	(0.19)	(0.44)	(0.21)
300	0.49^{b}	0.024^{a}	0.54^{b}	0.59^{c}	1.03^{b}	0.61^{c}
	(0.12)	(0.008)	(0.33)	(0.20)	(0.35)	(0.20)
60 d						
10	0.041^{a}	0.025^{a}	0.04^{a}	0.047^{a}	0.081^{a}	0.071^{a}
	(0.02)	(0.015)	(0.03)	(0.02)	(0.043)	(0.034)
100	0.58^{b}	0.022^{a}	0.54^{b}	0.18^{b}	1.12^{b}	0.20^{b}
	(0.33)	(0.024)	(0.09)	(0.077)	(0.33)	(0.09)
300	0.83^{b}	0.015^{a}	1.01^{c}	0.46^{c}	1.84^{c}	0.47^{c}
	(0.22)	(0.005)	(0.16)	(0.13)	(0.32)	(0.14)

Over the 60 d period there was a slight but insignificant decline in anemone body weight ($P>0.05$, ANOVA). Although the total lipid did not show a significant increase at the high light intensity, the storage lipids (wax esters + triglycerides) did increase significantly from 0.2 to 0.8% of anemone dry weight (Table 3.4). The main change was in the wax esters which increased significantly at the end of 60 d from 0.09 to 0.51% ($P<0.05$, ANOVA). The triglycerides increased only slightly, the difference not being significant ($P>0.05$, ANOVA).

Table 3.3. *Anemonia viridis*. Total lipids and dry weights of starved symbiotic anemones transferred from light intensity of 10 μE m^{-2} s^{-1} (Day 0) to light intensity of 300 μE m^{-2} s^{-1} for 30 d (Group 1) or 60 d (Group 2). Dry weights of anemones at Time 0 were derived from measurements of buoyant weight (see "Materials and methods - Experimental design"). Data for lipids expressed as % lipid in the dry weight of whole-anemone tissue. Standard deviations are shown in parentheses; $n = 5$.

Time interval	Total lipid (%)	Dry weight (mg)	
		Group 1	Group 2
0 d	7.62 (1.79)	577 (80.6)	605 (67.8)
30 d	6.85 (4.27)	562 (201.0)	
60d	9.27 (1.69)		574 (75.4)

Table 3.4. *Anemonia viridis*. Wax ester and triglyceride content of starved symbiotic anemones transferred from light intensity of 10 μE m^{-2} s^{-1} (Day 0) to light intensity of 300 μE m^{-2} s^{-1} for 30 or 60 d. Further details as in legend to table 3.2.

Time interval	Storage lipid (%)		
	Wax ester (WE)	Triglyceride (TG)	Total (WE + TG)
0 d	0.092^{a} (0.054)	0.116^{a} (0.087)	0.208^{a} (0.014)
30 d	$0.298^{a,b}$ (0.25)	0.254^{a} (0.195)	$0.552^{a,b}$ (0.423)
60 d	0.51^{b} (0.227)	0.294^{b} (0.169)	0.804^{b} (0.356)

Wax ester and triglyceride composition

After 30 d, fed symbiotic anemones contained more 32:1 and 34:2 and zooxanthellae more 32:0 wax ester at higher light intensities. There was no evidence for further changes in composition at 60 d (Table 3.5). Fed symbiotic anemones contained higher levels of C48 and C50 triglyceride (The numbers after "C" refer to the total number of carbon atoms in the fatty acids) at 100 μE m^{-2} s^{-1} when compared to anemones at 10 μE m^{-2} s^{-1}. However, there appeared to be no difference between 100 and 300 μE m^{-2} s^{-1} and between the 30 and 60 d treatments. There were no obvious differences in the triglyceride composition of zooxanthellae at each light level (Table 3.6). In contrast to fed anemones, starved anemones at 300 μE m^{-2} s^{-1} increased the amount of 34:2 wax ester while 32:1 levels remained constant over 30 and 60 d. There was also some evidence for an increase in the C50 triglyceride but C48 levels remained relatively constant (Table 3.7).

Table 3.5. *Anemonia viridis*. Wax ester composition (percent by weight) of whole-anemone tissue and isolated zooxanthellae. Anemones were fed on mussel and maintained for 30 or 60 d at light intensities of 10, 100 or 300 μE m^{-2} s^{-1}. Wax esters were identified by mass spectrometry and by comparison with authentic standards; $n = 5$, except for 60 d at 100 μE (*), where $n = 4$. Standard deviations omitted for clarity. nd: not detected (estimated to be <0.01 %); tr: trace (<0.1 %).

Compartment, time interval, light intensity (μE m^{-2} s^{-1})		Wax ester 30:0	32:1	32:0	34:5	34:2	34:1	34:0	36:1	36:0
Anemone										
30 d	10	6.59	17.9	16.4	9.44	11.8	19.5	1.30	10.7	6.46
	100	2.34	28.7	17.1	0.87	18.9	18.8	0.89	11.3	1.03
	300	2.36	30.9	17.7	1.11	18.8	19.3	0.91	8.21	1.01
60 d	10	5.92	15.4	17.5	7.77	9.42	18.9	5.84	14.8	4.47
	100*	0.19	28.3	15.1	1.45	21.2	18.8	3.03	10.3	1.61
	300	1.03	30.6	15.4	1.13	21.9	18.4	2.27	8.38	0.95
Zooxanthellae										
30 d	10	5.96	nd	39.1	nd	nd	nd	25.0	20.2	9.84
	100	tr	nd	27.8	nd	nd	nd	31.4	24.1	16.7
	300	tr	nd	50.1	nd	nd	nd	23.5	9.88	16.8
60 d	10	1.79	nd	35.4	nd	nd	nd	16.9	4.78	41.1
	100*	4.86	nd	40.0	nd	nd	nd	32.0	18.8	4.35
	300	1.95	nd	48.9	nd	nd	nd	23.4	12.2	13.6

Table 3.6. *Anemonia viridis*. Triglyceride composition (percent by weight) of whole-anemone tissue and freshly isolated zooxanthellae. Further details as in legend to table 3.5.

Compartment, time interval, light intensity (μE m^{-2} s^{-1})		TG C42	C44	C46	C48	C50	C52	C54
Anemone								
30 d	10	5.80	1.23	4.28	21.7	25.2	31.6	10.3
	100	0.64	0.29	2.55	26.4	37.6	26.5	6.10
	300	0.39	0.66	3.12	27.3	37.5	24.8	6.29
60 d	10	3.42	2.14	6.28	19.1	28.1	33.6	7.43
	100*	0.43	0.28	1.85	28.5	35.0	25.6	8.37
	300	0.32	0.24	2.34	29.7	36.2	23.9	7.20
Zooxanthellae								
30 d	10	7.30	nd	4.31	19.1	28.3	25.2	15.7
	100	2.43	nd	4.71	18.3	36.0	30.2	8.43
	300	1.34	nd	6.25	20.1	37.6	29.2	5.59
60 d	10	9.08	nd	5.41	21.3	34.4	20.1	9.78
	100*	0.70	nd	5.19	19.4	36.7	33.2	4.78
	300	1.94	nd	6.33	19.9	36.7	30.9	4.21

Table 3.7. *Anemonia viridis*. Wax ester and triglyceride composition (percent by weight) of starved anemones transferred from light intensity of 10 μE m^{-2} s^{-1} (Day 0) to light intensity of 300 μE m^{-2} s^{-1} for 30 or 60 d. Lipids were identified by mass spectrometry and by comparison with authentic standards. Data are means (n = 5). Standard deviations omitted for clarity.

Lipid	Time		
	0 d	30 d	60 d
Wax ester			
30:0	2.48	2.49	1.49
32:1	25.7	26.6	26.1
32:0	16.6	15.5	17.9
34:5	6.97	2.26	0.70
34:2	12.4	20.2	26.1
34:1	16.1	19.6	17.2
34:0	2.18	3.62	2.57
36:1	9.92	6.96	6.91
36:0	4.66	2.74	0.91
Triglyceride			
C42	1.95	0.72	0.65
C44	1.20	0.41	0.50
C46	4.29	3.37	0.81
C48	31.9	28.0	29.1
C50	34.9	40.1	41.7
C52	22.7	25.3	26.2
C54	2.93	2.15	0.98

Discussion

Fed symbiotic anemones have two sources of nutritional input, heterotrophic feeding and photosynthesis. During the course of the experiments they would be expected to grow, and a proportion of the carbon intake would be utilised in this way. Any excess carbon from photosynthesis and nutrition would be available for channelling into storage lipid. Hence with increasing irradiance, lipid levels would be expected to become progressively larger. After 30 d at 10, 100 and 300 μE m^{-2} s^{-1} there were no significant differences. However, by 60 d it was clear that lipids had increased in proportion to the irradiance levels. In *A. viridis*, saturation of photosynthesis occurs at about 190 μE m^{-2} s^{-1} (Tytler and Davies 1984) and so it seems unlikely that levels above the 300 μE m^{-2} s^{-1} used in this study would cause any increase in the values recorded.

Only part of the increase in total lipid at 60 d was due to increases in the storage lipids, wax esters and triglycerides. In both the animal tissues and the zooxanthellae, the amount of these storage lipids were proportional to light levels. However, there were differences in the type of storage lipid incorporated into each compartment. The zooxanthellae contained a relatively small amount of wax ester and levels were similar at

each light intensity. Other workers have shown that zooxanthellae contain only small amounts of wax ester (Patton et al. 1977, Blanquet et al. 1979, chapter 2). In contrast to the wax esters, there was a 10-fold increase in zooxanthellae triglycerides at 300 μE m^{-2} s^{-1}. In the animal tissues of *Anemonia viridis*, wax esters and triglycerides increased by a factor of 20 and 25 respectively after 60 d at 300 μE m^{-2} s^{-1}. Most of the wax ester and triglyceride was located in the animal compartment and this has also been demonstrated for other anthozoans. Wax esters and triglycerides occurred mainly in the host tissues of the anemone *Anthopleura elegantissima* (Blanquet et al. 1979) and the coral *Pocillopora capitata* (Patton et al. 1977).

One of the objectives of this study was to see if lipids would become replete after 60 d at saturating light values. However, it was clear that there was a gradual accumulation of total lipid with time. Thus differences were not discernable between the three light levels at 30 d, but the increased accumulation by 60 d produced significant differences. It was possible that the lipid stores were replete sometime before 60 d. Conversely, lipid stores may not have been replete at the end of the experiment and higher levels may have been recorded over a longer time period. For storage lipids, there was no further accumulation in the zooxanthellae after 30 d. However, in the animal tissues there was no evidence that storage lipids were replete at 60 d.

In starved anemones, it was anticipated that the rate of accumulation of total lipid at high light intensities would be faster than in fed anemones since none of the photosynthetically derived carbon would be channelled into growth. In fact, this was not observed and there was a very much smaller and non-significant increase in total lipid. A similar situation was observed in fed and starved *Astrangia danae* (Szmant-Froelich 1981). Nevertheless, for starved *Anemonia viridis*, the total storage lipids did increase significantly during the 60 d suggesting a decrease in non-storage and structural lipids. A decrease in the amount of structural lipids may not be unexpected since the anemones were starved and this would have reduced their ability to synthesise N-containing structural lipids. An alternative suggestion would be that starvation increased the catabolism of structural lipids although anemone dry weight did not decline significantly. Despite the significant increase in the storage lipids of starved anemones, they amounted to only 0.8% of anemone dry weight compared with 2.31% in fed anemones. Although increased heterotrophic carbon input may account for increased storage lipid in fed anemones there are other possible explanations for the differences in lipid content of fed and starved anemones: (1) In the starved anemones excess photosynthetically fixed carbon is excreted at a higher rate; (2) in starved anemones the net rate of photosynthesis is reduced. This may only be resolved by future research involving a complete carbon budget accounting for the carbon input from both autotrophic (photosynthesis) and heterotrophic sources.

The percentage composition of triglycerides of anemones at 10 μE m^{-2} s^{-1} was similar

to the values given in chapter 2. However, there were some differences in the wax ester composition with a decrease in the percentage of 36:1 in whole-anemone tissue and zooxanthellae, and an increase in 32:0 and 34:0 in the zooxanthellae. In the present study, compositional changes were observed at higher light intensities and it was clear that both wax esters and triglycerides were affected by the level of irradiance and hence algal photosynthesis. In fed anemones, the zooxanthellae 32:0 wax ester increased from 35 to 49% of the total wax esters. This change was not reflected in the whole anemone tissue where 32:1 and 34:2 increased. If any wax esters or wax ester fatty acids are transferred from zooxanthellae to the host, they are likely to be altered by the host-animal tissues. Patton et al. (1977) proposed that in *Pocillopora capitata*, unsaturated fatty acids from the zooxanthellae might be incorporated into the host tissues and subsequently saturated by the host. In the present study, the observed shift towards a more unsaturated wax ester profile would only result if any transferred saturated acids were desaturated by the host.

For triglycerides, higher light levels resulted in an increase in C48 and C50 and a decrease in C52 in the animal tissues of fed anemones. There were no discernible changes in the composition of zooxanthellae triglycerides. Where direct comparisons could be made, it was clear that starved and fed anemone compositional changes differed. In contrast to fed anemones, the levels of 32:1 wax ester in starved anemone tissues remained constant while 34:1 increased. For the triglycerides of starved anemones there was only evidence for an increase in C50. Some of the differences observed may be due to diet.

Although compositional changes were in some cases quite marked, it was difficult to draw any firm conclusions regarding the effect of different irradiances on the production of specific wax ester and triglyceride species. However, the changes observed in the composition of storage lipids may indicate that there are complex interactions between the zooxanthellae and animal cells which determine the composition of stored lipids. At higher irradiances there appeared to be an increase in the fed anemones of species composed of C16 and to some extent C18 fatty acids or alcohols. Although triglycerides were not separated according to degree of unsaturation, from the wax ester data it appears that there was a selective increase in 16:1 and 18:1 species.

The results of this study have shown that the levels of total lipid and storage lipid in *Anemonia viridis* are light dependent. In the tropical reef coral *Pocillopora damicornis*, Stimson (1987) showed that total lipid levels declined over a period of 17 d when irradiance was reduced by shading. This reduction in lipid was presumably the result of lipid catabolism resulting from a negative carbon budget (Davies 1991). Even after 60 d at light intensities exceeding photosynthetic saturation, the total lipid levels of *Anemonia viridis* at 16% of dry weight are well below the values of 30-46% of dry weight commonly observed in tropical symbiotic Anthozoa (Bergmann et al 1956, Patton et al 1977, Stimson 1987, Harland et al. 1992). Storage lipid levels may be genetically determined and the

comparatively large amounts of wax esters and triglycerides in tropical forms (eg. Patton et al 1977) may have been selected for in response to higher metabolic demands associated with higher temperatures when storage lipids are being depleted under adverse conditions.

Note: There may be a direct effect of light intensity on the animal lipid reserves.

Chapter 4

Incorporation of photosynthetically fixed carbon into the lipids of the sea anemone *Anemonia viridis* in the light and in the dark

Introduction

Previous studies have shown that photosynthetically fixed ^{14}C is translocated from the zooxanthellae to the host tissues of symbiotic anthozoans (Muscatine and Cernichiari 1969, Trench 1971 a) and that carbon may be translocated as glycerol (Muscatine and Cernichiari 1969) or as lipid (Patton et al. 1977). Zooxanthellae *in vitro* excrete glycerol and this is one of the first compounds to be labelled in the host tissue *in vivo* (Muscatine and Cernichiari 1969, Trench 1971 a, b). These authors suggested that glycerol was translocated from algae to host and incorporated intact into host lipids. Schmitz and Kremer (1977) examined ^{14}C uptake in corals using pulse-chase experiments followed by biochemical fractionation of tissues. In the tissues of whole coral, glycerol and glucose were rapidly labelled although this label was lost over a short period of time. As most of the ^{14}C accumulated was eventually incorporated in lipid, it was suggested that glycerol and glucose were translocated from the algae and used as intermediates for lipid biosynthesis. In contrast, several authors have provided evidence that lipids are translocated from symbiotic algae to host tissues. For example, Patton et al. (1977) found that triglyceride fatty acids of the zooxanthellae and host were identical in the coral *Pocillopora capitata* and it was proposed that these lipids were produced by the zooxanthellae and then translocated to the host (see chapter 2). It has also been proposed that both glycerol and lipid are translocated from the zooxanthellae to the host, the glycerol being catabolised rapidly while the lipid is stored (Battey and Patton 1987).

The majority of fixed ^{14}C accumulated by both the zooxanthellae and host is found in the lipids (Von Holt and Von Holt 1968, Trench 1971 a, Patton et al. 1977). In the previous chapter, it was shown that over a period of 60 d, both the total lipid and storage lipid (wax ester and triglyceride) content of *Anemonia viridis* was proportional to light level and therefore the rate of photosynthesis. Stimson (1987) also found that the total lipids of the reef coral *Pocillopora damicornis* were dependent upon light availablity. However, little is known about lipid carbon fluxes during periods of darkness. Davies (1991) suggested that corals may catabolise storage lipid on cloudy days when photosynthesis cannot meet the requirements for respiration and growth. Lipid stores would be replenished when light-levels were high and zooxanthellae were able to produce an excess of photosynthetically fixed carbon. Crossland et al. (1980 b) found that ^{14}C

incorporation into the lipids of the coral *Acropora acuminata* in darkness was only 1% of the noon maximum. Furthermore, several authors have suggested that there may actually be a reverse-carbon flux from host to zooxanthellae in the dark (Steen 1986, 1987, Streamer et al. 1986, Tytler and Davies 1986).

This chapter presents a preliminary attempt at examining the flux of fixed ^{14}C into the lipids of *Anemonia viridis* in the light and in the dark. The following questions were addressed:

1) What proportion of the fixed carbon which is retained by whole anemones is incorporated into the lipids and TCA-soluble compounds such as glycerol?

2) What is the pattern of fixed carbon incorporation into the lipids of the zooxanthellae and host tissues of *Anemonia viridis*?

3) What proportion of fixed carbon is incorporated into the wax esters, triglycerides and other lipid classes of whole-anemone tissues?

4) Is fixed ^{14}C excreted from the anemones?

To answer these questions, experiments were undertaken in which anemones were incubated in sea water containing $NaH^{14}CO_3$ and the accumulation of ^{14}C into the compartments and biochemical fractions derived from anemones was determined. Incubations were carried out with anemones which were firstly placed in darkness to allow all compartments to equilibrate with inorganic ^{14}C and then exposed to consecutive light and dark periods during which fixed ^{14}C accumulation was measured.

Materials and methods

Stock anemones

Anemonia viridis (Forskal) were collected from Loch Sween, Scotland and maintained for four months at 10 $\mu E\ m^{-2}\ s^{-1}$ under the same conditions as described in chapter 2. Low-light adapted anemones were used in this experiment because they would contain low levels of total and storage lipids (chapter 3) and by exposing them to a photosynthetically saturating light intensity, it was anticipated that high levels of carbon would be fixed and used in lipid biosynthesis.

Experimental design

$NaH^{14}CO_3$ (Amersham International PLC.) was added to sea water which had been sterilised with ultra-violet (UV) light and filtered (0.45 μm) to give a final concentration of 0.2 μCi $NaH^{14}CO_3$ ml^{-1}. 50 ml of radioactive sea water was added to each of 18 100 ml glass beakers which had previously been treated with Sigmacoat (Sigma Chemical Co.) to prevent mucus adhering to the glass. The beakers were placed in a water bath at 10°C under a bank of unlit fluorescent light tubes in the dark and each one was covered with a watch glass to reduce the exchange of labelled CO_2 with the atmosphere. One anemone was placed in each beaker and left for 2 h to allow the ^{14}C in the sea water to equilibrate with the anemone compartments (see below). The lights were then switched on to give a light intensity of 300 $\mu E\ m^{-2}\ s^{-1}$ at the level of the anemones. After 2 h, the lights were switched off and anemones were incubated for a further 2 h in darkness.

Two anemones were removed for analysis after the dark equilibration period and thereafter at 30 minute intervals for the duration of the experiment. Whilst one of the pair was being processed the other was kept in darkness to prevent further photosynthetic carbon fixation. Anemones were dissected on a steel block cooled to about 1 °C and at a background light intensity of 8 $\mu E\ m^{-2}\ s^{-1}$ in order to slow down metabolism .

Fixed carbon was measured in the following fractions:

1) Whole-anemone tissue and the total lipid and trichloroacetic acid (TCA) soluble fractions of whole-anemone tissue. The TCA-soluble fraction would be expected to contain low-molecular weight water-soluble compounds, including glycerol and glucose (Trench 1971 a, Szmant-Froelich 1981). No attempt was made to separate zooxanthellae from whole-anemone tissue in order to measure incorporation into the algal and host compartments. It was anticipated that a large proportion of ^{14}C would be present in water-soluble compounds and there would be exchange of labelled carbon between compartments during separation procedures (see below).

2) The total lipids of whole-anemone tissue and zooxanthellae. Incorporation of fixed ^{14}C into the total lipids of the host tissue compartment was determined by subtracting the values for zooxanthellae lipid from values for whole-anemone tissue lipid.

3) Whole-anemone lipid was also separated into the major lipid classes, which included the storage lipids wax esters and triglycerides.

Anemones were cut vertically into 3 approximately equal portions, each of which was rinsed thoroughly in fresh sea water, blotted dry, wet weighed and then frozen at -20°C. One portion (1) was freeze dried to constant weight before analysis of ^{14}C in whole-anemone lipid. A second (2) was used for the analysis of ^{14}C in zooxanthellae lipids. The remaining portion (3) was used for the analysis of total ^{14}C incorporated into the whole-anemone tissue and the TCA-soluble fraction. Each pair of specimens took less than 5 minutes to process.

Zooxanthellae were separated from portion 2 by homogenising the frozen anemone tissue in 40 ml of sea water and centrifuging the mixture at high speed (3,000 rpm, RCF 1,560 x g, Heraeus Minifuge RF) for 15 minutes at 10°C. The supernatant was then poured off and the resulting pellet resuspended in sea water before repeating the centrifugation procedure. This crude method of separation resulted in some contamination of the zooxanthellae pellet with animal cell debris and mucus. However, virtually all of the zooxanthellae were recovered in the pellet as microscopic inspection of supernatants showed that very few algal cells were lost. The isolated zooxanthellae were then frozen at -20°C.

Whole anemone and TCA-soluble fractions

Whole-anemone tissue (portion 3) was placed in a graduated centrifuge tube and sea water was added to give a total volume of 20 ml. The tissue was homogenised with an Ultra Turrax homogeniser for 30 sec. and a 0.25 ml aliquot removed for counting. A further 0.8 ml sample of the homogenised tissue was placed in an eppendorf tube and 0.2 ml of 20% TCA (Sigma Chemical Co.) was added. The mixture was shaken and then centrifuged (1,300 rpm, RCF 11,600 x g, MSE Microcentaur) for 5 minutes and a 0.25 ml sample of the supernatant counted.

Lipid fractions

Total lipid was extracted from freeze-dried whole-anemone tissue (portion 1) and isolated zooxanthellae (portion 2) using the methods described in chapter 2, and ^{14}C incorporation determined. The samples of whole-anemone lipid were then separated into wax esters, triglycerides and polar lipids by TLC. Lipids were co-chromatographed with wax ester and triglyceride standards.

Carbon loss

After removal of an anemone from a beaker, the radioactive sea water was filtered through a Whatman GF/C glass-fibre filter paper to separate the carbon of the soluble and particulate fractions. The filter paper and a 1 ml aliquot of the filtered sea water were frozen (-20°C) prior to measuring the radioactivity in each fraction.

Equilibration of ^{14}C pools and counting procedures

To estimate how long it would take for the inorganic ^{14}C to equilibrate with the water in the coelenteron and with the tissue compartments, anemones (n = 3) were placed in sea water containing ^{14}C bicarbonate and the depletion of the label from the medium measured in darkness. The anemone was first placed in a pre-weighed beaker of fresh sea water at 10°C which was then re-weighed to give an estimate of anemone volume, assuming the density of the anemone to be similar to that of sea water. The anemone was then placed in a 100 ml beaker containing 50 ml radioactive sea water (0.2 μCi ml^{-1} $NaH^{14}CO_3$) which was maintained at 10°C in a water bath in darkness. Radioactive sea water was used as a control. 0.25 ml samples of sea water were removed for counting at intervals of 0,5,10,15,20,30,60 and 90 minutes and the time at which equilibrium was reached noted. At equilibrium, the concentration of label (e) would be equal in sea water and anemone tissue and hence e would have the value:

$$e = \frac{\text{volume of sea water}}{\text{volume of sea water + volume of anemone}} \times \text{initial dpm ml}^{-1}\text{ sea water}$$

Equilibrium was reached between 60 and 90 minutes. There was no change in the counts in the control sea water.

All samples were counted with 5 ml scintillation fluid (Ecosint A, National Diagnostics) using a Philips PW 4700 liquid scintillation counter. When determining the radioactivity in the fixed carbon, samples were acidified to remove inorganic carbon as CO_2 by adding 200 μl 1N HCl to the scintillation vials and leaving them uncapped for 12 h in darkness. Counts per minute (cpm) were converted to disintegrations per minute (dpm) after correcting for sample blanks, counting efficiency and quenching.

Statistical procedures

In the experiment described in this chapter, only two anemones were sampled at each time interval and therefore interpretation of data based upon statistical analyses should be viewed with caution. All data were normalised to the dry weight of whole-anemone tissue and comparisons made using either a Student's *t*-test, analysis of variance or two-way analysis of variance. Data were arcsine-transformed (Sokal and Rohlf 1981).

Results

Incorporation of fixed ^{14}C into whole anemone-tissue

The total amount of fixed ^{14}C incorporated into whole-anemone tissue, the TCA-soluble fraction and the total-lipid fraction are shown in figure 4.1. and table 4.1.

Table 4.1. *Anemonia viridis*. Fixed ^{14}C incorporated into whole-anemone tissue and the TCA-soluble and lipid fractions of whole-anemone tissue. Data are dpm g^{-1} dry weight of whole-anemone tissue x 10^{-6}. Standard deviations are shown in parentheses; $n = 2$ except for whole-anemone tissue and TCA-soluble fractions at 0 and 3.5 h (*) where $n = 1$. ND: not determined.

Photoperiod	Time (h)	^{14}C incorporation dpm g^{-1} x 10^{-6}		
		Whole anemone	TCA-soluble	Lipid
Dark	0	ND	ND	ND
	2	0.3*	0.5*	0.01 (0.01)
Light	2.5	4.64 (0.40)	3.76 (0.46)	0.41 (0.08)
	3	7.15 (1.60)	4.78 (1.13)	1.44 (0.57)
	3.5	10.90*	6.48*	2.15 (0.66)
	4	12.80 (0.59)	6.89 (0.19)	2.80 (0.97)
Dark	4.5	14.07 (0.78)	6.79 (2.0)	5.04 (1.84)
	5	14.65 (0.77)	8.61 (0.76)	3.42 (0.90)
	5.5	18.96 (4.64)	10.39 (1.81)	2.96 (0.48)
	6	19.82 (3.28)	11.0 (2.07)	3.43 (1.49)

Whole-anemone tissue

The amount of fixed ^{14}C incorporated into whole-anemone tissues increased with time during the light and following dark period. Differences in the amount incorporated at each time interval in the light or the dark were found to be statistically significant (ANOVA, $P<0.05$). Dark fixation during the equilibration period was about 2% of the level of carbon fixed after 2 h in the light.

TCA-soluble fraction

Similar ^{14}C incorporation patterns to those of whole anemones were observed in the TCA-soluble fraction and differences were found to be statistically significant (ANOVA, $P<0.05$). The TCA-soluble fraction contained 55% of the total ^{14}C incorporated by whole anemones at the end of the 6 h experiment.

Total lipid fraction

Incorporation of ^{14}C into the total lipids increased in the light and differences at each time interval were statistically significant (ANOVA, $P<0.05$). However, there was no statistical difference between anemones sampled during the following dark period (ANOVA, $P>0.05$). The lipid fraction contained 17% of the total ^{14}C incorporated by anemones after 6 h.

The percentage of the total ^{14}C fixed by anemones and incorporated into the TCA-soluble and lipid fractions during the experimental period is shown in figure 4.2. After 30 minutes exposure to light, there was a large difference between the proportions of ^{14}C in the TCA-soluble and lipid fractions. However, the proportion of ^{14}C in the TCA-soluble fraction subsequently declined while that in the lipids gradually increased over time until 30 minutes had elapsed of the following 2 h dark period.

Incorporation of fixed ^{14}C into the lipids of zooxanthellae and host

Incorporation of fixed carbon into the zooxanthellae and host lipid compartments of *Anemonia viridis* is shown in figure 4.3. and table 4.2. In the light, the amount of ^{14}C in the algal lipid increased over time and differences in the amount incorporated at each time interval were statistically different (ANOVA, $P<0.05$). However, there were no differences in the amount incorporated at each time interval in the dark (ANOVA, $P>0.05$). In the host-tissue lipid, there was an increase in the amount of ^{14}C incorporated

over time in the light (ANOVA, $P<0.05$) and a decrease during the following dark period (ANOVA, $P<0.05$). There was no difference between the level of ^{14}C in the zooxanthellae and host lipids in the light (two-way analysis of variance, $P>0.05$) but in the dark, zooxanthellae contained more ^{14}C than the host tissues, in which the levels declined to zero at 6 h (two-way analysis of variance, $P<0.05$).

Table 4.2. *Anemonia viridis*. Fixed ^{14}C incorporated into the zooxanthellae and host-tissue lipids. Values for host-tissue lipids were derived by subtraction of zooxanthellae values from those of whole anemones (table 4.1) and the figure for the host tissues at 6 h appears negative. Data are dpm g^{-1} dry weight of whole-anemone tissue x 10^{-6}. ND not determined. Figures in parentheses are standard deviations, $n = 2$.

Photoperiod	Time (h)	^{14}C incorporation dpm g^{-1} x 10^{-6}	
		Zooxanthellae	Host tissue
Dark	0	ND	ND
	2	0.01 (0.002)	0 (0.003)
Light	2.5	0.27 (0.002)	0.13 (0.006)
	3	0.79 (0.24)	0.65 (0.33)
	3.5	1.40 (0.74)	0.75 (0.31)
	4	1.45 (0.30)	1.35 (0.66)
Dark	4.5	3.16 (2.49)	1.88 (0.32)
	5	2.49 (0.97)	0.93 (0.06)
	5.5	2.92 (1.58)	0.05 (0.11)
	6	3.44 (1.90)	-0.01 (0.41)

Incorporation of fixed^{14}C into the lipid classes of whole-anemone tissues

Incorporation of ^{14}C into the lipid classes of whole-anemone tissue is shown in figure 4.4. and table 4.3. Of the lipid classes which were identified, most of the ^{14}C was found in the triglycerides and wax esters. On average, there was about four times as much ^{14}C in the triglycerides as in the wax esters. In all lipid classes which incorporated fixed ^{14}C in the light, there was an increase in levels over time (ANOVA, $P<0.05$). In the following dark period, the amount of ^{14}C in these lipid classes remained relatively constant and no significant differences were observed (ANOVA, $P>0.05$). The n-alkanes did not become labelled until 6 h exposure to ^{14}C.

Table 4.3. *Anemonia viridis*. ^{14}C incorporated into the lipid classes of whole anemones. Data are dpm g^{-1} dry weight of whole-anemone tissue x 10^{-6}. WE: wax esters; TG: triglycerides; ND: not determined. Standard deviations are shown in parentheses, $n = 2$.

Photoperiod	Time (h)	^{14}C incorporation dpm g^{-1} x 10^{-6}				
		n-alkanes	polar lipids	WE	TG	other lipids
Dark	0	ND	ND	ND	ND	ND
	2	0	0.003 (0.003)	0.001 (0.001)	0.01 (0.01)	0
Light	2.5	0	0.04 (0.001)	0.04 (0.01)	0.15 (0.04)	0.18 (0.01)
	3	0	0.08 (0.01)	0.09 (0.01)	0.48 (0.11)	0.78 (0.04)
	3.5	0	0.14 (0.01)	0.12 (0.02)	0.82 (0.14)	1.05 (0.05)
	4	0	0.21 (0.08)	0.29 (0.16)	1.13 (0.17)	1.12 (0.09)
Dark	4.5	0	0.25 (0.11)	0.35 (0.30)	2.02 (0.79)	2.43 (0.14)
	5	0	0.40 (0.25)	0.67 (0.12)	0.99 (0.13)	1.36 (0.10)
	5.5	0	0.29 (0.10)	0.29 (0.19)	1.26 (0.18)	1.12 (0.01)
	6	0.07 (0.05)	0.42 (0.29)	0.42 (0.01)	1.22 (0.38)	1.29 (0.10)

Loss of fixed ^{14}C

The amount of fixed particulate ^{14}C lost from anemones which was collected on GF/C filter papers is shown in table 4.4. There were no significant differences in the amount of particulate carbon excreted during the 2 h light and following 2 h dark period (ANOVA, $P>0.05$). Losses during the equilibration period were about 8% of the average lost during the 2 h light period and the following 2 h dark period. The average CPM in the soluble carbon fraction over the light and following dark period were 27.5 and this was not significantly different from sea-water controls which contained 25 CPM (Student's *t*-test, $P>0.05$).

Table 4.4. *Anemonia viridis.* Fixed ^{14}C excreted by anemones and collected on GF/C filter papers after filtration of the sea-water incubation medium (see methods). Data are dpm g^{-1} dry weight of whole-anemone tissue x 10^{-3}. ND not determined. Standard deviations are shown in parentheses, $n = 2$.

Photoperiod	Time (h)	^{14}C incorporation dpm g^{-1} x 10^{-3}
Dark	0	ND
	2	1.37 (1.22)
Light	2.5	9.67 (2.46)
	3	7.67 (5.37)
	3.5	14.16 (14.59)
	4	36.50 (21.01)
Dark	4.5	6.69 (0.04)
	5	16.10 (5.28)
	5.5	28.63 (32.40)
	6	8.91 (10.64)

Discussion

Labelled carbon was fixed and incorporated into whole-anemone tissues in the dark and in the light. The carbon fixed during the dark equilibration period was equivalent to 2% of that fixed after 2 h in the light and similar values have been reported for other symbiotic anthozoa (Muscatine and Cernichiari 1969, Trench 1971 a, Patton et al. 1983, Crossland et al. 1980 a, b). In the light, fixed ^{14}C levels increased over time and this appeared to continue at about the same rate in anemones which were incubated for a further 2 h of darkness. It had been anticipated that the levels of ^{14}C fixation would be similar in both dark periods but during the second dark period it was on average about 25 times higher than during the equilibration period. This pattern of ^{14}C incorporation in the whole-anemone compartment was also reflected in the TCA-soluble fraction but not in the total lipid fraction. It is possible that the ^{14}C fixed by anemones in the second dark period simply reflects experimental variability due to the small sample size used. However, continued incorporation of ^{14}C by anemone tissue and the TCA-soluble fraction in the

second dark period could also be explained by a switch to dark fixation of $^{14}CO_2$. In certain plants, carbon is fixed in the dark by crassulacean acid metabolism (CAM) (Hall and Rao 1981). CAM plants fix CO_2 in the dark to produce the C_4 compounds, oxaloacetic and malic acids and this depends upon the build up of an energy source and pyruvic acid which is the precursor of oxaloacetic and malic acid. If the zooxanthellae of *Anemonia viridis*, during exposure to saturating light, accumulate pyruvic acid and compounds which could be used to produce energy, they may continue to fix CO_2 in the dark as malic acid. Malic acid would be found in the TCA-soluble fraction. If at 10 μE m^{-2} s^{-1} the levels of pyruvate and energy source are low, there would be little subsequent dark CO_2 fixation. Clearly, dark-carbon fixation by *A. viridis* needs to be investigated thoroughly.

After exposure to saturating light, more fixed ^{14}C was incorporated into the TCA-soluble fraction than the lipid fraction. In contrast, Trench (1971 a) found that more fixed ^{14}C was incorporated into the lipid fraction of *Anthopleura elegantissima* after 24 h incubations in either the light or the dark. In this study, about 80% of the total ^{14}C was initially found in the TCA-soluble fraction. This percentage steadily declined and at the same time the ^{14}C content of the lipids increased suggesting that TCA-soluble compounds such as glycerol and acetate were being used for fatty acid and lipid biosynthesis. Battey and Patton (1987) found that the glycerol pool turned over rapidly in *Condylactis gigantea* during a 1 h incubation with labelled carbon resulting in the accumulation of translocated carbon as lipid. However, the data in the present study do not support rapid turnover of glycerol as the loss of the label from the TCA-soluble compounds was slow.

Labelled carbon was fixed and incorporated into the lipids of both the zooxanthellae and host-tissue compartments of *Anemonia viridis*. In the light, the amount of ^{14}C accumulated in zooxanthellae lipids increased with exposure time. Although this increase continued in anemones sampled after 30 minutes of the following dark period, dark levels remained constant over the 2 h suggesting no further incorporation of fixed carbon into the lipids. In the host-tissue compartment, there was also a gradual increase in lipid ^{14}C during the light and for the first 30 minutes of darkness. However, levels then fell until there was virtually no ^{14}C left in host-tissue lipid by the end of the 2 h dark period. This pattern of carbon fixation suggested that the ^{14}C recently incorporated into the host lipid in the light was rapidly metabolised in the dark. Part of the loss of label from host lipid in the dark could also be explained if there was back-flux of carbon ^{14}C from host to zooxanthellae and there is some evidence to suggest that this could occur. Steen (1987), working with cultured zooxanthellae, observed that the algal cells could grow in darkness by assimilating exogeneous organic compounds and it was suggested that zooxanthellae would also have the potential to be heterotrophic in the intact symbiosis. Further evidence that zooxanthellae can assimilate host carbon has been provided from studies on the freshwater

hydra-*Chlorella* symbiosis. Douglas and Smith (1983) showed that the growth rate of symbiotic hydra declined more rapidly than aposymbiotic hydra in the dark suggesting that the symbionts represented a cost to the host. Furthermore, algae continued to grow and divide while the host was being fed and maintained in darkness indicating that nutrients were supplied to the algae from the host (Douglas and Smith 1984).

Each lipid class showed an essentialy linear, light dependent, accumulation of ^{14}C which continued for 30 minutes into the following dark period although levels remained relatively constant in darkness. About half of the total labelled lipid comprised wax esters and triglycerides in anemones sampled at the begining of the light period, and more ^{14}C was incorporated into triglycerides than wax esters. However, the amount of fixed carbon incorporated into non-storage lipids (polar lipids + other lipids) gradually increased until they formed the major labelled lipid components. These data agree well with those of Crossland et al. (1980 b) who observed that in *Acropora acuminata*, ^{14}C was mainly incorporated into phospholipids, sterols and fatty acids. It was suggested that non-storage lipids were synthesised immediately after dawn and that wax esters and triglycerides were synthesised later in the day. In the present study, wax ester ^{14}C was probably present only in the host-tissue compartment as it was shown in chapter 2 that zooxanthellae contain only small quantities of this lipid.

Less than 1% of the maximum amount of carbon fixed by *Anemonia viridis* was detected in the incubation medium. Similar results were shown by Lewis and Smith (1971) for a range of 12 symbiotic coelenterate species incubated in labelled bicarbonate in the light for periods of 2 to 4 h. However, Crossland et al. (1980 b) estimated that 40% of the net carbon fixed by *Acropora acuminata* could be lost as mucus and mucus-lipid. In the present study, anemones were adapted to low light and were assumed to contain low levels of storage lipid at the start of the experiment (chapter 3). All the excess carbon fixed by the zooxanthellae may therefore have been used in lipid biosynthesis. If anemones had been pre-adapted to high light, they would have contained higher levels of storage lipid and it is likely that more fixed carbon would have been lost. This hypothesis is supported by the observations made in chapter 6 which showed that high-light adapted anemones lost a large proportion of the total carbon fixed over a 24 h period.

Excreted ^{14}C was only detected in the particulate fraction of the filtered sea-water incubation-medium. These results appear to contradict those of experiments described in chapter 6 in which direct measurement showed that most of the organic carbon lost from anemones was dissolved in the sea water. If the fixed ^{14}C had been excreted as mucus (Crossland 1987), then the small amount of ^{14}C loss may reflect the length of time taken for the fixed ^{14}C to equilibrate with the mucus pool. However, all the excreted ^{14}C detected in this study (< 1% of the total fixed) could also be accounted for by the loss of

zooxanthellae (Hoegh-Guldberg et al. 1987). Zooxanthellae and zooxanthellae pellets (see Steele 1977) were observed on some filter papers and it was noted that these samples gave comparatively high counts which resulted in the large standard deviations observed in the data. Mucus and mucus-lipid excretion are investigated in more detail in the next chapter.

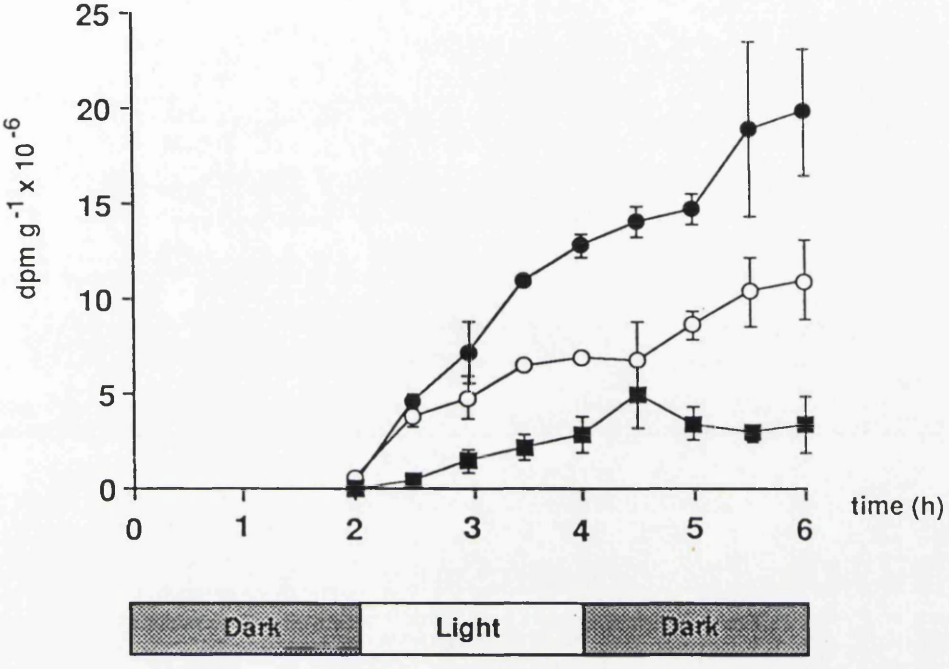


Figure 4.1. *Anemonia viridis*. Fixed ^{14}C incorporation into whole-anemone tissue (•) and the TCA-soluble (o) and lipid fractions (▪) of whole-anemone tissue. Anemones were maintained at 10 μE m^{-2} s^{-1} for 2 months before exposure to $NaH^{14}CO_3$ in the dark for 2 h to equilibrate all compartments with inorganic ^{14}C. Anemones were then exposed to 300 μE m^{-2} s^{-1} for 2 h followed by 2 h in darkness. Data are dpm g^{-1} of whole-anemone dry weight x 10^{-6}. Error bars are standard deviations. Values for *n* are given in table 4.1.

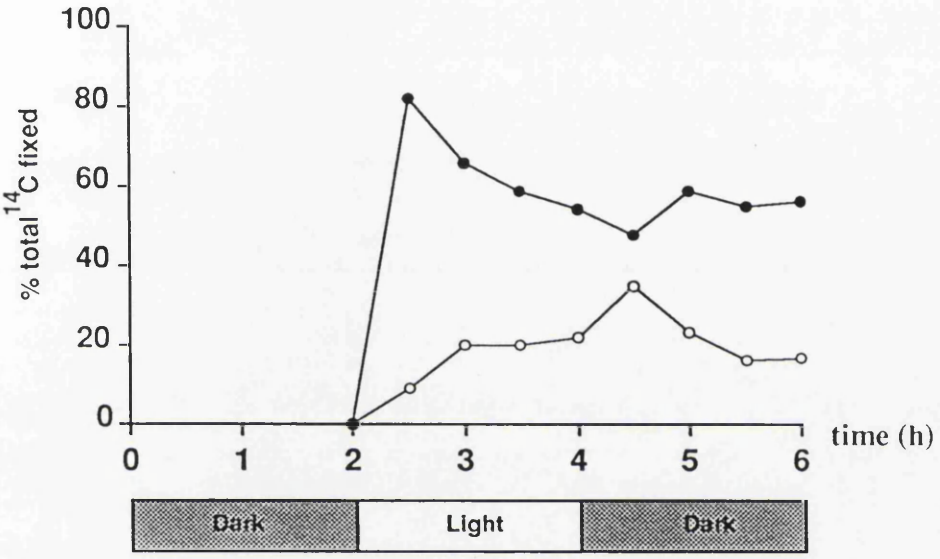


Figure 4.2. *Anemonia viridis*. % of total ^{14}C fixed by whole anemones and incorporated into the TCA-soluble fraction (•) and the lipid fraction (o). Further details as in legend to figure 4.1.

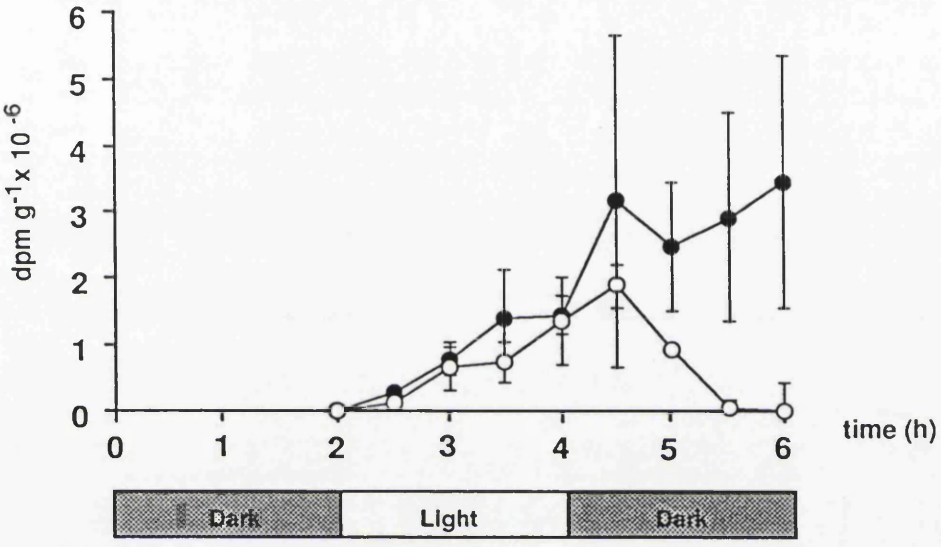


Figure 4.3. *Anemonia viridis*. Fixed ^{14}C incorporated into the lipids of the host (o) and zooxanthellae (•) compartments. Data for the host compartment were estimated by subtracting the values of zooxanthellae lipid ^{14}C from those of whole-anemone lipid ^{14}C. Further details as in legend to figure 4.1. $n = 2$.

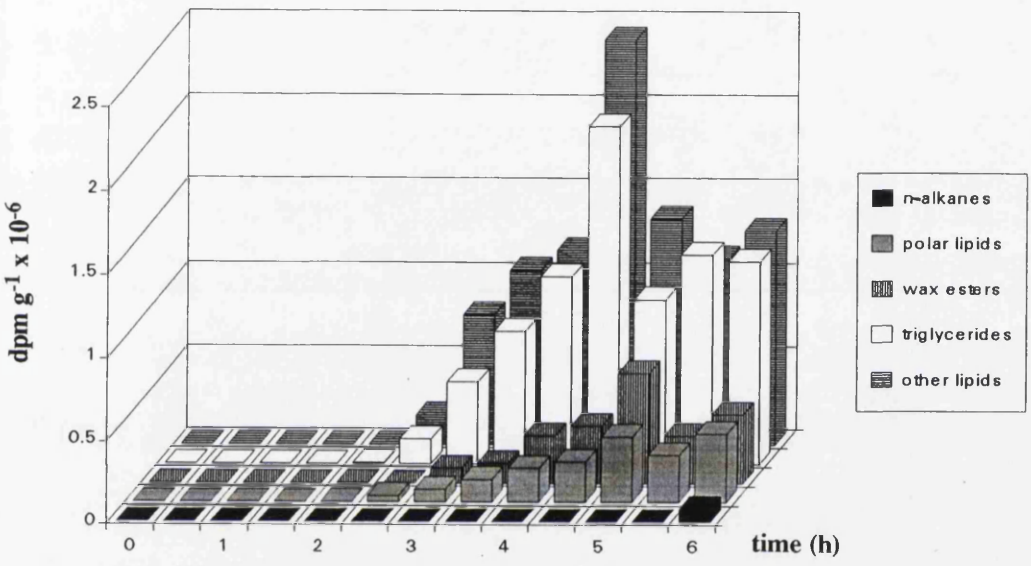


Figure 4.4. *Anemonia viridis*. Fixed ^{14}C incorporation into the lipid classes of whole anemone tissue. Lipid classes were separated by TLC (chapter 2). The solvent system used leaves polar lipids on the origin of the TLC plate. Wax esters and triglycerides were identified by comparing R_f's with authentic standards. All lipids running between the polar lipids and the triglycerides were termed "other lipids". A lipid group located above the wax esters in anemones at 6 h probably contained n-alkanes. Data are means (n = 2). Standard deviations were omitted for clarity. Further details as in legend to figure 4.1.

Chapter 5

Location of lipid in the symbiotic anemone *Anemonia viridis* and the movement of photosynthetically fixed carbon between the tentacles and mesenteries.

Introduction

Carbon fixed in photosynthesis may be incorporated into the lipids of both the zooxanthellae and host tissues of *Anemonia viridis* (chapter 4). Davies (1991) suggested that any fixed carbon in excess of the requirements for respiration and growth would either be stored as lipid, or if the lipid stores were replete, would be excreted. In the previous chapter, it was shown that under the conditions of the experiment, small amounts of fixed ^{14}C were excreted from *A. viridis* although the actual ^{14}C compounds released were not determined. However, it has been shown that carbon may be lost as "mucus", mucus and mucus-lipid from corals and anemones (Benson and Muscatine 1974, Ducklow and Mitchell 1979, Crossland 1980 b, Krupp 1981, Crossland 1987). (The terms "mucus", mucus and mucus-lipid have been defined on page 9). Anthozoan mucus is composed of polysaccharides and proteins (Krupp 1985) but it may also contain zooxanthellae (Harland and Nganro 1990), bacteria (Ducklow and Mitchell 1979) sediment debris (Lewis 1973) and lipid (Crossland et al. 1980 b, Crossland 1987). In the present study, "mucus" is the particulate and dissolved organic carbon (P/DOC) excreted from the host and this may include mucus, organic contaminants and mucus-lipid. The mucus-lipid may be covalently or non-covalently bonded to the "mucus".

The distribution of lipids appears to be similar in symbiotic anemones and corals (Kellog and Patton 1983, Stimson 1987). Kellog and Patton (1983) found that most of the storage lipid was located in the polyp column of the anemone *Condylactis gigantea*. Stimson (1987) showed that storage lipid deposits were mainly present in the lower part of the body wall and basal disc of three species of coral and suggested that this distribution was an adaptation to predation of polyp tissue by grazing fish which could remove the tentacles but not the inaccessible lipid-rich body tissues.

Although most lipid appears to be stored in the polyp body, up to 95% of the zooxanthellae may be present in tentacles (Kellog and Patton 1983) and therefore the majority of carbon is probably fixed in these tissues. In the previous chapter it was demonstrated that ^{14}C may be incorporated directly into lipid in the zooxanthellae and this has also been shown for other anthozoans (Patton et al. 1977, 1983). However, carbon could also be incorporated into lipid in the gastrodermal cytoplasm of the tentacles, or lipid precursors could be transported to other parts of the anemone for lipid biosynthesis. If incorporation of fixed carbon into lipid does take place mainly in the zooxanthellae or host-

tentacle tissue, then transport as lipid to other regions of the anemone must take place. Kellog and Patton (1983) observed lipid droplets within the gastrodermal cells, adjacent to the zooxanthellae and also free in the tentacle lumen. These authors suggested that the free droplets originated from the tentacles and were circulated in the coelenteron to transport lipid to other tissues within the polyp. Lipids would therefore be excreted and re-absorbed by the gastrodermal cell layer. Crossland et al. (1980 b) showed micrographs in which crystals of wax ester were situated adjacent to mucous gland cells of the gastrodermis of *Acropora acuminata* and it was suggested that when mucus was released, the wax esters could also be lost from the coral.

These observations on lipid distribution and carbon excretion raised several questions:

1) How is lipid distributed in the tissues of *Anemonia viridis* and where are the lipid stores located?

2) What is the distribution of zooxanthellae and hence the sites of photosynthetic carbon fixation?

3) Are carbon compounds transported within anemone tissues? If carbon is fixed in the tentacles is it then transported to the mesenterial gastrodermis, a possible site from which mucus-lipid could be excreted?

2) Is carbon lost from anemones as "mucus" and mucus-lipid and how are the mucous gland cells distributed?

Materials and method

Maintenance of anemones

Anemonia viridis were collected from the Isle of Cumbrae and maintained at 10 μE m^{-2} s^{-1} at 10°C for about 1 year (chapter 2).

Distribution of total lipid

Five anemones were placed in beakers containing 100 ml of sea water and narcotised by the gradual addition of 100 ml of 0.36M $MgCl_2.6H_2O$ over a period of 1 h. Relaxed anemones were dissected and divided into tentacles, oral disc, mesenteries and body wall.

These were then placed into separate pre-weighed glass vials and freeze dried. The dry weight of the tissues was determined and lipids extracted using the methods described in chapter 2.

Location of storage lipids

To determine the location of the storage lipids (wax esters and triglycerides) tissue sections were stained with Sudan IV for neutral lipid (Bancroft and Stevens 1990). Several small pieces of tentacle, oral disc, body wall and mesentery were removed from a narcotised anemone which had been previously adapted to a light regime of 300 μE m^{-2} s^{-1} as described in chapter 3. It was anticipated that large quantities of storage lipid would be present in a high-light adapted anemone. Pieces of tissue were fixed in formol calcium for a minimum of 24 h. Formol calcium was prepared as follows:

10% formalin	100 ml
10% calcium chloride	100 ml
Sea water (33°/oo)	900 ml

The fixed tissues were mounted and supported on cork tiles with cryostat embedding medium (Tissue-Tek O.C.T., Miles Scientific). The preparation was floated specimen side down in liquid nitrogen for about 10 s to freeze the tissues. The preparation was fixed to a pre-cooled (-30°C) cryostat chuck with O.C.T., placed in the cryostat cabinet (Bright Starlet 2212, Bright Instrument Co. Ltd.) at -30°C and left for about 1 h before cutting 12 μm sections. Sections were picked up on coverslips prior to staining. Sudan IV stains other tissue components as well as storage lipids but it can be removed from tissues by differentiating in 70% alcohol until only the storage lipids remain stained. In order to determine the length of time for differentiation, sections which had all lipid removed by delipidisation were stained with Sudan IV and differentiation was carried out until no colour remained in these sections. Positive controls were provided by using both rat adipose tissue and cod liver oil applied directly to cover slips. All sections were processed simultaneously.

Delipidisation

Lipids were extracted from control sections by placing them in the following solvent for 15 minutes (Bancroft and Stevens 1990).

Chloroform	66 ml
Methanol	33 ml
Distilled water	4 ml
Conc. HCl	1 ml

Staining method

1 A saturated solution of Sudan IV in 70% alcohol was made
2 Sections were immersed in the stain for 24 h
3 Differentiation was carried out with 70% alcohol until a delipidised section appeared colourless
4 Coverslips were mounted on glass slides in Aquamount (BDH Chemicals)

Sudan IV stains neutral lipids, including wax esters and triglycerides red.

Translocation of lipid

Location of zooxanthellae

Several pieces of tentacle, oral disc, body wall and mesentery were dissected from 3 narcotised anemones and fixed in formol calcium. They were then dehydrated, cleared and embedded in paraffin wax. Sections (6 μm) were cut, stained with haematoxylin and eosin and examined for the presence of zooxanthellae.

Staining method

1	Histoclear I	5-10 min.
2	Abs. alcohol	3-5 min.
3	Sections to water through graded alcohols	
4	Harris's haematoxylin	3 min.
5	Wash in running water until clear	
6	Differentiate in acid alcohol	4-5 s
7	Wash in running tap water	
8	Scott's tap water until blue	
9	Wash in running tap water	
10	Examine under microscope	
11	Differentiate nuclear stain further if necessary	

(continued)

12	Counterstain in Pott's eosin (aqueous)	30 s
13	Differentiate in running tap water	
14	Dehydrate through graded alcohols	1 min. each
15	Histoclear II	2 min.
16	Histoclear III	2 min.
17	Mount sections in Histomount	

^{14}C autoradiography

Macro-autoradiography was used to ascertain if the carbon fixed by the zooxanthellae in the tentacles could be translocated to the mesenteries. Anemones were exposed to ^{14}C for 2 and 4 h in the light. At the end of each period, pieces of tentacle and mesentery were removed from the anemone and examined for the appearance of the label.

Experimental procedure

Three anemones were incubated in sea water containing 1 μCi ml^{-1} $NaH^{14}CO_3$. Each anemone was placed in a 100 ml beaker containing 50 ml of the labelled sea water and maintained at 10°C in a water bath. The beakers were kept in darkness for 2 h in order to allow all compartments to equilibrate with inorganic carbon and then illuminated at 300 μE m^{-2} s^{-1} for 4h. Anemones were sampled after 2 h in darkness (control) and then after 2h and 4h in the light. Sampled anemones were rinsed in fresh sea water and then narcotised (see above) in unlabelled sea water in darkness. Pieces of tentacle and mesentery were removed from each anemone. To preserve ^{14}C labelled lipid, tissues were fixed in glutaraldehyde and osmium tetroxide. This method fixes unsaturated lipid and was chosen in preference to the use of frozen sections in order to minimise the radioactive contamination of equipment.

Method:

Fixative

25% glutaraldehyde	6 ml
0.2M phosphate buffer	50 ml
0.1% calcium chloride	2 ml
distilled water	42 ml
sucrose	1 g

Buffer

0.2M phosphate buffer	50 ml
0.1% calcium chloride	2 ml
distilled water	48 ml
sucrose	1 g

Tissues were fixed for 2 h and transferred to phosphate buffer:

1	Phosphate buffer rinse I	overnight
	Phosphate buffer rinse II	3 x 5 min.
2	1% osmium tetroxide	1 h.
3	Distilled water	3 x 10 min.
4	0.5% uranyl acetate	1 h. in dark
5	Rinse in distilled water	
6	Bring to 70% alcohol	10 min. each
7	1% *p*-phenylenediamine	1 h.
8	Bring to absolute alcohol	10 min. each
9	Epoxypropane	3 x 10 min.
10	1:1 araldite embedding mixture:epoxypropane	overnight
11	Araldite, vacuum embed	8 h
12	Polymerise at 60°C	48 h.

Sections (1 μm) were cut with a glass knife on an ultra microtome (Reichart OME3) and mounted on glass slides. Sections were counterstained with toluidine blue so that they could be examined for the presence of zooxanthellae.

Autoradiographic detection

Double coated Kodak X-OMAT AR film (Imperial Biochemical Industries (IBI) Ltd.) was placed on the glass slide in direct contact with the labelled specimen. The film was secured to the slide with PVC tape at each end and the preparation placed in a light tight box at 4°C. After exposing the films for 4 weeks, they were processed using Kodak LX-24 developer (5 min.) and Kodak FX-40 fixer (4 min.). As emulsion was present on both sides of the film, the PVC tape was removed from one end during processing to allow solutions to come in contact with both surfaces. This procedure did not alter the position of the autoradiograph with respect to the specimen. After processing, autoradiographs were removed from the slides and photographed and the remaining tissue sections examined for

the presence of zooxanthellae.

Lipid excretion

Detection of lipid in "mucus" samples

Six anemones were buoyant weighed (Davies 1988) and then placed in 150 ml beakers containing 100 ml filtered and sterilised sea water. The beakers were placed in a water bath at 10°C under a bank of fluorescent light tubes at 300 μE m^{-2} s^{-1} for 12 h. At the end of this period, the "mucus" and mucus-lipids were collected by filtering the sea water through a Whatman GF/C filter paper. The beaker was also swabbed with a second GF/C paper. Lipid was extracted from the filters using the methods described in chapter 2. Total lipids were weighed on a Sartorius Supermicro balance and normalised to anemone dry weight which was estimated from a regression of anemone buoyant weight against dry weight (chapter 3). As there was only a small amount of total lipid, the 6 samples were combined before separating the lipid classes by thin-layer chromatography (chapter 2).

Location of mucous gland cells

Tissue sections were prepared as described in "location of zooxanthellae" and stained for neutral and acid mucins with alcian blue, periodic acid and Schiff's reagent.

Staining solutions

Alcian Blue

1	Alcian blue 8GS	0.5 g
2	Glacial acetic acid	3 ml
3	Distilled water, make up to	100 ml
4	Filter and add a crystal of thymol	

Periodic acid

1	Periodic acid	1 g
2	Distilled water	200 ml

Schiff's reagent

1 1g basic fuschin dissolved in 200 ml boiling water
2 Allow to cool to 50°C
3 Add 2 g potassium metabisulphite and mix
4 Allow to cool to room temperature
5 Add 2 ml conc. hydrochloric acid and 2 g activated charcoal
6 Allow to stand overnight in the dark
7 Filter (Whatman no. 1)

Staining procedure

1	Bring to water through graded alcohols	
2	Alcian blue	5 min.
3	Periodic acid	7 min.
4	Wash in running tap water	7 min.
5	Schiff's reagent	10 min.
6	Wash in running tap water	10 min.
7	Haematoxylin	10 sec.
8	Wash in running tap water	2 min.
9	Scott's tap water	5 sec.
10	Wash in running tap water	2 min.

Acid mucins appeared blue, neutral mucins appeared magenta and mixtures of acid and neutral mucins appeared purple.

Results

Distribution of total lipid

The total lipid content of the tentacles, oral disc, mesenteries and body wall of *Anemonia viridis* is shown in table 5.1. As a percentage of dry tissue weight, the tentacles, oral disc and body wall contained between about 8 and 11% lipid. The mesenteries contained lower levels with about 3% of dry weight as lipid. When the total anemone lipid was partitioned, it was found that about 40% was located in the tentacles and about 20% in each of the oral disc, mesenteries and body wall.

Table 5.1. *Anemonia viridis*. Location of total lipid in anemones maintained under a light regime of 10 μE m^{-2} s^{-1}. Anemone tissues were divided into tentacles, oral disc, mesenteries and body wall, n = 6. Standard deviations are shown in parentheses.

Tissue	Lipid	
	% dry weight	% total lipid
Tentacle	7.83 (3.82)	43.08 (6.35)
Oral disc	10.79 (1.72)	18.53 (1.42)
Mesenteries	2.22 (1.37)	20.86 (2.41)
Body wall	8.20 (2.84)	17.54 (3.35)

Location of storage lipids

Sudan IV stained neutral lipids red in positive controls and anemone sections. Neutral lipid was located almost exclusively in the gastrodermal cell layer of the oral disc (figure 5.1a), mesenteries and body wall. It was not detected in tentacle tissues (figure 5.1b).

Translocation of lipid

Location of zooxanthellae

Zooxanthellae were located in the gastrodermal cell layer of the tentacle, oral disc and body wall. They were absent from mesenteries. A section of tentacle is shown in figure 5.2., and sections of tentacle and mesenteries in figure 5.4.

^{14}C autoradiography

Photographs of the exposed autoradiographic films are shown in figure 5.3. Control sections of both tentacle and mesentery did not incorporate ^{14}C. The tentacles from anemones maintained for 2 h in the light incorporated ^{14}C which is shown on the film by the dark images of the tentacle sections. After 4 h in the light, the images appear to be darker and more diffuse suggesting that more label was incorporated into tentacle tissue after this time. There was no image in sections of mesentery after 2 h in the light but a faint image after 4 h. Zooxanthellae were present in all autoradiographed sections of tentacle but not in the sections of mesentery (Figures 5.4 a, 5.4 b).

Lipid excretion

Detection of mucus-lipid in "mucus" samples

The mucus-lipid content of the "mucus" collected from anemones is shown in table 5.2. Mucus-lipid was not detected in one sample. Qualitative TLC separation of mucus-lipids showed that they were were composed of wax esters, triglycerides, phospholipids and some unidentified pigments.

Table 5.2. *Anemonia viridis*. Mucus-lipid excreted by 6 anemones which were adapted to 10 μE m^{-2} s^{-1} and then exposed to 300 μE m^{-2} s^{-1} for a period of 12 h. "Mucus" and mucus-lipid were collected on GF/C filter papers. Anemones were buoyant weighed (chapter 3) and the dry weight calculated from a regression of buoyant weight against dry weight. ND: not detected.

Sample number	Anemone dry weight (mg)	Mucus-lipid (mg)	%anemone dry weight
1	970	0.88	0.091
2	750	1.44	0.192
3	1030	0.72	0.070
4	1050	0.73	0.070
5	690	ND	ND
6	830	0.81	0.098
mean		0.76	0.087
±SD		(0.46)	(0.062)

Location of mucous gland cells

The majority of the mucous gland cells were located in the epidermal cell layer of the tentacles, oral disc and body wall. However, they were also present in the gastrodermis of the mesenteries although they were less numerous than in the epidermal cell layers. Examples of mucous cells in the the body wall and mesentery are shown in figures 5.5 a and 5.5 b respectively.

Discussion

In *Anemonia viridis*, approximately 60% of the total lipid was found in the polyp column (oral disc plus body wall and mesenteries) and 40% in the tentacles. Kellog and Patton (1983) demonstrated that about 90% of the total lipid in the tropical symbiotic anemone *Condylactis gigantea* was located in the polyp column and only 10% in the tentacles. This lower level of tentacle lipid may simply reflect differences in the proportion and location of

storage lipid in each anemone. *C. gigantea* contained 43% of dry weight as lipid of which 86% was storage lipid and this was located mainly in the coral polyp. The tentacle lipid contained mainly phospholipid, probably from zooxanthellae membranes as 95% of algal cells were located in the tentacles. Assuming that *A. viridis* contained only 11% total lipid of which 6% was storage lipid (chapter 2), and that both species of anemone had similar amounts of zooxanthellae and structural lipid in the tentacles, this may account for the comparatively high proportion of lipid found in the tentacles of *A. viridis*.

The major storage lipids in *Anemonia viridis* are the wax esters and triglycerides (chapters 2 and 3). Staining with Sudan IV showed that storage lipids were located almost exclusively in the gastrodermal cell layer of the polyp although they were not present in tentacles. Stimson (1987) found that storage lipids were mainly present in the lower half of the coral polyp in five species of coral. In two species investigated in more detail, storage lipids were also found only in the gastrodermal cell layer. Circumstantial evidence was presented to show that storage lipids were also present in the mesoglea of *Pocillopora damicornis* and *P. meandrina*. This evidence was based upon the vacuolated appearance of the mesoglea, and the assumption that this was caused by the solution of lipids by the solvents used in wax embedding. Sudan IV stained storage lipids were not found in the mesoglea of *A. viridis*.

Stimson (1987) suggested that for corals, it would be adaptive to store lipid in the lower half of the polyp which is protected from predators by the skeletal calyx. If the majority of lipid is produced in the tentacles as suggested by Kellog and Patton (1983) and Battey and Patton (1984), then this lipid may be translocated from the tentacles of *Anemonia viridis* to the rest of the polyp where it can be stored. To explain how the lipid could be transported, Kellog and Patton (1983) suggested that the zooxanthellae of the tentacles packaged lipid droplets into membrane bound vesicles which were then transported to the host tissues and then passed by exocytosis into the lumen of the tentacles. Contraction of the tentacles and the beating of the gastrodermal cell flagella would result in lipids being passed into the coelenteron of the column where they could be re-absorbed by the gastrodermal cells. The mesenterial tissues are known to transport other metabolites and they are the main region for phagocytosis and the excretion of faecal and zooxanthellae pellets (Van-Praet 1985).

In *Anemonia viridis*, zooxanthellae were found in the gastrodermis of the tentacles, oral disc and body wall whereas the storage lipids were found in the gastrodermis of the oral disc, body wall and mesenteries. If storage lipid is produced mainly by the zooxanthellae in the tentacles and is not stored in this region, then it may be moved to other tissues, such as the mesenteries which were shown to contain storage lipid but no zooxanthellae. Evidence from the autoradiography experiment suggested that fixed ^{14}C could be translocated within the anemone.

Analysis of "mucus" samples by TLC showed that mucus-lipid was lost from anemones. Davies (1984) estimated that 48% of the photosynthetically-fixed energy may be lost from *Pocillopora eydouxi*. It was suggested that corals may not be able to use all the carbon which can be produced during photosynthesis and therefore must secrete mucus or mucus-lipid in order to get rid of the excess. For *Anemonia viridis*, it has been shown that although carbon may be excreted continuously, the rate of particulate carbon excretion is light dependent (chapter 6). Similarly, Crossland (1987) demonstrated that mucus and DOC-lipid secretion rates in two coral species increased during the day. This author also suggested that the release of carbon was an inevitable by-product of phototrophic nutrition. Therefore, as most carbon is fixed during the day, and mucus-lipid is also released during this period, it is thought that the excretion of mucus-lipid is a way in which excess photosynthetically fixed carbon is eliminated.

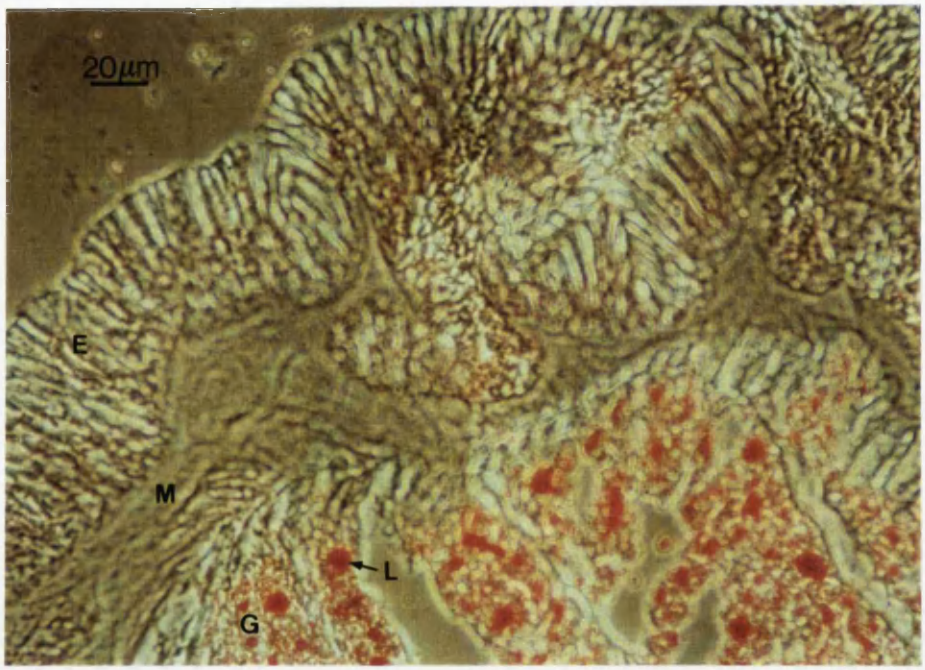
The mucus-lipid content of "mucus" samples have been shown to be highly variable and may range from 0 to 42% of the "mucus" dry weight (Ducklow and Mitchell 1979). The mucus-lipid excreted by *Anemonia viridis* was also variable and in one sample, no mucus-lipids were detected. The mucus-lipid contained mainly wax esters, triglycerides and phospholipids. However, other studies have shown different mucus-lipid compositions. Benson and Muscatine (1974) found that *Goniastrea retiformis* mucus-lipid contained mainly wax esters with some triglyceride. Crossland et al. (1980 b) analysed mucus-lipid from *Acropora acuminata* which contained mainly wax esters and triglycerides but also phospholipids, sterols and fatty acids. However, Crossland (1987) found only wax esters and phospholipids in the mucus-lipids of *Acropora variabilis* and *Stylophora pistillata*.

The variability in the composition of mucus-lipid may depend upon previous light history, the rates of photosynthesis and the methods of "mucus" collection. In the present study, mucus-lipid was collected by filtering the sea water from the experimental incubation chambers. Crossland et al. (1980 b) and Crossland (1987) collected only mucus-lipid which adsorbed to celite particles. Corals or anemones have also been exposed to air, broken into pieces, placed in chilled sea water or had jets of water directed onto tissues to stimulate mucus production for biochemical analyses (Benson and Muscatine 1974, Ducklow and Mitchell 1979, Krupp 1981, Meikle et al. 1988, Coffroth 1990). It is likely that the "mucus" released after stress will mainly consist of mucus-polysaccharide and that release of mucus-lipid may be more dependent upon the rate of photosynthesis.

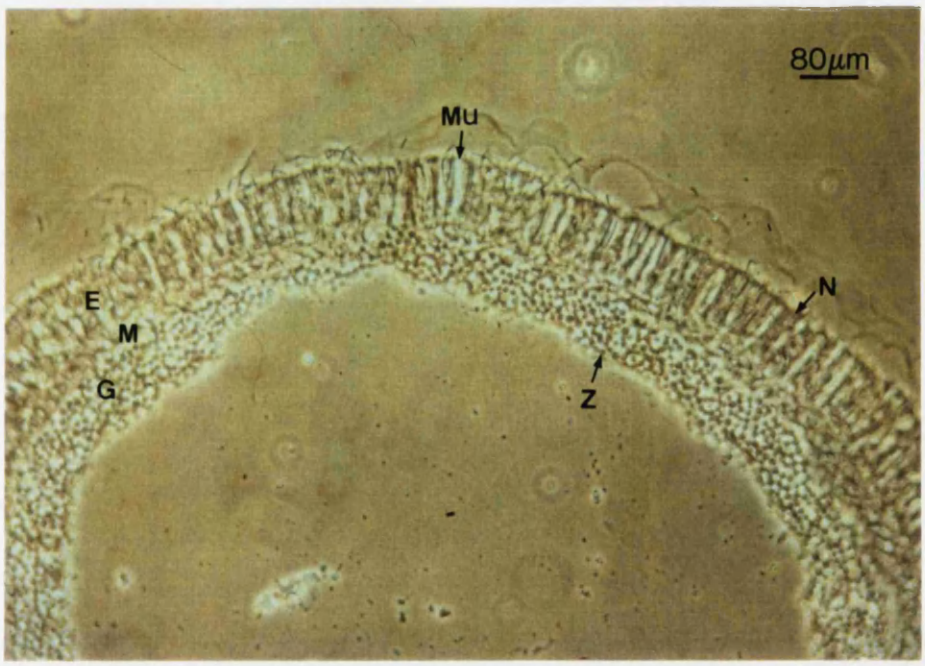
Storage lipid was observed only in the gastrodermal cell layer of *Anemonia viridis*. Muscatine and Hand (1958) and Taylor (1969) exposed anemones to ^{14}C in sea water for periods of up to 240 h and then tissue sections were analysed for the presence of the label using high-resolution autoradiography. Fixed ^{14}C was found exclusively in the gastrodermis with no trace of the label in the epidermis. However, Trench (1971 a) separated the gastrodermal and epidermal layers of tentacles from *Anthopleura*

elegantissima after incubation in labelled bicarbonate. The amount of label in each tissue section was then measured directly. In contrast to the above observations, between about 18 and 31% of the carbon fixed by the zooxanthellae moved into the epidermis during a 10 h incubation in the light and this was incorporated mainly into the total lipid and protein fractions. Therefore, it is possible that mucus-lipid could be excreted from the epidermis. Benson and Muscatine (1974) detected fixed ^{14}C in the mucus-lipid of the coral *Pectinia lactuca*. Corals were incubated in labelled bicarbonate and mucus was collected by directing a water jet onto the coral surface which would have resulted in the secretion of mucus from epidermal gland cells. If this interpretation is correct, then it would be neccessary for lipid or a lipid precursor to be transported from the gastrodermis, across the mesoglea and into the epidermal mucous gland cells. Lipid would then be lost as mucus-lipid when the cells discharged.

Alternatively, mucus-lipid may be lost with mucus secreted from gland cells which are located in the gastrodermal layer. Crossland et al. (1980 b) found what appeared to be crystals of wax ester adjacent to gastrodermal mucous cells and it was suggested that when the contents of these cells were discharged, the wax esters would also be excreted. Mucous cells are present in the gastrodermis of the mesenteries of *Anemonia viridis*. Since ^{14}C, which was probably fixed in the tentacles and body wall, accumulates in the mesenteries, it is suggested that this carbon could be excreted into the coelenteron as lipid together with mucus from the mesenterial mucous gland cells.



a



b

Figures 5.1a and 5.1b. *Anemonia viridis*. Frozen section of oral disc (fig. 5.1a) and tentacle (fig. 5.1b) stained for neutral lipids which appear red. Abreviations: **G** gastrodermis; **E** epidermis; **M** mesoglea; **Z** zooxanthella; **Mu** mucous-gland cell; **N** nematocyst; **L** neutral lipid.

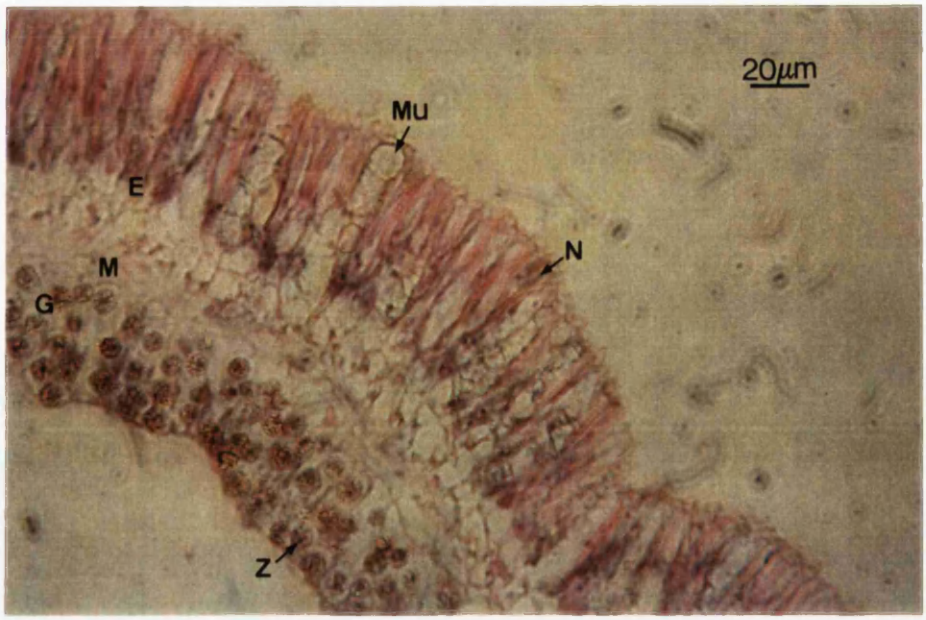


Figure 5.2. *Anemonia viridis*. Section of tentacle showing the location of zooxanthellae in the gastrodermal cell layer. Wax sections were stained with haematoxylin and eosin. Abreviations as in legend to figure 5.1.

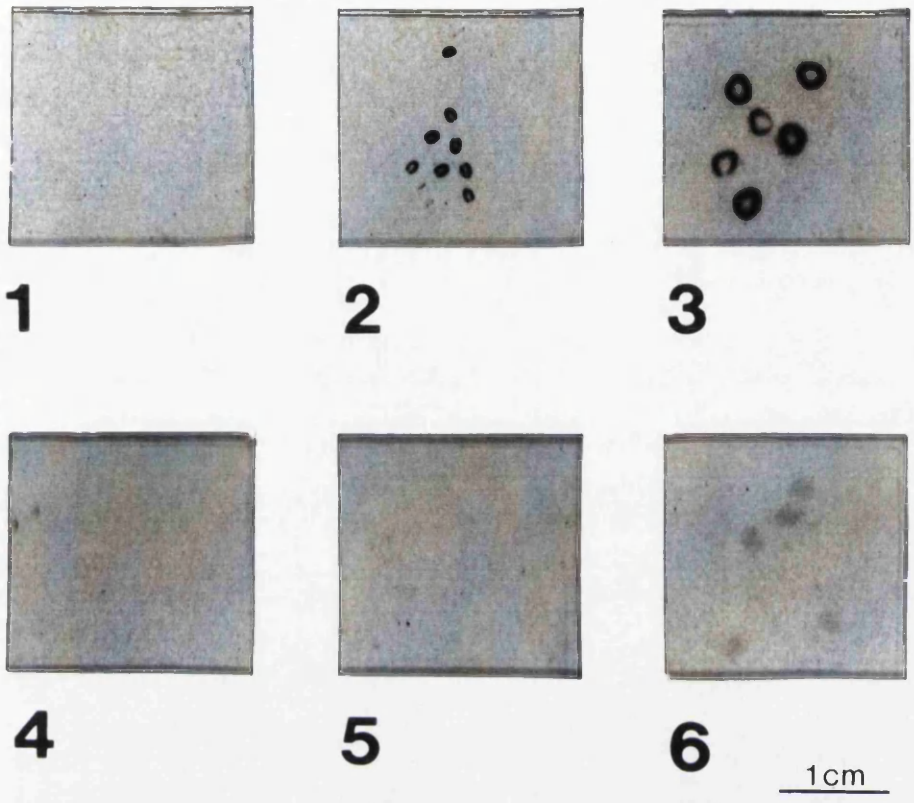
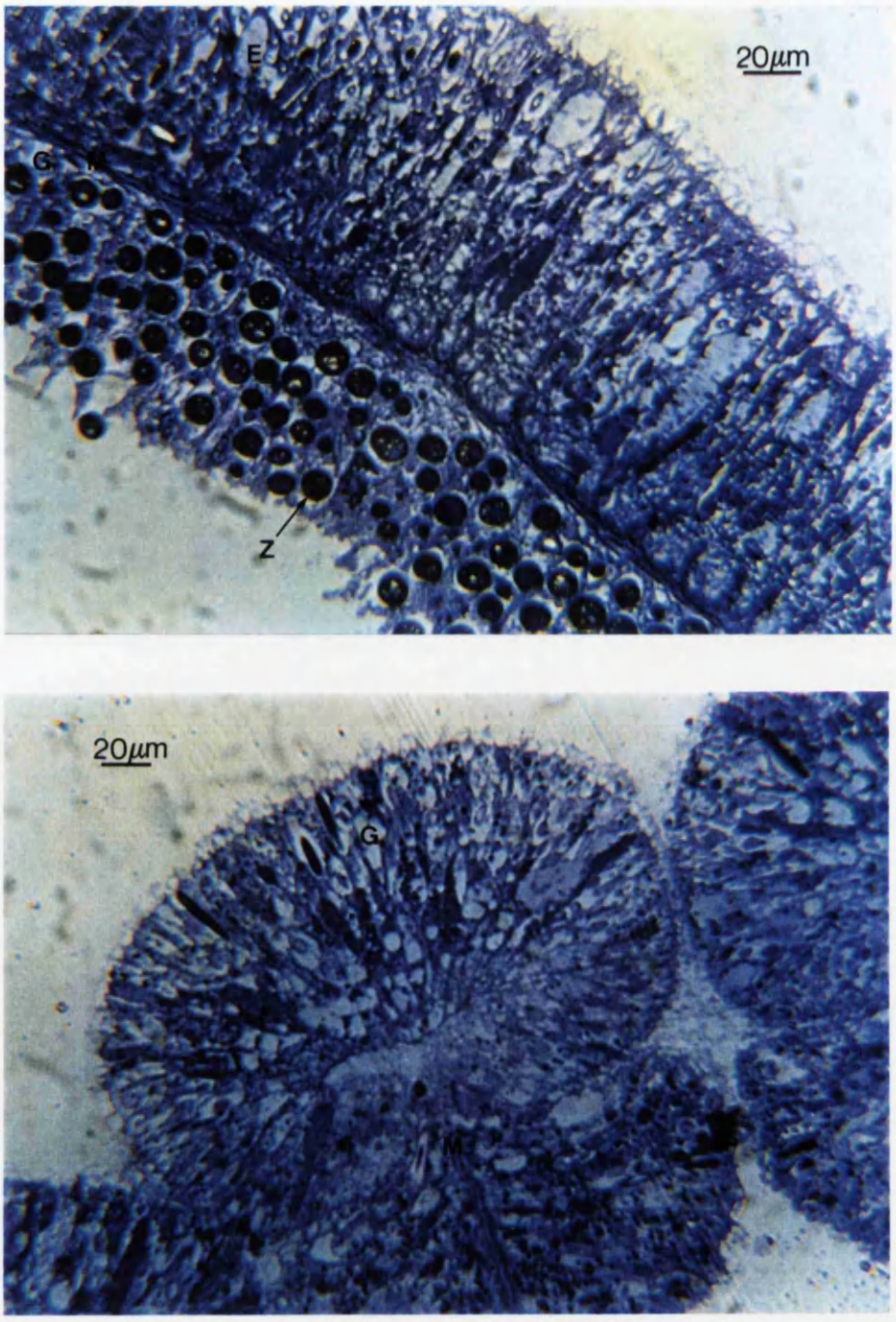


Figure 5.3. *Anemonia viridis*. Autoradiographic film of anemone tissues exposed to $NaH^{14}CO_3$ in sea water. Three anemones were maintained in labelled sea water for 2 h in the dark (control) after which time one was sacrificed. Lights were then switched on to give an intensity of 300 $\mu E\ m^{-2}\ s^{-1}$ and one anemone sacrificed after 2 and 4 h exposure. Films were placed in direct contact with a minimum of 5 tissue sections of tentacle or mesentery from each anemone. Autoradiographs 1,2 and 3 are from tentacles and 4,5 and 6 from mesenteries. Plates 1 and 4 are controls, 2 and 5 were exposed to the label for 2 h, and 3 and 6 for 4 h.



Figures 5.4a. and 5.4b. *Anemonia viridis*. Sections of tentacle (fig. 5.4a) and mesentery (fig. 5.4b) from the autoradiography experiment to show presence or absence of zooxanthellae. Abreviations as in legend to figure 5.1.

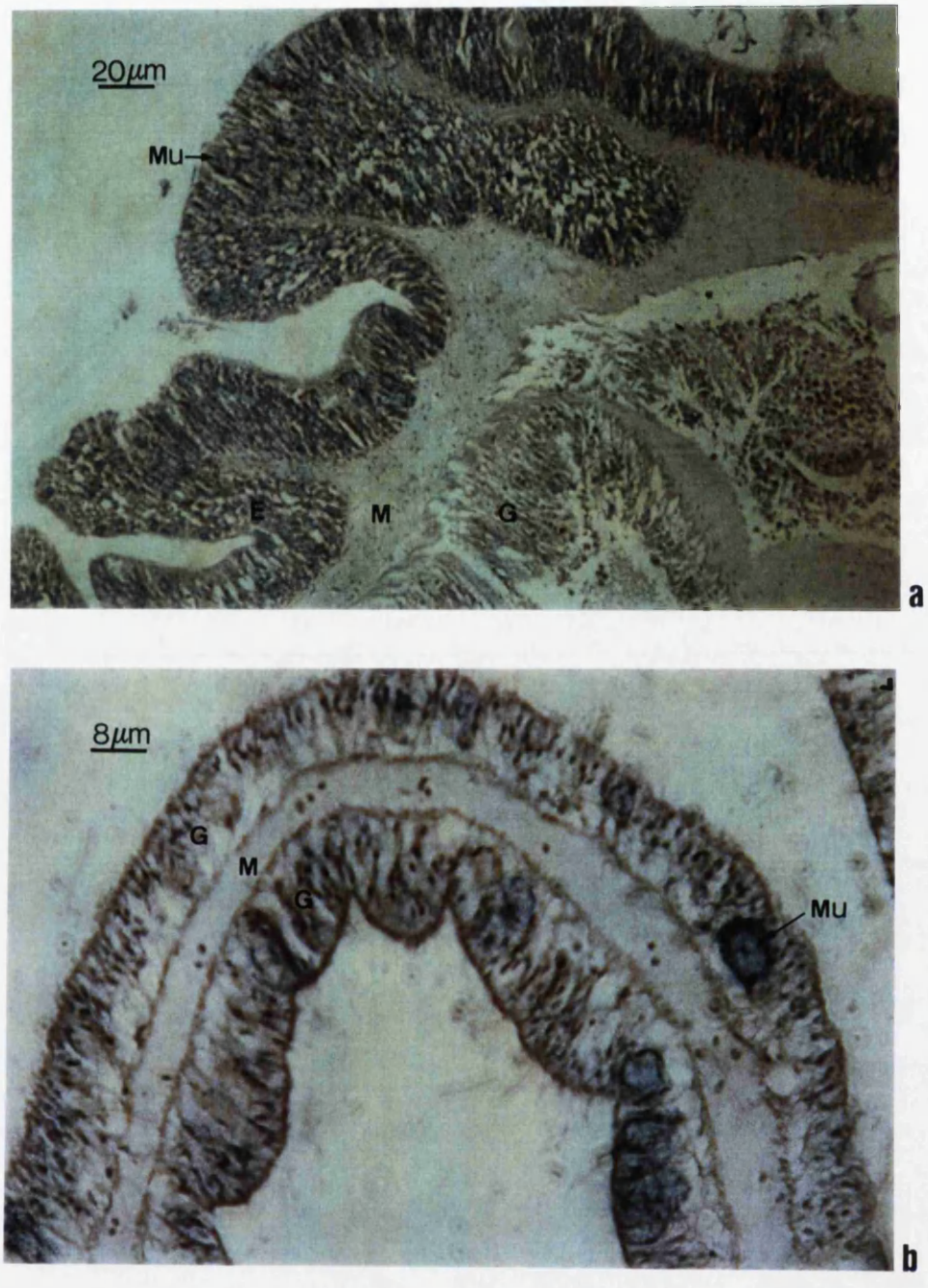


Figure 5.5a and 5.5b. Location of mucous-gland cells in the body wall (fig. 5.5a) and mesentery (fig. 5.5b). Acid mucins appear blue, neutral mucins magenta and mixtures of acid and neutral, purple. Abreviations as in legend to figure 5.1.

Chapter 6

A carbon budget for the symbiotic sea anemone *Anemonia viridis*

Introduction

To determine the quantitative importance of the carbon translocated from the zooxanthellae to the host tissues, a number of authors have constructed carbon or energy budgets (Davies 1977, Davies 1984, Muscatine et al. 1984, Edmunds and Davies 1986, Stambler and Dubinsky 1987). Conceptually, a budget should equate carbon input with carbon use. However, measuring all the components of a budget has proved difficult. Different approaches have been adopted when partitioning and balancing budgets, but to date, all have relied on at least one parameter which has been determined by subtraction to ensure a balanced budget. Furthermore, many budgets are based upon measurements determined over short time intervals and then extrapolated to 24 h.

Despite these shortcomings, carbon budgets have proved to be an extemely useful tool in quantifying the carbon fluxes in symbiotic coelenterates. Davies (1977) measured the hourly rate of photosynthesis (P) and respiration (R) in corals. In shallow water and at high-light intensities, the photosynthesis rate was more than double the respiration rate and it was estimated that zooxanthellae would be able to supply enough carbon to satisfy the total respiratory requirement over a 24 h period. Extending this approach, Davies (1984), Edmunds and Davies (1986) and Tytler and Davies (1986) developed more detailed budgets in which energy input and use were partitioned between zooxanthellae and host. These authors assumed that energy from photosynthesis of the zooxanthellae would be used for their own respiration and growth. Any excess would then be translocated to the host where it would be used for respiration and growth. Energy was assumed to be lost in reproduction, zooxanthellae expulsion and as mucus. All these parameters were measured except for losses, which were estimated by subtraction to balance the budget. Muscatine et al. (1984) calculated a daily carbon budget for the coral *Stylophora pistillata* and measured the amount of ^{14}C released from labelled corals. However, this budget was also balanced by subtraction and the value for carbon assimilated by the animal was estimated.

A carbon budget is produced by balancing the carbon input and the carbon utilised over a fixed period, normally 24 h. For a symbiotic anemone, the main components of a 24 h budget would be:

Carbon input	Carbon use
Photosynthetic carbon fixation	Respiration
Heterotrophic feeding	Growth
Dissolved organic matter	Storage (lipid)
	Reproduction
	Losses -mucus secretion
	-lipid excretion

For *Anemonia viridis* within the circulatory sea-water aquaria, under non-feeding conditions, the budget can be simplified. Since the concentration of low-molecular weight nutrients such as monosaccharides and amino acids is likely to be very low, effectively the only carbon input will be from photosynthetic fixation of carbon in the light. Similarly, since the anemones are not feeding, the only source of nitrogen for growth would come from dissolved ammonia in the sea water which would be taken up during the day (Davies 1988). However, previous studies (Tytler and Davies 1986) showed that anemones which were stored in the light lost body mass. Therefore under non-feeding aquarium conditions, carbon use for growth can be discounted. The budget therefore simplifies to:

Carbon input	Carbon use
Photosynthetic carbon fixation	Respiration
	Storage (lipid)
	Reproduction
	Losses -mucus secretion
	-lipid excretion

Photosynthetic carbon fixation is normally measured indirectly from determinations of the rate of oxygen evolution. However, the rate of oxygen production which is measured is a net value (Pnet) since some of the gross oxygen production (Pgross) is used for respiration of both the zooxanthellae and the animal tissue. Conventionally, Pgross is estimated by adding to the Pnet value the rate of respiration which is measured in darkness. An assumption is therefore made that the rate of respiration in the light is the same as that in darkness. This assumption has been challenged by Edmunds and Davies (1988) who showed that in the coral *Porites porites*, the respiration rate measured immediatly after exposure to light was higher than that measured after dark exposure. In order to circumvent this, it is possible to consider a net carbon budget. The full budget during light exposure would be:

Carbon input	**Carbon use**
Net photosynthetic carbon fixation	Respiration Storage (lipid) Reproduction Losses -mucus secretion -lipid excretion

From this equation it is clear that net photosynthetic fixation represents the carbon which is available for other purposes. In this case, the problem of respiration rate measurement is eliminated since the gross photosynthetic rate has been automatically corrected for the respiration rate. Under the aquarium conditions mentioned above, the equation can be simplified further to:

Carbon input	**Carbon use**
Net photosynthetic carbon fixation	Storage (lipid) Reproduction Losses -mucus secretion -lipid secretion

However, this budget applies only to the daytime period when the zooxanthellae are photosynthesising. Over the night-time period of darkness there would be no carbon input and the carbon requirements for respiration would have to be met from carbon which has been stored during the day. The 24 h budget based on net photosynthesis would therefore be:

Carbon input	**Carbon use**
Net photosynthetic carbon fixation	Night time respiration Storage (lipid) Reproduction Losses -mucus secretion -lipid excretion

No method is yet available to measure the change in carbon for storage or reproduction over time intervals as short as 24 h. A carbon budget was therefore determined for *Anemonia viridis* under non-feeding conditions in which the remaining three components were measured.

Materials and methods

Stock anemones

Anemonia viridis were collected from the Isle of Cumbrae in 1990 and maintained as described in chapter 2.

Experimental design

Anemones were adapted to either a low-light intensity of 10 μE m^{-2} s^{-1} or a high-light intensity of 300 μE m^{-2} s^{-1}, with a 12 h light: 12 h dark photoperiod. Two groups of 6 similar sized anemones were placed into individual chambers within 2 glass tanks which were supplied with recirculating sea water at 10°C. The volume of each chamber was about 200 ml. A bank of fluorescent tubes provided an irradiance of 300 μE m^{-2} s^{-1} at the level of the anemones in one tank. The other tank was covered with fine plastic netting which reduced the light intensity to 10 μE m^{-2} s^{-1}. Anemones were fed weekly and left to adapt to these conditions for 4 months.

Respiration and photosynthesis

Respiration and photosynthesis were measured in a 275 ml perspex respirometer. Magnetic spin bars ensured an even distribution of oxygen within the chamber. A Radiometer E5046 oxygen electrode was inserted into the chamber and connected to Strathkelvin Instruments 781 oxygen meter and a BBC SE 120 chart recorder. The chamber was placed in a water bath at 10°C under a bank of fluorescent lights. Light intensity could be varied by altering the voltage supplied to the fluorescent tubes with a Zenith variable transformer. Sea water was pumped through the respirometer. When the pump was switched off, a system of valves sealed the chamber. In all experiments, sea water (salinity $33^{o}/_{oo}$) was filtered (0.45μm), sterilised with UV light and maintained at 10°C and 100% oxygen saturation before use.

The anemone was placed in the chamber with circulating oxygen-saturated sea water for 1 h. The chamber was then sealed and respiration and photosynthesis were measured. Respiration was measured in darkness, towards the end of the normal 12 h dark period, for a maximum of 15 minutes or until about 80% oxygen saturation was reached. During this period, the respiration rate was constant.

Following a respiration run, saturated sea water was pumped through the chambers until the sea water in the chamber returned to 100% oxygen saturation. At the start of the normal 12 h light period, for anemones adapted to low light, the lights were switched on to

give an irradiance of 10 μE m^{-2} s^{-1} and after 10 minutes the chamber was resealed. The net photosynthesis rate was measured for a maximum of 15 minutes or until the sea water had reached 120% saturation. The photosynthesis rate was constant during this period. This procedure was then repeated at a light intensity of 300 μE m^{-2} s^{-1}. For anemones adapted to high light, photosynthesis was measured first at 300 and then at 10 μE m^{-2} s^{-1}.

After measuring respiration and photosynthesis, anemones were immediately returned to the appropriate experimental tank at high- or low-light intensity, for a minimum of 3 days before carbon excretion was measured in the same anemones.

Carbon loss

Anemones were placed in 150 ml pyrex beakers containing 100 ml filtered and sterilised sea water which was gently aerated. Beakers were placed in a water bath at 10°C under the fluorescent lights. Six low-light adapted anemones were each placed in a separate beaker for 12 h at 10 μE m^{-2} s^{-1}. At the end of this period they were transferred to new beakers containing freshly prepared sea water for 12 h in the dark. This procedure was repeated for 12 h at 300 μE m^{-2} s^{-1} and then 12 h darkness. Treatment of the six high-light adapted anemones was identical except that the experiment was started with a light intensity of 300 μE m^{-2} s^{-1} followed by darkness, then 10 μE m^{-2} s^{-1} followed by darkness. The incubation sea water was not homogeneous in composition because of the presence of "mucus" aggregates and it was therefore impractical to measure the total organic carbon in this sea water by analysing aliquots. It was therefore divided into soluble and particulate fractions by filtration. The sea water was filtered through a pre-combusted (500°C for 24 h) 25 mm Whatman glass fibre GF/C filter paper. The empty beaker was then swabbed with a second GF/C paper and the two filter papers combined to provide the particulate fraction. Filter papers were not treated with acid to remove inorganic carbon from the sample as preliminary analyses using TOCA (see below) had shown that the particulate fraction contained only 6% inorganic carbon. For soluble carbon, a 1 ml sample of the filtered sea water was pipetted into an eppendorf tube. Beakers containing only sea water provided experimental controls. All samples were frozen (-20°C).

Filter papers were dried at 60°C for 48 h before analysis of particulate carbon using a LECO CHN-900 carbon hydrogen nitrogen (CHN) elemental analyser. 2 mg EDTA (Elemental Microanalysis Ltd.) was used as a carbon standard. The CHN analyser was equiped to accept only solid samples and so the filtered sea water was analysed for carbon using a Shimadzu TO500 total organic carbon analyser (TOCA) using potassium hydrogen pthalate or sodium bicarbonate/sodium carbonate as total carbon and inorganic carbon standards respectively at a concentration of 400 mg C l^{-1}. Inorganic carbon was subtracted from total carbon to give the value for organic carbon. During the course of the TOCA

analyses, the samples from the low-light adapted anemones were unfortunately lost.

At the end of the experiment, anemones were freeze dried to constant weight and this biomass measurement was used to normalise all data.

Carbon equivalents

From knowledge of the temperature and the salinity of the sea water, the volumetric rates of oxygen change were calculated by reference to tables of oxygen solubility in sea water (Green and Carritt 1967). Carbon equivalents were calculated by the method described by McCloskey et al. (1978). The general formula for conversion of ml O_2 to mg C is:

$$\text{mgC} = 1.428 \times \text{ml } O_2 \times 12/32 \; [/ \; PQ \text{ or } \times \; RQ]$$

where *PQ* is the molecular photosynthetic quotient defined as the ratio of oxygen produced to carbon dioxide consumed during photosynthesis. The *RQ* is the ratio of oxygen consumed to carbon dioxide produced during respiration. As the main photosynthetic products and substrates for respiration are unknown, a *PQ* and *RQ* of 1.0 was used on the assumption that the primary product of photosynthesis was glucose and that glucose was the primary substrate for respiration. If glycerol was the major product of photosynthesis then the *PQ* would be 1.167 (McCloskey et al. 1978).

Results

Respiration and photosynthesis

The rates of respiration and Pnet in low- and high-light adapted anemones are shown in table 6.1. There was no significant difference in the respiration rates of anemones adapted to either low or high light ($P > 0.05$, Student's t test). However, for low- and high-light adapted anemones, Pnet was higher when measured at 300 μE m^{-2} s^{-1} than at 10 μE m^{-2} s^{-1} ($P < 0.05$, Student's t test). There were significant differences in Pnet between low- and high-light adapted anemones at both 10 and 300 μE m^{-2} s^{-1}, with low-light adapted anemones exhibiting higher rates at each light intensity ($P < 0.05$, Student's t test).

Table 6.1. *Anemonia viridis*. Respiration and net photosynthesis rates were measured as oxygen consumption and oxygen evolution respectively and normalised to dry tissue weights. Anemones were adapted to either low light (10 μE m^{-2} s^{-1}) or high light (300 μE m^{-2} s^{-1}) for a period of 4 months. Respiration was measured in darkness and net photosynthesis at 10 and 300 μE m^{-2} s^{-1}. n = 6. Standard deviations are shown in parentheses.

	Respiration ml O_2 g^{-1} h^{-1}	Net photosynthesis ml O_2 g^{-1} h^{-1}	
		Irradiance μE m^{-2} s^{-1}	
		10	300
Low-light adapted	0.24 (0.08)	0.37 (0.17)	1.99 (0.41)
High-light adapted	0.25 (0.12)	0.11 (0.16)	1.22 (0.15)

Carbon loss

Losses of particulate and dissolved carbon are shown in table 6.2.

Table 6.2. *Anemonia viridis*. Loss of particulate and dissolved carbon from anemones adapted to either low light (10 μE m^{-2} s^{-1}) or high light (300 μE m^{-2} s^{-1}) for a period of 4 months. For low-light adapted anemones (n = 4), samples were collected from 4 consecutive 12 h periods of 10 μE m^{-2} s^{-1}, dark, 300 μE m^{-2} s^{-1} and dark. For high-light adapted anemones (n = 5), the periods were 300 μE m^{-2} s^{-1}, dark, 10 μE m^{-2} s^{-1} and dark. Where superscripts differ for either low-light or high-light adapted anemones and for particulate or dissolved carbon loss, data are statistically different (SNK, $P<0.05$). Data are mg C g^{-1} dry weight of anemone tissue. Standard deviations are shown in parentheses. ND: no data.

	Low-light adapted anemones		High-light adapted anemones	
	Particulate C loss mg C g^{-1}	Dissolved C loss mg C g^{-1}	Particulate C loss mg C g^{-1}	Dissolved C loss mg C g^{-1}
Irradiance μE m^{-2} s^{-1}				
300	0.74 (0.36)[a]	ND	0.71 (0.19)[a]	2.0 (0.70)[a]
Dark	0.69 (0.21)[a]	ND	0.99 (0.46)[b]	2.25 (0.94)[a]
24 h total	1.43 (0.53)		1.70 (0.56)	4.25 (1.09)
10	0.45 (0.14)[a]	ND	0.43 (0.05)[c]	1.54 (1.08)[a]
Dark	0.56 (0.27)[a]	ND	0.31 (0.05)[d]	2.91 (0.48)[a]
24 h total	1.01 (0.36)		0.74 (0.07)	4.45 (1.12)

Particulate carbon loss. There were no significant differences in the amount of particulate carbon lost from anemones adapted to either low light or high light over the 48 h experimental period ($P > 0.05$, two-way ANOVA). For low-light adapted anemones there were no significant differences in the amount of carbon excreted in the light or dark ($P > 0.05$, ANOVA). For high-light adapted anemones, significantly more carbon was excreted at 300 μE m^{-2} s^{-1} than at 10 μE m^{-2} s^{-1}, and during the 12 h dark period following exposure to 300 μE m^{-2} s^{-1} than in the 12 h dark period following exposure to 10 μE m^{-2} s^{-1} ($P < 0.05$, ANOVA, $P < 0.05$, SNK).

Dissolved carbon loss. The amount of dissolved carbon lost by high-light adapted anemones was relatively constant over the 48 h experimental period and there were no significant differences at each irradiance and in darkness ($P > 0.05$, ANOVA). There were significantly higher levels of dissolved carbon lost than particulate carbon ($P < 0.05$, two-way ANOVA).

Carbon budget

As data for dissolved carbon loss from low-light adapted anemones were lost, it was only possible to construct a carbon budget for high-light adapted anemones (table 6.3).

Table 6.3. *Anemonia viridis.* 24 h carbon budget for high-light adapted anemones with a 12 h light and 12 h dark photoperiod. Components of the budget were measured at 10 ($n = 4$) or 300 μE m^{-2} s^{-1} ($n = 5$) (see materials and methods for details). Before starting the experiment, anemones were adapted to a light intensity of 300 μE m^{-2} s^{-1} for 4 months. Data are mg C g^{-1} dry weight of anemone. Standard deviations are shown in parentheses.

	net photosynthesis (12 h light)	=	respiration (12 h dark)	+	losses (24 h)	(balance)
light intensity μE m^{-2} s^{-1}						
10	0.71 (1.03)		1.61 (0.77)		5.19 (1.19)	(- 6.09)
300	7.84 (0.96)		1.61 (0.77)		5.96 (1.54)	(+0.27)

An assumption was made that photosynthesis and respiration rates would be constant over time. At an irradiance of 10 μE m^{-2} s^{-1}, these anemones could not provide enough carbon from photosynthesis to meet either the respiratory demand or losses. The deficit was 6.27 mg C g^{-1} over the 24 h experimental period. However, when the same anemones were exposed to 300 μE m^{-2} s^{-1}, they were essentially autotrophic with respect to carbon with a surplus of 0.56 mg C g^{-1} unaccounted for.

Discussion

Respiration and photosynthesis

The respiration rates of *Anemonia viridis* adapted to either high- or low-light intensities were similar and these data agree well with values previously published for the same species maintained at a low light intensity (Tytler and Davies 1984). However, the respiration rate of the coral *Stylophora pistillata* adapted to shade conditions was lower than in the same species adapted to high light intensities (Muscatine et al. 1984, Porter et al. 1984). It has also been shown that respiration rates decrease with depth and therefore reduced irradiances (Davies 1977, Davies 1980, Al-Sofyani 1991). Davies (1980) suggested that this decrease was a phenotypic response to environment since corals which were transplanted from shallow- to deep-water also showed a similar decrease in respiration rate. It was suggested that the higher respiration rates of shallow water corals were due to faster growth rates. It was also proposed that if food intake from zooplankton feeding was the same at all depths, then differences in respiration and growth could be due to the higher input of carbon from zooxanthellae as light values and the rates of photosynthesis would be higher in shallow-water. Similarly, Falkowski and Dubinsky (1981), Porter et al. (1984) and Muscatine et al. (1984) found that the respiration rates of *S. pistillata* were higher in light-adapted than shade-adapted corals and that more carbon was translocated from the zooxanthellae in those living in the high-light regime.

However, not all studies have shown a correlation betweeen respiration rate and depth. Svoboda (1978) measured respiration in a number of symbiotic anthozoans over a 40 m depth range and found that the rate did not change with depth, and Al-Sofyani (1991) found that the respiration rate of the coral *Echinopora gemmacea* was lower in deeper water in winter but not in summer. In the present study, the respiration rates of high-light and low-light adapted *Anemonia viridis* were measured in starved anemones and any carbon translocation from zooxanthellae would not have been used for growth as N_2 was limiting. This may explain why low-light and high-light adapted anemones were expending energy at a similar rate.

The data for net photosynthesis (Pnet) recorded for low-light and high-light adapted *Anemonia viridis* showed similar trends to that recorded by Porter et al. (1984) for shade- and light-adapted *Stylophora pistillata*. Maximum rates of net photosynthesis (Pnet max) were found to be higher in shade-adapted specimens when photosynthesis was measured at either high- or low-light intensities. In the present study, since respiration rates were the same for low- and high-light adapted anemones, then gross photosynthesis (Pgross) would be higher in the low-light adapted group at Pgross max suggesting that these anemones were more efficient at utilising available light energy in photosynthesis thereby showing a

photoadaptive response. Photoadaptation has been reported for a number of symbiotic coral species as a response to variations in light intensity (Falkowsky and Dubinski 1981, Chang et al. 1983, Dustan 1982, Wyman et al. 1987, Battey and Porter 1988), but this is the first report for *A. viridis*. Falkowski and Dubinsky (1981) compared the photosynthetic characteristics of shade- and light-adapted *Stylophora pistillata*. It was found that light-adapted corals appeared to be lighter in colour than shade-adapted corals and that this was due to a reduction in the chlorophyll *a* content of zooxanthellae and not to a reduction in the number of algal cells. It was also shown that the numbers of photosystem I reaction centres (P_{700}) remained constant while the size of the photosynthetic unit (PSU) was reduced. Light-adapted corals had lower Pnet max when data were normalised to coral surface area. High-light adapted anemones in the present study were also lighter in colour than those adapted to low-light. The dark colouration and higher Pnet of low-light adapted anemones suggested that photoadaptation may have been mediated by similar mechanisms as those described for *S. pistillata*.

Chang et al. (1983) measured photosynthesis in cultured zooxanthellae isolated from a coral, an anemone and a clam. The cultures were adapted to light intensities ranging from 22 to 248 $\mu E\ m^{-2}\ s^{-1}$. For the anemone *Aiptasia pulchella*, algal respiration rates were exteremely small and Pnet approximately equalled Pgross. When data were normalised to algal cell number, it was shown that Pgross max decreased with increasing light intensity. This photoadaptive response is in agreement with the observations made for *Anemonia viridis*. Although data obtained from algal cell cultures may not be directly comparable to those of algae in symbiosis, they do provide information on the physiological capabilities of the algae.

Carbon loss

Carbon is lost from symbiotic anthozoans as both particulate and dissolved organic carbon (P/DOC). Carbon is lost mainly as mucus and mucus-lipid (Crossland et al. 1980 b). Carbon may also be lost when zooxanthellae are expelled (Hoegh-Guldberg et al. 1987, Harland and Nganro 1990). In the present study, the sea water in which an anemone was incubated was passed through a GF/C filter paper and both the filter paper and the filtrate were analysed for released carbon. The former fraction was arbitrarily defined as particulate carbon and this contained visible "mucus" and expelled zooxanthellae.. The latter fraction was the dissolved carbon and it is suggested that this may have contained soluble mucus.

Carbon was released from anemones during both the light and the dark periods. Both low- and high-light adapted anemones appeared to release similar amounts of particulate carbon. However, more carbon was released from both groups of anemones when they

were exposed to 300 μE m^{-2} s^{-1} and during the following dark period than at 10 μE m^{-2} s^{-1} followed by darkness. Data for release of dissolved organic carbon were available only for high-light adapted anemones. However, these anemones lost similar amounts of carbon in the light and dark and this pattern was independent of light intensity. Muscatine et al. (1984) incubated *Stylophora pistillata* in ^{14}C in the light and measured the release of labelled organic carbon over 24 h. Secretion of dissolved organic ^{14}C was relatively constant. In contrast, Crossland (1987) found a diurnal pattern of carbon release when *in situ* measurements were made for *Acropora variabilis* and *Stylophora pistillata*. Both mucus and DOC-lipid were released continuously although maximal rates of release were during the afternoon. It was suggested that carbon fixed in the morning was used to replenish tissue carbon that had been metabolised during the night and that carbon fixed later in the day was incorporated into mucus and lipid. However, this study contradicted earlier work conducted in the laboratory (Crossland et al. 1980 b) in which it was shown that mucus was released at the same rate over 24 h with mucus-lipid being released mainly during the day. To explain this apparent contradiction, Crossland (1987) suggested that as corals were exposed to ^{14}C for relatively short periods in the laboratory study, this may not have been of sufficient duration to allow photosynthetically fixed carbon to be incorporated in mucus. Therefore, carbon may be released at a continuous rate as mucus (Crossland et al. 1980 b) and dissolved organic carbon (Muscatine et al. 1984, present study), or the rates of release may depend upon light intensity as shown for the release of mucus-lipid (Crossland et al. 1980 b), mucus and DOC-lipid (Crossland 1987) and particulate carbon (present study). The release of carbon as mucus and mucus-lipid could be an area for further research.

Carbon budget

At an irradiance of 300 μE m^{-2} s^{-1}, high light adapted *Anemonia viridis* could supply all the carbon needed for respiration and losses over 24 h, and were therefore potentially photo-autotrophic with respect to carbon. However, at an irradiance of 10 μE m^{-2} s^{-1}, these anemones fixed less carbon than was required and the budget was in deficit. The surplus carbon produced at high light would be available for growth and storage as lipid and the deficit at low light would have been met by drawing upon lipid stores (Davies 1991) or through tissue catabolism (Crossland 1987). Under natural conditions, anemones with low rates of photosynthesis could also replace lost carbon through intake of zooplankton and particulate organic matter.

About 63% of the total carbon fixed was lost from anemones at 300 μE m^{-2} s^{-1}. These figures are somewhat higher than those given for *Acropora acuminata* by Crossland et al. (1980 b) who estimated that 40% of the net ^{14}C fixed was lost as mucus and mucus-lipid.

For the coral *Pocillopora eydouxi* it was shown that under high light conditions, only a small proportion of the daily fixed carbon (Pgross) was used for growth and it was predicted that there would be a daily energy surplus of 48% which would be lost (Davies 1984). Tytler and Davies (1986) showed that for *Anemonia viridis* maintained at 140 μE m^{-2} s^{-1}, there was a 40% surplus of the photosynthetically fixed energy (Pgross) which was assumed to be excreted. Davies (1984) suggested that the reason why excess carbon may not be channelled into growth is nitrogen limitation. Without a nitrogen source, carbon in excess of respiratory and storage requirements could not be utilised for growth and would have to be "excreted". However, anemones at 10 μE m^{-2} s^{-1} also lost similar amounts of carbon to those at 300 μE m^{-2} s^{-1} and Muscatine et al. (1984) also found that carbon released from shade-adapted *Stylophora pistillata* exceeded the level of carbon fixed.

Some of the losses in the present study may have been caused by handling anemones and confining them in respirometry chambers during the experiment. Although anemones did not appear to be "stressed" and handling was kept to a minimum, zooxanthellae were present in all samples of "mucus" and stress-induced mucus secretion and zooxanthellae expulsion can not be totally discounted. However, it was not possible to separate zooxanthellae from the mucus to see if expulsion rates were above normal (Harland and Nganro 1990) and to estimate the loss of carbon as algal cells.

The composition of the organic carbon lost by *Anemonia viridis* was not ascertained although viscous mucus was observed in the incubation sea water and anemones have been shown to excrete mucus-lipid (chapter 5). However, as it is unlikely that anemones would excrete mucus-lipid at low-light intensities and as the total carbon losses were similar at both 10 and 300 μE m^{-2} s^{-1}, this suggested that the major loss of carbon was mucus. Mucus secretion is a normal physiological function for anthozoans and mucus may have to be secreted independently of the rate of zooxanthellae carbon fixation although Crossland (1987) has shown that the rate of mucus release was generally higher during the day than the night.

In this chapter, the data for photosynthesis, respiration and carbon loss have shown that at high light, anemones may fix more carbon in photosynthesis than is required for respiration and mucus secretion. However, at low-light levels, photosynthesis is not adequate to meet these requirements and anemones may have to draw upon lipid stores.

Conclusions

1) The symbiotic anemone *Anemonia viridis* acquires carbon by feeding and from translocation of photosynthetically fixed carbon from the symbiotic zooxanthellae. In the present study, carbon budget calculations indicated that under conditions of high light for 12 h of a 24 h cycle, anemones had the potential to be photo-autotrophic with respect to carbon.

2) Fixed ^{14}C was used for lipid biosynthesis in both the host and zooxanthellae although the majority was incorporated into TCA-soluble compounds, such as glycerol, in whole anemone tissues. ^{14}C was incorporated into several lipid classes including the wax esters and triglycerides which are considered as storage lipids, providing a source of energy for the symbiotic association. The rate of total and storage lipid biosynthesis was directly proportional to light intensity and hence the level of photosynthesis. However, in terms of the 24 h carbon budget, the utilisation of carbon as lipid was relatively small when compared to utilisation for respiration and losses.

3) Measurements of photosynthesis and respiration in low-light adapted and high-light adapted anemones showed that zooxanthellae were capable of photoadaptation. Both groups of anemones had similar respiration rates but those adapted to low light had higher rates of gross photosynthesis.

4) Carbon was lost from anemones during mucus secretion, mucus-lipid excretion and zooxanthellae expulsion. It was proposed that if the zooxanthellae fix more carbon than can be used for respiration and growth of the symbiotic association, then the excess may have to be excreted as mucus and mucus-lipid. Dissolved organic carbon loss appeared to be continuous and it was suggested that this would contain soluble mucus. Although no measurements were made, the quantity of mucus-lipid excreted might be expected to vary depending upon the rate of photosynthesis and the size of the lipid store.

5) The tentacle gastrodermis contains zooxanthellae which fix carbon in the course of photosynthesis. However, storage lipids were not present in the tentacles and were only detected in the gastrodermis of the oral disc, body wall and mesenteries. As there was evidence that carbon could be translocated from the tentacles to the mesenteries, it was suggested that carbon compounds, such as lipid, may also be translocated to the mesenteries where they could then be excreted into the coelenteron with mucus when the mucous-gland cells of the mesenterial gastrodermis discharged. However, mucous gland

cells were numerous in the epidermal cell layer and it is possible that excess storage lipid or lipid precursors could also be moved across the mesoglea to be excreted with epidermal mucus. Autoradiography at the cellular level may provide a useful technique for resolving these questions.

6) During the course of this work it was anticipated that some insight might be gained into the nature of the carbon compounds translocated from the zooxanthellae to the host. Indirect evidence from the biochemical analysis of zooxanthellae and host lipids suggested that triglycerides may be translocated. However, the labelling patterns of the TCA-soluble and lipid fractions of whole anemones suggested that fixed carbon may be incorporated into water-soluble compounds and subsequently used in lipid biosynthesis. Identification of the carbon compounds translocated from zooxanthellae to host is a key area for future research.

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