

**Nutritional Interactions between the Alga *Symbiodinium*
and Sea Anemone *Aiptasia pulchella***

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Abstract

Many marine cnidarians obtain photosynthetic carbon compounds from symbiotic algae of the genus *Symbiodinium*. This study explores the mechanism by which the algae are induced to release photosynthetic products, and the impact of translocated photosynthate on nitrogen metabolism in the animal: ammonium assimilation and essential amino acid biosynthesis.

Taurine and animal extract exhibited broadly comparable effects on photosynthate metabolism and photosynthate translocation by *Symbiodinium* derived from the sea anemone *Aiptasia pulchella*. Taurine-stimulated photosynthate release is inferred to be the signalling process from the evidence that (1) the k_m of taurine-stimulated photosynthate release is low (21 μM); (2) taurine is not intensively metabolized by *Symbiodinium*; and (3) part of taurine uptake by *Symbiodinium* is reversible. Kinetics and inhibitor studies further revealed that the putative *Symbiodinium* receptor for taurine-stimulated photosynthate release is distinct from the taurine transporter.

The translocated photosynthate has a considerable impact on the nitrogen metabolism in the animal host. The ammonium concentration in the animal tissues of *A. pulchella* was elevated by incubation in darkness or depletion of *Symbiodinium*, and the effect was partially or completely reversed by supplementing the medium with organic carbon (e.g. fumarate, α -ketoglutarate). The effect of these treatments on the ammonium concentration was inversely correlated with their impact on

the activity of glutamine synthetase. The concentration of free protein amino acids in the animal fraction was depressed in alga-depleted animals; but restored when the animal was repopulated with algal cells or when the medium was supplemented with 10 mM α -ketoglutarate. It is concluded that organic carbon, whether derived from algal photosynthate or exogenous sources, promotes the assimilation of ammonium by the animal, via glutamine synthetase, and reduces the rate of amino acid degradation (and ammonium production). These processes would tend to conserve nitrogen in the animal tissues, but they confound the interpretation of experimental designs traditionally adopted to explore the putative role of algae in nitrogen recycling.

Radiotracer analysis of amino acid biosynthesis revealed synthesis of all essential amino acids in control animals and of 2 essential amino acids (methionine and threonine) in alga-depleted animals. It is concluded that *A. pulchella* has an amino acid biosynthetic capability greater than most animals, but apparently less than Scleractinian corals, which have been reported to synthesize all essential amino acids (except threonine). With the capability to synthesize essential amino acids, the nutritional value of nitrogen recycling by *Symbiodinium* to the animal host has probably been over-estimated.

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Declaration

The material presented in this thesis is the product of my own research. The results presented in Chapter 3 are published in *Plant Physiology* (1997, 114: 631-636), and part of the material presented in Chapter 4 has been accepted by *Journal of Experimental Biology*.

Chapter 1. General Introduction

1.1 Symbiosis as a source of novel metabolic capabilities

The original meaning of the term symbiosis is "the living together of differently named organisms" (de Bary 1879). Through symbiosis, the eukaryotes have been proposed to be able to acquire novel metabolic capabilities from symbiotic partner (Douglas 1994). The eukaryotes are the only group involving the major radiation of multicellular organisms (the animals, the terrestrial plants, and the fungi). However, despite their remarkable morphological diversity and complexity, the eukaryotes were, ancestrally, of limited metabolic capabilities. They could not respire aerobically, photosynthesize, or fix nitrogen. Some eukaryotic group have further lost a variety of biochemical capabilities. For example, most animals cannot synthesize 10 of 20 amino acids which contribute to protein synthesis (so-called essential amino acids), or many coenzymes required for normal metabolisms (e.g. B vitamins).

Eukaryotes have overcome various metabolic limitations by forming symbiosis with other organisms that possess the appropriate biochemical capabilities. The ability of all eukaryotes to respire aerobically is believed to be derived from a symbiosis with Proteobacteria which evolved into mitochondria. Similarly, the symbiosis with Cyanobacteria is believed to be the origin of photosynthesis in the plastids of algae and plants (Margulis 1993). Other examples of symbiosis providing novel metabolic capabilities to eukaryotes include: (1) many leguminous plants (e.g. pea and clover) can utilize atmospheric nitrogen through the association with nitrogen-fixing bacteria called rhizobia; (2) aphids acquire the essential amino acids from the symbiosis with the intracellular bacteria *Buchnera*; (3) most

Table 1.1 Photosynthetic associations with the animal host (Smith 1991 and Douglas 1995)

Photosynthetic symbionts	Animal host
Unicellular algae	
<i>Symbiodinium</i>	Marine Cnidaria: especially Anthozoa, e.g. hermatypic corals, sea anemones
	Marine Molluscs : tridacnid clams nudibranchs
<i>Chlorella</i>	Freshwater Porifera (sponges)
	Freshwater Cnidaria : hydra
	Freshwater Molluscs : e.g. <i>Unio</i>
<i>Tetraselmis</i>	Platyhelminth : <i>Convoluta roscoffensis</i>
<i>Licmophora</i>	<i>Convoluta convoluta</i>
Cyanobacteria	Marine Porifera (sponges)

herbivorous mammals can digest cellulose by symbiosis with cellulolytic micro-organisms in the guts; (4) and some aquatic animals (see Table 1.1) can assimilate inorganic carbon through forming endosymbiosis with photosynthetic micro-organisms. This thesis concerns the last group of symbiotic association, the alga-invertebrate symbiosis.

1.2 Translocated photosynthate as nutrients for microalga-symbiotic animals

A range of aquatic animals are found containing photosynthetic microbes (Smith 1991; Douglas 1995), and the larger non-photosynthetic partner is conventionally termed the host and the smaller algal partner as the symbiont. Most of the animal hosts still retain the capability for holozoic feeding. However, the ability to assimilate inorganic carbon (e.g. bicarbonate) through symbiotic microalgae or photosynthetic bacteria enables the animals to tolerate low food supply under photosynthesizing conditions. The evidence for the algal contribution to the survival and/or growth of their hosts has been obtained mostly from the studies on *Chlorella* symbioses. For example, despite decline in size, illuminated cultures of symbiotic hydra can survive longer (>10 weeks) than those incubated in the dark or aposymbiotic hydra (whose algal population had been removed from the animal host) incubated in both darkness or illumination (<2 weeks), when these associations are starved (Douglas & Smith 1984). Similarly, when the neorhabdocoel turbellarian is starved, animals bearing *Chlorella* cells decline in length more slowly in the light than in the dark (Douglas 1987) or slower than the aposymbiotic animals in the illuminated cultures (Young & Eaton 1975). Comparable results are available for *Anthopleura elegantissima-Symbiodinium* (Muscatine 1961; Sebens 1980) and *Anemonia viridis-Symbiodinium* symbiosis (Taylor

1969). The light-dependence of symbiont contribution to host survival and maintenance of size suggests that photosynthetic products of the symbiotic algae are made available to the host tissues.

Estimation of the algal contribution to the animal host is principally derived from two different types of studies, radiotracer experiments and calculations of the balance between carbon fixation, respiration and biomass gain of the algae. For radiotracer techniques, the amount of ^{14}C -carbon dioxide fixed by the association and in the animal fraction is quantified. The results obtained from *Cnidaria-Symbiodinium* associations revealed that about 20-60 % of fixed ^{14}C by symbiotic algae is transferred to the host (e.g. Trench 1971b; Sutton & Hoegh-Guldberg 1990). However, the error caused by dis-equilibrium between ^{14}C and ^{12}C will significantly underestimate photosynthate transfer (Smith 1982). In order to avoid the error caused by radiotracer technique, Muscatine *et al.* (1984) have developed an alternative method to estimate the transfer of photosynthate. This approach quantifies the total oxygen flux (in the light), respiration rate (in the dark) of the intact association, and the changes in biomass (i.e. growth rate) of the symbiotic algae. The contribution of algal photosynthate to animal respiration is calculated from the difference between total photosynthetic input (derived from total oxygen flux) and total consumption of photosynthetic product by symbiotic algae (derived from the net respiration and growth of algae). This technique has been applied in many *Cnidaria-Symbiodinium* associations (e.g. Muscatine 1990; Davy *et al.* 1996). The calculated values of photosynthate transfer can exceed 90 % for shallow-water, tropical symbioses; but may be below 20 % for temperate symbioses adapted to low irradiance.

Despite the investigations of photosynthate transfer, questions still remain about the extent to which the animal host benefits from the translocation of algal photosynthate. For example, the protein content of *Aiptasia pulchella* does not change with an increased irradiance, in which the photosynthetic rate is elevated and the photosynthate transfer is expected to be higher under high irradiance than under low irradiance; but well-fed *Aiptasia* exhibit a significantly higher protein content than the starved groups (Muller-Parker 1985). From the study with *Anemonia viridis* (= *Anemonia sulcata*), Tytler and Davies (1986) also suggested that photosynthesis of symbiotic algae might provide excess of energy to the animal's respiration; but animal growth requires input from heterotrophic nutrition. Moreover, only 16 % and 4 % of alga-derived carbon is incorporated into the mineral portion of the light- and shadow-adapted *Stylophora pistillata* skeleton, respectively (Falkowski *et al.* 1984). These data indicate the photosynthate release by symbiotic algae may be a supplementary source of carbon to the animal hosts under certain circumstances.

1.3 Induction of photosynthate release by symbiotic algae

In the animal-microalga symbiosis, there is abundant evidence that algal symbionts provide the animal hosts with substantial amounts of photosynthetic products (e.g. Muscatine & Cernichiari 1969; Schmitz & Kremer 1977; Sutton & Hoegh-Guldberg 1990). The translocation of photosynthetic products from symbiotic algae to the animal in Cnidaria-*Symbiodinium* symbioses has been suggested to be induced by the host; but, the evidence for host control of photosynthate release by *Chlorella* is obscure (Hinde 1988). The release of photosynthetic products in Cnidaria-*Chlorella* symbioses has been suggested to be controlled by low pH (e.g.

Mews 1980; Douglas & Smith 1984). However, investigations of the pH within the peri-symbiont space which is the immediate environment enclosing *Chlorella* indicates the content of this space is not markedly acidic (Rands *et al.* 1992).

Exploration of the mechanism underlying host-induced photosynthate release by *Symbiodinium* requires identification of: (1) the specific component(s) in the host cell that induces photosynthate release; (2) the component(s) that influence intracellular metabolism of the algae to release photosynthetic carbon; (3) the component(s) that mediate photosynthate release in the intact symbiosis.

A typical experiment to demonstrate the host control of photosynthate release by symbiotic algae is to incubate freshly isolated algae with host homogenate and ^{14}C -bicarbonate. Consistent with many marine phytoplankton (Sharp 1977), the symbiotic alga *Symbiodinium* releases very little fixed carbon into seawater medium, only about 2-5 % of total carbon fixation (Hinde 1988). But, the percentage of photosynthate released is elevated to 20-60 % of total carbon fixation when the algae are incubated in seawater supplemented with host homogenates (e.g. Trench 1971b; Sutton & Hoegh-Guldberg 1990). The dependence on host material to induce the release of photosynthetic products from symbiotic algae suggests that the process of photosynthate release is triggered by a soluble factor(s) present in the animal tissues. This factor is generally named host factor or host releasing factor (Muscatine 1967; Hinde 1988). Furthermore, the "pulse and chase" experiments reveal that host factor induces the *Symbiodinium* to release only recently-fixed carbon to the host (Trench 1971b; Sutton & Hoegh-Guldberg 1990).

However, the chemical nature of the host factor has not been identified so far. Using host homogenate with an unknown concentration of host factor gives rise to the difficulty of comparing the results between different symbiotic associations on the same basis. The study of host impact on the photosynthetic metabolism in symbiotic algae is also made difficult by the biochemical complexity of the host homogenate. This may also be the reason for controversy about the properties of the host factor, for example, some "host factors" were found to be heat labile (Muscatine 1967, Trench 1971b, Sutton & Hoegh-Guldberg 1990) or heat stable (Muscatine 1967, Muscatine *et al.* 1972); enhancing carbon fixation (Trench 1971b) or not affecting carbon fixation (Sutton & Hoegh-Guldberg 1990); absent from alga-depleted animal and inducible by re-infection with its algal symbiont (Trench 1971b) or constitutive in the alga-depleted animals (Cook & Orlandini 1992); stimulating photosynthate release by cultured *Symbiodinium* and free-living non-symbiotic dinoflagellates or not being able to (Cook & Orlandini 1992, Masuda *et al.* 1994); displayed the molecular weight >10 kDa (Sutton & Hoegh-Guldberg 1990) or near 1 kDa (Cook & Orlandini 1992).

Gates *et al.* (1995) used synthetic chemicals to explore the activity of host factor, and they showed that amino acids at high concentrations (50 mM) might induce the photosynthate release by *Symbiodinium* isolated from a coral, *Pocillopora damicornis* and a sea anemone, *Aiptasia pulchella*, to a similar extent as the host homogenates. The effect was not specific to amino acids of particular chemical properties (e.g. acidic, nonpolar) or a biosynthetic family.

The use of isolated algae to study the host-factor effect also raises a fundamental question: how the effect relates to the conditions experienced

by *Symbiodinium* in the symbiosis. *Symbiodinium* cells are surrounded by a membrane of animal origin called the symbiosome membrane, which separates the algae from the animal cytoplasm (Roth *et al.* 1988). This membrane is considered to control both the conditions experienced by the algae in the symbiosome (e.g. pH and ionic composition) and the flux of compounds between algal and the animal cells (Douglas 1994). Moreover, the symbiosome membrane is formed during the endocytosis by the animal cell, and the contents of perisymbiont space and animal cytoplasm are unlikely to have a similar composition. Therefore, the simplest conclusion of the host-factor effect requires direct demonstration that the photosynthate release by symbiotic algae is mediated by the presence of factor(s) in the perisymbiont space. However, direct demonstration of the host factor effect in the intact association is almost technically impossible.

1.4 Identification of the photosynthetic compounds released from symbiotic algae

The determination of the chemical nature of the photosynthetic compounds released by symbiotic algae is mostly obtained from *in vitro* experiments (e.g. Muscatine 1967; Trench 1971b; Sutton & Hoegh-Guldberg 1990), and it is widely accepted that the released compounds are the same as the mobile compounds in the symbiosis. The mobile photosynthate of the symbiotic algae bear a common feature that it is dominated by carbon compounds. For example, in the lichen symbiosis, cyanobacterial symbiont release glucose and the algal symbiont release polyols (also known as polyhydric alcohols) (Smith 1980). Similarly, symbiotic *Chlorella* release maltose (Mews 1980); but results for *Symbiodinium* vary between associations, and may release a range of compounds including glycerol, organic acids, and lipid (e.g. Trench 1971b; Patton & Burris 1983).

The wide variation in the composition of translocated photosynthate derived from different *Symbiodinium* in different associations may be the result of different taxa of algae in the various animal hosts. Consistent with this proposal, several lines of evidence from molecular data of *Symbiodinium* 18S rRNA gene indicates a greater diversity of the algae in Cnidaria symbiosis than previous appreciated (Rowan & Powers 1991, McNally *et al.* 1994, Rowan & Knowlton 1995; Rowan *et al.* 1997; Billinghamurst *et al.* 1997).

1.5 The metabolic fates of photosynthate release in the Cnidaria bearing symbiotic algae

A proportion of photosynthetic compounds obtained by the animal hosts is used in animal's respiration and the remainder is incorporated into other compounds in the animal tissue. For example, the high light-adapted *Stylophora pistillata* uses up to 78 % of alga-derived carbon in respiration (Falkowski *et al.* 1984). Batty & Patton (1987) also proposed that the major fate of translocated glycerol (a major photosynthate release in several associations) in the animal tissue is rapidly mineralized to carbon dioxide. The carbon dioxide produced in the animal's respiration is believed to be taken up by algal partner for further photosynthesis. The photosynthesis-derived carbon that is not respired is converted to glycogen in hosts bearing *Chlorella* (Douglas 1994), and into lipid in hosts bearing *Symbiodinium* (Batty 1992). A proportion of the carbon is released as mucus into the surrounding environment (Falkowski *et al.* 1984; Batty 1992).

1.6 Nitrogen recycling in the Cnidaria-alga symbiosis

The nitrogenous nutrients required for the animal's growth and maintenance are traditionally considered to be derived from ingested organic nitrogen compounds, but plants and algae can utilize inorganic nitrogen. Animals have no general means of storing nitrogen as they store energy in glycogen or lipid. Amino acids or other organic nitrogen compounds in excess of the immediate requirements of animals are degraded, for example by gluconeogenesis to glucose with production of ammonia. Therefore, the traditional view about symbiotic algae has been that the algae are responsible for the assimilation of inorganic nitrogen, either as ammonium or nitrate from the environment or as ammonium from the animal cells (Lewis & Smith 1971). In particular, it has been widely accepted that the symbiotic algae may recycle the animal-derived waste ammonium. For the marine symbioses, *Symbiodinium* has been implicated in both the provision of photosynthesis-derived carbon and nitrogen recycling, and these processes are widely considered to underpin the ecological success of Cnidaria-alga symbioses in shallow, low-nutrient waters (Muscatine & Porter 1977; Falkowski *et al.* 1993).

However, nitrogen recycling has not been demonstrated directly in any alga-invertebrate symbiosis. Nitrogen recycling is a biochemically complex process, involving the bidirectional translocation of nutrients: first, the transfer of the animal's waste nitrogen compounds to the algal cells, which assimilate the nitrogen into compounds of nutritional value to the animal; and, second, the translocation of these latter compounds back to the animal (Douglas 1994).

It is accepted widely that symbiotic algae in culture or in Cnidaria host cell can assimilate ammonium. The chief evidence for ammonium

assimilation in the symbiosis is that animal bearing *Symbiodinium* can deplete ammonium from ammonium-supplemented seawater, whereas individuals deprived of their algae or incubated in darkness excrete ammonium (e.g. Wilkerson & Muscatine 1984; Kawaguti 1953; Cates & McLaughlin 1976; Szmant-Froelich & Pilson 1977). The green hydra-*Chlorella* symbiosis exhibits the same patterns (Rees 1986). The assimilation of ammonium by *Symbiodinium* has also been demonstrated by several studies (e.g. Summons & Osmond 1981; D'Elia *et al.* 1983; Wilkerson & Muscatine 1984; Anderson & Burris 1987; Dudler & Miller 1988). However, the evidence for the translocation of organic nitrogenous compounds back to animal host is poor. Only a few non-essential amino acids such as alanine and glutamate have been shown to be excreted from *Symbiodinium* in the presence of host tissue extracts, and always in low rates (von Holt & von Holt 1968; Trench 1971a,b; Sutton & Hoegh-Guldberg 1990). Evidence for the idea of nitrogen recycling in the Cnidaria-*Symbiodinium* symbiosis is, therefore, not persuasive.

A major study of nitrogen recycling in Cnidaria-*Symbiodinium* association was carried out by Muscatine *et al.* (1984). The nitrogen translocation rate estimated by the balanced-growth method suggested that more than 96 % of the inorganic nitrogen assimilated by the *Symbiodinium* in the coral *Stylophora pistillata* is transferred back to host. This estimate was based on the assumption that the animal host cannot assimilate ammonium, i.e. the inorganic nitrogen assimilated by the coral is exclusively through *Symbiodinium*. *Symbiodinium* is believed to have glutamine synthetase-glutamate synthetase complex (GS-GOGAT), which is an efficient ammonium assimilation system used in plants, algae and bacteria. But, the major ammonium assimilation enzyme detected in the coral tissue is glutamate dehydrogenase, whose K_m value is several orders

of magnitude higher than glutamine synthetase. Therefore, the capability for ammonium assimilation by the host cell was considered not to be able to compete with that by *Symbiodinium*, and could be ignored. However, the involvement of the host in ammonium assimilation has been clearly demonstrated in the hydra-*Chlorella* symbiosis (Rees 1986; McAuley 1995). Miller and Yellowlees (1989) also argued that higher glutamate dehydrogenase activity detected in the animal tissue might be able to compensate for the low affinity with substrate and to compete with *Symbiodinium* glutamine synthetase. Therefore, whether the symbiotic algae can recycle the ammonium derived from Cnidaria host is still an open question.

1.7 Aims of this study

Previous studies have provided a wide basis for understanding the symbiosis between animal and endosymbiotic microalga. Traditionally, endosymbiotic algae are believed to assimilate carbon dioxide derived from animal respiration and transfer photosynthetic products back to the host (carbon recycling); and to recycle the ammonium produced from the animal's nitrogen catabolism and transfer synthesized amino acids back to the host (nitrogen recycling). The closed nutrient recycling system enables the symbioses to thrive in the low food supply and oligotrophic (especially nitrogen limited) environment. However, two important questions about the nutrient interaction between symbiotic partners remained unresolved.

First, how does the Cnidaria host mediate photosynthate release from *Symbiodinium*? Even though there is abundant evidence that the marine Cnidaria host may contain so-called "host factor(s)" which induce the release of photosynthetic products from *Symbiodinium*, very little is

known about the mechanism underlying the process of photosynthate translocation. The key point of the limited information on this issue is our ignorance of the chemical identity of "host factor(s)". The recent finding that amino acids can exhibit "host factor" activity provides the basis for further study. The research in this thesis aims to examine whether amino acids are the host factor.

Second, does *Symbiodinium* recycle host-derived ammonium? In previous studies, carbon and nitrogen metabolism in the Cnidaria-microalga symbioses have always been considered separately, neglecting the impact of photosynthate release on nitrogen metabolism in the animal host (Miller & Yellowlees 1989). The extra carbon supplement from translocated photosynthate may have a crucial influence on the animal's nitrogen metabolism, for example to conserve the animal's amino acid pool from being consumed in respiration (Rees & Ellard 1989). This consideration gives rise to the possibility that *Symbiodinium* may provide photosynthetic carbon to help the animal host conserve its nitrogen rather than directly provide recycled amino acids to the host. The recent paper that Cnidaria can synthesize the whole set of protein amino acids (FitzGerald & Szmant 1997) further questions whether the algae recycle nitrogen. The second aim of this research was to examine the impact of photosynthate release on the animal's nitrogen metabolism as a way to explore the validity of nitrogen recycling

The experiments were conducted on *Aiptasia pulchella* for several reasons: (1) it has been used extensively for laboratory experimentation (e.g. Trench 1971b; Wilkerson & Muscatine 1984; Muller-Parker 1985; Weis 1993); (2) *Aiptasia pulchella* often reproduce asexually, so that the clone of

animals can be used for experiments; (3) unlike corals, sea anemones lack a calcareous skeleton, and are relatively easy for biochemical analysis.

In summary, the specific aims of the research in this thesis were to:

- (1) explore whether amino acids may be the "host factor" by examining the variation of photosynthate release with amino acid concentration, and comparing the impact of amino acid and host homogenate on photosynthetic metabolism in the *Symbiodinium* (chapter 3).
- (2) assess the impact of photosynthate release by *Symbiodinium* on the animal's nitrogen metabolism, with a view to establishing whether the algae recycle nitrogen (chapter 4).
- (3) evaluate the capability of the sea anemone to synthesize all the protein amino acids (chapter 5).

Chapter 2 Materials and Methods

2.1 Materials

Unless otherwise stated, chemicals and reagents were supplied by following companies: B.D.H. Chemicals Ltd., Poole, Dorset, UK (inorganic compounds, TLC plates, and HPLC solvents); Sigma Chemical Company Ltd. Poole, Dorset, UK (organic compounds, X-ray film and the diagnostic kits for aspartate aminotransferase, alanine aminotransferase, and ammonium); New Life Science Products Company, Thame, Oxon, UK (radiochemicals).

2.2 Maintenance of *Aiptasia pulchella*

All experiments were conducted on a clonal culture of *Aiptasia pulchella*, collected from a sea water dike at Tongkang (22° N), Taiwan.

2.2.1 "Control animal"

A stock of sea anemones were maintained in aerated artificial seawater (ASW, made from Instant Ocean salts, Aquarium Systems, Sarrebourg, France) at 25 °C under 12 h : 12 h light-dark regime with light intensity 30 $\mu\text{E m}^{-2} \text{s}^{-1}$ P.A.R., and fed with one-day-old *Artemia* nauplii twice a week. The sea anemones maintained in this standard condition were termed "control animal".

2.2.2 "Alga-depleted animal"

A sub-group of *A. pulchella* was treated by cold shock as described by Steen and Muscatine (1987), with the aim to generate aposymbiotic (alga-free) animals. The control animals were incubated in ice-cold artificial seawater

for 4 h, then returned to standard culture conditions with continuous darkness. By this treatment, the algal density in the animal's tentacles was reduced from $5-8 \times 10^6$ algae mg^{-1} animal protein in control animals to about 2×10^3 algae mg^{-1} animal protein within a month, and declined further, variably but slowly, during the following three months. These sea anemones were termed "alga-depleted animal". They were maintained in continuous darkness, except during maintenance and feeding, and used for experiments between 1 and 4 months after cold shock.

2.2.3 Animals with recovering algal population

To prepare the sea anemones with varied algal density, 30 alga-depleted animals which had been maintained in the continuous darkness for 3 months were returned to the standard culture condition. After 3 months of exposure to the standard light regime, the density of algae in the tentacles varied widely, ranged from 3×10^3 to 2×10^6 algae mg^{-1} animal protein. Because no *Symbiodinium* were fed to these animals during recovery of algal population, the increased algal density was exclusively derived from the endogenous algal population.

2.2.4 Aseptic culture

Aseptic culture of *Aiptasia pulchella* was obtained by antibiotic treatment as follows. All glassware used in this experiment were autoclaved in advance at 121°C for 20 min, and the artificial seawater containing 1 mg ml^{-1} ampicillin and $50 \mu\text{g ml}^{-1}$ streptomycin was filtered through $0.8\text{-}/0.2\text{-}\mu\text{m}$ membrane filter (Acrodisc PF, Gelman, Northampton, UK) before use. The sea anemones were washed with antibiotic supplemented ASW several times, and then each of three animals was incubated in 50 ml aseptic ASW medium. These animals were fed with autoclaved one-day-old *Artemia* nauplii every second day. Sterility of the cultures was tested

routinely on nutrient agar-ASW plate (code CM3, OXOID Ltd. Hants, England). About 500 μl ASW or 100 μl tissue homogenate of sea anemone were spread on agar plate, and incubated at 30 °C for 1-2 days to observe the formation of bacterial colonies. The routine sterility check showed that the bacterial counts were reduced from $7-8 \times 10^3$ CFU ml^{-1} in standard culture to zero within a week of antibiotic treatment.

All the experiments with "control animals" or isolated *Symbiodinium* were conducted in the light ($60 \mu\text{E m}^{-2} \text{s}^{-1}$ P.A.R.), and those with "alga-depleted animals" were in the dark, unless stated otherwise.

2.3 Photosynthetic carbon fixation and translocation by *Symbiodinium*

Two experimental systems were used to characterize the photosynthetic carbon fixation and translocation by *Symbiodinium*; intact *A. pulchella*, and the *Symbiodinium* freshly isolated from *A. pulchella*.

2.3.1 In the intact animal

Each of the duplicate control animals was incubated in 2 ml ASW supplemented with 4 μCi sodium [^{14}C] bicarbonate (Amersham International, Kent, UK), under (1) illumination (control); (2) illumination plus 10 μM dichlorophenyldimethylurea supplement in ASW; (3) in darkness. The incubations were terminated at 0.5, 2.5, 3.5, and 4.5 h, and the animals were homogenized in a hand-held glass tissue grinder with 6 ml ASW. Then, the homogenate was centrifuged at 247 g for 3 min to separate animal fraction and algae. The algal pellet was washed once by resuspension in 2 ml ASW followed with centrifugation at the same condition as above, and finally suspended in 5 ml ASW for the

determination of algal density (see section 2.6.1.2) and radioactivity. The supernatant obtained from centrifugation was pooled and the volume was measured. Acetic acid (200 μ l of 4 M) was added to each sample, 100 μ l of supernatant and 50 μ l of algal suspension, and shaken vigorously for 1 h, to remove unfixed ^{14}C . The radioactivity in each sample was measured as in section 2.8, and converted to dpm per algal cell.

Photosynthetic carbon fixation in the intact sea anemones was also examined with short term incubations, e.g. 5-150 sec. The sea anemones were incubated with sodium [^{14}C] bicarbonate as described above. At each time interval, the sample was dipped into liquid nitrogen to terminate the photosynthesis of *Symbiodinium*, then dried in a freeze-drier. The water soluble components and lipid in the dried tissue powder were extracted with 1 ml methanol:chloroform:H₂O:formic acid (12:5:2:1, v/v) three times, and the remaining precipitate was solubilized in 1 ml of 2 M NaOH by incubation at 100 °C for 15 min. The radioactivity in the solvent fraction and neutralized NaOH extract was quantified separately after the unfixed ^{14}C was driven off as described above, and the protein content in the neutralized NaOH extract was determined as in section 2.5.7. The algal density of each sample was estimated from total tissue protein content, using, as conversion factor, 5.9×10^6 algal cells mg^{-1} tissue protein (which was determined in a separate experiment), and the carbon fixation rate was expressed as dpm per algal cell.

2.3.2 In the isolated *Symbiodinium*.

The purpose of the experiment was to (1) compare the photosynthetic fixation rate between the freshly isolated *Symbiodinium* and in the intact

Table 2.1 Composition of SW buffer (pH 8.2).

contents	mM
NaCl	420
MgSO ₄	26
MgCl ₂	23
KCl	9
CaCl ₂	9
NaHCO ₃	2
HEPES	10

α: This buffer with 0.1 μM K₂HPO₄ supplement is termed SW-P buffer.

animal; and (2) establish whether photosynthate release by *Symbiodinium* was derived from recently-synthesized compounds.

2.3.2.1 Preparation of freshly isolated *Symbiodinium* and host extract

To isolate the *Symbiodinium*, excised *A. pulchella* tentacles were homogenized in a hand-held glass tissue grinder with SW buffer (see Table 2.1). The homogenate was passed through a 53- μm nylon mesh, and the filtrate was centrifuged at 247 g for 1 min. The supernatant ("host extract") was decanted and passed through a 0.8-/0.2- μm membrane filter to remove tissue debris and algal contamination. The pellet of *Symbiodinium* was washed three times by suspension in SW buffer and centrifugation, and finally filtered through a 15- μm nylon mesh to eliminate nematocysts. The algal density of isolated *Symbiodinium* was determined as described in section 2.6.1.2. The host extract was used within 30 min of preparation.

2.3.2.2 Photosynthetic carbon fixation and translocation by the freshly isolated *Symbiodinium*

Each sample of 10^6 *Symbiodinium* cells was incubated with 2 μCi sodium [^{14}C] bicarbonate in 1 ml SW buffer (the control) or SW buffer supplemented with host extract. At each time interval, the radioactivity of fixed ^{14}C in 20 μl of the suspension and 50 μl of supernatant (obtained by centrifugation of sample at 11,600 g for 2 min) of each duplicated sample was measured as described in section 2.3.1. The total carbon fixed during the incubation was calculated from ^{14}C in the suspension, and expressed as dpm cell $^{-1}$. The percentage of photosynthate released by *Symbiodinium* was calculated as below:

$$\% \text{ of fixed } ^{14}\text{C} \text{ release} = 100\% \times (\text{dpm in supernatant} / \text{dpm in suspension})$$

Table 2.2 Composition of a complex mixture of amino acid from Gates *et al.* (1995).

contents	mM
Aspartate	1.80
Glutamate	21.80
Serine	1.96
Glycine	11.27
Arginine	2.25
Alanine	12.89
Tyrosine	1.32
Histidine	1.84
Methionine	3.08
Valine	4.73
Phenylalanine	3.17
Isoleucine	2.26
Leucine	2.64
Taurine	3.85
Total	74.86

The experiments included a "dark control", i.e. samples incubated as above but in darkness; ^{14}C fixation by the dark control was consistently less than 1 % of the experimental samples incubated in the light.

2.3.2.3 Pulse-chase experiment

Nine replicates of 10^6 *Symbiodinium* cells were incubated in 1 ml SW buffer, supplemented with 2 μCi sodium [^{14}C] bicarbonate ("pulse"). Then, the release of pre-formed photosynthate was examined by incubating the algae (obtained by centrifugation at 11,600 g, 1 min followed with a wash in SW buffer) in the non-isotope SW buffer, FAA mixture (see Table 2.2), or host extract ("chase"). The duration in "pulse" and "chase" incubation was both for 1 h, and then the radioactivity in the algae and supernatant was quantified as above. The "pulse" experiment was also examined with FAA medium and host extract respectively, followed with the same "chase" condition as above. A control with 1 h pre-treatment in SW buffer prior to pulse experiment was also conducted to confirm that no detrimental effect to the isolated *Symbiodinium* had been made during pulse-chase procedure.

2.4 The impact of taurine on the photosynthate release by *Symbiodinium*

2.4.1 Effect of amino acids on the photosynthate release by *Symbiodinium*

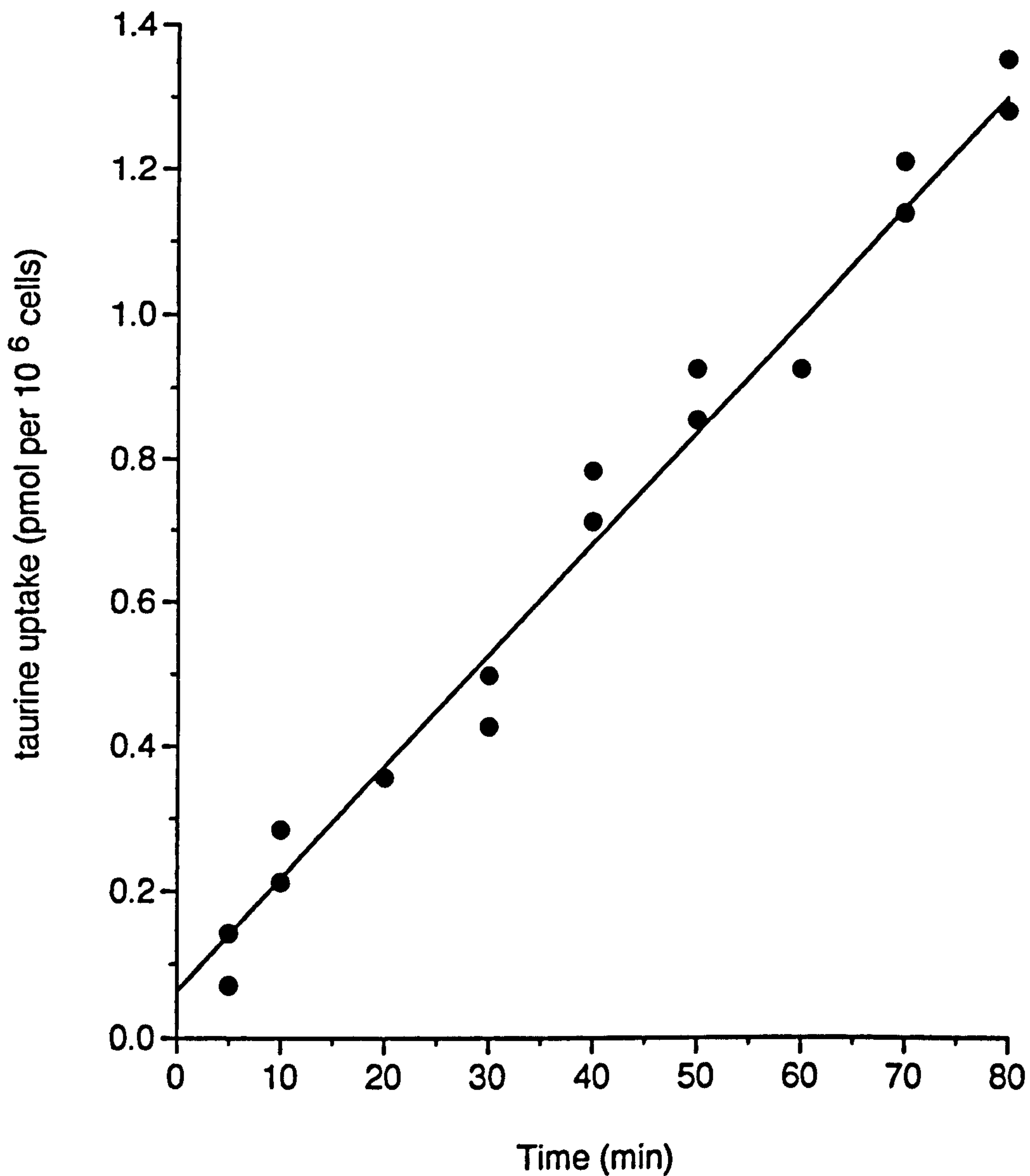
The freshly isolated *Symbiodinium* and host extract were prepared as described at 2.3.2.1. Each sample of 5×10^5 *Symbiodinium* cells was incubated with 2 μCi sodium [^{14}C] bicarbonate in 0.8 ml SW buffer (the control) or SW buffer supplemented with host extract, single amino acid or a complex mixture of amino acids (see Table 2.2). The various supplements had no detectable effect on the pH of the incubation medium,

reflecting the high buffering capacity of SW buffer. The duration of experiment was 1 h, and the radioactivity of fixed ^{14}C in 20 μl of the suspension and 50 μl of supernatant (obtained by centrifugation of sample at 11,600 g for 2 min) was measured as described in section 2.3.1. The total carbon fixation and the percentage of photosynthate release by *Symbiodinium* for each sample was determined as mentioned in section 2.3.2.2.

To identify the ^{14}C -labelled compounds, each algal and supernatant fraction was freeze-dried and extracted with methanol:chloroform:H₂O:formic acid (12:5:2:1, v/v) by the procedure of Redgwell (1980), and the radioactivity was quantified in the chloroform (lipid), aqueous and particulate fractions. The aqueous layer from the supernatant fraction was further separated into neutral, acidic, and basic fractions by Sephadex SP and QAE ion-exchange chromatography (Redgwell 1980). The eluted fractions from ion-exchange columns were dried in a rotary evaporator and re-suspended in 1 ml distilled water. The radioactivity of each sample from the concentrated eluate were quantified and standardized with cell numbers. The organic acid fraction from the ion-exchange column were separated by thin-layer electrophoresis (TLE) and chromatography (TLC) on cellulose plates by the method of Schürmann (1969) modified as follows. A 2 μl sample of the organic acid fraction which contained about 2,000-3,000 dpm was spotted onto the left corner of a cellulose plate and electrophoresed on the pre-chilled cold plate from cathode to anode at 450 V in pyridine:acetic acid:water (10:35:955, v/v) buffer, pH 3.9 for 35 min. The cellulose plate was dried and developed by isopropanol:formic acid:H₂O (6:2:1, v/v) in the second dimension. The radioactive spots were visualized by exposing to Kodak X-ray film for one week, and identified by

Figure 2.1 The time-course of [^{14}C]taurine uptake by isolated *Symbiodinium*.

Freshly isolated *Symbiodinium* (3×10^5) was incubated with $1 \mu\text{Ci}$ ($33 \mu\text{M}$) taurine in 0.5 ml SW buffer for a time course from 5 to 80 min, and the radioactivity of the cells in duplicate samples was measured every 10 min.



comparison with authentic organic acids: aspartate, citrate, fumarate, glutamate, glycolate, lactate, malate, oxaloacetate, pyruvate, and succinate.

2.4.2 Uptake of exogenous taurine by *Symbiodinium*

Isolated cells of *Symbiodinium* were incubated in 0.5 ml SW buffer supplemented with 5 μ M- 10 mM taurine and [1,2- 14 C]-taurine. To terminate taurine uptake, the algal suspension was applied to a filter (GF/C, Whatman), using a 12-place filtration manifold (Hoefer, San Francisco, CA, USA), and the filter was washed twice with 1 ml SW buffer. The filter was added to 4 ml scintillation cocktail for radioactivity determination. The metabolism of taurine taken up by *Symbiodinium* was checked by chromatography of the 70 % ethanol extract on a cellulose plate with chloroform:methanol:17.5 % NH_3 (2:2:1, v/v) as developing solvent. The non-specific binding of 14 C-taurine onto the surface of *Symbiodinium* was checked by incubating the cells at 100 °C for 10 min, which reduced the uptake rate by 96 %.

2.4.3 Kinetic study of taurine uptake and taurine-stimulated photosynthate release

Preliminary experiments with an incubation of isolated *Symbiodinium* in 33 μ M taurine indicated that taurine uptake was linear over at least 1 h (Fig. 2.1). For the parallel study of the kinetics of taurine uptake and taurine-induced photosynthate release, the *Symbiodinium* cells were incubated in SW buffer supplemented with 10-1,000 μ M taurine and either 14 C-taurine (0.01-0.2 μ Ci) or sodium [14 C] bicarbonate (2 μ Ci). A separate experiment was also conducted to explore the effect of an amino acid complex on taurine-stimulated photosynthate release by incubating *Symbiodinium* in the SW buffer supplemented with 2 μ Ci [14 C] bicarbonate and 10 μ M to 24 mM of amino acid complex diluted from FAA mixture (see Table 2.2). Taurine uptake and photosynthate release,

respectively, were assayed as above. The V_{\max} and kinetic constant of the half maximum rate of taurine uptake or taurine-stimulated photosynthate release (K_m) were determined from Eadie-Hofstee plots.

2.5 Effect of algal density on the nitrogen metabolism

In this study, the status of nitrogen metabolism in the animal tissue of *A. pulchella* was determined by several indices, e.g. the content of free amino acids, ammonium, and corresponding enzyme activities. The impact by algal symbionts was evaluated by comparing these indices between control animals and alga-depleted animals, and between the sea anemones with varied algal density derived from endogenous repopulation of the algae. All experiments mentioned in section 2.5 and 2.6 were conducted on tentacles of the animal, because the algal density was found to be more constant in this portion compared to in the body column (A. E. Douglas unpublished data). Precaution of avoiding the influence from food intake has been made by using the animal at the fourth day since feeding.

2.5.1 Sample preparation

The tentacles of each replicated sea anemone were excised and homogenized in a hand-held glass tissue grinder with 0.5 ml ice-cold 3.5% NaCl solution. The homogenate was centrifuged at 20,100 g, 4 °C for 5 min, and the supernatant was decanted for further analysis. The extraction for enzyme assay was used immediately after preparation, and that for ammonium and uric acid quantification was stored at -20 °C and used within 2 days. For free amino acid analysis, 230 μ l absolute ethanol was added to 100 μ l of supernatant, and then centrifuged at 20,100 g, 4 °C for 3 min to precipitate the protein. The supernatant obtained from centrifugation was stored at -20 °C before analysis.

Table 2.3 Reagents for glutamine synthetase and NADPH-glutamate dehydrogenase assay.

a. glutamine synthetase

imidazole buffer (final pH = 7.4)

imidazole-HCl	40 mM
MnCl ₂	0.6 mM
sucrose	0.5 M
dithiothreitol	20 mM
cetyltrimethylammonium-bromide	200 µg ml ⁻¹

substrate solution

imidazole-HCl (pH 7.0)	0.3 M
MnCl ₂	50 mM
potassium arsenate (pH 7.0, KOH)	0.26 M
hydroxylamine (pH 7.0, NaOH)	0.7 M
ADP (pH 7.0, NaOH)	0.1 M
L-glutamine	67 mM
cetyltrimethylammonium-bromide	10 mg ml ⁻¹

stop mix

10 % FeCl ₃	4 ml
24 % trichloroacetic acid	50 ml
H ₂ O	6.5 ml
6 N HCl	250 µl

standard

L-glutamic acid γ-monohydroxamate in imidazole buffer	0-3 µmol
--	----------

b. substrate solution for NADPH-glutamate dehydrogenase assay

NADPH	0.12 mM
α-ketoglutarate	10 mM
NH ₄ Cl	0.4 M
MOPS-KOH, pH 7.5	20 mM

2.5.2 Glutamine synthetase (EC 6.3.1.2)

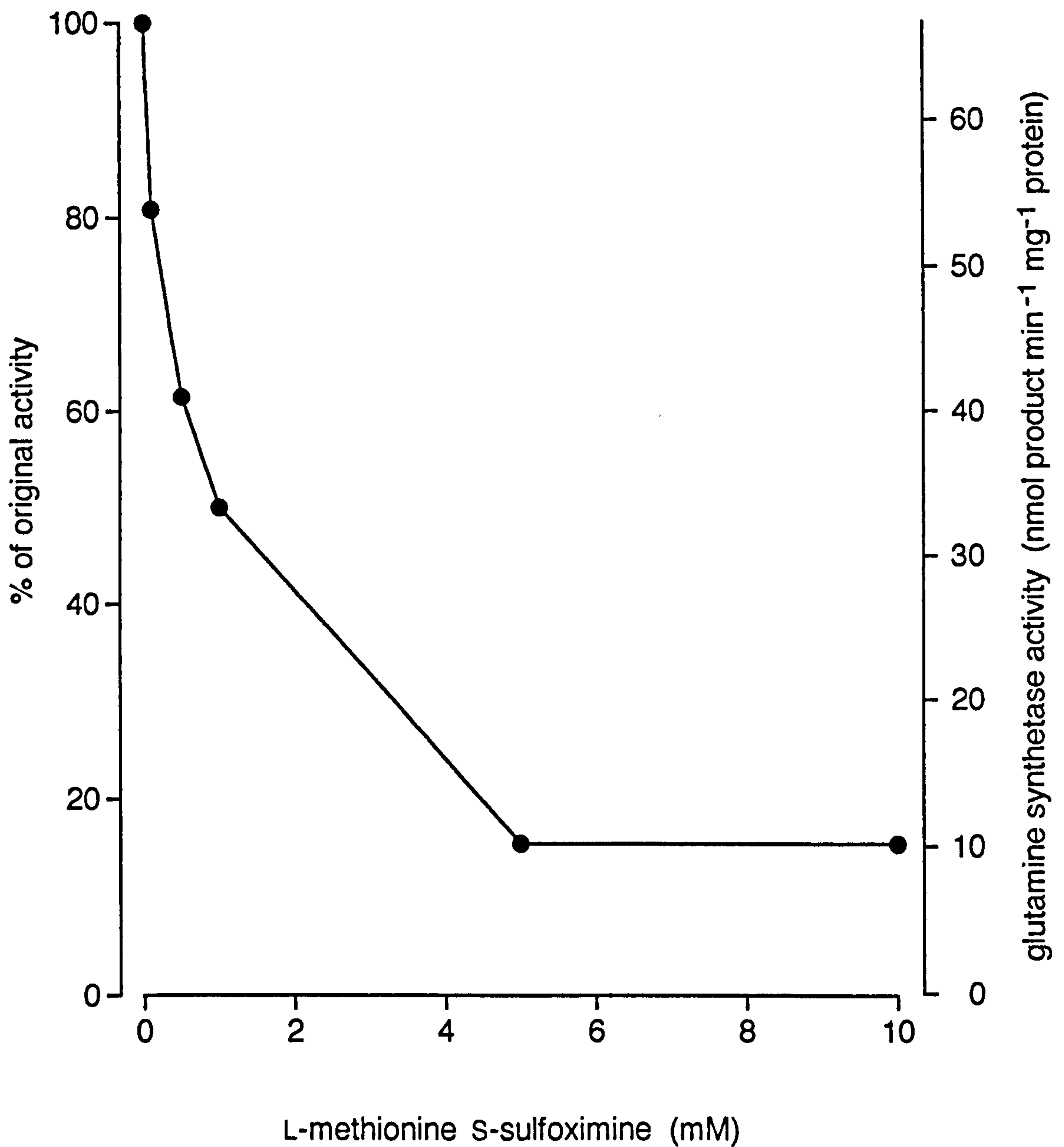
To assay the apparent glutamine synthetase activity, 250 μ l of tissue extract was diluted with an equal volume of imidazole buffer (see Table 2.3). Half of the diluted sample was incubated at 100 °C for 15 min, then centrifuged at 20,100 g, 4 °C for 3 min to collect supernatant for blank assay (i.e. sample blank). Glutamine synthetase activity was quantified by the transferase assay (Pahel *et al.* 1982), in which 250 μ l substrate solution (Table 2.3) was incubated with an equal volume of diluted tissue extract (or sample blank) at 30 °C water bath for 30 min, and then added with 1 ml of stop mix. After centrifugation at 11,600 g for 3 min, the synthesis of γ -glutamyl hydroxamate from glutamine is determined spectrophotometrically at 540 nm, and results were expressed as nmol γ -glutamyl hydroxamate formed per mg tissue protein per min at 30 °C. The specificity of enzyme reaction was confirmed by adding with the specific inhibitor, L-methionine S-sulfoximine (Fig. 2.2). The enzyme activity was reduced by 85 % when the enzyme extraction was pre-treated with 5 mM inhibitor for 10 min.

2.5.3 NADPH-glutamate dehydrogenase (EC 1.4.1.2)

The apparent activity of NADPH-glutamate dehydrogenase was determined in the aminating direction, in which 50 μ l of tissue extract was incubated with 1 ml of substrate solution (Table 2.3) at room temperature (20-22 °C) for 10 min. The enzyme activity was measured by the decrease of absorbance at 340 nm, and expressed as nmol NADP formed per mg tissue protein per min. The specificity of the enzyme reaction was confirmed by the co-incubation of tissue extract and substrate solution without ammonium chloride or α -ketoglutarate, which reduced the activity by 96-97 %.

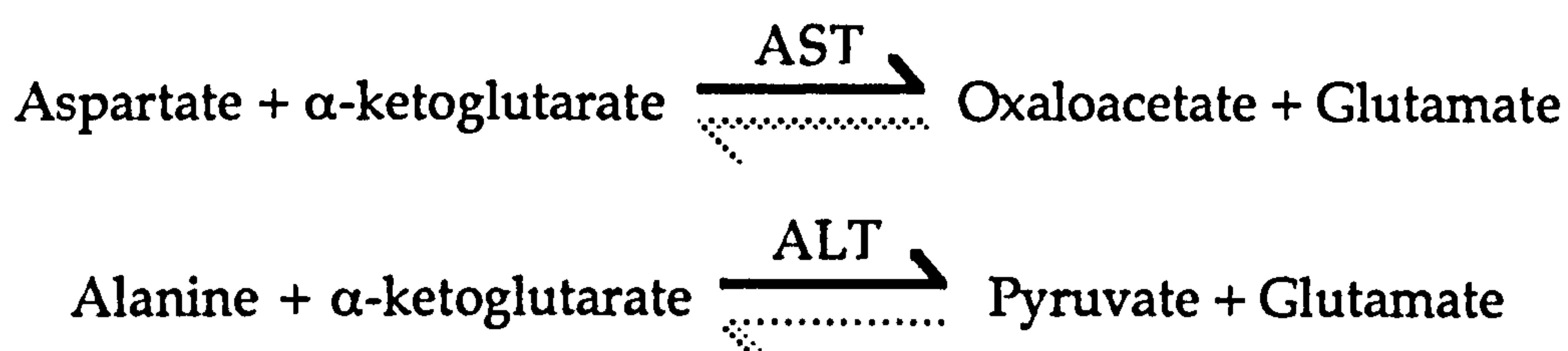
Figure 2.2 Inhibition of glutamine synthetase activity by L-methionine S-sulfoximine.

The tissue extract was incubated with a range concentration of L-methionine S-sulfoximine for 10 min at room temperature, then the glutamine synthetase activity was measured as described in section 2.5.2.



2.5.4 Aminotransferase

The apparent enzyme activities of aspartate aminotransferase (AST: EC 2.6.1.1) and alanine aminotransferase (ALT: EC 2.6.1.2) was quantified by using Sigma transaminase kit (Procedure No. 505, Sigma Co. USA). The reaction for the enzymes are as follows:



The oxaloacetate and pyruvate formed in the above reaction were reacted with 2,4-dinitrophenylhydrazine and determined spectrophotometrically at 500 nm. The total reaction volume was scaled down to 1.22 ml for microassay. By following manufacturer's instruction, the results of aspartate aminotransferase and alanine aminotransferase activity were multiplied by a factor 0.48, and expressed as nmol glutamate formed per mg tissue protein per min at pH 7.5 and 25 °C. Specificity of the enzyme reaction was also confirmed by omission of substrate (aspartate for aspartate aminotransferase and alanine for alanine aminotransferase), which reduced the activity by 97 %.

2.5.5 Amino acid composition

Free amino acids were quantified by reverse-phase HPLC after *o*-phthaldialdehyde derivitization (Jones *et al.* 1981). A sample of 10 μ l ethanol extract (see section 2.5.1) was reacted with the equal volume of *o*-phthaldialdehyde reagent (Sigma Co. USA) for 1 min at room temperature, and then 20 μ l of 0.1 M KH_2PO_4 was added to stop the reaction. The *o*-phthaldialdehyde derivatized amino acids were injected into a C_{18} -ultrasphere column with a Beckman System Gold solvent

delivery system and monitored with Shimadzu RF-551 fluorescence detector. The reference amino acid mixture was AA-S-18 supplemented with asparagine, glutamine, GABA, tryptophan, taurine; and ornithine. A standard separation profile is shown in Fig. 2.3. Note that proline and cysteine could not be detected by this method.

2.5.6 Nitrogenous waste compounds

The ammonium content of the sea anemones was quantified with an ammonia diagnostic kit (Procedure No. 171UV, Sigma Co.), in which the amination of α -ketoglutarate was measured by the oxidation of NADPH with a decrease in absorbance at 340 nm. The result of ammonium assay was converted to nmol per mg tissue protein, following manufacturer's instruction.

Prior to uric acid assay, 500 μ l tissue extract was diluted with an equal volume of 40 mM sodium borate buffer pH 8.5, and incubated at 100 °C for 10 min. The heat-denatured protein was removed by centrifugation at 11,600 g for 3 min, and then the supernatant was incubated with 0.01 unit of uricase for 30 min. The concentration of uric acid in the sample was determined by the decrease of absorbance at 292 nm with the molar extinction coefficient $12.3 \text{ cm}^{-1} \text{ mM}^{-1}$. The content of uric acid in animal tissue was converted to nmol per mg tissue protein.

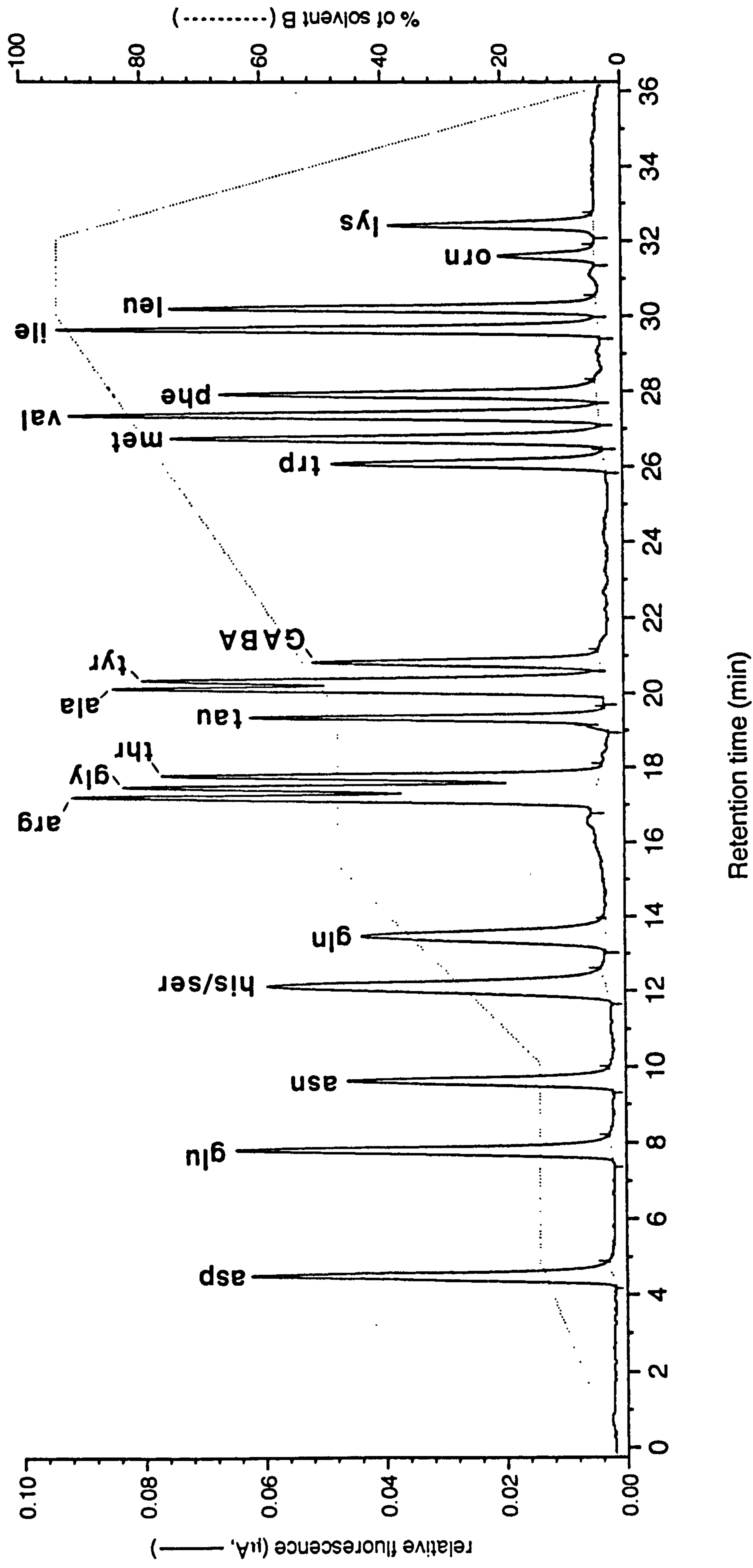
2.5.7 Protein content

The protein content in animal tissue extract was quantified by protein assay kit of Bio-Rad Chemical Co., following manufacturer's instructions for the microassay with 0-16 μ g bovine serum albumin as standard.

Figure 2.3 Elution profile of amino acid standards derivatized by the reaction with *o*-phthalaldehyde.

Each peak represents 62.5 pmol of amino acid. Non-standard abbreviations used: tau, taurine; GABA, γ -aminobutyric acid; orn, ornithine.

γ -aminobutyric acid; orn, ornithine.



2.6 Effect of light regime, exogenous organic carbon, and inorganic nitrogen supplement on nitrogen metabolism

2.6.1 Impact of light regime

Two separate experiments were conducted to explore the effect of light regime on the nitrogen metabolism in animal tissue of *A. pulchella*. First, a group of 30 control animals was incubated in 10 L aerated ASW under continuous darkness for 12 days. Another group of 30 animals maintained in the standard condition was used as the control. At each time interval, the wet weight of each animals was measured, and 2 animals from each group were collected and excised for quantification of algal density, chlorophyll *a* content, and nitrogen metabolic indices. Second, a group of 66 control animals was incubated in an aquarium tank containing 27 L aerated ASW. The dark treatment was conducted for 6 days, and then returned to standard light-dark regime. Six sea anemones were collected at each time interval for the quantification of enzyme activity (e.g. aspartate aminotransferase, alanine aminotransferase, and glutamine synthetase) and protein content. The tanks with dark treatment were covered with two layers of black plastic sheet. During the experiment, the animals were fed daily with one-day-old *Artemia* nauplii, and the ASW was changed every second day.

2.6.1.1 Wet weight of animals

The wet weight of sea anemone was determined in an analytical balance (precision: ± 0.1 mg, Precision Instruments, Leeds, UK) after removing excess seawater from the surface of sea anemones by filter paper. Error occurred in the weighing was checked with repeating measurement with the same animal for 6 times. The standard errors of repeating measurements on the same animal ranged from 3 mg to 26 mg obtained from 6 replicates with the mean weight ranged from 190 mg to 259 mg.

2.6.1.2 Algal density and the percent of dividing cells

Freshly isolated *Symbiodinium* cells were suspended homogeneously in 50 µl to 1.5 ml ASW depending on the algal density. Then 10 µl of algal suspension was applied to Neubauer improved haemocytometer, and the number of the cells was counted under light microscope at x400 magnification. The mean algal number per sample was obtained from 4-6 replicate counts, and the number of dividing cells per 1000 algal cells was scored.

2.6.1.3 Chlorophyll *a*

The chlorophyll *a* content in *Symbiodinium* cells was quantified by the spectrophotometric method (Jeffrey & Haxo 1968). The algal pellet was extracted in the dark overnight with 1 ml ice-cold 90 % acetone comprising 1 % of each of MgCl₂ and Na₂CO₃. The content of chlorophyll *a* in the acetone extract was determined by the equation as below:

$$\text{Chl. } a \text{ (}\mu\text{g ml}^{-1}\text{)} = 13.31 E_{663} - 0.27 E_{630}$$

2.6.2 Exogenous organic carbon supplement

To evaluate the effect of carbon supplement on host glutamine synthetase activity, each of three replicated control or alga-depleted sea anemones was incubated in 250 ml aerated ASW (control) or ASW supplemented with 10 mM carbon compound (fumarate, succinate, glucose, glycerol, or α -ketoglutarate) under continuous darkness. A separate set of control sea anemones maintained in standard light-dark regime without carbon supplement was used as positive control. The duration of all experiments was 7 days, and incubation dishes were cleaned and replaced with fresh medium daily. At the end of the experiment, the sea anemones were

collected and excised for the quantification of glutamine synthetase activity and ammonium content in animal tissue.

A further experiment explored the effect of α -ketoglutarate on the free amino acid profile of the sea anemones. Each of 12 control or alga-depleted animals was incubated in ASW or ASW supplemented with 10 mM α -ketoglutarate. After 7 days of incubation, amino acids of the tentacles in each sample were assayed as described as above.

2.6.3. Exogenous inorganic nitrogen supplement

The effect of inorganic nitrogen supplement on the *A. pulchella* was examined by three features; nitrogen metabolism (e.g. glutamine synthetase activity and ammonium content); photosynthetic carbon fixation in the symbiosis; and the dividing of algal cells.

In situ ammonium concentration of *A. pulchella* was elevated by incubating control and alga-depleted animals in 1.2 L SW-P buffer (Table 2.1) supplemented with 0, 10, 30, 60, 100 μ M ammonium chloride. After 24 h of incubation in regular light-dark regime, tentacles of six replicates from each treatment were excised for the quantification of glutamine synthetase activity and ammonium content as described in section 2.5.2 and 2.5.6, and the algal density and percentage of dividing algal cells in the control animals were determined (see section 2.6.1.2).

For quantifying photosynthetic carbon fixation rate, another six replicates of control animals from each treatment were incubated in 2 ml SW buffer with 4 μ Ci of sodium [14 C] bicarbonate (Amersham Co.) for 1 hr. The total carbon fixation and translocation by *Symbiodinium* were determined as described in section 2.3.1.

2.7 Biosynthesis of amino acids

2.7.1 Incubation with ^{14}C -labelled precursors

On the basis of preliminary experiments modified from FitzGerald and Szmant (1997), a single pulse-chase design was used to quantify amino acid synthesis, as follows: Individual *A. pulchella* in aseptic culture were incubated for 2 h in 1 ml aseptic ASW medium, supplemented with 4 μCi [^{14}C] glucose, 1 μCi [^{14}C] aspartate and 1 μCi [^{14}C] glutamate, and then transferred to non-radioactive sterile ASW for 2 days prior to analysis. The bleached animals were incubated in ASW supplemented with 10 mM α -ketoglutarate, a treatment that ensures that nitrogen assimilation and amino acid synthesis in these animals are not carbon-limited. At the end of each experiment, the animals were homogenized separately with 0.5 ml ice-cold 3.5 % NaCl solution, and centrifuged at 20,100 g 4°C for 5 min. Two 5 μl samples of the supernatant were used to quantify protein content and radioactivity respectively, and the rest of the supernatant was mixed with ice-cold 20 % trichloroacetic acid in 1:1 volume ratio to precipitate the protein. After 30 min on ice, the suspension was centrifuged at the same condition as above to obtain the trichloroacetic acid extract and the protein pellet. The protein pellet was washed twice with 10 % trichloroacetic acid and then with acetone to remove low molecular weight contaminants and lipids, and dried in the fume cupboard. The dried protein pellet was hydrolyzed in a vacuum-sealed ampoule with 6 M HCl at 110 °C for 24 h. The hydrolysate samples were dried in a Speed-Vac (SVC 100, SAVANT) to remove HCl, and then dissolved in 70 % ethanol. Because acid hydrolysis converts glutamine into glutamate and asparagine into aspartate, the pair of these amino acids are referred to as Glx and Asx respectively. Acid hydrolysis also breaks down tryptophan completely (FitzGerald & Szmant 1997), thus this method could not detect the synthesis of tryptophan.

Table 2.4 Preparation of 60 mM *o* -phthaldialdehyde reagent. The components are mixed in the order from top to bottom.

components	
<i>o</i> -phthaldialdehyde	8.0 mg
absolute methanol	100 μ l
β -mercaptoethanol	20 μ l
0.4 M boric acid (pH 10.4, NaOH)	880 μ l

Table 2.5 Cellulose acetate solution (Bieleski & Turner 1966).

components	
<u>solution A</u>	
cellulose acetate	1.8 g
diethylene glycol	0.9 g
camphor	0.6 g
H ₂ O	30 ml
<u>solution B</u>	
n-propanol	10 ml
<u>working solution</u>	
mix solution A and B	30 ml A and 10 ml B

Table 2.6 Preparation of epon araldite resin (Meg Stark's recipe).

components	
agar	8 g
dodecenyl succinic anhydride	18 g
araldite CY212	4 g
N-benzyl dimethylamine	0.7 ml

The trichloroacetic acid extract was further neutralized with 1 M NaOH, and then passed through Sephadex SP column (0.8 x 10 cm) (Redgwell 1980). The amino acid fraction bound to the SP resin was eluted with 0.2 M ammonium hydroxide, dried in a rotary evaporator, and reconstituted in 70 % ethanol.

2.7.2 Identification of ^{14}C -labelled amino acid

^{14}C labelled amino acids in the protein hydrolysate were identified by two dimensional thin layer chromatography (TLC), and confirmed by high performance liquid chromatography (HPLC). For TLC analysis, a 2 μl sample (5,000-10,000 dpm) was spotted on TLC plate (HPTLC, B.D.H. Co.) and developed with methanol/chloroform/17.5 % NH_3 (2/2/1, v/v) for the first dimension and phenol/ H_2O (75/25, w/v) for the second dimension. The plate was exposed with X-ray film for 4-6 days, and then sprayed with ninhydrin reagent to visualize amino acids. ^{14}C labelled amino acids were identified by matching the spots with radioactivity and ninhydrin reaction. The separation of amino acids on HPLC was modified from the method mentioned above. In order to obtain appropriate isotope counting, the concentration of amino acids was adjusted to 10-20 mM (2,000-6,000 dpm) and reacted with 60 mM *o*-phthaldialdehyde solution (Table 2.4) for 2 min. The eluate from the HPLC column was monitored by the fluorescence detector and collected by every 30 sec with a fraction collector (FC-100, Pharmacia LKB Biotechnology, USA) for radioactivity measurement. The ^{14}C labelled amino acids on each TLC plate were recovered with cellulose acetate (see Table 2.5) following the method described by Bielecki and Turner (1966). The radioactivity in the silica gel and HPLC eluate was quantified as described in section 2.8.

2.8 Radioactivity measurement

To measure the radioactivity in the sample, 4 ml Packard scintillation cocktail (Ultima Gold XR) was added, and the radioactivity was quantified in Packard Tri-carb β -counter with pre-set ^{14}C windows and quench curve. The radioactivity of ^{14}C -labelled amino acids obtained from the TLC plates or HPLC eluate were corrected with the background counting which was determined by non-radioactive amino acid run at the same condition as that of ^{14}C -labelled.

2.9 Electron microscopy examination

The sea anemones used for electron microscopy examination were relaxed in 7.5 % MgCl_2 (w/v) for 4 h. Then, a piece of tentacles was cut from the animal with a sharp scissors and pre-fixed in 4 % glutaraldehyde in sodium cacodylate buffer (pH 7.2) for 3-4 h. The specimen was washed for 10 min in each of three changes of 0.1 M phosphate buffer (pH 7.2), and left overnight in the phosphate buffer. Post-fixation of the specimen was conducted in 1 % osmium tetroxide in phosphate buffer for 1 h on ice, followed with dehydration through an ethanol series (25 %, 50 %, 70 %, then 90 % of ethanol for 30 min each), and finally dehydrated with three changes of 100 % of ethanol for 20 min each and two changes of epoxy propane for 5 min each. The specimen was infiltrated with following process: in epon araldite/ epoxy propane (25/75, v/v) for 1 h, dried in desiccator with silica gel overnight, and then in 37 °C epon araldite resin (Table 2.6) for 2 h; and finally embedded in moulds with polymeriser at 60 °C 48 h. Thin sections (60-90 nm) were cut with a diamond knife, and stained with saturated uranyl acetate in 50 % ethanol followed by Reynolds lead citrate for 15 min each. The grids were examined with a JEOL (JEM-1200X) electron microscope operating at 80 kV.

2.10 Statistical analysis

Parametric statistical tests were used to explore (1) the effects of SW buffer, amino acids, and host extract on the total carbon fixation of *Symbiodinium* and percentage of photosynthate released (after arc-sin square root transformation of the data); (2) the effects of exogenous carbon supplement on the glutamine synthetase activity, ammonium content and free amino acid content in animal tissue of sea anemones. Comparisons between pairs of treatments were made using Fisher's least significance difference test with significance level of 0.05. Student's t test was used to compare the biochemical indices between experiments. The impacts of treatments on the composition of ^{14}C -labelled components (chapter 3) and free amino acids (chapter 4) were analyzed by multivariate ANOVA and profile analysis (Morrison 1976, Seber 1984, Douglas 1996). In this case, univariate statistical analysis was not appropriate, because the elevation or depression in concentration of one fraction could not be assumed to be independent of change in other fractions. The slopes of linear regression lines obtained in this study were analyzed by a general linear model.

Chapter 3

Photosynthetic Carbon Fixation and the Induction of Photosynthate Release from *Symbiodinium*

3.1 Introduction

It is well-established that the dinoflagellate alga *Symbiodinium* in symbiosis with marine Cnidaria (sea anemones, corals, jellyfish etc.) exhibits high rates of photosynthesis, releasing much of the photosynthetic carbon to the animal cells (e.g. Muscatine & Cernichiari 1969; Schmitz & Kremer 1977; Sutton & Hoegh-Guldberg 1990); and that this provides part or all of the animal's carbon and energy requirements (Muscatine 1990; Gattuso *et al.* 1993; Dubinsky & Jokiel 1994). Little, however, is known about the processes underlying photosynthate release by *Symbiodinium*. Over the last 2 to 3 decades, most research on this latter topic has been shaped by the finding of Muscatine and Cernichiari (1969) and Trench (1971b & c) that algae isolated from the symbiosis release little photosynthetic carbon unless homogenate of the animal host tissues is added to the incubation medium. These observations have led to a widely-held view in the literature that: (1) specific component(s) of host homogenate (often known as "host factor") induce photosynthate release; (2) the host factor is a signal that results in the diversion of photosynthetic carbon from intracellular metabolism to release; and (3) the host factor mediates photosynthate release in the intact symbiosis.

Progress in the identification of the putative host factor and elucidation of its mode of action has been slow [reviewed in Hinde (1988) and Trench (1993)]. In part, this reflects the small volumes and biochemical complexity

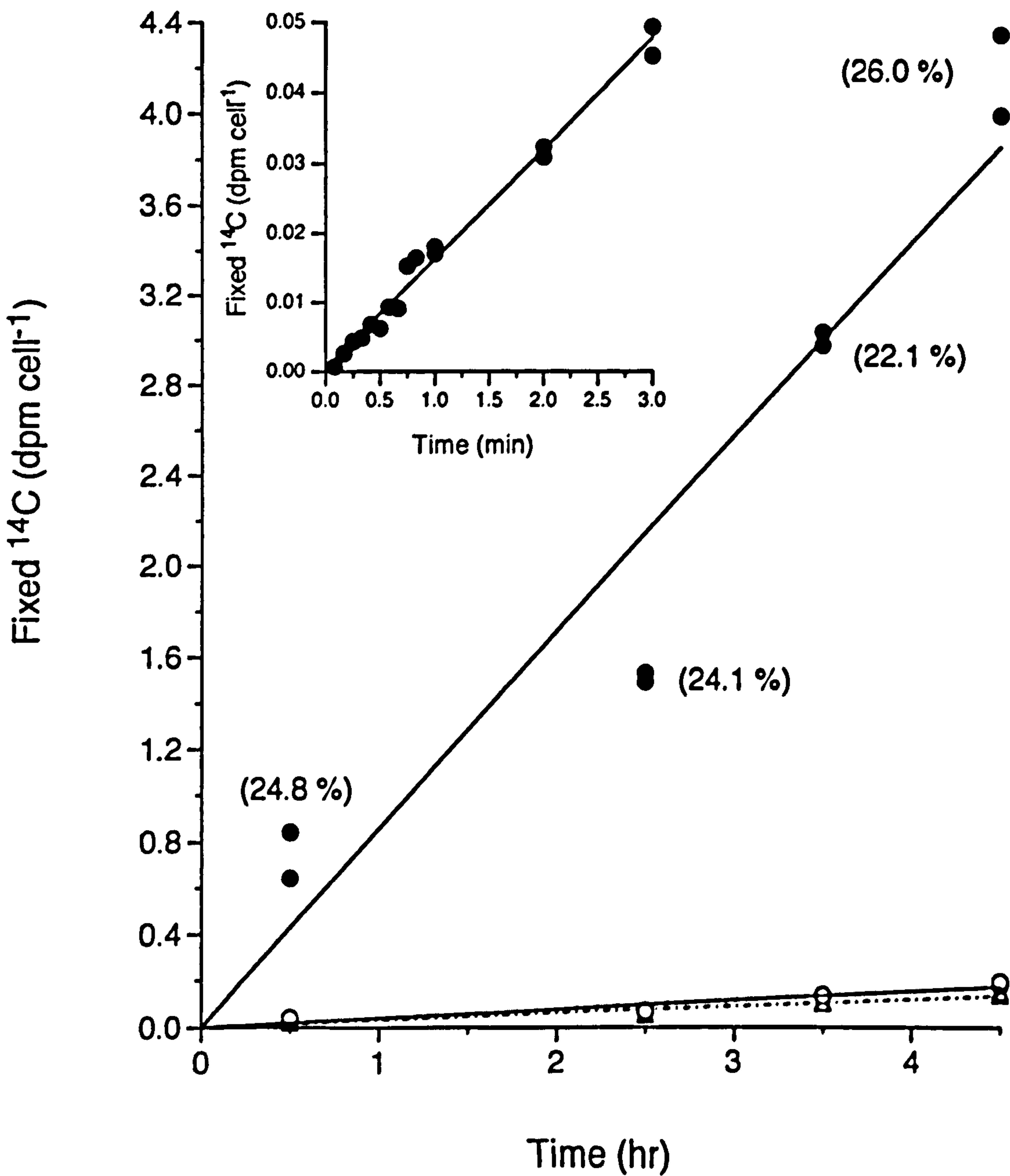
of host homogenates. A more fundamental difficulty with the studies of isolated algal cells is how the host factor effect relates to the conditions experienced by *Symbiodinium* in the symbiosis. As described in the section 1.3, *Symbiodinium* cells are enclosed in the symbiosome membrane rather than exposed directly to the animal cytoplasm. Although little is known about the conditions in the symbiosome (see Rands *et al.* 1993), they are not likely to resemble the composition of crude animal homogenates. Even so, there is a strong supposition in the literature that the host factor is an animal "signal" that results in the diversion of photosynthetic carbon from intracellular metabolism to release.

The recent paper of Gates *et al.* (1995) may provide the basis for significant advance in our understanding of photosynthate release by *Symbiodinium*. Gates *et al.* (1995) demonstrated that *Symbiodinium* release a high percentage of photosynthetically-fixed carbon when incubated with amino acids. The effect was not specific to the chemical nature of amino acids or lineage of the algae. This paper raises the possibility that the host factor may not be a signal, but alter photosynthetic metabolism of the algal cells through nutritional, or other metabolic, effects. Two unresolved aspects of the study are potentially important to the interpretation of these findings. First, there is no information on the variation in amino acid-induced photosynthate release with amino acid concentration. The experiments of Gates *et al.* (1995) were conducted with individual amino acids at the very high concentrations of 50 mM. Second, the impacts of amino acids and host homogenate on the photosynthetic metabolism of the algal cells were not compared, beyond the demonstration that both induced the release of glycerol from the algae isolated from *P. damicornis*. It is, therefore,

unclear whether the metabolic bases of photosynthate release induced by host homogenate and amino acids are strictly equivalent.

The initial purpose of the study described here was to explore the two unresolved issues considered above, using *Symbiodinium* isolated from *A. pulchella*. The results indicated that one amino acid, taurine, is a particularly effective inducer of photosynthate release; and the greater part of this study was therefore devoted to the impact of this amino acid on carbon fixation and release by *Symbiodinium*. My data have implications for the processes underlying photosynthate release by symbiotic algae.

Figure 3.1 Photosynthetic carbon fixation in the intact *A. pulchella* incubated in ASW with illumination (●), in the dark (Δ) or ASW supplemented with 10 μM dichlorophenyldimethylurea in the light (○) for 4.5 h. Percentage of the fixed carbon release is shown in the parentheses. The result of a separate short term experiment conducted from 5 sec to 3 min under illumination is also shown in inset.



3.2 Results

3.2.1 Preliminary study of photosynthetic ^{14}C fixation and translocation by *Symbiodinium*

The first experiment explored photosynthetic carbon fixation and the release of photosynthate by *Symbiodinium* either residing in the animal cells of *Aiptasia pulchella* or in the incubation medium. As shown in Fig. 3.1, photosynthetic carbon fixation by *Symbiodinium* in the animal cells increased linearly from 5 sec to 4.5 h, and it was depressed by up to 99 % by incubating the animal in the dark or 10 μM dichlorophenyldimethylurea supplemented ASW. The percentage of photosynthate release was nearly constant at 22-26 % along the period of incubation. Freshly isolated *Symbiodinium* also displayed a pattern of photosynthetic carbon fixation and export during the incubation between 5 min and 2 h comparable to that in symbiosis (Fig. 3.2). The percentage of photosynthate release by the isolated algae was consistently higher in the incubations with host extract (mean \pm s.d.; 20.2 ± 2.0 %) than in the SW buffer control (mean \pm s.d.; 5.6 ± 2.5 %). The carbon fixation rate of *Symbiodinium* either in the intact animal or incubation medium was also determined from the slope of linear regression, and the *Symbiodinium* in the animal cells displayed a higher carbon fixation rate ($0.84 \text{ dpm cell}^{-1} \text{ h}^{-1}$) than in the isolated algae [$0.54 \text{ dpm cell}^{-1} \text{ h}^{-1}$ for the incubation in SW buffer ($F_{1,32} = 11.76, p < 0.01$) and $0.60 \text{ dpm cell}^{-1} \text{ h}^{-1}$ for that in host extract ($F_{1,32} = 7.53, p < 0.01$)].

"Pulse-chase" experiments on photosynthate release indicated that host extract or free amino acids did not promote the *Symbiodinium* to release pre-fixed carbon, which had been formed either in the SW buffer, host extract or free amino acid supplemented medium (Table 3.1). These results suggest that *Symbiodinium* release only the newly-synthesized photosynthetic carbon.

Figure 3.2 Photosynthetic carbon fixation and translocation by freshly isolated *Symbiodinium* incubated in the SW buffer (a) or SW buffer supplemented with host extract (b). The total fixed ^{14}C (●) and ^{14}C in the supernatant (○) were quantified separately.

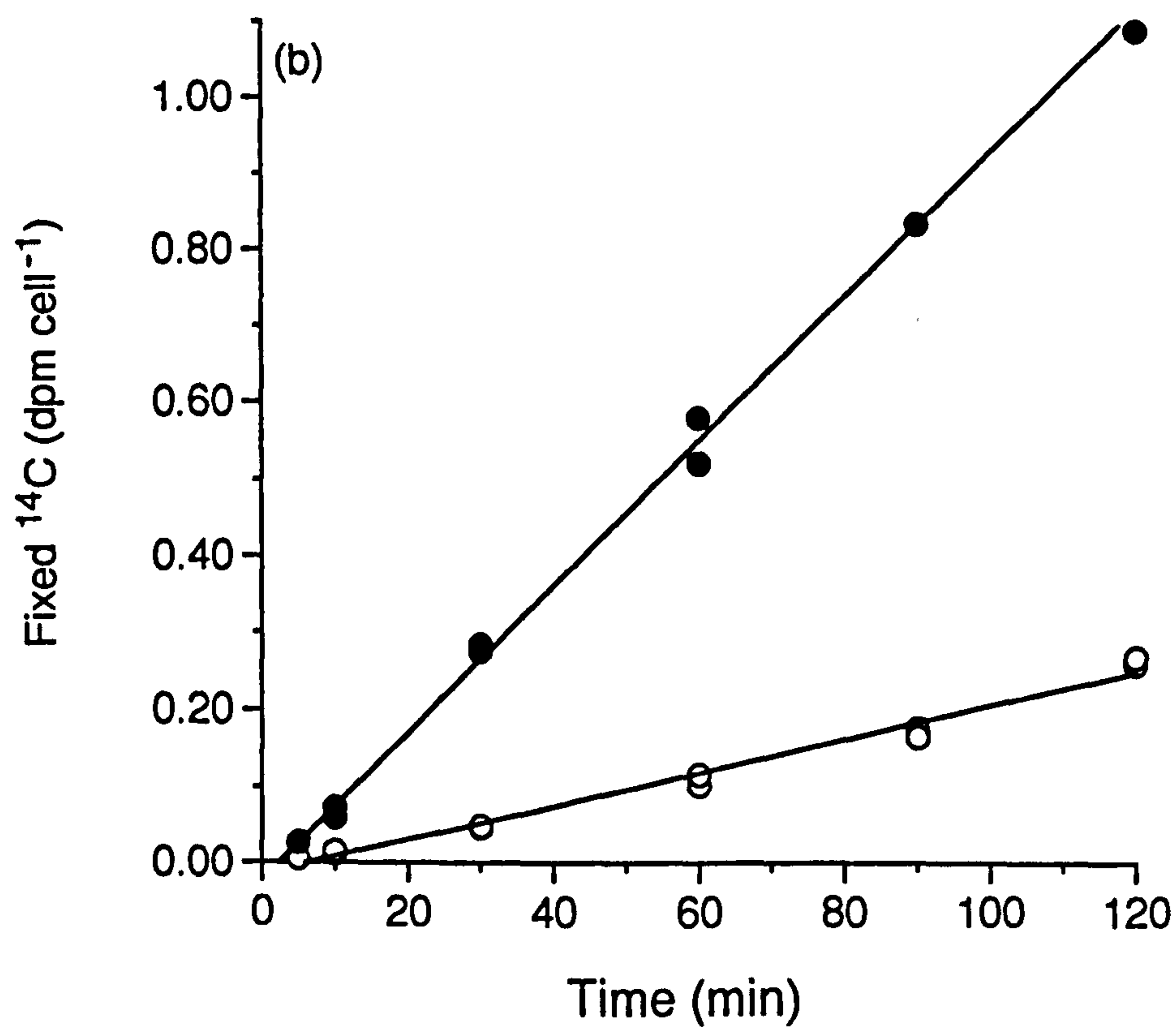
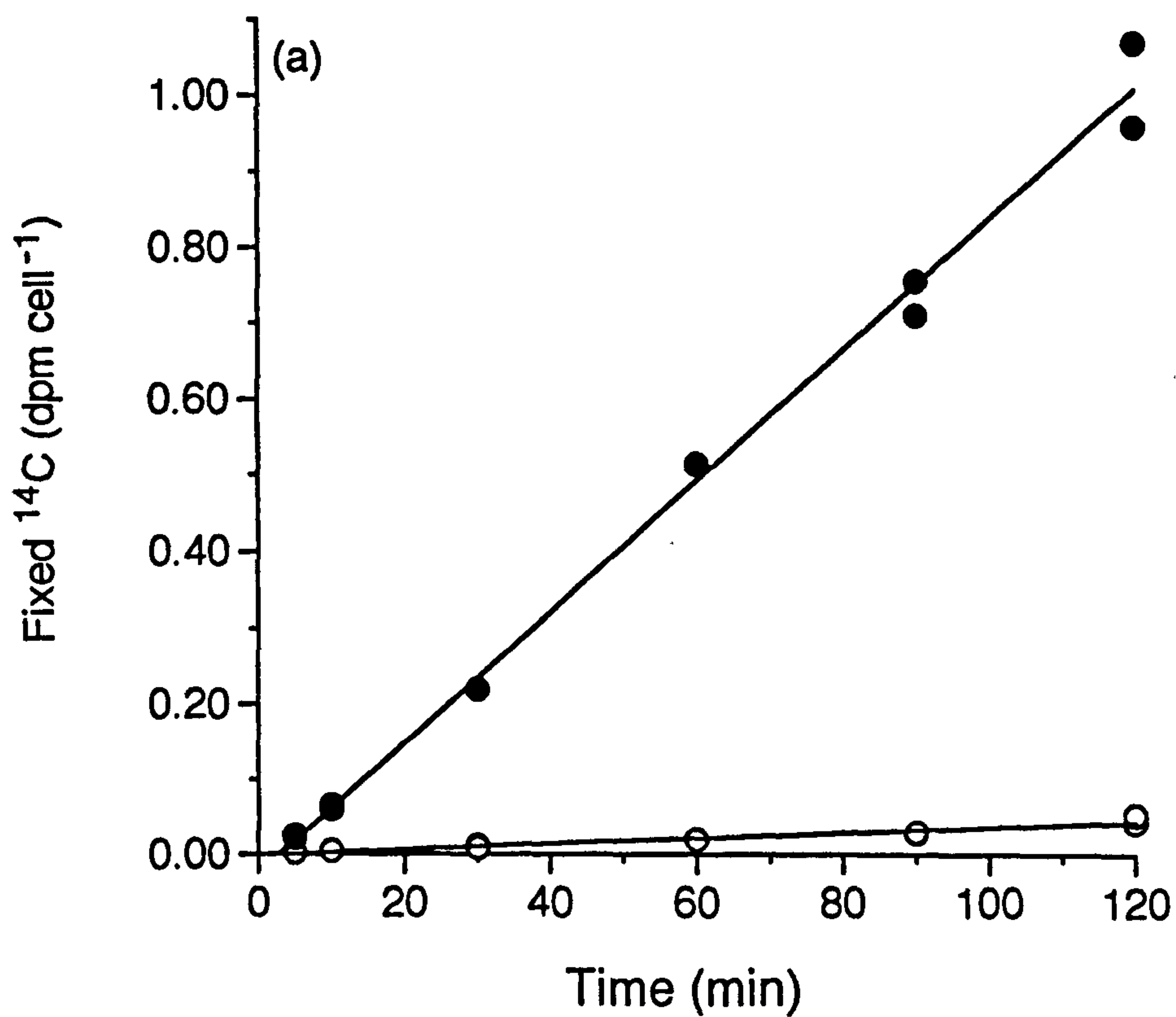


Table 3.1 "Pulse-chase" experiment on the release of photosynthetic carbon by *Symbiodinium*.

Symbiodinium cells were incubated in the SW buffer, FAA (see Table 2.2), or host extract supplemented with 2 μCi [^{14}C]bicarbonate at the first hour (pulse), and then transferred to isotope-free SW buffer, FAA or host extract at the second hour (chase). The *Symbiodinium* incubated in isotope-free SW buffer at the first hour and then in [^{14}C]bicarbonate supplemented SW buffer at the second hour was conducted as a control. The percentage of radioactivity in the medium was determined as described in section 2.3.2.2, and expressed as mean \pm s.d. (n=3, unless stated otherwise).

isotope supplemented to SW buffer		^{14}C in the medium	
[^{14}C]bicarbonate (1st h)	none (2nd h)	1st h	2nd h
(%)			
SW buffer	SW buffer	9.5 \pm 1.5 (n = 9)	4.7 \pm 0.2
	FAA		8.0 \pm 0.5
	host extract		6.6 \pm 0.3
FAA	FAA	21.7 \pm 0.8	13.6 \pm 1.8
host extract	SW buffer	20.1 \pm 3.5 (n = 6)	2.2 \pm 0.1
	host extract		5.1 \pm 0.4
isotope supplemented to SW buffer		^{14}C in the medium	
none (1st h)	[^{14}C]bicarbonate (2nd h)	1st h	2nd h
(%)			
SW buffer	SW buffer	0	10.2 \pm 0.7
	host extract		20.5 \pm 0.7

Table 3.2 The composition of free amino acids in a routinely prepared host extract inducing 25 % of fixed ^{14}C release (compared to 7 % derived from SW buffer incubation) from the isolated *Symbiodinium*.

amino acids	μM	%
Alanine+Tyrosine	10.90	5.2
Arginine	10.42	5.0
Asparagine	2.01	1.0
Aspartate	4.10	2.0
Glycine	3.32	1.6
Glutamine	5.23	2.5
Glutamate	30.44	14.6
γ -aminobutyric acid	4.53	2.2
Histidine+Serine	5.46	2.6
Isoleucine	2.90	1.4
Leucine	5.07	2.4
Lysine	3.91	1.9
Methionine	0.66	0.3
Phenylalanine	2.60	1.3
Taurine	112.75	54.2
Valine	3.63	1.7
Total	207.93	100.0

3.2.2 Effect of amino acids on the carbon fixation and translocation

Prior to examining the host factor effect of amino acids, the free amino acid composition in a routinely prepared host extract from *A. pulchella* was quantified by HPLC. As shown in Table 3.2, the host extract that was capable of inducing 24.5 % of fixed ^{14}C released from *Symbiodinium* comprised only 208 μM of amino acids, only 0.04 of that used in the study of Gates *et al.* (1995). Moreover, unlike the free amino acid composition in the mixture of Gates, more than 50 % of amino acids in *A. pulchella* extract is taurine.

To study the effect of amino acids on the carbon fixation and translocation by *Symbiodinium*, the experiments were designed to incubate *Symbiodinium* cells isolated from *A. pulchella* in the SW buffer, and SW buffer supplemented with host extract or amino acids, as indicated in Table 3.3. The photosynthetic ^{14}C fixation rates in the combined data set varied significantly between the treatments ($F_{7,154} = 5.03$, $p < 0.0001$), and the Fisher's least significance difference test revealed that the fixation rate was significantly elevated by the all supplements tested, compared to SW buffer control. As Figure 3.3 shows, the range of carbon fixation within the treatment varied widely between experiments, which is 0.1-0.7 $\text{dpm cell}^{-1} \text{h}^{-1}$ for the treatment of SW buffer or SW buffer supplemented with 75 mM FAA mixture, and 0.1-1.1 $\text{dpm cell}^{-1} \text{h}^{-1}$ for the treatment of SW buffer supplemented with host extract or 1 mM taurine. However, despite the variations in the carbon fixation between experiments, the corresponding release of fixed carbon by *Symbiodinium* increased linearly with the increase of carbon fixation. The slopes for linear regression of fixed carbon release on carbon fixation (i.e. ratio of photosynthate release to the total carbon fixation) were similar between the incubation with host extract,

Table 3.3 Effect of host extract and amino acids on photosynthate release by *Symbiodinium*.

The "75 mM FAA" supplement is the amino acid mixture of Gates *et al.* (1995), which includes 3 mM taurine; and "72 mM FAA (no taurine)" supplement is the amino acid mixture of Gates *et al.* (1995) from which taurine has been omitted. Means followed by the same letter are not significantly different at $p = 0.05$ (Fisher's least significance difference test). The data displayed are the combined results of multiple experiments, and expressed as mean \pm SE.

Supplements to SW buffer	n	Total carbon fixation dpm cell ⁻¹ h ⁻¹	¹⁴ C in medium %
none	82	0.39 \pm 0.02 a	9.2 \pm 0.2 a
host extract	24	0.62 \pm 0.06 b	21.6 \pm 0.7 c
75 mM FAA	28	0.47 \pm 0.03 b	22.4 \pm 0.5 c
1 mM taurine	16	0.52 \pm 0.06 b	21.0 \pm 0.7 c
72 mM FAA (no taurine)	3	0.62 \pm 0.01 b	15.3 \pm 1.1 b
25 mM glutamate	3	0.45 \pm 0.01 b	6.7 \pm 0.1 a
25 mM serine	3	0.48 \pm 0.01 b	9.3 \pm 0.1 a
25 mM lysine	3	0.47 \pm 0.02 b	8.5 \pm 0.6 a

Figure 3.3 The variability of photosynthetic carbon fixation and the release of fixed carbon by freshly isolated *Symbiodinium* incubated in the SW buffer (∇), SW buffer supplemented with host extract (\circ), 75 mM FAA (\blacktriangle), or 1 mM taurine (Δ). The equations of linear regression for the relationship between carbon fixation and fixed carbon release are: $y = 0.09x$ ($r^2 = 0.79$) for SW buffer control; $y = 0.20x + 0.01$ ($r^2 = 0.95$) for the incubations with host extract; $y = 0.25x - 0.01$ ($r^2 = 0.91$) for the incubation with 75 mM FAA; and $y = 0.26x - 0.02$ ($r^2 = 0.99$) for the incubations with 1 mM taurine.

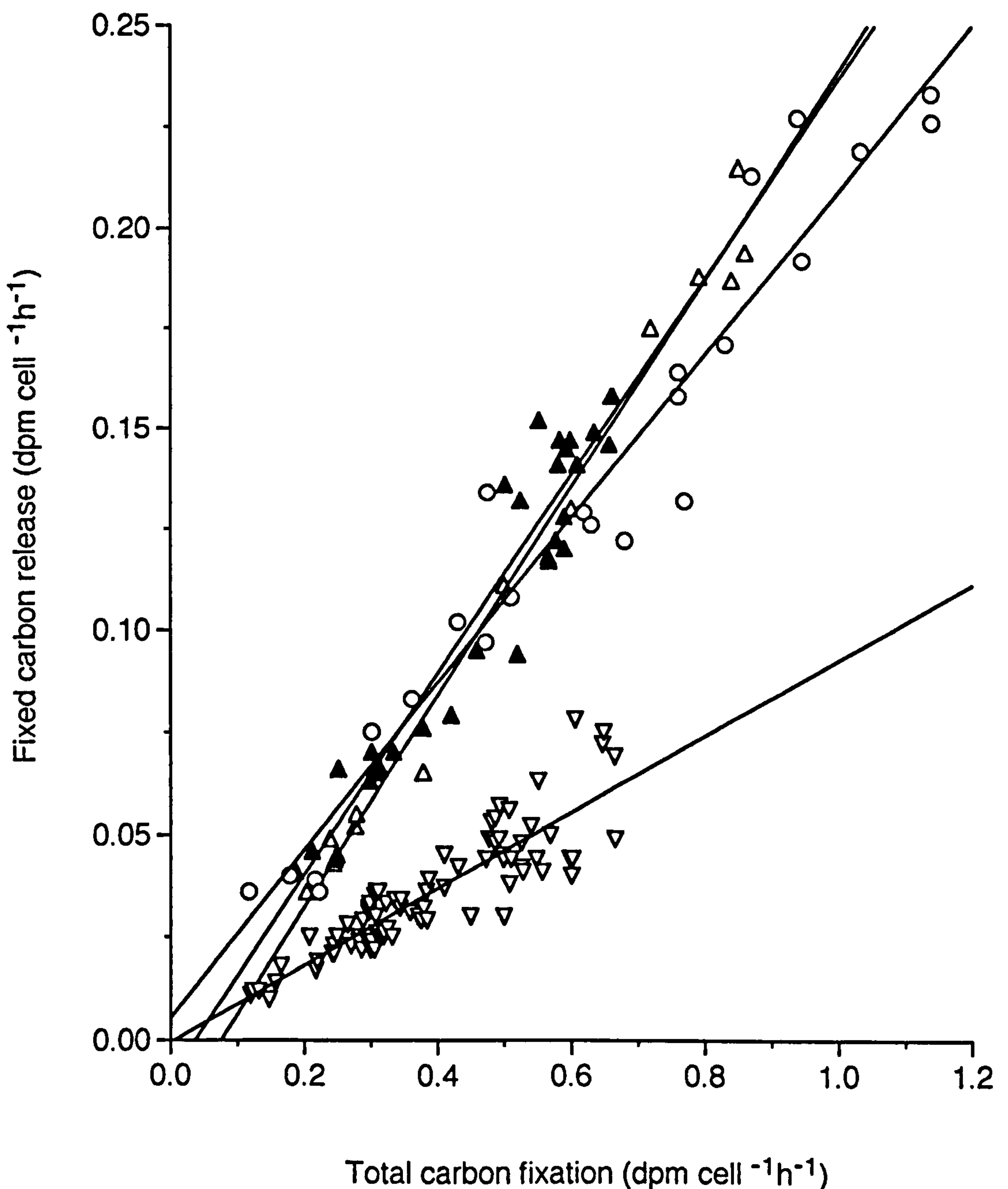
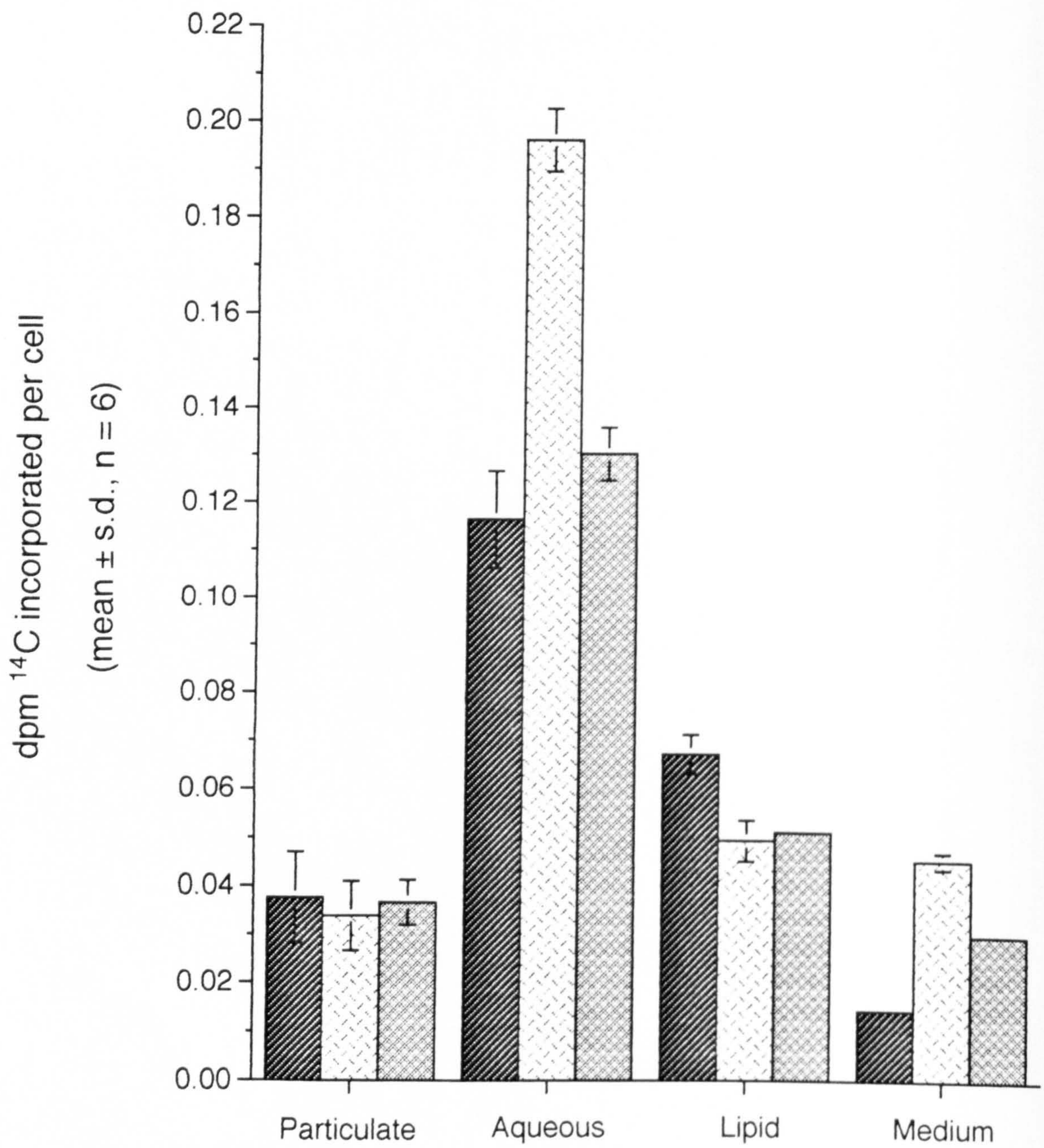


Figure 3.4 Incorporation of photosynthetically-fixed ^{14}C by freshly isolated *Symbiodinium* incubated in SW buffer (▨), and SW buffer supplemented with either host extract (▤) or 1 mM taurine (▩).



taurine, and FAA mixture, which were significantly higher than that for SW buffer control. This result suggests that the proportion of fixed carbon release is independent of the variation of carbon fixation rate exhibited by *Symbiodinium* cells.




Consistent with previous studies (e.g. Hinde 1988; Sutton & Hoegh-Guldberg 1990; Gates *et al.* 1995), the amount of ^{14}C released into the medium was <10 % of the total for cells in SW buffer, and between 20 and 30 % for cells incubated with host extract and with the 75 mM amino acid mixture of Gates *et al.* (1995). In the experiment displayed in Table 3.3, the percentage of photosynthate release varied significantly between treatments ($F_{7,154} = 211.36$, $p < 0.0001$), and 1 mM taurine was found to be as effective as host extract and 75 mM amino acid mixture in promoting release. The impact of three other amino acids, glutamate, serine and lysine, on photosynthate release was tested. Even at the concentration of 25 mM, none promoted ^{14}C release. Omission of taurine from the amino acid mixture of Gates reduced ^{14}C release by 34 %.

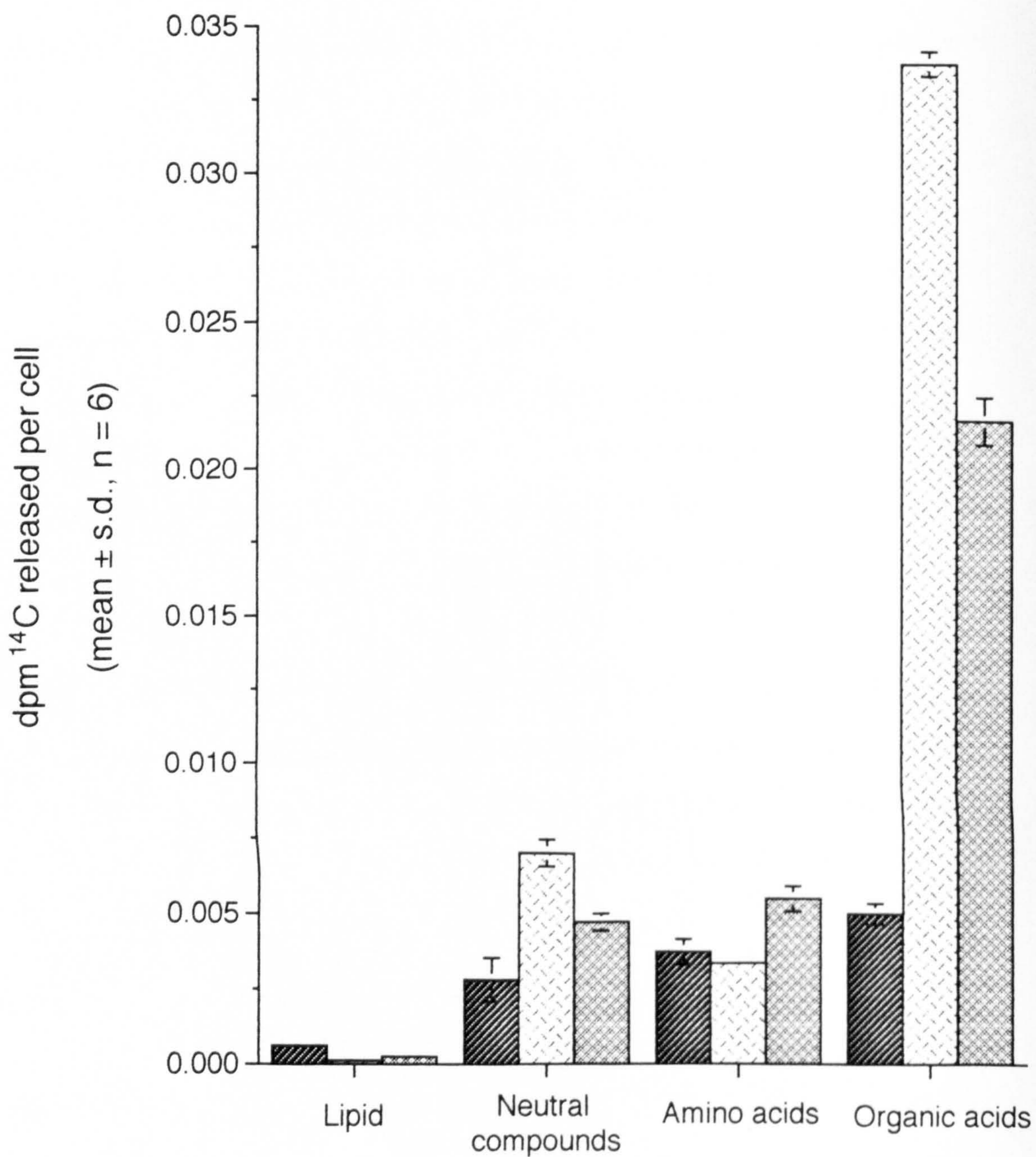
These results suggested that taurine contributed substantially to the effect of amino acids on photosynthetic metabolism by *Symbiodinium*.

Subsequent experiments compared the impacts of taurine and host homogenates on photosynthate release.

3.2.3 The metabolic fate of photosynthetically fixed ^{14}C

The effect of host extract and taurine on the metabolism of photosynthetically-fixed ^{14}C by the isolated *Symbiodinium* cells is shown in Fig. 3.4. For each treatment, the photosynthetically-fixed ^{14}C was recovered from the aqueous, lipid and particulate fractions of the cells, as well as the medium. In this experiment, the algal cells fixed more ^{14}C

Figure 3.5 Distribution of ^{14}C labelled compounds in photosynthate released from *Symbiodinium* into SW buffer (); and SW buffer supplemented with host extract () or 1 mM taurine ().



when incubated with host extract than with taurine and SW buffer. The distribution of photosynthetically-fixed ^{14}C between the fractions varied significantly with treatments (MANOVA, $F_{6,26} = 32.97$, $P < 0.0001$), and profile analysis revealed that the cells in the taurine and host extract treatments released more ^{14}C into medium, relative to the aqueous, lipid, and particulate fractions of the cells, than did the cells in SW buffer. No significant differences in the distribution of ^{14}C between host extract and taurine treatments ($p > 0.05$) were revealed by this analysis.

The ^{14}C -labelled compounds in the medium were studied further. As Fig. 3.5 shows, most of the ^{14}C was recovered from the water-soluble fraction, including neutral compounds (probably predominantly sugars), amino acids, and organic acids, while the lipid fraction accounted for less than 5% of the radioactivity for SW buffer incubation and less than 1% for both host extract and taurine incubations. The higher ^{14}C content in the medium of cells incubated with host extract and taurine than in SW buffer could be attributed primarily to an increase in organic acids. Analysis of the released ^{14}C -organic acids by TLE/TLC revealed two spots, (0.73, 2.3) and (0.85, 6.5) [(R_f on TLC, motility (cm) on TLE)], for the taurine incubations; and (0.77, 2.7) and (0.85, 6.2) for host extract incubation (Fig. 3.6). These were identified as fumarate for taurine treatment, and fumarate and succinate for the host extract treatment. A further minor spot (0.73, 2.3) for taurine treatment was identified in some experiments (e.g. Fig. 3.6). It was definitely not any of organic acids previously described as release products of *Symbiodinium*, including lactate (0.92, 3.8) and glycolate (0.76, 4.2) reported by Gates *et al.* (1995).

Figure 3.6 Identification of ^{14}C -labelled organic acids released by the *Symbiodinium*. The profile of authentic compounds (a); the incubation with host extract (b); and 1 mM taurine (c).

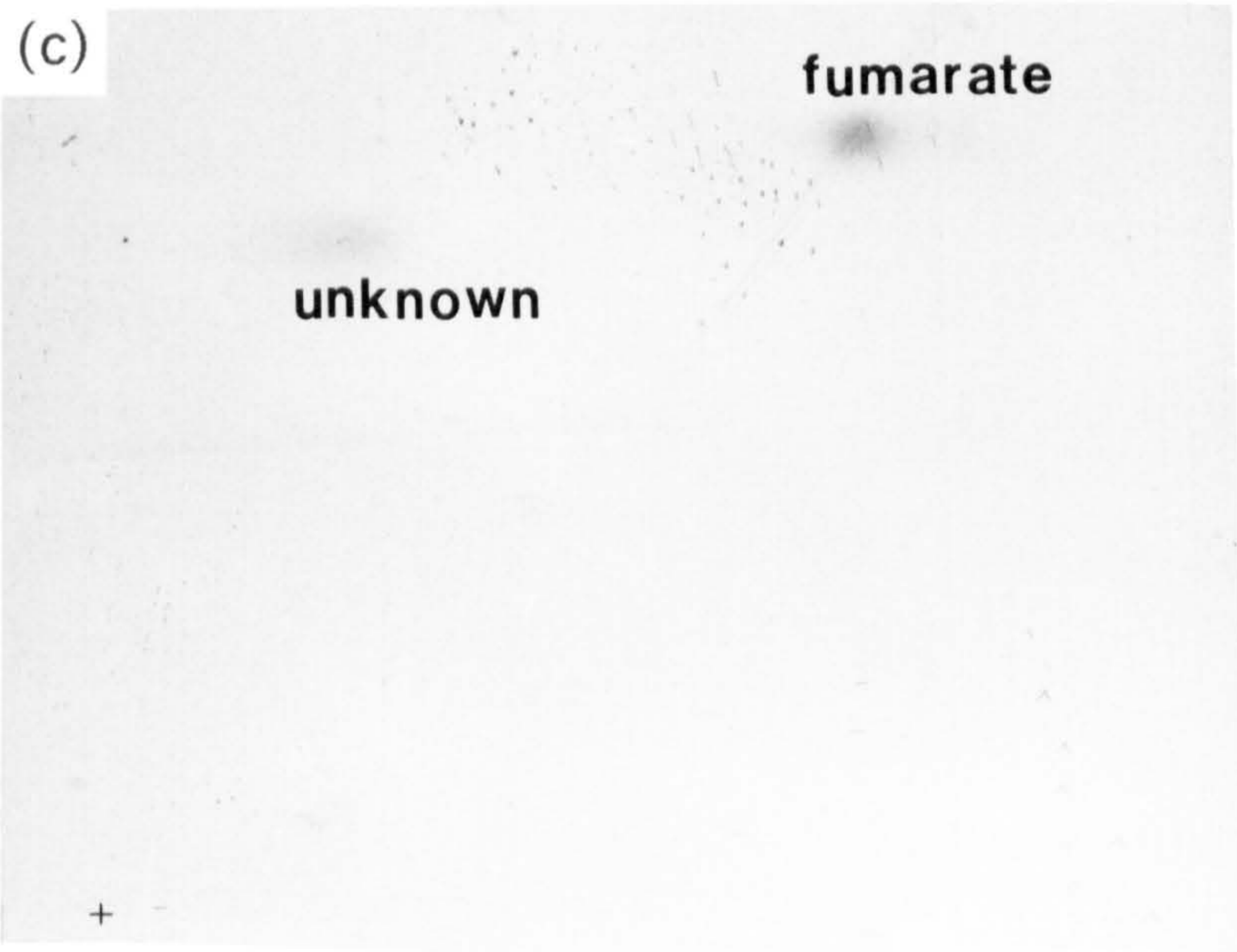
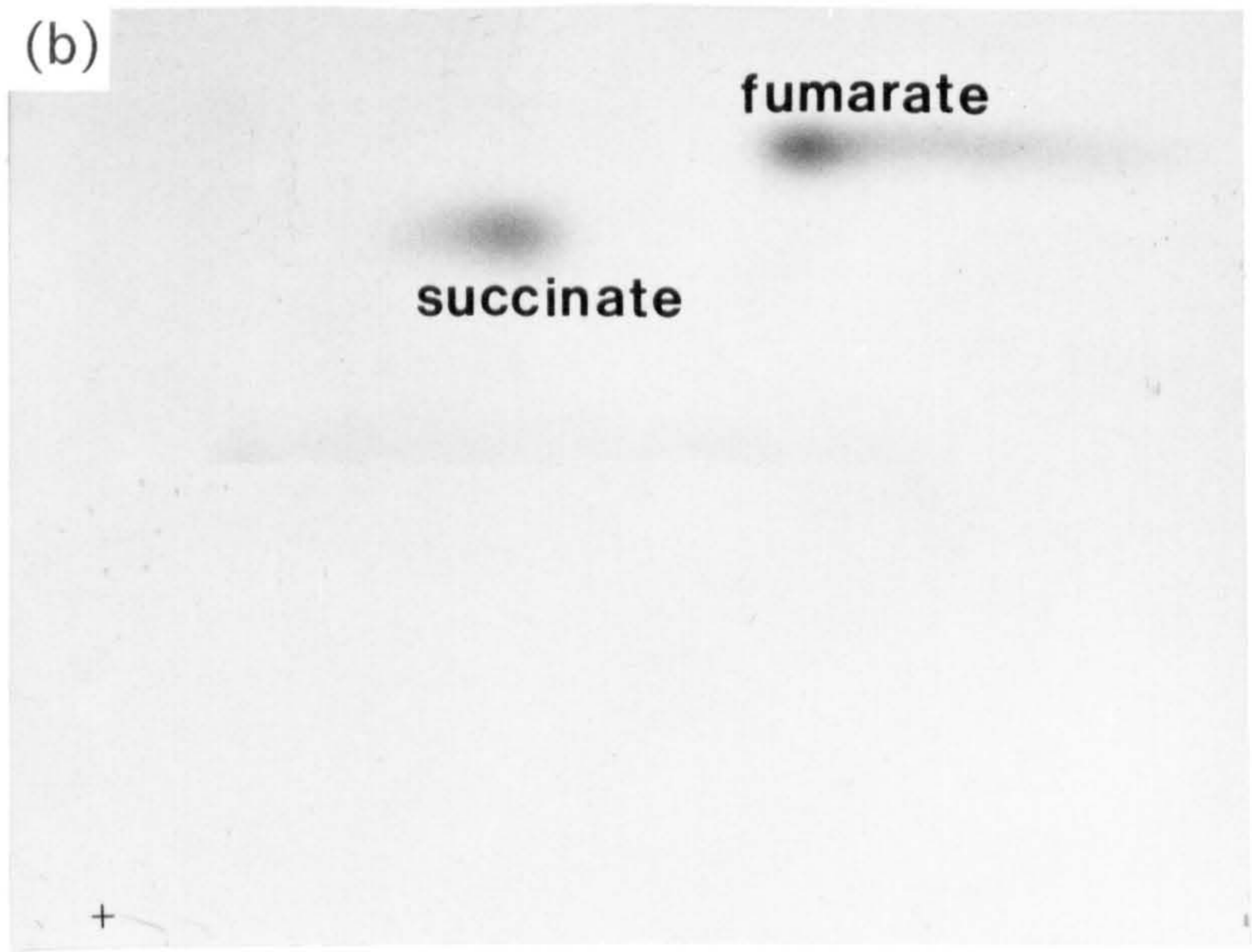
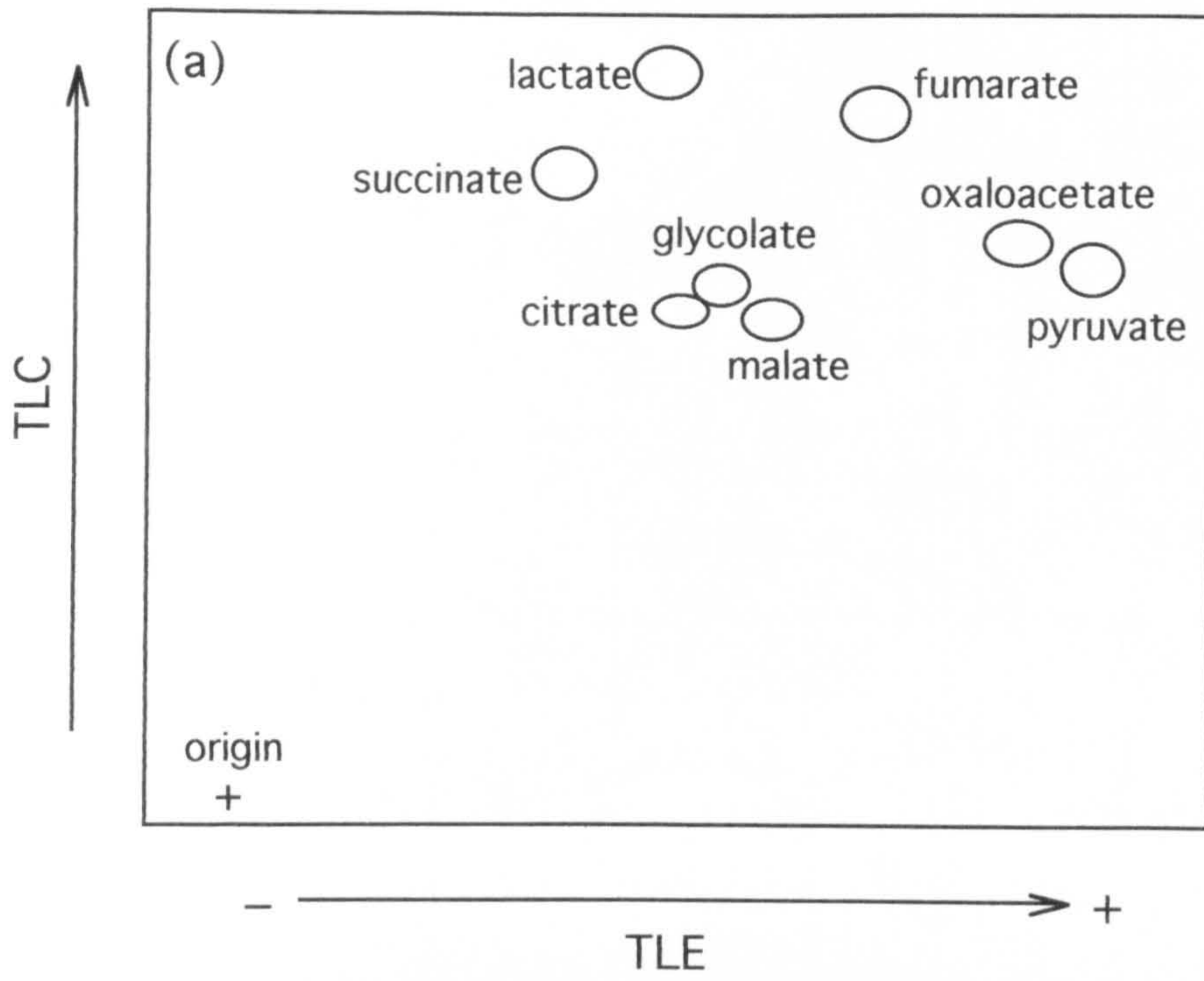
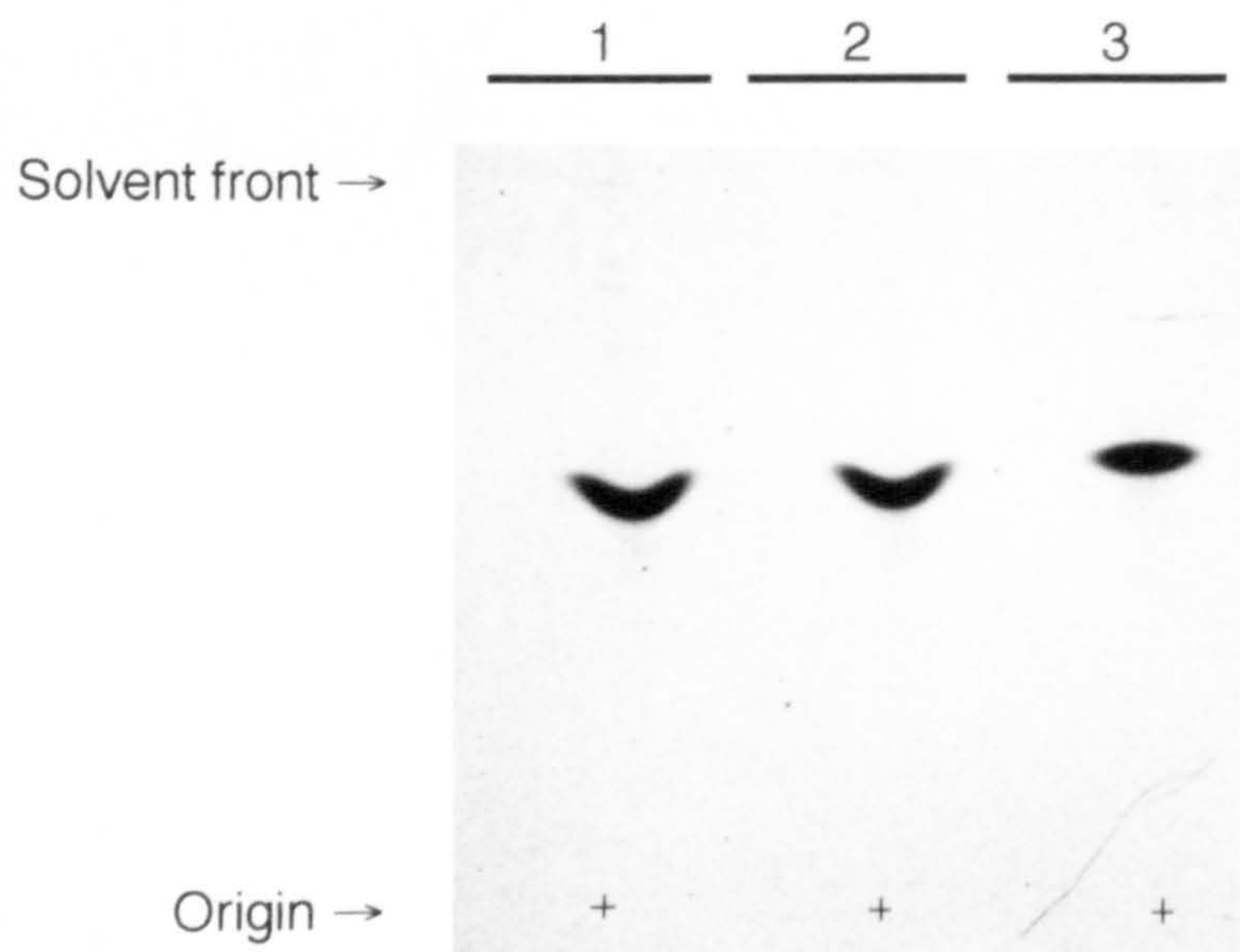


Figure 3.7 Thin-layer chromatography of incorporated ^{14}C -taurine by *Symbiodinium*. Lane 1 and 2 are two replicates of sample, and lane 3 is the standard ^{14}C -taurine.



In summary, these results indicate that host extract and taurine have similar, but not identical, effects on photosynthate metabolism and release by *Symbiodinium* cells.

3.2.4 Kinetics of taurine uptake and taurine-stimulated photosynthate release

The first experiments showed that isolated cells of *Symbiodinium* took up ^{14}C -taurine from SW buffer at linear rates for over one hour (see Fig. 2.1). More than 95 % of radioactivity was recovered from the ethanol fraction of the cells, and the sole ^{14}C product in this fraction was identified by TLC as taurine (Fig. 3.7), indicating that this amino acid was not extensively metabolized. Moreover, when the *Symbiodinium* cells were transferred to isotope-free medium immediately after uptake experiment, 70 % of incorporated ^{14}C in the algae released back to the medium within 1 h, but no further release had been detected in the following 6 h. Further, the incorporation of ^{14}C from exogenous taurine was reduced by >90 % in heat-killed cells.

The variation in initial taurine uptake rates with exogenous taurine concentration between 10 μM and 1 mM revealed saturation-kinetics (Fig. 3.8), with kinetic constants: K_m 68 μM , and V_{max} 271 pmol (10^6 cells) $^{-1}$ min $^{-1}$. Cells incubated with exogenous taurine at concentrations greater than 1 mM exhibited a further increase in taurine uptake rates, perhaps indicative of a low-affinity uptake system, but this was not investigated further in detail.

The effect of exogenous taurine concentration on ^{14}C -labelled photosynthate release was also studied. As shown in Fig. 3.10, taurine and taurine in the Gates FAA mixture both exhibited saturation-kinetics, and

Figure 3.8 Kinetics of taurine uptake by *Symbiodinium* .

The regression equation for the Eadie-Hofstee plots are: $y = 271 - 68x$,
 $r^2 = 0.84$.

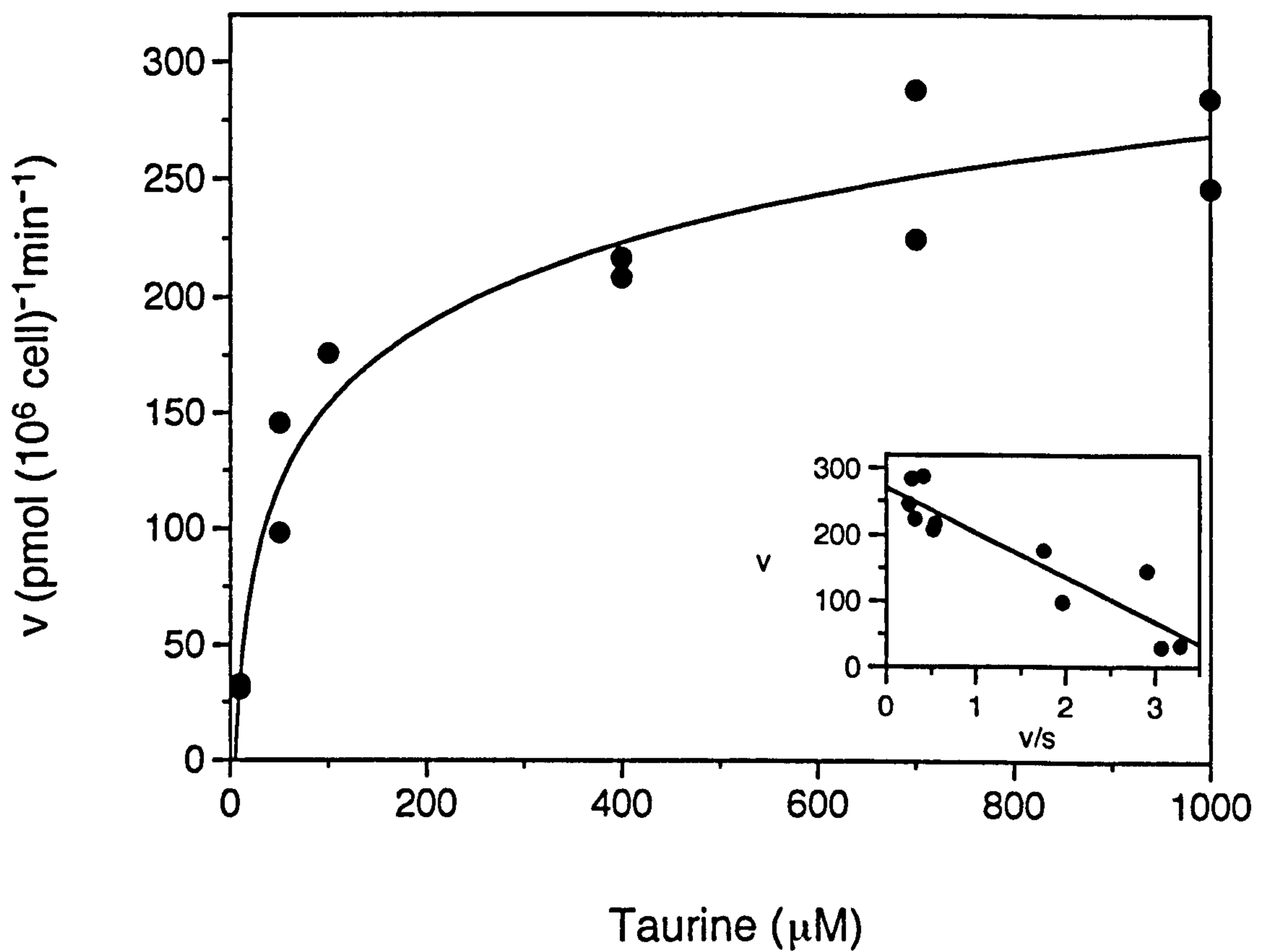


Figure 3.9 Kinetics of taurine-stimulated photosynthate release by *Symbiodinium*. The rate of photosynthate release was calculated by subtracting the amount of radioactivity in the SW buffer control from that in the treatment of taurine (a) and taurine in FAA mixture (b), respectively. The regression equations for the Eadie-Hofstee plots are: $y = 260 - 21x$, $r^2 = 0.78$ for the taurine treatment; and $y = 1331 - 21x$, $r^2 = 0.86$ for the treatment of FAA mixture which comprises 5 % of taurine.

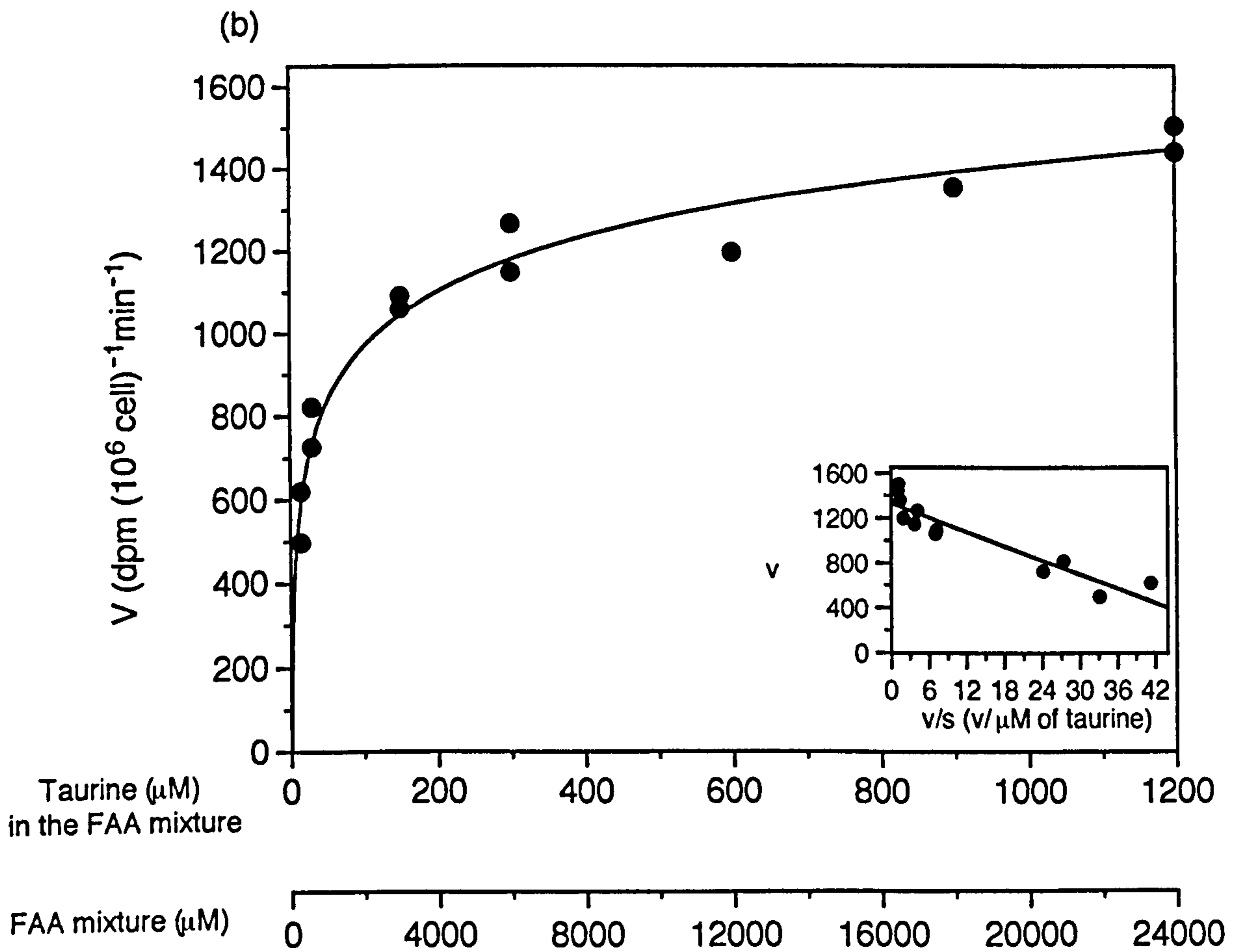
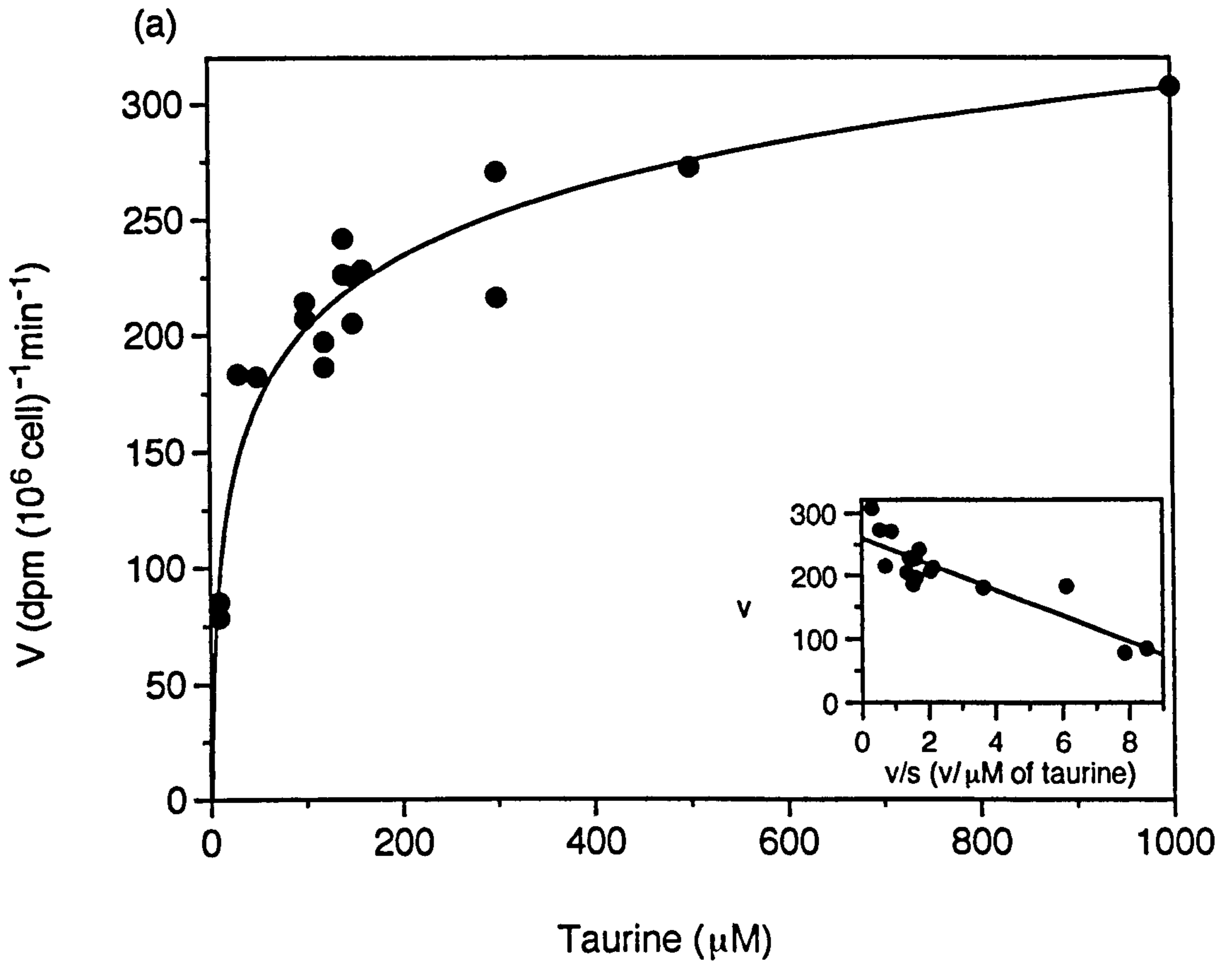


Table 3.4 The composition of free amino acids of *Symbiodinium* cells isolated from *A. pulchella*.

amino acids	mM	%
		n = 12
Alanine	1.3 ± 0.3	3.2 ± 0.5
Arginine	0.9 ± 0.1	2.4 ± 0.1
Asparagine	0.2 ± 0.0	0.5 ± 0.0
Aspartate	0.8 ± 0.2	2.2 ± 0.2
Glycine	2.7 ± 0.6	7.1 ± 0.6
Glutamine	8.9 ± 1.2	24.8 ± 1.4
Glutamate	2.2 ± 0.4	6.2 ± 0.7
γ-aminobutyric acid	5.0 ± 0.8	17.5 ± 3.2
Histidine+Serine	0.6 ± 0.1	1.7 ± 0.1
Isoleucine	0.5 ± 0.1	1.2 ± 0.1
Leucine	0.8 ± 0.1	2.4 ± 0.2
Lysine	0.6 ± 0.1	1.6 ± 0.1
Methionine	0.1 ± 0.0	0.4 ± 0.1
Ornithine	0.4 ± 0.1	1.0 ± 0.3
Phenylalanine	0.4 ± 0.1	1.1 ± 0.1
Taurine	6.9 ± 1.3	18.4 ± 1.5
Threonine	0.6 ± 0.1	1.6 ± 0.1
Tryptophan	0.1 ± 0.0	0.3 ± 0.0
Tyrosine	1.8 ± 0.3	4.6 ± 0.4
Valine	0.6 ± 0.1	1.6 ± 0.1

strikingly with the same K_M value (21 μM), but with the different V_{max} [260 dpm (10^6 cells) $^{-1}$ min $^{-1}$ for the treatment with taurine alone (Fig. 3.9a) and 1,331 dpm (10^6 cells) $^{-1}$ min $^{-1}$ for that with taurine in FAA mixture (Fig. 3.9b)]. The K_M for taurine-stimulated photosynthate release was significantly lower than that for taurine uptake ($F_{1,24} = 10.84$, $p < 0.005$ for taurine alone; and $F_{1,19} = 4.65$, $p < 0.05$ for taurine in the FAA mixture).

Symbiodinium cells isolated from *A. pulchella* exhibited a distinct composition of free amino acids (Table 3.4) from that in the animal extract (Fig. 4.2). Taurine concentration in the algae is 6.9 ± 1.3 mM (mean \pm s.e.) when estimated from assuming that the algae are spherical with diameter of 10 μm and contain 2.2×10^{-5} μg protein cell $^{-1}$. The high internal concentration of taurine in the algae implies that taurine is taken up by cells against a concentration gradient, and this is consistent with the conclusion of Deane and O'Brien (1981) that taurine uptake by *Symbiodinium* is energy-dependent.

3.3 Discussion

3.3.1 Host factor activity of taurine

The principal result of this chapter is that taurine and host extract have broadly comparable effects on the metabolism of photosynthetically-fixed carbon by *Symbiodinium* from *Aiptasia pulchella*. The evidence is that taurine and host extract exhibit the similar extent of carbon fixation rate and both promote two-fold increase in photosynthate release (Table 3.2), primarily in the form of organic acids (Fig. 3.5). However, their effects on algal metabolism are different. Although both treatments induced fumarate release, only the host extract caused succinate release (Fig. 3.6). The different effect is possibly attributable to components of host extract, for example animal derived enzymes, other than those responsible for the stimulation of photosynthate release. Further experiments are needed to establish the basis of these differences. However, it is concluded that taurine can be used with confidence as a chemically-defined alternative to host extracts in the study of photosynthate release by *Symbiodinium* from *Aiptasia pulchella*.

3.3.2 Taurine as a signal inducing photosynthate release

The value of experiments conducted with a chemically-defined inducer of photosynthate release is illustrated by the advances in our understanding of the processes underlying release obtained from the studies of Gates *et al.* (1995) and this study. There are three broad issues:

First, the demonstration in this study that exogenous taurine stimulates photosynthate release without being metabolized by the algal cells indicates firmly that the effect of amino acids on the photosynthetic metabolism of *Symbiodinium* is not linked to their role as nitrogenous nutrients.

Second, the results of this study are consistent with the conclusion of Gates *et al.* (1995) that amino acids do not induce the release of a uniform array of photosynthetic compounds from *Symbiodinium*. In the study of Gates *et al.* (1995), the principal compound released by algae from *Pocillopora damicornis* was glycerol, while the algae from *Aiptasia pulchella* released a mixture of glycerol, glycolate, lactate and succinate. However, in this study, *Symbiodinium* from *A. pulchella* released fumarate (but detectable amounts of none of the compounds identified by Gates *et al.*) when incubated with taurine. [Fumarate was also identified by Trench (1971b) as the major release product of the algae from *A. pulchella*.] It is also known that only recently-fixed photosynthetic carbon is released (see Table 3.1; Sutton & Hoegh-Guldberg 1990). A reasonable inference is that photosynthetic carbon is diverted from intracellular metabolism to release at point(s) in photosynthetic metabolism not far removed from carbon fixation; and that neither exogenous amino acids nor host extract determines the biochemistry of the subsequent metabolism of carbon destined for release. The identity of the compounds released may vary between lineage of *Symbiodinium* [as previously suggested by Gates *et al.* (1995)] and with the physiological condition of the algal cells in different symbioses.

Finally, the experiments on the variation in photosynthate release rates with taurine concentration indicate that the effect of taurine on the process(es) regulating photosynthate release is saturable and has a high affinity (K_M of 21 μM) for taurine. The lower K_M of taurine-stimulated photosynthate release than taurine uptake indicates that the process limiting taurine-stimulated photosynthate release is not internal to or dependent on taurine uptake. Further indications that these processes are

separate are from the demonstration that glycine and γ -aminobutyric acid substantially depress taurine uptake (L. Whitehead unpublished data), but do not either induce photosynthate release (L. Whitehead unpublished data) or influence the K_M of taurine-stimulated photosynthate release (Fig. 3.9). Moreover, only a proportion of incorporated-taurine (70 %) is reversibly released to the medium, also suggesting that the algae may have two separate pools interacting with taurine. Together these data suggest that *Symbiodinium* may bear a taurine signal transducer, responsible for photosynthate release, that is separate from the taurine transporter; and specifically that the taurine receptor on the algal surface does not bind glycine and γ -aminobutyric acid. These kinetic experiments illustrate the experimental value of a defined compound over host extracts in experiments on photosynthate release. The results obtained in Fig. 3.9 could not have been obtained with crude host extracts because the concentration of the putative host factor in a given volume of homogenate is unknown and likely to vary between preparations.

3.3.3 Unresolved issues

There remain the crucial, but still unresolved, issues of whether taurine is the host factor in host extracts of *Aiptasia pulchella* and whether photosynthate release by the algal cells in the symbiosis is induced by taurine. The involvement of taurine in the effects of host extract is very likely, given that the concentration of taurine in the *A. pulchella* homogenates used in this study was at least 0.1 mM, but, as yet, there is no firm information on the role of taurine in the intact symbiosis. If taurine is the inducer of photosynthate release in the intact symbiosis, one would predict that the symbiosome membrane bears a taurine-transporter, and that the rate of photosynthate release from the algae is correlated with the flux of taurine across this membrane from the animal cytoplasm. This

scenario provides a cellular basis for the possibility that taurine may be acting as a signaling molecule between the animal and algal cells. Following the classical paradigm for intercellular signaling, the binding of taurine to algal receptors may trigger intracellular signaling cascades that are directly responsible for the diversion of photosynthetic carbon from an intracellular to extracellular fate (as considered above). As a precedent for the putative role of taurine as a signal between the animal and *Symbiodinium* cells, taurine is implicated as an inhibitory neurotransmitter in some animals (Nistri & Constani 1976; Hue *et al.* 1979; Giles & Usherwood 1985; Huxtable 1989) and a stimulator of the motor nerve net of jellyfish (Carlberg *et al.* 1995). All the data obtained in this study, including the relatively low half-saturation constant for taurine-induced photosynthate release are compatible with this interpretation. However, further study is required to establish the role of taurine in the intact *A. pulchella* symbiosis and to establish whether this effect is widely distributed among *Symbiodinium* symbioses.

3.3.4 Difficulties of using the isolated *Symbiodinium* on the study of host factor activity

Because the translocated photosynthate is almost instantly converted into carbon dioxide by respiration and animal's components by biosynthetic pathways in the animal host (Battey & Patton 1987; Battey 1992), the composition of photosynthate release in the intact association is changed too fast to be distinguished from that biosynthesized *de novo* in the animal tissues. Therefore, composition analysis of the translocated photosynthate is inevitably to be obtained from experiments conducted on the isolated algae. Using isolated *Symbiodinium*, as revealed in Fig. 3.4 and other studies (e.g. Muscatine *et al.* 1972; Hinde 1997), the photosynthetic activity varied widely between experiments. Although the

% of photosynthate released seems to be uniform (see the legend of Fig. 3.3), the inconsistent photosynthetic activity of isolated *Symbiodinium* suggested the physiological condition of the algal samples may be varied. This wide variation in the physiological condition of the algal samples may affect the analysis of composition and absolute amount of translocated photosynthate and hinder comparison between the results of different experiments and different authors under certain circumstances. Using cultured *Symbiodinium* may avoid the difficulties derived from isolated algae, but the dilemma is that the algae in culture medium may have dramatic changes in the physiological response, as suggested for the *Symbiodinium* isolated from *Aiptasia pallida* (Stochaj & Grossman 1997). Furthermore, cultured *Symbiodinium* also may be the product of selective growth from a mixture of varied strains in the original colony (Goulet & Coffroth 1997). So far, there are no better strategy to study the host factor activity in the symbiotic associations than using algae, freshly isolated from the symbiosis.

Chapter 4

Impact of Symbiotic Algae on the Nitrogen Metabolism in *Aiptasia pulchella*

4.1 Introduction

Traditional concepts about the nutritional interactions between invertebrate hosts and their symbiotic algae are focused on carbon recycling and nitrogen recycling. It has been widely accepted that symbiotic algae can provide the animal hosts with photosynthetic carbon compounds by fixing animal-derived carbon dioxide and amino acids by assimilating ammonia derived from the animal's nitrogen catabolism, which underpin the ecological success of some associations, e.g. Cnidaria-algal symbioses, in the shallow and oligotrophic waters (Muscatine & Porter 1977; Falkowski *et al.* 1993).

This scenario about invertebrate-algal symbiosis has been established principally from studies that consider carbon and nitrogen metabolism separately in the symbiotic associations (e.g. Muscatine *et al.* 1984). Only a few studies (Rees & Ellard 1989; Miller & Yellowlees 1989; Szmant *et al.* 1990) had considered the impact of translocated photosynthate on the nitrogen metabolism in the animal hosts. However, ignorance of the impact of translocated photosynthate by algal symbionts on animal's nitrogen metabolism may result in misinterpretation of data. For example, the increased rates of ammonium efflux by the corals and sea anemones in darkness or in the animals with depleted algal population are interpreted as the evidence of nitrogen recycling, because the algae are considered as the major sink for animal-derived ammonium (e.g.

Kawaguti 1953; Cates & McLaughlin 1976; Szmant-Froelich & Pilson 1977; Wilkerson & Muscatine 1984). The surplus organic nitrogen compounds assimilated by the algae are believed to be translocated back, in the form of amino acids, to the animal cells (Muscatine *et al.* 1984; Falkowski *et al.* 1993). However, two further processes could contribute to, or even account for, these observations: (1) alga-stimulated assimilation of ammonium by the animal, and (2) alga-induced reduction in ammonium production by the animal. These putative processes are predicted by the hypotheses of nitrogen conservation by the animal and nitrogen-limitation of the algal population, respectively.

The nitrogen conservation hypothesis was first proposed for the hydra-*Chlorella* symbiosis (Rees 1986; Rees & Ellard 1989) and subsequently generalized to all algal symbioses in Cnidaria (Miller & Yellowlees 1989). It is proposed that the utilization of amino acids as respiratory substrates by the animal is reduced by the receipt, from the algal cells, of photosynthetic carbon compounds which are used preferentially in animal respiration. The resultant conservation of nitrogenous compounds in the animal tissues would promote the persistence of these symbioses in low nitrogen waters.

The chief evidence for nitrogen limitation of the symbiotic algae (Cook & D'Elia 1987) is that the algal proliferation rates and population size are increased by supplementing the medium with ammonium (e.g. Hoegh-Guldberg & Smith 1989; Falkowski *et al.* 1993; Muller-Parker *et al.* 1994). Rees (1986) has argued that the availability of ammonium to the algal cells is restricted by the activity of an ammonium-assimilating enzyme, glutamine synthetase, in the animal tissues. In the alga-depleted hydra, the activity of animal glutamine synthetase is depressed, raising the

possibility that this enzyme may be partly, or completely, responsible for elevated ammonium efflux in these animals. The impact of the algal population on the glutamine synthetase activity in the animal tissues of marine Cnidaria has, however, not been studied.

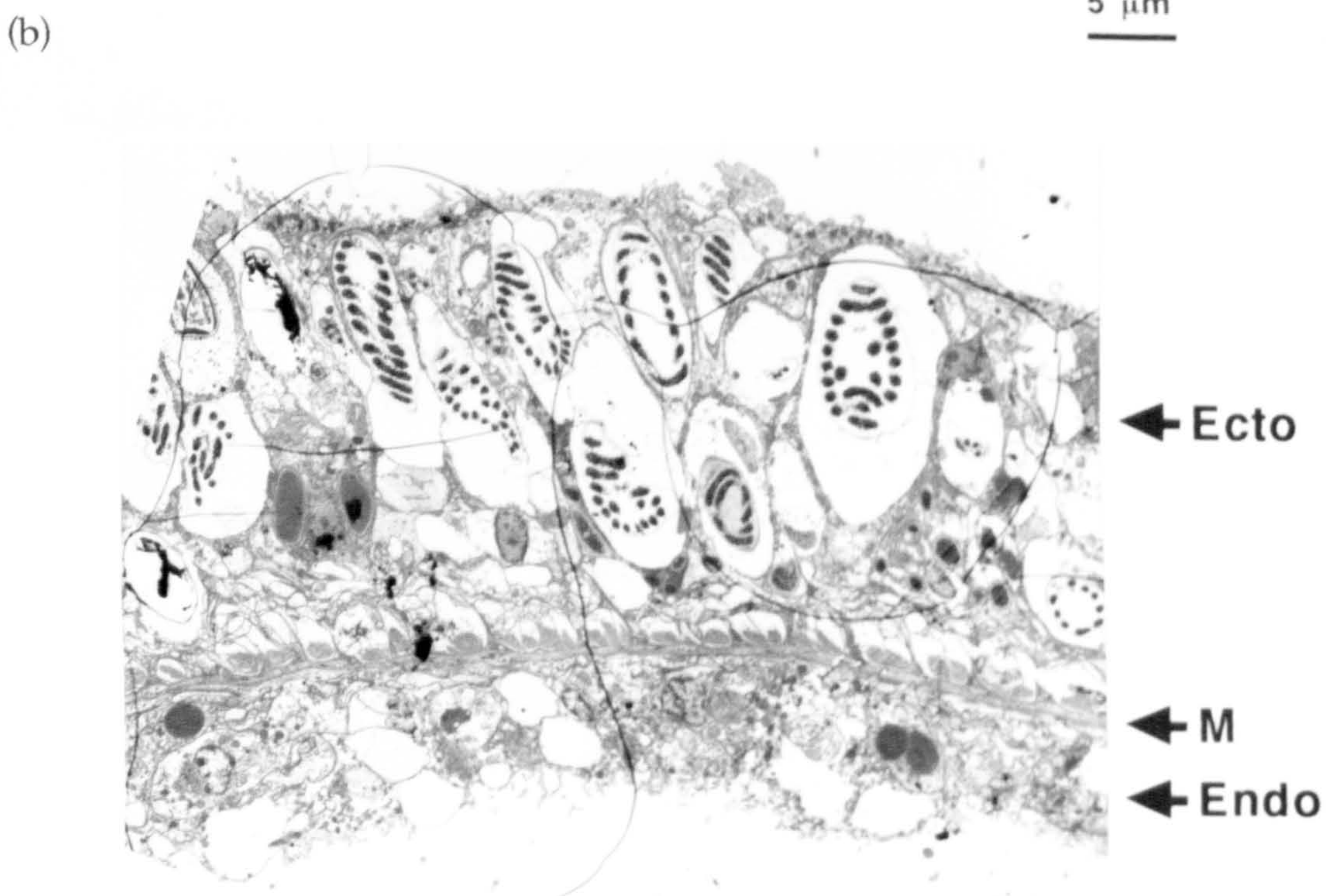
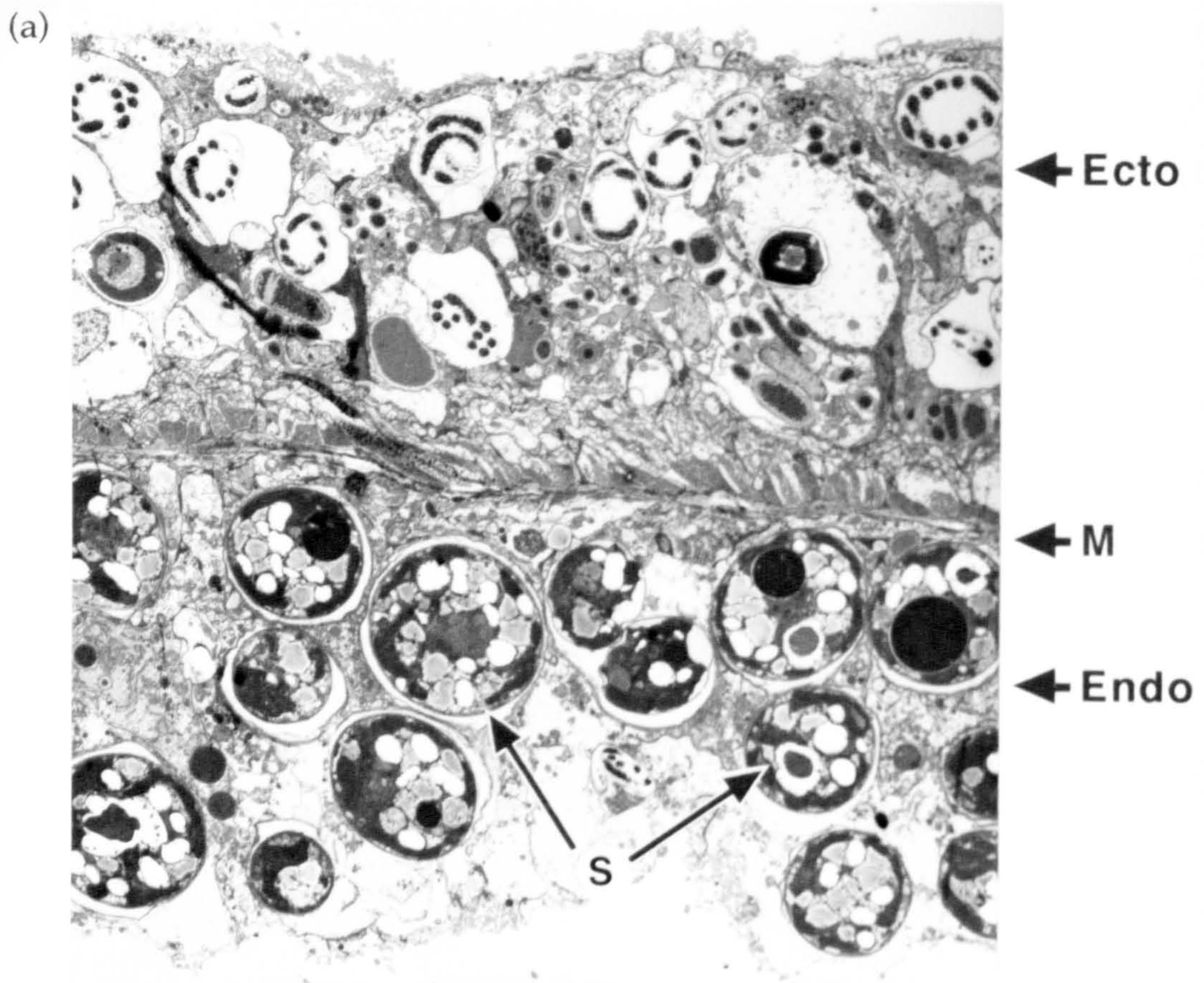
The hypotheses of nitrogen conservation and nitrogen limitation are broadly compatible with each other (Rees 1986), but neither is readily compatible with high rates of ammonium assimilation by the algae, as required by the nitrogen recycling hypothesis.

The experiments described in this chapter were designed to evaluate the impact of the symbiotic algae on the nitrogen metabolism in *A. pulchella*, and also to test the hypothesis of the nitrogen conservation in the animals with algal symbionts. The evaluation of nitrogen metabolism in *A. pulchella* was based on two issues. First of all, it was anticipated that the density of symbiotic algae would have a major impact on the nitrogen metabolism of the animals (an expectation that was amply confirmed by this study). The density of algal cells is highest and most uniform in the tentacles and oral disc of *A. pulchella*, and then declines irregularly with distance down the body column (A. E. Douglas, unpub. results). All assays were, therefore, conducted on the tentacles of *A. pulchella*. Second, the choice of the indices for nitrogen metabolic activity was influenced by the fact that: (1) Ammonium and uric acid are the two nitrogenous waste products in the sea anemones (Shick 1991); (2) In animals, glutamine synthetase is an important enzyme of ammonium assimilation; and glutamate dehydrogenase and transamination reactions are the major route for removing amino groups from amino acids (Lehninger 1970; Shick 1991); (3) The size of free amino acid pool in the animal tissue is usually proposed to be an indicator of nutritional level of the organisms

(Morris 1991). The evaluation of algal impact on the animal's nitrogen metabolism was explored by comparisons of the indices described above between the control animals (i.e. animals accommodating a "normal" population of symbiotic algae) and the animals bearing varied density of algal population (i.e. alga-depleted animals and the animals bearing a recovering algal population). Incubation of the control animals in darkness was also used to examine whether the algal population per se accounts for the effects.

An expectation of the nitrogen conservation hypothesis, but not of the nitrogen recycling hypothesis, is that exogenous organic carbon compounds and photosynthetic algae should have comparable effects on the nitrogen metabolism of symbiotic Cnidaria. Specifically, the nitrogen conservation hypothesis predicts that elimination of the algae or their photosynthetic activity should result in an increased concentration of ammonium in the animal tissues, and that this effect would be reversed by exogenous organic carbon compounds. The nitrogen conservation hypothesis also predicts that treatments which result in elevated ammonium concentration would also cause a decrease in the concentration of free amino acids in the animal tissues, and a decrease in the activity of enzyme(s) that assimilate ammonium and/or increase in activity of ammonium-producing enzymes. To test this hypothesis, these metabolic indices were also examined between the animals incubated in the presence and absence of carbon or ammonium-supplemented ASW. The choice of organic carbon supplements used in the experiments was influenced by the fact that the algae in *A. pulchella* release photosynthate as carboxylic acids, especially fumarate, and not as glycerol (Trench 1971b; chapter 3).

Figure 4.1 Electron micrograph of a cross section derived from the tentacle of (a) control *A. pulchella* and (b) alga-depleted *A. pulchella*. Ecto, ectoderm; M, mesoglea; Endo, endoderm; S, *Symbiodinium*. (x 1,000). Photography by Meg Stark.



4.2 Results

4.2.1 Effect of elimination of algal population

Transmission electron microscopy was used to examine tentacles derived from control animals, i.e. *A. pulchella* containing the 'normal' population of symbiotic algae, and alga-depleted animals, whose algal population was experimentally depressed 6-7,000 fold (see section 2.2.2). As shown in the Fig. 4.1, tentacle tissue exhibited no discernible changes in the ectoderm cells, for example nematocyst organization was comparable in the two treatments. However, the endoderm cell layer in the alga-depleted animals was much reduced compared to that in the control animals. With a much diluted algal population remaining in the tentacles of alga-depleted animals, it became very difficult to obtain a section with algal cells in it. However, by observing the algae in the tentacle homogenate under light microscopy, the algal cells remaining in the alga-depleted animals exhibited (1) smaller size, i.e. the diameter was $< 5 \mu\text{m}$ compared to $8-12 \mu\text{m}$ for that in control animals; (2) irregular shape compared to round shape in control animals; (3) pale in colour compared to brown in control animals.

Subsequent experiments explored the indices of nitrogen metabolism of the animal fraction in control and alga-depleted animals. As Table 4.1a shows, the concentration of nitrogenous waste, ammonium and uric acid, were significantly elevated in the alga-depleted animals, but the concentration of free protein amino acid in the alga-depleted animals were reduced by 57 % of that in control animals. The total free amino acid content, however, exhibited no significant difference between treatments.

In all samples, the concentration of each individual amino acid was also determined. As Fig. 4.2 shows, the composition varied significantly between the two treatments (multivariate analysis of variance $F_{19,5} =$

Table 4.1. Indices for nitrogen metabolism in the animal fraction of *Aiptasia pulchella*.

The control animals (containing symbiotic algae) were maintained under 12L:12D regime, and the alga-depleted animals in continuous darkness. The significance level for the t-tests was modified from 0.05 to 0.0125, following Bonferroni correction for multiple t-tests. Values of mean \pm s.e., with number of replicates in parentheses, are shown.

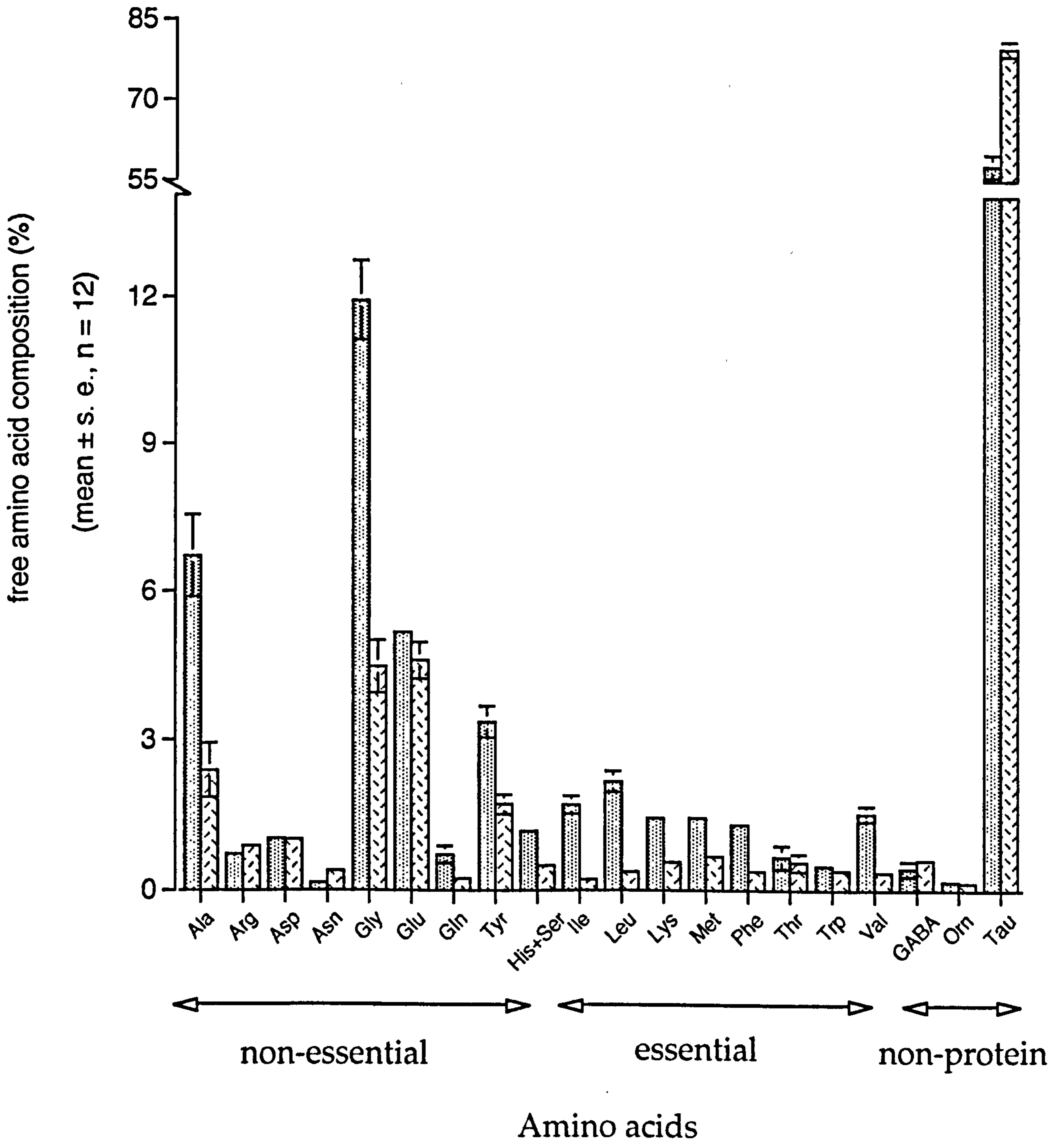
(a) Nitrogenous compounds (nmol mg⁻¹ animal protein)

The animals	ammonium	uric acid	free amino acids	
			total	protein
control	58 \pm 7 (14)	10 \pm 2 (12)	932 \pm 51 (12)	392 \pm 35 (12)
alga-depleted	149 \pm 10 (9)	31 \pm 6 (6)	843 \pm 48 (12)	168 \pm 17 (12)
	t ₂₁ = 7.53 p < 0.0001	t ₁₆ = 4.32 p < 0.001	t ₂₂ = 1.16 p > 0.05	t ₂₂ = 4.96 p < 0.001

(b) Apparent activity of enzymes in amino acid metabolism (nmol product mg⁻¹ animal protein min⁻¹)

The animals	glutamine synthetase	NADPH-glutamate dehydrogenase	alanine aminotransferase	aspartate aminotransferase
control	126 \pm 6 (18)	119 \pm 3 (6)	44 \pm 5 (6)	32 \pm 2 (6)
alga-depleted	27 \pm 2 (14)	150 \pm 9 (6)	85 \pm 2 (6)	31 \pm 1 (6)
	t ₃₀ = 14.08 p < 0.0001	t ₁₀ = 3.17 p < 0.0125	t ₁₀ = 7.95 p < 0.0001	t ₁₀ = 0.52 p > 0.05

Figure 4.2 The composition of free amino acids in the tissues of control (▨) and alga-depleted (▩) *A. pulchella*.



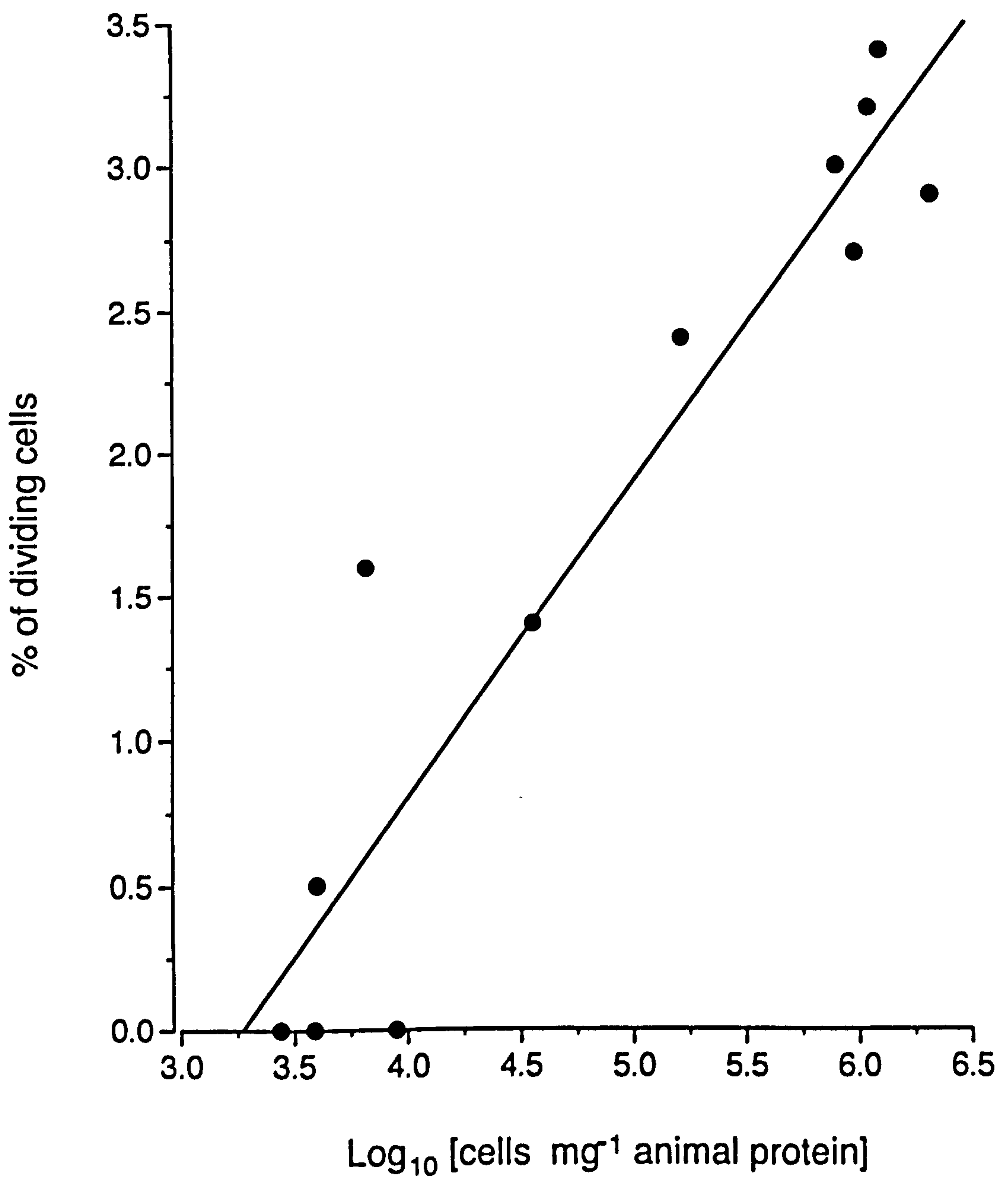
146.95, $P < 0.0001$), and profile analysis revealed that, among the free amino acids, the level of glycine, alanine, tyrosine, isoleucine, leucine, lysine, methionine, phenylalanine, and valine were elevated in control animals, relative to other amino acids. Conversely, the alga-depleted animals maintained similar total free amino acid pool by excess of taurine, a non-protein amino acid. Taurine is as well a dominant amino acid in all samples, which accounted for $57 \pm 2\%$ and $79 \pm 1.4\%$ (mean \pm s.e., $n = 12$) of the amino acids in the control and alga-depleted animals, respectively.

The apparent activity of four enzymes in the animal fraction is shown in Table 4.1b. The animals with depleted algal population showed depressed activity in glutamine synthetase, and increased activities in alanine aminotransferase and NADPH-glutamate dehydrogenase, relative to the control animals. But no significant change in aspartate aminotransferase activity was found between the two treatments.

4.2.2 Effect of repopulation of algae

When alga-depleted animals were transferred to the 12L:12D light regime, the residual population of symbiotic algae increased. Twelve weeks later, it was noted that the algal population in the tentacles varied between animals over three orders of magnitude: from animals with 3×10^3 cells mg^{-1} animal protein, just double the mean algal population in the dark-maintained alga-depleted animals, to animals with 2.1×10^6 cells mg^{-1} animal protein, 25-29% of the algal population in control animals. The cellular basis of this wide variation was not established, but it did provide the opportunity to investigate the impact of algal density on the nitrogen metabolic indices. Subsequent experiment compares the % of dividing algal cells, glutamine synthetase activity, free amino acid content and

Figure 4.3 Regression of the percentage of dividing cells on the algal density in alga-depleted *A. pulchella* after 12 weeks of repopulating endogenous symbiotic algae in 12L:12D. The regression equation is $y = 1.09x - 3.58$ ($r = 0.94$).



ammonium content of the animal fraction in 12 animals bearing the recovering algal population.

The experimental data (except the % of dividing algal cells) and algal density were transformed to a logarithmic scale, which increased correlation coefficients of the measurements with algal density. As the data shown in Fig. 4.3, the % of dividing algal cells increased linearly with the $\log[\text{algal density}]$. However, that in the animals with a algal density below 3.6×10^4 cell mg^{-1} animal protein exhibited a higher variability; three samples exhibited no dividing cells in 1,000 cell counts; 0.5 % was determined in one sample; and 1.3-1.6 % were determined in the another two samples, which is close to the level found in the control animal.

The algal density exhibited a reversed effect on the nitrogen metabolic indices between the animal's glutamine synthetase activity and ammonium concentration, i.e. glutamine synthetase activity of the animal fraction increased with algal density, but ammonium concentration decreased with it (Fig. 4.4). With the increase of $\log[\text{algal density}]$, the animals also displayed a linear increase in $\log[\text{protein amino acid content}]$ as well as $\log[\text{"essential" amino acid content}]$ (Fig. 4.5). As shown in Fig. 4.4, with regression equations in the legend, the slopes of the regressions of $\log[\text{glutamine synthetase activity}]$ and $\log[\text{ammonium concentration}]$ on $\log[\text{algal density}]$ were similar in magnitude, but opposite in sign, at +0.114 (s.d. 0.0177) and -0.120 (s.d. 0.0323), respectively. In other words, for a ten-fold increase in algal density, the animal is predicted to exhibit a 24-30% increase in glutamine synthetase activity and equivalent decrease in ammonium content. However, algal density showed less effect on the elevation of the animal's amino acid content, which would be only 10 %

Figure 4.4 Regression of glutamine synthetase activity (●) and ammonium concentration (○) on algal density in the animal fraction of alga-depleted *A. pulchella* incubated in 12L:12D for 12 weeks. The regression equations are: $\log[\text{glutamine synthetase activity}] = 0.114 \times \log[\text{algal density}] + 1.09$ ($r = 0.90$); and $\log[\text{ammonium concentration}] = -0.120 \times \log[\text{algal density}] + 2.43$ ($r = -0.76$). Enzyme activities are expressed as $\text{nmol product mg}^{-1} \text{ protein min}^{-1}$ and ammonium concentration as $\text{nmol mg}^{-1} \text{ protein}$.

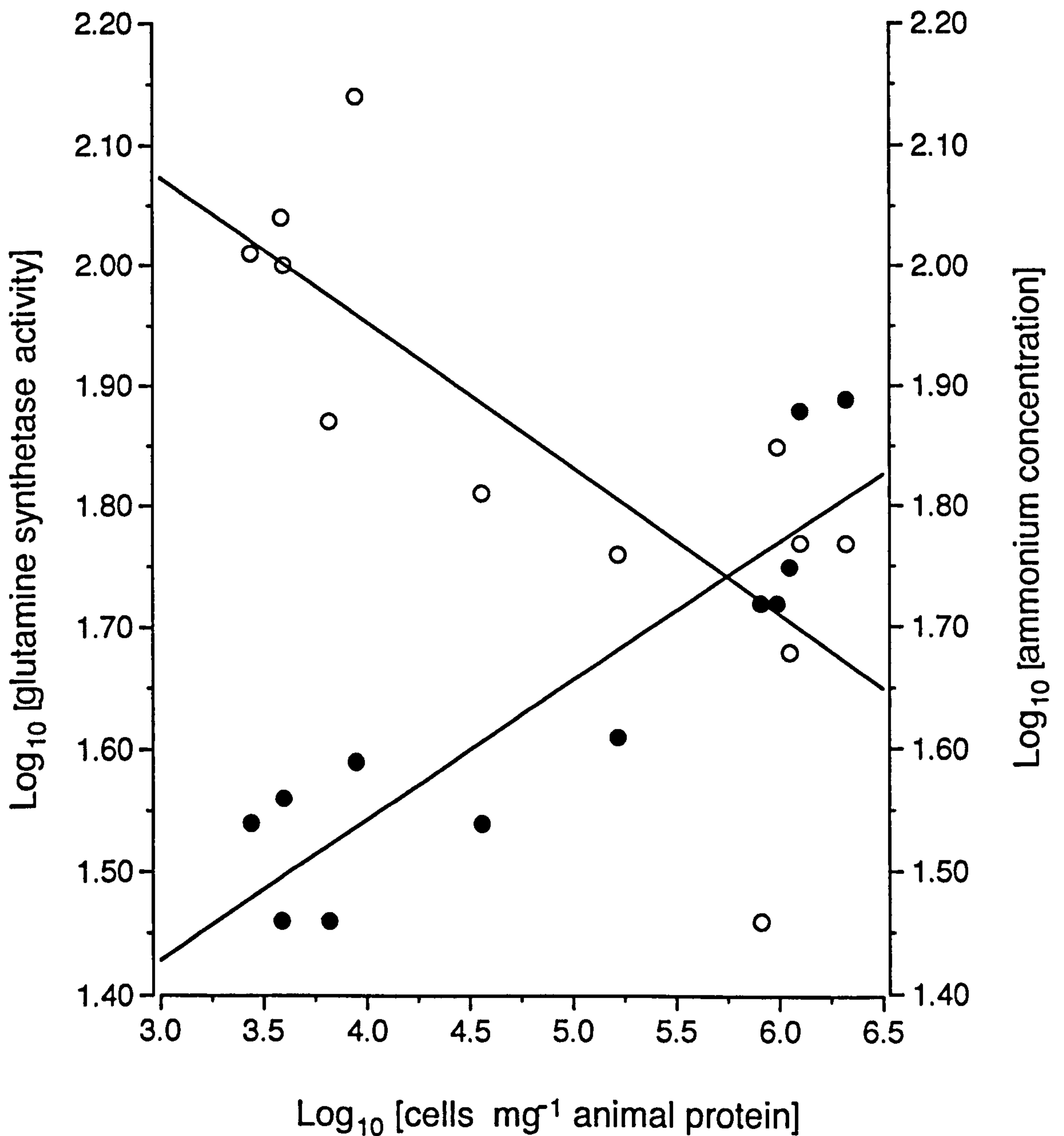


Figure 4.5 Regression of free amino acid concentration on algal density of alga-depleted *A. pulchella* in 12L:12D for 12 weeks. The equation for protein amino acids (a) is $\log[\text{amino acid}] = 0.04 \times \log[\text{algal density}] + 2.02$ ($r = 0.79$); and "essential" amino acids (b) is $\log[\text{amino acid}] = 0.06 \times \log[\text{algal density}] + 1.34$ ($r = 0.84$).

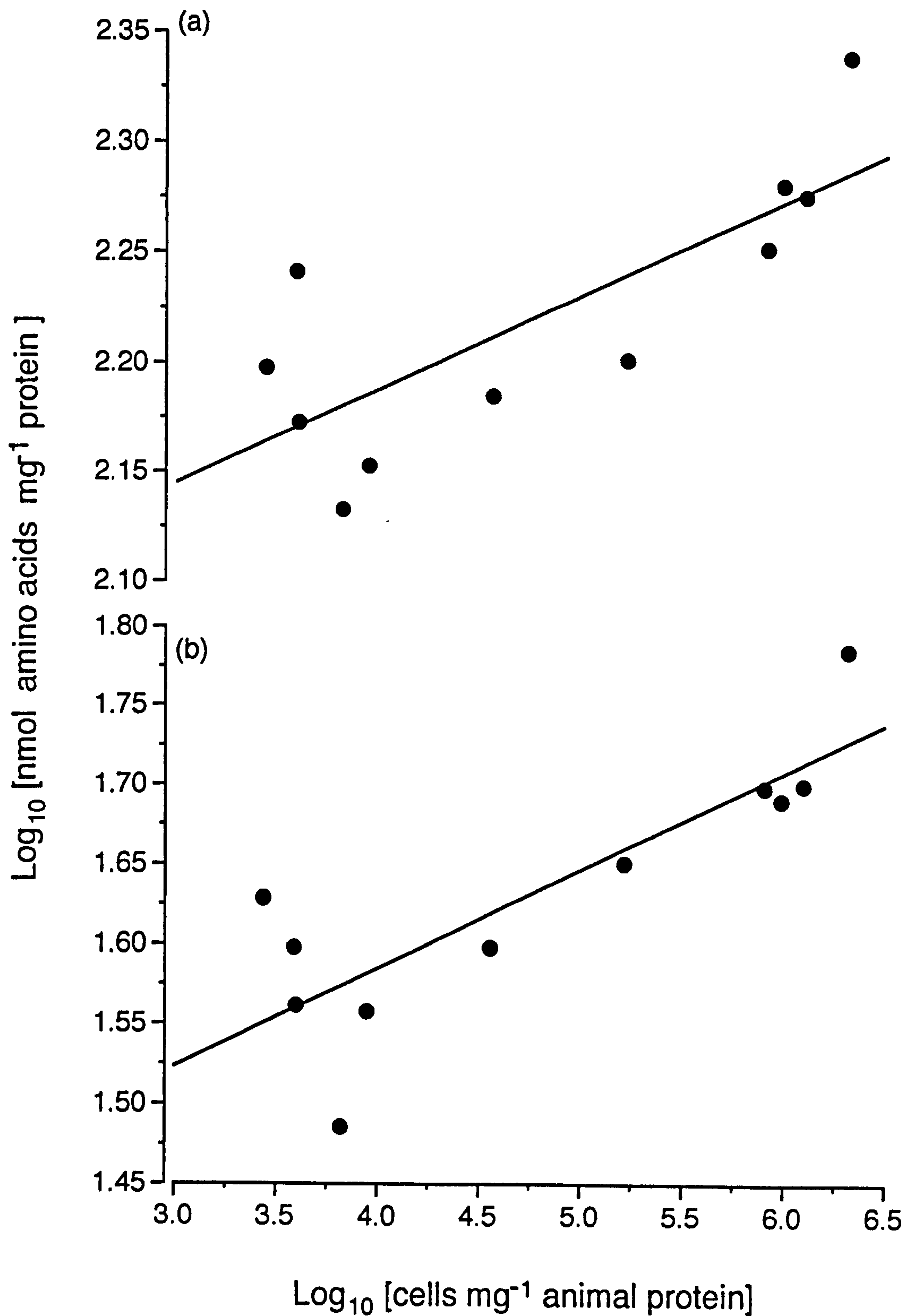
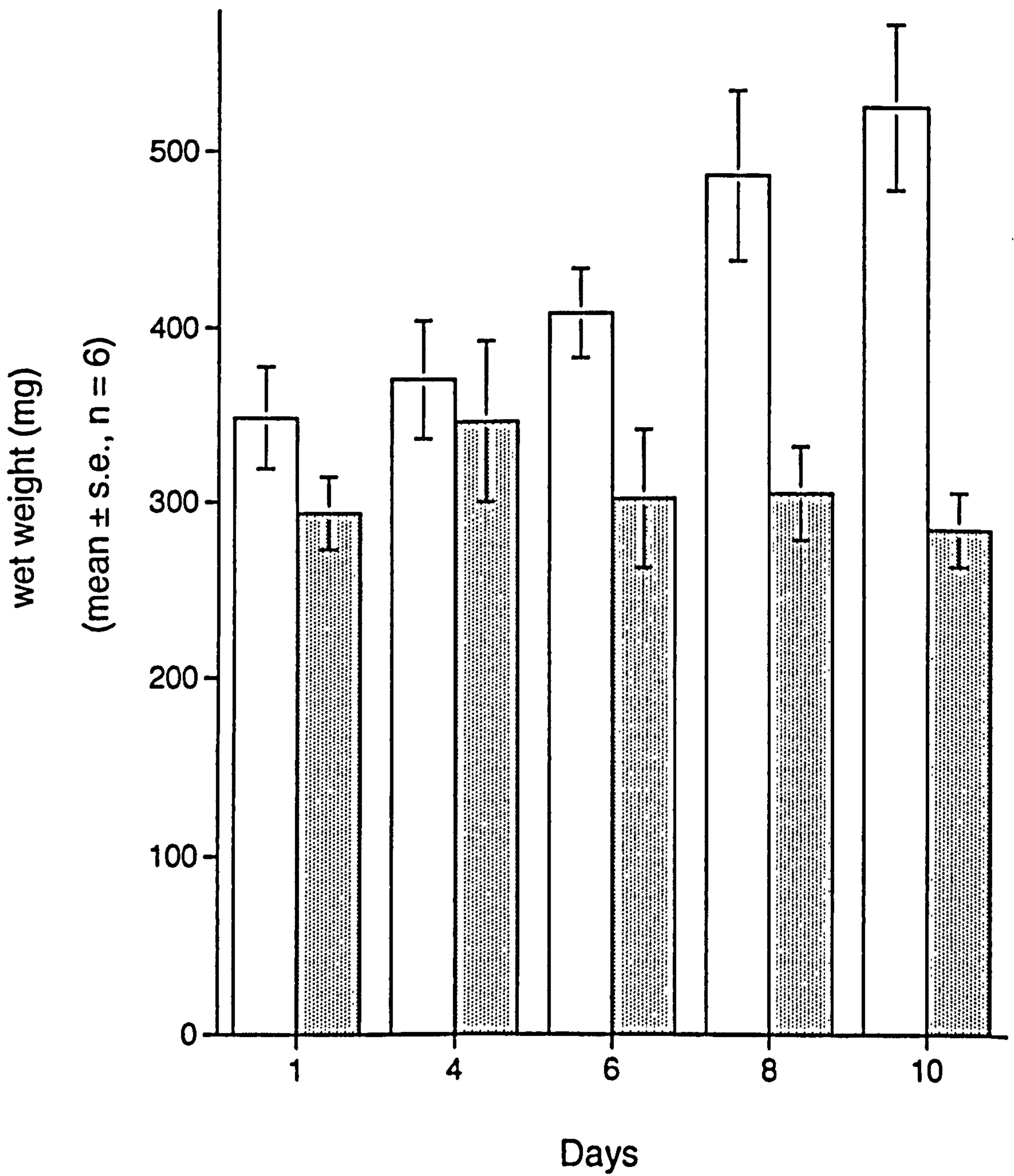


Figure 4.6 The blotted wet weight of symbiotic *A. pulchella* incubated in the 12L:12D (□) or continuous darkness (▨).



and 14 % increased in protein amino acid content and "essential" amino acid content for a ten-fold increase in algal density, respectively.

4.2.3 Impact of light regime

The control animals were maintained in continuous darkness for 12 days to explore the effect of light regime on the nitrogen metabolism, with a group of animals in 12L:12D as control. During the dark treatment, noticeable paling of the animal appearance was found at the fourth day, but no discernible difference in their feeding response and no mortality was observed.

Algal density, chlorophyll *a* content of the algal cells, and wet weight of the animals incubated in the dark were quantified and shown in Fig. 4.6-4.7.

The wet weight of the daily-fed animals in continuous darkness remained constant during the experiment, compared to a linear increase of weight in 12L:12D maintained animals (Fig. 4.6). The rate of weight gain in the animal maintained under 12L:12D light regime was determined by the slope of linear regression of wet weight on the incubation times, and revealed a growth rate of $29 \pm 8 \text{ mg day}^{-1}$. As shown in Fig. 4.7a, the algal density in the tentacle of the animals maintained in darkness exhibited a significant decline at the fourth day of incubation, and reduced by 73 % of the original density after 12 days of incubation. Conversely, the animals in the 12L:12D light regime maintained a constant algal population of $7\text{-}8 \times 10^6$ algal cell mg^{-1} animal protein in the tentacles. Comparable to the data determined in the *Symbiodinium* isolated from Hawaii *A. pulchella* (Muller-Parker 1987), the chlorophyll *a* content determined in this study ranged from 1.6 to 2.2 pg cell^{-1} for the samples derived from both control and darkness-treated animals (Fig. 4.7b). The constant chlorophyll *a* content suggests that the colour bleaching in the animals during dark

Figure 4.7 Algal density (a) and chlorophyll *a* content (b) of the *Symbiodinium* cells in control animal *A. pulchella*, incubated in the 12L:12D (□) or continuous darkness (▨). Each data point is the mean of two replicates.

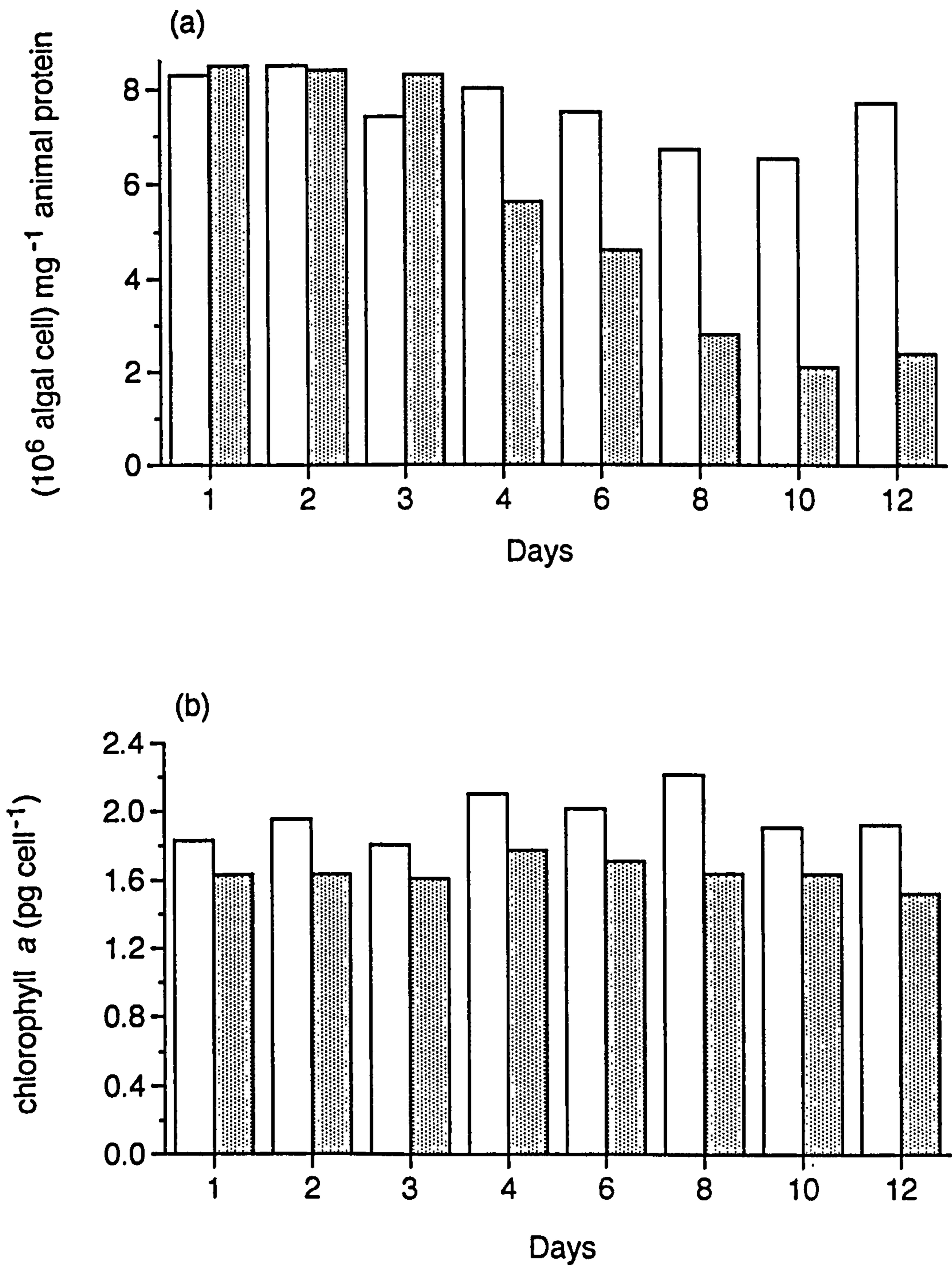
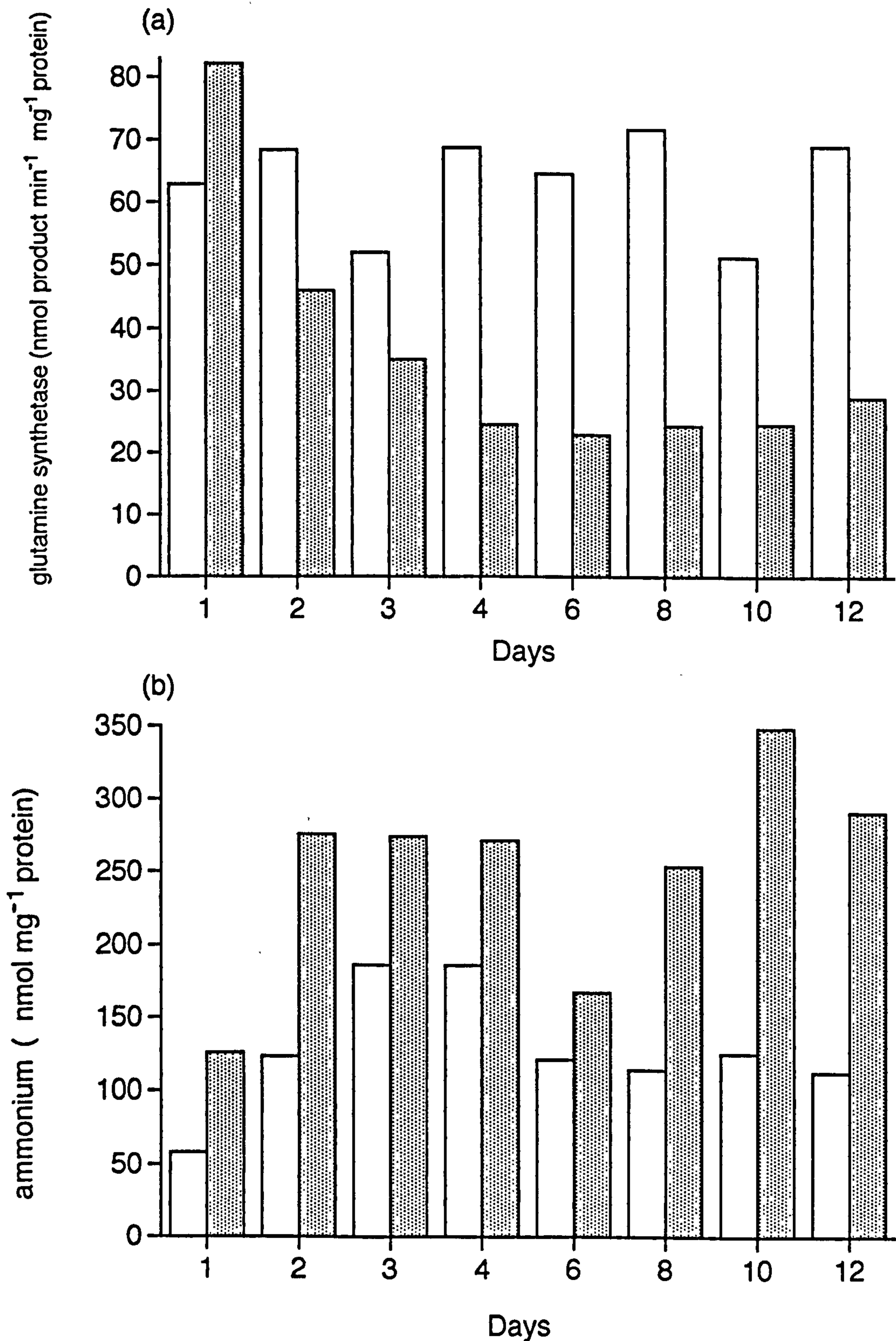


Figure 4.8 The changes of glutamine synthetase activity (a) and ammonium concentration (b) in the animal tissue of symbiotic *A. pulchella* incubated in 12L:12D (□) or continuous darkness (▨). Data are the mean of two replicates.



incubation is attributable to reduced algal density rather than to the loss of algal pigment.

The nitrogen metabolism in the animals incubated in darkness was evaluated by examining the activity of glutamine synthetase, ammonium concentration, and protein amino acid content in the free amino acid pool of animal fraction. As Fig. 4.8a shows, the glutamine synthetase activity declined dramatically when the animals were transferred to darkness, and stabilized at 25-30 nmol product mg⁻¹ protein min⁻¹ over 12 days during the experiment. It was also noted that the reduction in glutamine synthetase activity occurred 2 days before the decline in the density of symbiotic algae. The ammonium concentration was on average two times higher in the animals maintained in darkness than that in 12L:12D light regime, but both were elevated over the experimental period (Fig. 4.8b). The contents of protein amino acids in both treatments did not show obvious change in the first week of incubation, and then increased since 8th day (Fig. 4.9a). The elevation of protein amino acid content might be a result of the increase in the concentration of glycine (Fig. 4.9c), rather than to the "essential amino acid" (Fig. 4.9b).

The above experiment demonstrated that when control animals are transferred from the 12L:12D light regime to continuous darkness, the algal population in their tentacles remains unchanged at the first three days and only reduced by 30% after 6-7 days of incubation, values more than a thousand times greater than the population in the alga-depleted animals. This provided the basis to explore the contribution of darkness per se, and the very low algal density to the low glutamine synthetase activity of alga-depleted animals.

Figure 4.9 The free amino acid concentration in the animal tissue of symbiotic *A. pulchella* incubated in 12L:12D (□) or continuous darkness (■). Each data point is the mean of two replicates of protein amino acid (a), "essential" amino acid (b) and glycine (c).

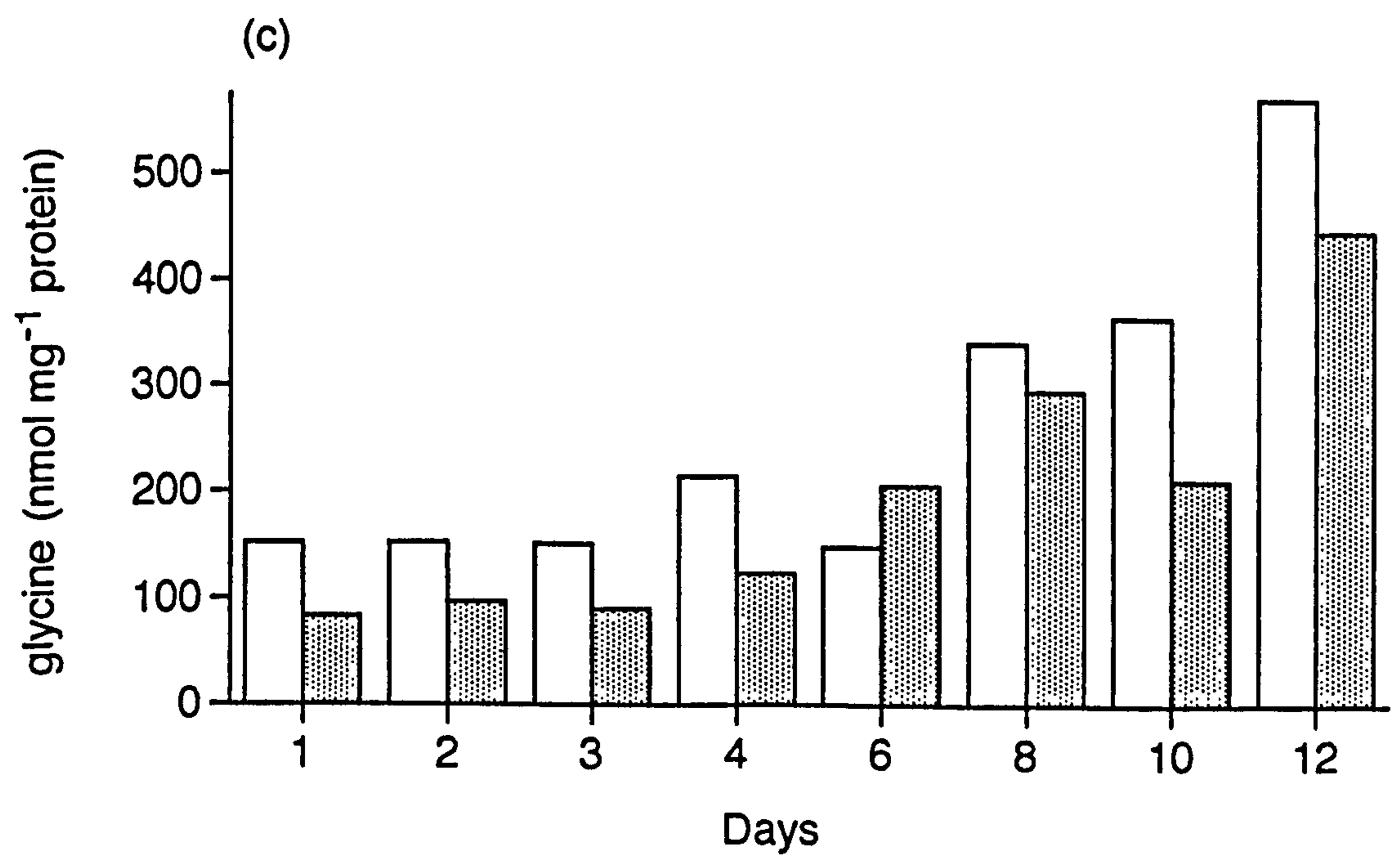
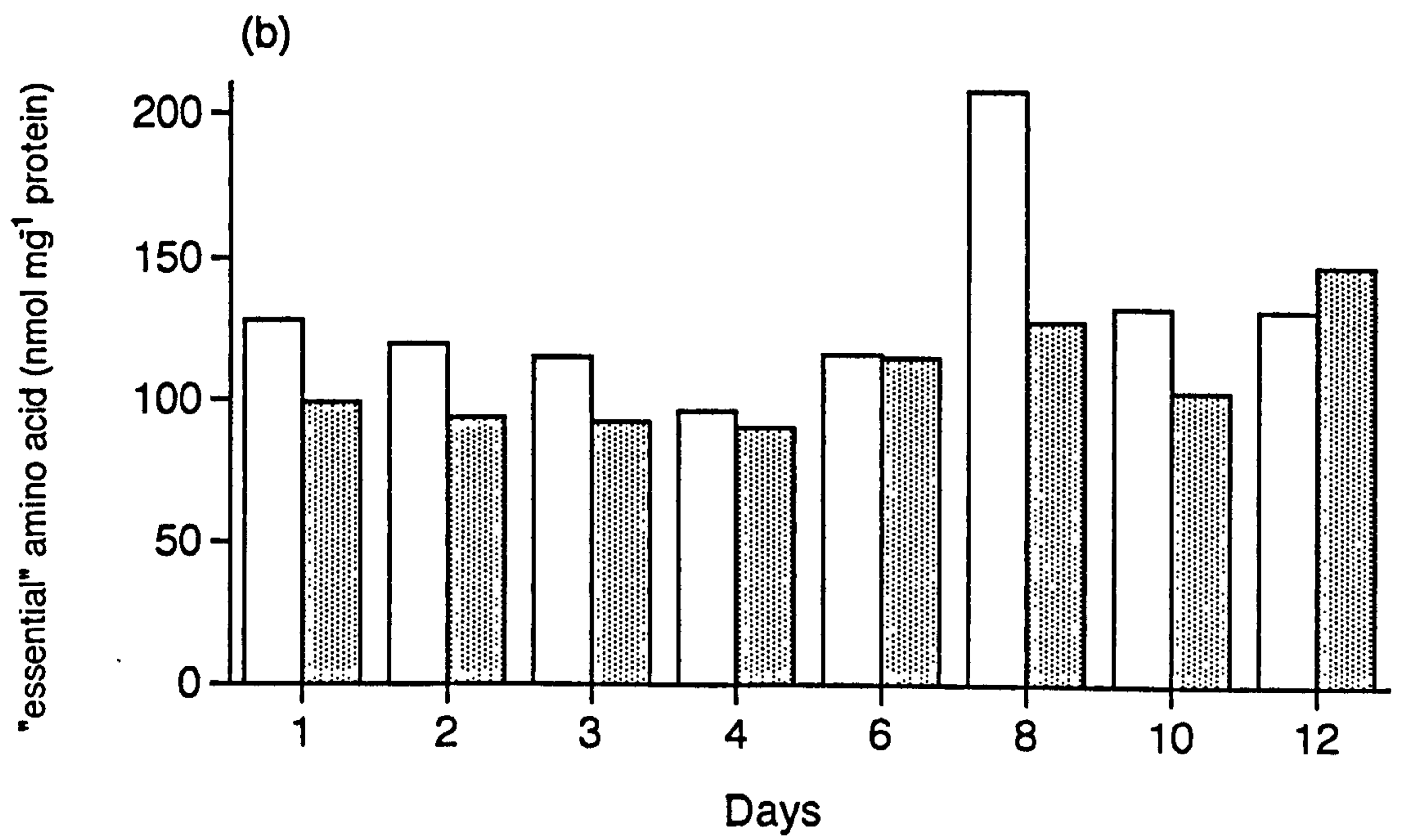
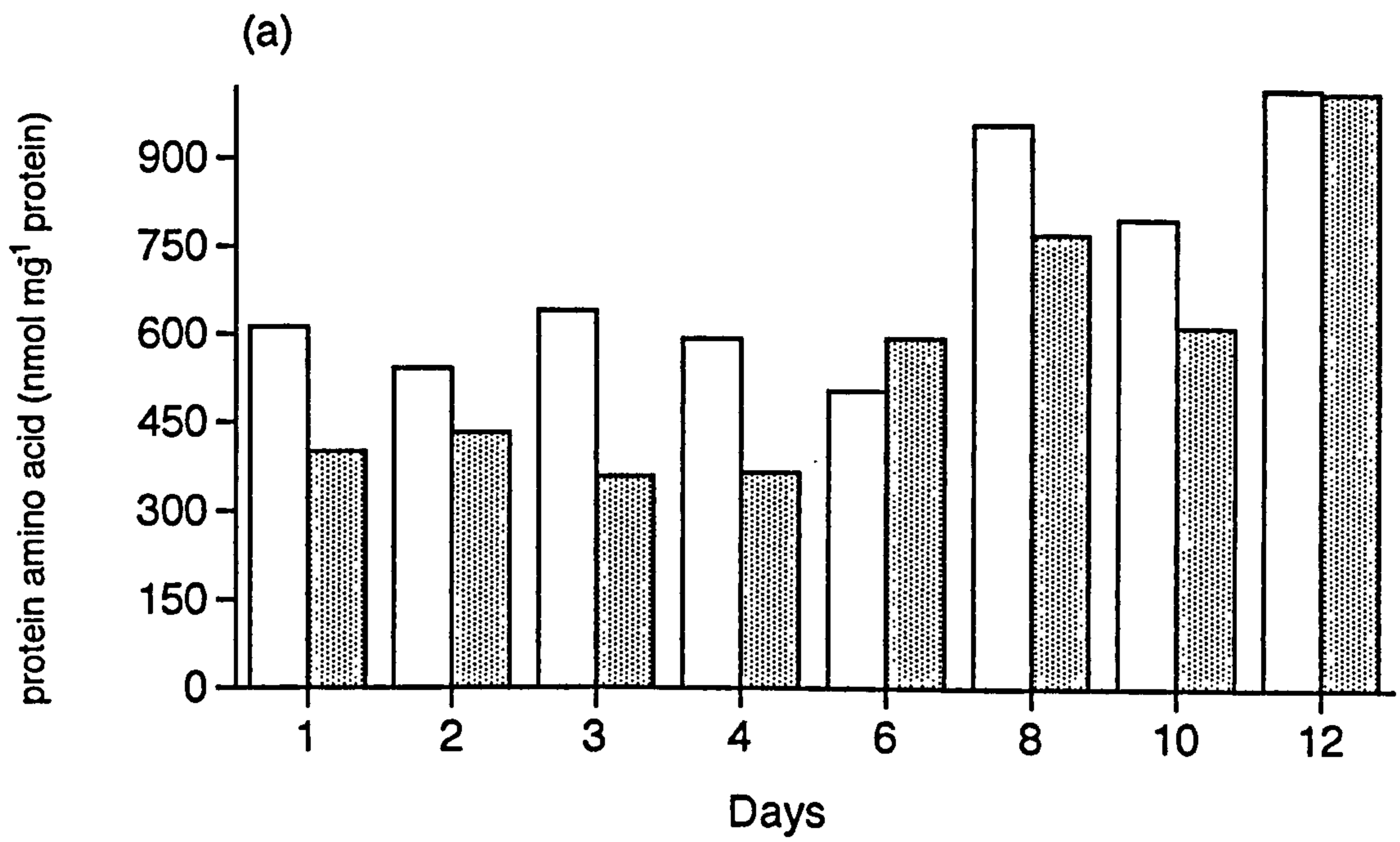


Figure 4.10 Impact of light regime on the activity of enzymes in amino acid metabolism in the animal fraction of control animals of *A. pulchella*, incubated in continuous darkness for 6 days, and then (arrow) transferred to 12L:12D. Enzyme activities are expressed as nmol product mg⁻¹ animal protein min⁻¹, and values of mean ± s.d. (6 replicates) are shown. Glutamine synthetase (a), aspartate aminotransferase (b), alanine aminotransferase (c).

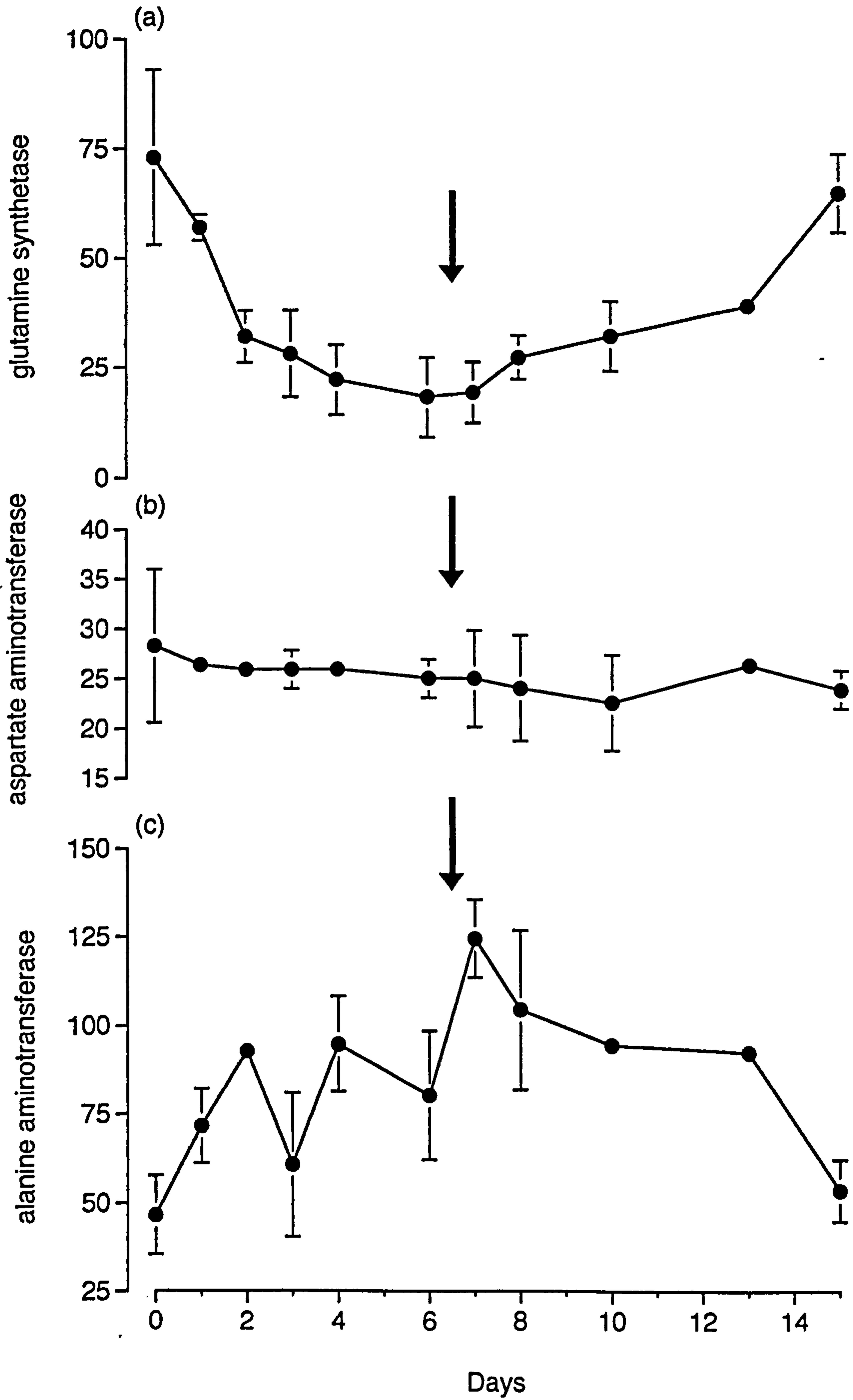


Table 4.2 The impact of organic carbon supplements on the ammonium content and glutamine synthetase activity in the animal fraction of *A. pulchella* for 7 days of incubation.

Values of mean \pm s.e. are shown, and means followed by the same letter are not significantly different at $p = 0.05$ (Fisher's least significant difference test). (n.d.: not determined)

organic carbon supplement (10 mM)	glutamine synthetase		ammonium	
	control (n=9)	alga-depleted (n=3)	control (n=3)	alga-depleted (n=3)
<u>12L:12D</u>				
none	114 \pm 7 ^a	n.d.	56 \pm 4 ^{a,b}	n.d.
<u>continuous darkness</u>				
none	24 \pm 2 ^b	28 \pm 2 ^a	78 \pm 10 ^c	113 \pm 8 ^a
succinate	62 \pm 5 ^c	n.d.	53 \pm 6 ^a	n.d.
fumarate	68 \pm 5 ^{c,d}	64 \pm 7 ^b	71 \pm 3 ^{b,c}	72 \pm 7 ^b
glycerol	69 \pm 7 ^{c,d}	112 \pm 2 ^c	n.d.	83 \pm 9 ^b
glucose	86 \pm 6 ^d	n.d.	n.d.	n.d.
α -ketoglutarate	110 \pm 10 ^a	123 \pm 2 ^c	43 \pm 2 ^a	74 \pm 8 ^b
ANOVA	F _{6,56} = 22.29	F _{3,8} = 37.94	F _{4,10} = 5.50	F _{3,8} = 5.64
	p < 0.0001	p < 0.0001	0.01 < p < 0.05	0.01 < p < 0.05

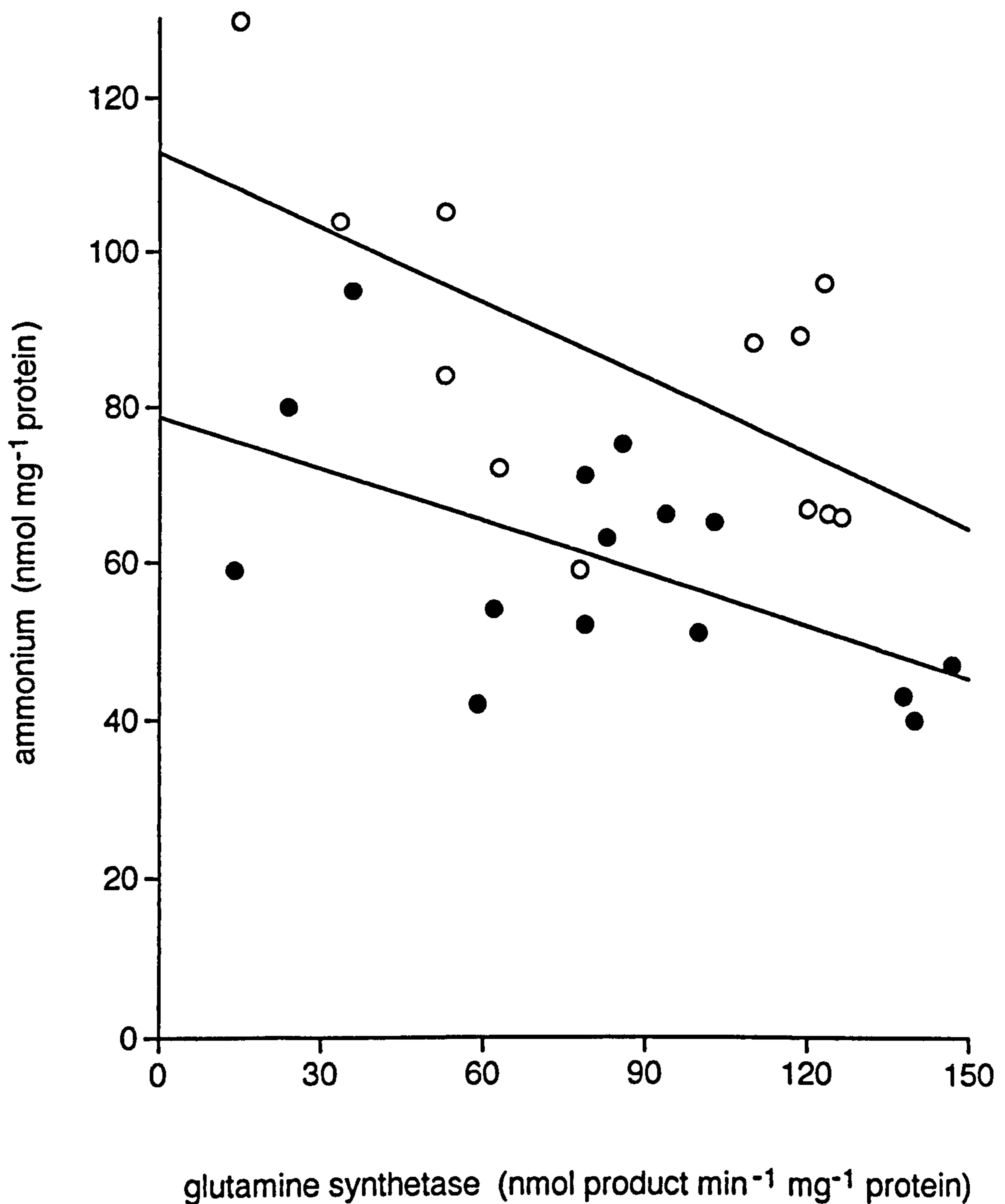
Consistent with the previous finding (Fig. 4.8a), control animals incubated in darkness for 6 days exhibited a rapid decline in glutamine synthetase activity to values comparable to those in alga-depleted animals, and a subsequent increase on return to the 12L:12D conditions (Fig. 4.10a). However, incubation in darkness had no discernible effect on aspartate aminotransferase and increased the activity of alanine aminotransferase of the animals (Fig. 10 b&c). The response of enzyme activity in the animals to the dark treatment, in which the animal still accommodated a substantial density of algae, is comparable to that in the animal with a depleted algal population (see also Table 4.1).

In summary, these results indicate that the glutamine synthetase activity and ammonium concentration of animal fraction in *A. pulchella* is remarkably influenced by the photosynthetic activity of symbiotic algae, and not algal density per se. Subsequent experiments explore the effect of organic carbon compounds, the crucial components in the photosynthate release by symbiotic algae, on nitrogen metabolism in *A. pulchella*.

4.2.4 Impact of exogenous organic carbon compounds

When *A. pulchella* was incubated in darkness for 7 days in the medium supplemented with an organic carbon compound, both the control and alga-depleted animals exhibited a significant increase of glutamine synthetase activity and reduction in ammonium concentration (Table 4.2). The effect varied between the compounds tested. Fumarate, for example, consistently increased glutamine synthetase to levels intermediate between those of animals incubated without a carbon supplement in continuous darkness and control animals in the 12L:12D regime; and α -ketoglutarate stimulated glutamine synthetase activity to values characteristic of control animals in 12L:12D light regime.

Figure 4.11 Regression of ammonium concentration on glutamine synthetase activity in the animal fraction of control (●) and alga-depleted (○) *A. pulchella*, incubated as described in Table 4.3. The regression equations for control animals is $y = -0.22x + 78.69$ ($r = 0.58$); and that for alga-depleted animals is $y = -0.33x + 112.99$ ($r = 0.63$), where y indicates the concentration of ammonium and x indicates the glutamine synthetase activity.



In these experiments, the ammonium content of the alga-depleted animals was generally higher than that of darkness-incubated control animals under the same treatment. To explore this further, the ammonium content for each sample was regressed on the corresponding value of glutamine synthetase activity (Fig. 4.11). Consistent with the differences observed between the animals bearing an algal population with different density (Fig. 4.4), the regression equations (see legend to Fig. 4.11) for the control and alga-depleted animals had negative slopes that were not significantly different from each other ($F_{1,25} = 0.28, p > 0.05$), but the intercept of the equation for alga-depleted animals was significantly greater than that for control animals ($F_{1,25} = 4.55, p < 0.05$). This result suggests that the control animals have an appreciable sink for ammonium, additional to glutamine synthetase, that is absent from alga-depleted animals. As considered in section 4.3.2, the additional sink may be the algal population.

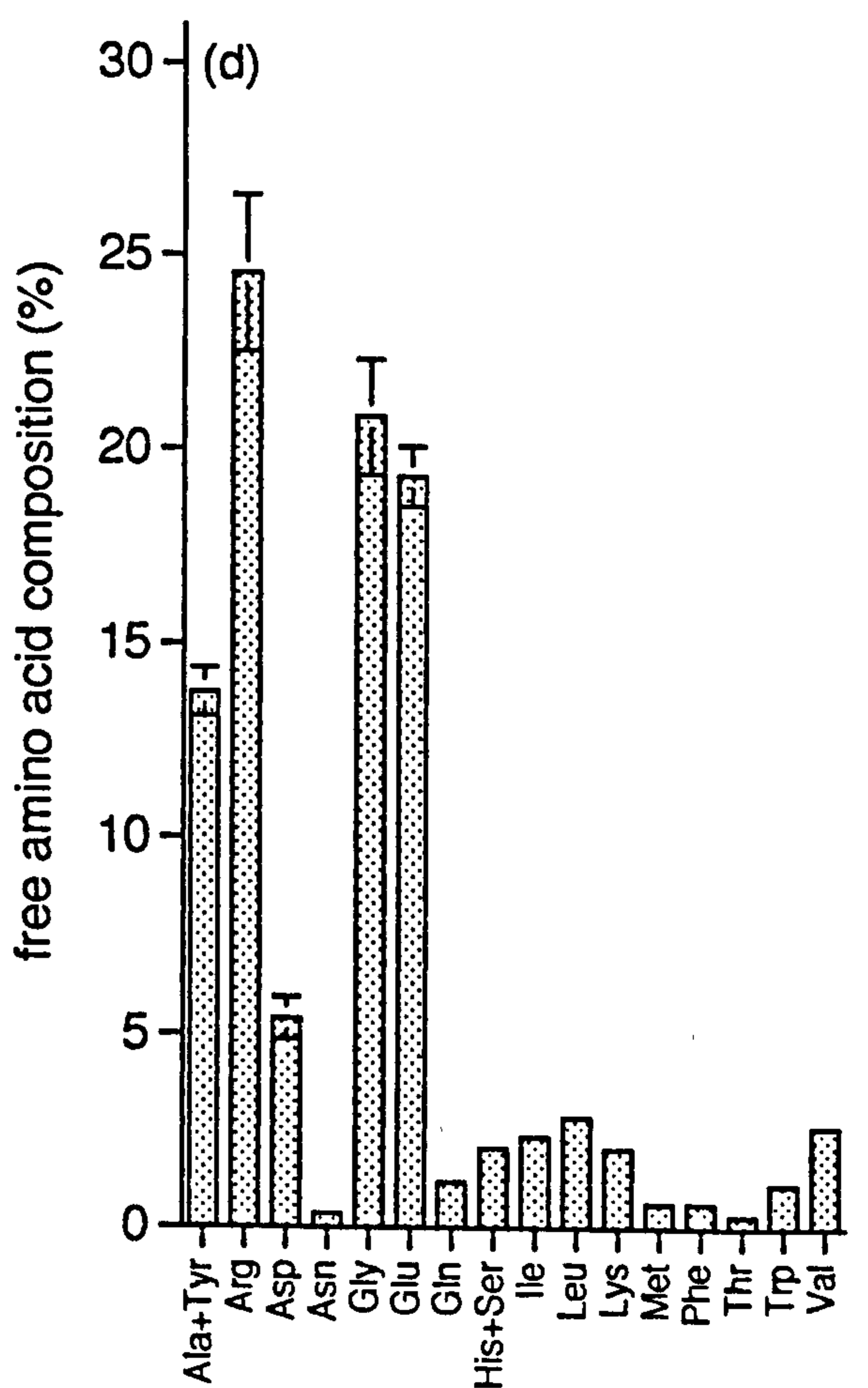
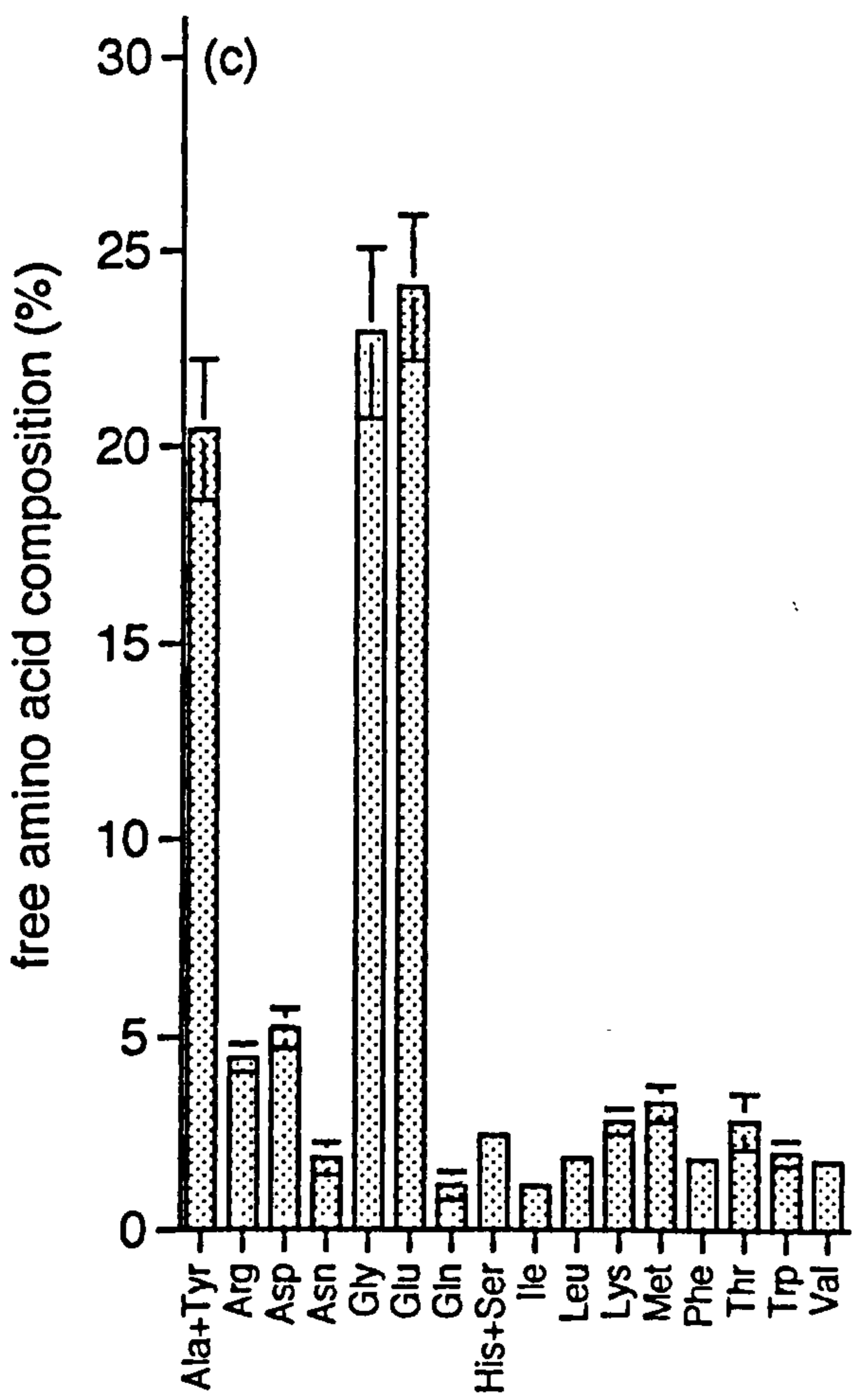
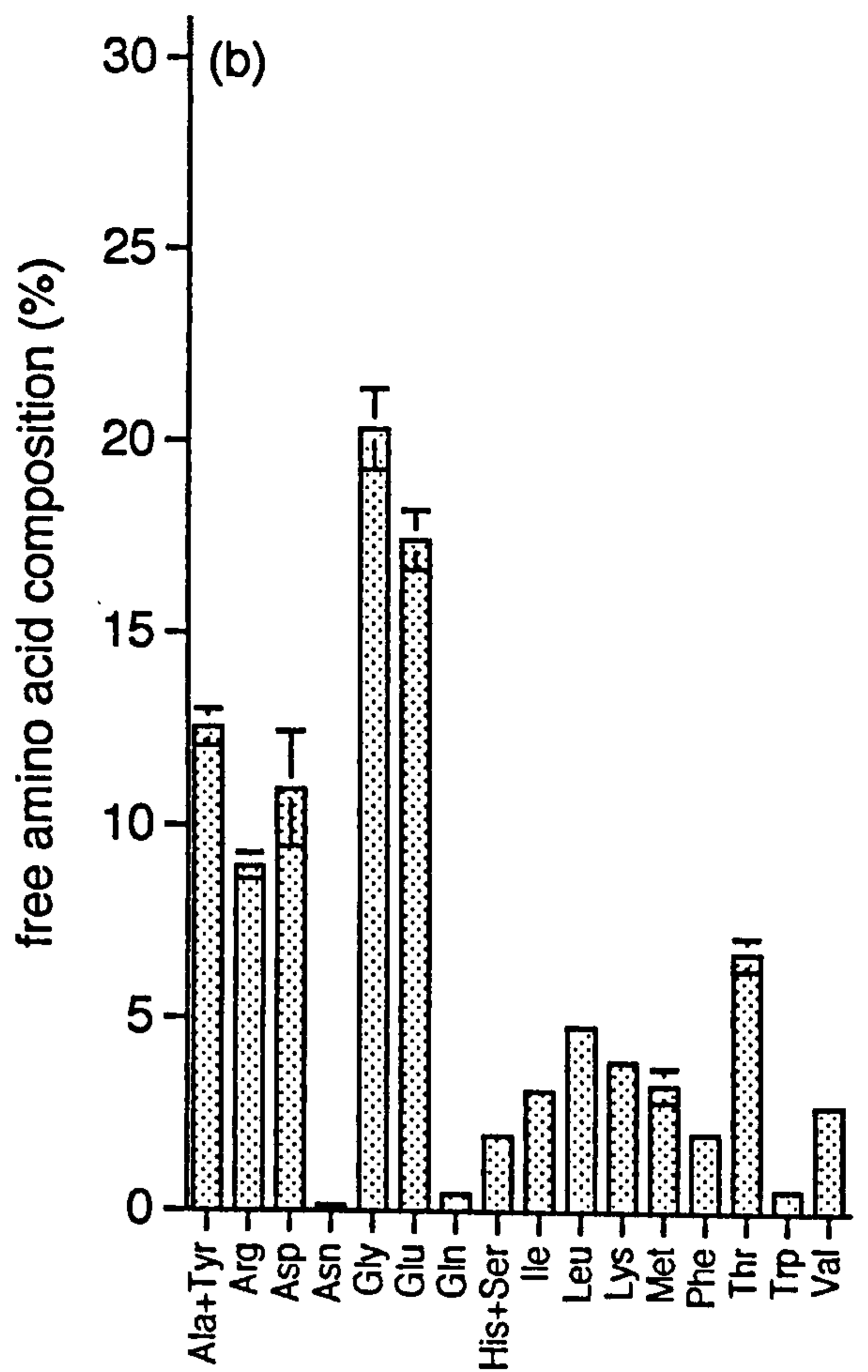
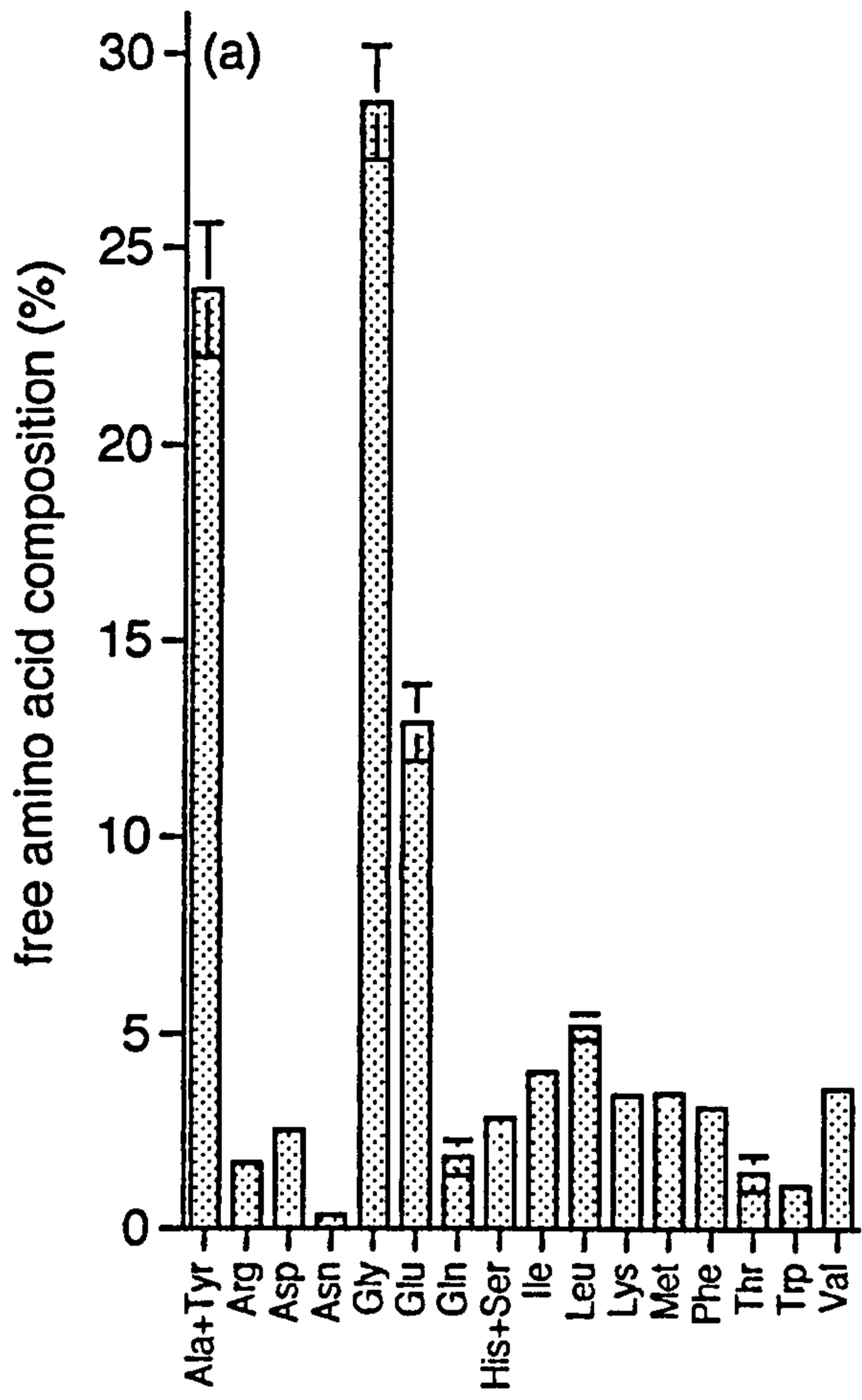
The impact of one organic compound, α -ketoglutarate, on the protein amino acid content in the free amino acid pool of *A. pulchella* was examined. As shown in Table 4.3, after 7 days of incubation with or without α -ketoglutarate, the carbon compound supplement showed reverse effect on amino acid content in the animal fraction of control and alga-depleted animals. With α -ketoglutarate supplement, the content of protein amino acids and "essential" amino acids in the control animals were depressed by 65 % and 59 % of that in ASW control, respectively; but that in the alga-depleted animals were elevated relative to that in ASW control (4.0 fold for protein amino acids and 2.7 fold for essential amino acids). Moreover, the analysis with 2-way ANOVA revealed that both depletion of symbiotic algae and α -ketoglutarate supplement significantly influenced the content of protein amino acids in the animal fraction of *A. pulchella*

Table 4.3 The effect of α -ketoglutarate on the content of free amino acids in the animal fraction of control and alga-depleted *A. pulchella*.

Each of 12 sea anemones was incubated in the aerated ASW, or ASW supplemented with 10 mM α -ketoglutarate in 12L:12D for control animals and continuous darkness for alga-depleted animals for 7 days. Results (nmol mg^{-1} animal protein) are mean \pm s.e. with 12 replicates.

supplement to ASW	protein amino acids		"essential" amino acids	
	control	alga-depleted	control	alga-depleted
none	392 \pm 35	168 \pm 17	102 \pm 12	31 \pm 5
α -ketoglutarate	138 \pm 14	667 \pm 35	42 \pm 6	83 \pm 7
ANOVA: algae	F _{1,44} = 31.6 p<0.001		F _{1,44} = 3.4 p>0.05	
α -ketoglutarate	F _{1,44} = 20.4 p<0.001		F _{1,44} = 0.2 p>0.05	
interaction	F _{1,44} = 193.5 p<0.001		F _{1,44} = 48.9 p<0.001	

Figure 4.12 The composition of free amino acids in the animal fraction of control and alga-depleted *A. pulchella*, incubated in the ASW or ASW supplemented with 10 mM α -ketoglutarate for 7 days. Control animals in ASW (a) and α -ketoglutarate supplemented ASW (b) were incubated in 12L:12D; alga-depleted animals in ASW (c) and α -ketoglutarate supplemented ASW (d) were incubated in continuous darkness. Results are mean \pm s.e. with 12 replicates.



($p < 0.001$), but that did not show a consistent influence on "essential" amino acid content in the animals.

As shown in Fig. 4.12, the amino acid composition between four treatments, i.e. with or without α -ketoglutarate supplement to the control animals and alga-depleted animals, also varied significantly (multivariate analysis of variance, $F_{15,45} = 375.53$, $P < 0.0001$), and the profile analysis revealed that the α -ketoglutarate supplement significantly elevated the level of arginine relative to other protein amino acids in both control and alga-depleted animals. Furthermore, the α -ketoglutarate supplement also elevated the level of aspartate in control animals, valine, isoleucine and leucine in alga-depleted animals, and depressed the level of asparagine in control animals, methionine and phenylalanine in the alga-depleted animals, relative to other amino acids. The conclusion of profile analysis is that the supplement of α -ketoglutarate might not fully restore the composition of protein amino acid in the alga-depleted animals to the characteristic level in the control animals, but some amino acids, for example valine, isoleucine and leucine, in alga-depleted animals were elevated by the supplement of this organic compound.

4.2.5 Effect of the supplement of exogenous ammonium

The interactions between glutamine synthetase activity and ammonium concentration in the animal tissue were further examined by using an exogenous supplement of ammonium ion. As shown in Fig. 4.13, over a 24 h incubation, ammonium concentration in the animal fraction was elevated when the concentration in the medium increased; and, consistent with data in Fig. 4.8, the ammonium concentration in the alga-depleted animals were on average two times higher than that in the control animals over a range of ammonium supplements. The glutamine

Figure 4.13 The impact of exogenous ammonium supplement on the glutamine synthetase activity (\square) and ammonium concentration (\boxtimes) in the animal fraction of control (a) and alga-depleted (b) *A. pulchella*, incubated in the SW-P buffer supplemented with a range of ammonium chloride concentration for 24 h. Results are mean \pm s.d. with three replicates.

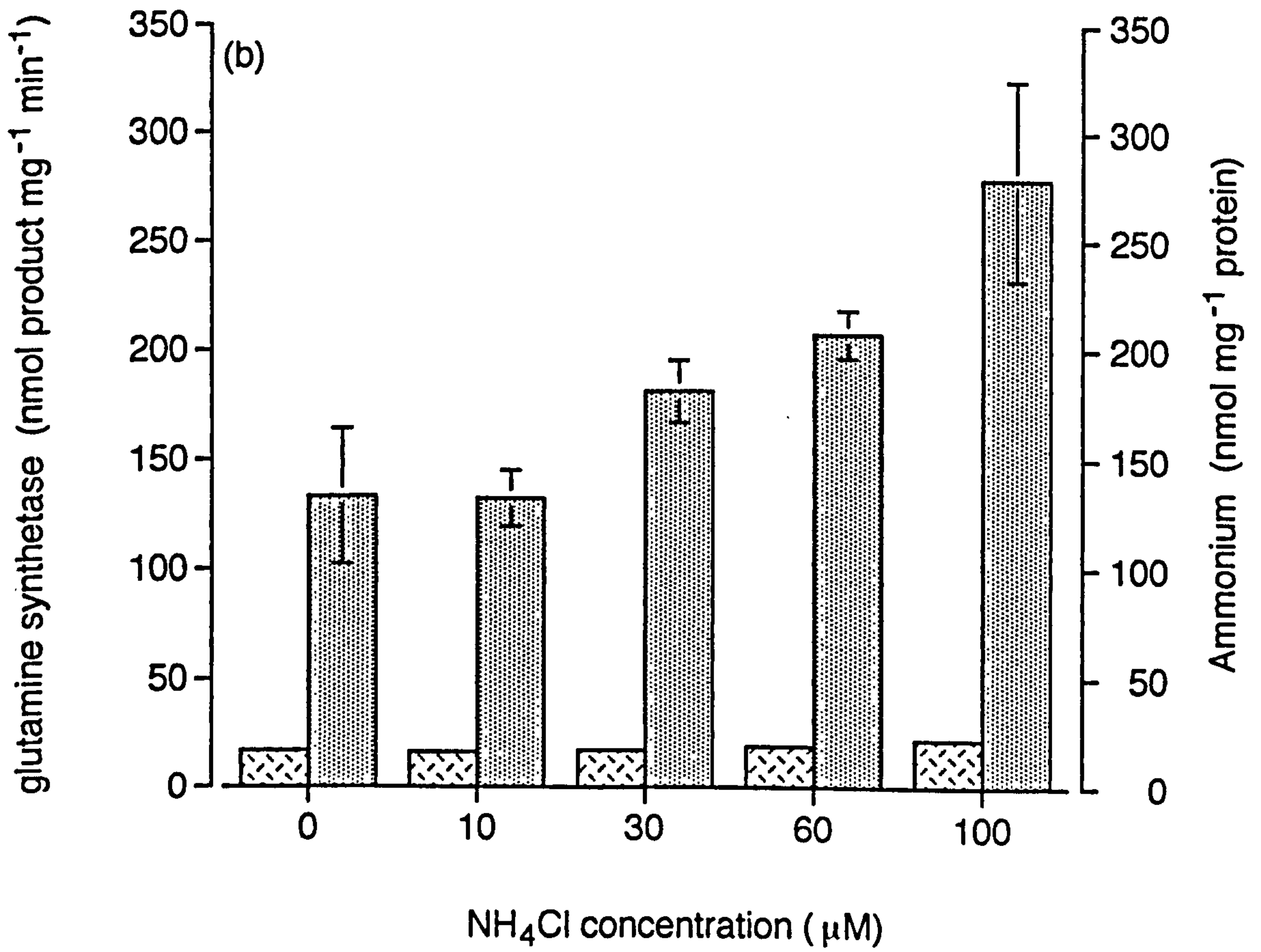
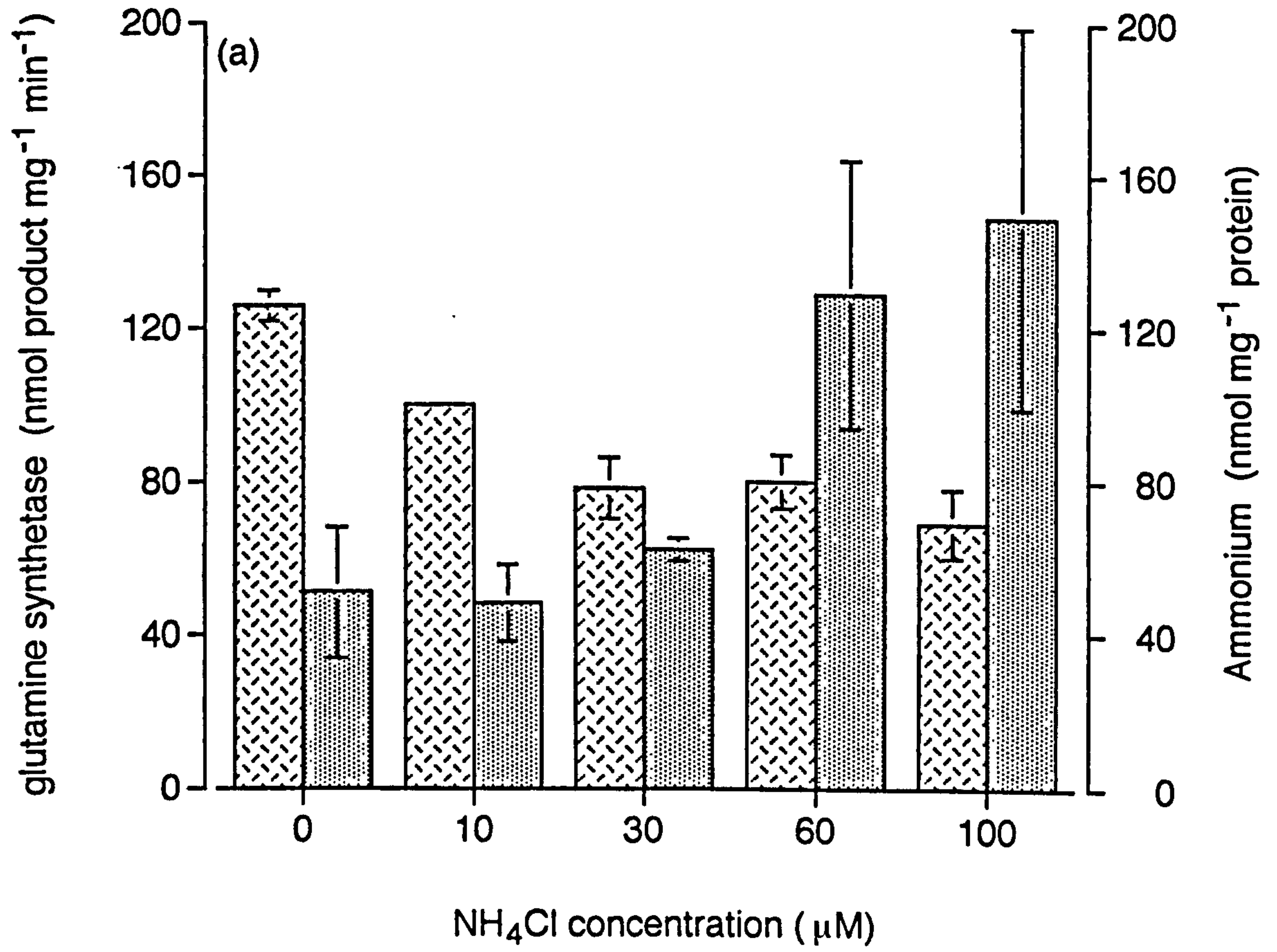
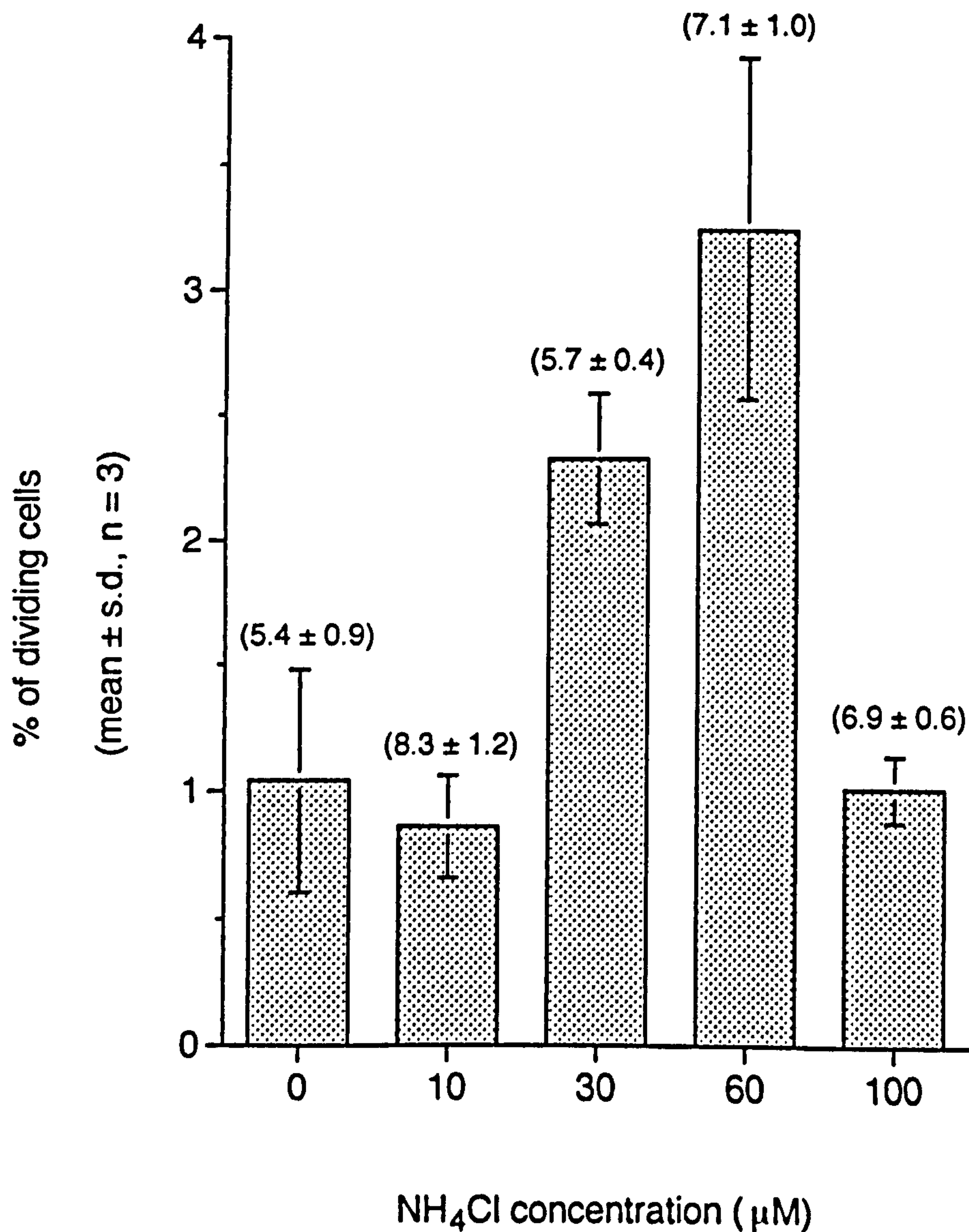


Figure 4.14 The impact of exogenous ammonium supplement on the percent of dividing algal cell in *A. pulchella*, incubated in SW-P buffer supplemented with a range of ammonium chloride concentration for 24 h. The algal density ($\times 10^6$) of each treatment is expressed as mean \pm s.d. in the parentheses.



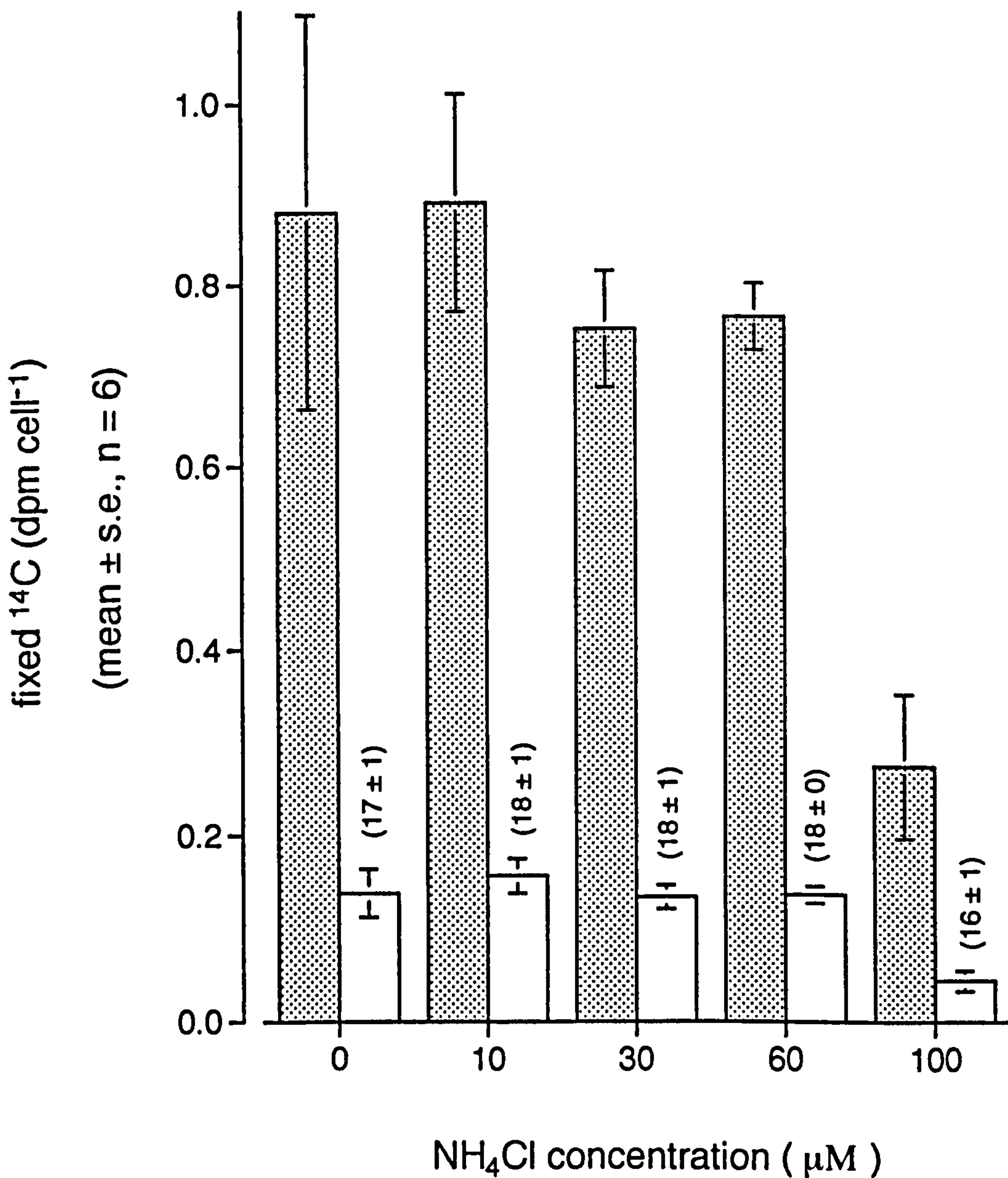
synthetase activity in the control animals was depressed by 31 % of that in the ammonium free medium, when the animals were incubated in 100 μM ammonium chloride supplemented medium; but the enzyme activities in the alga-depleted animals were constant at 20 nmol product mg^{-1} protein min^{-1} between the incubation in 0 and 100 μM ammonium supplemented medium.

The impact of ammonium supplement on the *Symbiodinium* cells *in vivo* was also examined. As Fig. 4.14 shows, the % of dividing algae in the animal cells increased by about two fold when the concentration of ammonium in the medium was elevated to 30 μM and 60 μM , but no difference was revealed between the incubations in 100 μM ammonium chloride and 0 or 10 μM ammonium chloride supplemented ASW.

However, the effect of ammonium supplement on the mean algal density (see the value in the parentheses) was not fully consistent with its effect on % dividing cell. This discrepancy might reflect the fact that long duration time at mitosis stage ($t_d = 11$ h as described in Wilkerson *et al.* 1983) would not result in an increase in algal density in the 24 h experiment.

The ammonium supplement exhibited no discernible impact on the photosynthetic carbon fixation by the algae, unless the concentration was increased to 100 μM (Fig. 4.15). When the animals were incubated in 100 μM ammonium supplemented medium, the photosynthetic carbon fixation by the algae were reduced by 67 % of that in ammonium free medium. The % of photosynthate release by *Symbiodinium* was not changed over the ammonium concentration tested.

Figure 4.15 The impact of exogenous ammonium supplement on the carbon fixation and photosynthate release by *Symbiodinium* in the *A. pulchella*, incubated in SW-P buffer supplemented with a range of ammonium chloride concentration for 24 h. The total ^{14}C fixed by the intact association (▨) and the fixed- ^{14}C detected in the animal fraction (□) are shown. The values in the parentheses are the % of photosynthate release by the algae and expressed as mean \pm s.d. with 6 replicates.



4.3 Discussion

4.3.1 Nitrogen metabolism in *Aiptasia pulchella*

By directly examining the indices for nitrogen metabolism, this study provides strong evidence that the *Symbiodinium* cause a significant impact on the overall nitrogen metabolism in the animal fraction of *A. pulchella* (Table 4.1). The animals bearing *Symbiodinium* exhibit higher level of net nitrogen assimilation than alga-depleted animals, i.e. maintaining higher concentrations of protein amino acids in the free amino acid pool and higher glutamine synthetase activity. When the symbiotic algal population is substantially reduced in the animal cells, the level declines by showing higher concentration of waste ammonium, uric acid and deamination enzyme activity, for example NADPH-glutamate dehydrogenase and alanine aminotransferase. These data are consistent with the conclusions of previous studies that zooxanthellate corals and symbiotic hydra have a lower amino acid catabolism rate than nonzooxanthellate corals and aposymbiotic hydra (Szmant *et al.* 1990; Rees 1986). The lower nitrogen catabolic activity in the control animals is proposed to be the result of the adaptation of zooxanthellate corals to a diet of low protein (less food intake) high carbohydrate and lipid (derived from translocated photosynthate) (Szmant *et al.* 1990), or preference in the hydra to respiration of the carbohydrate rather than of amino acids to conserve valuable nitrogen (Rees 1986; Rees & Ellard 1989).

4.3.2 Interactions between ammonium concentration and glutamine synthetase

When *A. pulchella* is incubated in darkness or its population of *Symbiodinium* substantially depleted, it exhibits increased ammonium concentration in its tissues (Table 4.1a and Fig. 4.8) and increased ammonium efflux into the medium (Wilkerson & Muscatine 1984). These

differences in ammonium content and efflux rates would, traditionally, be attributed to algal assimilation of animal-derived ammonium, and cited as evidence for nitrogen recycling by the algae (see section 4.1). This explanation is refuted by the demonstration here that the effects of darkness and depletion of the algal population on the ammonium concentration in the animal tissues are partially or completely reversed by exogenous carbon compounds (Table 4.2). The simplest explanation for the results of this study is that organic carbon, whether derived from algal photosynthate or exogenous sources, promotes the assimilation of ammonium in animal tissues and/or depresses its production.

This study has also obtained strong evidence for the stimulation of ammonium assimilation in the animal by organic carbon compounds, principally via glutamine synthetase, an enzyme which catalyses the ATP-dependent amination of glutamate to produce glutamine. The treatments that resulted in elevated ammonium concentration (darkness, depletion of the algal population) also depressed glutamine synthetase activity (Table 4.1; Fig. 4.8; Fig. 4.10); and either by recovering algal population, or resuming photosynthesis of the algae, or the organic carbon supplements, the glutamine synthetase activity was enhanced (Fig. 4.4; Fig. 4.10; Table 4.2). Further, the finding that the regressions of glutamine synthetase activity and ammonium concentration on algal density had closely similar slopes of opposite sign suggests that glutamine synthetase may play an important role in mediating the impact of algal density on the ammonium content of the animal.

The reduction in glutamine synthetase activity in Cnidaria deprived of their algae has been reported previously only in the freshwater hydra-*Chlorella* symbiosis (Rees 1986), and this study serve to generalize those

findings from freshwater to marine systems and across two classes (Hydrozoa to Anthozoa). The impact of algae on the glutamine synthetase activity in hydra was considered exclusively in terms of its proposed function to reduce the availability of ammonium to the algal cells. As described above, data from this study on *A. pulchella* indicate that glutamine synthetase is modulated by alga-derived photosynthate, and not by the presence of algae per se. The implication is that the translocation of algal photosynthate would tend, via increased glutamine synthetase activity, to depress the availability of ammonium to the algal cells. If the ammonium were the principal, or sole, nitrogen source utilized by the algae, then nitrogen limitation could be a direct consequence of the translocation of photosynthate to the animal tissues. The data shown in the Fig. 4.14 that exogenous ammonium supplement (30-60 μM) elevated the % of dividing algal cell are also consistent with this suggestion.

However, the relative importance of ammonium and organic nitrogen compounds to the nitrogen nutrition of the algae in symbiosis is uncertain. *Symbiodinium* can certainly assimilate both ammonium (e.g. Domotor & D'Elia 1984; Wilkerson & Muscatine 1984; McAuley & Smith 1995) and amino acids (e.g. Carroll & Blanquet 1984; Bester *et al.* 1997) efficiently from low external concentrations. The data in Fig. 4.11 provide circumstantial evidence that the algal cells in symbiosis utilize animal-derived ammonium. The significantly lower intercept for the regression of ammonium concentration on glutamine synthetase activity in control animals than in alga-depleted animals suggests that the former assimilate ammonium by a route, additional to glutamine synthetase, that is absent from the latter. This may be the algal cells, which are known to be capable of sustained ammonium assimilation, even in darkness (e.g. McAuley & Smith 1995). Moreover, the capacity of the animal hosts to restrict

ammonium availability, via glutamine synthetase activity, from symbiotic algae is not unlimited. When the animals were fed daily or incubated in the ammonium supplemented seawater, the ammonium concentration of the animal fraction was increased, but the glutamine synthetase activity was not changed or even depressed (Fig. 4.8; Fig. 4.13). The depressed glutamine synthetase activity is unlikely to have resulted from a reduction in supply of translocated photosynthate, because the total ^{14}C fixation rate and % of photosynthate release were not affected by the ammonium addition when the supplemented concentration is below $60\ \mu\text{M}$ (Fig. 4.15). Therefore, the *Symbiodinium* in the well-fed animals or the animals residing ammonium-eutrophicated environment may not be in a nitrogen-limited situation.

4.3.3 Free amino acid pool of the animal tissues

In development of the nitrogen conservation hypothesis, Rees and Ellard (1989) emphasized the importance of algal photosynthate as an alternative respiratory substrate to the amino acid pools of the animal. This study has also provided several lines of evidence to support this interpretation. The deprivation of symbiotic algae from *A. pulchella* resulted in a dramatic change in the composition of free amino acid pool of the animal fraction, in which the levels of 9 from 18 protein amino acids were found to be depressed by the treatment, but the level of taurine was elevated (Fig. 4.2).

Moreover, consistent with the hypothesis that algal photosynthate may protect the amino acid pools in the animal tissues of *A. pulchella*, the content of protein amino acids were elevated by receipt of photosynthate release in the control animals or exogenous carbon compounds in alga-depleted animals (Table 4.3), which contribute 42% and 50% of the total free amino acid pool, respectively. But protein amino acids just contribute

20% of the total in alga-depleted animals incubated in routine culture. The content of protein amino acids in the free amino acid pool of the control animals was found to be depressed when incubating with α -ketoglutarate supplement, but the reason is not clear. Perhaps, the exogenous carbon supplement plus the photosynthate received from symbiotic algae result in a remarkably higher C/N ratio, which promote the animal carrying on more gluconeogenesis rather than amino acid synthesis. Consistent with this interpretation, the percentage of nitrogen as amino acid displayed a reverse relationship with C/N ratio in the shoots of *Juncus acutiflorus* (Smolders *et al.* 1997).

Another expectation of the nitrogen conservation hypothesis is that darkness and depletion of the algal population would enhance animal transaminase activity (see section 4.1). In this study, only the alanine aminotransferase, but not aspartate aminotransferase, fits the rule (Table 4.1). This result might reflect the fact that alanine, compared to aspartate, is more dominant in the free amino acid pool of *A. pulchella* (Fig. 4.2); and the level of alanine was depressed from 7 % in the control animal to 2 % in the alga-depleted animals, compared to 1 % of aspartate in the two treatments.

4.3.4 Summary

In conclusion, this study has revealed the crucial importance of organic carbon to the nitrogen metabolism of the animal tissues in the *Aiptasia pulchella-Symbiodinium* symbiosis. Whether derived from algal photosynthate or exogenous sources, this organic carbon may serve to: first, promote animal assimilation of ammonium, via glutamine synthetase; and second, reduce the rates of amino acid degradation (and ammonium production) by providing an alternative respiratory substrate.

The impact of alga-derived photosynthate on the rates of ammonium production and consumption by the animal tissues confounds the interpretation of experimental designs traditionally used to explore the putative role of the algae in nitrogen recycling.

Chapter 5

"Essential" Amino Acid Synthesis in *Aiptasia pulchella*

5.1 Introduction

Animals generally lack the metabolic pathways for the synthesis *de novo* of 9 amino acids that contribute to protein: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine. These 9 amino acids, and any additional amino acids that are synthesized at rates lower than the animal's metabolic needs, are called essential amino acids (Morris 1991). The other amino acids that contribute to protein are termed non-essential or dispensable (Harper 1974).

Basically, animals rely on the supply of these essential amino acids in their diet. Some invertebrates bearing endosymbiotic micro-algae (e.g. corals and sea anemones) are further believed to obtain the essential amino acids from the provision of nitrogen-recycled products by algal symbionts (e.g. Wilkerson & Muscatine 1984; Falkowski *et al.* 1993). This nutritional basis for the invertebrate-alga symbiosis is partly generated from the concept described above that the animal hosts also cannot synthesize the 9 essential amino acids *de novo*.

The inability of lower animals to synthesize all protein amino acids is still in question. Various invertebrate species have been reported, especially in the early literature, to synthesize several of the 9 essential amino acids listed above. Most of these studies are, however, difficult to interpret because they do not exclude the possibility of essential amino acid synthesis by contaminating or symbiotic micro-organisms (e.g. Dadd 1985;

Morris 1991), and a variety of animals are now known to derive nutritionally-significant amounts of essential amino acids from associated micro-organisms (Douglas 1994 & 1998).

FitzGerald and Szmant (1997) have recently provided persuasive evidence that five species of scleractinian corals (three species bearing *Symbiodinium* algae; *Montastraea faveolata*, *Acropora cervicornis*, and *Porites divaricata*; and two species without symbiotic algae; *Tubastrea coccinea* and *Astrangia poculata*), can synthesize 8 of the 9 essential amino acids that animals, generally, cannot synthesize (all but threonine). By carefully cleaning the specimens with sterilized seawater, they minimized amino acid synthesis by contaminating bacteria. Noting that the Cnidaria is probably the most ancient phylum of animals, FitzGerald and Szmant (1997) suggest that cnidarians may have diverged from other animals before the "essential" amino acid biosynthetic pathways were lost. If the capability of synthesizing so-called essential amino acids is widely distributed among the Cnidaria, then the nutritional value of nitrogen recycling to the animal host in Cnidaria-alga symbiotic associations would have been over-estimated.

This study explores amino acid biosynthesis by a sea anemone, *Aiptasia pulchella*, a member of a different order (Actiniaria), of the same class (Anthozoa) of Cnidaria as the Scleractinia investigated by FitzGerald and Szmant (1997). This study not only extends the analysis of amino acid biosynthesis in Cnidaria, but also improves the incubation condition to further confirm that the amino acids were not derived from contaminating bacteria by using aseptic specimens derived from antibiotic pre-treatment.

Table 5.1 Incorporation of a mixture of ^{14}C labelled glucose, glutamate and aspartate into the animal fraction of *A. pulchella*.

The aseptic animals were incubated in ASW supplemented with ^{14}C labelled precursors for 2 h, and then chased in isotope-free ASW for 2 days. The ^{14}C incorporation into the animal fraction was $1.9 \pm 0.1 \%$ and $0.7 \pm 0.2 \%$ of total application for control and alga-depleted animals, respectively. Results are mean \pm s.d. with three replicates.

	total	free amino acid pool	protein
	(x 1,000 dpm) mg^{-1} animal protein		
control	210 ± 59	47 ± 11	35 ± 5
alga-depleted	83 ± 27	11 ± 2	15 ± 4
	$t_4 = 3.42$	$t_4 = 5.35$	$t_4 = 5.30$
	$p < 0.05$	$p < 0.01$	$p < 0.01$

5.2 Results

Aseptic Aiptasia pulchella incorporated radioactivity from a mixture of ^{14}C -labelled glucose, aspartate, and glutamate, and $1.9 \pm 0.1\%$ and $0.7 \pm 0.2\%$ (mean \pm s.d., $n = 3$) of total ^{14}C application was recovered from the animal fraction of control and alga-depleted animals, respectively. As Table 5.1 shows, the incorporation of ^{14}C into the total animal fraction, free amino acid pool, and protein are all significantly higher in control animals than alga-depleted animals.

5.2.1. ^{14}C labelled amino acids in the soluble fraction of trichloroacetic acid soluble extract

The free amino acids in trichloroacetic acid extract from both control and alga-depleted animals were isolated by ion-exchange column, and analyzed by HPLC and TLC. As shown in Fig. 5.1, the control and alga-depleted animals shared in common with seven ^{14}C labelled amino acid peaks, including aspartate (retention time; RT: 4.8 min), glutamate (RT: 7.8 min), asparagine (RT: 11.5 min), histidine/serine (RT: 12.5 min), threonine (RT: 17.5 min), taurine (RT: 19.5 min), alanine/tyrosine (RT: 20.5 min) by HPLC analysis. In the samples derived from control animals, the radioactivity was also detected in the amino acid peaks corresponding to isoleucine (RT: 29.5 min), and leucine (RT: 30.0 min). The HPLC analysis also revealed two *o*-phthaldialdehyde-positive peaks (RT: 2.0 min and 6.5 min) and two *o*-phthaldialdehyde-negative peaks (RT: 4.0 min and 16.0 min) in the sample from both treatments, which did not correspond to any amino acid standards (probably the primary amines and some trichloroacetic acid soluble peptides). TLC analysis confirmed some ^{14}C -labelled amino acids detected in the HPLC analysis, e.g. alanine, asparagine, glutamate, serine, threonine, and tyrosine, but no taurine was detected by TLC (Fig. 5.2). Further identification of histidine and cysteine, which cannot be detected

Figure 5.1 Example of HPLC analysis of *o*-phthaldialdehyde derivatized ¹⁴C-labelled amino acids and the radioactivity in HPLC eluate, prepared from the trichloroacetic acid extract of animal fraction of *A. pulchella*. HPLC profile (a); radioactivity in each 30 sec fraction of the sample derived from control animals (b) and alga-depleted animals (c). Non-standard abbreviations used: tau, taurine; orn, ornithine.

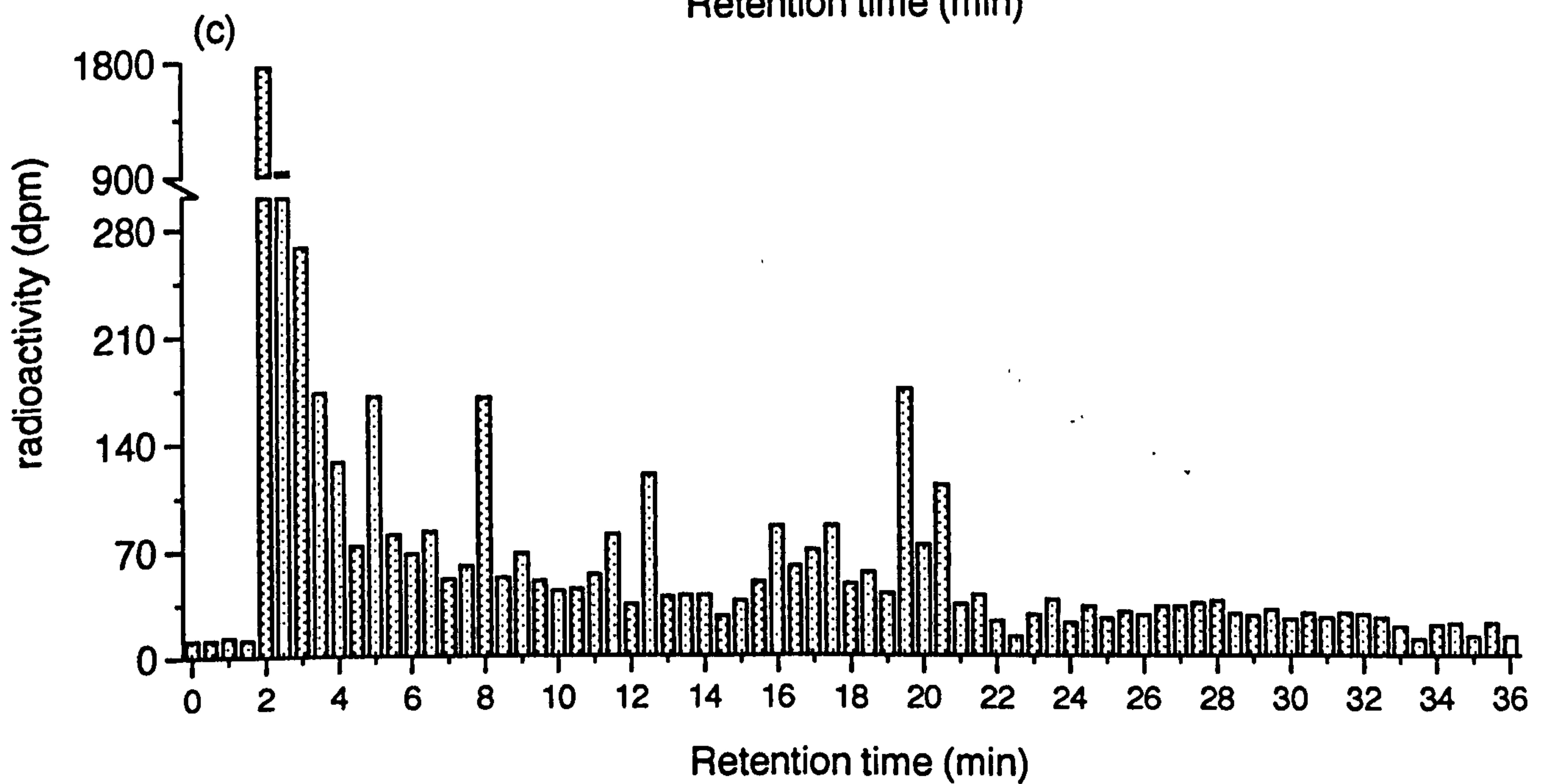
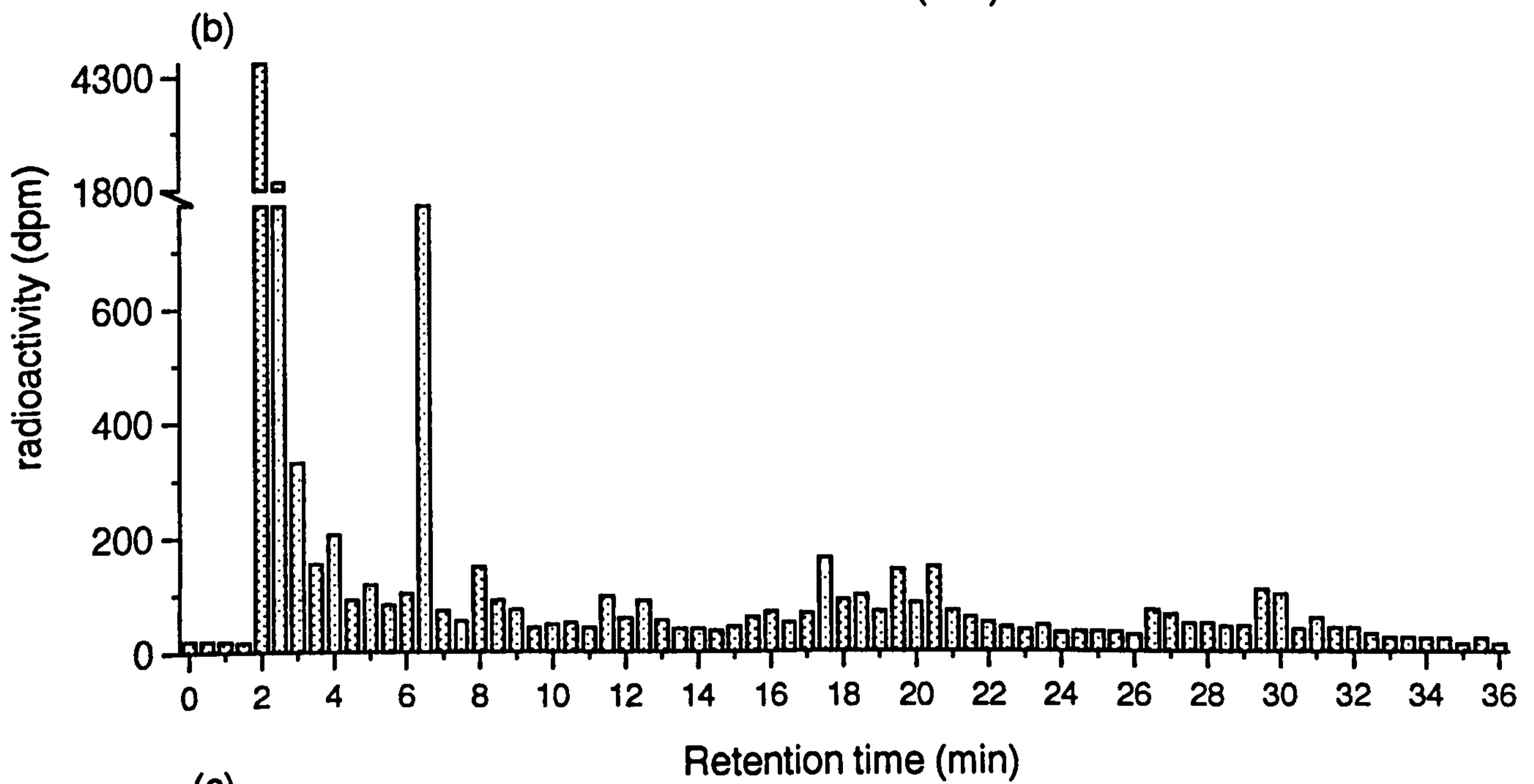
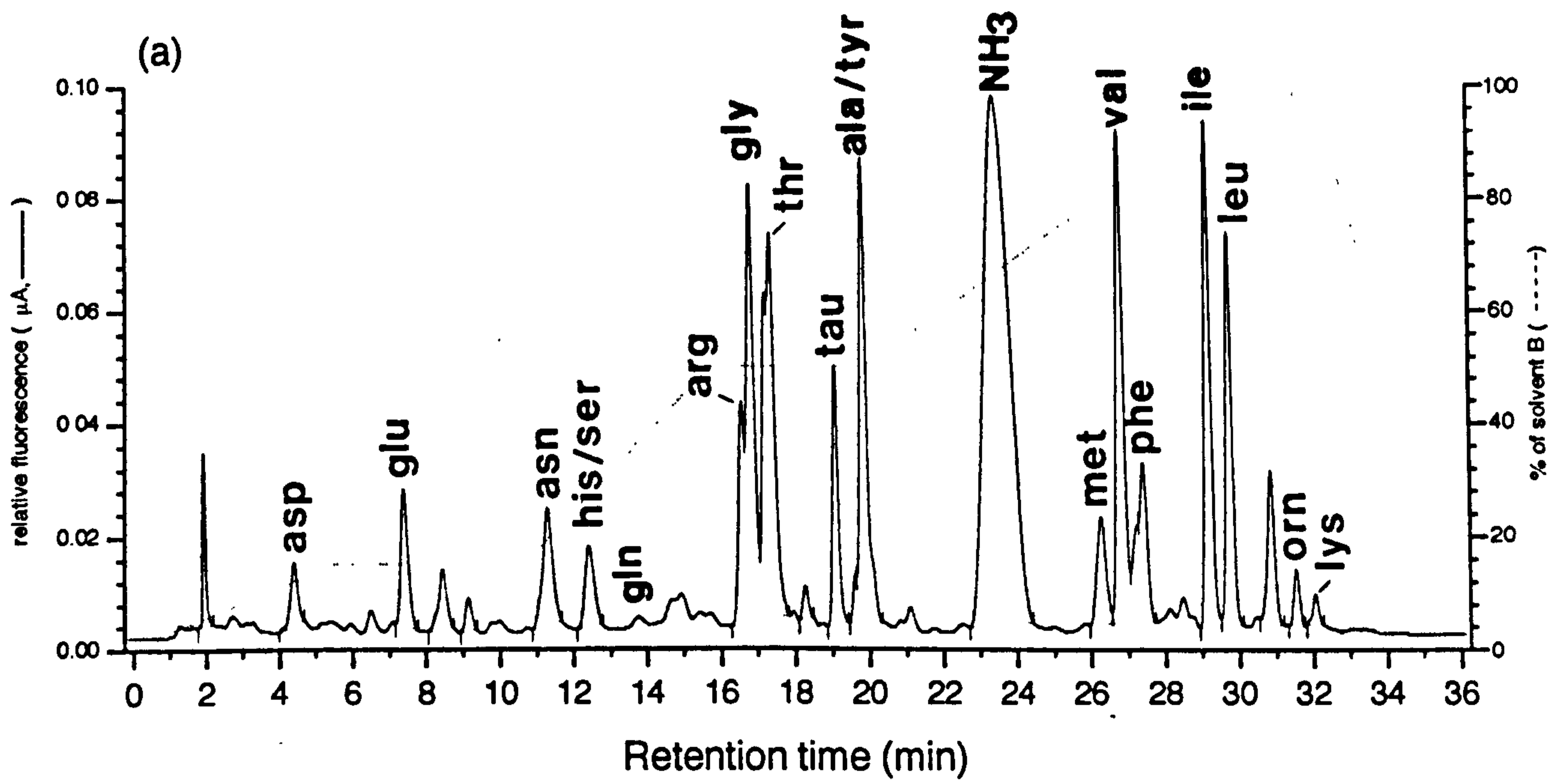
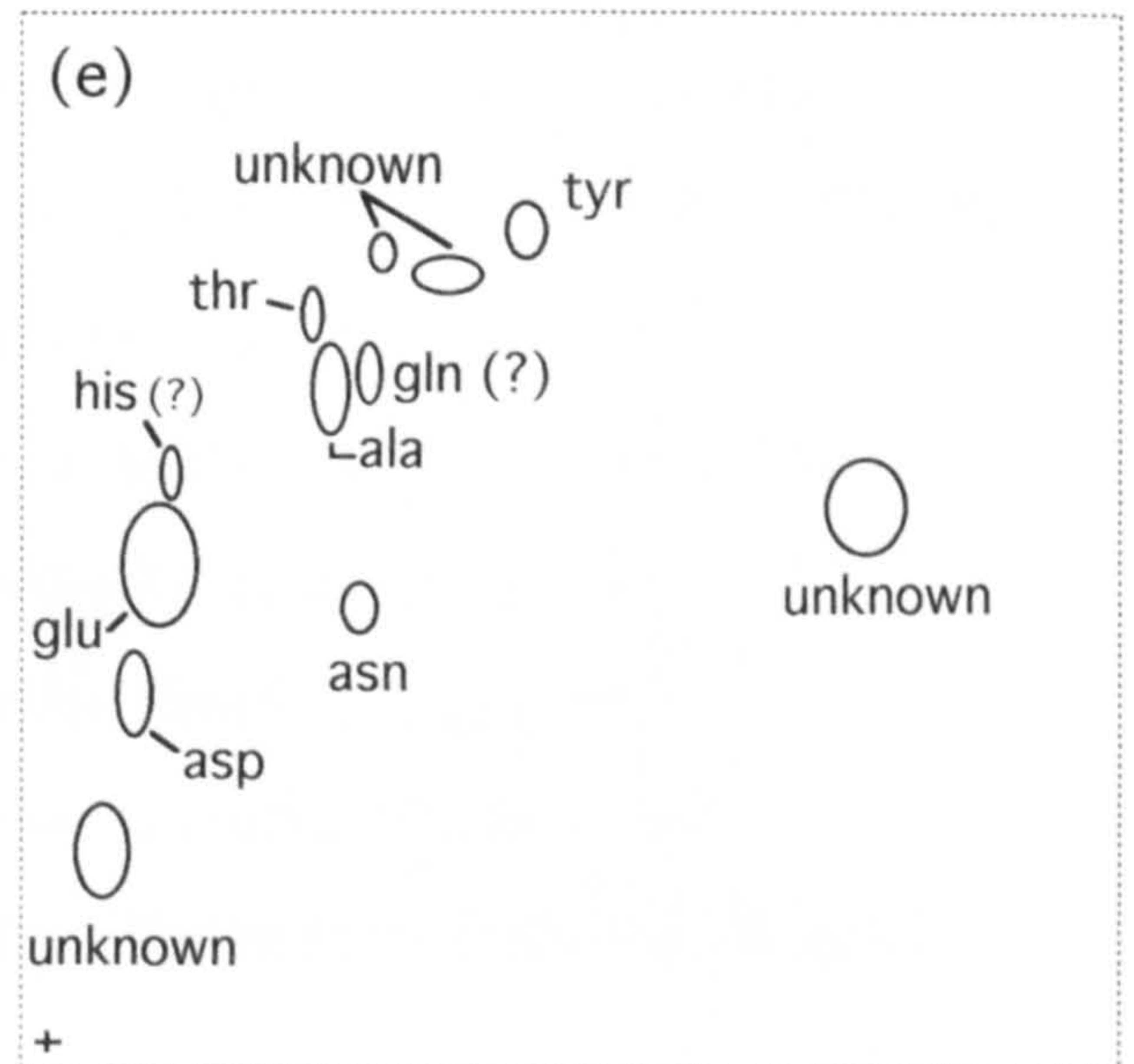
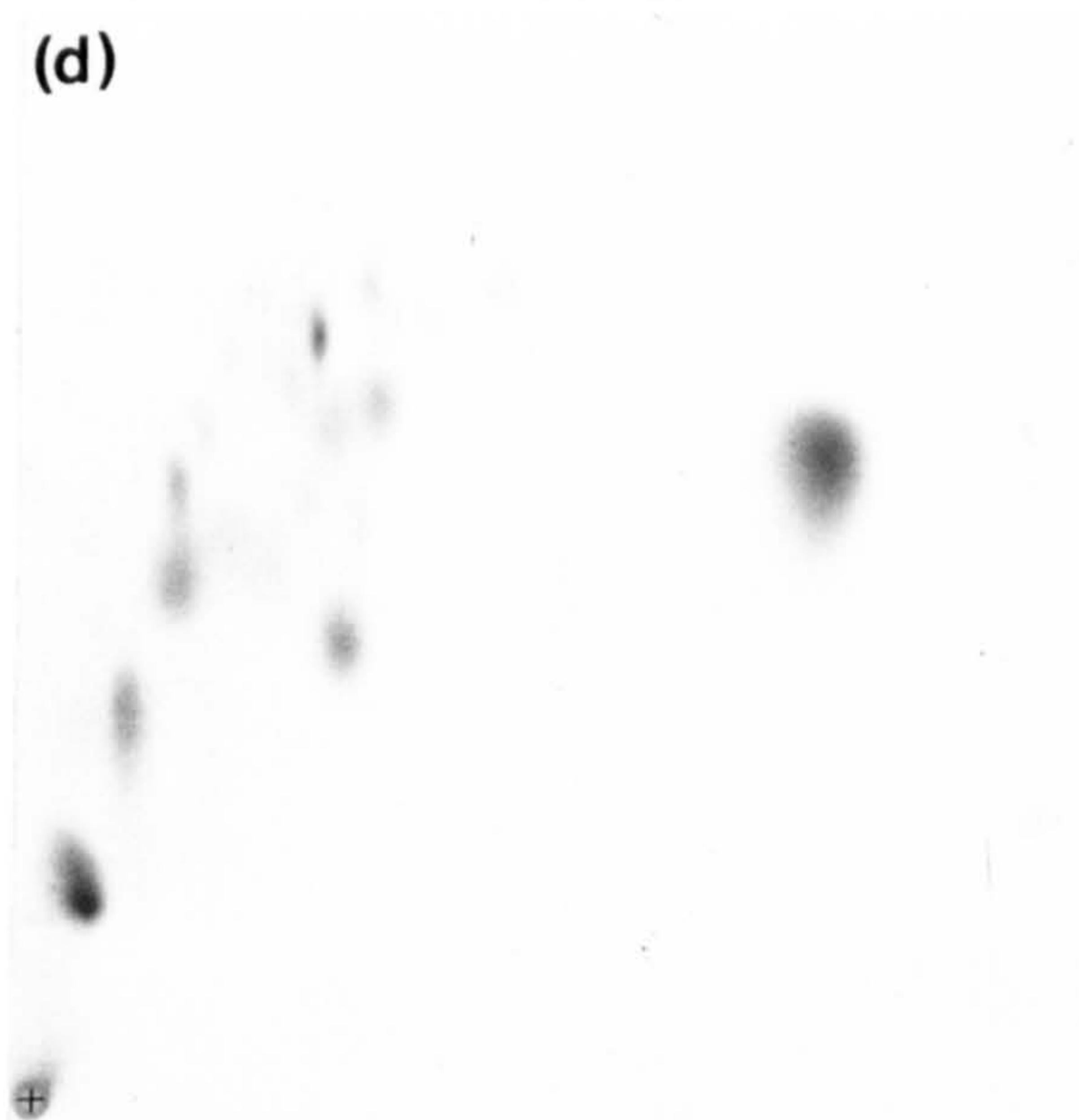
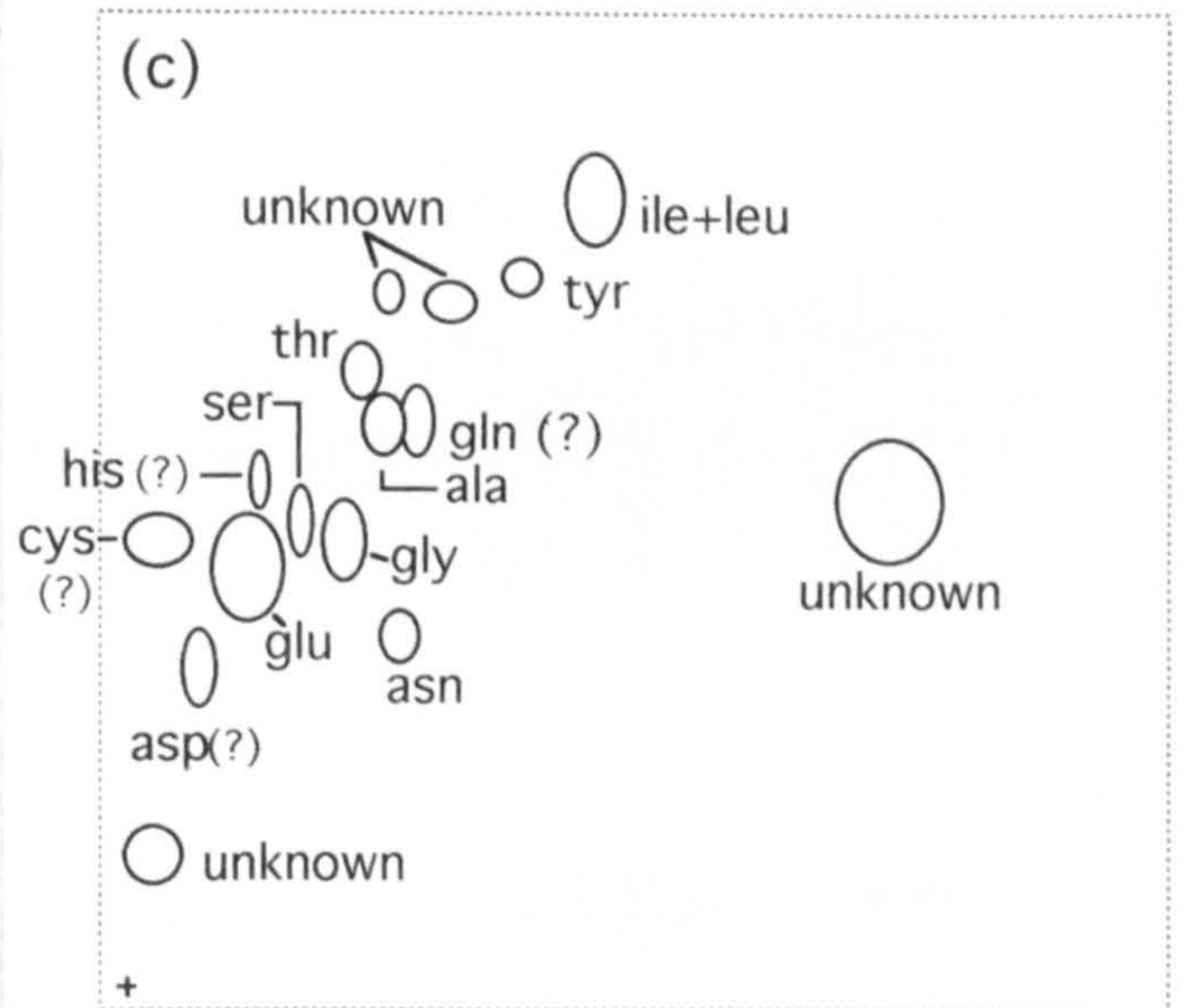
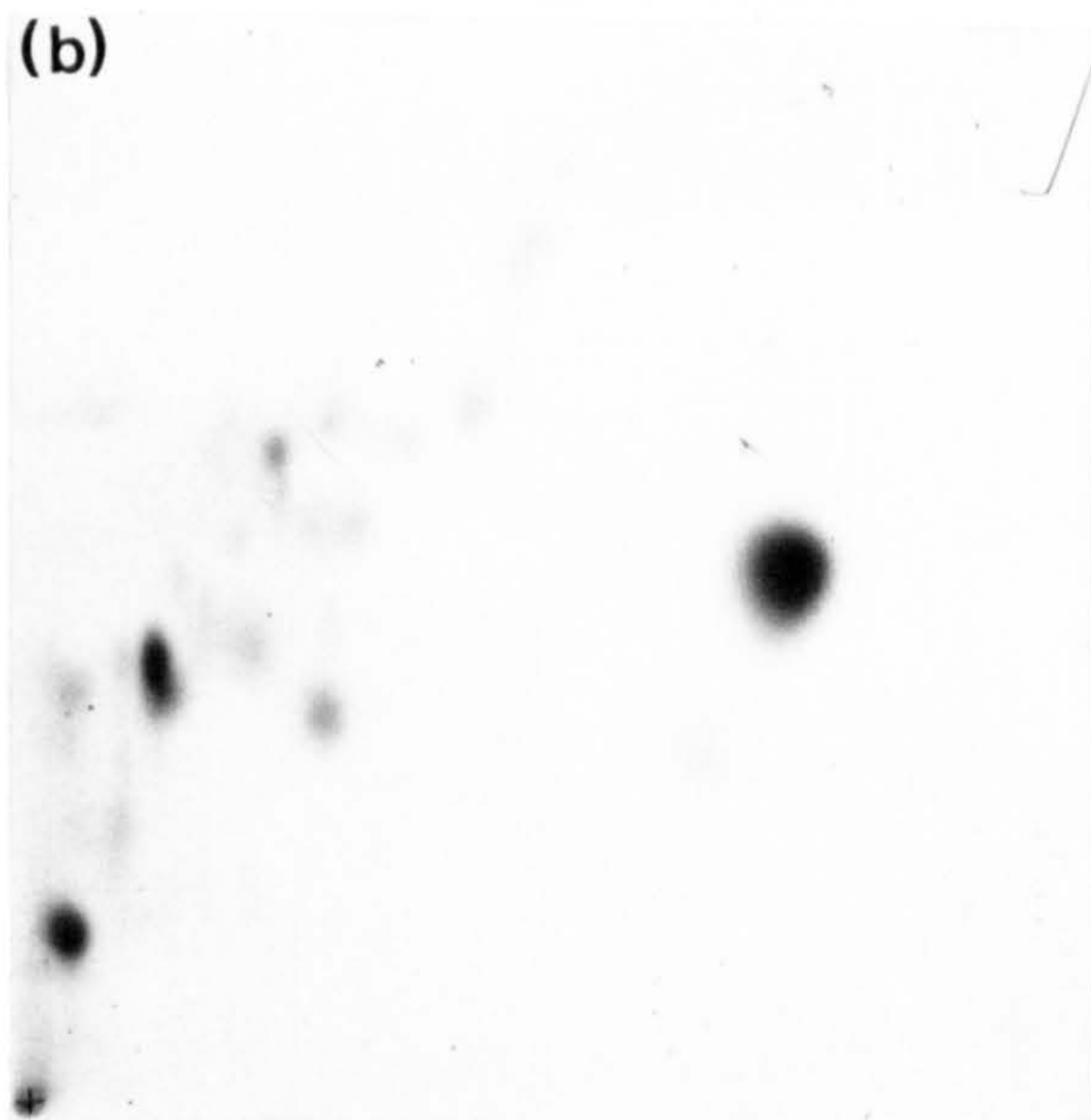
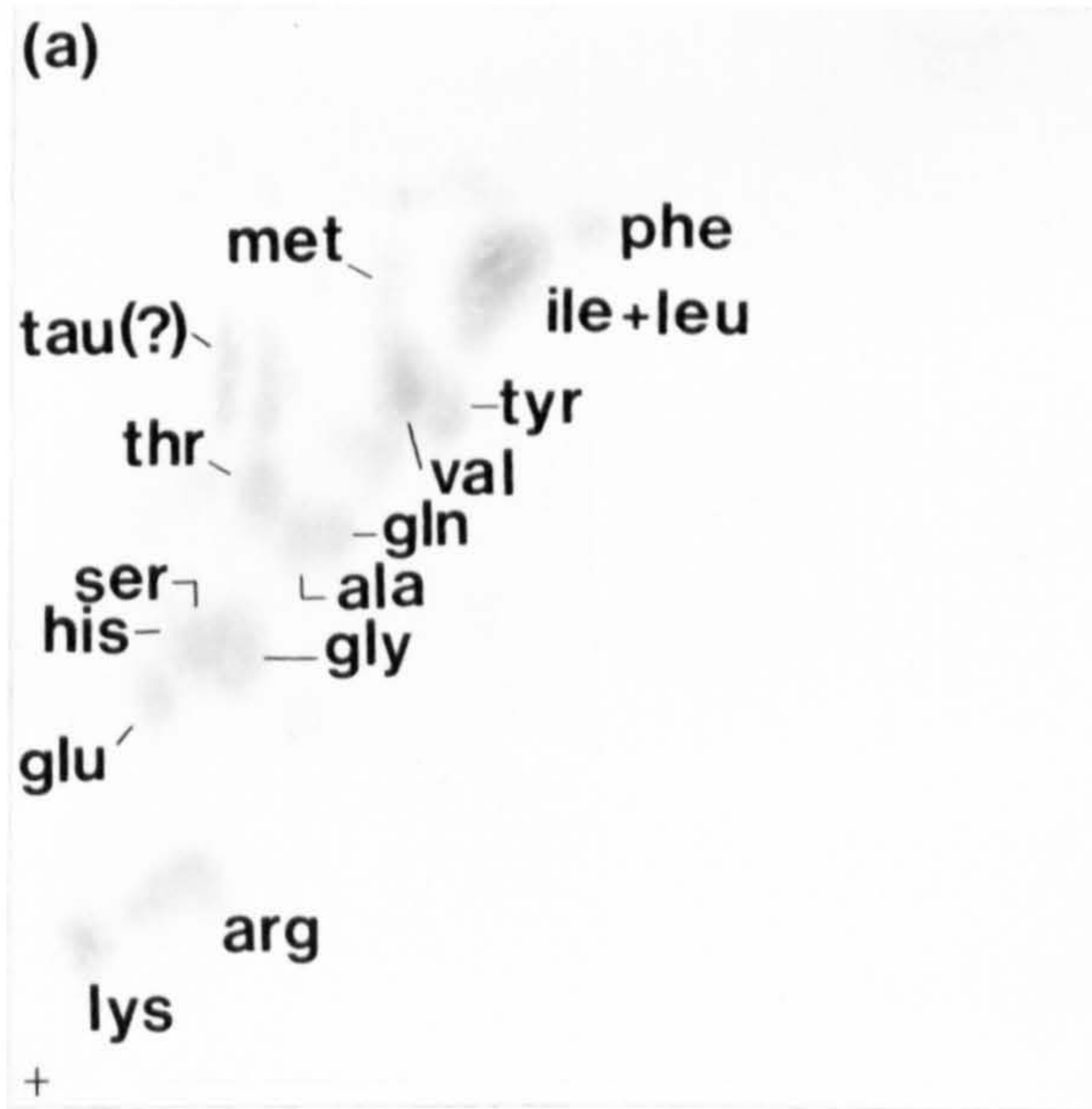


Figure 5.2 Example of TLC analysis of ^{14}C labelled low-molecular-weight components in the trichloroacetic acid extract derived from the animal fraction of *A. pulchella*. Ninhydrin reaction (a); autoradiography of ^{14}C -labelled compounds and the identification profile derived from control animals (b, c) and alga-depleted animals (d, e). Non-standard abbreviations used: tau, taurine.



by the HPLC technique used, by TLC method was not confirmed. The difficulty of the identification of amino acids and the inconsistent result between TLC and HPLC analysis (e.g. the incorporation into taurine) partly resulted from interference of contaminants and partly from the low radioactivity of these amino acids. The radioactivity of these non-identified spots was $50 \pm 6 \%$ and $55 \pm 11 \%$ (mean \pm s.d., $n = 3$) of total radioactivity in the free amino acid fraction for control and alga-depleted animals, respectively. In summary, only 6-7 amino acids in the free amino acid pool of animal fraction were confirmed to be synthesized by the animals using the method described in section 2.7. They are glutamate, asparagine, alanine, tyrosine, threonine, and isoleucine (and/or leucine) for control animals; and aspartate, asparagine, glutamate, alanine, tyrosine, and threonine for alga-depleted animals. Because of the difficulty on recovering ^{14}C labelled amino acids from the TLC plate with confidence and the low radioactivity of most amino acids detected, the quantification for radioactivity of each amino acid in the free amino acid pool was not explored further.

5.2.2 ^{14}C labelled amino acids in the insoluble fraction of trichloroacetic acid extract

Between 10-30% of the radioactivity incorporated into the animal fraction was recovered from trichloroacetic acid precipitate (primarily as the animal proteins). For the control animals, radioactivity was detected in all of 13 amino acid peaks by the HPLC analysis, including the "essential" amino acids isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine (Fig. 5.3b). TLC analysis, as shown in Fig. 5.4b, confirmed these identifications and further demonstrated ^{14}C incorporation into both amino acids of pairs histidine/serine and alanine/tyrosine, and into cysteine and proline which cannot be detected

Figure 5.3 Example of HPLC analysis of *o*-phthaldialdehyde derivatized ¹⁴C-labelled amino acids and the radioactivity in HPLC eluate, prepared from the protein hydrolysate of animal fraction of *A. pulchella*. HPLC profile (a); radioactivity in each 30 sec fraction of the sample derived control animals (b) and alga-depleted animals (c). Asx refers to aspartate + asparagine; and glx to glutamate + glutamine (acid hydrolysis converts glutamine to glutamate and asparagine to aspartate).

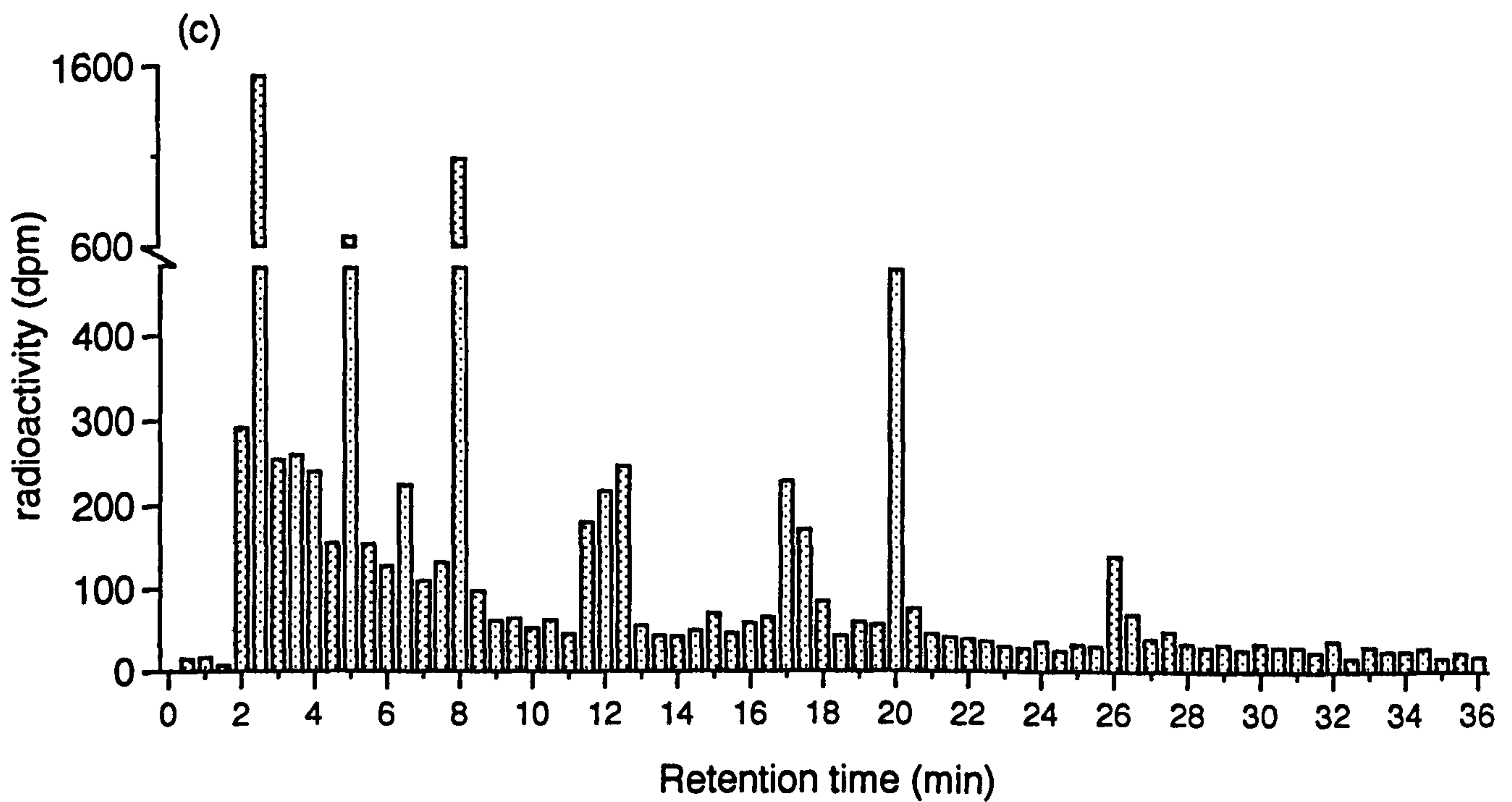
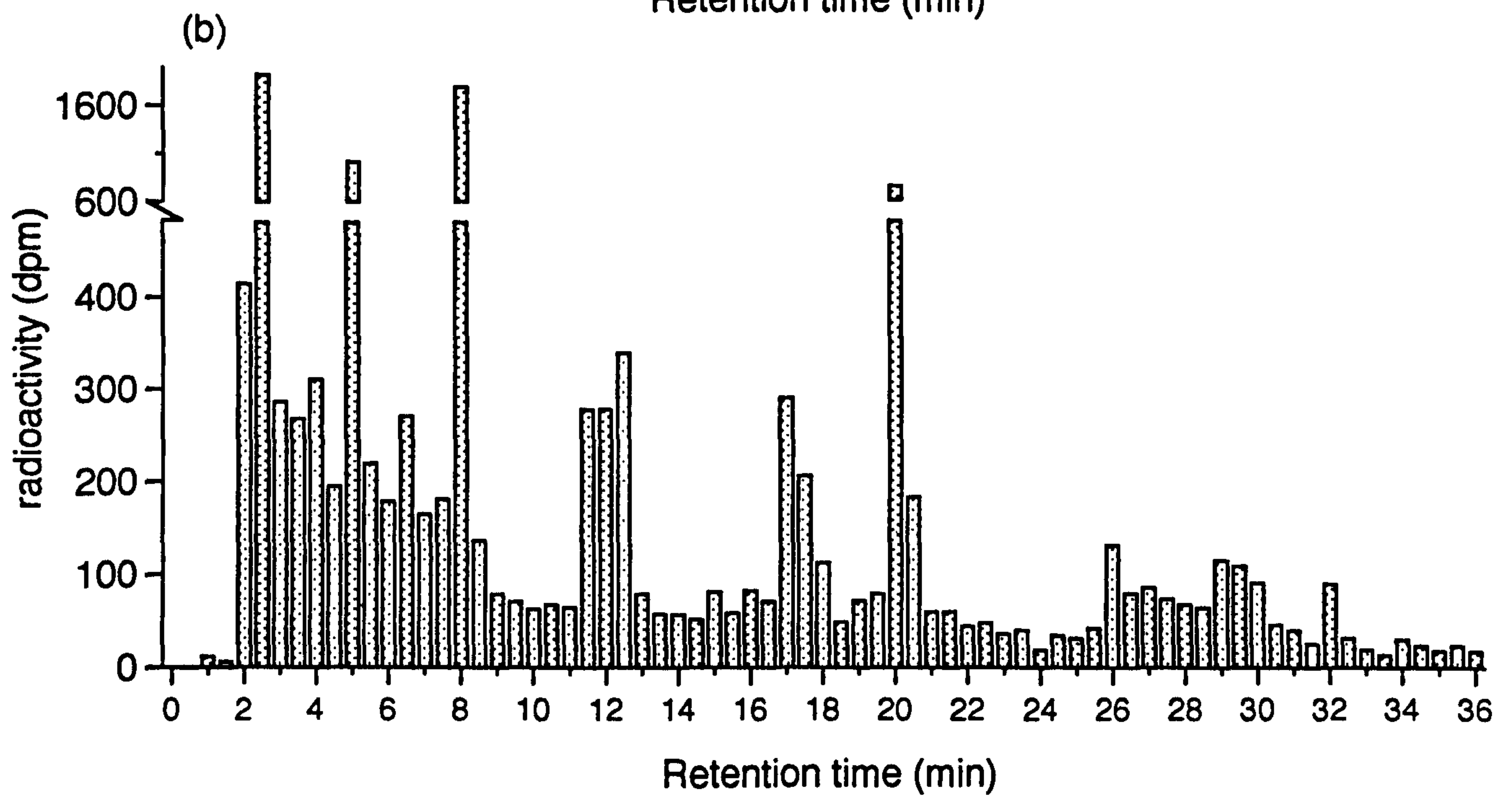
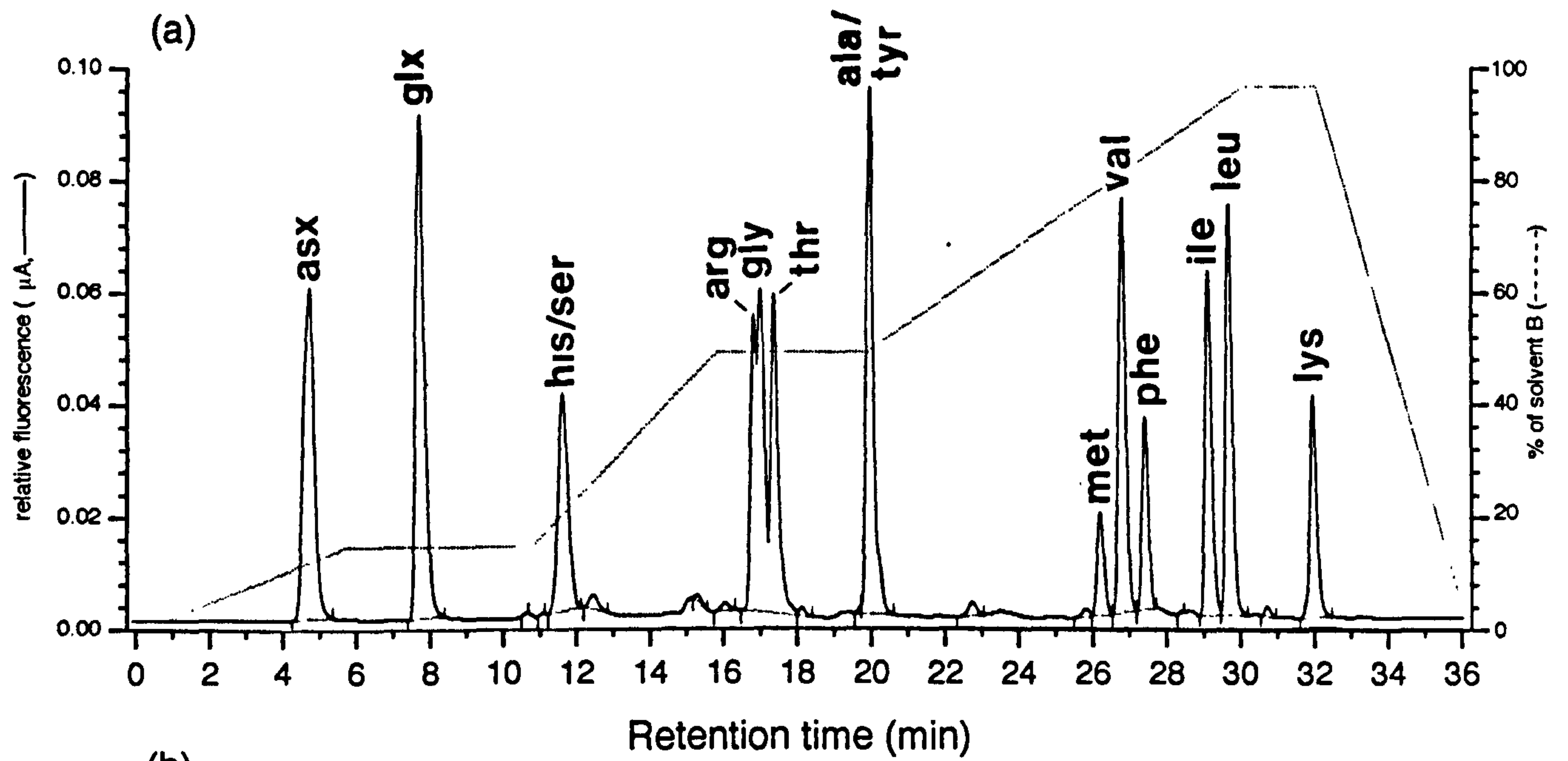


Figure 5.4 Example of TLC analysis of ^{14}C -labelled amino acids in protein hydrolysate derived from animal fraction of *A. pulchella*. Ninhydrin reaction (a); autoradiography of ^{14}C -labelled amino acids and the identification profile derived from control animals (b, c) and alga-depleted animals (d, e). Asx and glx refer to the same amino acids as described in the legend of Fig. 5.3. The "unknown" label represents the ^{14}C -labelled component with no ninhydrin reaction.

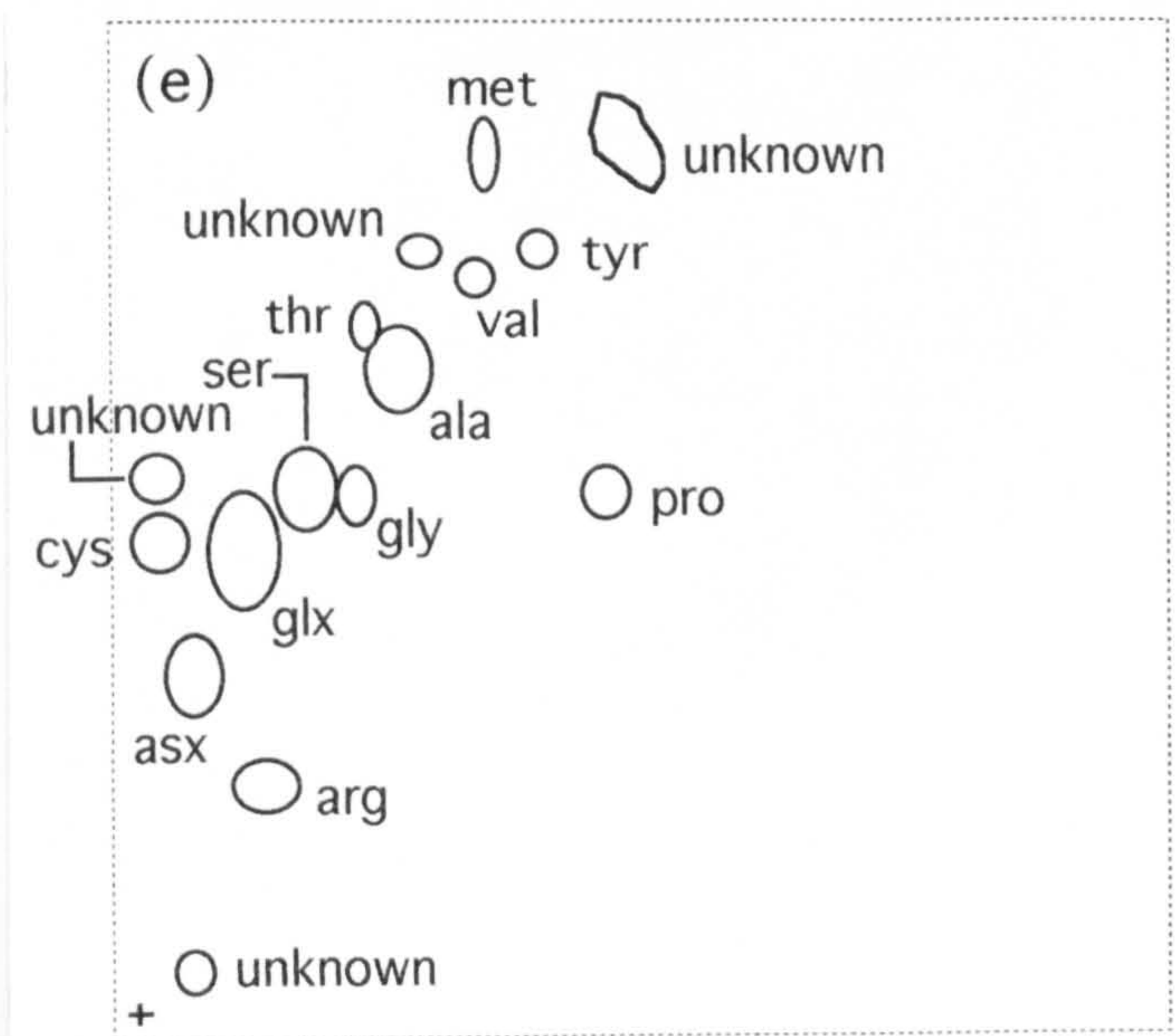
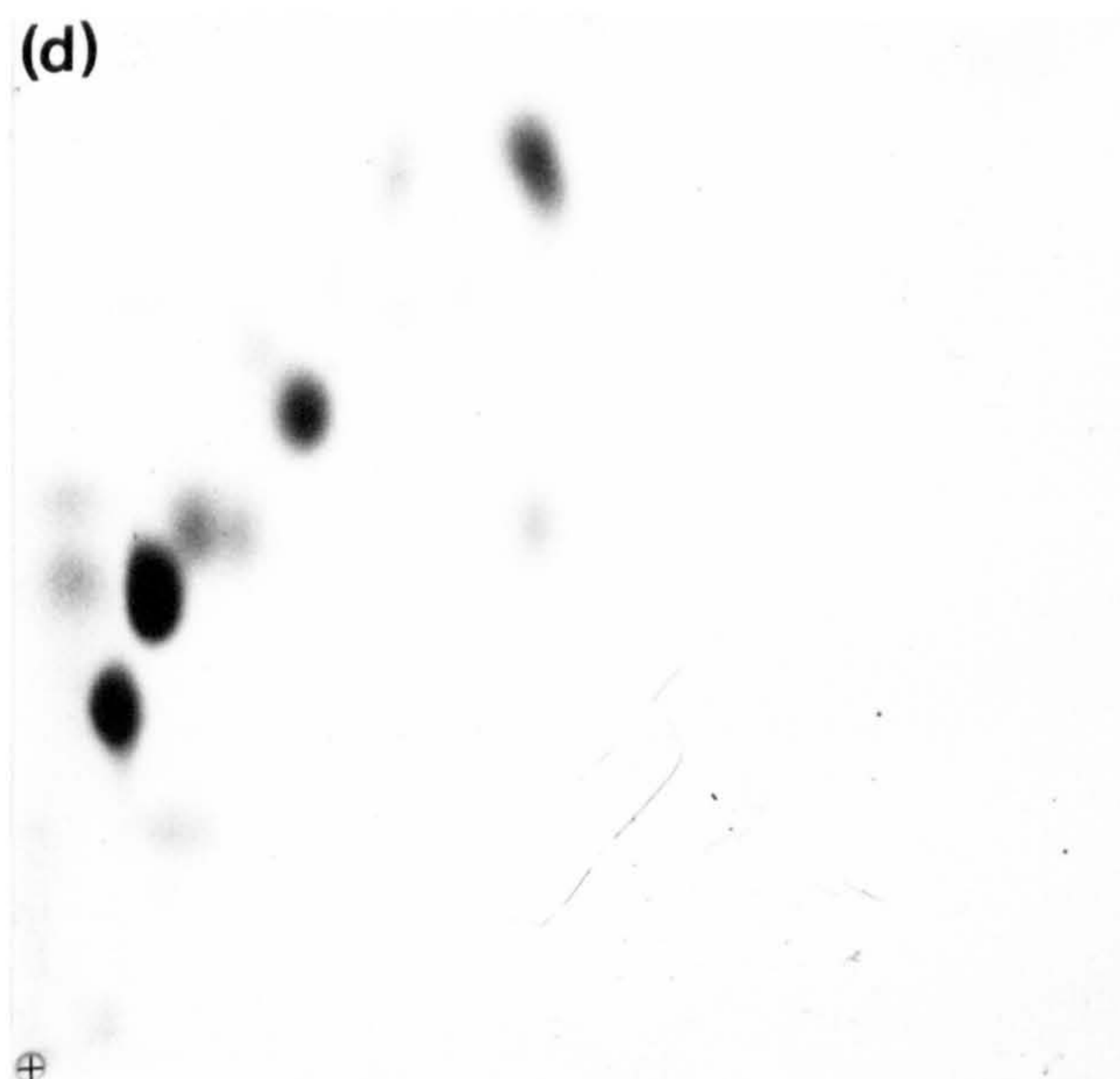
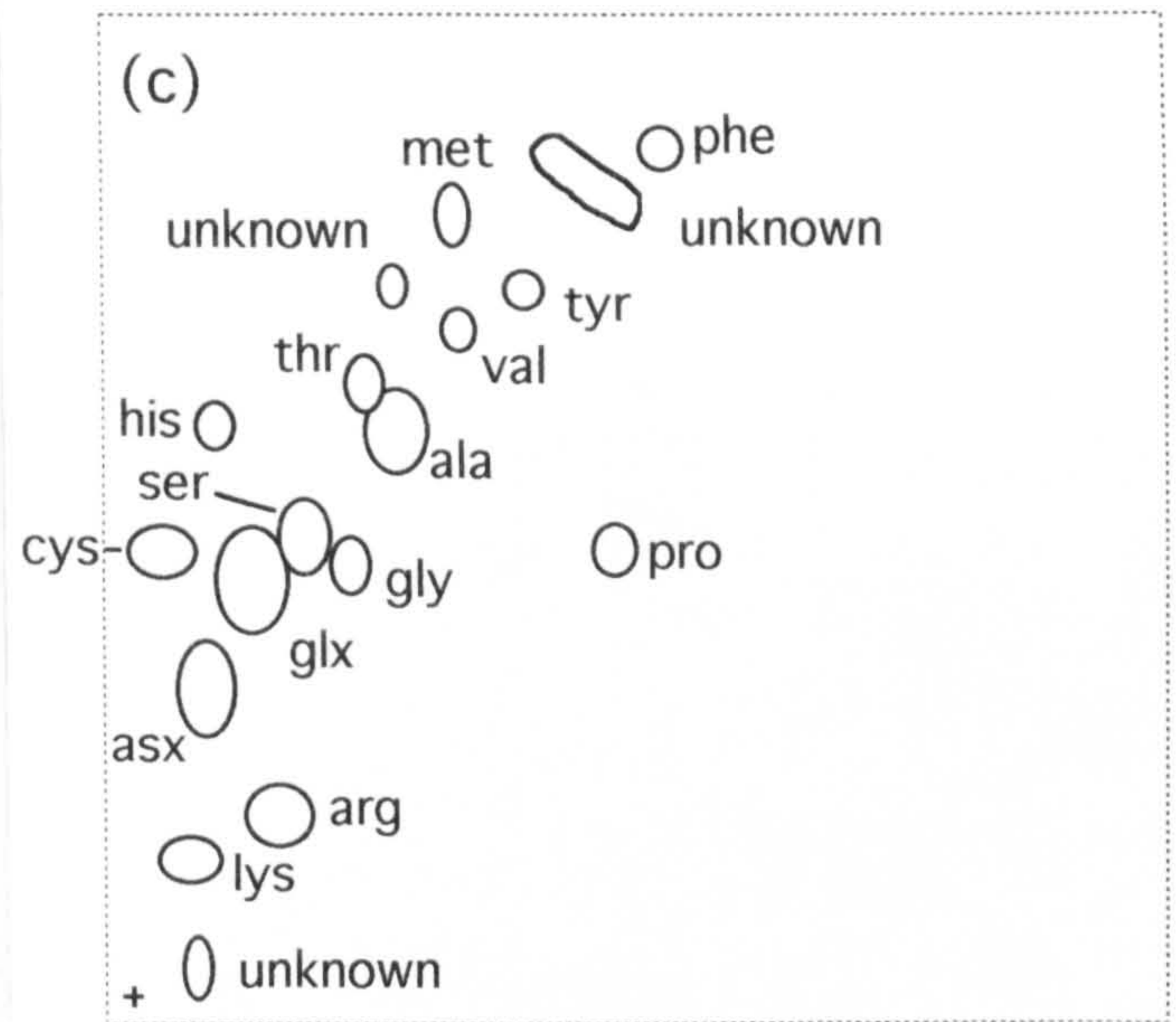
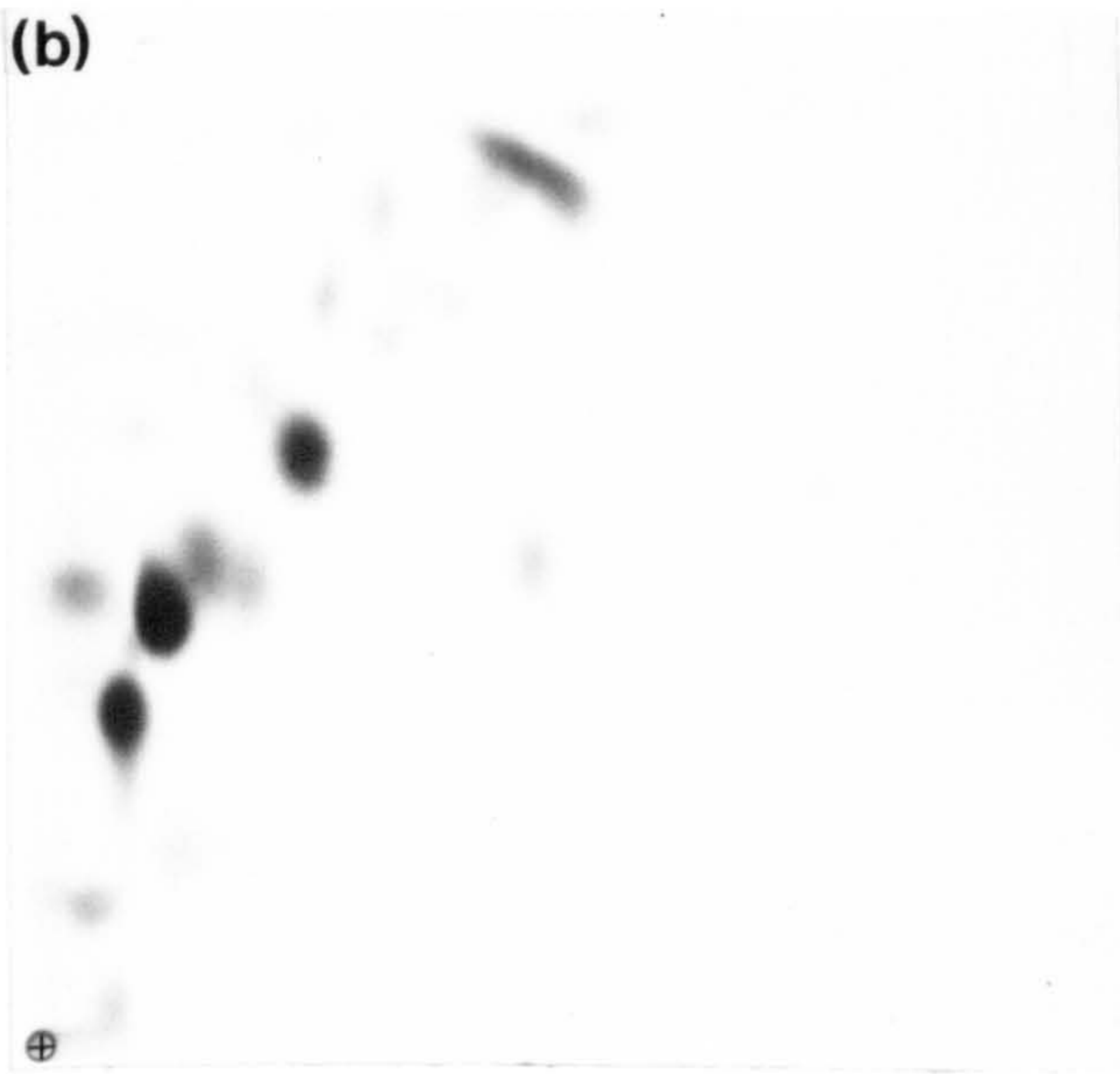
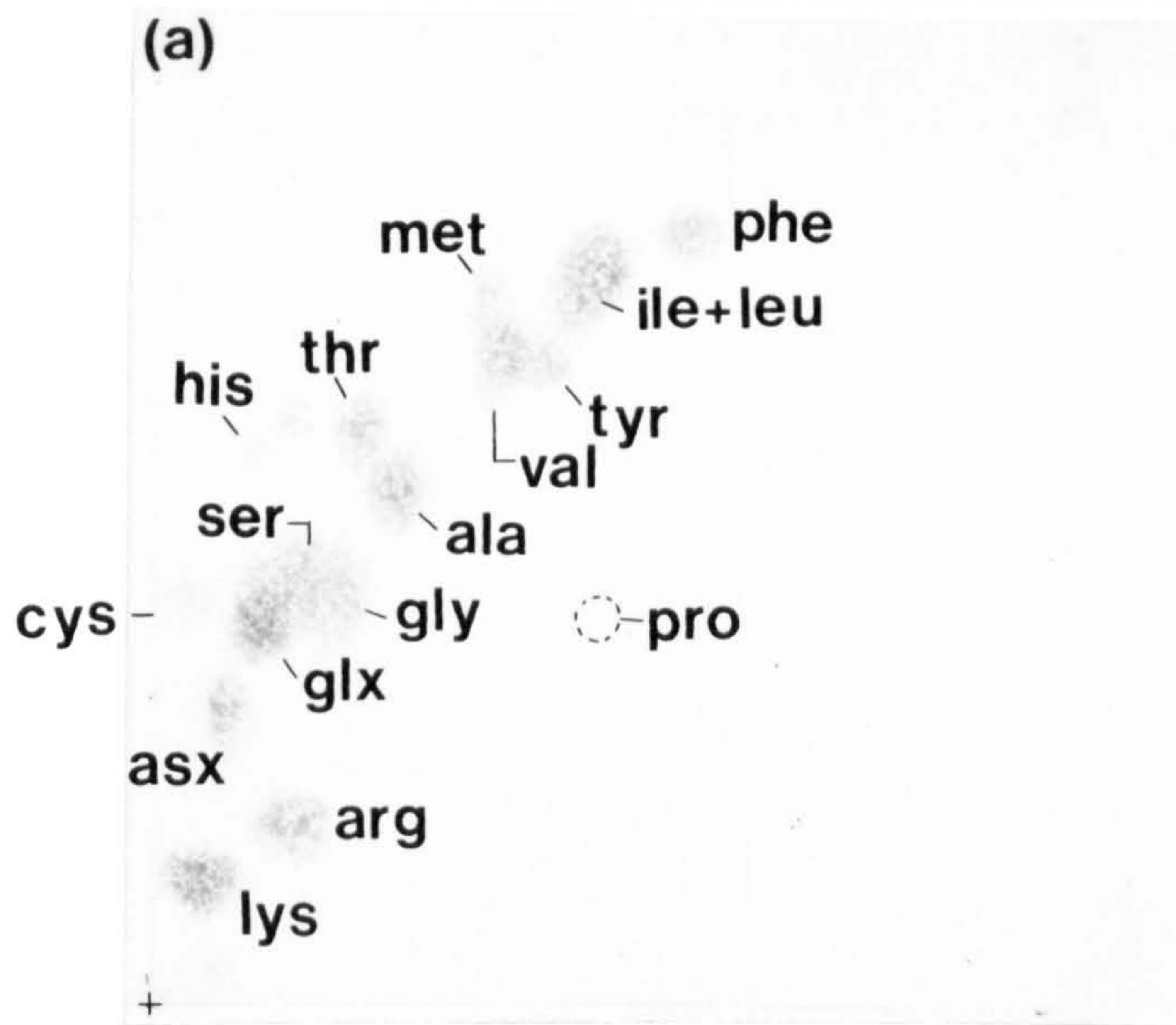
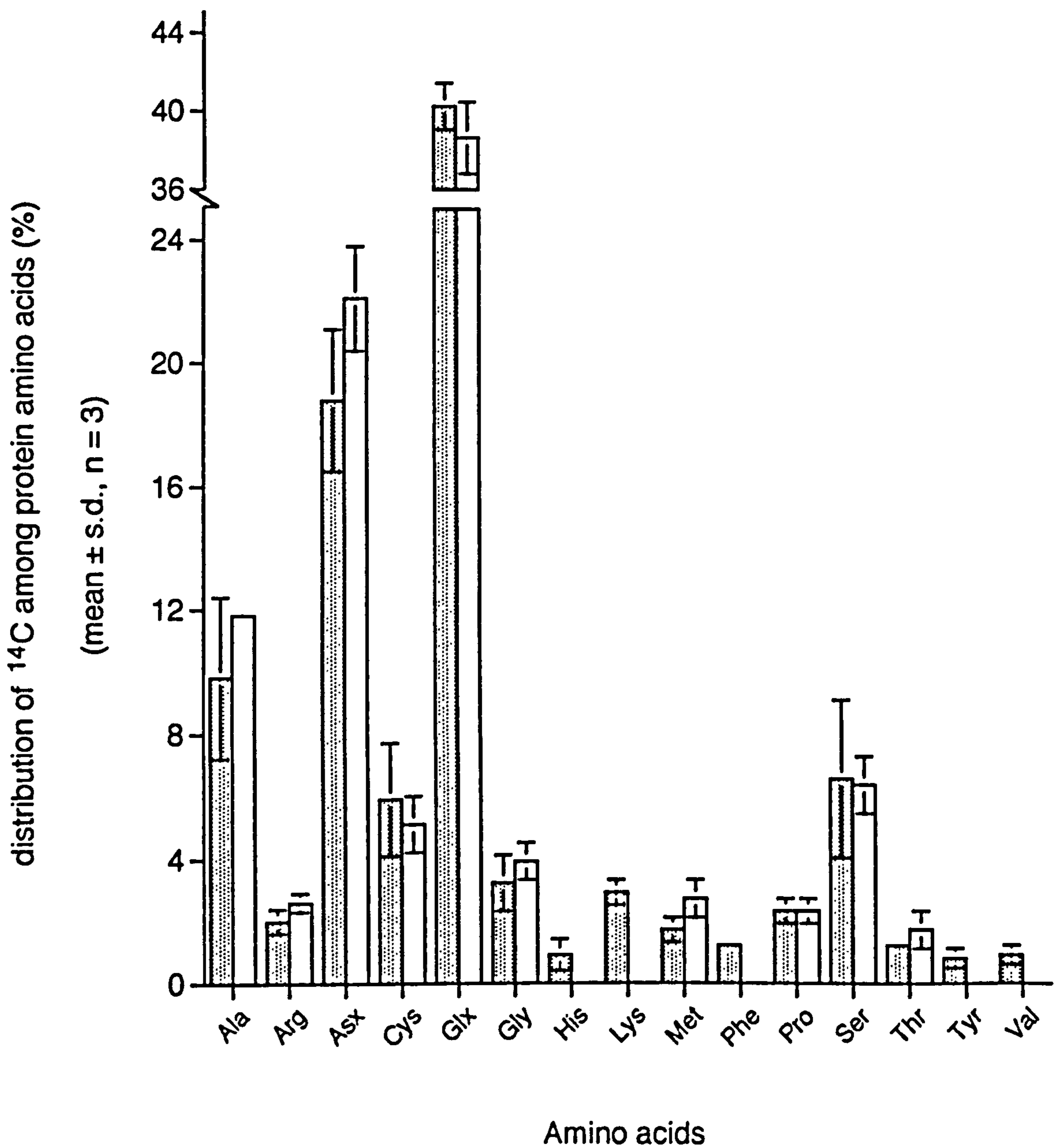


Figure 5.5 The distribution of ^{14}C -labelled amino acids isolated from the protein hydrolysates of control (\square) and alga-depleted animals (\square) incubated in ASW supplemented with a mixture of ^{14}C -labelled precursors for amino acid synthesis.



by the HPLC technique used. The unknown of ^{14}C -labelled components located at the similar position to that of isoleucine/leucine on TLC plate was determined experimentally to be not an amino acid or sugar by its lack of reaction with ninhydrin reagent and alkaline-silver reagent, but further identification was not undertaken. For the alga-depleted animals, only 8 amino acid peaks were detected with significant radioactivity by the HPLC analysis, including two "essential" amino acids threonine and methionine (Fig. 5.3c). As shown in Fig. 5.4c, TLC analysis confirmed these identifications and further demonstrated ^{14}C incorporation into serine, alanine, cysteine, and proline. When examining the radioactivity of each amino acid spot on the TLC plate, ^{14}C integration into the essential amino acids phenylalanine, valine, lysine, and histidine was still higher than background value (20-30 dpm), but lower than 100 dpm, the lowest value at which the radioactivity could be measured accurately.

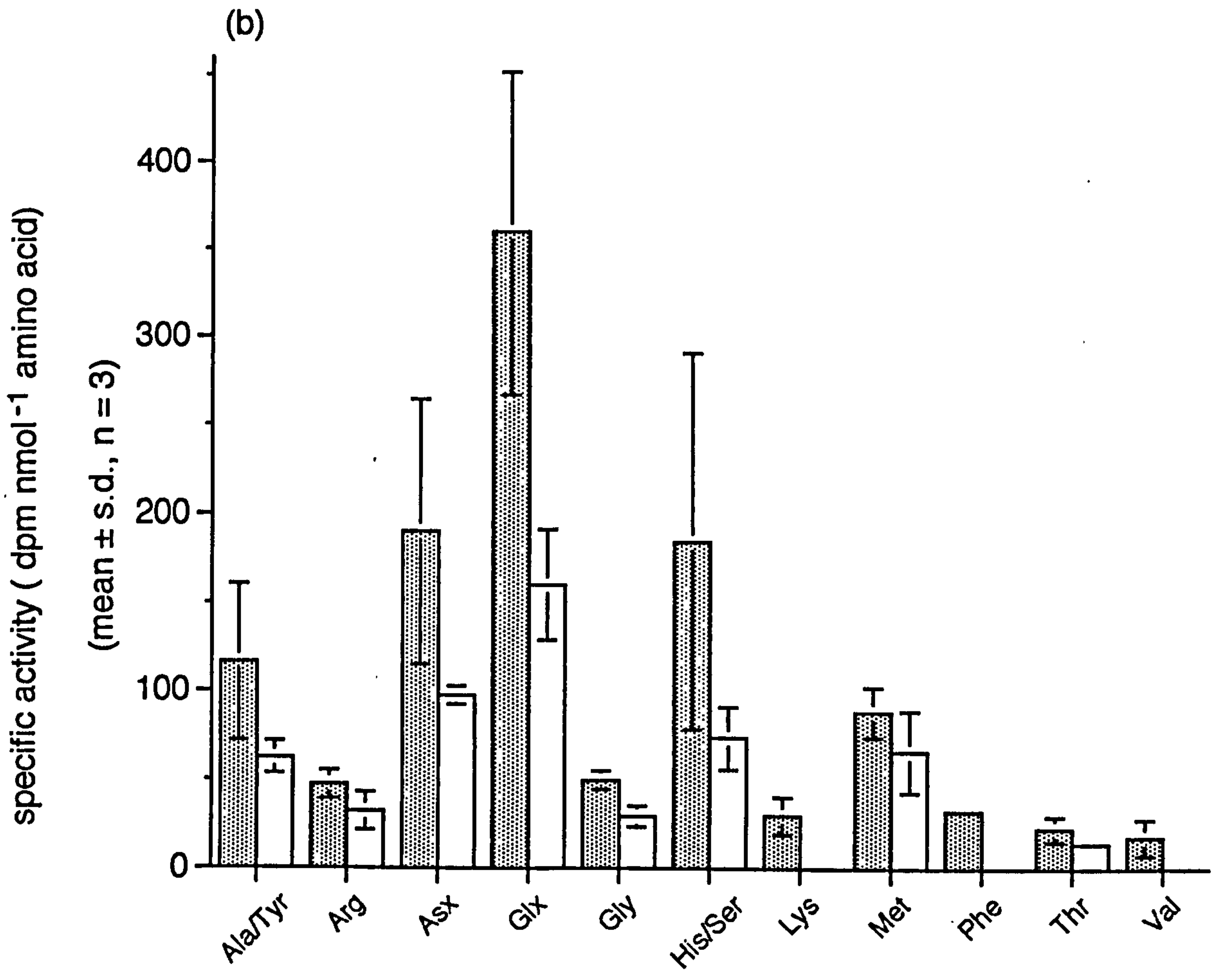
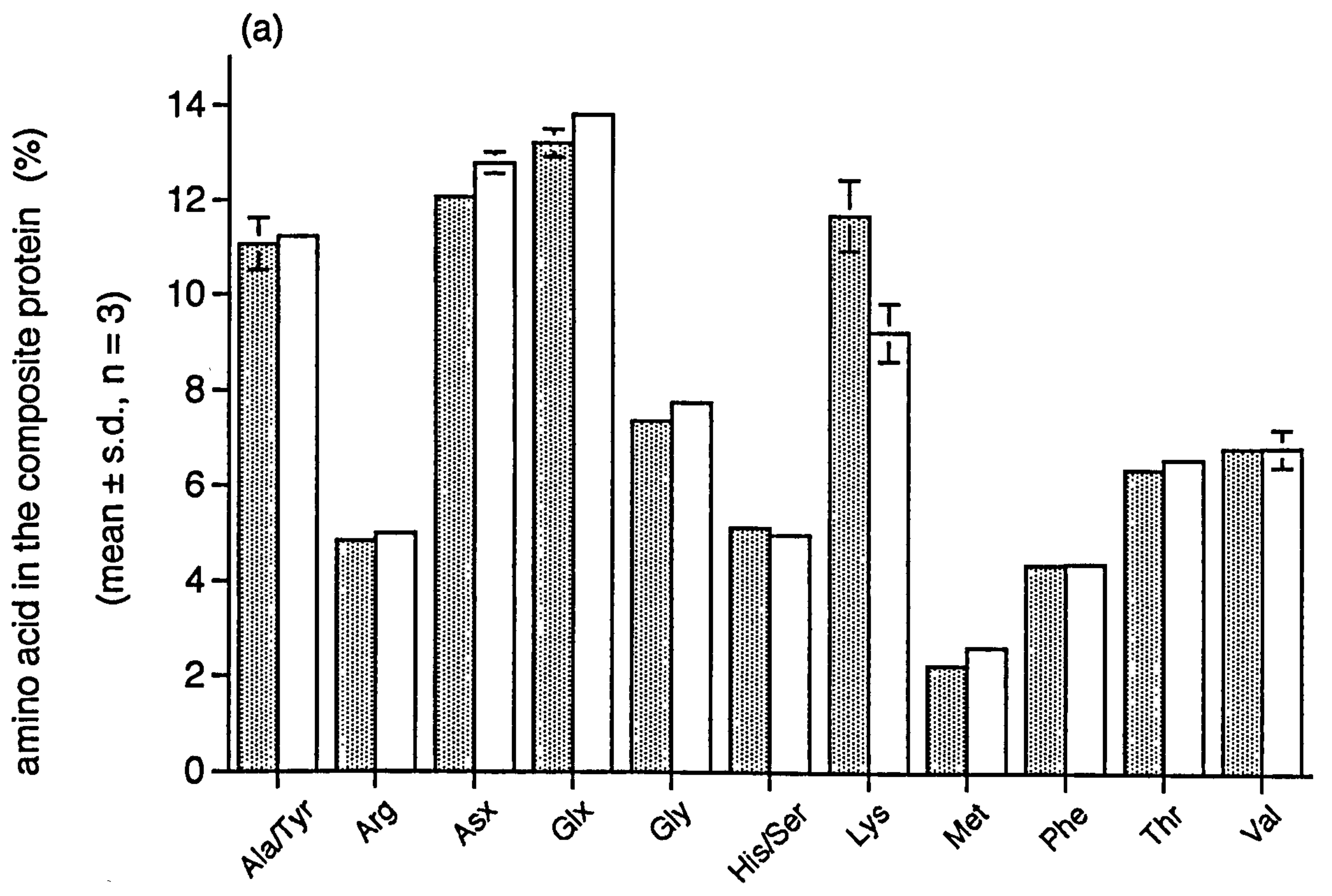
5.2.3 Analysis of ^{14}C distribution in the protein amino acids

Each amino acid in the protein hydrolysate was recovered from the ninhydrin-positive spots on TLC plate for the quantification of distribution of radioactivity (Fig. 5.5). In order to obtain quantitatively reliable results, the amino acids detected with radioactivity lower than 100 dpm were not included. As shown in Fig. 5.5, the samples from both treatments displayed the similar level of ^{14}C distribution in a set of amino acid (Ala, Arg, Asx, Cys, Glx, Pro, Ser, Met and Thr); and the ^{14}C precursor was dominantly incorporated into Glx and Asx which represented more than half of the ^{14}C incorporated into protein amino acids. The control animals, however, exhibited more ^{14}C incorporation into several "essential" amino acids including histidine, phenylalanine, lysine, and valine, and a non-essential amino acid tyrosine.

Figure 5.6 The % of amino acid composition (a) and specific activity of ^{14}C in amino acids (b) of protein hydrolysates from ^{14}C labelled *A. pulchella* .

Asx refers to aspartate + asparagine; and Glx to glutamate + glutamine.

Control animals (); and alga-depleted animals ().



Further, the specific activity of ^{14}C incorporation into each amino acid (dpm nmol $^{-1}$), as shown in Fig. 5.6b, was estimated from the radioactivity obtained from TLC analysis and the amino acid composition of animal protein from HPLC analysis (Fig. 5.6a). The peaks for, first, histidine and serine, and, second, alanine and tyrosine in the HPLC analysis could not be separated with confidence in some samples, and only their combined concentrations and specific activity are displayed. Consistent with previous study (FitzGerald & Szmant 1997), the highest specific activities were found for Glx, Asx, Ser, Ala. But, instead of finding high ^{14}C specific activity in the "essential" amino acid histidine as in corals (FitzGerald & Szmant 1997), the control *A. pulchella* has a comparable level of ^{14}C specific activity in methionine to other non-essential amino acids. For every amino acid, the mean specific activity was higher in the control animals than in the alga-depleted animals, in spite of the fact that both types of animals displayed nearly the same composition of protein amino acids. The specific activity of ^{14}C in the protein amino acids between the control and alga-depleted animals was different, but not in an uniform pattern. Both animals exhibited similar level of specific activity of ^{14}C in Arg, as well as in Gly, Met, and Thr. However, the specific activities of ^{14}C in Asx, Glx, Ala/Tyr, and Ser/His in alga-depleted animals were only 40-44 % of that in control animal, and that in Lys, Phe, and Val in alga-depleted animals were too low to be quantified with confidence.

5.3 Discussion

When *A. pulchella* is incubated with ^{14}C labelled glucose, aspartate, and glutamate, a set of non-essential amino acids (Ala, Arg, Asn, Asp, Cys, Gly, Glu, Pro, Ser, and Tyr) and two "essential" amino acids (Met, Thr) are recovered from the animal fraction of control and alga-depleted animals with varied radioactivity. The essential amino acids, e.g. histidine, isoleucine, leucine, lysine, valine and phenylalanine, were also recovered from the control animals, but that in alga-depleted animals were too low to be detected with confidence. The synthesis of tryptophan is still uncertain, because it is destroyed by acid hydrolysis of protein, and no ^{14}C labelled tryptophan was detected in the trichloroacetic acid soluble fraction. The amino acid synthesis cannot be attributed to contaminating bacteria, because (1) as far as I know, no endosymbiotic bacterium had been reported to reside in the tissue of *A. pulchella*; (2) light and electron microscopy studies of the specimens used in this study exhibited no sign of bacteria (see section of 4.2.1); and (3) the antibiotic treatment eliminated all readily-cultivable micro-organisms.

Though the specific activity of radioactivity of methionine and threonine is comparable to the control animals, the alga-depleted animals exhibited less ability to synthesize the most essential amino acids, e.g. lysine, valine and phenylalanine (Fig. 5.6b). These results are open to two alternative explanations. First, *A. pulchella* cannot synthesize most essential amino acids, and the ^{14}C incorporation into lysine, valine and phenylalanine detected in the control animals is derived from the provision of symbiotic algae. Second, metabolic activity for the alga-depleted animals to synthesize essential amino acid and incorporate into protein are too low to be detected. The higher specific activity of radioactivity detected in the amino acids derived from control animals probably reflects a generally higher level of metabolic activity and nitrogen conservation capacity in

these animals, when promoted by the algal provision of organic carbon. With the evidence obtained in this chapter, it is not appropriate to exclude the possibility of amino acid provision from symbiotic algae, even the *in vitro* radiotracer experiments reveal very low and limited variety of amino acids released by isolated *Symbiodinium* (chapter 3; von Holt & von Holt 1968; Trench 1971a,b; Sutton & Hoegh-Guldberg 1990). So far, no direct evidence demonstrating the translocation of amino acids from *Symbiodinium* to the animal host has been revealed. The glycoconjugated proteins released by *Symbiodinium* in culture has been suggested as a candidate for the amino acid provision to the animal host (Trench 1993); however, the evidence for demonstration of algal protein released *in vivo* is still unconvincing. On the other hand, the low total ^{14}C incorporation rate (Table 5.1) and the trace amount of radioactivity detected in the essential amino acids (e.g. phenylalanine, lysine, valine etc.) derived from alga-depleted animals (Fig. 5.4) indicate that there is still room for improving the detection technique to demonstrate the biosynthesis of essential amino acids in *A. pulchella*, for example using the precursor with high specific activity of radioactivity.

In summary, these data are not readily comparable to the results obtained by FitzGerald and Szmant (1997) for scleractinian corals. However, this study confirms that animals of the phylum Cnidaria can, maybe in varied capability, synthesize some essential amino acids which most animals general cannot (e.g. methionine). The implication is that the algae may not have a greater amino acid biosynthetic capability than their animal partner, and the expectation that the algae should enhance the amino acid nutrition of the animal should be re-evaluated. Further research is required to establish whether the algae contribute, directly or indirectly, to the nitrogen nutrition of their Cnidarian hosts.

Chapter 6 General Discussion

The significance of nutritional interactions in the Cnidaria-alga symbiosis for the success of these associations in oligotrophic environments has been studied for at least three decades. Previous studies have conclusively revealed that the algal symbionts provide the animal host with photosynthetic products. These carbon compounds provided by *Symbiodinium* cells, principally as glycerol and organic acids, contributes much or all of the animal's carbon requirements for basal respiration (Muscatine 1990). This study has extended our understanding of the nutritional basis of these symbioses as follows.

(1) A non-protein amino acid taurine which is also a major free amino acid of the animal fraction induces photosynthate release by *Symbiodinium*, and may be the signal *in vivo*.

(2) Photosynthate release by *Symbiodinium* provides an impact on the animal's nitrogen metabolism; i.e. the algal symbionts probably promote nitrogen conservation by the animals, rather than contribute through nitrogen recycling.

(3) *A. pulchella* can synthesize so-called essential amino acids, for example methionine and threonine.

6.1 Taurine as a signal to induce photosynthate release from *Symbiodinium*

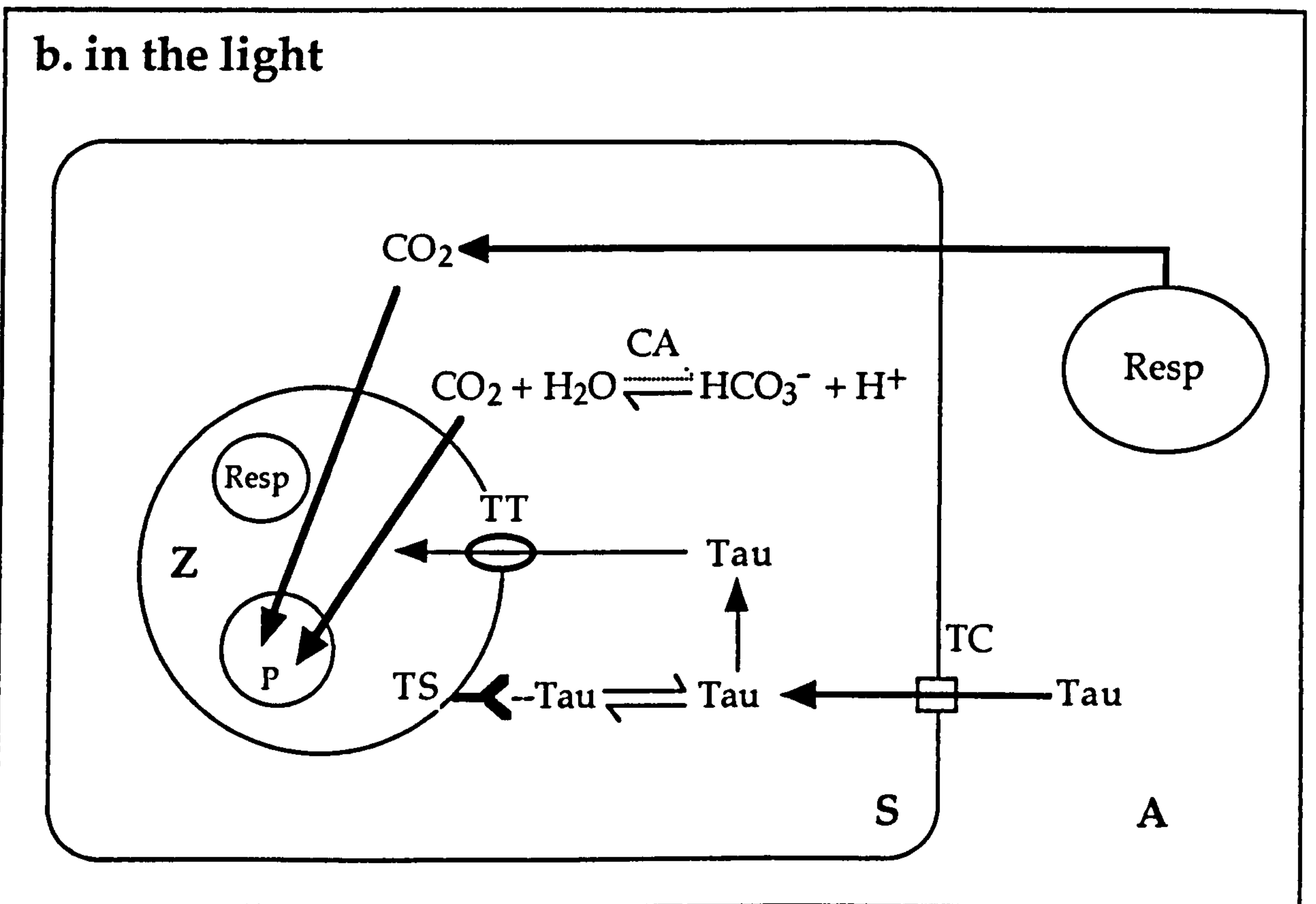
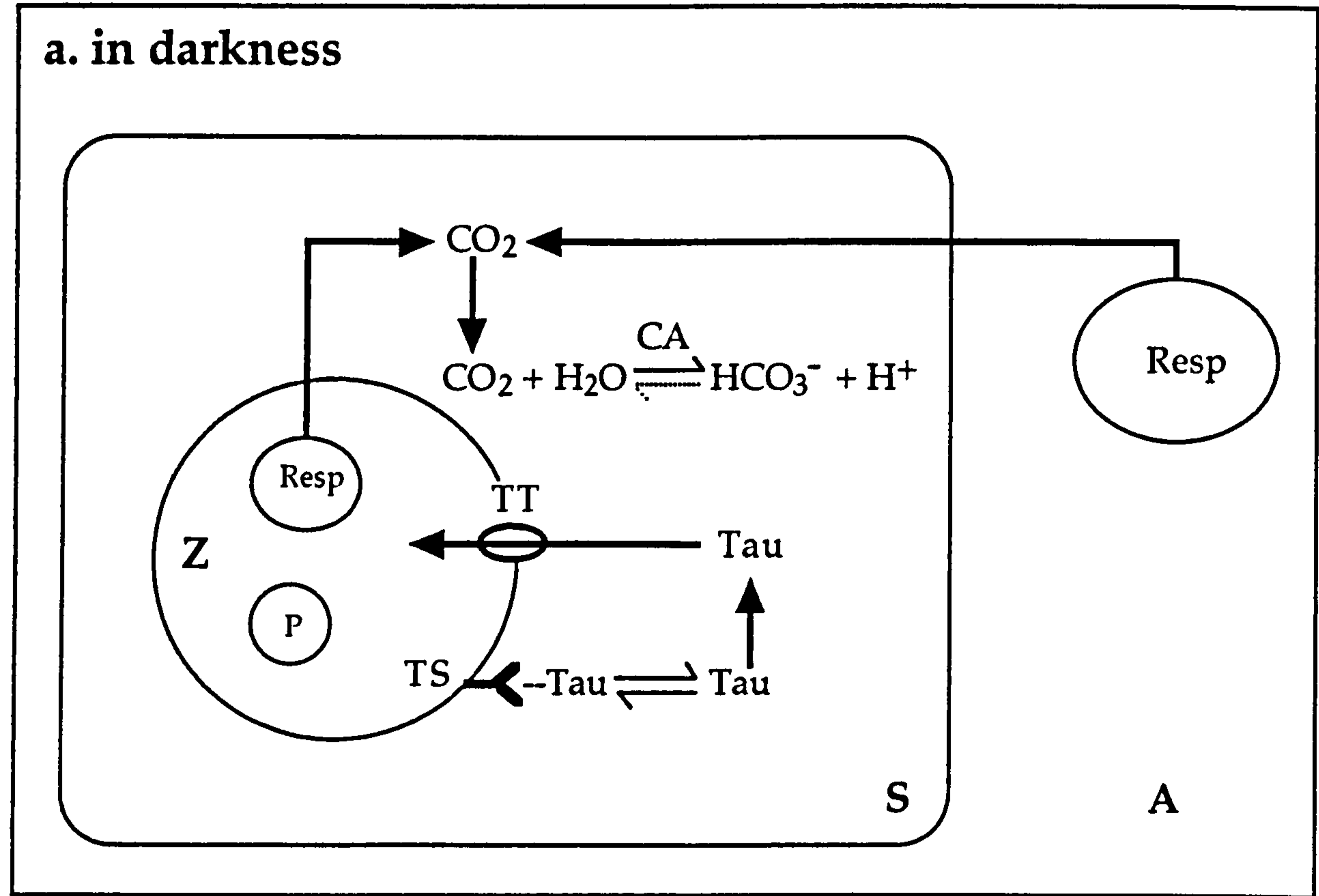
Conventionally, host extract is used to demonstrate the capability of some Cnidaria to induce photosynthate release by *Symbiodinium* (e.g. Trench

1971b, Sutton & Hoegh-Guldberg 1990, Grant *et al.* 1997). As shown in this study, taurine and host extract have broadly comparable impacts on photosynthetic metabolism and the induction of photosynthate release by *Symbiodinium* (see chapter 3). The low K_m value (21 μM) for taurine-stimulated photosynthate release also indicates the taurine may be a signal mediating photosynthetic metabolism in the *Symbiodinium*. Kinetic evidence, further, indicates that *Symbiodinium* might interact with taurine in two distinct ways, i.e. a taurine transporter and a taurine receptor for stimulating photosynthate release. If taurine is the signal for the induction of photosynthate release *in vivo*, then two questions arise. First, whether the concentration of taurine within the perisymbiont space is regulated. Second, how taurine affects photosynthetic metabolism and photosynthate release in the *Symbiodinium* cells. The following sections aim to address these questions in three parts: (1) taurine flux into the perisymbiont space may relate to the osmotic pressure change in the symbiosome; and a regulation model is provided; (2) general concept of nutrient translocation among varied symbioses; (3) an interpretation of how taurine may stimulate photosynthate release by *Symbiodinium*.

6.1.1 Osmotic model: mediating taurine transportation across symbiosome membrane

Taurine is a very hydrophilic amino acid over physiological pH, and can not penetrate across membranes without a carrier or specific channel (Huxtable 1992). So, if taurine is a signal that stimulates photosynthate release, a taurine transporter on the symbiosome membrane is to be expected, to generate the taurine in the perisymbiont space signaling to the *Symbiodinium* to release photosynthetic products. Due to the feature that *Symbiodinium* only release recently-fixed photosynthetic products to the animal host (e.g. Sutton & Hoegh-Guldberg 1990), taurine influx into

Figure 6.1 Model of taurine transport from animal cytosol into perisymbiont space. Abbreviations used: Resp, respiration; P, photosynthesis; CA, carbonic anhydrase; Tau, taurine; TS, receptor for taurine-stimulated photosynthate release; Z, *Symbiodinium* cell; S, perisymbiont space; A, animal cell; TT, taurine transporter; TC, taurine channel.



perisymbiont space may occur only when *Symbiodinium* are photosynthesizing, i.e. in the light. In the other word, taurine influx for signaling *Symbiodinium* to release photosynthetic products could be directly or indirectly mediated by illumination. A hypothetical model is considered as follows.

This model has several bases. First, as suggested by Streamer *et al.* (1993), the primary enzyme conducting the photosynthetic carbon fixation in the *Symbiodinium* is ribulose biphosphate carboxylase oxygenase (RuBisCo), which uses CO₂, but not HCO₃⁻ as substrate. Second, carbonic anhydrase is an important CO₂ concentrator in the algal symbiosis with Cnidaria (Weis *et al.* 1989; Weis 1991). Third, subcellular fractionation and in situ immunological localization data suggest that the carbonic anhydrase of *A. pulchella* is distributed in the soluble fraction of cell homogenate (i.e. not bound to any membrane), and located strictly at the algal side of the symbiosome membrane (Weis 1993). Fourth, *A. pulchella* can take up bicarbonate from the seawater medium (Weis 1993).

The hydration of carbon dioxide by carbonic anhydrase is a reversible reaction.



Therefore, as shown in the Fig. 6.1a, the carbon dioxide produced from respiration of the animal cell and algal symbiont diffuses into perisymbiont space in darkness; and the carbon dioxide is expected to be converted into bicarbonate and accumulated in this space by carbonic anhydrase. As described above, taurine is not necessary under these circumstances; and it is expected to be taken up by *Symbiodinium*. It is

Box 1. Estimation of reduction in osmotic pressure within perisymbiotic space resulted from the photosynthesis by *Symbiodinium* in the symbiosis.

Assumptions:

1. The shape of *Symbiodinium* is a uniform sphere with $10\ \mu\text{m}$ ($= 10 \times 10^{-4}\ \text{cm}$) in diameter.
2. The mean distance between symbiosome membrane and algal surface is $0.3\ \mu\text{m}$ ($= 0.3 \times 10^{-4}\ \text{cm}$) (see Fig. 4.1).
3. Photosynthetic fixation rate in vivo is equal to $0.84\ \text{dpm cell}^{-1}\ \text{h}^{-1}$ (see section 3.2.1)

Calculation:

The radius of symbiosome $= (10 \times 10^{-4} + 0.3 \times 10^{-4})/2 = 5.15 \times 10^{-4}\ \text{cm}$

The radius of the *Symbiodinium* $= (10 \times 10^{-4})/2 = 5 \times 10^{-4}\ \text{cm}$

Volume in perisymbiotic space $= \frac{4}{3} \times \pi \times (5.15 \times 10^{-4})^3 - \frac{4}{3} \times \pi \times (5 \times 10^{-4})^3$
 $= 5 \times 10^{-11}\ \text{mL}$
 $= 0.05\ \text{pL}$

Specific activity of ^{14}C -bicarbonate $= 674\ \mu\text{Ci mg}^{-1}$ (data from Amersham Co.)
($1\ \mu\text{Ci} = 1.5\ \mu\text{g C mL}^{-1}$)

The concentration of ^{14}C -bicarbonate was $2\ \mu\text{Ci mL}^{-1}$ (see section 2.3.1).

Then the specific activity of ^{14}C in the ASW is $28.7\ \mu\text{g C} (\mu\text{Ci})^{-1}$ (bicarbonate concentration in ASW $= 25.7\ \mu\text{g C mL}^{-1}$); i.e. $1\ \text{dpm} = 1.30 \times 10^{-5}\ \mu\text{g C}$.

So, photosynthetic carbon fixation rate $= 0.84 \times 1.30 \times 10^{-5}$
 $= 1.0 \times 10^{-5}\ \mu\text{g C cell}^{-1}\ \text{h}^{-1}$
 $= 8.3 \times 10^{-7}\ \mu\text{mol C cell}^{-1}\ \text{h}^{-1}$
 $= 0.01\ \text{pmol C cell}^{-1}\ \text{min}^{-1}$

If the carbon used in the *Symbiodinium* photosynthesis is principally derived from the dehydration of bicarbonate by carbonic anhydrase in the perisymbiotic space, then the enzyme reaction will reduce $0.02\ \text{pmol}$ ($= 0.4\ \text{M}$ in the volume of $0.05\ \text{pL}$) of ionic solute per min.

So, the reduction of osmotic pressure (Kirschner 1991) $= 0.4 \times 22.4 \times 76$
 $= 608\ \text{mOsmol L}^{-1}\ \text{min}^{-1}$

noted that *Symbiodinium* cells can take up taurine both in the light and darkness (Dean & O'Brien 1981; L. Whitehead unpublished results).

In the light (Fig 6.1b), the *Symbiodinium* cells photosynthesize. The CO_2 in the perisymbiotic space will be assimilated by the algal cell immediately for carbon fixation in the plastid, and the sustained supply of CO_2 in the perisymbiotic space may be mediated by the conversion of HCO_3^- to CO_2 by carbonic anhydrase in this space. When the reaction goes forward to CO_2 synthesis, this would cause a reduction in the concentration of HCO_3^- and H^+ in the perisymbiotic space. The volume of perisymbiotic space is very limited, so a tiny amount of reduction in the concentration of HCO_3^- and H^+ will be possible to cause an osmotic shock to the animal cell membrane. As shown by Ferrier (1992), *A. pulchella* releases taurine into the medium for osmotic regulation when transferred into medium of low salinity seawater. An estimate of the magnitude of osmotic shock caused by dehydration of bicarbonate is explored in Box 1. Based on several assumptions, the concentration reduction of HCO_3^- and H^+ might cause a drop of osmotic pressure by $608 \text{ mOsmol L}^{-1} \text{ min}^{-1}$. [It is noted that the osmotic pressure of 35 ‰ is equal to $1025 \text{ mOsmol L}^{-1}$.] Because the disequilibrium between ^{14}C and ^{12}C during algal carbon fixation may cause a significant effect on the assumed $^{14}\text{C}/^{12}\text{C}$ conversion factor used in Box 1 (Smith 1982), the estimation of carbon fixation rate derived from oxygen production data is also applied for comparison. Photosynthetic rate in *Aiptasia pulchella* maintained at $45 \mu\text{E m}^{-2} \text{ s}^{-1}$ is $0.0762 \mu\text{g O}_2 (\mu\text{E m}^{-2} \text{ s}^{-1})^{-1} \text{ h}^{-1} (10^6 \text{ cells})^{-1}$ (Muller-Parker 1985), which is equal to $0.0024 \text{ pmol C cell}^{-1} \text{ min}^{-1}$ at the $60 \mu\text{E m}^{-2} \text{ s}^{-1}$ (the irradiance used in this thesis). Then, the reduction of osmotic pressure will be up to $163 \text{ mOsmol L}^{-1} \text{ min}^{-1}$. However, these two estimates are still conservative, because the distance between the symbiosome membrane and algal surface shown on the

electron microscopic image might have been expanded by the shrinkage of algal cell during fixation. Therefore, the real osmotic pressure changes may be greater than the value estimated in the Box 1.

The high affinity receptor ($K_m = 21 \mu\text{M}$) for taurine-stimulated photosynthate release on the algal surface is expected to bind with taurine, and trigger photosynthate release by the *Symbiodinium*. The dissociated taurine from that receptor and the extra taurine in the perisymbiont space will be taken up by the *Symbiodinium* using the taurine transporter ($K_m = 63 \mu\text{M}$). The model with a high affinity receptor for the taurine signaling process, a lower affinity transporter for the taurine uptake process, and movement of the taurine signal from perisymbiont space by the signal receiver (e.g. *Symbiodinium*), is consistent with that occurring at the neurotransduction process mediated by amino acids (Teichberg 1980). Further, the reduced osmotic pressure in the perisymbiotic space may be sustained by the continuous uptake of bicarbonate from the seawater medium by the animals (Weis 1993). It is noted that the *Symbiodinium* must draw on dissolved inorganic carbon from the seawater pool to satisfy the high carbon demand (Goreau 1977a & b; Muscatine *et al.* 1989). As suggested by Weis (1993), the carbonic anhydrase might be only located in the perisymbiont space rather than in the animal's cytosol; then the internalized bicarbonate is expected to be transported into perisymbiont space.

In summary, this taurine influx model suggests the high demand for carbon dioxide during photosynthesis by *Symbiodinium* will trigger the dehydration of bicarbonate using carbonic anhydrase, reduce the osmotic pressure within the perisymbiont space, and then induce the influx of taurine from the animal cytoplasm. To test this hypothesis, it will be

needed to establish if taurine is present within the perisymbiont space when the animal is incubated in the light, but not in darkness or in seawater supplemented with acetazolamide [Diamox, a specific inhibitor for carbonic anhydrase (Maren 1985)] in the light. It is noted that an immunocytochemical method to localize taurine in cells has been developed (e.g. Carlberg *et al.*, 1995).

6.1.2 Control of nutrient translocation in symbiosis

The mechanisms underlying nutrient translocation between the host and symbionts have been well studied in the associations of plant-*Rhizobium* and insect-*Buchnera*. The mobile compound in most nitrogen-fixing symbioses in plants is ammonia, the product of nitrogen fixation (Douglas 1994). In the rhizobium-legume symbiosis, the transport of ammonia from rhizobial cells to the surrounding plant cell cytoplasm is promoted by a very steep concentration gradient (e.g. Streeter 1989), which may be sustained by repressing the expression of glutamine synthetase genes in the rhizobium and a higher glutamine synthetase activity in the plant cells (e.g. Nierzwicki-Bauer & Haselkorn 1986). In order to maintain the capability of nitrogen fixation in the high ammonia subcellular environment, the rhizobia also have a novel mechanism for the regulation of *nif* genes (nitrogen fixation genes), distinctly different from that in the non-symbiotic bacteria, such as *Klebsiella*. As demonstrated by de Philip (1990), the *nif* genes in rhizobia are expressed under low oxygen tension (as occur in root nodules) using membrane-bound haemoprotein (FixL) as oxygen sensors, and independent of ammonia concentration. Under low oxygen conditions, FixL phosphorylates a regulator protein (FixJ) resulting in the activation of *nif* genes.

The nutrients provided by *Buchnera* to the insect host aphid are essential amino acids (Mittler 1971; Douglas 1988; Febvay *et al.* 1995). The results from recent molecular analyses indicate that the translocated essential amino acids may be derived from the overproduction of these amino acids by *Buchnera* (Lai *et al.* 1994; Bracho *et al.* 1995); however, it is not clear yet whether the genes coding for the enzymes in the essential amino acid synthesis are uniformly amplified.

The underlying mechanism of how the animal host induces its algal symbiont to release photosynthetic compounds is still not well-understood. However, there are two mechanisms considered. The animal host might elevate the photosynthetic carbon fixation rate in the algal symbiont, then the overproduced photosynthetic products are translocated to the host, and/or stimulate the release of photosynthetic products (Hinde 1988). Sutton and Hoegh-Guldberg (1990) suggested, based on the results from *Plesiastrea versipora*, that the host might affect the photosynthetic metabolism in the algal symbiont by diverting the incorporation of newly-fixed carbon from lipid synthesis to neutral compounds, the principal compounds in the translocated photosynthate. This idea was further supported by the direct determination of glycerol and lipid content in the *Symbiodinium* isolated from *Plesiastrea versipora* (Grant *et al.* 1997). This study has also provided several lines of evidence to partly support this proposal. The isolated *Symbiodinium* incubated in ASW supplemented with host extract or taurine displayed a higher ^{14}C incorporation rate into aqueous fraction (a mixture of low molecular weight compounds), but a lower ^{14}C incorporation rate into the lipid fraction (Fig. 3.5). However, the evidence for enhanced photosynthetic rates by the animal host is confused. The effect of "host factor" on the photosynthetically carbon fixation rate varied widely between different symbiotic associations (Trench 1971c;

Muscatine *et al.* 1972; Sutton & Hoegh-Guldberg 1990) or even in the same animals (see Fig. 3.4).

6.1.3 A possible mechanism underlying taurine-stimulated photosynthate release

As mentioned in section 3.3, the identity of the compounds released may vary between lineages of *Symbiodinium* and with the physiological condition of the algae in different symbioses. A reasonable hypothesis about the mechanism underlying the taurine-stimulated photosynthate release by *Symbiodinium* might be that photosynthetic metabolism is diverted from the regular pathway to release at point(s) not far removed from carbon fixation. The dominant compounds in the taurine-stimulated photosynthate release by the *Symbiodinium* isolated from *A. pulchella* is fumarate (see section 3.2.3), which is an intermediate of citric acid cycle in mitochondria. The synthesis of fumarate in plant mitochondria is derived from succinate catalyzed by succinate dehydrogenase (Hanson and Day 1980). Fumarate is also a product in the pathway of arginine synthesis from arginosuccinate in the plant leaf (Kelly and Latzko 1980). Because the *Symbiodinium* cells only release recently fixed photosynthetic carbon, the most likely source of photosynthate release is expected to be derived from the incorporation of newly-synthesized dihydroxyacetone phosphate into the citric acid cycle in mitochondria. However, unlike the succinate which could be exported from mitochondria via a dicarboxylate transporter in exchange with malate from cytosol, there is no evidence that fumarate transport occurs in a typical plant mitochondria (Wiskich 1977). Therefore, unless the membrane of *Symbiodinium* mitochondria bears a special fumarate transporter, one would expect an enzyme or enzyme complex in the

Symbiodinium cytoplasm mediating the synthesis of fumarate from newly synthesized carbon precursors.

6.2 The role of taurine in *A. pulchella*

One feature of the "host factor" in the sea anemone *Anthopleura elegantissima* is that only the animal extract obtained from individuals bearing symbiotic algae stimulates photosynthate release by *Symbiodinium*, suggesting that the factor might be inducible (Trench 1971c). In this study, taurine was demonstrated as a signal to stimulate the release of photosynthetic products from *Symbiodinium*, and exhibited a broadly comparable effect with animal extract on the photosynthetic metabolism in the algae. However, the concentration of taurine in alga-depleted *A. pulchella* is even higher than that in control animals (see section 4.2.2), and the animal extract obtained from alga-depleted animal also could induce photosynthate release by *Symbiodinium* to a similar extent to that from control animals. So far, as described in the section 1.3, attempts to characterize the properties of the "host factor" in the Cnidaria-*Symbiodinium* association have been controversial. The inconsistent features of the "host factor" between symbiotic species suggest that a single compound may not be the host factor for all Cnidaria-alga symbioses. Moreover, it is noted that *A. elegantissima* may also accommodate unicellular green algae (Chlorophyceae) named zoochlorellae (Muscatine 1971; O'Brien 1978). Perhaps, for example, *A. elegantissima* may not use taurine as signal to induce the release of photosynthetic products from *Symbiodinium*.

The high concentration of taurine in the tissue of both the control and alga-depleted animals indicates that the role of taurine in *A. pulchella*

must not only behave as a signal to induce photosynthate release by *Symbiodinium*. As with some other sea anemone species (e.g. Male & Storey 1983; Deaton & Hoffmann 1988; Herrera *et al.* 1989), taurine may be the principal osmolyte in *A. pulchella*. Taurine meets the requirements for a biologically ideal osmolyte (Huxtable 1992). It can attain high concentration without affecting enzyme function, such as K_M or V_{max} (Somero & Bowlus 1983), and the availability of other amino acids in the cells (Huxtable 1992). Taurine is also highly soluble and zwitterionic over the physiological pH range. The property as a zwitterionic compound enables taurine to be accumulated without perturbing membrane potential and exhibit low lipophilicity, i.e. taurine can be maintained at a extremely high intra- to extracellular concentration gradient without loss by simple diffusion (Huxtable 1992). In this study, the concentration of taurine was found to be higher in the alga-depleted *A. pulchella* than in control animals (see section 4.2.1). The elevation of taurine concentration in alga-depleted animals could also be attributed to the involvement of taurine in osmotic regulation. Because the alga-depleted animals would consume more than half of the total protein amino acids from the free amino acid pool for respiration (Table 4.1), which would result in the lack of enough organic solutes for maintaining the osmotic pressure. This interpretation is consistent with the role of free amino acids in regulation of cell volume in sea anemones (Shick 1991), such that the total free amino acid content of the animal tissues is constrained to vary within very narrow limits, in a medium of a given salinity.

6.3 Nitrogen recycling or nitrogen conservation

Some animals can live on diets with limited level of particular nutrients, and are believed to acquire the supplementary supply of these nutrients

from symbionts. One of the best known models for nutrient supply by symbionts is nitrogen recycling. A wide range of insects may use nitrogen recycled by their symbiotic bacteria or yeasts to live on the diets with low-nitrogen quality (Douglas 1994). For example, aphids can thrive on plant phloem sap (in which the content of essential amino acids is very low) by converting nitrogenous wastes into glutamate and/or aspartate, which, in turn, are used by its intracellular symbiotic bacteria to reproduce essential amino acids (Whitehead & Douglas 1993).

The low nitrogen turnover rate between the Cnidaria-alga symbioses and their environment has been considered the consequence of nitrogen recycling by symbiotic algae (Muscatine & Porter 1977; Barnes & Hughes 1988), but some evidence suggested that it might be the result of nitrogen conservation in the animal hosts (Rees 1986; Rees & Ellard 1989).

The primary evidence used to interpret nitrogen recycling in the Cnidaria-alga symbioses was provided by ammonium uptake and excretion studies in the corals or sea anemones (e.g. Kawaguti 1953; Cates & McLaughlin 1976; Szmant-Froelich & Pilson 1977; Wilkerson & Muscatine 1984). The capability of *Symbiodinium* to assimilate ammonium has also been demonstrated by many studies (e.g. Summons & Osmond 1981; D'Elia *et al.* 1983; Wilkerson & Muscatine 1984; Anderson & Burris 1987; Dudler & Miller 1988). However, the attempts to directly demonstrate nitrogen recycling in Cnidaria-*Symbiodinium* symbioses have never been successful. One way to examine the capability of amino acid translocation from *Symbiodinium* is to trace the process with ^{14}C isotope. The results from the *in vitro* test showed that only 8 or 17 % of ^{14}C labelled photosynthate release was recovered as amino acids (Fig. 3.6), and the isolated *Symbiodinium* only released a limited amount of alanine and

glutamate (von Holt & von Holt 1968; Trench 1971a,b; Sutton & Hoegh-Guldberg 1990). Moreover, labelling the intact animals with ^{14}C -bicarbonate resulted in the recovery of ^{14}C labelled amino acid from animal fraction; but, as mentioned in section 5.1, they were not distinguishable from the amino acids synthesized in the animal cell using the ^{14}C labelled organic acid derived from the photosynthate release.

A prediction of the hypothesis of nitrogen conservation is that the elimination of the algae or their photosynthetic activity would result in: (1) increased concentration of ammonium; (2) decrease in the activity of enzyme(s) that assimilate ammonium and/or increase in activity of ammonium-producing enzymes; (3) decrease in the concentration of free amino acids. Moreover, these effects would be reversed by exogenous organic carbon compounds. In this study, the crucial importance of organic carbon to the nitrogen metabolism of the animal tissues in the *Aiptasia pulchella*-*Symbiodinium* symbiosis has been demonstrated. Whether derived from algal photosynthate or exogenous sources, this organic carbon may serve to: first, promote animal assimilation of ammonium, via glutamine synthetase; and second, reduce the rates of amino acid degradation (and ammonium production) by providing an alternative respiratory substrate. The impact of alga-derived photosynthate on the rates of ammonium production and consumption by the animal tissues confounds the interpretation of experimental designs traditionally used to explore the putative role of the algae in nitrogen recycling. This study has also confirmed that *A. pulchella* can synthesize most protein amino acids, including some "essential" amino acids (e.g. methionine and threonine) to most animals. This evidence may further discount the necessity of nitrogen recycling in this symbiotic system, in which the provision of essential amino acid to the animal host has been

considered the most important basis for the Cnidaria-alga symbioses to maintain nitrogen recycling (Trench 1993).

On available evidence, the persistence of these symbioses in low-nitrogen waters can be attributed principally to the efficiency with which the animal tissues retain nitrogen acquired from food or the medium, as promoted by the algal provision of organic carbon.

6.4 Regulation of glutamine synthetase in the *A. pulchella*

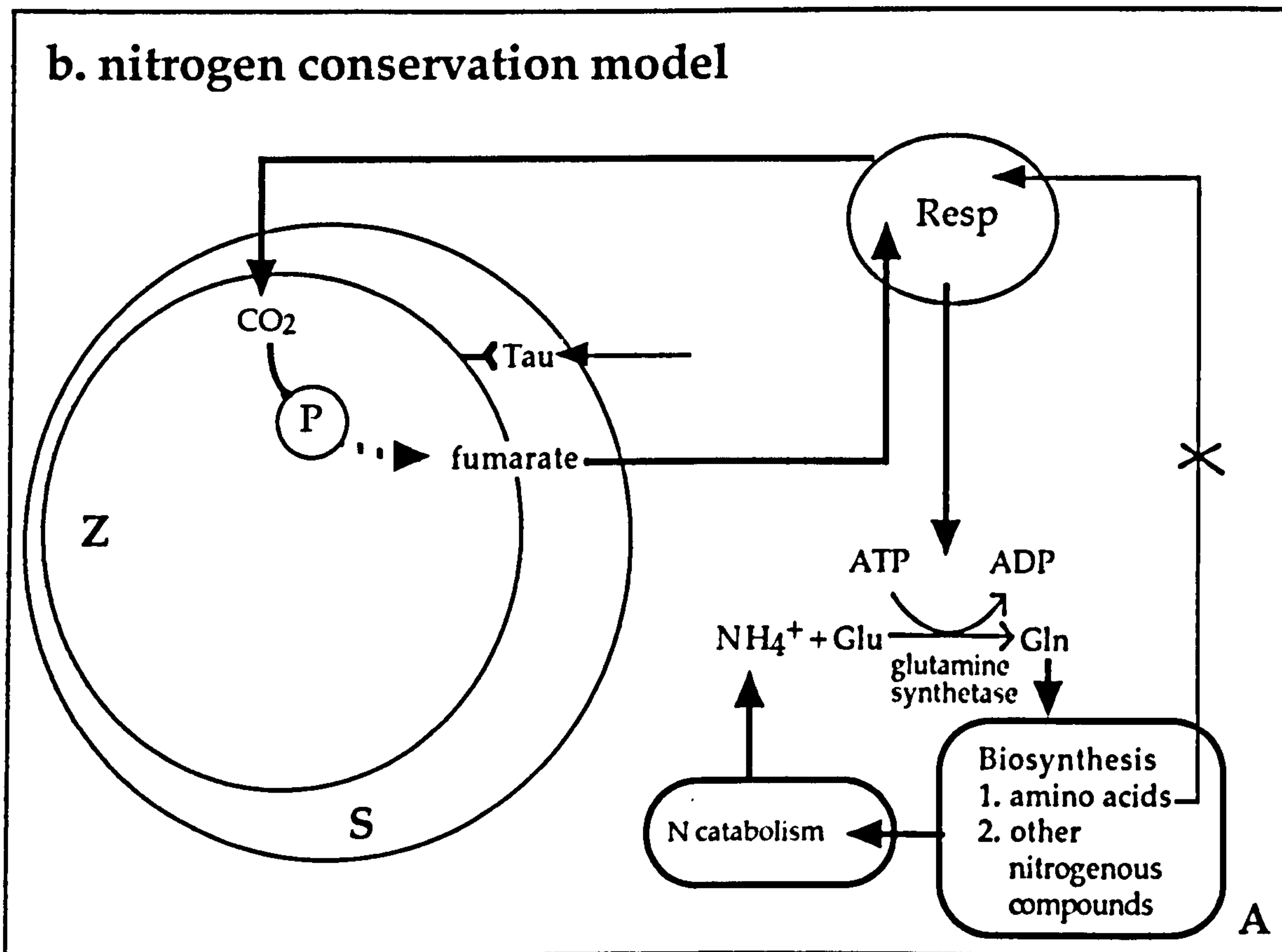
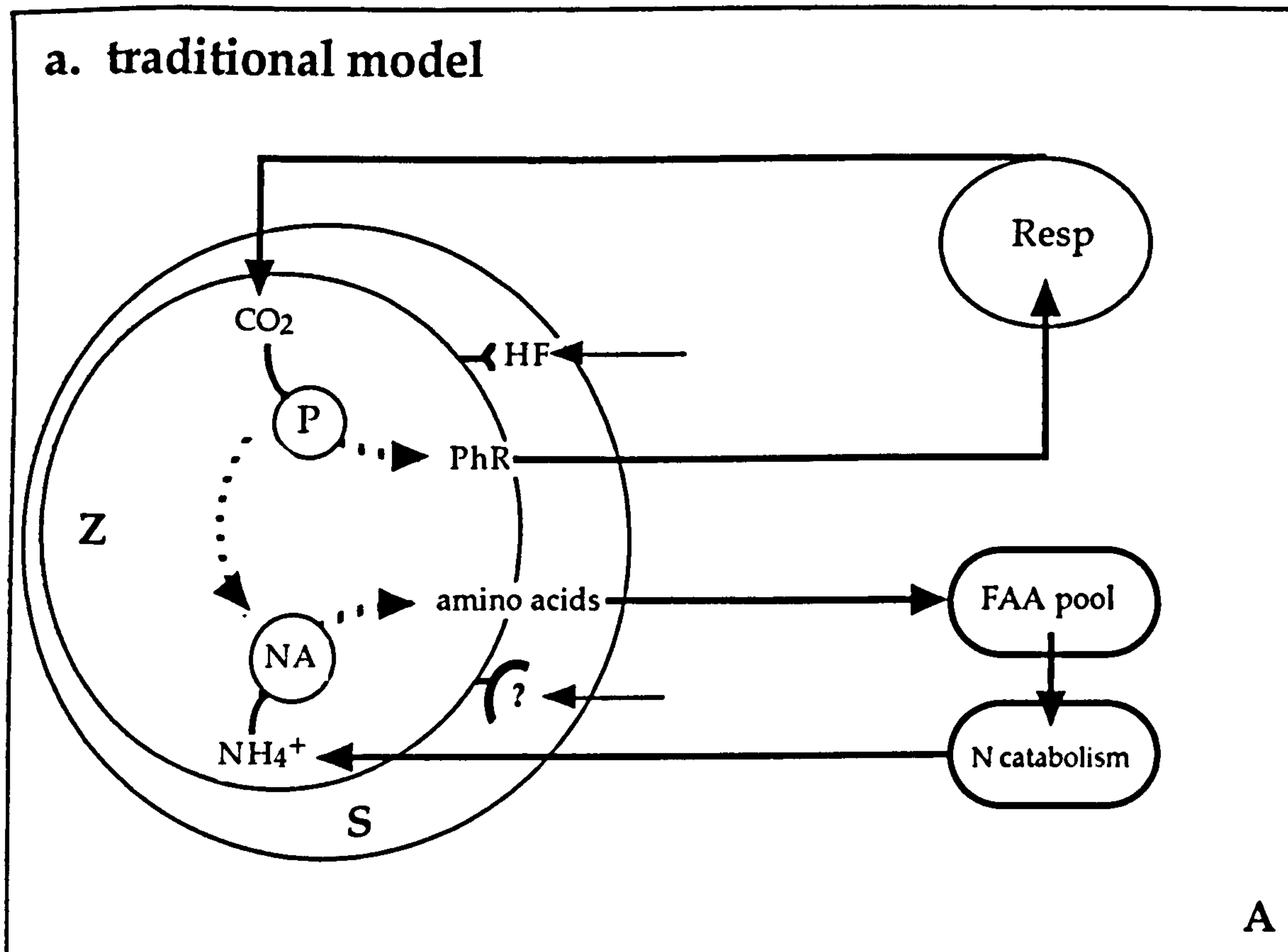
In this study, the glutamine synthetase activity was elevated in the animals receiving carbon supplement either from an exogenous source or photosynthate release by *Symbiodinium*. It is unlikely, however, that the alga-derived photosynthate contributes to the substrate pool of glutamate for glutamine synthetase in the animal tissues, because the principal fates of algal photosynthate translocated to the animal are respiration and incorporation into the lipid fraction, not the amino acid or protein fractions (e.g. Trench 1971a; Battey & Patton 1987; chapter 3). One possibility is that the algal photosynthate and exogenous carbon compounds promote the respiration rates of the animal, and the resultant increase in ATP content and energetic status of the animal tissues may result in increased glutamine synthetase activity. Consistent with this proposal, both feeding and the receipt of algal photosynthate are known to enhance respiratory oxygen consumption in Cnidaria (Muller-Parker 1984; Tytler & Davies 1984; Harland & Davies 1995); and a favourable energetic status promotes glutamine synthetase activity in some algae (e.g. García-Fernández *et al.* 1995).

However, in the determination of transferase activity of glutamine synthetase, a constant and relatively high concentration of ADP (0.4 mM) in the reaction medium provides each sample with essentially the same energy environment. It is hard to attribute the high level of glutamine synthetase activity directly to the high energy charge derived from the proportions of ATP or ADP in the sample animals. Further, the changes of glutamine synthetase activity in this study were the results of a long term effect, i.e. over a week rather than within several hours, indicating that the regulation of enzyme activity is probably mediated at transcription and/or translation level, not by modification of enzyme activity as described by Knight and Weissman (1982).

Glutamine synthetase is induced in bacteria such as *Escherichia coli* by depriving the cells of nitrogen (e.g. Rhee *et al.* 1989), in nodule-forming plants such as soybean by elevated ammonium input (e.g. Hirel *et al.* 1987), or in animals for ammonium detoxification (e.g. Campbell 1991). At present, nothing is known about the mode of regulation of glutamine synthetase in the eukaryotes, which is not controlled by the complex adenylylation/deadenylylation system found in most bacteria (White 1995). However, all the enzyme induction process, no matter what the underlying mechanism, are triggered ultimately by the metabolic needs of the cells. It should be noted that the symbiotic animals exhibit higher respiration rates (Muller-Parker 1984; Tyler & Davies 1984; Harland & Davies 1995) and growth rates than alga-depleted animals. To meet this more active status, the biosynthesis of DNA, ATP, and amino acids etc. in the symbiotic animals are expected to be higher than alga-depleted animals, and this should be reflected in increased activity of key enzymes, especially glutamine synthetase. Consistent with this hypothesis, glutamine did not accumulate in animals with high glutamine synthetase

Figure 6.2 Nutritional interaction models in the Cnidaria-alga symbiosis.

Abbreviations used: Resp, respiration; HF, host factor; P, photosynthesis; PhR, photosynthate release; Z, *Symbiodinium* cell; S, perisymbiont space; A, animal cell; NA, nitrogen assimilation; Tau, taurine; N, nitrogen.



activity, and its concentration was very low ($< 10 \text{ nmol mg}^{-1}$ protein). This was presumably because glutamine is utilized as a major donor of nitrogen in the biosynthesis of a wide range of important compounds, including some amino acids (e.g. asparagine, tryptophan, and histidine) and purine (Meister 1974). In conclusion, I propose that, by an unknown regulatory mechanism, the higher glutamine synthetase activity in the *A. pulchella* might be induced by the high demand for the synthesis of purines, pyrimidines, and amino acids in animals provided with carbon compounds.

6.5 Future directions

This study has provided evidence to modify the traditional understanding of Cnidaria-*Symbiodinium* association, and to support the role of algae in nitrogen conservation by the animal. In the traditional model (Fig. 6.2a), *Symbiodinium* assimilate carbon dioxide and ammonium derived from animal's respiration and nitrogen metabolism, and then provide the animal host with carbon compounds and amino acids. This recycling mechanism helps the animal hosts to survive in the low nutrient environment. The nitrogen conservation model (Fig. 6.2b) developed (after Rees and Ellard 1989) in this study suggests that animal hosts may use a signal (e.g. taurine in the *Aiptasia pulchella*-*Symbiodinium* symbiosis) to trigger the release of photosynthetic carbon (e.g. fumarate) from the algal symbiont. This extra carbon supplement provides the major substrates for animal's respiration, and reduces the extent of animal utilization of free amino acids as respiratory substrates. This model also suggests that it may be the recycling of nitrogen in the animals, principally via glutamine synthetase activity, and not nitrogen recycling by

Symbiodinium, that contributes to the persistence of Cnidaria-*Symbiodinium* association in low-nutrient habitats.

There are many interesting points worthy of further study. The unknown chemical identity of the so-called host factor(s) has always been a barrier to explore the nutritional interaction between the animal host and algal symbionts. In this study, the successful demonstration that taurine triggers photosynthate release by *Symbiodinium* has provided a basis for the study of host factor effect. Future research should address whether taurine may be the signal *in vivo*, and to explore how taurine diverts photosynthetic metabolism in *Symbiodinium* to the pathway for releasing photosynthetic products to the animal host. The capability of Cnidaria to synthesize essential amino acids, such as threonine and methionine will be another interesting point. Study of the genes coding for the biosynthetic enzymes for these amino acids in Cnidaria will help us understand more about the evolutionary history of nitrogen metabolism in these organisms. Unlike the studies on plant-*Rhizobium* or insect-*Buchnera* symbioses, nothing is clear about genes involved in symbiosis between Cnidaria and alga. There have been some attempts to explore animal protein profiles before and after depriving animals of algal population (Weis & Levine 1996), or algal protein profiles between freshly isolated *Symbiodinium* from symbiosis and cultured *Symbiodinium* (Stochaj & Grossman 1997); and several proteins had been found existing only in the symbiotic animals and *Symbiodinium* in symbiosis. The symbiosis-specific proteins could be the basis of searching for symbiosis-specific genes in the Cnidaria-*Symbiodinium* symbiosis.

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