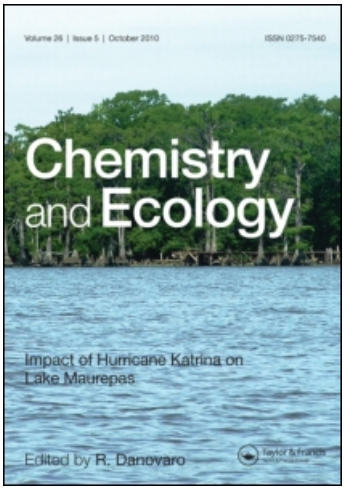

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The Effect of Altered Habitat on Nitrogen Metabolism in Some Free-Living and Symbiotic Relationships Involving Nitrogen Fixing Organisms

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Citrulline, one of the forms in which fixed nitrogen is assimilated in free-living blue-green algae and additionally in the blue-green algae/cycad symbiosis in *Macrozamia*, is similarly assimilated in the nitrogen fixing root nodules of *Alnus glutinosa*. By investigating the localisation of ornithine carbamoyl transferase in both cases it has been shown that in these symbiotic systems the ornithine carbamoyl transferase is only active in host tissue. This suggests that the host exerts an influence on the assimilation of fixed nitrogen in the microsymbiont resulting in the blocking of the enzyme trans-carbamylase with the subsequent excretion of the fixed nitrogen as ammonia to the host for further assimilation. This is discussed in the light of work on nitrogen metabolism in other symbiotic relationships involving nitrogen fixing organisms, where the effect of altering the habitat of the micro-organism has in fact resulted in a change in its metabolism.

INTRODUCTION

The factors responsible for the establishment of a symbiotic association between prokaryotic nitrogen fixing organisms and eukaryotic organisms are poorly understood. Stewart (1977) has speculated that the reason why blue-green algae are able to form symbiotic associations with a restricted but a very diverse group of plants is that the latter are capable of producing the requisite factor whereby the further metabolism of the nitrogen fixed in the prokaryote is switched to the eukaryote. In a series of papers dealing with the lichens and liverworts (1977) Stewart and his co-workers have shown that the key ammonia assimilating enzymes of the free-living blue-green algae were glutamine synthetase and glutamate synthase and that the former was largely inactivated when the blue greens were in symbiotic association with the eukaryotic host. The fixed nitrogen is thus excreted and

this transfer of nitrogen helps to explain the apparent anomaly that the symbiotic algae fix nitrogen at a high rate and yet appear, from various lines of evidence, to be nitrogen starved. The algal studies of the Stewart and Rowell (1977) group have been concerned in this context with the amino acid glutamine as one of the first products of nitrogen assimilation by the host species. Linko *et al.* (1957) had shown that free-living blue-green algae can produce high levels of citrulline as a primary product of nitrogen assimilation and Pate (1976) reported that this amino acid was also one of the principal nitrogenous compounds of the coralloid roots of the cycad *Macrozamia communis* and also that this compound was involved in the transport of fixed nitrogen from alga to host in this association. Citrulline has also been shown to be the first product of nitrogen assimilation in the nitrogen fixing root nodules of the alder tree, *Alnus glutinosa,* and to be the vehicle for the translocation of this fixed nitrogen from the nodules to the other parts of the plant (Leaf *et al.*, 1958: Gardner and Leaf, 1960). Leaf *et al.*, (*loc. cit.*) had assumed that the process of assimilation of this fixed nitrogen occurred within the endophyte.

The findings of Stewart and his co-workers on the lichen/liverwort symbioses with the switching off of the ammonia assimilation process in the prokaryote has encouraged us to investigate further the situation as it occurs in the cycad *Macrozamia* and in *Alnus glutinosa* with respect to the localisation of the synthesis of citrulline. The conversion of the fixed ammonia to citrulline involves the enzyme ornithine transcarbamoyl transferase and the availability of a technique for the localisation of this enzyme at the electron microscope level has made it possible to locate, with some degree of certainty, the site of the synthesis of the citrulline within the endophyte tissues and in this way to test the applicability of Stewart's findings to a wider group of eukaryotic symbioses.

MATERIALS AND METHODS

Coralloid roots from the cycad *Macrozamia communis* were obtained from the Royal Botanic Garden, Edinburgh. The root nodules of *Alnus glutinosa* were collected from alder trees growing near Milngavie, Glasgow.

The plant material on harvesting was immediately sliced and immersed in cold 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 to be later subdivided and fixed in fresh buffered fixative for 4 hours at 4°C in the laboratory. For localisation of the ornithine carbamoyl transferase (EC 2.1.3.3), the fixed tissue was rapidly washed and incubated in a modified Merker and Spors (1969) medium as follows: DL ornithine 2.6 mM, carbamyl phosphate (di lithium salt) 5 mM, lead nitrate 3 mM, tri-sodium citrate 5 mM, glucose 0.1 mM, Tris-maleate buffer, pH 7.2 50 mM. Incubation was at

25°C for 10 and 30 minutes and the reaction mixture minus ornithine was used as a control. After incubation the tissues were rapidly washed and post-fixed in 1% aqueous osmium tetroxide for 1 hour. The tissue then was routinely processed for electron microscopy using either Taab or Spurr's embedding resin. Sections cut on an LKB Ultratome III were viewed with or without staining in an AEI EM6B.

RESULTS

The symbiotic blue-green alga generally associated with *Macrozamia communis* is *Nostoc*. In the present investigations *Nostoc muscorum* has been used and in addition the localisation procedure was repeated with *Anabaena cylindrica*, a related filamentous and heterocystous species. The results obtained in the case of the latter species are indistinguishable from those obtained from *Nostoc*. In the vegetative cells of the free-living *Nostoc*, the reaction product of the enzyme localisation is seen to be associated with the plasma membrane and in addition with the plasma membrane of the cross wall region between adjacent cells (Figure 1). No free electron dense reaction product is discernible in the cytoplasm of these vegetative cells but it would appear to be present in the thylakoids and also in the cyanophycin granules (Figure 2). In control material (Figure 3) electron dense deposits can be seen within the cytoplasm but these are osmiophilic granules characteristic of the algal cells.

The localisation of the enzyme, as shown by the presence of reaction product, is shown to be similar in the heterocyst of *Nostoc muscorum* to that in the vegetative cells, being again clearly discernible on the plasma membrane (Figure 4). Vegetative cells in the free living state outnumber heterocysts by 33 to 1.

In the coralloid roots of *Macrozamia* the symbiotic *Nostoc* forms a distinct and obvious algal zone within the cortex, the algal cells themselves lying in intercellular space and thus assuming an extracellular location, and in this situation the vegetative-heterocyst cell ratio is altered to 3 to 1. In this symbiotic state, neither the vegetative cells (Figure 5) nor the heterocysts (Figure 6) appear to possess any active ornithine carbamoyl transferase since electron dense product was absent completely from both plasma membrane surfaces and from the thylakoids. These symbiotic algae were found to be deficient in cyanophycin granules, and this is in keeping with the view that in the symbiotic state these cells are nitrogen starved. The host cells in contrast show positive reaction product and this is most commonly associated with the elongate membrane stacks in the plastids (Figure 7).

The symbiotic actinomycete associated with the root nodules of *Alnus glutinosa* is intracellularly located and exhibits a variety of developmental

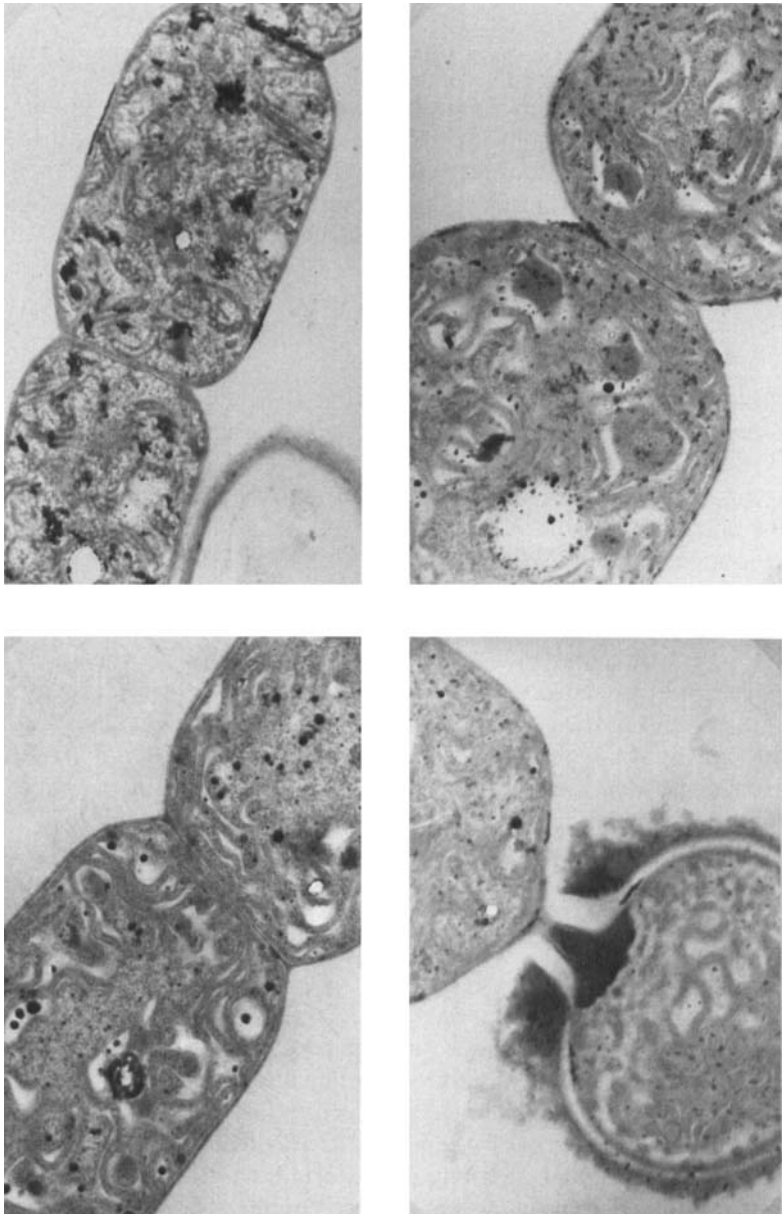

FIGURE 1 Free-living vegetative cells of *Nostoc muscorum* showing the reaction product associated with the lateral plasma membrane and with the plasma membrane of the cross wall region between adjacent cells. (× 15,000)

FIGURE 2 Part of the vegetative cells of *Nostoc* to show reaction product associated with the thylakoids and also with the cyanophycin granules. Again the enzyme is also localised on the plasma membranes. (× 23,300)

FIGURE 3 When ornithine was absent from the incubating medium no electron dense deposit was present, the osmiophilic granules are common to these cells. (× 20,250)

FIGURE 4 In the heterocyst of the free-living *Nostoc* the pattern of reaction product deposition is similar to that of the vegetative cells and can be seen on the plasma membrane and to a lesser extent on the thylakoids. (× 17,250)

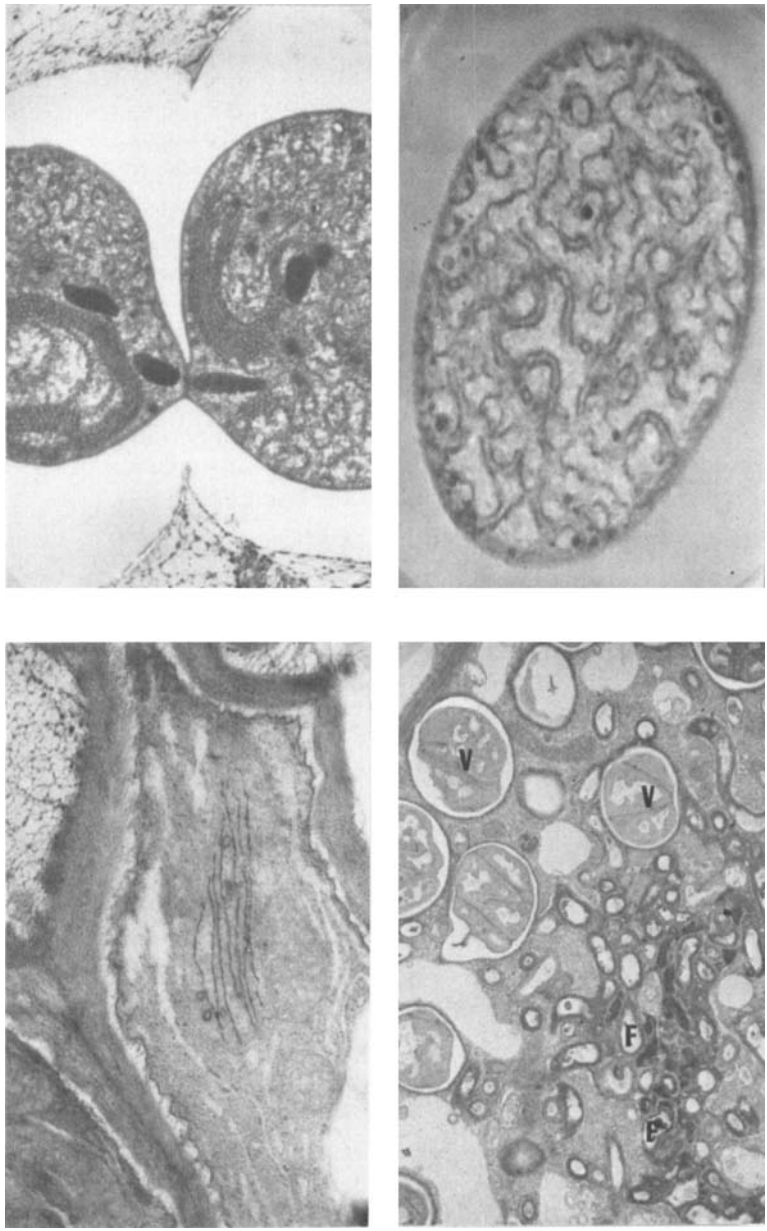

FIGURE 5 In the symbiotic algal cells in the coralloid roots of *Macrozamia* the vegetative blue-green cells show no reaction product on the plasma membrane or on the thylakoid lamellae. Dense osmiophilic deposits are obvious in the algal cytoplasm. (× 12,500)

FIGURE 6 No reaction product is seen in the endophytic heterocyst. Again osmiophilic deposit can be seen in the cytoplasm. (× 22,000)

FIGURE 7 Part of a host cell of *Macrozamia* showing reaction product associated with the elongate membranes of the plastids. (× 17,550)

FIGURE 8 Part of an infected cell of a root nodule of *Alnus glutinosa* showing the filamentous (F) and the vesicular (V) forms of the endophyte. (× 5,750)

stages in the infected cell, including hyphae and vesicles (Figure 8). In infected cells of the root nodule tissue electron dense reaction product was found to be absent from both hyphal and vesicular stages of the endophyte. In the host cell however, electron dense deposit was found only in the mitochondria after a 10 minute incubation period (Figure 9). After a 30 minute incubation, however, plastids in addition to mitochondria showed a positive reaction product. In this case the deposit was extremely fine in appearance and was associated with the central translucent regions (Figure 10). Host cell endoplasmic reticulum and Golgi were both singularly devoid of apparent enzyme activity. In the noninfected *Alnus* cell a similar distribution of enzyme reaction product was found as in the infected host cell. The results thus show that in this symbiosis the assimilation of the fixed nitrogen to citrulline takes place within the host cell.

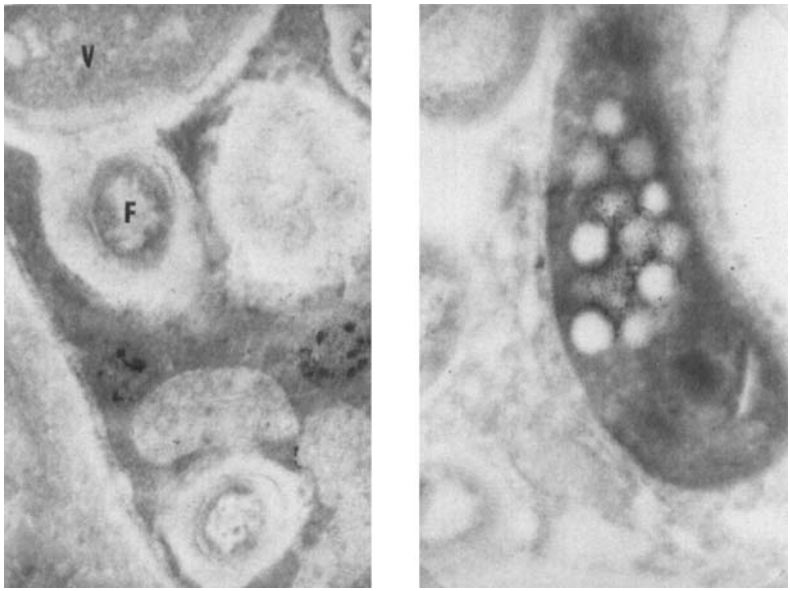

FIGURE 9 Part of an infected *Alnus* nodule cell after a 10 minute incubation for ornithine carbamoyl transferase activity. Reaction product is obvious in the host cell mitochondria but none is associated with either the filaments (F) or the vesicles (V) of the endophyte. (×22,000)
FIGURE 10 Part of an infected *Alnus* nodule cell after a 30 minute incubation for ornithine carbamoyl transferase activity, showing a finely granular deposit associated with the central translucent regions of the host cell plastids. (×37,500)

DISCUSSION

The findings of the present investigation show that the effect of the altered habitat i.e. the assumption of a symbiotic habit in both the blue-green algal/*Macrozamia* and in the actinomycete/*Alnus* association, would

appear to involve an alteration in the nitrogen metabolism of the microsymbiont but it must be borne in mind that in the latter case the symbiotic system only has so far been studied.

In both these associations it would appear that citrulline is a significant intermediate in the assimilation of the fixed nitrogen and further that the synthesis of this citrulline is effected only in the host cell. Earlier work by Gardner (1976) on alder nodules had shown that the enzyme ornithine transcarbamoylase was present in the host cytoplasm but that it also seemed to occur in the endophyte. This last however, was only found after longer incubation times and at higher temperatures that were employed in the present work and this might well have resulted in the removal of the host inhibition barrier which Stewart (1977) has suggested occurs in other symbiotic associations. Leaf *et al.*, (1958) and Gardner and Leaf (1960) have shown that in the alder citrulline in indeed the primary route of both assimilation and translocation of fixed nitrogen and that there would appear to be little evidence to suggest the involvement of glutamine in alder. Akkermans and Roeloesen (1980) have reported however that glutamine synthetase does occur in alder nodules and that it and glutamic dehydrogenase are detectable only in the cytoplasmic fraction of the host. In *Macrozamia* Pate (1976) has shown that both citrulline and glutamine are involved in the transport of nitrogen fixed in the coralloid roots. Stewart (1977) has shown that in the lichen and hepatic/blue-green symbiosis glutamine synthetase and thus the synthesis of glutamine is switched off in the microsymbiont and the fixed nitrogen excreted as ammonia to the host. Since citrulline, like glutamine, is synthesised directly from ammonia in *Nostoc* species in the free living state (Linko *et al.*, 1958) the absence of ornithine carbamoyl transferase from *Nostoc* in the symbiotic state while at the same time being present in the host organelles would suggest a parallel situation to that found for glutamine synthetase by Stewart.

The plastids would appear to be the site of citrulline synthesis in *Macrozamia*. Miflin (1974) has shown that the plastid fraction plays an extremely important role in nitrogen metabolism and amino acid synthesis in higher plant root cells and Miflin and Lea (1977) have stated that it is possible that plastids are the primary site of ammonia assimilation and amino acid biosynthesis in the plant cell. Shargool *et al.*, (1978) using fractionation procedures in soybean cells grown in suspension culture found ornithine carbamoyl transferase localised in a fraction composed primarily of plastids but their results also indicate detectable amounts of enzyme activity associated with the mitochondrial pellet. They further state that the relatively small amounts of ornithine carbamoyl transferase in the mitochondrial pellet can "be at least partly accounted for by some co-sedimentation of organelles and by the adsorption of unbound enzyme to

the mitochondria". Ornithine carbamoyl transferase has also been located within mitochondria in fungi (Weiss and Davies, 1973) and in animals (Merker and Spors, 1969; Gamble and Lehninger, 1973) and it may well be the case that in higher plants variation exists in the compartmentalisation of this enzyme.

It is of interest in this context that in free-living blue-green algae the enzyme ornithine carbamoyl transferase as well as having a location on the membrane system of the prokaryotic cell is also present in association with the cyanophycin granules. These last have been shown to be reserve material consisting of copolymers of arginine and aspartic acid (Simon, 1971), and a possible explanation for the presence of the reaction product in this location could well be that the citrulline is involved in the biosynthesis of arginine.

In the free-living state the prokaryote has been shown to exhibit normal nitrogen metabolism. In the symbiotic association the host is seen to exert its influence so that while the prokaryote still fixes nitrogen its ability to further metabolise this nitrogen is altered so that the fixed nitrogen is excreted to the host as ammonia. The exact manner in which the host further deals with this ammonia depends on the available enzymatic pathways and on the host species. The current findings for the blue-green/cycad symbiosis and the actinomycete/alder association together with those of Stewart (1977) for the lichen and the blue-green/hepatic symbioses and additionally the indirect evidence of Bergersen (1965) and Dilworth and Brown (1975) for the rhizobium/legume symbiosis would suggest that this induced change in the nitrogen metabolism of the prokaryote may well be of general occurrence in nitrogen fixing symbiotic associations.

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