Plant Physiol. (1978) 62, 463–467

Azolla-Anabaena Relationship

VII. DISTRIBUTION OF AMMONIA-ASSIMILATING ENZYMES, PROTEIN, AND CHLOROPHYLL BETWEEN HOST AND SYMBIONT[1, 2]

Received for publication March 22, 1978 and in revised form June 5, 1978

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ABSTRACT

The N_2-fixing *Azolla-Anabaena* symbiotic association is characterized in regard to individual host and symbiont contributions to its total chlorophyll, protein, and levels of ammonia-assimilating enzymes. The phycocyanin content of the association and the isolated blue-green algal symbiont was used as a standard for this characterization. Phycocyanin was measured by absorption and fluorescence emission spectroscopy. The phycocyanin content and total phycobilin complement of the symbiotic algae were distinct from those of *Anabaena cylindrica* and a free-living isolate of the *Azolla* endophyte. The algal symbiont accounted for less than 20% of the association's chlorophyll and protein. Acetylene reduction rates in the association (based solely on the amount of algal chlorophyll) were 30 to 50% higher than those attained when the symbiont was isolated directly from the fern. More than 75% of the association's glutamate dehydrogenase and glutamine synthetase activities are contributed by the host plant. The specific activity of glutamate dehydrogenase is greater than that of glutamine synthetase in the association and individual partners. Both the host and symbiont have glutamate synthase activity. The net distribution of these enzymes is discussed in regard to the probable roles of the host and symbiont in the assimilation of ammonia resulting from N_2 fixation by the symbiont.

The *Azolla-Anabaena azollae* symbiosis is an N_2-fixing association between a eukaryotic fern and a prokaryotic alga, both of which exhibit a higher plant type of photosynthesis (18, 19).

As an approach toward the characterization of the role of the host and symbiont[4] in the association, it was considered important to determine the contribution of the partners to the association's Chl, protein, and ammonia-assimilating enzymes. While the alga could be isolated free of the fern, its removal from the host was never complete. Thus it was necessary to find an independent quantitative estimate for the amount of alga in the association. While nitrogenase is unique to the alga (21) its lability makes it unsuitable for quantitation. Chl *a/b* ratios provided another estimate, but this was based on the absence of Chl *b* from the alga (20) and was therefore not as sensitive. A third method, the quantitation of phycobilins which are unique to the alga in the association, was employed. A procedure involving fluorescence emission spectroscopy at 77 K permitted quantitative measurement of phycobilins in the association, where the fern's Chl *b* would have precluded the use of absorption spectroscopy (1, 13).

The phycocyanin content of the isolated symbiont and that of the association were used to determine the contributions of the individual partners to the association's protein and Chl. It was then possible to estimate the distribution of principal ammonia-assimilating enzymes between the host and symbiont. This allowed an initial assessment of the probable roles of the individual partners in the assimilation of ammonia resulting from nitrogen fixation. For comparative purposes data are also presented on the nonsymbiotic blue-green alga *Anabaena cylindrica*, the free-living endophyte from *Azolla caroliniana* (16), and algal-free *Azolla* plants.

MATERIALS AND METHODS

GROWTH OF ORGANISMS

Plants. *A. caroliniana* Willd. was grown as described previously (18, 20) except that the N-free nutrient solution contained 0.03% NaCl. For growth of algal-free *Azolla* 4 mM No_3^- was added. All cultures were maintained using a cycle of 16 hr light (22–24 C) and 8 hr dark (18–20 C). Illumination of approximately 450 ft-c was provided by Daylight and Gro-lux fluorescent plus incandescent lights. Cultures used in experiments involving phycocyanin determinations were transferred weekly. This helped maintain a uniform frond growth pattern and assured that cultures were of a comparable age. Moreover, these cultures were maintained within a 0.5 m^2 area to minimize any gradients in temperature or light.

Algae. The symbiotic alga was isolated from *Azolla* plants immediately prior to use by the "gentle roller method" (20). *Anabaena* strain 1a, an isolate from *A. caroliniana* capable of growth in independent culture (16) was obtained from J.W. Newton. This isolate and *A. cylindrica* 1403/2a were grown on an N-free medium (14). The algal cultures were grown at 400 ft-c, and 24 C, with constant aeration and agitation.

PHYCOCYANIN DETERMINATIONS

Association. The *Azolla-Anabaena* association and, as a control, algal-free *Azolla* plants, suspended in buffer, were ground with a motor-driven Teflon homogenizer. The buffer, containing 50 mM phosphate (pH 7.3), 10 mM DTT, 20 mM ascorbate, and 1% (w/v) Polyclar AT, was used at 2.5 ml/g fresh weight of plant material. Homogenates were passed twice through a French press at 16,000 p.s.i., centrifuged at 72,000*g* for 30 min, and supernatants employed for phycocyanin determinations.

Algae. The symbiotic algae and free-living algal cultures were centrifuged, washed, resuspended in phosphate buffer, passed twice through the French press, centrifuged, and the final supernatants used as above.

The phycocyanin concentration of algal extracts was determined initially by absorption spectroscopy (13) with a Cary 118 recording spectrophotometer. A dilution series of known concentrations of phycocyanin in 50% glycerol was then used to obtain a plot of

[1] Supported in part by NSF National Science Foundation Grant DEB 74-11679 A01.
[2] Contribution No. 620 from the Charles F. Kettering Research Laboratory.
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[4] "Symbiont" is used throughout this paper in reference to the algal partner.

fluorescence intensity *versus* concentration. This was linear over at least two orders of magnitude and all samples were diluted so as to fall within this linear range. Excitation light at 557 nm was isolated from the output of a Sylvania DXM tungsten-halogen lamp with a Bausch & Lomb 0.25-m monochrometer. In order to minimize stray light, a 555 nm broad band (20 nm) Baird-Atomic filter and a Ditric Optics filter cutting off wavelengths greater than 660 nm were placed in the exciting beam. The fluorescence was measured between 600 and 740 nm using a B and L 500 monochrometer and an EMI 9558 photomultiplier tube. A Corning 2434 filter was placed in the analyzing monochrometer to remove scattered light. The sample cuvette had a path length of 0.17 mm. The spectra were corrected for the spectral response of the system. Fluorescence generated by 557 nm excitation of a Wratten No. 26 filter was used as an internal standard to obtain daily checks of the system's response.

ACETYLENE REDUCTION ASSAYS—NITROGENASE

Acetylene reduction assays were conducted as described previously using a gas phase of 10% C_2H_2, 0.03% CO_2 in argon (21, 23).

OTHER ENZYME ASSAYS

Azolla plants with and without the symbiont were ground as above in a buffer (5 ml/g fresh weight) containing 50 mM phosphate (pH 7.3), 10 mM DTT, and 1% (w/v) Polyclar AT. The homogenized material was passed twice through a French press at 16,000 p.s.i. and centrifuged at 30,000*g* for 15 min. Extracts of the symbiotic and free-living algae were prepared in a similar manner. Aliquots of the 30,000*g* supernatants were passed through Sephadex G-25 equilibrated with 50 mM phosphate (pH 7.3) and 3 mM DTT, and the desalted extracts used for enzyme assays. Activities were corrected for dilution on the Sephadex columns and the specific activities were based on the total protein measured in the extracts prior to centrifugation.

NADH-dependent GDH[5] was assayed according to Pruisner *et al.* (24). GS was assayed by both the coupled biosynthetic assay and the transferase assay (27). In the former assay, controls without glutamate were routinely run to correct for endogenous NADH oxidation. For the transferase assay the extracts were prepared as described above except the extraction buffer contained 2 mM EDTA, K_2HPO_4, $MgCl_2$, and $CaCl_2$, 20 mM KCl, 50 mM HEPES (pH 7.6), 20 mM ascorbate, 0.33 M sorbitol, 5 mM DTT, and 5% (w/v) Polyclar AT; the G-25 column elution buffer contained 10 mM imidazole and 1 mM $MnCl_2$.

GOGAT activity was determined by measuring [^{14}C]glutamate formation from [^{14}C]glutamine. Reaction mixtures contained 50 mM HEPES (pH 7.5), 5 mM α-ketoglutarate, 10 mM [^{14}C]glutamine (0.05 μCi/μmol, New England Nuclear), either 0.2 mg of spinach ferredoxin (type III, Sigma) or 1 mM methyl viologen, 9 mM $Na_2S_2O_4$, and enzyme in a total volume of 1 ml. Dithionite solutions were prepared under argon just prior to use. Sealed vials containing the reaction mixture less dithionite were evacuated, flushed several times with argon, and the reaction initiated by injection of the dithionite solution. Controls did not receive dithionite. Assays were at 30 C and terminated by adding 1 ml of acetone. The [^{14}C]glutamate was separated from the reaction mixture supernatant by ion exchange chromatography using Bio-Rad AG 1-X4 200–400 mesh Cl form resin (25) equilibrated with 10 mM imidazole (pH 7). The reaction mixture supernatants were passed through the resin and washed with 3 to 5 ml of 10 mM imidazole buffer (pH 7) prior to eluting the glutamate with 1 N HCl. Radioactivity in the fractions was determined by liquid scintillation counting. Reaction products were verified by cochromatography with glutamine and glutamate standards on Gelman ITLC type SA sheets with a solvent system of phenol-water-ammonia (75:21:4, v/v/v).

CHL AND PROTEIN

After ethanol extraction, Chl was determined according to the method of Wintermans and DeMots (30). Protein was precipitated with 10% trichloroacetic acid (w/v), washed several times with 95% ethanol, and dissolved in warm 0.1 N NaOH. Insoluble material was pelleted by centrifugation and protein determined on the supernatant by the method of Lowry *et al.* (12).

USE OF PHYCOBILIN CONTENT TO ESTABLISH SYMBIONT CONTRIBUTION TO ASSOCIATION'S CHL, PROTEIN, AND ENZYME ACTIVITY

Two methods were used to estimate the algal contribution to the association from which it was isolated. (*a*) The phycocyanin content of a known fresh weight of fronds (usually 2 g) was determined by fluorescence. The symbiont was then isolated from a known weight of fronds (usually 10–15 g) from the same culture and the total phycocyanin content of the algal isolate determined. The phycocyanin content of the association was used to calculate the phycocyanin content of the frond material from which the symbiont was isolated. The amount of phycocyanin actually recovered in the isolated algal fraction, divided by the calculated total phycocyanin in the frond material, is equivalent to the fraction of the symbiont isolated. This fractional value could then be used to estimate total algal Chl, protein, and enzyme activity in the association. (*b*) The amount of protein, Chl, and enzyme activity/mg of phycocyanin was determined for *A. azollae* isolated from the association. These values, multiplied by the amount of phycocyanin in extracts of the association, yield the fraction of protein, Chl, and enzyme activities which is algal.

RESULTS

Absorption and 77 K fluorescence emission spectra of phycobilins in 70,000*g* supernatants of cell-free extracts from the nonsymbiotic, free-living blue-green alga, *A. cylindrica,* a free-living isolate of the *Azolla* endophyte, the endophyte immediately after isolation from the host plant and the *Azolla-Anabaena* association are compared in Figure 1, a through d. While the absorption maxima of the phycobilins from the two free-living blue-greens occur at about 620 nm, those from the symbiotic algae and the association occur at 610 nm. The asymmetry or slight shoulder on the blue side of the absorption maxima, which is most apparent in the spectrum of the symbiotic alga (Fig. 1c), may result from a phycoerythrin-type phycobilin. In accord with the absorption maxima, there is a similar shift in the fluorescence emission maxima. While the major fluorescence of the phycobilins from the free-living algae (Fig. 1, b and d) occurs at 645 to 647 nm, agreeing well with the emission peak of C-phycocyanin (2), those from the symbiotic alga (Fig. 1c) and the association (Fig. 1a) exhibit a maximum at 635 nm with a shoulder at 655 nm. The major emission is presumably from a phycocyanin-type phycobilin while the shoulder may indicate a form of allophycocyanin. In comparing these spectra it is important to remember that the fern leaf acts as a green filter. Presumably the symbiotic alga is chromatically adapted to its niche and it may have phycobilins that differ slightly in their spectra from those of free-living species.

As shown in Figure 1, a and c, the fluorescence emissions of the extracts from the association and symbiotic alga removed from the fern are similar. While the absorption spectra show more residual Chl associated with extracts from the association than from the symbiont or other algae, due to the preponderance of

[5] Abbreviations: GDH: glutamate dehydrogenase (EC 1.4.1.2); GOGAT: glutamate synthase (EC 2.6.1.53); GS: glutamine synthetase (EC 6.3.1.2).

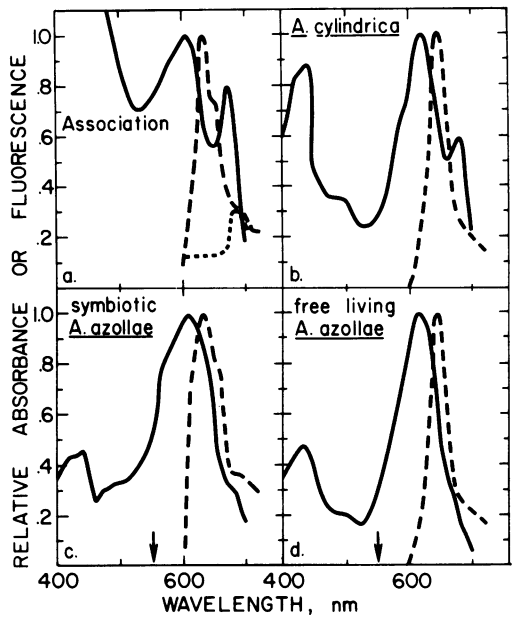

FIG. 1. Absorption (——) and fluorescence emission (- - -) spectra of phycobilins in 70,000*g* supernatant from cell-free extracts of: (a) *Azolla-Anabaena* association and fluorescence emission from algal-free *Azolla* (...); (b) blue-green alga, *A. cylindrica*; (c) symbiotic alga, *A. azollae* isolated from the association; (d) free-living endophyte of *Azolla (Anabaena* strain 1a). Arrows indicate the excitation wavelength (557 nm) used to obtain fluorescence emission spectra. Absorption maxima: (a) and (c), 610 nm; (b) and (d), 620 nm. Fluorescence emission maxima: (a) and (c), 635 nm; (b) and (d), 647 nm.

host material employed in the extraction, the host plant makes no contribution to the fluorescence between 600 and 660 nm. This is shown by the emission spectra of algal-free fern plants in Figure 1a. Thus, the fluorescence emission at 685 nm in these spectra is from Chl-containing membrane fragments which were not sedimented by centrifugation.

The phycobilin content of the association and isolated symbiont may be slightly underestimated in this study since phycocyanin alone was used to quantitate the fluorescence but lesser amounts of other phycobilins are indicated. In determining the symbiont's contribution to the association, any error in the actual phycobilin content will be the same for the association and the symbiont isolated from it.

Protein, Chl, and Phycocyanin Levels in Association and Symbiont. Chl, protein, and phycocyanin content of the *Azolla-Anabaena* association and the symbiont's contribution to them are given in Table I. Under the growth conditions employed, the symbiont contributes about 12 to 22% of the association's total protein and 11 to 21% of its total Chl. The average Chl contribution from the symbiont compares well with a previously reported value of 7 to 15% calculated from Chl *a/b* ratios (20). In the symbiont 4 to 10% of the total protein is phycobilin while a value of 30% was obtained with *A. cylindrica*. Previous workers have found similar amounts of phycocyanin in *A. cylindrica* (6).

Algal-free fronds require nitrate for growth and are morphologically distinct from plants containing the symbiont (10, 18). These plants contained 0.512 mg of Chl and 16.2 mg of protein/g fresh weight. They do not reduce acetylene and their extracts do not contain phycobilins.

Nitrogenase Activities-C_2H_2 Reduction. Results of three separate experiments in which C_2H_2 reduction was determined for the association and the symbiont isolated from the respective culture of the association are given in Table II. The values are the average of duplicate or triplicate analyses and the individual averages reflect the normal variation among cultures grown under the same conditions. Over-all, the average rate of C_2H_2 reduction by the association was 17.8 ± 5.2 nmol of C_2H_4/mg of Chl · min. Rates of C_2H_2 reduction by the symbiont parallel those of the respective culture of the association from which it was isolated, but are appreciably higher since they are based only on the algal Chl. These rates generally appear to be 50 to 70% of the actual rate of C_2H_2 reduction by the symbiont in the association. This is estimated by basing C_2H_2 reduction rates of the association on the algal contribution to the total Chl and suggests that there may be an inhibition of nitrogenase activity and/or other physiological processes during isolation of the symbiotic alga. Moreover, after isolation it is not possible to reestablish the environment of the host leaf chamber.

In companion studies the free-living *Azolla* endophyte and *A. cylindrica* had C_2H_2 reduction rates of 137 nmol of C_2H_4/mg of Chl · min and 98 nmol/mg of Chl · min, respectively. Although the assay conditions were the same, these rates are relevant only for the growth conditions employed and caution is warranted in comparing rates in the free-living organisms and the estimated rates of the association.

Ammonia-assimilating Enzymes. The relative roles of the host and symbiont in the utilization of ammonia produced during N_2 fixation were investigated by measuring GDH, GS, and GOGAT activities in the association and its symbiont. For comparative purposes, GDH and GS activities were also determined in extracts of the free-living *Azolla* endophyte and *A. cylindrica* grown under N_2-fixing conditions and algal-free *Azolla* plants grown on nitrate. Table III shows the specific activities of these enzymes, on a protein basis, in crude extracts of the various organisms. Activities of the isolated symbiont are from the same culture as the association. GDH activity was always higher than GS (coupled assay) in the association and the symbiont. This was also the case for algal-free *Azolla* plants grown on nitrate. Moreover the symbiotic algae had much higher GDH activity than either of the free-living algae. In N_2-fixing cultures of the free-living *Azolla* endophyte, GDH activity was below levels of detection while the GS activity (22 nmol/mg of protein · min) was about twice that found in the symbiont and equal to that found in *A. cylindrica*. Isolates of the symbiont contain some epidermal hairs which line the leaf cavity in the association and these hairs have GDH activity when freed of the algal symbiont. However, the symbiont freed of the hairs still exhibits comparable GDH activity. The symbiont appears to be distinct from the free-living isolate and nonsymbiotic species in the enzymes of ammonia assimilation as well as in its phycobilin content and complement.

Table I. Chlorophyll, protein, and phycocyanin in the association and isolated symbiont

Values are the mean of 3 determinations ± the standard deviation. The average percentage of chlorophyll and protein contributed to the association by the symbiont are in brackets.

	mg/g fresh wt of Association		
	Chlorophyll	Protein	Phycocyanin
Association	0.465 ± 0.05	13.4 ± 3.6	0.139 ± 0.07
Isolated Symbiont	0.075 ± 0.03 (16.1%)	2.2 ± 0.12 (16.4%)	- (100%)

Table II. Acetylene reduction in the Azolla-Anabaena association, the isolated symbiont, and in the association when based on the symbiont's chlorophyll content.

	nmoles C_2H_4/mg chl·min		
	Association	Isolated Symbiont	Association based on Symbiont Chlorophyll
Expt.			
1	11.9	79.6	131
2	19.9	87.7	199
3	21.6	109.0	137
Avg	17.8±5.2	92.1±15.1	155±37

In order to compare the specific activities of enzymes in the association, symbiont, and free-living algae, the activities were based on protein in the extracts prior to centrifugation. This was necessary since differing amounts of soluble protein were found in the association and symbiont. For example, the specific activity of GS in the association based on protein in the extract prior to centrifugation was 22 nmol/mg of protein · min but increased 4-fold to 88 nmol/mg of protein · min when based on protein in the G-25-treated extract after centrifugation at 30,000*g*. In the symbiont, however, the specific activity was increased only 1.5 times, from 16 to 24 nmol/mg of protein · min.

In addition to GS as measured by the coupled biosynthetic assay, crude extracts from all sources exhibited the γ-glutamyl transfer reaction (transferase) of GS. The ratio of transferase to coupled biosynthetic activity was from 12 to 20 for the association, isolated symbiont, *A. cylindrica,* and the free-living endophyte, and 33 for algal-free fronds. The cause of this variation is not yet known.

GOGAT activities of the association and symbiont were 51 and 33 nmol/mg of protein · min, respectively. Activity was dependent upon α-ketoglutarate, either methyl viologen or ferredoxin as a reductant, and dithionite. The formation of [^{14}C]glutamate was linear for at least 15 min and proportional to enzyme concentration to at least 0.9 mg of protein providing that anaerobic assay conditions were maintained. There was no detectable pyridine nucleotide-dependent activity.

The average specific activities of GS and GDH in the association, host, and symbiont as well as the percentages of the total activities in the association attributable to the individual partners are given in Table IV. Activities are based on total protein of the association and that part contributed by the individual partners. Host plant activities were established from the difference between the total protein and total activities in the association and those in the symbiont. The algal contribution to the association's protein was based on the phycocyanin content and the results are the average of three experiments. Although there is little difference in the specific activity of GS in the host and symbiont, the symbiont accounts for only 11% of the total GS activity in the association. In the case of GDH, the specific activity was somewhat higher in the symbiont than in the host and 21% of the association's GDH activity is contributed by the symbiont. Epidermal hair cells were not removed from these algal preparations and the values for the enzyme activities in the symbiont include their activities as well. Thus, the per cent of the total activities attributed to the symbiont may be slightly high. These values for enzyme activities are an average of those contributed by the host and symbiont in all stages of the association's development.

Table III. Specific activities of ammonia assimilating enzymes in extracts of the Azolla-Anabaena association; the symbiotic and free-living endophytes; A. cylindrica; and algal-free Azolla.

Enzyme	Glutamine Synthetase		GDH[a]	GOGAT[c]
	Coupled Biosynthetic Activity[a]	Transferase Activity[b]		
Association	26	417	46	51
Symbiotic A. azollae	11	154	45	33
Free-living A. azollae	22	440	N.D.*	-
A. cylindrica	22	281	5	-
Algal-free Azolla	6	197	41	-

* not detectable

[a] nmoles NADH oxidized/mg protein·min

[b] nmoles γ-glutamylhydroxamate formed/mg protein·min

[c] nmoles glutamate formed/mg protein·min

Table IV. Specific activities, total activities and percent of GS (biosynthetic) and GDH in the Azolla-Anabaena association, the Azolla host, and the Anabaena symbiont.

Specific activities are averages of 3 determinations ± the standard deviation

	Association	Azolla (host)	Anabaena (symbiont)
GS Activity			
Specific[a]	15±5	16±6	11±4
total[b]	188±35	168±31	20±13
% of total	100	89	11
GDH Activity			
Specific[a]	34±15	24±6	40±10
total[b]	277±16	219±20	58±22
% of total	100	79	21

[a] nmoles NADH oxidized/mg protein·min

[b] nmoles NADH oxidized/g fresh wt of association·min

DISCUSSION

Although symbiotic species of *Nostoc* or *Anabaena* occurring in associations with liverworts and the angiosperm *Gunnera* lack or have very low levels of phycobilin (26, 28), absorption and fluorescence emission spectra show that the phycobilins occur in the *Azolla* symbiont and differ from those of the free-living *Azolla* endophyte and *A. cylindrica.* Compared to free-living blue-green algae, both the absorption and fluorescence emission maxima of the symbiont's phycocyanin are displaced toward the blue, indicating an altered phycobilin complement. This difference may be due to a chromatic adaptation by the symbiont. Since in the *Azolla-Anabaena* association the symbiont occupies cavities in the dorsal leaf lobes of the fern, the light reaching the alga is altered by the filtering effect of the photosynthetic pigments in the leaf tissue surrounding the cavity.

Chromatic adaptation occurs in heterocystous blue-green algae (3). The observation that the phycobilins of the free-living isolate of the *Azolla* endophyte are more like those of *A. cylindrica* than those of the symbiont also suggests that the symbiont is chromatically adapted to its niche.

Fluorescence emission spectroscopy affords an extremely sensitive and specific means of quantitating small amounts of phycobilins in extracts of blue-green algae. However, the method is subject to limitations, and precautions were taken. Extracts were prepared in phosphate buffers of low ionic strength further disrupting phycobilisomes and minimizing internal energy transfer (7). The majority of the Chl-containing membrane fragments were removed by centrifugation, and measurements were conducted on dilute samples at 77 K.

Under the growth conditions used here, the phycocyanin contents of the free-living endophyte and *A. cylindrica* were 140 and 300 μg/mg of protein, respectively. These values apply only to the growth conditions employed since the phycocyanin content varies with culture density and growth conditions, *i.e.* light intensity (8) and nitrogen status (4, 29). Although the symbiotic algae contained only 70 μg of phycocyanin/mg of protein, the microenvironment within the plant leaf and other restrictions imposed by the symbiotic state preclude a direct comparison with the phycocyanin content of the free-living algae. Because heterocysts lack phycobilins (29) the diminished phycocyanin content of the symbiotic algae is consistent with its high average heterocyst frequency (10, 17).

These studies demonstrate that in the association both partners have the capacity to synthesize glutamate, either through GDH or by the GS-GOGAT pathways. How these enzymes are integrated into the assimilatory pathway of ammonia from N_2 fixation is still subject to speculation.

In the plant apex, relatively undifferentiated and actively dividing filaments of the symbiont occur among leaf primordia (11, 22). As leaf development proceeds, filaments of the symbiont colonize cavities formed in the aerial dorsal leaf lobe. In conjunction with this, multicellular hairs, some of which have transfer cell characteristics, are differentiated from host cells during cavity formation (5, 22) and the symbiont exhibits a decreased cell division along with cell enlargement and a large increase in heterocyst frequency (10). The latter is paralleled by an increase in C_2H_2 reduction capacity of the individual leaf lobes (11). Due to the developmental pattern of this association (11, 22) the values for enzyme activities as well as Chl and protein attributed to the host and symbiont necessarily represent an *average* of their respective total contributions. Although the enzymes of ammonia assimilation are present in the heterogeneous population of the isolated symbiont, they may be localized in algal filaments found in specific developmental stages of the association. For example, the excretion of recently fixed $^{15}N_2$ as NH_3 by isolated algal filaments (19) and the high intracellular levels of NH_3 found in the association under N_2-fixing conditions (15) suggest that filaments which actively fix N_2 may have a diminished capability to metabolize the resulting ammonia. These filaments would logically be those exhibiting a high heterocyst frequency and found in mature leaf cavities. If this is the case, algal GS with its high affinity for NH_3 may be localized in filaments associated with young leaves. Since these filaments have a low heterocyst frequency and nitrogenase activity (11), they may require fixed N_2. Furthermore, a complementary gradient may occur in the host. The increased levels of GDH found in the algal partner may be a characteristic of this symbiotic association.

As shown here (Table III) and previously (9), GDH activity is very low in free-living blue-green algae. Although hair cells are present in the isolated algal fractions, preliminary studies have shown that they contributed less than 5% of the total protein and there was no significant loss of GDH activity in algal fractions from which they were removed. In accord with the release of newly fixed N_2 as ammonia (19), GDH with its lower affinity for ammonia may regulate the utilization of ammonia by the symbiont and occur mainly in filaments which actively fix N_2 and are found in mature leaf cavities. A complete understanding of the assimilation of N compounds in this association will require the localization of enzymes of ammonia assimilation in relation to specific tissues, cells, and organelles, as well as developmental gradients in the association.

Acknowledgments—The authors wish to thank W. Evans, D. Keister, and J. Robertson for their helpful discussion and comments on the manuscript, and R. Neal for clerical assistance.

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