Aspects of Nitrogen and Carbon Interchange in the *Azolla–Anabaena* Symbiosis

G.A. Peters,* D. Kaplan,† J.C. Meeks,‡ K.M. Buzby,*
B.H. Marsh,* J.L. Corbin*

*Battelle-C.F. Kettering Research Laboratory, Yellow Springs, OH 45387, USA. †The Jacob Blaustein Desert Research Institute, Ben Gurion University, Sede Boquer Campus, Israel. ‡Department of Bacteriology, University of California, Davis, CA 95616, USA.

The free-floating aquatic pteridophytes in the genus Azolla contain an N_2-fixing cyanobacterium, Anabaena azollae, as a symbiont. The genus is widely distributed, occurring in relatively placid tropical and/or temperate freshwater environments. Six extant species usually are recognized. In each species the symbiotic Anabaena can provide the total N requirement of the association via N_2 fixation and the Azolla sporophytes are capable of prolific vegetative reproduction in the absence of a combined N source [1,2]. The practical significance of this attribute is demonstrated by their use as a biofertilizer for rice [3-6].

While vegetative reproduction and growth is more common, sexual reproduction occurs and presumably provides a means for survival during unfavorable environmental conditions. Azolla is heterosporous, producing both megasporocarps and microsporocarps on the same plant. Species demarcation is based primarily upon the morphology of these structures [6,7]. Anabaena filaments are partitioned into both types of sporocarps during their development. The endophyte within the megasporocarp provides an inoculum for the developing sporophyte, maintaining the symbiosis.

This report is restricted to studies conducted with the vegetatively propagated Azolla caroliniana - Anabaena azollae symbiosis grown on medium without combined N [2]. It focuses on recent approaches to further elucidate aspects of host-endophyte interaction and incorporates analyses of soluble amino acids and ammonium, assays of ammonia assimilating enzymes, the use of $[^{13}N]N_2$ and the analysis of soluble sugars as well as $^{14}CO_2$-pulse chase studies. For orientation purposes the results are preceeded by a description of the symbiosis.

Salient Features of the Symbiosis

A. caroliniana sporophytes have branched floating stems bearing deeply bilobed, alternately arranged leaves and adventitious roots. An ontogenetic sequence of the host and endophyte is expressed along each axis of the floating stems [8-10]. A colony of undifferentiated, or generative [9], Anabaena filaments is associated with the apical meristem of each stem. The meristem and Anabaena filaments are enclosed and protected by the developing bilobed leaves. As these leaves differentiate, some of the filaments from the Anabaena colony are partitioned into forming cavities at the base of the aerial dorsal leaf lobes. This process is facilitated by a specialized epidermal trichome termed the primary branched hair [PBH] which consists of a stalk cell, two or three body cells and several finger-like terminal cells [10,11]. The terminal cells of this rapidly differentiated trichome protrude into the Anabaena colony and Anabaena filaments become entwined around them. Subsequently the PBH and associated filaments are engulfed by the forming cavity [11]. As development and maturation proceed a second branched hair and a distinct population of up to 25 simple hairs, composed of a stalk cell and a single terminal cell, are formed in each cavity [10]. The two branched hairs occupy a well defined position in each cavity and are located on the path of the foliar trace. Simple hairs are randomly distributed around those portions of the cavity bordered by mesophyll [10]. Ultrastructural studies have shown that all hairs initially develop transfer cell ultrastructure (TCU), i.e. ramified cell wall elaborations and organelle-rich cytoplasm, in their terminal cells and

Copyright 1985 by Elsevier Science Publishing Co., Inc.
Paul W. Ludden and John E. Burris, editors
NITROGEN FIXATION AND CO_2 METABOLISM

exhibit a distinct basipetal pattern of TCU differentiation and senescence as a function of leaf age [11, Calvert, unpublished].

The occurrence of a parallel developmental profile in the endophyte as a function of leaf age was originally described by Hill [8] using sequentially dissected individual leaves from stem axes of A. filiculoides. These observations have been confirmed and extended in A. caroliniana [11-13]. The generative Anabaena filaments in the apical colonies lack heterocysts and nitrogenase activity. Heterocyst differentiation commences as the PBH and associated filaments are engulfed within the forming cavity. By the time a cavity has its full complement of epidermal trichomes, cell division in the endophyte is greatly diminished, its cells have enlarged, the heterocyst frequency approaches 30% and nitrogenase activity is maximal. This activity declines as the leaves begin to senesce.

The dry matter and N content are highest in the apical segments of stem axes and the C/N ratio increases with leaf age [13]. Pulse-chase studies using $^{15}N_2$ showed that N_2 fixed in mature cavities is transported toward the stem apices [13]. Heterocysts of the endophyte retain phycobiliproteins (PBP) which are effective in harvesting light energy for N_2 fixation [14]. The PBP content increases with leaf age and there is no significant alteration of the PBP complement [15] as a function of leaf age and heterocysts frequency [16].

Soluble Amino Acids and Ammonia

Insight on N metabolism and host-endophyte interaction has been sought by the analysis of ammonia or/and soluble amino acids in the association and its components. The latter included intact stem axes along with their dissection into the apical segment and the remainder of the axes [10,11] and preparations of the endophyte with associated cavity hairs [12] as well as the endophyte freed of the hairs and the simple and branched hairs themselves. Details of the procedure for isolation of the hairs, which includes sieving with nylon mesh and use of Percoll gradients, will be presented elsewhere along with micrographs illustrating purity of the preparations. Since the isolation procedure breaks the more fragile stalk cell of the hairs near the site of attachment to the cavity wall, the cell membrane has been ruptured in the majority of the stalk cells. This is readily demonstrated using the vital stain, fluorescein diacetate [17]. Thus the soluble amino acids and enzymatic activities of the hair cell preparations are attributed primarily to the terminal cells of the simple hairs and terminal cells plus body cells of the branched hairs.

Soluble amino acids were determined by HPLC of their OPT derivatives using the general approach of Jones and Gilligan [18]. Rapid analyses were conducted using a Brownlee Labs guard column (3 cm, OD-GU, 5 µm). When better resolution was desired, analyses were carried out with a 15 cm Ultrasphere column (ODS, 5 µm, Altex). Ammonia was determined separately with HPLC following reaction with OPT-mercaptoethanol at pH 7.3 [19]. Cellular material was extracted with boiling water for 10-15 min, centrifuged and the supernatants analyzed.

HPLC analysis was consistent with the results obtained using $^{15}N_2$ [20] and $^{14}CO_2$ [21] in showing that the endophyte isolated from the leaf cavities released ammonia but little or no organic N. Analysis of centrifuged media [22] at intervals during a 3 hr incubation under 0.03% CO_2 in N_2 and under 0.03% CO_2 in Ar revealed that ammonia increased at a rate of 135 nmoles/mg protein·min under N_2 in the light. No ammonia production occurred under N_2 in the dark or under Ar in light or dark. During the 3 hr incubation in the light only aspartate and glutamate increased significantly, about 3 fold, under both N_2 and Ar.

In the association, as well as in the intact stem axes and their segments, the major constituents of the nitrogenous pools are free ammonia, glutamine and glutamate (Table 1). As reported by others [23], nitrogenous pools vary with culture density. However, the material used here is all

from recently transferred cultures and the increased levels of ammonia and glutamine in the stem axes and segments relative to the association are attributed to the selective trimming. The association values include contributions from roots and old leaf tissues. Stem axes were restricted to segments of 10-12 leaves with roots removed and on a fresh weight basis are enriched in leaf cavities where the endophyte exhibits maximal N_2ase activity [12,13]. Glycine and threonine were not completely resolved by the rapid analysis used in these determinations and have been combined for purposes of quantitation. The component denoted X is the third most abundant amino acid and preliminary evidence indicates that it is a glutamate derivative. Although Newton and Cavins (24) found cystathionine to be the third most abundant amino acid in extracts of A. caroliniana, we have not obtained evidence for its occurrence with our procedures.

Since apical segments have a higher dry matter content than the rest of the axes [13], the higher concentrations of cell amino acids on a fresh weight basis is not entirely unexpected. However, as the endophyte in these segments lacks any appreciable nitrogenase activity [12,13] the levels of ammonia, glutamine, X, and perhaps to a lesser extent glutamate, are noteworthy and will be considered below in conjunction with $[^{13}N]N_2$ pulse-chase studies. The apical segments account for about 25% of the fresh weight of the axes. Thus, the concentrations of individual soluble amino acids, ammonia and protein in the axes are approximated by 0.25 (apical value) + 0.75 (remainder value). For glutamine, this approximation yields 2.73 µmoles/g fr wt for the sum of the segments compared to 2.66 ± 0.65 for the segment itself.

Table II gives the relative amounts of indivdual amino acids expressed as a percent of the total from three preparations of the endophyte with and without cavity hairs and in the two types of hairs. The calculations are based on the integrated areas of the individual amino acids. While not quantitative, they do allow a comparison of the composition in the individual fractions. Quantitative values (nmoles/mg protein) are shown for two of the endophyte preparations ± cavity hairs and the simple hairs from a single preparation in Table II. Protein values were not attained for any fraction in one preparation and availability of material has precluded obtaining replicate protein determinations for any of the other hair fractions.

The extracts from both hair types are distinct from the endophyte in containing a greater proportion of serine and the unknown. Branched hair extracts have generally contained more arginine but less glutamine than those from simple hairs. Glutamate was consistently the major constituent of the endophyte extracts followed by aspartate and arginine. The cyanophycean granule is composed of copolymers of the latter two amino acids [25]. While it is not known if there is any direct connection, cyanophycean granules do occur in vegetative cells of the endophyte. Relative to the other amino acids glutamine is much less prevalent in the endophyte and two hair types than it is in the leaf tissues. The concentrations of amino acids in the endophyte and hairs are much lower than those in the intact plant tissues when the latter are expressed on a protein basis.

Ammonia Assimilating Enzymes

Ray et al. [26] reported that the association and endophyte preparations containing cavity hairs exhibited glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) activities. Preliminary studies revealed that cavity hairs obtained from endophyte preparations exhibited GDH activity. However, the GDH activity in endophyte preparations from which the hairs were removed was not significantly diminished and the endophyte was estimated to contribute 10% of the associations' GS activity and 20% of its GDH activity. The specific activities of the endophyte GS were half of those obtained with free-living cyanobacteria. Subsequently, Orr and Haselkorn [27] reported that using

TABLE I. Soluble amino acids, ammonia, and protein in Azolla caroliniana fronds, stem axes and segments (apex plus first two leaves and remainder) of the axes. Data are µmoles/g FW for amino acids and ammonia, mg/g FW for protein. Individual amino acids are expressed as a % of the total in parentheses. All data are based on 4-8 replicates.

	Asp	Glu	Asn	Ser	Gln	Gly/Thr	Ala	"X"*	NH_3	Protein
Fronds	.32±.01 (12±1)	.86±.11 (32±2)	.03±0 (1±0)	.17±.02 (6±0)	.46±0 (17±1)	.13±.02 (4±1)	.23±.08 (8±2)	.53±0 (20±1)	1.3±.2	4.3±1.1
Axes	.28±.08 (6±2)	.82±.11 (17±3)	.08±.01 (1±1)	.25±.02 (5±1)	2.66±.65 (55±6)	.13±.01 (3±0)	.17±.05 (4±1)	.49±.06 (10±3)	2.1±.5	4.1±.5
Apical Segments	.44±.02 (5±1)	1.26±.23 (14±2)	.08±.03 (1±0)	.34±.07 (4±1)	5.07±.38 (57±1)	.28±.05 (3±1)	.32±.06 (3±1)	1.25±.20 (14±2)	2.8±.8	4.9±.7
Remainder of Axes	.22±.08 (6±2)	.82±.03 (21±1)	.07±.02 (2±1)	.25±.04 (6±1)	1.95±.18 (49±4)	.13±.01 (3±0)	.24±.04 (6±1)	.33±.03 (8±1)	2.2±.4	4.0±.1

* "X" may be a glutamate derivative and was estimated using the average response factor of the ten amino acid standards in Table II.

TABLE II. Soluble amino acids in Anabaena azollae preparations before and after removal of cavity hairs and in the two hair types. Data are percentages of the individual amino acids based on integrated areas. Available data as nmoles/mg protein are in parentheses. n = number of determinations

	n	Asp	Glu	Asn	Ser	Gln	Gly	Thr	Arg	Ala	Tyr	"X"*
Anabaena and hairs	3 2	15±5 (2.6)	30±10 (6.7)	2±0 (.3)	4±4 (.5)	8±2 (1.2)	2±2 (.9)	6±2 (.8)	19±5 (4.3)	9±1 (1.5)	5±2 (.8)	0±0 (.2)
Anabaena	3 2	17±6 (5.7)	30±6 (12.8)	2±0 (.6)	4±2 (1.6)	7±2 (2.2)	6±6 (4.8)	6±1 (1.8)	16±8 (5.4)	9±2 (3.6)	4±2 (2.1)	0±0 (.2)
Simple Hairs	3 1	6±0 (.6)	14±4 (1.6)	6±2 (.5)	20±7 (2)	9±1 (.9)	10±7 (1.3)	6±2 (.3)	6±3 (.4)	8±3 (1.1)	3±1 (.3)	4±1 (.5)
Branched Hairs	3	8.6±3	15±14	3±1	23±10	4±3	11±7	7±3	12±1	10±3	4±1	2±1

*As per Table I.

antiserum to Anabaena 7120 GS and a sensitive radioimmunoassay, the Azolla endophyte exhibited only 5-10% of the antigen found with Newton's [28] free-living isolate of the endophyte. However, in studies using $^{13}NH_4^+$ (63 μM) the amount of label incorporated by endophyte preparations and Newton's isolate were very similar when normalized to protein content (Meeks, unpublished). Incorporation was inhibited 99% by 100μM MSX in both organisms and inclusion of unlabeled ammonia (1 mM) during incubation of endophyte preparations with and without MSX did not significantly alter the pattern of incorporation. These results are consistent with both the endophyte and Newton's isolate assimilating exogenous ammonium by the GS-GOGAT pathway with little biosynthetic GDH activity. Incubation of endophyte preparations under $[^{13}N]N_2$ also indicates incorporation of N_2 derived ammonium by the GS-GOGAT pathway and no significant contribution by GDH or alanine dehydrogenase. For example, in the presence of 100 μM MSX ^{13}N incorporation into glutamine, glutamate and alanine was inhibited 99% or more. However, the analysis of soluble amino acids in the endophyte (Table II) showed that glutamate was consistently the major pool component. In order to ascertain whether or not the endophyte itself contained GDH and GS or if these activities might be attributed to the presence of epidermal hairs in the preparations, we initiated a new investigation of these activities in the association, the endophyte preparations before and after removing the hairs, and the two types of hairs.

Whole fronds were homogenized in buffer (10 mM MOPS, 2% Polyclar AT, 10 mM DTT except where noted that MOPS was replaced by 50mM phosphate) passed twice through a french press at 16K psi and centrifuged at 20000g for 15 min. The supernatant was passed through a Sephadex G-25 column equilibrated with the extraction buffer and used for analysis. Endophyte and cavity hair preparations were resuspended in buffer without Polyclar AT or DTT, subjected to three passes through the pressure cell, centrifuged for 10 min at 20000g and the supernatant used directly for assays. Preliminary studies showed no change in activity after passing endophyte plus cavity trichome extracts through Sephadex G-25. NADH dependent GDH (E.C. 1.4.1.2) was assayed according to Pruisner et al. [29]. GS was assayed routinely by the ATP dependent formation of γ-glutamylhydroxymate [30] and the transferase assay [31]. A few assays also were conducted using the coupled biosynthetic assay [31].

The specific activities, on a protein basis, from crude extracts of the various components of the symbiosis are summarized in Table III. Each value is based on at least three separate preparations with duplicates for each GS assay. The specific activities obtained for the association are similar to those reported previously [26]. However, the GDH activity previously attributed to the endophyte obviously resulted from incomplete removal of cavity hairs. The high GDH activity in the hairs is consistent with their containing numerous mitochondria. In contrast to GDH activities, removal of the hairs did not have a significant effect on the GS activity attributed to the endophyte and the specific activities in Table III are in accord with those reported by Ray et al. [26], namely 11 nmoles NADH oxidized/mg protein·min using the coupled biosynthetic assay and 154 nmoles γ-glutamylhydroxymate formed/mg protein·min in the transferase assay. There was no loss of endophyte GS activity following further purification using sucrose density gradients [1] to remove any possible chloroplast contamination. The possibility remains that the GS activity exhibited by the endophyte is associated primarily with the undifferentiated filaments in apical segments [26,32]. It is not clear why the endophyte preparations exhibit specific activities half those of free-living cyanobacteria but only 5-10% of the GS protein [27]. Simple hairs exhibited reproducible GS activities but the branched hairs did not. For example, with branched hairs the controls (minus ADP and arsenate) for the transferase assay exceeded the complete mixture in 5 of 8 determinations and the absorbance changes in the biosynthetic assay were negligible.

TABLE III. Specific activities of ammonia assimilating enzymes in extracts of the Azolla-Anabaena association, preparations of Anabaena with and without cavity hairs, and the two hair types.

Enzyme	Glutamine Synthetase[a] Biosynthetic Activity	Glutamine Synthetase[a] Transferase Activity	GDH[b]
Association	44 ± 1	921 ± 10	31 ± 4
Anabaena with cavity hairs	14 ± 3	192 ± 11	48 ± 15
Anabaena	10 ± 1	170 ± 39	2 ± .5
Simple hairs	28 ± 7	106 ± 41	469 ± 80
Branched hairs	N.D.	N.D.	397 ± 67

[a] nmoles γ-glutamylhydroxamate formed/mg protein·min
[b] nmoles NADH oxidized/mg protein·min
N.D. not detectable, see text.

Although purified preparations of simple and branched hairs each have accounted for approximately 1% of the protein in the endophyte freed of hairs, a number of simple hairs are lost in the purification procedures. Based solely on the differences in specific activity for GDH in the endophyte with hairs and the isolated hair types, it is estimated that the hairs account for about 5% of the total protein in the endophyte plus hair preparations. Based on their preliminary studies Ray et al. [26] roughly estimated this contribution at less than 5%.

The use of phosphate in the isolation and column buffer did not significantly affect GDH activities or GS activities based on the coupled biosynthetic assay. It did, however, result in lower activities for the biosynthetic and transferase assays in the association and was extremely inhibitory to both these assays in the endophyte preparations. Although phosphate and arsenate enhance deamination of glutamine [33] and Pi inhibits Mg dependent GS activity in peas [34], the explanation for this differential effect is not clear. The possibility that the diminished GS activity in the association with phosphate might be attributed solely to the endophyte is being explored.

$[^{13}N]N_2$ Time Course and Pulse-Chase Studies

Products of N_2 fixation in the association were determined using $[^{13}N]N_2$ at the Crocker Nuclear Laboratory U.C., Davis. The generation of ^{13}N by the $^{16}O(p,\alpha)^{13}N$ nuclear reaction, its concentration and conversion to $^{13}NH_3$ [35] were followed by hypobromite oxidation to yield $[^{13}N]N_2$ [36]. The association was incubated under $[^{13}N]N_2$ at room temperature and 15 Wm^{-2} of tungsten illumination. Methanolic extracts were processed for thin-layer electrophoresis [36]. Results from initial 10 min time course studies are shown in Fig. 1. The distribution of label is consistent with ammonium from the endophyte being assimilated by the GS-GOGAT pathway, the primary organic products being glutamine and glutamate in a precursor-product relationship. Thus, this data supports that obtained for soluble amino acids and ammonia assimilating enzymes in the association.

Since $^{15}N_2$ pulse-chase studies with stem axes had shown that N_2 fixed in mature cavities was transported to the apical segments, a series of $[^{13}N]N_2$ pulse-chase studies were initiated in an effort to resolve the transported compound(s). While still in progress, the results are consistent with the time course studies in demonstrating the predominance of the GS-GOGAT pathway and have confirmed transport to the apex. Identification of the transported substance(s) has been more complex than

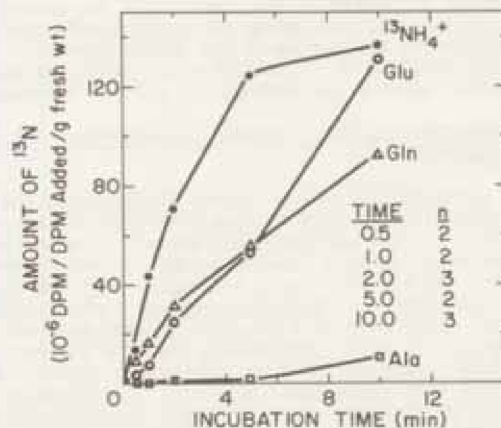

FIG. 1. Time course of fixation of $[^{13}N]N_2$ by the *Azolla caroliniana*-*Anabaena azollae* symbiosis.

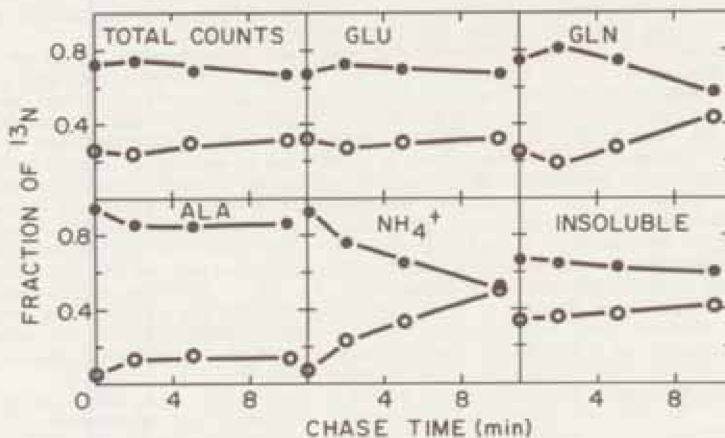

FIG. 2. Distribution of ^{13}N in apical segments plus the first two leaves (o) and in the remainder of the axes (●) expressed as the fraction of the total recovered in the individual compound at each time point.

expected, possibly due to pools of soluble amino acids, including "X" which has only recently been found, and ammonium as well as the distribution of enzymes involved in nitrogen metabolism. While all available ^{13}N data are currently being analyzed with this in mind, Figure 2 shows examples of the type of results obtained in $[^{13}N]N_2$ pulse-chase studies. Stem axes were exposed to $[^{13}N]N_2$ for five minutes followed either by immediate dissection or by dissection after air chase periods of 2, 5, and 10 mins. When plotted as the fraction of the radioactivity recovered in the individual compound at each time point as in Figure 2 the

data imply enrichment of glutamine and ammonium in the apical segments. In terms of actual counts recovered, these two constituents plus glutamate increase as a function of chase time in the apical segment. Combining these data with the information on soluble amino acids, it appears that glutamine, glutamate, ammonia and perhaps "X" all may be transported.

Interaction in Carbon Metabolism: Soluble Carbohydrates and Evidence for Cross-Feeding of Sucrose

Using $^{14}CO_2$ pulse-chase and time course studies with the association and endophyte, respectively, it was shown that both exhibited Calvin cycle intermediates [21]. Sucrose was the major product of photosynthesis in *Azolla* but was not detected as a labeled product in the endophyte during a 10 min time course. We had previously postulated that the *Azolla* endophyte might undergo a transition from photoautotrophic to photoheterotrophic [1] or mixotrophic [37] metabolism as a function of leaf age and increasing heterocyst frequency. It seemed reasonable that the endophyte might require an exogenous carbon source from its host to maintain adequate reductant levels for high nitrogenase activity. While sucrose or a metabolized product of it seemed to be a likely candidate [21], we had no evidence for this being the case.

In a related but separate series of studies, soluble carbohydrates in boiling water extracts were analyzed from the association, endophyte-free *Azolla*, and the *Anabaena* isolated from the leaf cavities [38]. A series of steps involving centrifugation, 0.45μ Millipore and Amicon PM-10 diaflow membrane filtration and desalting on a Bio-Rad AG11A8 ion retardation column were employed to obtain three fractions which corresponded to polysaccharides, oligosaccharides and mono- and disaccharides based on their elution profiles from a Bio-Gel P-2 column. The alditol-acetate [39] derivatized sugar residues of the hydrolzed polysaccharide fractions were consistent with its containing primarily solubilized cell wall components, while the hydrolyzed oligosaccharide fractions were greatly enriched in glucose residues and considered to be comprised primarily of starch and/or a glycogen-like compound. Mono- and disaccharide fractions were analyzed by GLC following both the alditol-acetate derivatization procedure and as the TMS-oxime derivatives using the general procedure described in Pierce Catalogue, Method 18. Results obtained for the TMS-oxime derivatized mono- and disaccharide fraction from the N_2-grown association and the endophyte isolated from it are shown in Figure 3 a and b. Although no sucrose synthesis had been detected in the endophyte during the 10 min $^{14}CO_2$ time course studies [21], sucrose accounted for approximately 50% of the mono- and disaccharide fraction of the isolated endophyte. While this result suggested that the endophyte might receive sucrose from the *Azolla*, it was necessary to exclude the possibilities that: a 10 min time course with $^{14}CO_2$ was not long enough to enable the detection of sucrose synthesized in the endophyte; and that the hair cells lining the leaf cavities and occurring in the endophyte preparation [12] were the source of the sucrose. Subsequently, the hair cells were removed without any appreciable loss of sucrose in the endophyte preparations and $^{14}CO_2$ time course studies of up to 90 min with the isolated endophyte, employing 2-dimensional chromatography and autoradiography of the soluble fraction at 15 min intervals, revealed no sucrose synthesis by the endophyte.

In order to demonstrate the transfer of sucrose from the *Azolla* plant to the endophytic *Anabaena*, the association was given a pulse of $^{14}CO_2$ (0.5 to 2 min) and the endophyte was isolated rapidly at intervals throughout an air chase (60 to 120 min). In these studies the total ^{14}C in the association decreased slightly during the air chase, probably due to $^{14}CO_2$ loss through respiratory processes. However, the ^{14}C-label in the endophyte fraction increased, indicating a transfer of fixed carbon from the *Azolla* to the *Anabaena*.

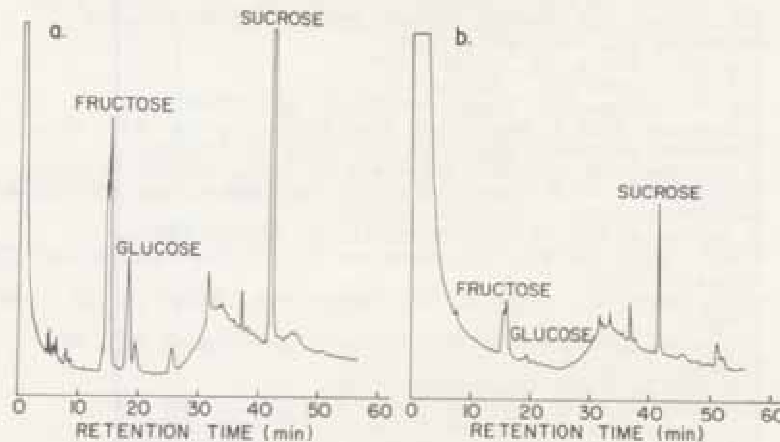

Figure 3a,b. Chromatographic analysis of the TMS-oxime derivatized mono- and disaccharide fractions from: (a) the N_2 grown Azolla caroliniana-Anabaena azollae association; and (b) the endophyte isolated from the association.

Although the ^{14}C-label in the ethanol soluble fraction of the endophyte isolated from the Azolla during the chase was lower than that in the insoluble material, which is the reverse of that obtained with the $^{14}CO_2$ time course studies on the isolated endophyte, 2-dimensional chromatography and autoradiography, along with elution and rechromatography with sucrose standards at the 45 and 90 min chase intervals, conclusively demonstrated the appearance of ^{14}C labeled sucrose in the endophyte. Thus, we feel quite confident in concluding that sucrose is cross-fed from the Azolla to the Anabaena in the association. A detailed account of these results is in preparation. Current activities include autoradiography of sectioned material in an attempt to ascertain the paths of carbon flow in main stem axes and analysis of invertase activities in the Azolla and the Anabaena.

Acknowledgment: This work was supported in part by USDA-CRGO grant nos. 79-59-2391-1-330-1 and 83-CRCR-1-1296 to GAP and 79-59-2063-1-1-276-1 and 83-CRCR-1-1295 to JCM. The excellent technical assistance and team work of Dr. J.S. Chapman, Ms. C.S. Enderlin, Ms. C.M. Joseph, Mr. M. Lollar and Mr. P. Jorgensen in conjunction with the ^{13}N studies is gratefully acknowledged as is the skillful clerical assistance of Ms. D.M. Patten. This is contribution number 840 from the Battelle-C.F. Kettering Research Laboratory.

REFERENCES

1. G.A. Peters and B.C. Mayne, Plant Physiol. 53, 813-819 (1974).
2. G.A. Peters, R.E. Toia, Jr., W.R. Evans, D.K. Crist, B.C. Mayne and R.E. Poole, Plant Cell Environ. 3, 261-269 (1980).
3. I. Watanabe, C.R. Espinas, N.S. Berger and B.V. Alimagno, IRRI Research Paper Series No. 11, 1-5 (1977).
4. P.K. Singh in: Ann. Rev. Plant Sciences, C.P. Malik, ed., (Kalyani, New Delhi 1979) pp. 38-65.
5. S.R. Talley and D.W. Rains, Agronomy J. 72, 11-18 (1980).

6. T.A. Lumpkin and D.L. Plucknett, Economic Bot. 342, 11-153 (1980).
7. H.E. Calvert, S.K. Perkins and G.A. Peters, Scanning Electron Microscopy III, 1499-1510 (1983).
8. D.J. Hill, Planta 133, 237-242 (1975).
9. D.J. Hill, New Phytol. 78, 611-616 (1977).
10. H.E. Calvert and G.A. Peters, New Phytol. 89, 327-335 (1981).
11. G.A. Peters and H.E. Calvert in: Algal Symbiosis, L.J. Goff, ed., (Cambridge University Press, New York 1983) pp. 109-146.
12. G.A. Peters, T.B. Ray, B.C. Mayne and R.E. Toia, Jr. in: Nitrogen Fixation, Vol. 2, W.E. Newton and W.H. Orme-Johnson, eds. (University Park Press Baltimore 1980) pp. 293-309.
13. D. Kaplan and G.A. Peters, New Phytol. 89, 337-346 (1981).
14. V.V.S. Tyagi, T.B. Ray, B.C. Mayne and G.A. Peters, Plant Physiol. 68, 1479-1482 (1982).
15. V.V.S. Tyagi, B.C. Mayne and G.A. Peters, Arch. Microbiol. 128, 41-44 (1980).
16. D. Kaplan, G.A. Peters and H.E. Calvert, Plant Physiol. 67S, 156 (1982).
17. P.J. Larkin, Planta 128, 213-216 (1976).
18. B.N. Jones and J.P. Gilligan, J. Chromat. 266, 471-482 (1983).
19. J.L. Corbin, Applied Environ. Microbiol. 47, 1027-1030 (1984).
20. G.A. Peters, in: Genetic Engineering for Nitrogen Fixation, A. Hollaender, ed. (Plenum Press, New York 1977), pp. 231-258.
21. T.B. Ray, B.C. Mayne, R.E. Toia, Jr. and G.A. Peters, Plant Physiol. 64, 791-795 (1979).
22. A. Neilson, R.R. Rippka, and R. Kunisawa, Arch. Mikrobiol. 76, 139-150 (1971).
23. H.F. Tung and I. Watanabae, Plant Soil, 73, 413-419 (1983).
24. J.W. Newton and J.F. Cavins, Plant Physiol. 58, 798-799 (1976).
25. R.D. Simon, J. Bacteriol. 114, 1213-1216 (1973).
26. T.B. Ray, G.A. Peters, R.E. Toia, Jr. and B.C. Mayne, Plant Physiol. 62, 463-467 (1978).
27. J. Orr and R. Haselkorn, J. Bacteriol. 152, 626-635 (1982).
28. J.W. Newton and A.I. Herman, Arch. Microbiol. 120, 161-165 (1979).
29. S. Pruisner, R.E. Miller and R.C. Valentine, Proc. Nat. Acad. Sci. 69, 2922-2926.
30. K.J.F. Farnden and J.G. Robertson, in: Methods for Evaluating Biological Nitrogen Fixation, F.J. Bergerson, ed. (John Wiley & Sons, New York 1980), pp. 283-285.
31. B.M. Shapiro and E.R. Stadtman, in: Methods in Enzymology, Vol. XVIIA, H. Tabor and E. White, eds. (Adademic Press, London 1970), pp. 910-922.
32. R. Haselkorn, B. Mazur, J. Orr, D. Rice, N. Wood and R. Rippka, in: Nitrogen Fixation, Vol. 2, W.E. Newton and W.H. Orme-Johnson, eds. (Univ. Park Press, Baltimore 1980), pp. 259-278.
33. J. P. Greenstein and M. Winitz, Chemistry of the Amino Acids, Vol. 3 (John Wiley & Sons, New York 1963).
34. T.D. O'Neal and K.W. Joy, Plant Physiol. 55, 968-974 (1975).
35. J.C. Meeks, in: Short-Lived Radionuclides in Chemistry (Amer. Chem. Soc., Washington DC 1981), pp. 269-294.
36. J.C. Meeks, C.S. Enderlin, C.M. Joseph, J.S. Chapman and M.W.L. Lollar, Planta (in press).
37. G.A. Peters, Arch. Microbiol. 103, 113-122 (1975).
38. G.A. Peters and D. Kaplan, Plant Physiol. 67S, 37 (1981).
39. A.J. Mort and W.D. Bauer, Plant Physiol. 66, 158-163 (1980).