Interaction of Carbon Metabolism in the *Azolla-Anabaena* Symbiosis

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Abstract

The *Azolla-Anabaena* symbiosis is an association between the water fern *Azolla* and the heterocystous, N_2-fixing cyanobacterium, *Anabaena azollae*. The *Anabaena* is associated with all stages of the fern's life cycle, and in the sporophyte the two organisms undergo a parallel pattern of differentiation and development. Undifferentiated *Anabaena* filaments are associated with the apical regions of the fern. These filaments are partitioned into cavities formed in the dorsal leaf lobes where they rapidly differentiate heterocysts and synthesize nitrogenase. Filaments in the cavities of mature leaves exhibit a high heterocyst frequency and dinitrogen fixed by these filaments is transported to the apices. Dinitrogen fixed by the *Anabaena* can meet the total N requirement of the association and when the symbiosis is provided with an exogenous source of combined nitrogen, such as ammonium or nitrate, fixation of dinitrogen continues to account for about 50% of the total nitrogen input.

The association, the endophyte-free *Azolla*, and the *Anabaena* freshly isolated from the leaf cavities are photosynthetical competents. Rates of carbon dioxide fixation by the freshly isolated *Anabaena*, which are similar to those of free-living cyanobacteria, appear to be considerably greater than the rate of CO_2 fixation by the *Anabaena* in the leaf cavities. Our data indicate that the *Anabaena* contributes very little to the total carbon fixation of the association and that the fern actually provides fixed carbon to the *Anabaena*.

1.   Introduction

The aquatic fern *Azolla* harbors a heterocystous cyanobacterium, *Azolla*, as a symbiont. The endophytic *Anabaena* can supply the association with its total N requirement by fixing atmospheric N_2 (Peters and Mayne, 1974a; Peters et al., 1980b), but the association also can use combined nitrogen and retain nitrogenase activity (Peters et al., 1981, 1982; Ito and Watanabe, 1983). The *Anabaena* occupies special cavities formed in the fern's aerial dorsal leaf lobes (Calvert and Peters, 1981; Konar and Kapoor, 1972; Peters and Calvert, 1983). The symbiont is associated with all stages of the fern's development, including *Azolla* sporulation and sexual reproduction (Becking, 1978; Calvert et al., 1983) and undergoes a pattern of development and differentiation which parallels that of the fern (Hill, 1975, 1977; Peters et al., 1980a; Kaplan and Peters, 1981; Calvert and Peters, 1981). Undifferentiated *Anabaena* filaments are associated with the fern's apical region. As the fern's leaves develop, some of the algal filaments of the apical colony are partitioned into the forming leaf cavities. The *Anabaena* filaments in the apical colony lack heterocysts and nitrogenase activity. During leaf maturation and cavity closure, heterocyst differentiation commences, cell division in the endophyte is greatly diminished and its cells markedly enlarge. The heterocyst frequency in filaments occupying mature leaf cavities approaches 30% (Hill, 1975, 1977; Peters, 1975), while in free-living N_2-fixing cyanobacteria heterocysts frequency seldom exceeds 10%. Nitrogenase activity is maximal in filaments present in mature leaf cavities and decreases as leaves enter senescence (Kaplan and Peters, 1981; Hill, 1977; Peters et al., 1979; Shi et al., 1981; Kaplan et al., 1986). Nitrogen fixed by the endophyte removed from mature leaf cavities is released as ammonium (Peters et al., 1979; Meeks et al., 1987) which is assimilated via the GS-GOGAT pathway and/or transported to the fern's apical region (Ray et al., 1978; Kaplan and Peters, 1981; Peters et al., 1985a; Meeks et al., 1985, 1987). Thus aspects of the interaction of nitrogen metabolism in the *Azolla-Anabaena* symbiosis have been the subject of a number of investigations. The extent of interaction of carbon metabolism in the symbiosis has received much less attention.

Both partners of the symbiosis are photosynthetical competents and their pigments are complementary. The fern contains chlorophyll a, b and carotenoids and the alga chlorophyll a, carotenoids and the phycobiliproteins phycoerythrocyanin, phycocyanin and allophycocyanin (Peters and Mayne, 1974a, Tyagi et al., 1980, 1981). The action spectrum of photosynthesis for the isolated endophyte is maximal in the phycobilin region (580–630 nm)

but a contribution by the endophyte is not apparent in the action spectrum for photosynthesis in the association. There is no clearly discernible difference in the action spectra for photosynthesis of the association and the endophyte-free fronds (Ray et al., 1979). The association and the endophyte-free *Azolla* both fix CO_2 via the Calvin cycle, exhibit an O_2-dependent CO_2 compensation point, and have sucrose as the major fixation product. When removed from the fern, the endophyte also fixes CO_2 by the Calvin cycle. However, rates of CO_2 fixation and the CO_2 compensation point of the isolated *Anabaena* are the same in 2 and 20% O_2, and labelled sucrose is not detected in short-term $^{14}CO_2$ time course studies of 10 min (Ray et al., 1979). Although the N_2 fixing cyanobacteria in symbioses with green plants usually lack phycobiliproteins and/or the ability to fix CO_2 (Duckett et al., 1977; Rodgers and Stewart, 1977), phycobiliproteins are retained by the endophytic *Anabaena azollae* and are effective in harvesting light energy for O_2 evolution (Ray et al., 1979) and N_2-fixation (Tyagi et al., 1981). The phycobiliprotein content of the cyanobacteria filaments increases with leaf age and there is no significant alteration of the phycobiliprotein complement with increasing heterocysts frequency (Kaplan et al., 1986).

The fact that the endophyte fixes CO_2 when removed from leaf cavities was interpreted to imply, but not prove, that the endophyte fixes CO_2 when in the leaf cavities. Some approaches used in attempts to estimate the endophyte's contribution to the total photosynthetic capacity of the association, and to demonstrate the existence of interaction of carbon metabolism, carbon cross-feeding and identification of the metabolite transported are presented.

2. Materials and Methods

Plant material and growth conditions

Azolla caroliniana Willd. with the endophyte was grown on N-free medium (Peters et al., 1980b). For endophyte-free *Azolla* plants, which were obtained as previously described (Peters and Mayne, 1974), 4 mM NO_3^- was added to the medium. All cultures were maintained using a cycle of 16 hr light ($26\pm1°C$) and 8 hr dark ($19\pm1°C$). A combination of cool-white fluorescent and incandescent lights provided a photosynthetic photo flux density (PPFD) of 200 μE $m^{-2}s^{-1}$. For isolation of the endophytic *Anabaena*, fronds grown in nitrogen-free medium were harvested and their fresh weight determined. Fronds were then put in the nitrogen-free medium containing 1% soluble PVP (8 ml/gr fr. wt) and ground for 30–60 sec in a motor-driven, ice cold, teflon homogenizer. The homogenized material was sequentially passed through 4

and then 8 layers of cheesecloth and a 110 μ nylon mesh, followed by rinsing with the grinding medium. The filtrate was centrifuged at full speed in a clinical centrifuge for 20–30 sec and the supernatant decanted. The pellet, containing the endophyte filaments, was resuspended in 2.0 ml of the growth medium without PVP and recentrifuged prior to final resuspension in the same medium for use in specific studies.

CO_2 exchange rates under air and 2% O_2

Whole fronds of the association and the endophyte-free plants were harvested and major roots were removed. Samples of 0.3 gr (fr. wt.) were placed on 5 ml of growth medium in petri-dishes (5.0 cm diameter) under fluorescent and incandescent light at a PPFD of 600 μE m^{-2}s^{-1}. Rates of carbon fixation by the association and endophyte-free plants were determined in air and 2% O_2 under white light and under monochromatic light at 610 and 660 nm, obtained using interference filters, at 25°C. CO_2 exchange rates as a function of temperature under air and 2% O_2 were measured in plants placed in petri dishes and maintained at PPFD of 600 μE m^{-2}s^{-1} for 2 hr at 20, 24, 28, 32, 36 and 40°C prior to measurements at the indicated temperatures. All determinations were made using Horiba IRGA in a closed system, using a custom made sample chamber. White light and monochromatic light from interference filters, was obtained from a Variac controlled halogen source and the intensity measured with Kettering Radiometer.

$^{14}CO_2$ fixation and identification of labelled compounds

Time course studies of $^{14}CO_2$ incorporation by the isolated *Anabaena* were conducted as described by Ray et al. (1979), using small vials placed in a water jacketed reaction vessel at 27°C. Vials containing 3 ml of a stirred suspension of the endophyte were placed in the reaction vessel and illuminated with white incandescent light at an intensity of 300 μE m^{-2}s^{-1}. After a 15 min equilibrium period the vial was sealed with a serum cap and 1.0 ml of a solution of NaH$^{14}CO_3$ containing 50 μCi of $^{14}CO_2$ (specific activity 8.5 μCi/mM) was injected. The final bicarbonate concentration was 12.5 mM in a reaction volume of 4.0 ml. Samples of 0.5 ml were removed immediately after addition of the NaH$^{14}CO_3$ and after 5, 10, 15, 30, 45, 60 and 90 min. These were added to 2.0 ml of boiling 95% ethanol and centrifuged. Supernatants were decanted and pellets extracted again using 2.0 ml of boiling 80% ethanol. The extracts were combined, acidified and after removing aliquots for liquid scintillation counting, concentrated and subjected to standard two-dimensional chromatography and autoradiography (Bassham and

Calvin, 1957). The two-dimensional chromatography was repeated with the addition of sucrose, glucose and fructose as internal standards.

Labelled compounds in regions of specific interest were eluted from the paper chromatograms not containing internal standards and rechromatographed on polyamide sheets (TLC) following the procedure of Marais (1967), using unlabelled glucose, fructose and sucrose as standards. For visual detection the chromatograms were sprayed with the alinine-ethanol-phosphoric acid-benzene reagent of Marais (1967), and then cut into strips for measuring radioactivity. Ethanol insoluble material from each time point was resuspended in 1.0 ml H_2O and the radioactivity was measured by liquid scintillation counting using a Packard Tri-Carb, model 3255, liquid scintillation spectrometer. The scintillation cocktail was Aquasol-universal from New England Nuclear.

$^{14}CO_2$ pulse-chase

The $^{14}CO_2$ pulse-chase experiments with the *Azolla-Anabaena* symbiosis were performed as described by Ray et al. (1979) using a plexiglass chamber similar to the one described by Wynn et al. (1973). Known weights of *Azolla* plants containing the endophyte were placed in small stainless steel baskets and allowed to equilibrate at 25°C and 300 μE m^{-2}s^{-1} PAR for 60 min. Then the chamber was sealed and 1 mCi of $^{14}CO_2$ (specific activity 58.5 mCi/m mol) was injected. After 2 min the top of the chamber was removed to initiate the air ($^{12}CO_2$) chase. Baskets with plant material were removed at specific intervals and placed on ice in near darkness. As rapidly as possible two to three frond were weighed and dropped into boiling 80% ethanol for extraction and fractionation into ethanol soluble and ethanol insoluble fractions. The rest of the frond material in each sample was immediately used for isolation of the endophyte as described previously. Aliquots of the isolated endophyte preparations were removed for chlorophyll determination. The rest was fractionated into the ethanol soluble and insoluble fractions. After taking aliquots for radioactivity counting, the ethanol soluble fractions were subjected individually to two-dimensional chromatography and autoradiography as described for time course studies. Total counts at each time point were determined for the fronds and the endophyte. Ethanol insoluble fractions were subsequently hydrolyzed for 2 hr in 2N trifluoroacetic acid at 100°C. Hydrolyzates were analyzed by polyamide TLC as described for time course studies, and by GLC using alditol-acetate derivatives (Mort and Bauer, 1982). Radioactivity was measured by liquid scintillation counting.

Other determinations

Total sugar was determined with the phenol sulfuric acid method (Ashwell, 1966) and chlorophyll was determined in 95% ethanol (Wintermans and Demots, 1965).

3. Results

As no labelled sucrose was detected in the endophyte preparations during 10 min $^{14}CO_2$ time-course experiments (Ray et al., 1979), but sucrose was found to account for about 50% of the mono- and disaccharides fraction extracted in boiling water extracts of the endophyte (Peters et al., 1985a), the postulate that sucrose produced by the fern might be transported to the endophyte to support the high rates of nitrogenase activity (Ray et al., 1979) seemed to gain credence. However, the aspect of the fern providing sucrose to the endophyte seemed somewhat at odds with the demonstrated ability of the endophyte to fix CO_2 immediately after its isolation from the leaf cavities. Thus it was deemed desirable to attempt to estimate the endophyte's contribution to the total CO_2 fixation of the association and to see if evidence could be obtained to show that the fern provided sucrose to the endophyte.

The differences in the action and absorption spectra of the two partners along with the difference in the effect of O_2 partial pressure on their photosynthesis was selected as an initial approach to obtain an estimate of the endophyte's contribution to the total photosynthetic activity of the association. Rates of CO_2 fixation by the association and endophyte-free plants were determined in atmosphere of air and 2% O_2 under white light, and monochromatic light at 610 nm and 660 nm. The results are shown in Table 1.

In white light the inhibition of photosynthesis by air versus 2% O_2 is 34% for the association and 38% for the endophyte-free *Azolla*. Thus O_2 inhibition is 4% greater in the endophyte-free plants than in association. A similar difference in the amount of O_2 inhibition exhibited by the association and endophyte-free plants, is observed at 610 nm the region where light harvesting by the endophyte's phycobiliproteins is maximal. There is effectively no difference between the association and endophyte-free plants in the amount of inhibition at 660 nm where photosynthesis is attributed to the fern. These results were interpreted to suggest that the endophyte's contribution to the association's total CO_2 fixation is less than 5%.

The differences in the sensitivity of the two partners in the symbiosis to temperature also was used in an effort to estimate the endophyte's contribu-

Table 1. Carbon fixation rates (μ moles CO_2 hr^{-1} mg Chl^{-1}) in the association and endophyte-free plants in air and 2% O_2 under white light (10^5 ergs cm^{-2} sec^{-1}) and 610 nm and 660 nm (10^4 ergs cm^{-2} sec^{-1}). (Each value is the average of 10 experiments $\pm$S.D.).

| | Association | | | Endophyte-free | | |
	air	2% O_2	% inh	air	2% O_2	% inh
white light	89.45	136.24	34	71.14	114.75	38
	±5.16	±8.23	±3.3	±12.21	±15.20	±3.5
610 nm	17.64	26.22	33	13.86	22.86	39
	±6.08	±7.91	±3.8	3.85	±6.35	±4.0
660 nm	31.10	50.89	39	25.98	43.53	40
	±8.50	±13.81	±4.3	±5.42	±9.93	±3.5

tion to the total photosynthetic capacity of the association. Photosynthetic activity (CO_2 fixation rates) of the association and endophyte-free *Azolla* were measured at temperatures from 20 to 40°C under an atmosphere of air and 2% O_2. Photosynthetic rates of the association were consistently slightly higher than those of the endophyte-free plants (data not shown), and the inhibition by O_2 more pronounced in the endophyte-free *Azolla* as the temperature was increased. These differences were attributed to the presence or absence of the endophyte (Fig. 1). These results also indicate that while the endophyte is capable of fixing CO_2 when part of the symbiosis, its contribution to the total CO_2 fixation of the association under its optimal growth conditions is no more than 5%.

Subsequently we employed long-term $^{14}CO_2$ time course studies of 90 min, to assess further the ability of the endophyte to fix carbon when removed from the association. The distribution of label in the ethanol soluble and insoluble fractions obtained in the $^{14}CO_2$ time course are shown in Fig. 2. Results of 2-dimensional chromatography and autoradiography were in agreement with the 10 min time-course of Ray et al. (1979). No evidence of sucrose was detected in the fixation products of the endophyte, even after 90 min.

As stated previously sucrose was found to be a major constituent of the boiling water extract obtained from the endophyte immediately after separation from the association (Peters et al., 1985a). Since no evidence was obtained in the long term $^{14}CO_2$ time-course studies to indicate that the endophyte itself synthesized sucrose, experiments were conducted in which the *Azolla-Anabaena* association was given a 2 min pulse of $^{14}CO_2$ followed by an air chase. At the onset and at intervals of the chase period the endo-
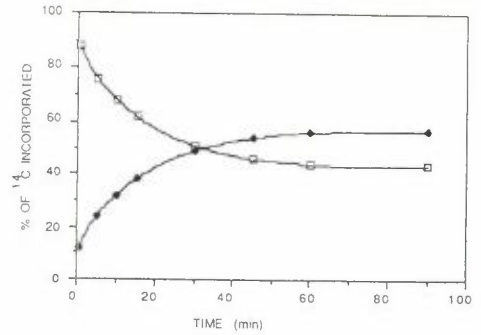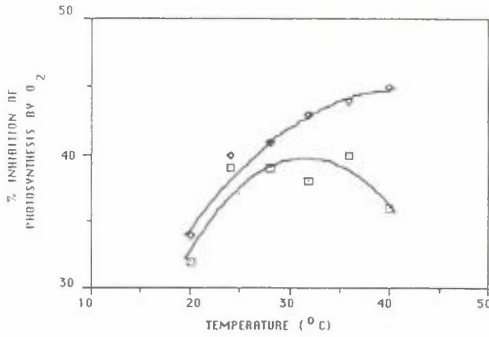
Figure 1. Effect of oxygen inhibition of photosynthesis as a function of temperature in the association (□) and endophyte-free *Azolla* (◊).

Figure 2. Distribution of label in ethanol-soluble (□) and insoluble (◆) fractions during time-course studies of $^{14}CO_2$ fixation by the endophytic *Anabaena*.
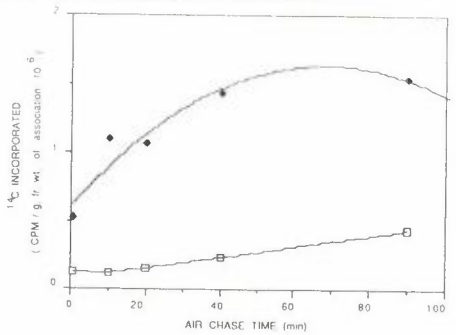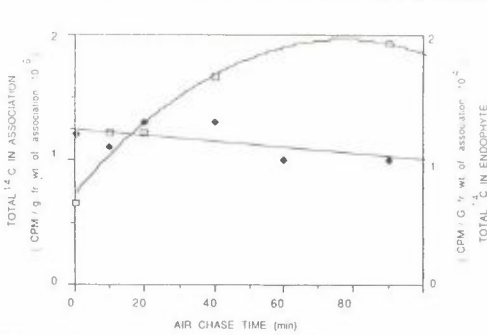

Figure 3. Kinetics of total ^{14}C-labeling in the association (◆) and the endophytic *Anabaena* isolated from it (□) at intervals during an air chase following a 2 min exposure to $^{14}CO_2$.

Figure 4. Distribution of ^{14}C-label in the ethanol-soluble (□) and insoluble (◆) fractions from the endophyte isolated from the association at indicated periods of the air chase after a 2 min pulse with $^{14}CO_2$.

phyte was isolated and analyzed to see if evidence could be obtained for the transfer of fixed carbon from the fern to the endophyte. The total ^{14}C label observed in the association and the isolated endophyte at intervals during the air chase are presented in Fig. 3. The slight decrease in the total label in the association during the air chase probably reflects the loss of radioactivity due to respiration. The total label in the endophyte increases slightly during the chase period (note difference in scales) indicating that some of the $^{14}CO_2$ fixation products of the fern are transported to the endophyte. From the distribution of label between the soluble and insoluble fractions in

the endophyte (Fig. 4) it can be seen that when it is in the leaf cavities, the ethanol soluble sugars are a small portion of the total sugars found in the endophyte. This is in agreement with other studies indicating that the mono- and disaccharides fraction in the endophyte immediately after its isolation account for about 10–20% of the total sugars, while this fraction accounts for about 40% of the total in the association (Kaplan, unpublished data). It can also be seen in Fig. 4 that when the endophyte is in the leaf cavities, the label fraction is incorporated very rapidly into the ethanol insoluble fraction. This is followed by a plateau at about 60 min which may indicate a dilution of labelled compounds by newly fixed unlabelled material transported from the fern into the endophyte and incorporated into the ethanol insoluble fraction as carbon fixation continues during the chase period.

In these experiments the total label in the endophyte accounted for only about 1% of that in the association after a 2 min pulse and a 0.5 min chase. When the association was pulsed for 30 sec and the endophyte was isolated from it after 30 sec of chase (not shown), the label found in the isolated endophyte was 2–3% of that in the association. Assuming that very little transport occurs during this time (total of 1 min before sampling), we inter- pret these values to represent the carbon fixation capacity of the endophyte in the intact association. These values are remarkably similar to those ob- tained with other procedures (Table 1 and Fig. 1). They again indicate that while the endophyte, isolated directly from the association is capable of fixing CO_2, in the intact association its contribution to the total CO_2 fixation ca- pacity of the symbiosis is very small, on the order of 5% or less, even though it accounts for about 16% of the association's total protein and chlorophyll.

Ethanol soluble fractions from the endophyte removed from the associa- tion during the air chase following the two minute labeling with $^{14}CO_2$ were subjected to 2-dimensional chromatography and autoradiography. Samples from each time point were analyzed, and labelled spots were eluted from the chromatograms for further analysis. When rechromatographed on polyamide sheets using sucrose, glucose and fructose standards, labelled sucrose was found to occur in the 0.5 min and subsequent chase samples. Data for the 0.5 and 15 min chase samples are shown (Fig. 5A&B respectively). Label in the sucrose region decreased in samples from chase periods of longer than 15 min. Labelled glucose was detected in all chase samples. Very low label was detected in fructose. These results support the postulate that sucrose is transported from the fern to the endophyte.
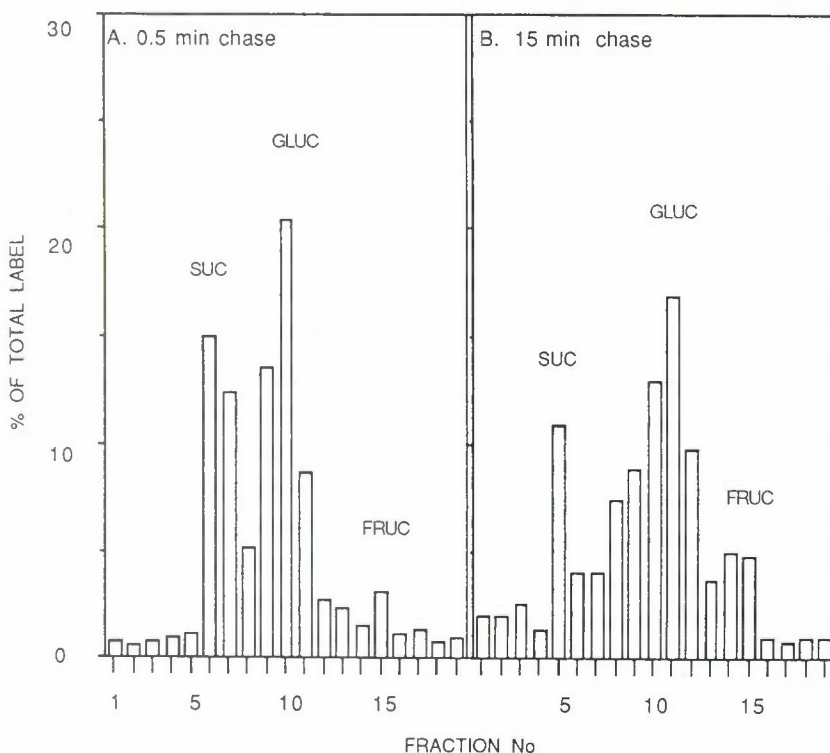
Figure 5A&B. ^{14}C-label in individual sugars of the ethanol-soluble fractions of the en-
dophyte isolated from the association at the 0.5 min (A) and 15 min (B)
intervals of the air chase initiated after a 2 min [14]CO_2 pulse.

4. Discussion

Differences in the action spectra, O_2 inhibition of photosynthesis and tem-
perature sensitivity in the individual partners of the *Azolla-Anabaena* sym-
biosis were used to estimate the contribution of the endophyte to the total
carbon fixation capacity of the association. Rates of CO_2 fixation were always
slightly higher in the association than in the endophyte-free plants (Table 1).
Oxygen inhibition of photosynthesis was consistently greater in *Azolla*-free
plants than in the association, indicating that the endophyte does contribute
to the CO_2 fixation capacity of the association. The relative contribution of
the cyanobacteria to the association's total CO_2 fixation was shown to vary
with temperature. At temperatures above 30°C the endophyte's contribu-
tion increases markedly as is indicated by the difference in O_2 inhibition of
photosynthesis in the association and endophyte-free plants (Fig. 1). That

the endophyte is capable of fixing CO_2 when it is part of the association also is demonstrated by the $^{14}CO_2$ pulse-chase experiments. However, in contrast to studies with the endophyte after its removal from the fern, each of the approaches used to assess its contribution to the association's total CO_2 fixation when it is in the leaf cavities indicates that its contribution is very low, even under conditions known to be optimal for the association's growth. While the endophyte accounts for about 16% of the association's total protein and chlorophyll, its photosynthetic capacity is estimated, from the experiments described here, to account for about 5% or less of that of the total photosynthetic capacity of the association. This finding suggests that the endophyte is dependent on carbon compounds fixed by the fern to maintain high rates of nitrogenase activity associated with high heterocyst frequency. It is possible that the endophyte activity fixes CO_2 in the apical region, where the filaments contain only vegetative cells, but becomes dependent on the photosynthetic activity of the fern in the mature leaves, where every third cell in the filaments is a heterocyst, and nitrogenase activity is maximal. Attempts to determine whether the endophyte's contribution varied with increasing leaf age were undertaken using sequential leaf segments but the results were inconclusive. Based on $^{14}CO_2$ pulse-chase studies in which material was embedded using non-aqueous techniques to prevent loss of water-soluble sugars, sectioned and coated with nuclear tract emulsion, we do know that $^{14}CO_2$-labelled compounds move into the mature cavities and that $^{14}CO_2$-labelled compounds also are transported to the apex. Thus both the mature cavities and the apex appear to be sinks for fixed carbon. The apical region has been shown previously to be a sink for fixed nitrogen (Kaplan and Peters, 1981).

As no labelled sucrose was detected in the endophyte in short-term $^{14}CO_2$ time-course studies (Ray et al., 1979) but sucrose was found to be a major constituent of the mono- and disaccharides fraction in boiling water extracts of the endophyte (Peters et al., 1985), long-term time course studies of up to 90 min were conducted on the isolated endophyte. As there was no evidence for the ability of the endophyte to synthesize sucrose, it was concluded, as postulated previously, (Ray et al., 1979), that *Azolla* must be the source of sucrose detected in the boiling water extracts of the isolated endophyte.

The $^{14}CO_2$ pulse-chase studies of the association with rapid isolation and analysis of the endophyte showed that ^{14}C fixed by the fern is transported to the endophyte where it is rapidly incorporated into an ethanol insoluble fraction. Moreover, identification of the ^{14}C labelled carbon compounds of the ethanol soluble fraction from the endophyte, indicate that cross-feeding of

carbon compounds, including sucrose, occurs in the *Azolla-Anabaena* symbiosis. The decrease in radioactivity detected in sucrose in the endophyte fractions after long periods of chase suggest that there is a continuous transport of newly fixed sucrose into the endophyte, which can be either immediately cleaved and used to support nitrogenase activity or incorporated into the ethanol insoluble fraction. The latter consists mainly of glucose residues, as indicated from the chromatography of the trifluoroacetic acid hydrolyzates on TLC.

In conclusion, long-term $^{14}CO_2$ time-course studies confirmed that sucrose is not a fixation product of the endophyte. The $^{14}CO_2$ pulse-chase studies in which the endophyte was rapidly isolated from the association as well as the measurements of CO_2 fixation rates under different O_2 regimes and different light qualities indicate that the endophytic *Anabaena*, which is capable of actively fixing CO_2 immediately after its removal from the fern, fixes very little CO_2 when in the leaf cavities. In fact, the low values obtained for our estimates of the endophyte's contribution to the association's total CO_2 fixation capability suggests that the endophyte in the leaf cavities is almost completely dependent on the fern for carbon compounds. The foregoing, along with the identification of the labelled carbon compounds of the ethanol soluble fraction from the endophyte, indicates that as in other symbioses between N_2 fixing cyanobacteria and green plants (Stewart and Rodgers, 1977), cross-feeding of carbon compounds occurs in the *Azolla-Anabaena* symbiosis. The results also strongly indicate that sucrose is a (if not the) major carbon compound transported from the fern to the cyanobacterium in mature leaf cavities.

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