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Azolla-Anabaena azollae Relationship

V. $^{15}N_2$ FIXATION, ACETYLENE REDUCTION, AND H_2 PRODUCTION[1, 2]

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ABSTRACT

In order to characterize the reactions catalyzed by nitrogenase in the *Azolla-Anabaena* association, $^{15}N_2$ fixation, C_2H_2 reduction, and ATP-dependent H_2 production were measured in both the *Azolla-Anabaena* complex and in the alga isolated from the complex.

The rate of reduction of substrates and of ATP-dependent H_2 evolution was determined at various partial pressures of C_2H_2 and N_2. A pC_2H_2 of 0.1 atm was nearly optimal for C_2H_4 production and inhibited H_2 production by 95%. The ratio of C_2H_2 reduced to N_2 fixed was determined as a function of constant pC_2H_2 (0.1 atm) and variable pN_2. This ratio decreased with increasing pN_2 and the decrease was correlated with less H_2 production. Ratios obtained at N_2 partial pressures of approximately 0.3, 0.6, and 0.8 atm, respectively, were 3.2, 2.0, and 1.7 for the association and 4.4, 3.0, and 2.5 for the isolated symbiont. Rates obtained for C_2H_2 reduction, N_2 fixation, and H_2 production were used to obtain an expression of the electron balance *in vivo*.

Azolla is a genus of small aquatic ferns which, under natural conditions, invariably contain the heterocystous blue-green alga, *Anabaena azollae,* as a symbiont in an enclosed chamber in the dorsal leaf lobes (10, 12). In the intact association, the alga can provide the *Azolla* plant with its total nitrogen requirement. Previous studies showed that the symbiont contained nitrogenase and was capable of C_2H_2 reduction, ATP-dependent H_2 evolution, and excretion of ammonia (10, 11, 13).

Although C_2H_2 reduction is a simple and sensitive assay of nitrogenase activity, C_2H_2 is not the biologically important substrate, and the assay is an indirect measurement of nitrogen fixation. Since the reduction of N_2 to $2NH_3$ requires six electrons while reduction of C_2H_2 to C_2H_4 requires two electrons, a theoretical conversion factor of $3C_2H_2$ reduced per N_2 fixed is frequently used in estimating nitrogen fixation from C_2H_2 reduction assays. Studies on isolated nitrogenase have shown that when provided with a source of ATP and reductant the rate of electron flow through the enzyme is independent of the substrate (7, 20). Moreover, while normal assay levels of C_2H_2 almost totally suppress H_2 production, some electrons continue to be utilized in reducing protons to H_2 when N_2 is the substrate and employed at saturating levels (16, 20). Thus, the C_2H_2/N_2 ratio is usually closer to 4 for nitrogenase *in vitro*. However, *in vivo* studies have produced a range of ratios from less than 2 to greater than 8 (8), and it would appear that the ratio may be dependent upon both the organism and experimental conditions employed.

This is a report on $^{15}N_2$ fixation by the fern-algal association and the symbiont isolated directly from the leaf cavities. In order to determine whether a specific conversion factor can be used, and to assess some factors capable of affecting it, the effect of pN_2 on N_2 fixation and ATP-dependent H_2 evolution was determined in parallel with measurements of C_2H_2 reduction. Ratios of C_2H_2 reduction to N_2 fixation and the effect of H_2 evolution on both this ratio and on calculated values of the electron balance for nitrogenase-catalyzed reactions *in vivo* are presented for the experimental conditions employed.

MATERIALS AND METHODS

Growth and maintenance of *Azolla caroliniana* Willd. cultures, algal isolation, and chlorophyll determinations were essentially as described previously (10, 12).

C_2H_2 Reduction and H_2 Evolution. These assays were conducted as described previously (10, 11) except that various pC_2H_2 or pN_2 were employed. For assays with the isolated alga, the final resuspension was in a modified Kratz and Myers medium (9). Flasks were evacuated, flushed, and filled to a slightly positive pressure with Ar-0.3% CO_2. An appropriate volume of Ar-CO_2 was removed and replaced with C_2H_2 to give the desired pC_2H_2. Various pN_2 were obtained by means of the manifold used for $^{15}N_2$ studies (see below). Serum caps and necks of flasks were sealed with rubber cement, covered with a layer of Saran Wrap and aluminum foil, and tightly bound with rubber bands at the neck of the flask. All cylinder gases were obtained from Matheson Gas Products. The actual pN_2 in individual flasks was monitored as described previously for H_2 and O_2 (11). Room air (pN_2 = 0.78 atm, pO_2 = 0.21 atm) was used as a standard. "Mini-Cal" calibration standards (Applied Science Laboratories) were employed for quantitation of C_2H_4 and H_2.

$^{15}N_2$ Determinations. A gas manifold equipped with valved inlets for three gases in addition to a bulb of $^{15}N_2$ was connected to a Toepler pump, a Hg manometer, an adapter for insertion of small gas bulbs, and a glass-stopcocked capillary sample manifold equipped with syringe needles. Incubations were conducted in either 10-ml Erlenmeyer flasks or 15-ml serum vials stoppered with serum caps and sealed as described above. The flasks contained either 2 or 3 ml of incubation medium and three or four fronds or 2 or 3 ml of a suspension of the algal symbiont.

The flasks were evacuated, flushed six times with Ar-0.3% CO_2, and, after a final evacuation, gas mixtures containing the desired pN_2 and atom % excess ^{15}N were prepared. (The $^{15}N_2$ as obtained from ICN Pharmaceuticals was 99 atom % and free of oxides of nitrogen. Mass spectrometer analysis of individual gas bulbs gave values between 98.6 and 99.2 atom % ^{15}N.) Predetermined quantities of $^{15}N_2$, $^{14}N_2$-0.3% CO_2 and/or Ar-0.3% CO_2 were added to the flasks by measuring pressure changes in the Hg manometer which had been calibrated employing P/V relationships for the system. Diluent gas (usually Ar-0.3% CO_2) was added to yield a final pressure of 1 atm. Gases were mixed with a Toepler pump and flasks incubated as described below.

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The atom % ^{15}N in the gas phase was determined at the onset and termination of the experiments by mass spectrometry employing the equation for a nonequilibrium mixture of mass (M) 28, M29, and M30 (1, 3). The contribution of CO^+ from CO_2 to M28 was found to be negligible and not routinely corrected for. The presence of M32 was used as an index of leakage at the onset but was not used at the end of light incubations since O_2 was evolved photosynthetically. The consistency of the calculated atom % ^{15}N at the onset and termination of the experiments was the best index of whether or not any leaks occurred.

Incubation of Samples. Samples were incubated in a New Brunswick Scientific Gyrotory water bath, model 677, at 28 C. The shaking rate was 50 or 100 rpm and overhead illumination (500–600 ft-c) was provided by a bank of fluorescent lights.

Inactivation, Kjeldahl Digestion, Distillation, and Conversion of Samples to N_2. At the end of the incubation period, algal samples were quantitatively transferred to Kjeldahl flasks, cooled in liquid N_2, and stored in a freezer. Flasks containing whole fronds were placed in an ice bath in the dark and the fronds were removed rapidly and ground in 80% acetone. The acetone extracts were centrifuged and the chlorophyll content determined on the supernatant fraction. The supernatant and pellet fractions were combined in Kjeldahl flasks. Acetone was removed by heating in a water bath and the samples were stored in a freezer. Digestion, distillation, and conversion of samples to N_2 by hypobromite oxidation were essentially according to published procedures (2, 3, 5). After distillation, ammonia was determined with either Nessler's reagent or the nitroprusside reagent of Chaykin as described in Burris (2). Hypobromite oxidation was run on duplicate or triplicate samples with or without carrier N. A stock solution of NH_4Cl with a known atom % ^{15}N was employed as carrier. Carrier N did not exceed sample N by more than a factor of 4 and was usually less than twice the sample's N.

Analysis of Samples. A Finnigan quadrapole model 1015 mass spectrometer was used for gas analysis. Standards of known atom % ^{15}N from 0.365 to 1.010 were employed for calibration. Regression analysis of measured *versus* known values of samples in this range gave a coefficient of determination of 0.997 and any amount of N between 0.1 and 1.0 mg was found to yield sufficient gas pressure for accurate analysis (J. Johnson, personal communication).

The atom % excess ^{15}N was determined by subtracting the natural abundance of unexposed samples and, when carrier N was employed, the values were corrected for the dilution effect. The amount of N_2 fixed was determined from the atom % excess of the incubation gas and that incorporated by the sample (3).

Small amounts of air contamination in samples after hypobromite oxidation, indicated by a peak at M32 (O_2) and confirmed by M40 (Ar) (15), were corrected for by determining the ratios of M28/M32 and M29/M32 in room air. These ratios were 6.0 and 0.04, respectively. The correction of M28 and M29 from air contamination was based on the amount of M32. The atom % ^{15}N of the individual samples was determined from the ratio (R) of M29/M28 and by employing the equation

$$\text{atom \%} = 100\ R/(2+R)$$

RESULTS

The effect of pC_2H_2 on the rate of C_2H_4 and H_2 production (Fig. 1) demonstrated that the maximum rate of C_2H_4 production occurred at 0.1 atm C_2H_2 in this association and, in accord with previous observations (11), 0.1 atm C_2H_2 inhibited H_2 production by 95 to 97%. Although the inhibition of H_2 production at any pC_2H_2 is somewhat greater than reported for *in vitro* studies (16), the pattern of inhibition is very similar. Subsequently, in attempts to determine the stoichiometric relationship between C_2H_2 reduced and N_2 fixed under various pN_2, C_2H_2 was employed at 0.1 atm.

Initial studies with $^{15}N_2$ demonstrated that the increase in the atom % excess ^{15}N was linear with time, that fixation under anaerobic conditions was light-dependent in both the intact association and isolated alga, and as previously shown for C_2H_2 reduction in *Azolla* (11), 2% CO also inhibited $^{15}N_2$ fixation. Subsequently, studies were conducted on the time course relationships of C_2H_2 reduction, $^{15}N_2$ fixation, and H_2 production under both Ar and several partial pressures of N_2. Results obtained in experiments of this type with the intact association are presented in Figure 2 and with the isolated symbiont in Figure 3 and Table I.

H_2 production under Ar is consistently less than C_2H_2 reduction in both the intact association and isolated alga and is quite

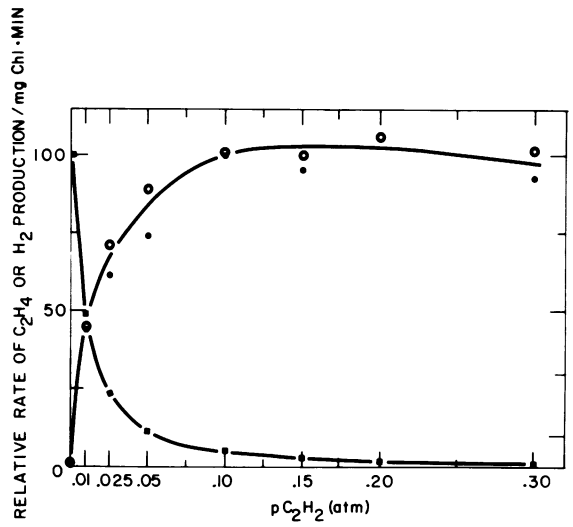

FIG. 1. Effect of acetylene concentration on the rate of ethylene production (●, ○) and nitrogenase-catalyzed H_2 evolution (■) by the fern-algal association. Ethylene production data are a composite of duplicate experiments. Individual points are average of duplicate samples. Incubation period was 4 hr at 28 C and 550 ft-c.

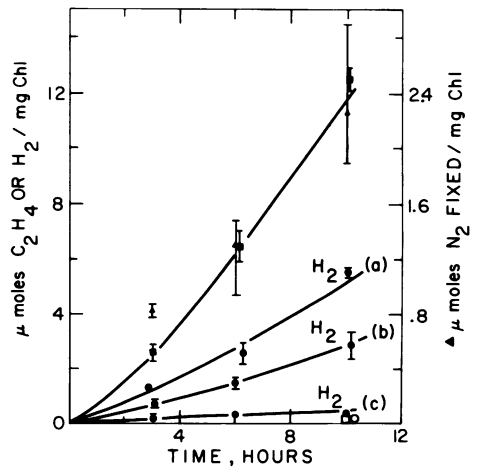

FIG. 2. Time course relationships of C_2H_2 reduction (■), $^{15}N_2$ fixation at a pN_2 of 0.26 atm (▲), and H_2 evolution (●) under (a) Ar (pN_2 <0.005 atm); (b) 0.1 atm N_2; and (c) 0.26 atm N_2 in the *Azolla-Anabaena azollae* association. Dark C_2H_2 reduction (□) and H_2 production (○) are indicated at 10 hr. Incubations for intervals indicated at 500 ft-c, 28 C, and very mild agitation. The 0.26 atm N_2 contained 22.79 atom % ^{15}N. Atom % excess ^{15}N in the samples at 3, 6, and 10 hr were 0.113, 0.198, and 0.326, respectively, and C_2H_2/N_2 ratios 3.2, 5.0, and 5.6, respectively.

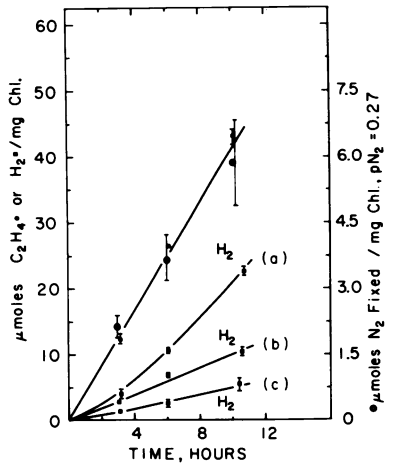

FIG. 3. Time course relationships of C_2H_2 reduction (●), $^{15}N_2$ fixation (●) at a pN_2 of 0.27 atm and H_2 production (■) under (a) Ar (pN_2 <0.005 atm); (b) 0.1 atm N_2; and (c) 0.27 atm N_2 in the algal symbiont. Atom % excess ^{15}N in the gas phase at 0.27 atm N_2 was 23.53. Incubations as described in Table II.

Table I. A comparison of $^{15}N_2$ fixation and the C_2H_2/N_2 ratio as a function of time in two preparations of Anabaena azollae.

The atom % ^{15}N in the gas phase was 95.11 at 0.1 atm N_2, 40.52 at 0.2 atm N_2 and 23.53 at 0.27 atm N_2. Incubations were at 500 ft-c, 28 C. The data at 0.27 atm complement Fig. 3.

Time	atom % excess ^{15}N at pN_2 (atm)			C_2H_2/N_2 at pN_2 (atm)		
Hr	0.10	0.21	0.27	0.10	0.21	0.27
3	0.442	0.374	0.424	31.2	18.1	5.4
6	0.879	0.808	0.860	36.7	17.6	7.7
9 or 10*	1.547	1.249	1.187*	31.3	17.7	8.2*

variable (11). Values from as low as 30% to as high as 97% of the rate of C_2H_2 reduction have been obtained with an average value around 60%. The relationship of C_2H_2 reduction to H_2 production under Ar shown in Figures 2 and 3 is consistent with this observation. These data also demonstrate that with increasing pN_2, less H_2 is produced. This is in accord with a competition for electrons between the two substrates of nitrogenase, *i.e.* N_2 and protons (16, 20), and is manifested as a decrease in the C_2H_2/N_2 ratio as a function of increasing pN_2 in both the symbiont (*cf.* Table I) and the association. An increase in the C_2H_2/N_2 ratio as a function of time was often observed in experiments with the intact association (Fig. 2), and less frequently with the symbiont (Table II), at a pN_2 of 0.25 atm and above. This may arise from the absence of a utilized metabolic product from C_2H_2 reduction, *i.e.* the absence of feedback control on the nitrogenase. In contrast, normal metabolic products and inherent control mechanisms are presumed to exist when N_2 is the substrate and is provided at levels approaching saturation.

The effect of varying pN_2 on H_2 production is demonstrated in Figure 4. This figure is a composite of the data from experiments in which H_2 production under Ar and various partial pressures of N_2 was measured at approximately 3-, 6-, and 9-hr intervals. In order to express the inhibitory effect of a given pN_2 on H_2 production, the average value for H_2 production under Ar at a specific interval was designated 100% and the average value of H_2 production under a specific pN_2 at the same interval was expressed as a percentage of the Ar control. Therefore, the data presented in Figure 4 represent the observed inhibition by a particular pN_2 relative to the H_2 production under Ar, regardless of how H_2 production under Ar compared to C_2H_2 reduction. There is considerable variation in the amount of inhibition at a specific pN_2 (there was no difference in the inhibition by $^{14}N_2$, $^{15}N_2$, or a mixture at the same pN_2 in the same preparation). However, the variation was consistent with the C_2H_2/N_2 ratios obtained in individual experiments. In general, the lower the rate of H_2 production was under a specific pN_2, the lower the C_2H_2/N_2 ratio was at that pN_2. As with the inhibition of H_2 production by C_2H_2 (Fig. 1), the inhibition at any pN_2 is somewhat greater than is observed in *in vitro* nitrogenase studies (16). Figure 4 also demonstrates that at the same pN_2, inhibition of H_2 evolution in the symbiont is generally less than that observed in the association.

Time course studies showed that the rates of C_2H_2 reduction, N_2 fixation, and H_2 production were relatively linear after a 4- to 6-hr incubation period, and during this interval $^{15}N_2$ incorporation from gas atmospheres with atom % excess ^{15}N as low as 20% was sufficient to provide confidence in the accuracy of analysis. Therefore, we used a 5-hr incubation with a minimum of three replicate samples in attempts to demonstrate an electron balance *in vivo*. H_2 production and $^{15}N_2$ fixation were determined as a function of pN_2. This information plus the rate of C_2H_2 reduction (pC_2H_2 = 0.1 atm) under identical conditions were employed to determine the electron balance. It was assumed that all electron flow through the nitrogenase was utilized in C_2H_2 reduction (a $2e^-$ process) and that as *in vitro* (7, 20) total electron flow was constant regardless of the substrate. There-

Table II. A comparison of C_2H_2 reduction, H_2 production under Ar and 0.29 and 0.60 atm N_2, the C_2H_2/N_2 ratios and electron balance, i.e., $C_2H_4/(3N_2 + H_2)$, for the association and isolated symbiont.

The incubation period was 5 hr at 500 ft-c, 28 C. The atom % excess ^{15}N in the alga was 0.413 at 0.29 atm N_2 and 0.386 at 0.60 atm; for the association it was 0.219 at 0.29 atm N_2 and 0.258 at 0.6 atm N_2, based on an average of 4 to 6 determinations with and without carrier N.

Product (or ratio)	Association	Isolated Symbiont
	nmol/mg Chl·min	
C_2H_4 (light)	13.96	92.97
C_2H_4 (dark)	0.06	0.35
H_2 (pN_2<0.005 atm)	8.58	89.97
H_2 (pN_2 0.29 atm)	2.02	35.90
H_2 (pN_2 0.60 atm)	1.31	20.21
N_2 fixed (pN_2 0.29 atm)	4.35	21.30
N_2 fixed (pN_2 0.60 atm)	6.91	30.76
C_2H_2/N_2 (pN_2 0.29 atm)	3.21	4.36
C_2H_2/N_2 (pN_2 0.60 atm)	2.02	3.02
$C_2H_4/(3N_2 + H_2)$ (pN_2 0.29 atm)	0.93	0.93
$C_2H_4/(3N_2 + H_2)$ (pN_2 0.60 atm)	0.63	0.83

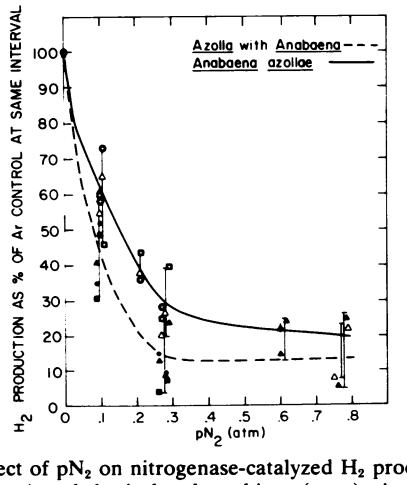

FIG. 4. Effect of pN_2 on nitrogenase-catalyzed H_2 production in the association (- - -) and the isolated symbiont (——). Average rates of duplicate or triplicate samples at 3 hr (●, ○), 5 or 6 hr (▲, △) and 9 or 10 hr (■, □) are expressed as a percentage of the rate of H_2 production under Ar (pN_2 <0.005 atm). Marker lines indicate range at comparable partial pressures. Solid symbols represent the association, open symbols the isolated alga.

fore, the total electrons employed in C_2H_2 reduction should equal the sum of the electrons employed in the reduction of N_2 (a $6e^-$ process) and protons (a $2e^-$ process) at any pN_2. Expressing this as an equation yields

$$\frac{\text{moles of } C_2H_4 \text{ produced}}{3\ (\text{moles } N_2 \text{ fixed}) + \text{moles } H_2 \text{ evolved}} = 1$$

The effect of H_2 evolution on the C_2H_2/N_2 ratio and electron balance *in vivo* is apparent in the comparison of the association and isolated alga in Table II. At a pN_2 of 0.29 atm, the expression of the electron balance is equal to 0.93 for both, but the C_2H_2/N_2 ratio is greater in the alga, *i.e.* 4.4 *versus* 3.2 for the association. The difference in the C_2H_2/N_2 ratios is clearly the result of more H_2 evolution in the alga than in the association at this pN_2. At a pN_2 of 0.6 atm, the C_2H_2/N_2 ratio for the alga is 3.0 while in the association a value of 2.0 is obtained. The value for the expression of the electron balance has decreased in both, the decrease being greater in the association than in the alga. Although the increase in N_2 fixed at 0.6 atm *versus* 0.29 atm N_2 is comparable in the association and alga, the inhibition of H_2 is slightly greater in the association. In this comparison, it should also be noted that in the association H_2 evolution under Ar was about 61% of the rate of C_2H_2 reduction, while in the alga it was about 97%, *i.e.* the highest value attained.

While the values obtained at a pN_2 of 0.29 atm were within the expected range, as was the value at 0.6 atm with the alga, the values for the C_2H_2/N_2 ratio and electron balance for the association at 0.6 atm N_2 were suspiciously low. Therefore, comparable studies were conducted at a pN_2 of 0.8 atm with the alga and at a pN_2 of 0.8 atm ± 0.2 atm O_2 with the association (Table III). During the time course employed, O_2 had no effect on C_2H_2 reduction or N_2 fixation in the association. However, the C_2H_2/N_2 ratio and the expression of the electron balance in both the association and alga decreased again. In all cases, the rate of H_2 production under Ar was approximately 70% of the rate of C_2H_2 reduction (70 and 77% in the association with and without O_2 respectively, and 67% in the alga). Compared to H_2 production under Ar, a pN_2 of 0.8 atm resulted in 96% inhibition of H_2 production in the association and a 93% inhibition in the alga. In other experiments at this pN_2, less inhibition of H_2 was observed (Fig. 4).

The values obtained for both the expression of the electron balance and the C_2H_2/N_2 ratio at a pN_2 of 0.29 atm in the association, and at pN_2 of 0.29, 0.6, and 0.8 atm in the alga are considered reasonable. As shown in Figure 4, the inhibition of H_2 production at pN_2 greater than 0.3 atm became more or less asymptotic. Therefore, the lower values obtained in the association at 0.6 and 0.8 atm N_2 initially suggest either too low a determination of the C_2H_2 reduction or too high a value for N_2 fixation. However, there was a constant trend in both the symbiont and association toward lower C_2H_2/N_2 ratios with increasing pN_2.

Table III. A comparison of the C_2H_2 reduction, H_2 production under Ar and 0.8 atm N_2, and the C_2H_2/N_2 ratios and electron balance in the association (± O_2) and the alga at 0.8 atm N_2.

The incubation period was 5 hr at 500 ft-c, 28 C. The atom % excess ^{15}N in the incubation gas for the alga was 22.31 and for the association ± O_2, 22.10. The measured pO_2 was 0.18 atm and the pN_2 0.75 atm.

Product (or ratio)	Association		Isolated Symbiont
	nmol/mg Chl·min		
	$+O_2$	$-O_2$	$-O_2$
C_2H_4	14.84	14.53	58.46
H_2(pN_2<0.005 atm)	10.31	11.12	39.25
H_2(pC_2H_2 0.1 atm)	0.42	0.51	1.40
H_2(pN_2 0.75 atm)	0.42	0.46	2.91
N_2 fixed(pN_2 0.75 atm)	9.05	8.80	23.80
C_2H_2/N_2	1.64	1.65	2.46
$C_2H_4/(3N_2 + H_2)$	0.54	0.54	0.79

DISCUSSION

Without determinations of H_2 production and a demonstration of electron balance, it may be fortuitous to obtain conversion factors in the range of 3 to 4 *in vivo*. We have found that with increasing pN_2, both the C_2H_2/N_2 ratio and the expression for the electron balance decrease. While we cannot totally exclude experimental error in the present work, it may be appropriate to pose two questions. (*a*) Can it be assumed that the rate of electron flow to the nitrogenase *in vivo* is constant, regardless of the substrate and its concentration? (In the case of photosynthetic organisms, this question must be extended to encompass whether the rate of photosynthesis and coupled metabolic process are constant under various gas atmospheres.) (*b*) If an uptake hydrogenase is present, is its activity constant under varying pN_2 and does its activity vary from one preparation to another? For example, in our studies, conversion factors of less than 3 could easily arise if the relative rate of photosynthesis increased with increasing pN_2 up to 0.8 atm. Similarly, the ratio would likewise be affected if the activity of an uptake hydrogenase is variable or affected by the pN_2 employed, and if C_2H_2 were to inhibit net photosynthesis in any way, such as a slight uncoupling of photophosphorylation.

In free living blue-green algae, C_2H_2/N_2 values of 2.8 to 3.6 (19), 1.42 to 2.47, averaging 1.9 (4), and 2.7 to 6.5, averaging 4.4 (14) have been reported. The exposure periods were from 30 min (4, 14) to 1 hr (19) and the partial pressures of N_2 were 0.25 atm (the data corrected to account for an apparent 83% saturation at this pN_2) (19), 0.36 atm (14), and 0.50 atm (4). C_2H_2 was used at 0.13 to 0.14 (4, 14) and 0.25 atm (19). There was *no* information provided on H_2 production. In those instances in which we used a pN_2 of 0.25 atm or greater, the C_2H_2/N_2 ratios reported in this paper fall within the range of values reported for nonsymbiotic blue-green algae. Moreover, the present studies demonstrate the variation encountered as a function of the pN_2, within the same organism and under the same experimental conditions.

H_2 production under Ar is variable and consistently lower than C_2H_2 reduction when this fern-algal association is grown solely on N_2 and our studies implied the existence of an uptake (unidirectional) hydrogenase in the symbiont (11). H_2 evolution by fronds grown on N_2 was greatest under 0.1 atm C_2H_2, 0.02 atm CO in Ar (11), but we failed to associate this observation directly with uptake hydrogenase activity. A recent report has shown that C_2H_2 inhibits the activity of an uptake hydrogenase (18) and that in the presence of C_2H_2 plus CO, *all* nitrogenase-catalyzed H_2 production, including that portion normally reoxidized by the hydrogenase, appears in the gas phase. Therefore, it seems probable that much of the variability between the rates of C_2H_2 reduction and H_2 evolution under Ar in our studies can be attributed to variations in hydrogenase activity.

Since the reoxidation of H_2 by the uptake hydrogenase provides additional reducing power, the efficiency of nitrogen fixation is presumably increased (6, 17, 18). Applying the relative efficiency expression of Schubert and Evans *i.e.*

$$1 - \frac{H_2\ (\text{Air})}{H_2\ (\text{Ar})} \text{ or } 1 - \frac{H_2\ (\text{Air})}{C_2H_2}$$

(17), to our published data (11), the relative efficiency of the *Azolla-Anabaena azollae* symbiosis grown on N_2 is 0.94 to 0.99, which is comparable to what they reported for nonlegume symbiotic associations. It is apparent that there should be a correlation between the C_2H_2/N_2 ratio and the relative efficiency value obtained for any organism. In essence, low efficiency values should correspond to high C_2H_2/N_2 ratios and *vice versa*. (In the absence of experimental error it is nevertheless impossible to account for C_2H_2/N_2 ratios of less than 3.0 in even the most efficient organisms unless there are differences in the rate of electron flow to the nitrogenase under different gas atmospheres

or other unknown factors are involved.) It is worth noting, however, that the C_2H_2/N_2 ratio cited for Alnus (8), which was reported to have a very high efficiency value of 0.99 (17), ranged from 2.0 to 2.8. This is in relatively good agreement with the results reported here for *Azolla*. In legumes, the *over-all* average of the conversion factor is reported as 3.9 but average values for individual species range from 2.3 to 6.6 and individual determinations from 1.5 to 8.4 (8). In light of recent findings (17), it is probably inappropriate to refer to an average C_2H_2/N_2 ratio for legumes as a group and an average value of 3.9 for the species studied may be fortuitous. For example, 75% of the leguminous species studied by Schubert and Evans (17) were indicated to expend 40 to 60% of their ATP and reductant on nitrogenase-catalyzed H_2 production under an air atmosphere (0.78 atm N_2). Excluding experimental error, it would be impossible to attain a C_2H_2/N_2 ratio of less than 5 if electron flow were constant *in vivo*, regardless of the substrate, and there was this much H_2 evolved. Clearly, parallel determinations of H_2 production are highly desirable in conjunction with C_2H_2/N_2 ratios.

We suggest that variations obtained in C_2H_2/N_2 ratios may well reflect differences in both the activity of an uptake hydrogenase and in the electron flow under the various gas atmospheres employed. As stated by Burris (4), one is faced with either employing the theoretical value of 3.0 or using an experimentally derived constant. We would conclude that for *in vivo* studies, a valid conversion factor is obtained only in a comparison of organisms grown under exactly the same conditions and under a pN_2 approximating that of air. We must conclude from this study that the conversion factor most consistent with natural conditions is between 1.6 and 2.0 for the association and 2.5 and 3.0 for the algal symbiont. The higher values for the isolated symbiont may be due to the concentration of dissolved nitrogen in the medium being lower than that which occurs in the leaf cavity when the intact association is exposed to the same partial pressure of N_2. This is suggested by less inhibition of H_2 production in the alga than in the association at the same pN_2.

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