THE QUANTITATIVE RELATIONSHIP BETWEEN NITROGEN FIXATION AND THE ACETYLENE-REDUCTION ASSAY

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Summary

Acetylene-reduction activity of detached soybean nodules was positively correlated with the nitrogen content of the host plants grown in nitrogen-free media. Acetylene reduction by nodulated root systems was related to light intensity preceding sampling; activity of detached nodules was lower and less influenced by light. Comparison of acetylene reduction and $^{15}N_2$ uptake by detached soybean nodules gave ratios of acetylene reduced to nitrogen fixed (C_2H_4 : NH_3 ratio) ranging from 2·7 to 4·2 in different experiments. The mean value for four experiments was 3·3±0·6 (S.E.). Different effects of oxygen were obtained with detached soybean nodules when 0·1 atm nitrogen and 0·1 atm acetylene were used as substrates. When the acetylene concentration was reduced to 0·005 atm the effects upon both assays were similar but the ratio of the two assays was still affected by pO_2.

The C_2H_4 : NH_3 ratio in a growing culture of *Azotobacter vinelandii* fell with time. It was concluded that this was probably due to the failure to match the oxygen-solution rates in the culture and in the acetylene-reduction assays.

Growth and nitrogen fixation, in a culture of *Klebsiella aerogenes*, were linear with time for 8 hr following the logarithmic growth phase. During this 8-hr period, the ratio C_2H_4 : NH_3 was 1·5±0·4 (S.E.). During the following 8 hr, uptake of nitrogen declined but acetylene reduction did not. The acetylene-reducing activity of this culture was destroyed by brief exposure to air during sampling.

It is concluded that caution should be observed in the application of the acetylene-reduction assay to quantitative determinations of nitrogen fixation. Major errors are likely to result when the conditions in the assays are not carefully matched with the conditions under which nitrogen fixation is occurring.

I. Introduction

Since the work of Dilworth (1966) and Schöllhorn and Burris (1967) showed that acetylene was reduced to ethylene by nitrogen-fixing systems, a number of authors have developed the phenomenon as a sensitive assay for nitrogen-fixing activity (e.g. Koch and Evans 1966; Stewart, Fitzgerald, and Burris 1967; Hardy *et al.* 1968; Roughley and Dart 1969; Sprent 1969; Schwinghamer, Evans, and Dawson 1970). It has been found that acetylene reduction by nitrogenase has the same requirement for reductant, Mg^{2+}, and ATP as does the reduction of nitrogen to ammonia or of H^+ to H_2. The activities in terms of the reduction of acetylene or nitrogen were parallel during purification of nitrogenases from free-living bacteria and from legume nodule bacteroids (Hardy *et al.* 1968; Klucas *et al.* 1968). Experiments such as these have established the validity of the acetylene-reduction assay for nitrogenase activity. Although other systems producing ethylene in nature might be expected, they have not proved to be a hazard as long as suitable controls are included in experiments.

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The measurements of the input of nitrogen into ecosystems has been a major difficulty in the measurement of nitrogen balance. It has been hoped that the acetylene-reduction method would provide an adequate measurement for this purpose and it has been used for nitrogen-fixation studies in field situations. Before the potential of the method can be realized it must be established that there is a constant quantitative relationship between these two reactions of nitrogenase and the numerical value of the relationship must be accurately known. If substrate, energy supply, and reductant supply are not limiting, the ratio of the products for equal numbers of electrons transferred should be 1·5 : 1 according to the reactions:

$$3C_2H_2+6H^++6\epsilon \rightarrow 3C_2H_4,$$

or

$$N_2+6H^++6\epsilon \rightarrow 2NH_3.$$

The alternative of expressing the relationship as the ratio of nitrogen to acetylene reduced (3 : 1 above) has been commonly used (e.g. Hardy *et al.* 1968; Klucas *et al.* 1968). Values close to this theoretical value have been obtained by a number of workers with cell-free extracts of nitrogen-fixing organisms but there is little information available from intact living systems. Preliminary experiments in our laboratory indicated that considerable variations in the ratio could be encountered. These may have been due to some fundamental differences between the two reactions. For example, although acetylene is iso-electronic with nitrogen and has similar molecular dimensions, it is about 65 times more soluble in water than nitrogen (at 1 atm and 25°C, acetylene in solution is 41·9 mM and nitrogen in solution is 0·64 mM). In whole cell systems, fixed nitrogen enters the nitrogen pools of the cells and contributes to protein synthesis, while acetylene reduction measures the activity of the nitrogenase system only and the product makes no contribution to the metabolism of the cells. The two substrates may also have differing abilities to enter living cells through lipoprotein membranes. In addition, nitrogenase is a fairly unstable enzyme system and failure to match adequately environmental conditions in the acetylene-reduction assay with those of the nitrogen-fixing system in nature may have a differential effect.

This paper presents results from a number of experiments in which factors affecting the reduction of nitrogen to ammonia and of acetylene to ethylene have been examined in three different types of nitrogen-fixing systems.

II. Materials and Methods

(*a*) *Legumes*

Soybeans (*Glycine max* Merr. cv. Lincoln) inoculated with *Rhizobium japonicum* strain CB1809 were grown in pots of vermiculite : sand (1 : 1 v/v) watered daily with nitrogen-free nutrient solution (McKnight 1949, diluted 1 : 1 with tap water). The glasshouse temperature was controlled between a minimum of 20°C at night and a maximum of 26°C during the day. In experiments in which different host varieties and bacterial strains were compared, the same medium was used in aseptic jar assemblies (Leonard 1944, quoted in Norris and t'Mannetje 1964).

Nodules were detached from a number of plants, washed, blotted dry, divided into subsamples, and used immediately for most experiments. In experiments comparing nodulated roots with detached nodules, each sample consisted of the root system of a single plant decapitated

at the hypocotyl or the nodules detached from a single plant. In other experiments, all of the nodules from single plants were used separately and the results related to the nitrogen content (Williams and Twine 1967) of the tops of the same plants. The activity measured was expressed in terms of nodule fresh weight for both nodulated roots and detached nodules. Alternatively, it was expressed on a per plant basis.

(*b*) *Nitrogen-fixing Cultures*

Cultures of *Azotobacter vinelandii* strain O (P. W. Wilson, University of Wisconsin) and *Klebsiella aerogenes* strain H26 (Bergersen and Hipsley 1970) were grown in 4 litres of Burk medium (Burk and Lineweaver 1930) at 30°C in the 7-litre culture vessel of Microferm equipment (New Brunswick Scientific Co., New Jersey). The *A. vinelandii* cultures were stirred at 800 r.p.m. while air was passed in at a rate of 8 litres/min. These conditions had been found to be optimal in preliminary experiments. The *K. aerogenes* cultures were stirred at 500 r.p.m. and oxygen-free nitrogen was passed in at a rate of 0·5 litres/min. Samples of the cultures were removed at intervals for measurement of growth, nitrogen content, and acetylene reduction. For the *Klebsiella* cultures this was done anaerobically by syringe through a rubber-capped port. Growth was measured by recording optical density at 600 nm and total nitrogen was determined by digesting 10-ml samples with 5 ml of concentrated sulphuric acid containing 1 g Se and 100 g sodium sulphate per litre, and distilling and titrating the ammonia. The rates of oxygen solution in cultures and assays were measured by the sulphite oxidation method (Cooper, Fernstrom, and Miller 1944).

(*c*) ^{15}N *Methods*

Nitrogen fixation by detached root nodules was measured as follows. Nodule samples (1 g fresh weight) were incubated at 25°C without shaking, in gas mixtures containing 0·05, 0·10, or 0·20 atm $^{15}N_2$ (*c.* 60 atoms % ^{15}N), with 0·20 atm oxygen and the balance argon, for 1 hr. The nodules were then digested with 10 ml of digestion mixture (see above) and total nitrogen measured by distillation and titration. The ^{15}N content of this nitrogen was then determined from the measurement of the mass 29 : mass 28 ratio of nitrogen in an Atlas M86 mass spectrometer, after oxidation of the NH_4^+ with sodium hypobromide. The amount of nitrogen fixed was calculated from the total nitrogen, the ^{15}N enrichment of the sample, and the ^{15}N content of the gas phase during the incubation. From these data, for each experiment, the regression of $1/v$ upon $1/s$ was determined ($v = \mu$moles ammonia $hr^{-1} g^{-1}$; $s = pN_2$ in atm, 1 atm = 700 mm Hg). From this the nitrogen-fixation rate at atmospheric nitrogen concentration (0·78 atm) was calculated. This method was used to conserve $^{15}N_2$. Nitrogen fixation was linear with time for the period of the assay.

(*d*) *Measurement of Acetylene Reduction*

Acetylene-reduction assays were done in rubber-capped glass vials of 13·5 ml capacity and incubated for 1 hr at 23°C for nodules or 30°C for cultures. Unless otherwise stated, gas phases contained 0·20 atm acetylene, 0·20 atm oxygen, and 0·60 atm argon for aerobic assays. For anaerobic assays the mixture was 0·25 atm acetylene in argon. Aerobic assays of cultures contained 2 ml of culture and they were shaken at 250 cycles/min. Anaerobic assays also contained 2 ml of culture but were shaken at 120 cycles/min. Nodule assays contained no free liquid and were not shaken. In all cases, reduction rates were linear with time for the period of the assays. Analysis of acetylene and ethylene was done with a Philips P4000 gas chromatograph equipped with flame-ionization detectors and matched columns (2 m by 2 mm) of Poropak R at 50°C. Samples of gas (200 μl) were withdrawn from the assay vials by gas-tight syringe and injected into the gas chromatograph in a standard manner. Peak heights of acetylene and ethylene were directly proportional to concentration; acetylene was retained for 1·8 min and ethylene for 2·4 min. Control vials containing the starting gas mixture or a standard ethylene : argon mixture (0·053%) (Commonwealth Industrial Gases, Sydney) were included with all assays. Analyses whose acetylene peak height departed by more than 5% from the analysis of the starting gas mixture were discarded.

III. Results and Discussion

(a) *Experiments with Nodules*

(i) *Relationship between Plant Nitrogen and Acetylene Reduction by Nodules*

Four strains of *R. japonicum*, representing different degrees of symbiotic effectiveness, were used to inoculate four varieties of soybean. After 5 weeks of growth, the plants were carefully removed from the Leonard jars, the tops dried, weighed, and analysed for nitrogen and the nodules subjected to the acetylene-reduction assay. The results showed that there was a good correlation between the shoot nitrogen at harvest and the assays. The data for the host varieties Shelby and Wills are presented in Figure 1. These results were similar to those of Schwinghamer, Evans, and Dawson (1970) with peas and red clover. The slopes of the regression lines in Figure 1 would be expected to vary with time of harvest. Unreliable relationships between the two measurements would be likely early in growth, before nitrogen fixation had significantly increased plant nitrogen, and later in the growth of the plants, when some nodules may be ceasing to function.

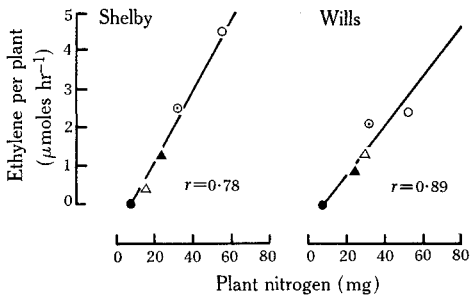

Fig. 1.—Relationship between total nitrogen in the tops of soybean plants grown in nitrogen-free medium and the acetylene-reduction assays of nodules detached from these plants. Each point is the mean for two plants inoculated with each strain of *R. japonicum*. The correlation coefficients were calculated from the data for individual plants. ● Uninoculated control. Bacterial strains CB1809 (○), CC711 (⊙), CC705 (▲), and CC703 (△).

(ii) *Effects of Illumination*

The activity of nodules detached from uniformly aged, glasshouse-grown soybeans was influenced by illumination preceding harvest as was suggested from data presented by Hardy *et al.* (1968). Samples of nodules, assayed on 22 days between November 21, 1968, and February 11, 1969, showed that those taken on days which were bright and sunny for at least 2 hr before harvest had a mean activity of $6 \cdot 7 \pm 1 \cdot 7$ (S.E.) μmoles ethylene $hr^{-1}\,g^{-1}$. Those taken on days with more than 5/10 cloud during 2 hr or more preceding harvest had a mean activity of $3 \cdot 0 \pm 0 \cdot 5$ (S.E.) μmoles ethylene $hr^{-1}\,g^{-1}$. In no case was a cloudy-day sample as active as any bright-day sample.

These effects were investigated further in an experiment in which acetylene reduction by nodulated roots and nodules detached from individual plants was measured at intervals during a 28-hr period (2 days and 1 night). Four replicates of each treatment were used at each sampling time. Diffuse light intensity was measured with an EEL photometer in the shadow of a white screen placed on the glasshouse bench surface between the photometer and the direction of the sun. The results are presented in Figure 2. During the day, the activity of nodulated roots was higher and more variable than that of detached nodules and the higher activity was maintained for about 2 hr after the onset of darkness. After about 10 hr of darkness the

activity of nodulated roots had fallen close to the value for detached nodules. Activity of nodulated roots continued to rise for 3 hr after the onset of bright sunny conditions. The activity of detached nodules was also influenced by periods of bright light but the effect was smaller and more transient than that observed with nodulated roots. These results are interpreted as showing that the nodules have a basal endogenous rate of activity, upon which is superimposed activity stimulated by photosynthetic products which can readily move into the nodules from the roots. The detached nodules in this experiment were more active than the bright-day nodules assayed previously. This may have been due to a prolonged spell of favourable growing conditions which preceded the experiment, allowing the endogenous nodule activity to increase to a high level.

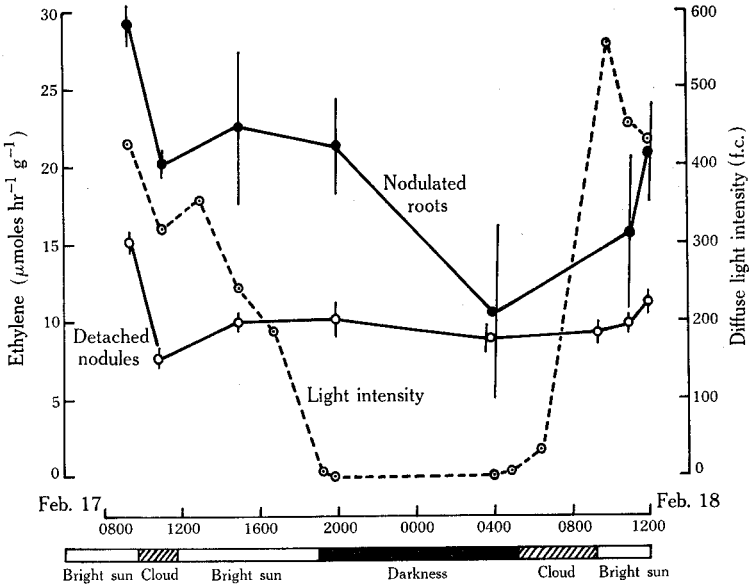

Fig. 2.—Relationship between light intensity and the acetylene-reduction assay for detached nodules and nodulated roots (both expressed in terms of nodule weight) of glasshouse-grown soybeans. Vertical lines show the magnitude of the standard errors of each determination. Assays were made at 25°C.

The results also show that the quantitative estimation of nitrogen fixed by legumes, by means of the acetylene-reduction assay, will require the integration of results obtained at several sampling times each day. Sampling at a fixed time each day, as used by Hardy *et al.* (1968), cannot be expected to provide very accurate measurement of nitrogen fixation.

(iii) *C_2H_4 : NH_3 Ratio for Nodules*

Four experiments compared nitrogen fixation with acetylene reduction by detached soybean nodules. As far as could be determined the experiments were similar. The nodules were picked at the same time of day from plants of the same age during a period of 10 days in February 1969. Five replicates were used in the

acetylene-reduction assays. In two experiments the standard error of the means was about 5%. For unknown reasons, in the other two experiments the standard errors were 11·6 and 13·1% of the means. This variability in the accuracy of acetylene-reduction assays has been a feature of the work with nodules in this laboratory and has also been encountered elsewhere (e.g. Roughley and Dart 1969). In one of the experiments reported herein, fresh weight and dry weight data suggested that the nodules may not have been in moisture equilibrium during the measurements and this may have contributed to the increased variation. It was not possible to calculate standard errors for the nitrogen-fixation measurements because of the method employed. However, examination of the individual values obtained at each pN_2 showed a maximum variation of about 6% between duplicates. The apparent K_m (N_2) values in these experiments varied between 0·06 and 0·08 atm (42–56 mm) indicating that the experiments were kinetically similar. However, the actual activity (v) of the nodules in both assays varied between experiments. The results, summarized in Table 1, show that the C_2H_4 : NH_3 ratio varied between 2·7 and 4·2. Over all, the

TABLE 1

RELATIONSHIP BETWEEN NITROGEN FIXATION AND ACETYLENE REDUCTION BY DETACHED SOYBEAN ROOT NODULES

Measurements were made with samples of 1 g (fresh weight) of nodules detached from plants aged 40 days. Five replicates used for acetylene assays. For details of the $^{15}N_2$ uptake tests, see text

Expt. No.	Acetylene Reduction (μmoles C_2H_4 hr^{-1} g^{-1})	Nitrogen Fixation			C_2H_4 : NH_3 Ratio*
		V_{max} (μmoles NH_3 hr^{-1} g^{-1})	K_m (atm)	$v_{0.78\ atm}$ (μmoles NH_3 hr^{-1} g^{-1})	
1	6·31±0·27	2·51	0·072	2·26	2·79
2	8·33±1·09	3·43	0·080	3·08	2·71
3	5·83±0·68	1·92	0·084	1·74	3·36
4	7·62±0·39	1·95	0·059	1·81	4·20

* Mean 3·3±0·6.

mean ratio was 3·3±0·6 (S.E.). The variation was such that it could not be assumed in any one experiment that this ratio would apply.

In these experiments, gas mixtures nominally containing 0·2 atm oxygen (by manometer) were carefully prepared in reservoirs. Mass spectrometer analyses revealed that actual oxygen concentrations varied between 0·21 and 0·24 atm. Although these differences would be acceptable in many experiments, the activity of nodules responds very sharply to increased pO_2 in this range and relatively small differences may produce quite large differences in nitrogen fixation (Bergersen 1962). An increase from 0·20 to 0·24 atm oxygen could be expected to increase activity by up to 28%. More careful matching of pO_2 between the atmosphere in which nitrogen fixation is occurring and those used in the acetylene assays will be necessary in order to reliably use these as accurate quantitative assays of nitrogen fixation by detached

nodules. It is difficult to devise further improvements in gas mixture preparation which would be suitable for biological experiments.

Other effects of oxygen must also be considered. Koch and Evans (1966) found that acetylene reduction by detached soybean nodules remained constant from 0·2 atm oxygen upwards, when the acetylene concentration in the gas phase was 0·1 atm. It is known that nitrogen fixation is inhibited at supra-optimal pO_2 in a nearly competitive fashion (Bergersen 1962). We therefore examined the effects of oxygen upon acetylene reduction by detached soybean nodules. It was found that when 0·1 atm of acetylene was used ethylene production remained unaffected by pO_2 above 0·5 atm [Fig. 3(*a*)] in partial agreement with Koch and Evans (1966). However, when the acetylene concentration was reduced to 0·005 atm, pO_2 values above 0·5 atm were inhibitory, as they also were for $^{15}N_2$ uptake [Fig. 3(*b*)]. The

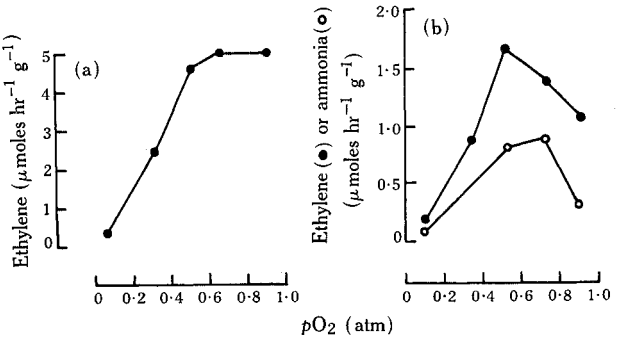

Fig. 3.—Effects of pO_2 upon acetylene reduction and nitrogen fixation by detached soybean root nodules. Data from two experiments: (*a*) 0·1 atm acetylene (●); (*b*) 0·005 atm acetylene (●), 0·1 atm $^{15}N_2$ (○).

earlier results (Bergersen 1962) predicted that oxygen inhibition could be reversed by increasing the nitrogen concentration. This is not possible at total pressures of one atmosphere because of the limited solubility of nitrogen in water. However, with the alternative substrate, acetylene, with its much higher solubility, there was no oxygen inhibition when 0·1 atm acetylene was used [Fig. 3(*a*)]. Furthermore, the shapes of the oxygen response curves were different, thus causing a variable relationship between the two assays when the lower acetylene concentration was used. At 0·2 atm oxygen, the $C_2H_4 : NH_3$ ratio was 3·1 : 1 but at 0·7 atm oxygen it was 1·5 : 1.

The K_m (C_2H_2) for nodulated soybean roots has been reported to be about 0·007 atm (Hardy *et al.* 1968). In this laboratory the value for soybean bacteroid nitrogenase has been found to be about 1 mmHg, which is equivalent to 54 μM acetylene in solution at 25°C (Turner and Bergersen, unpublished data). The K_m (N_2) of about 0·07 atm (Table 1) is similar to values obtained previously for nodules and bacteroid extracts (Bergersen and Turner 1968). It is equivalent to about 45 μM nitrogen in solution. The K_m (C_2H_2) and K_m (N_2) for the soybean nitrogenase are thus very similar in terms of substrate concentration in solution. It follows that, for calibration of the relationship between acetylene reduction and nitrogen fixation, the acetylene concentration should be adjusted to give equimolar concentrations of acetylene and nitrogen in solution. When this is done, the effects of oxygen upon both assays are similar and the $C_2H_4 : NH_3$ ratio remains constant over a wider

range of pO_2. If in some legume nodules the K_m (C_2H_2) and the K_m (N_2) differ in terms of substrate in solution, the acetylene concentration should be adjusted accordingly.

(*b*) *Experiments with Growing, Nitrogen-fixing Cultures*

(i) *Azotobacter vinelandii*

In two experiments, samples of culture were withdrawn from the culture vessel at 2-hr intervals and examined for acetylene-reducing activity and nitrogen content. One experiment dealt with the exponential phase of growth and the other with the phase of declining growth rate. Figure 4 presents the data expressed as nmoles ethylene hr^{-1} ml^{-1} and as nmoles ammonia hr^{-1} ml^{-1} in relation to growth. The nitrogen increments were measured at 2-hr intervals and are plotted as the mid-points between samplings. It can be seen that the relationship between acetylene reduction and nitrogen fixation changed as the culture aged. Acetylene reduction

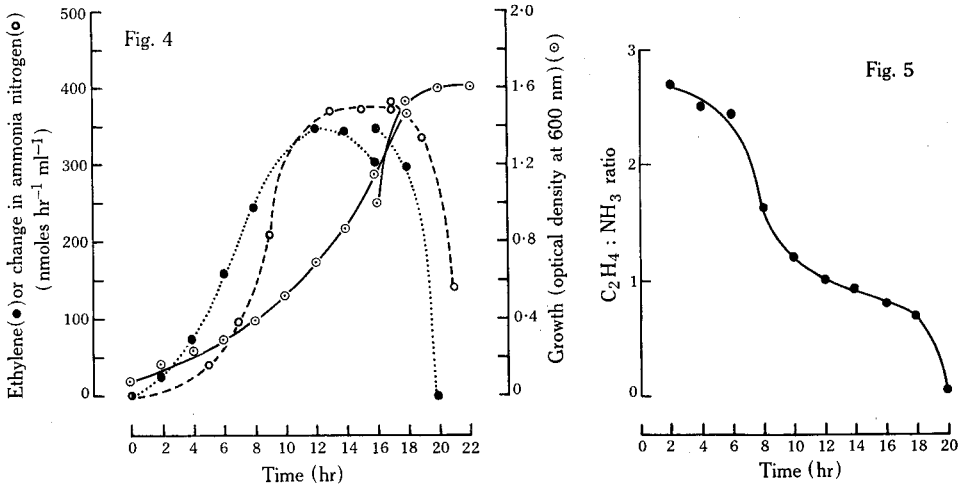

Fig. 4.—Growth, nitrogen fixation, and acetylene reduction by *Azotobacter vinelandii* in culture. Data for two comparable experiments. The culture contained 14·2 μg nitrogen per millilitre at time 0 and 46·9 μg nitrogen per millilitre after 16 hr growth.

Fig. 5.—*A. vinelandii* culture : the change in C_2H_4 : NH_3 ratio with time. Values were calculated from the nitrogen curves of Figure 4 and the acetylene-reduction assays at the times shown.

declined before nitrogen fixation. The ratio of the assays was close to 3 : 1 early but fell sharply between 6 and 10 hr and again between 18 and 20 hr (Fig. 5). Preliminary experiments had established that the growth conditions which were used were optimal and sulphite oxidation measurements of the oxygen solution rates established that the rate was higher in the acetylene-reduction assay (0·17 ml oxygen ml^{-1} min^{-1}) than it was in the culture (0·03 ml oxygen ml^{-1} min^{-1}). Therefore, the change in ratio as the culture grew was not due to increasing oxygen limitation in the acetylene assay caused by increasing cell density. Optimum efficiency of nitrogen fixation by *Azotobacter* cultures is achieved at low pO_2 values or aeration rates (Parker and Scutt 1960; Dalton and Postgate 1969). It is therefore possible that the fall in the

$C_2H_4 : NH_3$ ratio (Fig. 5) was due to nitrogen fixation becoming increasingly efficient as growth proceeded and oxygen became increasingly limiting in the culture. No similar effect upon acetylene reduction would be expected because of the higher rates of oxygen solution in these assays. This explanation was not supported by the results of preliminary experiments which showed that growth rates were reduced when the culture aeration rates were reduced below that used in the experiments of Figure 4. It is also possible that the sensitivity of the cells to oxygen increased as the culture aged, resulting in a decline in acetylene reduction (where aeration was higher) relative to nitrogen fixation. Whatever the true explanation of these results, the experiments emphasized the need for careful matching of aeration rates used for acetylene-reduction assays with those applying under the conditions in which nitrogen fixation is occuring. In order to avoid this complication in the comparison of the two reactions, an anaerobic nitrogen-fixing bacterium was studied.

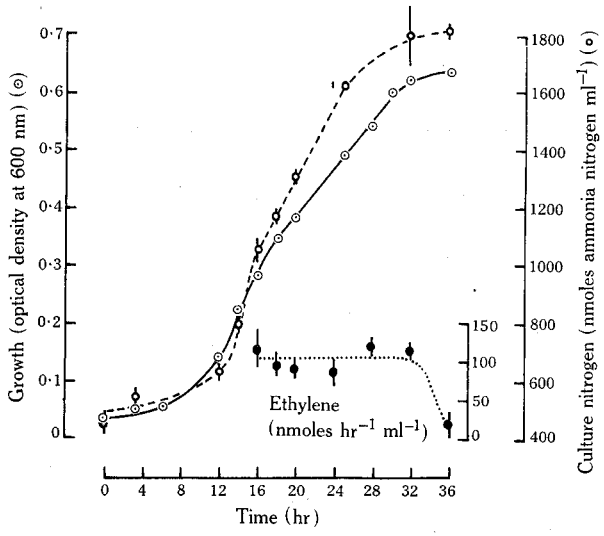

Fig. 6.—Growth, nitrogen fixation, and acetylene reduction by *K. aerogenes* in anaerobic culture. Vertical lines show the ranges of duplicate observations.

(ii) *Klebsiella aerogenes*

In this experiment, acetylene-reduction assays were made on aliquots of a culture which was growing in a linear manner with time from 16 to 25 hr from inoculation, following a period of exponential growth (Fig. 6). During linear growth, nitrogen fixation was also linear with a slope of $68 \cdot 5 \pm 12 \cdot 5$ (S.E.) nmoles ammonia hr^{-1} ml^{-1}. (It should be noted that the expression of culture nitrogen is cumulative in Figure 6 and not change in ammonia nitrogen as in Figure 4.) During this phase of growth (16–25 hr) eight acetylene-reduction assays were made which gave a mean value of $102 \cdot 5 \pm 19 \cdot 0$ (S.E.) nmoles ethylene hr^{-1} ml^{-1}. The $C_2H_4 : NH_3$ ratio was thus $1 \cdot 50 \pm 0 \cdot 39$ (calculated from the large sample formula for the standard error of a ratio).

From Figure 6 it may also be seen that, although nitrogen fixation declined between 25 and 32 hr, acetylene reduction did not, indicating again that conditions in the assay were not matched to conditions in the culture.

During this work it was noted that the *K. aerogenes* culture was very sensitive to oxygen. Samples which were exposed to air for 30 sec before injection into evacuated assay bottles were almost completely inactive. Slight air contamination during sampling may thus have contributed to the relatively large standard error in the acetylene-reduction assays.

IV. Conclusions

The experiments reported here show that it will be necessary to establish a calibration for the relationship between nitrogen fixation and acetylene reduction for every condition in which the latter assay is to be used as a quantitative estimate of nitrogen fixation. Rigid standardization of the assay will also be necessary and the procedures should take account of such factors as the matching of oxygen concentrations in the assay and in the system being studied. Variations of significant magnitude have been frequently encountered in the acetylene-reduction assays. In the experiments reported above, these assays were done very carefully by experienced operators and only some of the variability was attributable to slight variations in procedures or conditions. It is concluded that caution should be observed in the use of the acetylene-reduction assay for precise measurements of nitrogen being fixed by free-living or symbiotic systems in nature. This conclusion in no way invalidates the use of the method for comparisons of nitrogen-fixing systems or of the effects of imposed conditions, within experiments in which the absolute amount of nitrogen fixed is not required to be measured. However, the work presented herein indicates that the acetylene-reduction method, as presently developed, is not adequate for the measurement of nitrogen input in ecosystem studies of nitrogen balance, where several different types of nitrogen-fixing systems may be involved.

V. Acknowledgments

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