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NITROGENASE

I. REPRESSION AND DEREPRESSION OF THE IRON–MOLYBDENUM AND IRON PROTEINS OF NITROGENASE IN *AZOTOBACTER VINELANDII*

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SUMMARY

Evidence is presented that the dilution effect on nitrogenase from *Azotobacter vinelandii* can be overcome by the addition of an optimal amount of Component I (iron–molybdenum protein) or Component II (iron protein); and this optimized activity parallels the activity obtained by applying the dilution-factor correction. The synthesis of both of the nitrogenase components, after exhaustion of ammonia from the medium, seems to be coordinate. The degradation of both of the nitrogenase components after repression was found to be coordinate and neither component was found to be in excess at any time after repression. For the initial one-half generation the nitrogenase activity falls at approximately the same rate as the increase in cell mass, suggesting simple dilution. After this point, however, activity falls more rapidly and more than 95 % of the activity is lost in two generations.

INTRODUCTION

Previous studies[1–7] on the conditions for synthesis of the nitrogen-fixing enzyme system have depended on measurement of the growth characteristics of the organisms used, or on the levels of activity detectable in crude extracts of the cells. When cells are grown in batch culture[2,3], no synthesis of the nitrogenase system is detectable until after exhaustion of free ammonia from the growth medium, whereas in a chemostat[4–6] very low levels of ammonia stimulates the production of the nitrogen-fixing enzyme system. In batch cultures similar beneficial effects of added amino acids have been observed and the results have been explained on the basis of the need for pools of amino acids for the synthesis of the nitrogen-fixing enzyme system and the action of relatively high levels of ammonia causing repression of the system[7,8].

It has been established for several organisms[9–14] that the nitrogenase system consists of at least two separable components. SILVERSTEIN AND BULEN[15] have used computer simulation to correlate the rate and product data with a proposed reaction pathway involving a dynamic equilibrium between an associated enzyme species and its components.

We have re-examined the repression and derepression of the nitrogen-fixing enzyme system in *Azotobacter vinelandii*, using the procedures of STRANDBERG AND WILSON[2] for crude extracts and some additional techniques with purified components of nitrogenase. These data provide evidence for the coordinate synthesis during derepression and coordinate loss of the nitrogenase components after repression.

MATERIALS AND METHODS

A. vinelandii OP was used throughout these studies. The organism was grown in a modified Burk's nitrogen-free medium as described by STRANDBERG AND WILSON[2]. Growth was followed by measuring turbidity with a Klett–Summerson photoelectric colorimeter with a No. 640 filter. Proper dilutions were made before reading when turbidity readings were higher than 150 Klett units. One Klett unit is equal to $3.4 \cdot 10^6$ cells that have been growing on N_2. For large-scale repression and derepression experiments, cells were grown in 15 l of medium in a 5-gallon carboy with vigorous aeration. Samples were removed periodically and the cells were harvested in a refrigerated centrifuge at 12000 × *g* for 10 min and washed by resuspending in 0.025 M Tris–HCl buffer (pH 7.4), followed by recentrifugation.

ATP, creatine phosphokinase (ATP:creatine phosphotransferase, EC 2.7.3.2), creatine phosphate, deoxyribonuclease I (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5), dithiothreitol and Tris base were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of analytical grade available commercially.

Ammonium acetate, when used, was sterilized by filtration through a 0.45-μm Millipore filter. Ammonia utilization was followed by spot-testing samples of the medium with Nessler's reagent. Quantitative determinations[2] of ammonia in the medium and in nitrogen fixation experiments were done using Nessler's reagent from Paragon C and C Co., New York.

Extracts were prepared by the following modification of the osmotic-shock disruption method of ROBRISH AND MARR[16]. The cells were suspended in 4 vol. of 4 M glycerol in 0.025 M Tris–HCl buffer at pH 7.4 for 30 min followed by centrifugation at 12000 × *g* for 10 min at 0–4°. After the glycerol was decanted, the pellet was loosened and the cells were lysed by vigorous shaking with 3 or 4 vol. of 0.025 M Tris–HCl buffer (pH 7.4) containing 0.1 mg dithiothreitol per ml. 5–10 μg deoxyribonuclease I were added per ml and the contents were flushed with N_2. The lysate was centrifuged at 27000 × *g* for 30 min. All buffers were thoroughly sparged with N_2 before use.

Assays of nitrogen fixation were carried out in 20-ml serum bottles; 8.5-ml serum bottles were used for acetylene-reduction assays. For both of the assays, 1 ml reaction mixtures contained 2.5 μmoles ATP, 30 μmoles creatine phosphate, 0.2 mg creatine phosphokinase, 5 μmoles $MgCl_2$, 20 μmoles Tris–HCl buffer (pH 7.4), 20 μmoles $Na_2S_2O_4$, and the specified protein. Bottles containing ATP, creatine phosphate, creatine phosphokinase, $MgCl_2$ and buffer were evacuated and flushed 3 times with He for acetylene-reduction assays and with N_2 for nitrogen-fixation assays. Dithionite solution, prepared anaerobically in 0.013 M NaOH prior to use, was added just before the addition of enzyme fraction. Assays were run at 30° for 15 min in a water-bath shaker. The reaction is linear during this time, and a more rapid shaking rate does not increase the observed activity. In acetylene-reduction

assays, 0.7 ml of the gas phase was removed before injecting 0.5 ml acetylene. Acetylene-reduction reactions were terminated by injecting 0.1 ml of 30 % (w/v) trichloroacetic acid to the solution. Ethylene production was measured after 30 min with a Varian Aerograph 600-D gas chromatograph with Porapak R column as described by STEWART *et al.*[17].

H_2 evolution was measured by dithionite oxidation followed in anaerobic cuvettes in a Gilford spectrophotometer by a modification of the method of LJONES AND BURRIS[18]. For sensitive assays at low levels of dithionite, the decrease in absorbance at 315 nm was followed. For use at high concentrations of dithionite, it was necessary to follow the oxidation at 350 nm. The absorbance at both wavelengths is proportional to concentration and there is a constant ratio of the absorbancies at the two wavelengths. Both ATP-dependent H_2 evolution (nitrogenase) and ATP-independent H_2 evolution (hydrogenase) were assayed in this way. However, as shown by HYNDMAN *et al.*[19], there is very little ATP-independent H_2-evolving activity in extracts of Azotobacter.

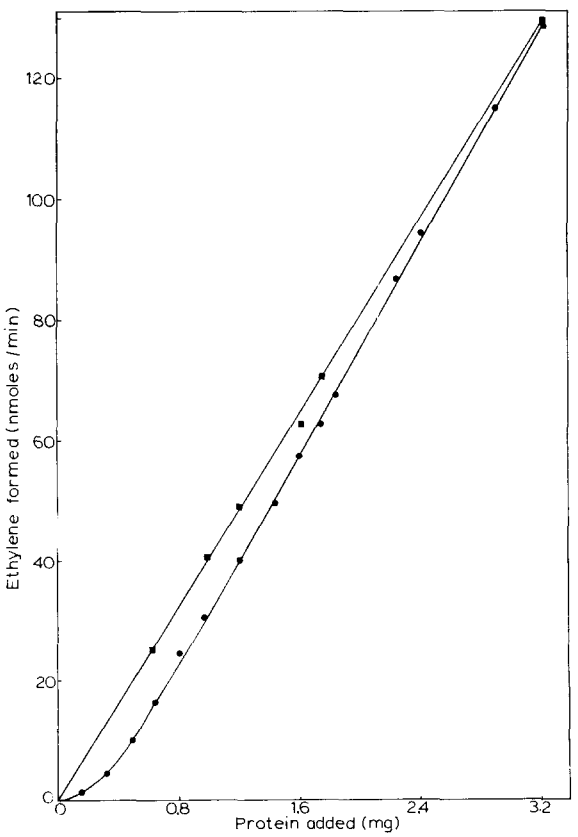

Fig. 1. Variation in observed enzyme activity with protein concentration in the assay. Assays were carried out under standard conditions as described in MATERIALS AND METHODS. Corrections were made for salt concentrations in those assays to which purified Component I was added (see Table I for the correction factors). ●—●, extract alone; ■—■, extract + Component I added to optimum activity.

Chromatography on DEAE-cellulose was performed by a method similar to that of BULEN AND LECOMTE[9] with modifications as indicated. Whatman DE-52 (microgranular) was equilibrated with 0.025 M Tris–HCl buffer (pH 7.4), then thoroughly degassed and packed into 1.5 cm × 10 cm column. Routinely the enzyme was applied to the column fully reduced with 300 ml of Tris–HCl buffer (pH 7.4) containing 0.3 mg/ml $Na_2S_2O_4$. The column was eluted successively with 0.025 M Tris–HCl buffer (pH 7.4) and 0.15, 0.25 and 0.5 M NaCl in the same buffer. All the eluting buffers contained 0.1 mg/ml $Na_2S_2O_4$. Iron–molybdenum protein (I) appears in the 0.25 M NaCl fraction and iron protein (II) in the 0.5 M NaCl fraction. All the treatments throughout the purification were performed under an atmosphere of H_2, N_2 or He.

Protein concentrations were determined by the method of BUCHER[20] or LOWRY *et al.*[21]. Fractions containing $Na_2S_2O_4$ were exposed to air for some time before protein determinations to destroy the dithionite which otherwise interferes with the assay.

RESULTS AND DISCUSSION

As reported by STRANDBERG AND WILSON[2], derepression of the nitrogen-fixing system in Azotobacter occurs after the exhaustion of ammonia in the medium. The rate of appearance of nitrogen-fixing ability varies with the medium used and the conditions of growth. As shown by DALTON AND POSTGATE[6], and MUNSON AND BURRIS[5], limiting ammonia in a chemostat increases the level of activity, whereas in the batch culture used by STRANDBERG AND WILSON[2] no activity is detectable until 1 h after complete exhaustion of ammonia from the medium.

With the acetylene-reduction assay it is possible to detect levels of less than 0.1 nmole/min per mg of extract protein, but quantitation is made difficult by the component concentration dependence of the assay. As shown in Fig. 1, the observed activity is not proportional to enzyme concentration and activity drops sharply at lower enzyme concentrations. The rate of acetylene and N_2 reduction is related to nitrogenase concentration in a sigmoidal fashion and the specific activities drop with decrease in enzyme concentration but the ratio of acetylene/N_2 reduction remains

TABLE I

EFFECT OF PROTEIN CONCENTRATION IN THE ASSAY ON ACETYLENE AND N_2 REDUCTION

Protein concn. (mg/assay)	*Acetylene reduction (nmoles/min per mg protein)*	*N_2 reduction (nmoles/min per mg protein)*	*Acetylene → ethylene / $N_2 \rightarrow 2NH_3$*
0.4	16.2	5.0	3.24
0.8	27.9	8.2	3.40
1.2	24.7	8.0	3.08
1.6	32.5	9.9	3.28
2.4	33.4	10.0	3.34
3.2	36.4	10.4	3.50
			Average 3.30

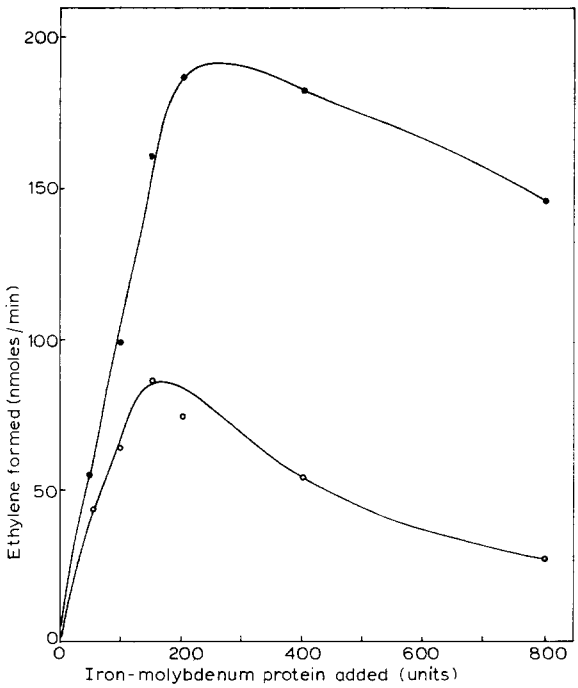

Fig. 2. Variation in observed activity with ratio of DEAE-cellulose column Components I and II. Assays were carried out as indicated in MATERIALS AND METHODS, with corrections applied for salt concentration in each case. The Component II used for these assays contained less than 5% as much activity when assayed alone, as when assayed in the presence of an optimum amount of Component I. Unit of iron–molybdenum protein is defined as the amount of Component I required to produce 1 nmole of ethylene per min when titrated with optimal amount of Component II. ●—●, activity obtained with 50 μl of Component II; ○—○, activity obtained with 25 μl of Component II.

TABLE II

EFFECT OF NaCl CONCENTRATION IN THE ASSAY ON THE ACTIVITY OF NITROGENASE IN CRUDE EXTRACT AND RECOMBINED FRACTIONS

Average of two experiments using different preparations of enzyme. Acetylene reduction assays were carried out as described in MATERIALS AND METHODS, with a final volume of 1.0 ml after addition of NaCl.

NaCl concn. (mM)	*Relative activity*	
	Crude extract	*Recombined fractions**
0	100	100
12.5	—	95
25.0	88	87
37.5	—	73
50.0	71	—
62.5	—	63
75.0	63	56
100.0	48	—

* DEAE-cellulose column fractions were recombined in ratio 1:1. The lowest NaCl tested was 0.05 M.

almost constant, on the average 3.3 (Table I). Investigators[22-28] have observed that the nitrogen-fixing system exhibits a dilution effect and at low extract concentrations there is no detectable enzyme activity. This is to be expected if the components of the nitrogenase are freely dissociable and the degree of association of a system in dynamic equilibrium is concentration dependent as suggested by SILVERSTEIN AND BULEN[15]. However, accepting this model, it is possible to determine empirically a correction factor to be applied to all observed activities if the ratio of components in the assay is constant and only their concentration is varied simultaneously.

An additional implication of this model, as observed for the clostridial system[29], is that the detected activity in an assay will vary with the ratio of the two components in the assay, as well as with their concentrations. The data in Fig. 2 indicate that the amount of Component I required for maximum activity depends upon the levels of

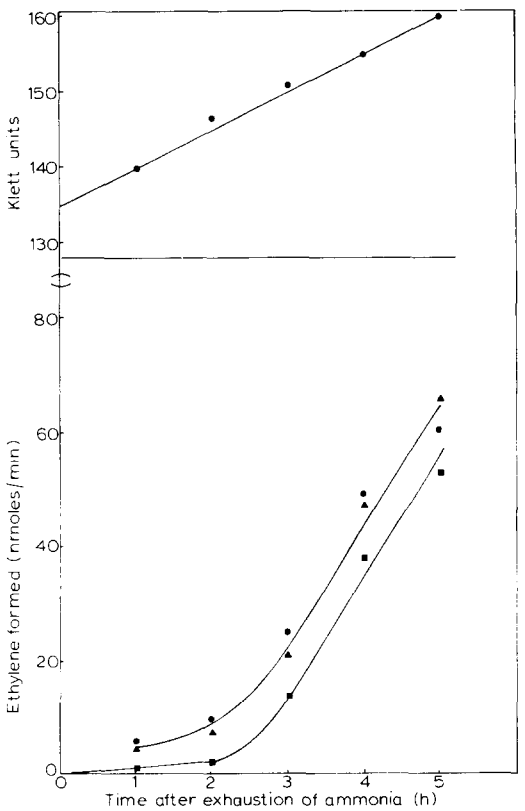

Fig. 3. Derepression of nitrogenase in *A. vinelandii*. Cells were grown in 15 l medium in a 5-gallon carboy at 25–28° with 90 μg N per ml as ammonium acetate, under the conditions described by STRANDBERG AND WILSON[2]. The ammonia was exhausted from the medium at 133 Klett units and this was designated time zero. 1-l samples were withdrawn at the times indicated and extracts prepared as described in MATERIALS AND METHODS. ■—■, activities observed when 0.2 ml aliquots of crude extract were assayed as described in MATERIALS AND METHODS; ●—●, activities corrected for dilution using a factor obtained from results similar to those of Fig. 1; ▲—▲, optimum activities obtained on addition of varying amounts of purified Component I to the assay of the crude extract, as illustrated in Fig. 2. In the insert are shown the Klett units of the experiment.

Component II. Recently SORGER[30] suggested that the dilution effect is due to the limiting amount of Component II and that Component I does not overcome the dilution effect of nitrogenase. The data presented in Fig. 1 clearly indicate that the dilution effect can be overcome by Component I. As evident from Fig. 2, if an excess of Component I is added, activities in the inhibition range may be observed and the stimulation effect would consequently be masked. Whenever a crude extract is assayed, the ratio of components is presumably constant as the concentration is varied, but when dealing with separated or purified components the ratio depends on the relative amounts of each component added. Usually the amount of one component or the other is somewhat in excess of the optimum and the observed activity depends on the availability of the other component. In attempting to quantitate the activities of Components I and II in crude extracts during the early stages of synthesis of the nitrogenase, determination of the optimum is crucial, for it cannot be assumed that both components are synthesized in a strictly coordinate fashion, and hence a simple correction factor for observed activity as shown in Fig. 1 cannot be applied. Instead, varying amounts of Component I which is completely free of Component II activity can be added and the activity of Component II present in the crude extracts

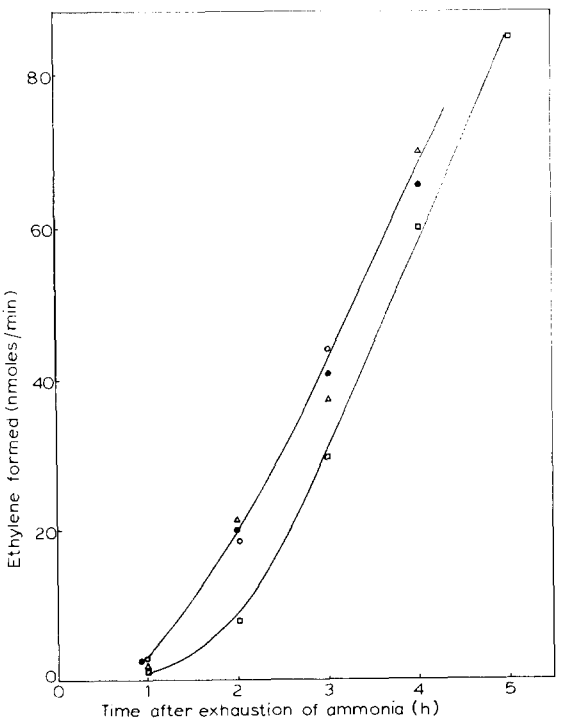

Fig. 4. Activities of Components I and II during derepression of nitrogenase. The extracts whose activities are shown in Fig. 3 were fractionated on DEAE-cellulose column as described in MATERIALS AND METHODS. The activities of the components were determined by assay of 0.1 ml of each Fraction I and II from the DEAE-cellulose column. □—□, Fractions I and II from the indicated times of derepression; △—△, Fraction I from the indicated times of derepression + Fraction II from fully derepressed cells; ○—○, Fraction II from the indicated times of derepression + Fraction I from fully derepressed cells; ●—●, activities corrected for dilution using a factor obtained from results similar to those of Fig. 1.

determined. If the effect of added Component I is the same with extracts obtained early in the synthesis of nitrogenase as for dilutions of fully derepressed cultures containing the same total amount of activity, then we may assume that the ratio of components in the extracts is very nearly the same. A further test of this is possible by purification of the components on a DEAE-cellulose column and determination of the total units of each component present in a given amount of extract. If this ratio of recovery of Components I and II is constant for all the stages of the derepression, we have further evidence that the synthesis of the components is coordinate.

BULEN AND LECOMTE[9] reported that NaCl inhibits N_2 fixation in extracts of Azotobacter at levels above approx. 0.05 M. Within the activity range we tested, there is 50 % inhibition of activity with each 0.095 M increase in NaCl concentration in crude extracts when tested up to 0.15 M in the acetylene-reduction assay and up to 0.3 M in the ATP-dependent dithionite-oxidation assay. The observed inhibition is somewhat lower with higher activities. A correction factor for salt concentration can be easily applied whenever purified fractions are used in the assay (Table II). $MgCl_2$ shows a similar effect in both acetylene-reduction and dithionite-oxidation assays.

The synthesis of nitrogenase activity as a function of time after exhaustion of ammonia from the medium is shown in Fig. 3. The cells were grown at 25–28°, somewhat lower temperature than optimum so that the finer kinetics of enzyme synthesis could be followed. The activity in the crude extracts was measured and a

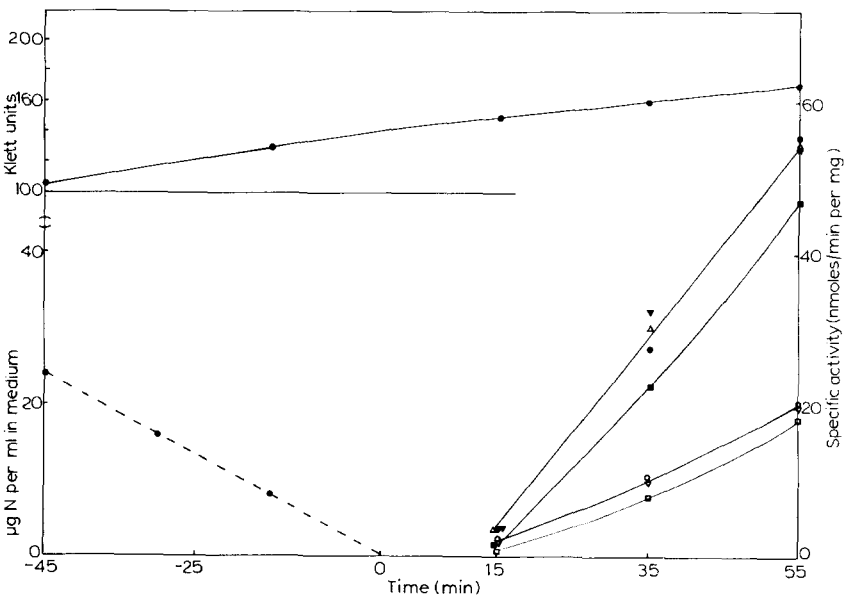

Fig. 5. Effect of added Component I or II on the activity of nitrogenase during derepression. Cells were grown and harvested as indicated in legend of Fig. 3, except that the temperature was maintained at 32°. Samples were taken at times indicated and extracts prepared as mentioned in MATERIALS AND METHODS. Time zero is defined as when ammonia is exhausted from the medium. ●---●, ammonia N in the medium. N_2 fixation: □—□, extract alone; ○--○, extract + Component I; ▽—▽, extract + purified Component II. Acetylene reduction: ■—■, extract alone; ●—●, extract + Component I; ▼—▼, extract + Component II; △--△, corrected for dilution. In the insert are shown the Klett units of the experiment.

correction for dilution applied. There is approximately a 1-h lag after the loss of ammonia from the medium before any detectable activity. Application of the dilution correction indicates that the synthesis lags for an additional hour before becoming linear with time. Addition of Component I to obtain optimum activity agrees well with this correction.

Components were separated by DEAE-cellulose chromatography and recombined for assay (Fig. 4). When corrected for the variation in salt concentration in different fractions, the results are in good agreement with those obtained using crude extracts. In the early stages of derepression when the amount of each component is small relative to that found in a fully derepressed culture, the activities observed on recombination of components are low. Addition of purified Component I or II from fully-derepressed cells leads to an apparent stimulation of the activity. Alternatively, if the ratio of components is assumed to be constant and a correction for concentration in the assay is applied, a similar increase in activity is observed. The fit is not perfect

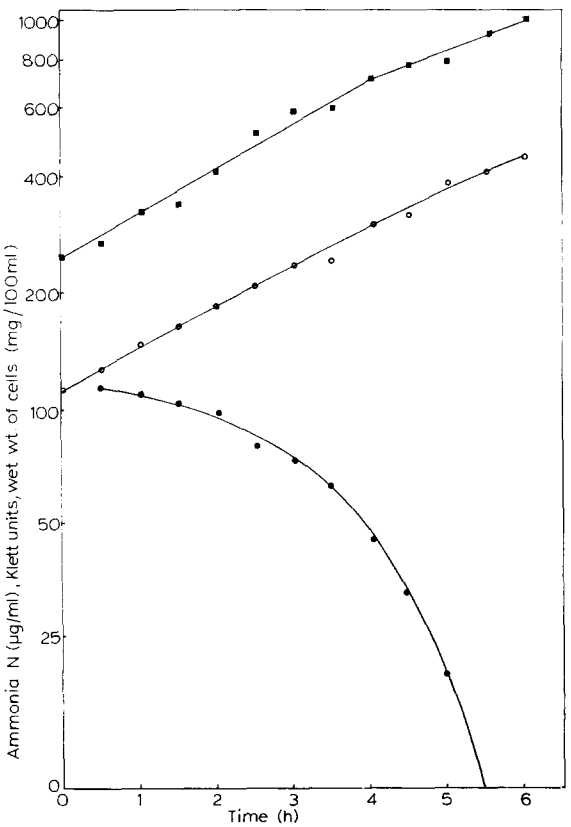

Fig. 6. Growth and ammonia utilization during transition from N_2 to NH_3 as nitrogen source. Experiment was carried out in a 5-gallon carboy as described in MATERIALS AND METHODS. When cell density reached 110 Klett units, 130 µg of N as ammonium acetate per ml was added to 15 l of actively growing cells at 32°. Aliquots of cells were withdrawn immediately before and every half hour after the addition of ammonia. The supernatant fluid after harvesting the cells was assayed for ammonia. Time zero is defined as when ammonia is added to the medium. ●—●, ammonia N in the medium; ○---○, Klett units; ■—■, wet weight of the cells.

since there are several variables influencing the exact yield and concentration of components obtained from the columns, but there does not appear to be any large excess of one component over the other at any time during the derepression. The synthesis of the components thus seems to be coordinate.

At 32° derepression is much more rapid (Fig. 5). Under these conditions, the lag period before the beginning of synthesis of the nitrogenase was less than 15 min, and there was no lag in the increase in cell number as measured by the increase in Klett units. Wet weight of cells per liter stopped increasing for more than an hour during which time the nitrogenase increased rapidly in specific activity until it reached a value comparable to that of cells grown continuously on N_2. Although the time required to reach full specific activity was much shorter under these conditions, the increase in activity of Components I and II was identical to that observed with cultures at a low temperature. Nitrogen reduction and acetylene reduction increased

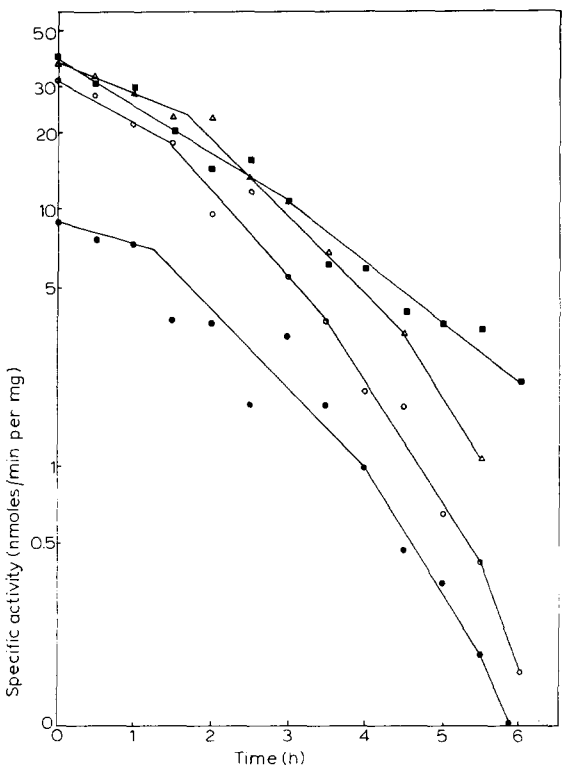

Fig. 7. Decrease in N_2- and acetylene-reduction activity during repression of nitrogenase by ammonia. Cells obtained as indicated in the legend to Fig. 6 were broken as described in MATERIALS AND METHODS. Activities toward N_2 and acetylene reduction were determined in the coupled assay system as described in MATERIALS AND METHODS. Dithionite oxidation rates were extrapolated to v_{max} by using several concentrations of ATP with constant amounts of creatine phosphate and creatine phosphokinase in the assay as described in MATERIALS AND METHODS. Corrected activity values for acetylene reduction were obtained using an activity correction curve obtained from data similar to that shown in Fig. 1. Time zero is defined as when ammonia is added to the medium. ●—●, N_2 reduction; ○—○, acetylene reduction; △—△, dithionite oxidation; ■—■, acetylene reduction corrected for dilution using a factor obtained from results similar to those of Fig. 1.

in exactly the same manner, maintaining a constant ratio of 3 acetylene reduced per N_2 reduced.

Similar experiments were performed to determine whether the degradation of enzyme after repression was coordinate or specific for one or the other component. The decrease in the activity was followed after addition of ammonium acetate to a culture growing rapidly with N_2 as its source of nitrogen. The activity of extracts was determined by addition of varying aliquots of extracts to the assay, and by supplementing with varying amounts of Component I to the assay, so that the activities per assay determined were in a comparable range, making the correction factors more reliable. There was no observable lag on addition of ammonia to the medium.

As may be seen in Fig. 6, the growth rate as measured either by Klett units or wet weight of cells at harvest is a good approximation to logarithmic growth even at very high yields of cells. Ammonia utilization by the cells over this time increases rapidly and as may be seen by comparison to Fig. 7, the increase in utilization of ammonia from the medium corresponds with the decrease in the nitrogenase activity

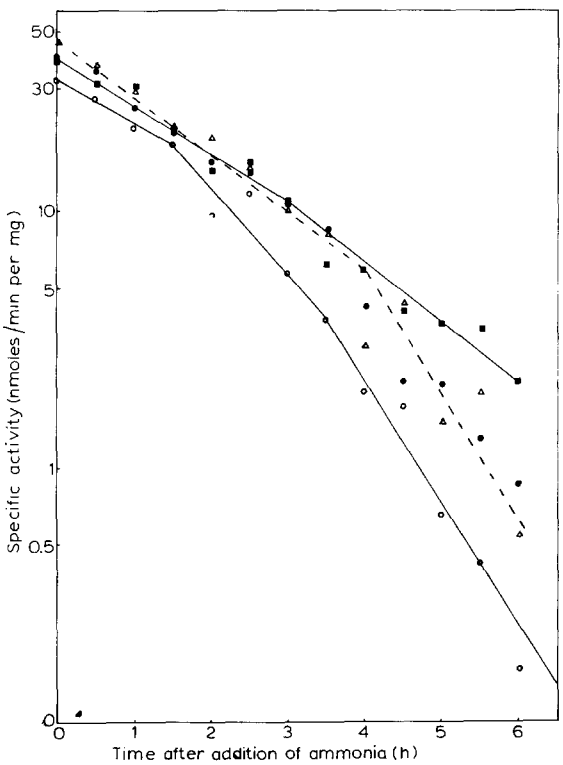

Fig. 8. Effect of added Component I or II on acetylene reducing activity at various times during repression. Activities of extract alone or with added Component I or II were determined using the coupled system as indicated in MATERIALS AND METHODS. No salt correction was needed since the components used were in 0.025 M Tris buffer. Component II was a Sephadex G-200 column fraction containing less than 0.1% Component I. ○—○, extract alone; ●---●, extract + Component I; △---△, extract + Component II; ■—■, acetylene reduction activity corrected for dilution as indicated for Fig. 7.

of the cells. For the initial one-half generation the nitrogenase activity, as measured by N_2 or acetylene reduction or dithionite oxidation, falls at approximately the same rate as the increase in mass of cells, suggesting simple dilution. After this point, however, the activity falls more than twice as fast as cell mass increase and more than 95 % of the activity is lost in two generations when simple dilution would predict a loss of only 75 %. As seen from Figs. 8 and 9, addition of optimal amounts of component I or II stimulates the activity, but at any stage after repression the effect of added Component I or II is practically the same, showing that both the components are inactivated simultaneously. STRANDBERG AND WILSON[2] observed a somewhat higher rate of loss of activity as a function of time, using activities uncorrected for dilution in the assay. The data presented clearly indicate that synthesis and degradation of both the components of nitrogenase is coordinate.

The observation that the activity of nitrogenase is diluted out during the initial half generation after repression suggests that there is no specific mechanism fur turning off the enzyme activity, such as proteolytic degradation or O_2 denaturation. This relative stability indicates that repression is rapid and highly effective, not

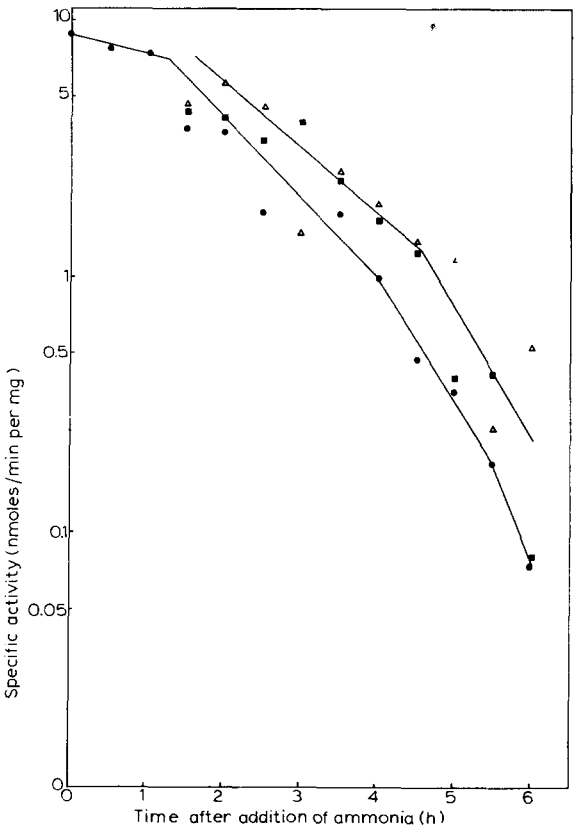

Fig. 9. Effect of added Component I or II on N_2-reducing activity at various times during repression. Conditions were the same as described for Fig. 8. ●—●, extract alone; ■—■, extract + Component I; △—△, extract + Component II. NH_3 production was measured with Nessler's reagent after micro-diffusion as in MATERIALS AND METHODS.

simply a shift in equilibrium between synthesis and degradation, since the specific activity of the enzyme begins to fall immediately after addition of the ammonia. The accompanying paper[31] demonstrates that the EPR signal, seen in purified Component I and in whole cells, as well as various measures of activity, decay in parallel after repression, whereas the cross-reacting material to Component I declines more slowly. The cross-reacting material declines by simple dilution, while after the first half generation the activity and EPR signal decline more rapidly.

The observation that dithionite-oxidizing ability as well as acetylene- and nitrogen-reduction activities fall in parallel provides another demonstration that these reactions are all catalyzed by the intact nitrogenase complex[32], with no activities attributable to either component alone[33]. Further support is provided by the parallel rise in acetylene- and nitrogen-reducing activities during derepression of nitrogenase.

It will be of interest to examine the derepression of mutants such as those described by FISHER AND BRILL[34] and SORGER AND TROFIMENKOFF[28], which are unable to grow on N_2 but which can be derepressed when grown on $He–O_2$ and limiting ammonia. With the ability to quantitate the activities of Components I and II in crude extracts it may be possible to isolate classes of mutants which have partially active components or for which the synthesis of the two components is no longer apparently coordinate. Quantitative techniques for measuring the activities of components should be applicable also to other systems for which relatively less is known about repression and derepression[7,35].

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