EFFECT OF NH_4^+ ON ACETYLENE REDUCTION (NITROGENASE) IN *AZOTOBACTER VINELANDII* AND *BACILLUS POLYMYXA**,†

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ABSTRACT

In vivo *acetylene reduction* (*nitrogenase*) *is inhibited by* NH_4^+ *immediately in* Azotobacter vinelandii, *but not in* Bacillus polymyxa. *In addition to the repression of enzyme synthesis,* NH_4^+ *has two types of inhibitory effects on acetylene reduction in* A. vinelandii (*i*) *reversible inhibition in the initial stages of incubation with* NH_4^+, (*ii*) *irreversible loss of activity in the later stages of incubation with* NH_4^+.

Key words : Ammonia, nitrogenase, *Azotobacter*, *Bacillus polymyxa*.

1. INTRODUCTION

The synthesis of nitrogenase in nitrogen fixing organisms is repressed by combined nitrogen.[3,4,7,8,12,17,19] Apart from its repressive effect, NH_4^+ causes immediate inhibition of *in vivo* nitrogenase activity in *Azotobacter vinelandii*[17], *A. chroococcum*[11], *Anabaena cylindrica*[10] and *Rhodospirillum rubrum*[13]. This immediate inhibitory effect of NH_4^+ on nitrogenase is not due to : (*a*) direct inactivation of nitrogenase by NH_4^+ as the activity is retained in the extracts of *A. vinelandii* for several hours after the addition of NH_4^+ [17], (*b*) feedback inhibition at the level of nitrogenase as neither NH_4^+ nor its metabolites inhibit nitrogenase activity in cell-free extracts of *A. vinelandii*[1], (*c*) the effect of NH_4^+ on the electron

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carriers as *Azotobacter* electron carriers are constitutive components of the cells[20].

The present paper gives evidence that apart from the repression of the enzyme in *A. vinelandii* cells, NH_4^+ exerts two types of inhibitory effects on nitrogenase activity. The paper also indicates that in *Bacillus polymyxa*, NH_4^+ does not have any effect other than the repression of the enzyme synthesis.

2. Materials and Methods

Growth conditions

Azotobacter vinelandii OP (obtained from Professor R. H. Burris, University of Wisconsin) was grown in a modified Burk's nitrogen-free medium.[17] The culture was grown in 200 ml of media in 500 ml Erlenmeyer flasks, inoculated with 5% (v/v; OD 1·5) inoculum and incubated at 30° C on a rotary shaker (250 rpm). Growth was followed by measuring turbidity in a Spekol colorimeter (PGH Radio, Ferneschen-Electro, Marienberg/Sa, DDR) at 540 nm. All experiments were carried out with 14h cultures (OD 0·37, Dry weight 0·15 mg/ml, 30° C).

B. polymyxa (obtained from Professor J. R. Postgate, University of Sussex) was grown anaerobically in 5 litre nitrogen-free medium[15] in a 7 litre Erlenmeyer flask at 30° C under constant nitrogen sparging (0·05–0·15 litres of N_2/min/litre culture). A 20% v/v inoculum was used. The inoculum was prepared by growing the cells aerobically with limited amount of ammonia (100 mg ammonium sulphate/litre) for 18 h. The experiments were carried out with 18 h cultures.

Assay of nitrogenase

Nitrogenase activity was assayed by following acetylene reduction. Conical Büchner flasks (100 ml) with serum stoppers were used for the assays. The assay mixture contained 20 or 40 ml culture with a gas phase of 10% oxygen, 10% acetylene and 80% purified argon for *A. vinelandii*, an aerobic nitrogen fixing organism, and 10% acetylene and 90% argon for *B. polymyxa*, which fixes nitrogen anaerobically. The flasks were evacuated and flushed with argon four times before injecting acetylene and/or oxygen.[16] The flasks were incubated at 30° C on a rotary shaker (120 rpm). At various time intervals, one ml of gas phase was removed and the ethylene produced measured in a F11 model Perkin-Elmer gas chromatograph with Porapak N column as described by Stewart *et al.*[16] Peak heights of ethylene and acetylene were measured and compared with standards.

Inhibition of incorporation of [^{14}C] labelled amino acids by chloramphenicol and rifampicin in A. vinelandii

Twenty ml of cultures, harvested and resuspended in 20 ml of fresh medium were placed in a 100 ml conical flask. Chloramphenicol (75 μg/ml) or rifampicin (20 μg/ml) and (^{14}C) labelled chlorella protein hydrolysate (0·05 μ Ci/ml; obtained from B.A.R.C., Bombay) were added together and the culture was shaken in a waterbath at 30° C. At various time intervals, 2 ml of the culture was removed into 2 ml of 10% TCA, then heated at 90° C for 30 min, cooled and filtered on Whatman No. 3 filter paper. These filters were washed with 5% TCA several times and then with a mixture of ethanol-ether (3 : 1) and finally with ether. They were dried, and the radioactivity measured in a Beckman LS-100 liquid scintillation spectrometer.[14]

Effect of NH_4^+, glutamine, asparagine on nitrogenase activity

To determine the effect of these metabolites on nitrogenase activity they were added to the medium prior to the assay. The concentration of ammonium acetate used was 28 μg N/ml and that of the amides, 140 μg N/ml.

Acetylene reduction of NH_4^+ treated A. vinelandii *cells in the absence of new protein synthesis*

Ammonium acetate (280 μg/N/ml) was added to 40 ml of culture and incubated statically for one hour. Chloramphenicol (75 μg/ml) was added after 45 min. incubation. The cells were harvested using a refrigerated centrifuge (10,000 rpm for 5 min.), washed twice with fresh nitrogen-free medium containing chloramphenicol, resuspended in medium containing chloramphenicol and assayed for acetylene reduction. When rifampicin (20 μg/ml) was used to inhibit the enzyme synthesis, it was added 30 min. after the addition of NH_4^+. The culture was incubated for a further period of 30 min. and then harvested, washed, resuspended and assayed for acetylene reduction as described earlier. The total period of incubation with NH_4^+ was 60 min.

Effect of pyruvic acid and citric acid cycle intermediates on NH_4^+, glutamine and asparagine inhibition of nitrogenase in A. vinelandii

The cells were preincubated for 15 min. with pyruvic acid (40 or 80 mM) or citric acid cycle intermediates (40 mM) before the addition of ammonium acetate (14 μg N/ml), glutamine or asparagine (140 μg N/ml).

Kinetics of disappearance of acetylene reduction (nitrogenase activity) in A. vinelandii cells treated with NH_4^+ or chloramphenicol or rifampicin

Cultures (200 ml, 14 h old) were treated with ammonium acetate (280 μg N/ml) or chloramphenicol (75 μg/ml) or rifampicin (20 μg/ml) and kept on the shaker. At different time intervals 20 ml of culture was removed, washed and assayed as described earlier for 45 min. NH_4^+ treated samples were washed and assayed either with chloramphenicol or rifampicin. Cells initially treated with chloramphenicol or rifampicin were assayed in the presence of the same antibiotic.

Effect of NH_4^+, chloramphenicol and rifampicin on acetylene reduction in B. polymyxa

One of the following, ammonium acetate (280 μg N/ml); chloramphenicol (75 μg/ml); or rifampicin (20 μg/ml) were added to an 18 h culture and sparging with nitrogen was continued. At different time intervals samples were removed and assayed for acetylene reduction for 30 minutes. Simultaneously the OD was also recorded to follow the effect of these compounds on the growth of the organism.

Concentration of ammonium acetate, glutamine and asparagine

Ammonium acetate was used as the source of NH_4^+ since earlier results have shown that the extracellular concentration of NH_4^+ in *A. vinelandii* remains constant with respect to time in the presence of ammonium acetate unlike ammonium sulphate or ammonium chloride.[9] In the experiments designed to show the reversibility of the immediate inhibition the concentration of ammonium acetate used was 28 μg N/ml so as to give 90% inhibition of acetylene reduction, whereas when pyruvate or Kreb's cycle intermediates were tested for reversing the effect of NH_4^+ the concentration of ammonium acetate was reduced to 14 μg N/ml. In the experiments done to determine the disappearance of enzyme activity in the presence of NH_4^+ the concentration of ammonium acetate was increased to 280 μg N/ml as the cells were incubated for a longer time. When glutamine or asparagine were used to inhibit the enzyme activity the concentration used was adjusted to give 90% inhibition *viz.*, 140 μg N/ml.

3. Results and Discussion

The results indicate that ammonium acetate immediately inhibited nitrogenase activity in *A. vinelandii* (Fig. 1), but not in *B. polymyxa* (Fig. 2).

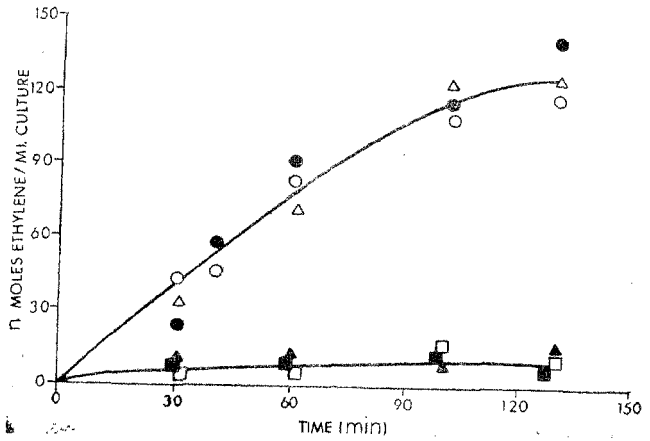

FIG. 1. Effect of ammonium acetate (▲—▲), glutamic acid (○—○), aspartic acid (△—△) glutamine (■—■) and asparagine (□—□) on acetylene reduction in *A. vinelandii*. The concentration of ammonium acetate was 28 μg N/ml and that of amino acids and amides, were 140 μg N/ml. The compounds were added prior to the assay. No addition (●—●).

The estimation of free amino acids in the cells of *A. vinelandii* after NH_4^+ addition showed increased levels of glutamic acid, whereas no such change was seen in *B. polymyxa*. In the above estimations the levels of free glutamine and asparagine in the cells could not be determined separately. Hence it is possible that the immediate effect of NH_4^+ on nitrogenase activity may be due to increased levels of free glutamine or glutamic acid.

Glutamic and aspartic acids did not cause any immediate inhibitory effect on nitrogenase activity, but asparagine and glutamine inhibited nitrogenase activity in *A. vinelandii* cells (Fig. 1). None of the above compounds inhibited nitrogenase activity in *B. polymyxa* (Fig. 2).

To determine whether the inhibition is reversible, experiments were performed using inhibitors of protein and mRNA synthesis, chloramphenicol and rifampicin, respectively. Chloramphenicol inhibited (^{14}C) amino acid incorporation by about 75% in 15 min. and completely in 35 min. Rifampicin did not show any inhibition of amino acid incorporation in the initial stages. However, it inhibited protein synthesis within 30 min. (Fig. 3).

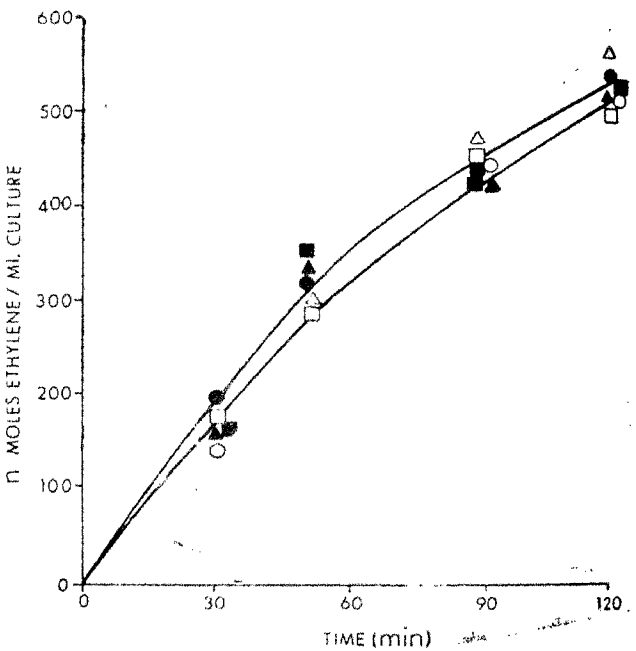

FIG. 2. Effect of ammonium acetate (▲—▲), glutamic acid (○—○), aspartic acid (△—△), glutamine (■—■) and asparagine (□—□) on acetylene reduction in *B. polymyxa*. Ammonium acetate (28 μg N/ml), amino acids or amides (140 μg N ml) were added prior to the assay. No addition (●—●).

After determining the time taken to inhibit protein synthesis by the antibiotics, the reversible nature of the inhibitory effect of NH_4^+ was investigated. The results (Fig. 4) showed that NH_4^+-treated *A. vinelandii* cells reduced acetylene when washed free of NH_4^+ even in the absence of new protein synthesis. Identical results were obtained when rifampicin was used to inhibit the protein synthesis. The inhibition of nitrogenase activity was reversible when the cells were incubated with NH_4^+ for a maximum period of one hour. As the aim of the experiment was to investigate the reversibility of the immediate

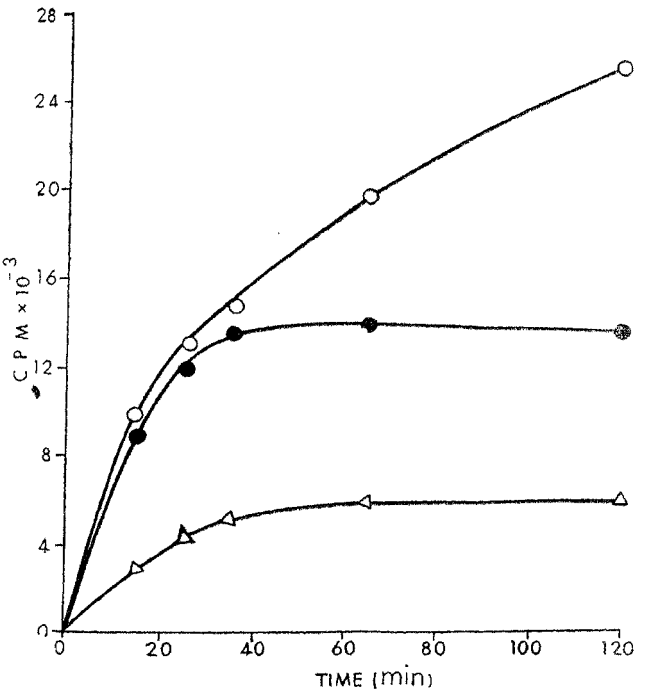

FIG. 3. Inhibition of [^{14}C] amino acid incorporation by chloramphenicol and rifampicin in *A. vinelandii*. No inhibitor (○—○); chloramphenicol 75 μg/ml (●—●), rifampicin, 20 μg/ml (△—△).

inhibitory effect, the cells were not aerated in order to avoid the changes due to the partial pressure of O_2.[5] This indicates that the immediate inhibitory effect of NH_4^+ is reversible. From experiments with continuous culture, Kleiner[9] also has suggested that the immediate inhibitory effect of NH_4^+ on nitrogenase activity is partially reversible in *A. vinelandii*. A similar situation has been suggested in the case of *Rhodospirillum rubrum*.[13]

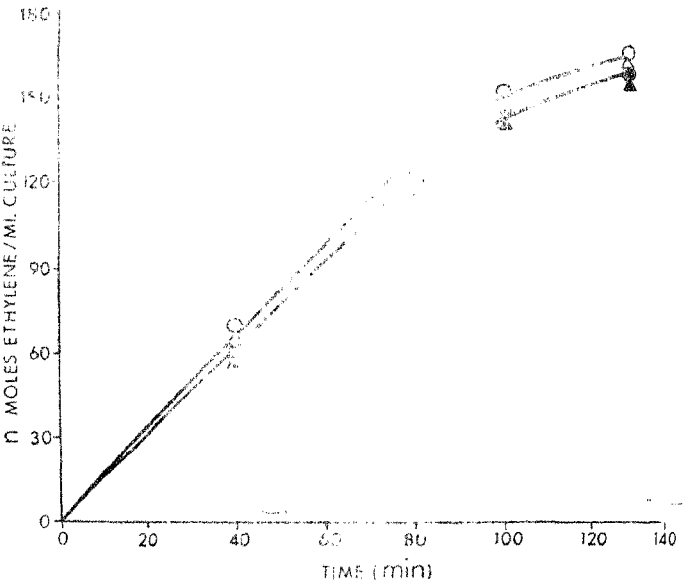

FIG. 4. Acetylene reduction by NH_4^+ treated *A. vinelandii* cells in the absence of new protein synthesis. Ammonium acetate (280 μg N/ml), or chloramphenicol (75 μg/ml) or rifampicin (20 μg/ml) were added simultaneously to different flasks. Cells treated with ammonium acetate and at different time intervals washed and assayed for 45 min with nitrogen-free medium containing chloramphenicol (● - ●). Cells treated with ammonium acetate washed and assayed with fresh nitrogen-free medium (▲ ▲). Cells, which received no ammonium acetate, washed and assayed with fresh medium containing chloramphenicol (○ ○). Cells, which received no ammonium acetate, washed and assayed with fresh medium (□ - □).

The immediate inhibitory effect of NH_4^+ on nitrogenase activity has been observed in several aerobic nitrogen-fixing organisms, but not in the anaerobic nitrogen fixers.[5] It has been suggested that this immediate inhibition in aerobic organisms could be due to depletion of ATP/NADPH resulting from NH_4^+ assimilation.[10, 12] It is also known that *A. vinelandii* cells reduce acetylene in the presence of NH_4^+, if glutamate analogues like L-methionine-DL-sulfoximine and L-methionine sulfone are present in the

medium.[6] This observation supports the idea that the metabolism of NH_4^+ into glutamine is necessary for such an inhibition.

If the immediate inhibitory effect of NH_4^+ is due to depletion of the ATP/NADPH that is used for the assimilation of NH_4^+, metabolites capable of providing these energy sources should reverse this inhibition. The results (Table I) showed partial reversibility of the inhibition when *A. vinelandii* cells were incubated with pyruvate, DL-isocitrate, α-ketoglutarate, or succinate, prior to the addition of NH_4^+. When the concentration of pyruvate was increased, enhanced rate of acetylene reduction was observed in NH_4^+ treated *A. vinelandii* cells. Similarly, the immediate inhibition of glutamine or asparagine was also found to be partially reversible with pyruvate (Table I).

TABLE I

Effect of pyruvic acid and citric acid cycle intermediates on NH_4^+ glutamine or asparagine

	Inhibition of Nitrogenase				
	Inhibitors				
Expt. No.	NH_4^+ (μg N/ml)	Glutamine (μg N/ml)	Asparagine (μg N/ml)	Inhibition reversing metabolite (mM)	*n* mols of ethylene 90 min/ml culture
1.	..	..	..	..	130
2.	14	..	..	..	10
3.	,,	..	..	Pyruvic acid (40)	39
4.	,,	..	..	Pyruvic acid (80)	72
5.	,,	..	..	DL-isocitric acid (40)	38
6.	,,	..	..	Succinic acid (40)	36
7.	,,	..	..	α-Ketoglutaric acid (40)	45
8.	...	140	..	..	12·5
9.	..	140	..	Pyruvic acid (40)	63
10.	..	..	140	..	12
11.	..	..	140	Pyruvic acid (40)	54

A. vinelandii cells were preincubated for 15 min. with pyruvic acid or TCA cycle intermediates before the addition of ammonium acetate or glutamine or asparagine ; ..No addition.

These observations indicate that the immediate inhibitory effect of NH_4^+, asparagine and glutamine could be due to the drainage of ATP or NADPH.

Kinetics of disappearance of nitrogenase activity in cells of A. vinelandii *treated with NH_4^+, chloramphenicol or rifampicin. Evidence for non-immediate irreversible inhibition by NH_4^+*

Rifampicin inhibits enzyme synthesis at the transcriptional level.[14] Similarly, if NH_4^+ also inhibits nitrogenase synthesis at the transcriptional level, the disappearance of nitrogenase activity in the cells treated with NH_4^+ should mimic that of rifampicin treated cells. In addition to repression, there is an immediate inhibition of nitrogenase by NH_4^+ in *A. vinelandii*. Hence a direct comparison of nitrogenase activity in NH_4^+ treated cells with that of chloramphenicol or rifampicin treated cells is not possible. We have observed that the immediate effect of NH_4^+ in *A. vinelandii* can be reversed by washing the cells free of NH_4^+. This has enabled us to study the kinetics of NH_4^+ repression of the enzyme and to compare the results with that of rifampicin-treated cells. When the cells were treated with rifampicin and assayed for acetylene reduction at different time intervals, 50% loss of activity was observed only after 7 h. But when the cells were initially treated with NH_4^+ and washed free of NH_4^+ at different time intervals in the presence of rifampicin 50% loss of activity was noticed within 4·1 h (Fig. 5). The disappearance of the enzyme activity was faster in the cells treated with NH_4^+ and washed with rifampicin than the cells initially treated with rifampicin. Hence the site of inhibition of nitrogenase by NH_4^+ is at a later stage than that of rifampicin, *i.e.*, at the post-transcriptional stage.

In *K. pneumoniae* NH_4^+ is believed to block nitrogenase synthesis midway between mRNA synthesis and translation.[2] If the situation in *A. vinelandii* were similar to that of *K. pneumoniae*, *A. vinelandii* cells treated with NH_4^+, washed and assayed in the presence of chloramphenicol should show a slow disappearance of enzyme activity than the cells initially treated with chloramphenicol and assayed similarly. In these experiments too, the disappearance of the enzyme activity was faster in the cells treated with NH_4^+ than the cells treated with chloramphenicol (Fig. 5). This shows that the inhibition by NH_4^+ is not at the site where chloramphenicol inhibits the synthesis, *i.e.*, at the translational level, but at a post-translational stage. These experiments indicate that there is a faster degradation or inactivation of nitrogenase in NH_4^+ treated cells during the later stages of incubation with NH_4^+. It may be noted that these observations have been made with

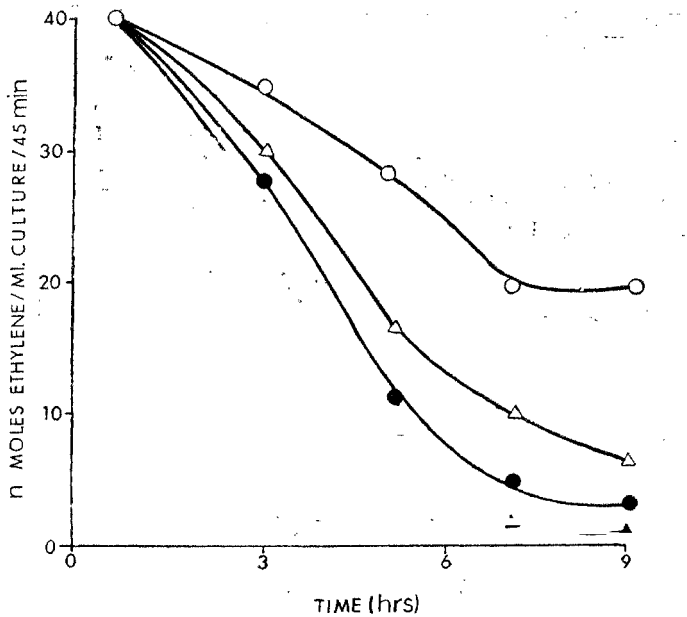

FIG. 5. Kinetics of disappearance of acetylene reduction in *A. vinelandii* cells treated with NH_4^+ (280 μg N/ml) chloramphenicol (75 μg/ml) or rifampicin (20 μg/ml). The inhibitors were added simultaneously. At different time intervals, samples were removed, washed and assayed for 45 min. NH_4^+ treated samples were washed and assayed with nitrogen-free medium containing either chloramphenicol (▲—▲) or rifampicin (●—●); cells initially treated with chloramphenicol (△—△) or rifampicin (○—○) and were assayed in the presence of the same drug.

cells which were aerated, to determine the stability of the enzyme after the inhibition of its synthesis.

Our observations suggest that NH_4^+ has two types of inhibitory effects on nitrogenase activity in *A. vinelandii*. (*a*) A rapid inhibitory which is reversible if NH_4^+ is removed within 1 h by washing, (*b*) An irreversible loss of activity during the later stages of incubation with NH_4^+.

Kinetics of disappearance of nitrogenase activity in B. polymyxa *cells treated with NH_4^+, chloramphenicol or rifampicin*

In *B. polymyxa* there is no immediate inhibition of nitrogenase by NH_4^+. Hence it is possible to compare the disappearance of nitrogenase activity in the cells treated with NH_4^+ to that of chloramphenicol or rifampicin treated cells. The effect of NH_4^+ and the antibiotics on nitrogenase activity and growth of *B. polymyxa* is shown in Fig. 6. Both chloramphenicol and rifampicin caused a rapid inhibition of growth and nitrogenase activity in *B. polymyxa*. Nitrogenase activity was retained for a longer time in NH_4^+

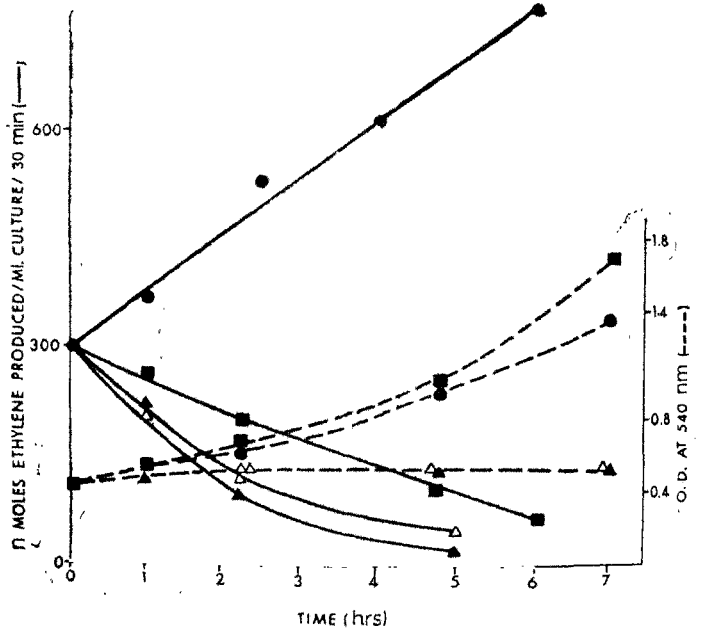

FIG. 6. Effect of NH_4^+ (280 μg N/ml), chloramphenicol (75 μg/ml) and rifampicin (20 μg/ml) on acetylene reduction and growth in *B. polymyxa*. After the addition of NH_4^+ or the drug sparging of nitrogen was continued. At different time intervals samples were removed and assayed for acetylene reduction for 30 minutes. Control (●—●); ammonium acetate (■—■); chloramphenicol (▲—▲); rifampicin (△—△).

treated cells. NH_4^+ causes the inhibition of nitrogenase synthesis, while chloramphenicol and rifampicin inhibits the synthesis of all proteins. Hence it can be suggested that though nitrogenase is still present, the enzyme is not able to express in chloramphenicol and rifampicin treated cells. It may be possible that the half-life of some other proteins required for *in vivo* nitrogenase expression is shorter than the half-life of nitrogenase ; the antibiotics block their synthesis and hence an early disappearance of nitrogenase activity in the cells treated with chloramphenicol and rifampicin.

It is clear from the above results that *A. vinelandii* and *B. polymyxa* cells respond to NH_4^+ differently.

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