Control of Nitrogenase Synthesis in *Klebsiella pneumoniae*

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SUMMARY

Sulphate-limited continuous cultures of *Klebsiella pneumoniae* showed a proportional repression of nitrogenase activity with increasing concentrations of ammonium ion in the influent medium. A fully repressed population had a 50 % greater bacterial density than a fully derepressed one. On derepression, synthesis of nitrogenase lagged for 90 min after exhaustion of NH_4^+ from the medium but was complete within one doubling time. Casamino acids did not decrease the pre-fixation lag obtained under sulphate-limited conditions in the chemostat. Longer lags were obtained when NH_4^+ was exhausted under N-limited conditions. Such lags were decreased by Casamino acids, yeast extract, or L-aspartate. Aspartate-N completely repressed nitrogenase under sulphate- or carbon-limited conditions but not under nitrogen limitation. In the initial stages of repression of nitrogenase synthesis by NH_4^+ , apparent production of active enzyme continued for a short time in the absence of protein synthesis *de novo*; nitrogenase was then diluted out as the organisms multiplied. Repression by NH_4^+ was at the level of mRNA transcription according to studies with rifampicin and chloramphenicol. 'Coding capacity' for nitrogenase synthesis declined with a half-life of about 4.5 min following inhibition of RNA synthesis with rifampicin. NH_4^+ did not influence the decay rate but stimulated translation of such nitrogenase-specifying mRNA as had been initiated.

INTRODUCTION

The regulation of nitrogenase synthesis by sources of fixed nitrogen in *Azotobacter chroococcum* in continuous culture was described by Drozd, Tubb & Postgate (1972). In *Azotobacter*, derepression of nitrogenase followed rapidly once NH_4^+ became exhausted from the medium. Nitrogenase activity was detected immediately (Drozd *et al.* 1972) or after a delay of up to 1 h (Strandberg & Wilson, 1968; Shah, Davis & Brill, 1972) depending upon growth conditions. In *Klebsiella pneumoniae*, Pengra & Wilson (1958) found no significant $^{15}\text{N}_2$ incorporation during the period immediately following exhaustion of NH_4^+ . Inductive lags of 13 h (Mahl & Wilson, 1968) and 15 h (Lindsay, 1963) have been reported. Yoch & Pengra (1966) observed a diauxic growth lag of about 2 h after exhaustion of limiting NH_4^+ . This shorter lag could have arisen from the use of nitrogen-fixing organisms as inocula because the length of such a lag in *Clostridium pasteurianum* depended on the number of generations of growth on NH_4^+ (Daesch & Mortenson, 1972), a shorter lag ensuing where residual nitrogenase was present. Long pre-fixation lags recorded for *K. pneumoniae* may have arisen from depletion of amino acid pools (Yoch & Pengra, 1966; Parejko & Wilson, 1970). Yeast extract, peptone, Casamino acids and various single amino acids shortened this lag (Yoch & Pengra, 1966; Patil, Pengra & Yoch, 1967). Parejko & Wilson (1970) claimed that amino acids increased the nitrogenase content of a derepressed culture above that of one grown on N_2 as sole nitrogen source. They also showed that

K. pneumoniae synthesized nitrogenase *in vacuo*, so induction by substrate (N_2) does not appear to be involved in the regulation process.

We now report a study of the repression and derepression of nitrogenase activity by chemostat cultures of *Klebsiella pneumoniae* grown in defined nutrient conditions. This facultative anaerobe, which only fixes nitrogen at low or zero concentrations of dissolved oxygen, is more convenient than *Azotobacter* for repression–derepression studies with whole organisms: assays are performed anaerobically and the problem of defining an optimal pO_2 for each assay (see Drozd & Postgate, 1970; Drozd *et al.* 1972) is eliminated. We also provide evidence from the use of rifampicin and chloramphenicol for the locus of ammonium repression and the longevity of the mRNA specifying nitrogenase.

METHODS

Organism and culture. *Klebsiella pneumoniae* M5a1, a gift from Professor P. W. Wilson, was maintained at 20 °C in air on 2 % nutrient agar slopes and subcultured monthly. Continuous cultures (200 ml; Baker, 1968) were inoculated with 10 to 20 ml of an overnight culture grown on thioglycollate medium (Oxoid Ltd, London). The medium used for continuous cultures contained per litre: 20 g sucrose, 100 mg $MgCl_2 \cdot 6H_2O$, 36 mg ferric citrate, 7.6 mg $Na_2MoO_4 \cdot 2H_2O$, 10.4 g Na_2HPO_4 and 3.4 g KH_2PO_4 . Except where mentioned, growth was limited by sulphate, present as 0.05 mM- Na_2SO_4 . Ammonium succinate was included to repress nitrogenase synthesis without a drastic pH shift (Drozd *et al.* 1972) when required. Phosphate, Na_2SO_4 and ammonium succinate solutions were sterilized independently and subsequently added aseptically. Organisms were grown at 30 ± 0.5 °C under an atmosphere of N_2 flowing at about 200 ml/min. Medium reservoirs were also maintained under an atmosphere of N_2 . The buffering capacity of the medium was sufficient to maintain the pH at 6.7 ± 0.1 under all conditions employed; automatic pH control was therefore omitted. The criteria of nutrient limitation were those of Hill, Drozd & Postgate (1972). At dilution rates, D , of $0.20\ h^{-1}$ or above on nitrogen-free medium, N_2 -limitation and sparse populations were inevitably obtained, so slower growth rates were used.

Batch (10 ml) cultures were grown in Pankhurst tubes (Campbell & Evans, 1969, modified by Postgate, 1972) at 30 °C. The medium used was as above except that 100 mg $MgSO_4 \cdot 7H_2O$ /l replaced $MgCl_2 \cdot 6H_2O$, 20 g glucose/l replaced sucrose and Na_2SO_4 was omitted.

Assay of nitrogenase activity. Acetylene reduction by whole organisms under anaerobic conditions was accepted as a measure of nitrogenase activity. The assumption was therefore made that, during derepression, the rate of acetylene reduction *in vivo* was limited by the amount of nitrogenase proteins present rather than by the availability of reductant or ATP. Conical flasks (25 ml) were flushed with argon and capped with Suba-seal (Griffin & George, Wembley, Middlesex) closures. Acetylene (2 ml) was injected ($pC_2H_2 = 0.06$ atm) and flasks were equilibrated at 30 °C for 5 min. The culture sample (2 ml) was injected to start the assay, which was performed with gentle shaking at 30 °C. For time course studies, gas samples (0.5 ml) were removed at intervals by syringe for gas chromatography and replaced by 0.5 ml argon. Dilution of ethylene in this procedure was corrected for by measuring the concomitant dilution of acetylene. Where this correction was used the actual acetylene reduced was always < 1 % of the total acetylene present. Generally, assays were stopped after 30 min with 0.2 ml of 40 % (w/v) NaOH. Gas samples (0.5 ml) were injected into a Pye 104 gas–liquid chromatograph fitted with a 45 cm Porapak R column (1 mm internal

diam.) at 37 °C with N₂ as the carrier gas flowing at 7 ml/min. Ethylene and acetylene peak heights were recorded and compared with standards.

Acetylene reduction by samples from sulphate-limited populations was linear for at least 5 h without further addition of sulphate; N₂-limited populations similarly required no further addition. Carbon-limited populations were assayed in the presence of 10 mg additional glucose/ml. In contrast to the partial inhibition observed in *Azotobacter* (Drozd *et al.* 1972), repressive concentrations of ammonium succinate had no significant effect on acetylene reduction rates assayed over 30 min. Since acetylene reduction by whole organisms of *Klebsiella* can be inhibited by oxygen (Klucas, 1972), anaerobic handling of culture samples was maintained throughout. Activities thus measured agreed well with acetylene reduction rates measured *in situ* in the chemostat.

Analyses. Ammonia was estimated directly on supernatant fluid from centrifuged culture samples using the indophenol method of Chaney & Marbach (1962). Standard amounts of ammonia in culture medium or in distilled water gave identical absorbances when assayed and nitrogen-free medium itself gave a very low blank. Microdiffusion was therefore unnecessary.

Dry weights were determined by washing organisms twice with distilled water and drying to constant weight at 80 °C. Dried material was cooled and weighed in the presence of desiccant.

Bacterial densities were also recorded as A_{540} measurements. A_{540} for populations grown with NH₄⁺ or facing N₂ was linearly related to dry wt organism/ml up to a value of 0.5, corresponding to 0.58 mg dry wt/ml.

Protein was estimated on washed organisms by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

Derepression techniques. Derepression of nitrogenase synthesis in continuous cultures *in situ* was performed as described by Drozd *et al.* (1972). In some experiments described here a 'small-scale' method, developed for derepressing enzyme synthesis in several samples simultaneously under various conditions, was used. Samples (2 ml) of fully repressed sulphate-limited continuous cultures were injected into 25 ml conical flasks containing argon and acetylene as for an acetylene reduction assay (see above), except that flasks contained excess (0.5 mM) Na₂SO₄ plus any other required additions. Under these conditions excess NH₄⁺ was exhausted and derepression of nitrogenase synthesis proceeded. A time course of acetylene reduction was recorded for each sample. Since the rate of acetylene reduction gradually increased during derepression, a curve was obtained. Acetylene reduction rates (nitrogenase activities) were calculated by drawing tangents at suitable intervals.

RESULTS

Dependence of steady-state nitrogenase activity on the influent NH₄⁺ concentration

An increase in the concentration of ammonium succinate in the medium supplied to a sulphate-limited, nitrogen-fixing continuous culture brought about a proportional decrease in the steady-state nitrogenase activity (Fig. 1); steady states with partially repressed populations were thereby obtained. Free ammonium ion was only detected in the culture supernatant of fully repressed populations, although a narrow range of ammonium succinate concentrations gave complete repression without detectable free ammonium. When growth in the absence of fixed nitrogen was limited to 0.32 mg dry wt bacteria/ml by 0.05 mM-Na₂SO₄, 58 µg N/ml was sufficient to effect complete repression of nitrogenase synthesis; for a popu-

Fig. 1. Effect of increasing concentrations of ammonium succinate in the medium inflow of a SO_4^{2-} -limited chemostat culture ($0.05 \text{ mM-Na}_2\text{SO}_4$, $D = 0.11 \text{ h}^{-1}$) of *Klebsiella pneumoniae* on the steady-state parameters. ■, Bacterial concentration; ○, nitrogenase activity of culture; ●, concentration of NH_4^+ in the culture supernatant.

lation at the same dilution rate limited to $0.18 \text{ mg dry wt bacteria/ml}$ by $0.025 \text{ mM-Na}_2\text{SO}_4$, repression was just complete with $29 \mu\text{g N/ml}$. The concentration of NH_4^+ required for 100 % repression therefore depended on the population density, nitrogenase activity being absent where the influent concentration of ammonium ion satisfied the total nitrogen requirement of the population. These findings are essentially similar to those of Drozd *et al.* (1972) for *Azotobacter chroococcum*.

Bacterial yields calculated from the biomass data in Fig. 1, assuming effectively complete assimilation of sulphate, were $6.4 \times 10^3 \text{ g bacteria/g-atom S}$ for a fully derepressed population and 9.8×10^3 for a fully repressed one. This difference is larger than in *Azotobacter chroococcum* which, corrected for a misprint in Drozd *et al.* (1972), changed from 6.4×10^3 to $7.7 \times 10^3 \text{ g/g-atom S}$. Organisms from fully derepressed, partially repressed or fully repressed populations of *Klebsiella pneumoniae* all contained 75 % dry wt as protein.

To check that the increased yield with ammonium succinate was not an effect due simply to the succinate added, $1.5 \text{ mg disodium succinate/ml}$ were added simultaneously to the culture vessel and medium reservoir of a fully derepressed continuous culture (Na_2SO_4 , 0.05 mM ; D , 0.11 h^{-1} ; $0.32 \text{ mg dry wt bacteria/ml}$). No detectable increase in yield was observed after allowing the culture to regain steady state.

Derepression of nitrogenase in sulphate-limited continuous culture

The medium supply, containing $70 \mu\text{g N/ml}$ as ammonium succinate, to a fully repressed population was replaced with one free of ammonium ions at zero time (Fig. 2). Nitrogenase activity was first detected about 65 min after the exhaustion of free ammonium ions from the culture. After a further 30 min, enzyme activity increased linearly with time before giving a characteristic 'overshoot' and falling back to an activity which was subsequently maintained as the steady-state nitrogenase activity of the derepressed population. The total time from exhaustion of NH_4^+ to this steady-state activity was 340 min, which is 90 % of the doubling time (378 min) at the dilution rate used.

Fig. 2. Kinetics of derepression of nitrogenase activity in a chemostat culture of *Klebsiella pneumoniae*. The medium supply to a SO_4^{2-} -limited culture ($0.05 \text{ mM-Na}_2\text{SO}_4$) growing under N_2 at $D = 0.11 \text{ h}^{-1}$ with $70 \mu\text{g N/ml}$ as ammonium succinate, was abruptly changed to one free of NH_4^+ . ○, Acetylene reduction activity; ●, free ammonium ions in the culture; ■, A_{540} of culture.

The effect of Casamino acids on the derepression of nitrogenase

In experiments otherwise similar to that shown in Fig. 2, $10 \mu\text{g N/ml}$ as vitamin-free Casamino acids, whether present all the time or added to the culture vessel 20 min before exhaustion of NH_4^+ , did not decrease the delay before detecting activity nor increase the rate of nitrogenase synthesis.

To test a wider variety of conditions the 'small-scale' procedure was used (see Methods). Derepression of nitrogenase in the absence of amino-acid N by this procedure ceased at a specific activity of about $10 \text{ n-mol C}_2\text{H}_4 \text{ formed/min/mg bacterial protein}$. This was low, in agreement with Parejko & Wilson's (1970) report of only limited synthesis of nitrogenase under an inert gas phase. Fig. 3 shows the effect on derepression of various amounts of Casamino acids added after NH_4^+ had been exhausted. At low concentrations Casamino acids did not affect the lag, the rate of enzyme synthesis or the final activity; higher concentrations ($16 \mu\text{g N/ml}$ and above) were repressive. Comparable derepression experiments with different initial excesses of NH_4^+ are shown in Table 1. Where high levels of ammonium had to be exhausted before derepression could start, the subsequent lag before linear synthesis of nitrogenase activity was longer. Although Casamino acids had no effect on the shorter lags, when derepression was imminent they shortened a longer lag appreciably. In conditions similar to those of experiment 5, Table 1, Casamino acids also increased the rate of enzyme synthesis after derepression. The final activity was increased by 50 % to $15 \text{ n-mol C}_2\text{H}_4 \text{ formed/min/mg bacterial protein}$. Yeast extract ($10 \mu\text{g N/ml}$) and aspartate ($10 \mu\text{g N/ml}$) were in this instance as effective as Casamino acids in shortening the lag but had little effect on the rate of nitrogenase synthesis or final activity.

In derepression experiments in the chemostat (e.g. Fig. 2) during which sulphate-limitation was maintained throughout, the time taken before acetylene reduction activity increased

Fig. 3. Effect of various amounts of Casamino acids on derepression of nitrogenase. At zero time, culture samples from a fully repressed, sulphate-limited chemostat culture ($0.05 \text{ mM-Na}_2\text{SO}_4$; $D = 0.11 \text{ h}^{-1}$) were injected into flasks gassed with C_2H_2 in argon, and containing excess sulphate. At A, free ammonium ion was $< 1 \mu\text{g N/ml}$, and Casamino acids were added to 0.8 (●), 8 (○), 16 (△), 80 (▲), and 160 (■) $\mu\text{g N/ml}$. □, Control with no addition.

Table 1. Influence of the time required to exhaust excess ammonium ion on the subsequent lag in derepressing nitrogenase by the 'small-scale' method (see Methods)

Experiment	Excess NH_4^+ ($\mu\text{g N/ml}$)	Time at which NH_4^+ exhausted (min)	Lag* before derepression of nitrogenase (min)	
			No addition	Casamino acids added ($10 \mu\text{g N/ml}$)
1	n.d.	—	86	84
2	0.5	—	106	103
3	3.2	40	82	—
4	8.0	100	110	—
5	23.0	154	138	90
6	40.0	190	175	—

n.d., Not detected; —, not determined.

* 'Lag' was the time between exhaustion of NH_4^+ and the point to which linear derepression of nitrogenase activity could be extrapolated.

linearly with time was independent of the amount of excess NH_4^+ to be exhausted. The longer lags recorded in Table 1 only arose where sulphate-limitation had been relieved for at least 2 h. Under those conditions it seems most likely that nitrogen became growth-limiting. The major component of these lags was the time taken for the rate of nitrogenase synthesis to become linear; the delay before first appearance of acetylene reduction, obtained by extrapolation of the non-linear part of the curve to zero, was almost invariant at $40 \pm 5 \text{ min}$. The length of the lag and its relief by Casamino acids therefore depended on nutritional status.

Table 2. *Effect of various nitrogen supplements on acetylene reduction by batch cultures of Klebsiella pneumoniae*

NH₄-grown inocula were cultured anaerobically in Pankhurst tubes (see Methods). Nitrogen supplements to the nitrogen-free medium were as shown. Acetylene ($pC_2H_2 = 0.03$) was added to the N₂ gas phase 5 h after inoculation.

Supplement	$\mu\text{g N/ml}$	Twenty-four hours after inoculation	
		Growth*	n-mol C ₂ H ₄ /ml gas phase
Diammonium succinate	140	++++	0
KNO ₃	140	++++	0
L-Aspartate	160	++++	290
Vitamin-free			
Casamino acids	100	++++	213
Yeast extract	100	++++	330
None	0	+	87

* + + + +, Very good growth; +, growth just detectable.

Repression by aspartate

Certain amino acids (e.g. L-aspartate) eliminated the diauxic growth lag following the exhaustion of a limiting concentration of NH₄⁺ from batch cultures of *Klebsiella pneumoniae* (Yoch & Pengra, 1966; Patil *et al.* 1967). Aspartate appeared to stimulate adaptive formation of the nitrogenase system; assimilation of aspartate and N₂ occurred simultaneously. In batch cultures, we have confirmed these observations. L-Aspartate (160 $\mu\text{g N/ml}$), Casamino acids (100 $\mu\text{g N/ml}$) or yeast extract (100 $\mu\text{g N/ml}$) allowed good growth without substantial repression of nitrogenase activity (Table 2). Parallel cultures provided with NH₄⁺ or NO₃⁻ grew well but showed no acetylene reduction activity. Batch cultures of nitrogen-fixing anaerobes in nitrogen-deficient media are N₂-limited, in that μ_{max} is determined by the rate of nitrogen fixation (Hill *et al.* 1972). A chemostat culture at $D = 0.11 \text{ h}^{-1}$ was made N₂-limited by increasing the influent Na₂SO₄ to 0.30 mM giving, in the absence of fixed nitrogen, a population at steady state of 0.69 mg dry wt/ml, which reduced 28 n-mol acetylene/min/mg bacterial protein. The addition of 95 $\mu\text{g N/ml}$ as L-aspartate to such a population increased the biomass to 0.98 mg dry wt/ml whereas the nitrogenase specific activity was virtually unchanged at 24 n-mol acetylene/min/mg bacterial protein. Therefore, although aspartate-N increased the yield of organisms obtained it did not repress nitrogenase. By elimination of other possibilities, this culture was shown to be N-limited.

However, L-aspartate (95 $\mu\text{g N/ml}$) added to the medium reservoir of a sulphate-limited, nitrogen-fixing continuous culture (Na₂SO₄, 0.05 mM; D , 0.11 h⁻¹) completely repressed nitrogenase: no activity was present after allowing the culture to regain steady state, and the biomass increased to 0.48 mg dry wt/ml (which is characteristic of a similar population completely repressed by ammonium; see Fig. 1). This concentration of L-aspartate was equivalent in terms of N/ml to a fully repressive concentration of ammonium.

When 142 $\mu\text{g N/ml}$ as L-aspartate were added to the medium supply to a glucose-limited, nitrogen-fixing continuous culture (5 g glucose/l; D , 0.10 h⁻¹), the steady-state nitrogenase activity was undetectably low, and 14 $\mu\text{g N/ml}$ ammonium ion were present in the culture supernatant. Clearly the ability to effect repression from exogenous aspartate was determined by the nutritional status of the population (see Discussion).

Fig. 4. Kinetics of repression of nitrogenase activity of *Klebsiella pneumoniae* in sulphate-limited continuous culture ($0.05 \text{ mM-Na}_2\text{SO}_4$; $D = 0.14 \text{ h}^{-1}$). (a) Decline of nitrogenase activity/mg bacterial protein. (b) Decline of activity/ml of culture. ●, At zero time ammonium succinate ($120 \mu\text{g/ml}$) was added to the culture vessel and the medium inflow changed to one introducing a similar NH_4^+ concentration. The same data are plotted in (a) and (b); a difference arises from the small increase in bacterial protein/ml during the experiment. ○, A second experiment in which $100 \mu\text{g/ml}$ chloramphenicol were added with repressive ammonium. Dashed lines represent nitrogenase activities of cultures before additions were made.

Kinetics of ammonium repression of nitrogenase synthesis in sulphate-limited continuous culture

Ammonium succinate ($120 \mu\text{g N/ml}$) was added to the culture vessel of an N_2 -fixing chemostat culture limited by Na_2SO_4 and the medium input at once changed to one introducing a medium containing $120 \mu\text{g NH}_4^+\text{-N/ml}$. For about 75 min no decrease in nitrogenase activity was observed, indicating continued production of active enzyme (Fig. 4a). Subsequently nitrogenase activity declined logarithmically faster than 'wash-out' with a half-life of 190 min compared with a doubling time for the population ($D = 0.14 \text{ h}^{-1}$) of 288 min. When chloramphenicol ($100 \mu\text{g/ml}$) was added simultaneously with repressive NH_4^+ and the dilution of the culture continued as normal, a delay of about 30 min (Fig. 4b) was still observed, suggesting that active enzyme was produced without protein synthesis *de novo*. Here we have assumed that chloramphenicol produced its usual response – a rapid and substantial inhibition of protein synthesis. This appeared to be so since both bacterial density (A_{540} of culture) and bacterial protein/ml declined immediately following the addition of the inhibitor (not shown in the Figure). In this experiment the total nitrogenase activity subsequently declined by wash-out at a similar logarithmic rate to the total protein content of the culture (see Table 3). In the absence of further protein synthesis, therefore, nitrogenase turnover was small.

Separation of transcription and translation during derepression of nitrogenase

In principle, if rifampicin (an inhibitor of initiation of RNA synthesis: see Sippel & Hartman, 1968) and chloramphenicol (an inhibitor of mRNA translation) are added to parallel

Table 3. *Half-lives of parameters during ammonium repression of nitrogenase activity in continuous culture*

Parameter	$t_{0.5}$ (min)
Nitrogenase activity (n-mol C_2H_4 formed/min/mg bacterial protein) after addition of NH_4^+ , see Fig. 4(a)	190
Nitrogenase activity (n-mol C_2H_4 formed/min/ml culture) after addition of NH_4^+ , see Fig. 4(b)	195
Nitrogenase activity (n-mol C_2H_4 formed/min/ml culture) after addition of NH_4^+ and chloramphenicol, see Fig. 4(b)	240
Bacterial protein in culture after addition of NH_4^+ and chloramphenicol	264
Theoretical $t_{0.5}$ of 'wash-out' in continuous culture at $D = 0.14\text{ h}^{-1}$	288

cultures just beginning to derepress, chloramphenicol should curtail derepression more abruptly than rifampicin. A higher final activity should arise with rifampicin as a consequence of translation of pre-formed mRNA. Comparison of the effect of these inhibitors with that of NH_4^+ during derepression of nitrogenase should indicate the level at which NH_4^+ acts. The experiments described here are based on studies by Kepes (1969) and Jacquet & Kepes (1969) on the expression of the lactose operon of *Escherichia coli*.

Samples were removed at intervals following the exhaustion of NH_4^+ from the culture in a derepression experiment carried out as for Fig. 2. Portions (4×2 ml) were treated as in the 'small-scale' derepression method; flasks finally contained: (i) 200 μg rifampicin/ml, 8 μg N/ml as Casamino acids, 0.5 mM- Na_2SO_4 ; (ii) as (i) but with 100 μg chloramphenicol/ml instead of rifampicin; (iii) as (i) but with 200 μg N/ml as ammonium succinate instead of rifampicin; (iv) no additions. Flask (iv) was therefore a normal acetylene reduction assay and contained no additional sulphate. Linear rates of acetylene reduction were recorded; these were obtained immediately with treatments (ii) and (iv) but only after about 40 min with treatments (i) and (iii), during which time the nitrogenase activity increased, presumably due to continued enzyme synthesis. Acetylene reduction rates finally obtained for treatments (i) to (iv) at intervals during derepression are plotted in Fig. 5, which permits three conclusions: since rifampicin added shortly after the exhaustion of NH_4^+ did not block eventual appearance of some nitrogenase activity, nitrogenase mRNA must already have been initiated at this time; initiation of nitrogenase mRNA preceded appearance of enzyme activity by about 20 min; and since NH_4^+ paralleled the effect of rifampicin, in that it allowed further enzyme synthesis for a short period, repression by NH_4^+ was apparently at the level of mRNA synthesis.

Complementary results were obtained from a further experiment in which either chloramphenicol, rifampicin, or ammonium succinate were added to organisms which were just beginning to derepress. Here, the 'small-scale' derepression method was employed (see Methods). Chloramphenicol, rifampicin and ammonium succinate all curtailed derepression. Chloramphenicol was almost immediate in its effect whereas more enzyme was synthesized

Fig. 5. Separation of transcription and translation during derepression of nitrogenase. The medium supply, containing $70 \mu\text{g N/ml}$ as ammonium succinate, to a sulphate-limited chemostat culture ($0.05 \text{ mM-Na}_2\text{SO}_4$; $D = 0.11 \text{ h}^{-1}$), was changed to one free of ammonium ions at zero time. Δ , Free ammonium ions in culture. At intervals following exhaustion of NH_4^+ , culture samples were removed and were either untreated ($\bullet$), or treated with Casamino acids ($8 \mu\text{g N/ml}$) and Na_2SO_4 (0.5 mM) in addition to: $\blacksquare$, rifampicin ($200 \mu\text{g/ml}$); $\square$, ammonium succinate ($200 \mu\text{g N/ml}$); or $\circ$, chloramphenicol ($100 \mu\text{g/ml}$). Final acetylene reduction rates obtained after allowing any further enzyme synthesis to occur are plotted against the time of sampling.

in the presence of rifampicin or ammonium over about 40 min (Fig. 6). The accelerated derepression and higher final activity with ammonium suggested that translation of residual message was stimulated. This was further substantiated by showing that ammonium succinate and rifampicin added together gave the same effect as ammonium succinate alone, whereas ammonium succinate and chloramphenicol together paralleled the result of chloramphenicol alone. In the experiment shown in Fig. 6, the inhibitors were added during the lag, i.e. the period before translation of message reached steady state (see Kepes, 1969;

Fig. 6

Fig. 7

Fig. 6. Effects of inhibitors on derepression using the 'small-scale' procedure (see Methods). Parallel samples from a fully repressed, sulphate-limited continuous culture ($0.05 \text{ mM-Na}_2\text{SO}_4$; $D = 0.11 \text{ h}^{-1}$) had exhausted excess NH_4^+ in the presence of additional (0.5 mM) Na_2SO_4 and were in an early stage of derepression at time zero. Ammonium succinate ($200 \mu\text{g N/ml}$), ■; rifampicin ($200 \mu\text{g/ml}$), □; or chloramphenicol, ○; were added at 5 min (arrowed), and further derepression of nitrogenase activity followed in each case. ●, Control to which no addition was made.

Fig. 7. Decay of 'coding capacity' for nitrogenase synthesis following inhibition of mRNA initiation by rifampicin. (a) A sample from a fully repressed, sulphate-limited continuous culture ($0.05 \text{ mM-Na}_2\text{SO}_4$; $D = 0.11 \text{ h}^{-1}$) had exhausted excess NH_4^+ and reached linear derepression under the conditions of the 'small-scale' procedure. $200 \mu\text{g}$ rifampicin/ml were added at time zero and further derepression recorded. (b) Logarithmic decay of message specifying nitrogenase. Each point represents the increment in nitrogenase activity for each successive two-minute interval in (a).

Coffman, Norris & Koch, 1971). Consequently, apparent coding capacity continued to increase even in the presence of rifampicin. When rifampicin was added during the linear phase of derepression, enzyme activity increased to a maximum over 20 min (Fig. 7a). The semi-log plot of successive increments of activity (Fig. 7b) gave a half-life for the coding

capacity of pre-formed message of 4.4 ± 0.2 min. A similar half-life of 4.7 ± 0.2 min was obtained in a comparable experiment in which ammonium succinate and rifampicin were added together.

DISCUSSION

Yield coefficients in sulphate-limited continuous cultures of Klebsiella pneumoniae. The 50 % increase in yield for an ammonium-assimilating population over a nitrogen-fixing one is unlikely to be explained merely by the known high sulphur content of nitrogenase. It may be related to the fact that carbon-limited populations show a lowered efficiency of conversion of energy source into biomass when N_2 replaces NH_4^+ as the nitrogen source – a phenomenon more pronounced in *Klebsiella pneumoniae* than in *Azotobacter chroococcum* (see Hill *et al.* 1972).

Derepression of nitrogenase synthesis. Under conditions of sulphate limitation, derepression of nitrogenase proceeded with a shorter lag following exhaustion of ammonium ion than has previously been reported (Lindsay, 1963; Mahl & Wilson, 1968). Even so, a comparable lag was absent in similar experiments with *Azotobacter chroococcum* (Drozd *et al.* 1972). With *Klebsiella pneumoniae* the shortened lag in expression of nitrogen fixation under sulphate-limited conditions was not further decreased by exogenous Casamino acids. A longer lag, however, was observed when ammonium was exhausted under conditions where presumably nitrogen became growth limiting; conditions which, for example, lead to a high degree of catabolite repression (see Mandelstam, 1962). Such a lag was decreased by Casamino acids; this is consistent with the observation of Yoch & Pengra (1966) that the amino acid pool of *K. pneumoniae* is depleted during derepression of nitrogenase. Synthesis of nitrogenase can therefore be influenced by the nutritional status of the bacterial population.

During derepression under an argon–acetylene gas phase, ammonium stimulated enzyme synthesis before the repressive effect was observed (Fig. 6). The derepression rate doubled transiently, yet we know that ammonium did not stabilize existing message since its half-life was unaltered in the presence of ammonium succinate. Hence ammonium stimulated translation to such an extent that twice as much enzyme was obtained from the amount of mRNA formed or initiated before repressor was added. A similar effect of NH_4^+ *in vivo* was observed by Rigano & Violante (1973) during derepression of nitrate reductase synthesis by *Cyanidium caldarium*. Ammonium ions stimulate cell-free amino acid incorporation (Lubin & Ennis, 1964); high NH_4^+ concentrations are required for ribosomal stability *in vitro* (Hartman, Nolan & Amaya, 1971) and for the peptidyl transferase reaction (Miskin, Zamir & Elson, 1970). Under argon, only a limited amount of nitrogenase was derepressed, as previously observed by Parejko & Wilson, 1970; Casamino acids added prior to derepression increased the final activity obtained in one instance. *Klebsiella pneumoniae*, therefore, did not synthesize nitrogenase completely when only endogenous nitrogen sources were available.

Repression of nitrogenase synthesis. During the initial stages of repression by NH_4^+ (Fig. 4) we have inferred continual production of active enzyme in the absence of protein synthesis *de novo*. Whether activation of inactive nitrogenase proteins is continually taking place in fully derepressed populations remains to be tested critically. During purification of nitrogenase from *Clostridium pasteurianum* an inactive derivative of the iron–molybdenum protein which lacks molybdenum and much of its normal complement of iron has been separated (Zumft, Cretney, Huang, Mortenson & Palmer, 1972). If such a species occurs *in vivo*, reactivation in the absence of further protein synthesis may be possible. To be

involved in derepression, such an activation process must be rapid, since derepression was curtailed immediately by chloramphenicol (see Fig. 5).

Nitrogenase activity was primarily diluted out during repression. The small amount of nitrogenase turnover depended on continued protein synthesis (Table 3). In agreement with Shah *et al.* (1972), there appears to be no specific mechanism for rapidly degrading or inactivating nitrogenase following repression of its synthesis by ammonium.

Under certain conditions, other nitrogen sources may be partially or wholly as effective as NH_4^+ in repressing nitrogenase synthesis. These include glutamine in *Klebsiella pneumoniae* (Parejko & Wilson, 1970), asparagine (Wilson, Hull & Burris, 1943; St John & Brill, 1972), urea (Wilson *et al.* 1943; Hardy & Knight, 1967) and nitrate (Wilson *et al.* 1943; Drozd *et al.* 1972) in *Azotobacter*. This diversity suggests that a common intermediate, the most obvious being NH_4^+ , is the effector of repression. Here, L-aspartate repressed nitrogenase under sulphur- or carbon-limited conditions of growth but not under nitrogen limitation. We therefore suggest that, under sulphur and carbon limitation, L-aspartate is deaminated at a sufficient rate to maintain fully repressive concentrations of NH_4^+ , whereas under nitrogen limitation NH_4^+ produced from aspartate does not accumulate.

When the nitrogen fixation genes were transferred from *Klebsiella pneumoniae* to *Escherichia coli* (Dixon & Postgate, 1972) repression of nitrogenase synthesis by ammonium was conserved, indicating that regulatory and structural genes are closely linked. This concept of a *nif* operon has more recently been expanded by Streicher, Gurney & Valentine (1972). Here we have provided direct evidence that ammonium repression is effective at the level of mRNA synthesis. During derepression of nitrogenase in *Klebsiella pneumoniae*, however, considerable lags were observed even after acetylene reduction became just detectable (see Fig. 2, 3, 5 and 6). Such lags have been associated with the time taken for messenger RNA translation to come to steady state (see Kepes, 1969). Here the length of the lag depended on nutritional status, demonstrating that even though the repressive effect of NH_4^+ on the *nif* genes was relieved, efficient and rapid synthesis of nitrogenase did not necessarily follow.

The coding capacity of pre-formed message for nitrogenase synthesis decayed with a half-life of about 4.5 min with or without NH_4^+ . Decay rates of different mRNAs in *Escherichia coli* have been summarized by Blundell, Craig & Kennel (1972) and are generally more rapid than the figure reported here. These authors, however, quote decay rates which vary considerably with different strains or with growth conditions. Here we have measured a decay rate during derepression at 30 °C under non-growing conditions. Nitrogenase of *Klebsiella pneumoniae* comprises two component proteins resembling those from other organisms (Eady, Smith, Cook & Postgate, 1972). Since the larger iron-molybdenum component consists of two non-identical classes of subunit, there are at least three structural genes involved in nitrogenase synthesis. Shah *et al.* (1972) have demonstrated that the component proteins are synthesized co-ordinately in *Azotobacter vinelandii*. Presumably the decay rate of coding capacity for nitrogenase synthesis measured here represents the decay rate of the fastest decaying cistron.

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