Nitrogen Fixation in *Klebsiella pneumoniae*: Nitrogenase Levels and the Effect of Added Molybdate on Nitrogenase Derepressed under Molybdenum Deprivation

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Klebsiella pneumoniae had low levels of nitrogenase activity and antigenic cross-reacting material to both nitrogenase components when grown, or when the *nif* operons were de-repressed, under Mo-deficient conditions. When molybdate (100 μM) was added to Mo-deprived cultures shortly after initiating derepression of nitrogenase, activity was rapidly restored and reached up to 60% of that of control cultures supplied with molybdate throughout de-repression. Restoration of activity was prevented by chloramphenicol (200 $\mu\text{g ml}^{-1}$) but not by tetracycline (100 $\mu\text{g ml}^{-1}$), although both compounds inhibited general protein synthesis at these concentrations. Expression of the *nif* structural genes was not rapidly stimulated by added molybdate, so the rapid stimulation of activity was due to the activation of a low level of apoprotein present in Mo-deprived cultures. After 16 h of Mo-deprivation, suspensions no longer responded rapidly to added molybdate, consistent with the finding that, when further protein synthesis was inhibited by the addition of chloramphenicol after 4 h Mo-deprivation, polypeptides of the MoFe protein (Kp1) but not the Fe protein (Kp2) of nitrogenase were degraded within the following 18 h.

INTRODUCTION

Biological N_2 fixation is an energy-dependent process requiring ATP and a source of low-potential reducing equivalents. About 16 mol ATP are consumed per mol N_2 fixed *in vivo* (for a review, see Andersen *et al.*, 1977). In view of this high energy requirement it is not surprising that nitrogenase is repressed when organisms have a plentiful supply of fixed N, or when this O_2 -sensitive enzyme system would be inactivated by free dissolved O_2 (Eady *et al.*, 1978; Robson, 1979).

The presence of molybdenum in the MoFe protein of nitrogenase (see Eady & Smith, 1979) explains the long-known essential requirement for Mo in biological N_2 fixation (Bortels, 1930). Speculation that Mo may regulate nitrogenase synthesis has led to studies of the effect of Mo-deprivation on nitrogenase in a number of organisms, and no uniform response has been found: the cyanobacteria *Plectonema boryanum* and *Anabaena cylindrica* synthesize both the Fe protein and the MoFe protein of nitrogenase (Nagatani & Haselkorn, 1978; Hallenbeck & Benemann, 1980); *Azotobacter vinelandii* synthesizes only the Fe protein (Nagatani & Brill, 1974); *Clostridium pasteurianum* synthesizes neither component (Cardenas & Mortenson, 1975). For *Klebsiella pneumoniae* there are conflicting data concerning the involvement of molybdate in nitrogenase synthesis. Since only low levels of antigenic cross-reacting material to either nitrogenase component were detected under Mo-deprived conditions, Brill *et al.* (1974) concluded that molybdate was required for nitrogenase synthesis. However, Kennedy & Postgate (1977) detected significant levels of

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nitrogenase polypeptides by sodium dodecyl sulphate (SDS) gel electrophoresis of extracts of a *narD* strain of *Escherichia coli* carrying the *Klebsiella* N_2 fixation (*nif*) gene cluster on the plasmid pRD1; this strain was deficient in Mo-processing (Sperl & DeMoss, 1975) and consequently the nitrogenase was inactive unless a sufficiently high concentration of molybdate (which relieved the Nar^- phenotype) was subsequently supplied. Experiments using gene fusions in which the *lacZ* gene was under the control of various promoters of the *K. pneumoniae* *nif* gene cluster (Dixon *et al.*, 1980) indicated that none of the *nif* operons had an absolute requirement for molybdate for expression, but that full expression of the structural genes (as determined from measurement of β -galactosidase activity) required the presence of molybdate together with another *nif*-encoded polypeptide.

In this paper we report a reinvestigation of the involvement of molybdate in regulating nitrogenase synthesis in *K. pneumoniae*, using immunochemical and ^{14}C pulse-labelling techniques to determine nitrogenase levels directly. In addition, data are presented on the effects of chloramphenicol and tetracycline on the ability of molybdate to restore nitrogenase activity to Mo-deprived organisms. A preliminary report of some of this work was given by Postgate *et al.* (1981).

METHODS

Organisms and culture. The *Klebsiella pneumoniae* strains used in this work were the wild-type strain M5a1, and strain UNF619(pMF183), a *nif*⁺ $\Delta lac/nifH2783::Mud(Aplac)$ merodiploid (Dixon *et al.*, 1980), which carries the *lacZ* gene (encoding β -galactosidase) fused in the structural gene (*nifH*) of Kp2. The stock strains were maintained at 20 °C in air on 2% (w/v) nutrient agar slopes and subcultured at 2 month intervals. Cultures were grown anaerobically at 30 °C in N-free medium (NFDM; Cannon *et al.*, 1974) supplemented with $(NH_4)_2SO_4$ (1.5 mg ml⁻¹) to repress nitrogenase synthesis. Traces of Mo in the medium were removed by extraction of the glucose and sodium and potassium phosphates with 8-hydroxyquinoline and chloroform (Kelly & Lang, 1970), and by washing glassware with nitric acid (50%, v/v, AnalaR). Organisms were collected by centrifuging and resuspended under Ar in Mo-depleted NFDM without added NH_4^+ but containing aspartate (100 μ g ml⁻¹). Nitrogenase activity was derepressed at 30 °C with or without sodium molybdate (100 μ M) or sodium tungstate (100 μ M) as indicated.

Radioactive labelling. Samples (1 ml) were pulse-labelled with a mixture of ^{14}C -labelled amino acids essentially as described by Eady *et al.* (1978). Uptake of radioactivity was stopped after 5 min by addition of 0.15 ml of unlabelled Difco Casamino acids (2 mg) and 0.15 ml of phenylmethylsulphonyl fluoride in 2-propanol (0.3 mg). The saline washing step of Eady *et al.* (1978) was omitted and the samples were stored on ice before the organisms were harvested by centrifuging.

Electrophoresis and autoradiography. The pelleted organisms were resuspended in 0.2 ml of Ortec sample buffer (Eady *et al.*, 1978) and lysed by heating in a boiling water bath for 4 min. Polyacrylamide gels containing 10% (w/v) acrylamide and 0.16% (w/v) bisacrylamide were loaded with samples containing approximately 40000 c.p.m. Electrophoresis was carried out as described by Kennedy *et al.* (1976), and autoradiography of the dried gels and scanning of the X-ray films as described by Eady *et al.* (1978). To determine the relative rates of synthesis of Kp1 and Kp2, the relevant peaks were cut out and their weights expressed as a percentage of that of the complete scan (Eady *et al.*, 1978).

Protein synthesis. The effectiveness of chloramphenicol and tetracycline in preventing general protein synthesis was checked by their ability to inhibit incorporation of ^{14}C -labelled amino acids into trichloroacetic acid-precipitable material. Derepressing cultures were treated with chloramphenicol (200 μ g ml⁻¹) or tetracycline (100 μ g ml⁻¹). At intervals samples were removed, pulse-labelled for 1 min, and lysed with sample buffer as described above. Samples (5 μ l) of the lysates were added to 1 ml of carrier bovine serum albumin (100 μ g) before precipitation with 1 ml of 10% (w/v) trichloroacetic acid. The precipitate was collected by filtration, washed with 5% (w/v) trichloroacetic acid and, after drying, the radioactivity was determined by scintillation counting.

Enzyme assays. Nitrogenase activity was measured in cultures (1 or 2 ml containing approx. 0.2 mg protein ml⁻¹) in conical flasks (25 ml nominal volume) capped with a rubber closure (Suba-seal) and maintained under Ar containing 10% (v/v) acetylene. The flasks were shaken in a water bath at 30 °C and at intervals gas samples (0.5 ml) were removed by syringe for measurement by g.l.c. of the ethylene formed. Specific activities were calculated from the increment in ethylene between samples, using the acetylene peak as an internal standard. Nitrogenase activity in crude extracts was measured as described by Eady *et al.* (1972). The maximum potential activity of the individual nitrogenase components Kp1 and Kp2 in extracts was measured by the addition of purified Kp2 or Kp1, respectively, to the assay.

β -Galactosidase activity was measured as described by Dixon *et al.* (1980) under the same conditions as

above except that acetylene was omitted. One unit of activity is defined as the amount of enzyme that will hydrolyse 1 nmol *o*-nitrophenyl- β -D-galactoside min⁻¹ at 30 °C.

Nomenclature. Nitrogenase is comprised of two redox proteins one of which contains both Fe and Mo, and one which contains only Fe (see Eady & Smith, 1979). The nomenclature used in this paper for the *K. pneumoniae* proteins is as follows: the MoFe protein (Kp1) is an $\alpha_2\beta_2$ tetramer, the subunits of which are encoded by *nifD* and *nifK* respectively; the Fe protein (Kp2) is a dimer, the subunit of which is encoded by *nifH* (Roberts *et al.*, 1978).

Serological assay. Kp1 and Kp2 proteins, purified by repetitive (NH₄)₂SO₄ precipitation (Rennie *et al.*, 1978), were used as antigens to induce specific antibody formation in New Zealand rabbits. Quantification of antibody cross-reacting material present in crude extracts was performed by the Laurell immunoelectrophoresis technique (Laurell, 1972), using calibration curves prepared from dilutions of antigens of known concentrations. Linear precipitin cones were obtained up to 3 μ g Kp1 and 0.5 μ g Kp2.

RESULTS

Nitrogenase levels in Mo-deprived organisms

Both the nitrogenase activity of extracts of organisms supplied with molybdate throughout derepression, and the maximum potential Kp2 activity, were comparable with nitrogenase activity *in vivo*. The maximum potential Kp1 activity, as determined with saturating amounts of Kp2, was approximately 3-fold higher than the nitrogenase activity of unsupplemented extracts (Table 1). The concentration of active Kp2 was therefore rate-limiting for nitrogenase activity of extracts.

Typically, the specific activity of nitrogenase after 7 h derepression under conditions of Mo-deprivation was approximately 2% of that of cultures supplied with molybdate throughout derepression (Fig. 1). In extracts prepared from Mo-deprived organisms, Kp1 activity was approximately 3% of that in extracts of cultures supplied with molybdate, and no Kp2 activity was detected (Table 1). Rocket immunoelectrophoresis of extracts after 6 h derepression under Mo-deprived conditions showed that the levels of cross-reacting materials to both anti-Kp1 and anti-Kp2 were considerably lower than in control suspensions (Table 1). After 16 h under non-repressive but Mo-deprived conditions, the levels of cross-reacting material to both anti-Kp1 and anti-Kp2 were reduced to the limit of detection. The addition of tungstate (100 μ M) in the Mo-deficient medium did not significantly alter the nitrogenase activity or the levels of cross-reacting material. Consistent with these serological data, extracts of 18 h Mo-deprived suspensions showed neither Kp1 nor Kp2 bands in stained SDS polyacrylamide electrophoresis gels.

Table 1. *Effects of Mo-deprivation on nitrogenase activity and levels of antigenic cross-reacting material to nitrogenase components in extracts of K. pneumoniae*

NH₄⁺-grown cultures were harvested and derepressed for 6 h in Mo-deficient medium with the additions indicated (100 μ M). The nitrogenase activity *in vivo* was then determined, before the suspensions were harvested by centrifuging and extracts were prepared by disruption in a French pressure cell. The protein concentrations of the extracts were, respectively, 7.7, 8.8 and 7.7 mg ml⁻¹ for organisms derepressed in medium containing molybdate, tungstate and no addition; the 100% values correspond to 0.44 mg ml⁻¹ for Kp1 and 0.38 mg ml⁻¹ for Kp2, determined by immunoelectrophoresis. The nitrogenase activity was measured both in the normal extract (the intrinsic activity) and after the addition of purified Kp1 or Kp2 protein to assess the maximum potential activity of nitrogenase components Kp2 and Kp1, respectively.

Addition to Mo-deficient NFD	Antigenic cross-reacting material		Nitrogenase activity [nmol ethylene formed min ⁻¹ (mg protein) ⁻¹]			
			Extracts			
	Kp1 (%)	Kp2 (%)	Cultures	Intrinsic activity	Maximum Kp2 activity	Maximum Kp1 activity
Molybdate	100	100	14	17.5	18.9	48.7
Tungstate	3.6	13.7	0.4	0	0	1
None	3.6	10.5	0.35	0	0	1.5

Fig. 1

Fig. 2

Fig. 1. Effect of Mo-deprivation on derepression of nitrogenase activity, and the restoration of activity by added molybdate. NH_4^+ -grown organisms were resuspended in NFDM containing aspartate ($100 \mu\text{g ml}^{-1}$) with or without added molybdate ($100 \mu\text{M}$). The gas phase was Ar containing acetylene (10%, v/v), and gas samples were removed at intervals for measurement of nitrogenase activity [expressed as $\text{nmol ethylene formed min}^{-1} (\text{mg protein})^{-1}$]: ○, molybdate present throughout derepression; △, molybdate added after 4 h 10 min derepression; ●, no added molybdate.

Fig. 2. Effect of tetracycline on the restoration of nitrogenase activity by added molybdate. Cultures were derepressed under Mo-deprived conditions for 4 h (a) or 6 h (b). Where indicated, tetracycline ($100 \mu\text{g ml}^{-1}$) was then added, followed 5 min later (zero time) by molybdate ($100 \mu\text{M}$). Samples were taken at intervals during the following 50 min for determination of nitrogenase activity [expressed as $\text{nmol ethylene formed min}^{-1} (\text{mg protein})^{-1}$]: ○, △, molybdate added at zero time (no tetracycline); ●, ▲, tetracycline added 5 min before molybdate; □, no added molybdate or tetracycline (control). The dotted curves are corrected for the contribution of newly-synthesized nitrogenase to the observed activity by subtraction of the linear rate of increase which occurs in the absence of tetracycline.

Effect of molybdate on nitrogenase activity in Mo-deprived cultures

When molybdate ($100 \mu\text{M}$) was added to Mo-deprived organisms soon after derepression had been detected in control Mo-sufficient suspensions, nitrogenase activity was rapidly restored (Fig. 1) yet the levels of cross-reacting material to both anti-Kp1 and anti-Kp2 remained unchanged for at least 45 min. The response was biphasic, with a rapid burst followed by a slower increase in activity (Fig. 2). The initial increase was at least 10 times faster than the rate of derepression of activity in control suspensions.

In contrast, prolonged Mo-deprivation resulted in the loss of the ability to respond rapidly to added molybdate. Cultures grown for 16 h under Mo-deprivation with aspartate, a non-repressive N source, showed a 50 min lag following addition of molybdate before nitrogenase activity gradually increased.

The rapid increase in nitrogenase activity following addition of molybdate could arise either from the activation of Kp1 apoprotein synthesized by the organisms during Mo-deprivation or by stimulation of transcription or translation of the *nif* structural genes, these being the only genes of the *nif* cluster whose expression responds to added molybdate (Dixon *et al.*, 1980). These two alternatives were investigated further.

Fig. 3. Effect of molybdate and tungstate on the rate of nitrogenase synthesis by *K. pneumoniae* M5a1 during derepression of *nif*. An NH_4^+ -grown culture of *K. pneumoniae* was harvested and resuspended in Mo-depleted NFDm with the following additions at $100\ \mu\text{M}$: (a) none; (b) molybdate; (c) tungstate. After 5 h 45 min derepression, suspensions were pulse-labelled for 5 min with a mixture of ^{14}C -labelled amino acids. Labelled organisms were lysed, and extracts were subjected to SDS gel electrophoresis and autoradiography.

Fig. 4. Effect on nitrogenase synthesis of molybdate added to cultures of *K. pneumoniae* derepressed under conditions of Mo-deprivation. Nitrogenase synthesis was measured by scanning autoradiograms of SDS gels of extracts of labelled cells (see Methods). An NH_4^+ -repressed control culture was derepressed in the presence of molybdate ($100\ \mu\text{M}$) and samples were removed and pulse-labelled at various times of derepression: ○, Kp1; □, Kp2. To a parallel suspension, derepressed under conditions of Mo-deprivation, molybdate was added at 3 h (indicated by the arrow) and at intervals samples were removed and pulse-labelled to assess the effect of molybdate on nitrogenase synthesis: ●, Kp1; ■, Kp2.

Effect of molybdate on the rate of expression of the nif structural genes

The expression of operons in the *nif* gene cluster can be monitored from the levels of β -galactosidase activity in various *nif-lac* gene fusions (Dixon *et al.*, 1980); during derepression the *initial* rate of expression of the *nif* structural genes was the same in the presence or absence of molybdate, but decreased after 3.5 to 4 h in the absence of molybdate.

The β -galactosidase activities were measured before and after the addition of molybdate to a merodiploid *nif⁺/nifH-lac* fusion strain UNF619(pMF183), which showed normal derepression kinetics for nitrogenase activity in the presence of molybdate. After 4 h derepression of suspensions under Mo-deficient and Mo-sufficient conditions, molybdate was added to the Mo-deprived cells. In the 1 h following molybdate addition the specific activity of β -galactosidase in the Mo-deprived cells increased from 1223 to only 1541 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$, compared with an increase from 1451 to 3733 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ in the Mo-sufficient cells.

Substantial differences were also observed in the rates of synthesis of nitrogenase polypeptides in suspensions derepressed in the presence and absence of molybdate;

tungstate (100 μ M) did not stimulate expression of *nif* genes (Fig. 3). There was no burst in the rate of synthesis when molybdate was added to Mo-deprived cultures (Fig. 4). No significant increase in the rate of expression occurred until 60 min after the addition of molybdate, during which time the specific activity of nitrogenase had increased at least six-fold (Fig. 2). These data indicate that the rapid effect of molybdate is not due to a rapid stimulation of expression of the *nif* structural genes.

Effect of inhibitors of protein synthesis on reactivation by added molybdate

Both chloramphenicol and tetracycline produced a 95% inhibition of protein synthesis within 30 s of addition to cultures, and neither stimulated nor inhibited the activity of pre-formed nitrogenase. Chloramphenicol, added 10 min before molybdate, usually completely prevented the stimulation of nitrogenase activity, although approximately 5% stimulation by molybdate was observed in some experiments. However, in the presence of tetracycline which inhibited protein synthesis equally effectively, a significant restoration of activity was observed (Fig. 2). Therefore, *de novo* protein synthesis was not required for rapid activation by molybdate and the process must involve activation of Kp1 apoprotein synthesized during Mo-deprivation.

Figure 2 also shows that the degree of activation increased with the duration of previous derepression under Mo-deprivation.

Stability of nitrogenase polypeptides in Mo-deprived organisms

The inability of cultures deprived of Mo for 16 h to respond rapidly to added molybdate, the very low levels of antigenic cross-reacting material, and the absence of nitrogenase polypeptides in extracts of such organisms on SDS gel electrophoresis, could be accounted for if the nitrogenase components synthesized early in the derepression period had been degraded. The stability of nitrogenase polypeptides under Mo-deprivation was therefore investigated by labelling derepressing cultures with 14 C-labelled amino acids for 10 min, inhibiting further protein synthesis by the addition of tetracycline or chloramphenicol, and monitoring the fate of labelled proteins by SDS gel electrophoresis and subsequent autoradiography.

When chloramphenicol was added after 4 h derepression, the bands corresponding to Kp1 polypeptides decreased in intensity with time. Degradation of Kp1 polypeptides occurred more rapidly in the absence of molybdate, and after 18 h they were barely detectable in extracts of Mo-deprived cultures. In contrast, Kp2 polypeptide was only slowly degraded in the presence or absence of molybdate. When tetracycline was used in place of chloramphenicol, the bands corresponding to the Kp1 and Kp2 polypeptides decreased in intensity during the 18 h following synthesis, although the extent of degradation was 2- to 3-fold lower than when chloramphenicol was used to inhibit protein synthesis.

DISCUSSION

In *K. pneumoniae* there are 17 genes in the *nif* cluster, the *nifB*, *nifN* and *nifE* gene products being involved in molybdenum processing (Roberts *et al.*, 1978). Studies using *nif-lac* gene fusions (Dixon *et al.*, 1980) showed that the levels of expression of *nifB*, *nifN* and *nifE* are independent of added molybdate but that the nitrogenase operon *nifHDKY* is subject to autoregulation: maximum rates of expression require both molybdate and one or more polypeptides specified by that operon, although nitrogenase activity *per se* is not required. The requirement for molybdate for maximum rates of synthesis of nitrogenase polypeptides is further substantiated by our pulse-labelling data which show that after 3 h derepression synthesis is stimulated approximately two-fold by the addition of molybdate (Fig. 4).

We have confirmed the observations of Brill *et al.* (1974) that only low levels of antigenic cross-reacting material to both nitrogenase components are found in *K. pneumoniae* after long derepression times and that, unlike *A. vinelandii* (Nagatani & Brill, 1974), tungstate does not elevate levels of the MoFe protein. Since only very low levels of nitrogenase polypeptides could be detected by SDS gel electrophoresis, these data indicate that Kp2 and Kp1 apoprotein were not present at significant levels after prolonged Mo-deprivation, consistent with the failure of such cultures to respond rapidly to added molybdate.

The addition of molybdate to Mo-deprived cultures after short periods of derepression resulted in a biphasic restoration of nitrogenase activity. Because transcription and translation of the structural genes were not stimulated rapidly, we attribute the rapid tetracycline-insensitive reactivation to activation of demolybdo-Kp1 protein, and the slower tetracycline-sensitive reactivation to the incorporation of added molybdate into newly synthesized Kp1 protein. Since the degree of tetracycline-insensitive reactivation increased with time in early derepression, and amounted to 60% of that of cultures derepressed in the presence of molybdate, the low levels of antigenic cross-reacting material detected (see Table 1) are surprising. We have no satisfactory explanation of this phenomenon. The low levels are unlikely to be due to weak reaction of antibody with demolybdo-Kp1 protein for several reasons. Firstly, Kp1 purified from a *nifB* mutant of *K. pneumoniae* shows a strong antigenic reaction of identity with Kp1 antibody (T. Hawkes, personal communication); secondly, no change in the levels of cross-reacting material to Kp1 protein was observed in the 45 min following the addition of molybdate, during which time reactivation was complete; thirdly, low levels of cross-reacting material to both Kp1 and Kp2 proteins were observed under conditions of Mo-deprivation.

In suspensions supplied with molybdate throughout derepression, nitrogenase activities *in vivo* were comparable with the intrinsic activities measured in extracts (Table 1). However, the activity of Kp1 protein was only 37% of its maximum potential activity when assayed with an optimum excess of Kp2 protein (see Eady *et al.*, 1972) indicating that levels of active Kp2 limit nitrogenase activity in these organisms. In contrast, under Mo-deprivation, Kp1 apoprotein levels decreased rapidly relative to Kp2 hence the ratio of Kp2:Kp1 increased and this may have caused the marked increase in nitrogenase activity when Kp1 was reactivated.

The factors which regulate intracellular protein degradation in the *Enterobacteriaceae* are complex (see Goldberg & St John, 1976). *Escherichia coli* has two degradative systems, one which protects the cell from the accumulation of abnormal proteins, and a second inducible system which is activated on deprivation of a required nutrient (e.g. NH_4^+) and whose activity is correlated with the intracellular levels of guanosine 3'-diphosphate 5'-diphosphate (ppGpp).

The apparent stability of Kp2 polypeptide under conditions of Mo-deprivation, when further synthesis was prevented by the addition of chloramphenicol or tetracycline after 4 h derepression, conflicts with the serological (Table 1) and SDS gel electrophoresis data which indicate that Kp2 cannot be detected after prolonged Mo-deprivation in the absence of inhibitors of protein synthesis. Since Kp2 polypeptide is unstable in the absence of Kp1 (Roberts & Brill, 1980), and Kp1 polypeptides are degraded under prolonged Mo-deprivation, it seems that chloramphenicol and tetracycline inhibit the activation of, or prevent the synthesis of, a protease involved in degradation of Kp2 under these conditions. In contrast, the data for Kp1 are consistent with the apoprotein being degraded under conditions of Mo-deprivation, even in the presence of inhibitors of *de novo* protein synthesis, presumably because of an altered conformation which renders it susceptible to a different (constitutive) protease involved in degrading proteins with abnormal conformations (see Goldberg & St John, 1976). The greater stability of Kp1 polypeptides under both Mo-sufficient and Mo-deprived conditions when tetracycline was used in place of chloramphenicol to inhibit further protein synthesis may be a consequence of the rapid inhibition of ppGpp synthesis

(and hence decreased proteolytic activity) by tetracycline (see Goldberg & St John, 1976). It has been shown that during NH_4^+ -starvation of *K. pneumoniae*, such as is required for the derepression of nitrogenase, the levels of ppGpp are elevated (Kleiner & Phillips, 1981; D. Kahn, M. Hawkins & R. Eady, unpublished).

Differences in the stability of the nitrogenase peptides under various conditions may also account for detection of Kp1 and Kp2 polypeptides in a *narD* mutant of *E. coli* carrying *nif* genes (Kennedy & Postgate, 1977). In *K. pneumoniae*, as in both *Plectonema boryanum* (Nagatani & Haselkorn, 1978) and *Anabaena cylindrica* (Hallenbeck & Benemann, 1980), synthesis of protein *de novo* was not required for the restoration of activity by added Mo. However, in the latter organisms, unlike *K. pneumoniae*, restoration of activity occurred in the presence of chloramphenicol. The apparent requirement for protein synthesis for the restoration of activity by molybdate added to *narD* mutants of *E. coli* carrying *K. pneumoniae nif* genes (Kennedy & Postgate, 1977) might well be a consequence of the use of chloramphenicol as an inhibitor of protein synthesis, since we have shown that chloramphenicol, but not tetracycline, inhibits the restoration in *K. pneumoniae*. Since chloramphenicol did not inhibit acetylene reduction in control cultures, it may exert a specific effect on molybdate uptake or processing, which makes it an unsuitable inhibitor for studies of this type in *K. pneumoniae*.

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