A Chemostat Study of the Effect of Fixed Nitrogen Sources on Nitrogen Fixation, Membranes and Free Amino Acids in *Azotobacter chroococcum*

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(Received 15 May 1972)

SUMMARY

Increasing concentrations of ammonium ions in the medium of nitrogen-fixing, sulphate-limited continuous cultures of *Azotobacter chroococcum* caused a proportionate repression of nitrogenase activity; free NH_4^+ could be detected in the extracellular culture fluid only when nitrogenase activity was wholly repressed. The NH_4^+ concentrations giving 50% or 100% repression were proportional to the population density. Nitrate ions repressed with similar stoichiometry; glutamate, glutamine and aspartate did not repress and were not metabolized; repressed and derepressed populations contained equal amounts and proportions of glutamate-forming enzymes. Repressed populations lacked both enzymatic components of nitrogenase. The intracellular free amino acid pools were typical of Gram-negative bacteria; an increase in the degree of repression was associated with an increase in the pool levels of ammonia, aspartate and glutamate. Nitrogen-fixing populations possessed a convoluted intracytoplasmic membrane system which was absent from ammonia-assimilating organisms, but the phospholipid contents of the two types of population were similar. All members of a half-repressed population possessed these membranes, but to a lesser extent than fully derepressed populations.

When N_2 -fixing chemostat populations were abruptly exposed to repressive concentrations of ammonium succinate, repression occurred exponentially and nitrogenase activity disappeared from the culture faster than wash-out of stable enzyme. Repression was not alleviated by exogenous cyclic AMP. Derepression was complete, according to the acetylene test, within half a doubling time of disappearance of free ammonium ions from the culture.

INTRODUCTION

The fact that sources of fixed nitrogen, such as ammonia, nitrate or urea, repress synthesis of nitrogenase by nitrogen-fixing bacteria has been known for many years (see Wilson, 1958). *Klebsiella pneumoniae* (Goerz & Pengra, 1961; Yoch & Pengra, 1966; Mahl & Wilson, 1967) showed a diauxic growth pattern on transfer from ammonia-assimilating to nitrogen-fixing conditions and a decreased lag in nitrogenase synthesis when amino acids were supplied; there was evidence that ammonia exerted its effect on synthesis rather than on activity of the enzyme. Parejko & Wilson (1970) showed that *K. pneumoniae* synthesized nitrogenase *in vacuo*, thus providing strong circumstantial (but not conclusive) evidence that nitrogenase synthesis was a derepression and not an induction phenomenon, a conclusion also indicated by chemostat studies in which ammonia-limited populations of *Clostridium pasteurianum*

(Daesch & Mortenson, 1968) and *Azotobacter chroococcum* (Dalton & Postgate, 1969*b*) synthesized nitrogenase under helium or argon. Nitrogenase synthesis under an inert gas has also been shown with *Rhodospirillum rubrum* (Munson & Burris, 1969) and the blue-green algae *Anabena cylindrica* (Smith & Evans, 1970; Neilson, Rippka & Kunisawa, 1971) and *Anabena flos-aquae* (Bone, 1971).

Strandberg & Wilson (1968) observed diauxic growth on derepression in *Azotobacter vinelandii*; nitrogenase activity appeared about 45 min after exhaustion of ammonia. Sorger (1968) claimed that 2-methylalanine or methylamine acted as co-repressors in this species but St John & Brill (1972) showed that this was a growth inhibition effect specific to glucose media. Shah, Davis & Brill (1972) studied repression and derepression of nitrogenase in *A. vinelandii* and showed that both components of the enzyme were repressed co-ordinately; electron-paramagnetic-resonance signals attributable to the iron-molybdenum protein appeared or disappeared according to the state of repression (Davis, Shah, Brill & Orme-Johnson, 1972). Hardy, Holsten, Jackson & Burns (1968) mentioned that, besides repressing synthesis, ammonium ions had an immediate inhibitory effect on nitrogenase activity in whole organisms which did not occur with extracts. Oppenheim & Marcus (1970*a*) reported that the elaborate cytoplasmic membrane network of *A. vinelandii* (Pangborn, Marr & Robrish, 1962) was absent from presumptive repressed populations: those grown with nitrate or ammonia.

Chemostat culture has certain advantages for studying control of nitrogenase syntheses: nutritional status, which can lead to considerable differences in relevant assimilating enzymes, can be controlled; so can growth rate, and this is important since $\mu_{\max}$ is usually different as between ammonia-assimilating and nitrogen-fixing cultures of the same bacteria. Steady states may be obtained with partially repressed populations and population density may be controlled independently. We report here a study, making use of this technique, of the regulation of nitrogenase activity in *Azotobacter chroococcum*.

METHODS

Organism and culture. *Azotobacter chroococcum* (NCIB8003) was grown in continuous culture (Baker, 1968) at 30 ± 0.5 °C and pH 6.8 to 7.2 as described by Drozd & Postgate (1970); automatic pH control was used with cultures assimilating NH_4Cl ; oxygen tension was monitored with a Clark type oxygen electrode.

A medium allowing growth limitation in the nitrogen-assimilating pathway was inappropriate for the present study. Carbon and phosphate limitation would involve complication due to hypersensitivity of the population to oxygen inhibition (Dalton & Postgate, 1969*a*) so sulphate limitation was chosen for chemostat culture because such populations were not hypersensitive. The mannitol medium of Drozd & Postgate (1970) was prepared but without sulphate and all trace elements as their chlorides; Na_2SO_4 was then added to 0.05 mM. Media with sucrose as carbon source or with different sulphate concentrations were used occasionally where mentioned. KNO_3 , NH_4Cl or diammonium succinate solutions were sterilized independently and added aseptically. Automatic pH control was not necessary when ammonium succinate was used in place of NH_4Cl . The atmosphere was either air or mixtures of $\text{N}_2 + \text{O}_2$ from cylinders, flowing at about 200 ml/min. The oxygen content of the atmosphere, which ranged from 0.1 to 0.3 atm according to the population density, was adjusted to avoid both oxygen limitation and oxygen toxicity by sustaining 5 to 15 μM dissolved O_2 . The criteria of nutrient limitation were those of Hill, Drozd & Postgate (1972).

Acetylene reduction was measured in culture samples and bacterial extracts as described by Drozd & Postgate (1970). Measurements were made at five different pO_2 values to obtain the maximum rate of acetylene reduction for each sample. No additional sulphate was needed with samples from the sulphate-limited chemostat cultures provided the assay was completed in 1 h.

Bacterial extracts. Sonic disruption was used as described by Drozd & Postgate (1970). Osmotic lysis was based on the procedure of Oppenheim & Marcus (1970): the centrifuged culture sample was resuspended in 3 to 4 ml 4M-glycerol which had been sparged with N_2 . After 1 h at room temperature (18 to 20 °C) the suspension was centrifuged at 12000 g (10 min, 5 °C) and the supernatant liquid discarded. The pellet was 'loosened' under a stream of N_2 on a mechanical agitator and sonicated with 4 to 8 ml of N_2 -sparged 0.025 M-tris-HCl buffer (pH 7.4), sometimes containing 0.09 M-MgCl₂ + 0.2 mg dithiothreitol/ml, with a small crystal of DNase to prevent the extract becoming viscous. Sedimentation at 38000 g for 20 min gave a nitrogenase-containing supernatant fluid and a membranous pellet.

Chromatography. Anaerobic column chromatography of the extracts at 5 °C on DEAE-cellulose 32 was based on the method of Kelly, Klucas & Burris (1967). Elution was with 0.15 M-NaCl, 0.26 M-NaCl and 0.09 M-MgCl₂ respectively, all in 0.025 M-tris-HCl buffer (pH 7.4).

Electron microscopy. Membrane preparations were fixed by the method of Kellenberger, Ryter & Sechaud (1958) with 1 % OsO₄ in veronal acetate buffer (pH 6.0). Fixed preparations were dehydrated in a graded series of mixtures of ethanol and water with a final wash in acetone, and then embedded in Epon 812 by the method of Luft (1961). The blocks were cured for 48 h at 60 °C and then thin sections were cut with glass knives using a LKB Ultratome ultramicrotome and were mounted on copper grids. Sections were stained with saturated uranyl acetate in 50% ethanol for 15 to 30 min and then soaked in lead citrate (Reynolds, 1963). Samples were examined in a A.E.I. 6B electron microscope operating at 60 kV.

Lipid phosphorus analyses were based on the method of Lascelles & Szilagyi (1965). Trichloroacetic acid was added to culture samples containing 5 to 20 mg protein until its concentration reached 5%. After centrifugation, the pellet was resuspended in 10 ml 5% trichloroacetic acid and recentrifuged. The supernatant was completely removed and the pellet homogenized in 5 ml of chloroform + methanol (2:1, v/v). After centrifuging the supernatant was collected and the pellet re-extracted with a further 5 ml of extraction mixture. The combined supernatants were made up to 10 to 15 ml and extracted with aqueous calcium chloride (Folch, Lees & Sloane-Stanley, 1957) to remove non-lipid material. Total phosphorus was determined in 0.5 to 1.0 ml samples of the lipid extract by the method of Bartlett (1959). In one experiment mentioned in the text, total extractable lipid and phospholipid were determined for us.

Amino acid pools. A volume of culture containing approximately 30 mg equivalent dry wt organisms was centrifuged at 3000 g for 3 min. The pellet was resuspended in 5 ml of water and the tube capped and placed in boiling water for 5 min. Further centrifugation at 3000 g for 3 min gave a clear supernatant, samples of which were applied to the column of a Locarte amino acid analyser previously calibrated with a standard solution of mixed amino acids. For estimating NH₃, a small correction was made for NH₃ in the buffers; the interstitial and cell-bound water was taken to be four times the dry wt (Roberts *et al.* 1955).

Analyses and assays. Ammonia was estimated with Nessler's reagent. Respiratory activity was measured directly on culture samples in Warburg manometers (Drozd & Postgate, 1970); protein was estimated as described by those authors, culture samples and membrane

preparations being first digested with N-NaOH for 5 min in boiling water. Nitrite was assayed with α -naphthylamine and nitrate with 1,2,4-phenoldisulphonic acid (Boltz, 1958).

Glutamate dehydrogenase (GDH) and GOGAT [glutamine(amide)-2-oxoglutarate amino-transferase oxido-reductase; glutamate synthetase] (NADP) were assayed as described by Meers, Tempest & Brown (1970).

RESULTS

Sulphate-limited culture of Azotobacter chroococcum. Chemostat cultures grown without ammonium salt and with 2.35 mM-NH₄Cl (sufficient to repress nitrogenase synthesis completely – see below) showed little dependence of biomass on dilution rate above $D = 0.1 \text{ h}^{-1}$ but a marked increase in biomass at lower growth rates; at such growth rates most organisms contained large, refractile granules, probably poly- β -hydroxybutyrate. The critical wash-out point occurred at a faster dilution rate (0.45 h^{-1}) when ammonia was being assimilated than when nitrogen was being fixed (0.33 h^{-1}). Curves illustrating these features, with cultures grown at $p\text{O}_2 = 0.3 \text{ atm}$ and 0.025 mM-Na₂SO₄, were given by Hill *et al.* (1972). The yield coefficients at $D = 0.2 \text{ h}^{-1}$ for such populations were 200 mg dry wt/g-ion SO₄²⁻ when fixing N₂, 242 with NH₃. The difference may partly reflect the known high sulphur content of nitrogenase, which was absent from the population grown with NH₃ (below); the yields were in the range (200 to 240) recorded for *Klebsiella aerogenes* (syn. *Aerobacter aerogenes*) in sulphate-limited chemostat cultures (Postgate & Hunter, 1962).

Membrane and phospholipid content of Azotobacter chroococcum. An internal membrane network specific to nitrogen-fixing *A. chroococcum* analogous to that in *A. vinelandii*, was reported by Hill *et al.* (1972). The network was not satisfactorily visible in thin sections of whole organisms because other structures interfered, but it was clear in osmotically lysed preparations. Hill *et al.* (1972) published electron micrographs, which will not be repeated here, and reported that nitrogen-fixing organisms contained some 70% more phospholipid than ammonia-repressed populations. We have not been able to confirm this report. In one of several further experiments, the phospholipid contents of sextuplicate samples of populations grown at $D = 0.25 \text{ h}^{-1}$, $p\text{O}_2 = 0.2 \text{ atm}$ and Na₂SO₄ = 0.05 mM was 92 ($s = 5.5$) nmol phospholipid P/mg bacterial protein when fully derepressed and 94 ($s = 8.0$) when growing with a fully repressive concentration (6.4 mM-NH₄⁺) of ammonium succinate. However, values ranging from 74 to over 200 have been obtained in our hands and the reasons for this variability, which may well be an analytical artifact, still elude us. Samples of such cultures were kindly analysed by Dr D. Ellwood of the Microbiological Research Establishment, Porton, Wiltshire, who confirmed that the phospholipid contents of wholly repressed and derepressed populations were similar. He also reported that the amounts of total extractable lipids were similar to within 3%.

Steady states with partially repressed populations. Preliminary experiments showed that 0.1% (18.7 mM) NH₄Cl added to the sulphate-limited medium completely repressed nitrogenase synthesis according to the acetylene test. Graded concentrations of NH₄Cl were added to a culture limited with Na₂SO₄ and steady states in which nitrogenase was partially repressed according to the acetylene test were obtained. Data were collected from such populations only after at least four doublings in the steady state. The optimal $p\text{O}_2$ for acetylene reduction was determined for each steady state because this shifted to lower $p\text{O}_2$ values as the steady-state nitrogenase content declined. Automatic pH control corrected for increased acidity in the environment as the shift to ammonia utilization proceeded. Fig. 1 records a more detailed series of experiments following those provisionally reported by Hill *et al.* (1972); it shows (i) that free NH₃ only became detectable in the medium when

Fig. 1. Effect of increasing concentrations of NH_4Cl in the medium inflow of a SO_4^{2-} -limited chemostat culture growing in $\text{N}_2 + 0.3 \text{ atm O}_2$ (50 mM- Na_2SO_4 , $D = 0.2 \text{ h}^{-1}$) of *Azotobacter chroococcum* on the steady-state parameters. $\circ$, Bacterial concentration; $\bullet$, nitrogenase activity of the culture; $\blacksquare$, concentration of NH_4Cl in the culture supernatant.

Table 1. Minimum repressive concentration of NH_4Cl for sulphate-limited chemostat cultures of *Azotobacter chroococcum*

Organisms grown in mannitol medium at 30°C under $\text{N}_2 + 0.3 \text{ atm O}_2$, limited by $0.05 \text{ mM-Na}_2\text{SO}_4$.

Dilution rate (h^{-1})	NH_4Cl (mM) required for suppression of nitrogenase activity	Biomass (mg dry wt/ml)
0.09	3.05	0.52
0.20	2.67	0.40
0.29	2.48	0.37

nitrogenase synthesis was wholly repressed; (ii) that, contrary to our earlier impression, biomass did increase measurably over the transition from nitrogen fixation to ammonia assimilation, but that the increase was very slight and entirely consistent with the increased yield of ammonia-assimilating organisms mentioned earlier.

Electron microscopy of thin sections of osmotically lysed bacteria from a half-repressed population showed that all had a moderate amount of internal membrane structure. Hence the population was uniformly half repressed, not a mixture of fully repressed and fully derepressed organisms.

Effect of dilution rate. Minimum concentrations of NH_4Cl required for complete repression of nitrogenase at various dilution rates are given in Table 1. Biomass increased about 40% while the minimum repressive ammonia concentrations increased by about 20% with decreasing growth rate over the range tested; the latter increase was paralleled by the N-content of the culture.

Effect of population density. Preliminary experiments suggesting that the concentrations of ammonia required to repress fully or to half-repress nitrogenase activity depended on population density were mentioned by Hill *et al.* (1972). Chemostats were set up at a fixed dilution rate with different sulphate concentrations so as to obtain different population densities; the $p\text{O}_2$ of the atmosphere was adjusted to avoid oxygen inhibition at low sulphate concentration (a $p\text{O}_2$ of 0.1 atm was used at the lowest population densities). Fig. 2 shows that the concentration of NH_4Cl required to half-repress populations and the minimum concentration needed to repress fully were proportional to population density.

Fig. 2. The effect of the bacterial population density (controlled by the concentration of Na_2SO_4 in the inflowing medium) in SO_4^{2-} -limited chemostat culture, $D = 0.2 \text{ h}^{-1}$, of *Azotobacter chroococcum* on the concentration of repressor required to 100 % repress (open symbols) or 50 % repress (closed symbols) the nitrogenase activity of the culture. $\circ, \bullet$, NH_4Cl ; $\square, \blacksquare$, KNO_3 .

Table 2. Influence of the degree of repression of nitrogenase synthesis by KNO_3 or NH_4Cl on the free ammonia and amino acid pool contents of chemostat cultures, $D = 0.2 \text{ h}^{-1}$, of *Azotobacter chroococcum* limited by $0.05 \text{ mM-Na}_2\text{SO}_4$ under $\text{N}_2 + 0.3 \text{ atm O}_2$

For details see Methods. Concentrations (mM) of amino acids in extracts assessed as peak areas of traces from the automatic amino acid analyser. Water content assumed to be four times the bacterial dry weight. Iso-leucine, leucine, methionine, proline, phenylalanine and tyrosine were not detected (less than 0.11 mM).

Pool material	Repressor					
	None	NH ₄ Cl			KNO ₃	
		Degree of repression				
		0	50 %	100 %		50 %
NH ₃	2.0	3.6	5.0	3.8	5.7	
Glutamate	1.2	3.6	4.6	3.9	7.3	
Aspartate	0.23	0.48	1.0	1.2	3.2	
Alanine	0.84	0.81	0.54	0.60	1.0	
Arginine	0.21	0.22	0.11	0.18	0.22	
Glycine	0.21	0.27	0.16	0.38	0.38	
Histidine	0.49	0.52	0.36	0.80	1.4	
Lysine	0.28	0.23	0.18	0.30	0.50	
Serine	0.21	0.35	0.60	0.38	0.80	
Threonine	—	—	0.22	—	1.6	
Valine	0.35	0.32	0.40	0.23	0.27	

— = not detected (less than 0.11 mM).

Potassium nitrate as repressor. Comparable experiments were performed with KNO_3 in place of NH_4Cl using a chemostat population growing at 0.2 h^{-1} limited by $0.05 \text{ mM-Na}_2\text{SO}_4$ (0.31 to $0.32 \text{ mg dry wt/ml}$). The effect of repressor concentration on degree of repression was similar to that observed with NH_4Cl ; free KNO_3 was only detected in the culture fluid when the population was fully repressed and the concentrations of N/ml required for complete repression were similar: $37 \pm 1 \mu\text{g N/ml}$. The N -content of the completely repressed culture was also $37 \pm 1 \mu\text{g N/ml}$.

Comparable experiments on the effect of population density are included in Fig. 2: as with NH_4Cl , the repressive effect of KNO_3 depended on population density.

Nitrogenase in extracts. The contents of the effluent vessels from several completely repressed, NH_4Cl -grown, SO_4^{2-} -limited cultures were harvested, and extracts prepared by sonic disruption. The resulting crude extract was devoid of acetylene-reducing activity. It was chromatographed on DEAE-cellulose 32 and fractions were assayed for acetylene-reducing activity alone, in combination and with or without different amounts of substantially purified *Azotobacter chroococcum* nitrogenase proteins 1 or 2 (Kelly, 1969). In no case was activity, or enhancement of the low endogenous activity of the protein 1 or 2 preparations, obtained. Thus completely NH_4^+ -repressed populations were devoid of both nitrogenase proteins.

Amino acid pools. Table 2 shows the free intracellular amino acid and NH_3 pool-levels in populations limited by $0.05 \text{ mM-Na}_2\text{SO}_4$ grown at $D = 0.2 \text{ h}^{-1}$, in which nitrogenase synthesis was unrepressed, half and fully repressed with NH_4Cl or KNO_3 . As the degree of repression with either repressor increased the major changes were increases in the levels of NH_3 , glutamate, aspartate and serine. Glutamine appeared as a small shoulder on the serine peak and could not be measured accurately; however, the shoulder size was small and independent of the degree of repression. Threonine was only detected in fully repressed populations. Casamino acids (Difco), aspartate, glutamate or glutamine at 1 g/l had no effect on nitrogenase synthesis nor on biomass when added to the medium reservoir. Replacement of the N_2 in the gas phase by A caused the cultures to wash out, which indicated that none of the compounds tested would serve as a nitrogen source at $D = 0.2 \text{ h}^{-1}$. Tests with batch cultures under $\text{A} + 0.2 \text{ atm O}_2$ confirmed that they did not serve as nitrogen sources for growth.

Ammonia-assimilating enzymes. GDH and GOGAT were present in almost equal amounts in nitrogen-fixing populations. Growth at the same dilution rate in ammonia- or nitrate-repressed conditions made no substantial difference to the ratio or amount of these enzymes (Table 3).

Table 3. *Contents of GDH and GOGAT in Azotobacter chroococcum growing at $D = 0.2 \text{ h}^{-1}$ in chemostats limited by $0.05 \text{ mM-Na}_2\text{SO}_4$ under $\text{N}_2 + 0.3 \text{ atm O}_2$*

Units: nmol NADPH oxidized/mg protein/min.

Added N-source (mM)	GDH	GOGAT
None	40.7	37.6
NH_4Cl (3.7)	30.8	28.0
KNO_3 (2.1)	36.4	32.4

Mono- and trimethylamine and 2-methylalanine as repressors. Sorger (1968) reported that monomethylamine and 2-methylalanine acted as gratuitous co-repressors of nitrogenase synthesis in *Azotobacter vinelandii* grown with glucose. Either of these compounds, as well as trimethylamine, at 1 g/l increased the lag phase of N_2 -fixing, sucrose- or mannitol-grown batch cultures of *A. chroococcum*, *A. vinelandii* (NCIB 8660) and *Azomonas macrocytogenes* (NCIB 8700) in air, compared with NH_4^+ -assimilating cultures. But samples from exponentially growing cultures showed strong acetylene-reducing activity. None of the test compounds, at 1 g/l , caused a decrease in population density or acetylene-reducing activity in a continuous culture of *A. chroococcum* ($D = 0.2 \text{ h}^{-1}$) growing under $\text{N}_2 + 0.3 \text{ atm O}_2$ and limited by $0.05 \text{ mM-Na}_2\text{SO}_4$. The populations washed out when A replaced N_2 in the gas phase, so these compounds, like the amino acids, were not utilized.

Kinetics of NH_4^+ repression. Diammonium succinate was added to the culture vessel of a

Fig. 3. Kinetics of repression of nitrogenase activity in a chemostat culture of *Azotobacter chroococcum*. Diammonium succinate (9.3 mM-NH_4^+) added simultaneously to influent medium and culture vessel of an SO_4^{2-} -limited nitrogen-fixing chemostat culture ($50 \text{ mM-Na}_2\text{SO}_4$, $D = 0.25 \text{ h}^{-1}$) growing in air. Free NH_4 declined from 9.3 to 5.4 mM during the experiment; bacterial protein/ml culture increased from 0.17 to 0.21 mg/ml .

Fig. 4. Kinetics of derepression of nitrogenase activity in a chemostat culture of *Azotobacter chroococcum*. The medium supply to a SO_4^{2-} -limited culture ($50 \text{ mM-Na}_2\text{SO}_4$) growing in air at $D = 0.25 \text{ h}^{-1}$ with 0.93 mM-NH_4^+ as ammonium succinate was abruptly changed to one free of ammonium salts. ○, Rate of acetylene reduction; ●, free ammonium ions in culture.

N₂-fixing chemostat culture limited by Na₂SO₄, and the medium input at once changed to one introducing a medium containing a similar ammonium concentration. Nitrogenase activity declined logarithmically at a rate faster than predicted if the enzyme simply diluted out (Fig. 3): the half life of the enzyme in Fig. 3 was 72 min compared with a replacement time for the culture of 240 min. Shah *et al.* (1972) reported logarithmic decline of nitrogenase activity on repression of *Azotobacter vinelandii*, preceded by a lag. In our chemostat experiments no lag in repression occurred, provided transition to the NH₄-containing medium was abrupt (no residual N-free medium in the medium line) and provided the pH value remained constant. If NH₄Cl was used and pH control was omitted, repression appeared to accelerate after a lag as the pH declined. Ammonium ions also caused an immediate decline of some 30% in nitrogenase activity as reported by Hardy *et al.* (1968) even at subrepressive concentrations (0.37 to 0.55 μ M-NH₄⁺); KNO₃ had no such effect. In a single experiment, exogenous 3'5'-cyclic AMP (5 mM) did not prevent ammonia repression; the enzyme activity declined with a half life of 45 min compared with 60 min when tested four generations later without cyclic AMP.

Kinetics of derepression. Fig. 4 shows the rate of resynthesis of nitrogenase following replacement of a medium input containing repressive concentrations of ammonium succinate by one free of available combined nitrogen. Resynthesis started as soon as free ammonium ions could not be detected, proceeded at a linear rate and was complete in 120 min: about 75% of the doubling time of the population (165 min) at the dilution rate used.

DISCUSSION

The characteristics of *Azotobacter chroococcum* in sulphate-limited chemostat culture, the relation of biomass to dilution rate and lack of hypersensitivity to oxygen inhibition, were commented on by Hill *et al.* (1972) and their discussion will not be amplified here.

Regulation of nitrogenase synthesis. In continuous culture the rule seems to be that when sufficient repressor (ammonium or nitrate ions) is added to satisfy the total nitrogen requirements of the population, then nitrogenase synthesis is fully repressed; so the concentration of repressor in the influent medium required for complete repression depends on population density. In SO₄²⁻-limited culture, population density and, therefore, critical repressor concentration is independent of dilution rate. In many natural micro-environments the population densities of azotobacters are low and it may be that the addition of large amounts of NH₄⁺ and NO₃⁻ fertilizers, by repressing nitrogenase synthesis, will deny azotobacters their principal ecological advantage and their numbers might then decline to a very low level. Macura (1966), in soil perfusion experiments, noted such a decrease in number of azotobacters on adding ammonium salts to a glucose medium otherwise free of fixed nitrogen. The kinetics of synthesis and repression of nitrogenase – its appearance within less than one doubling time and disappearance faster than wash-out – indicates that it is subject to relatively rapid control and turnover in the living organism. We obtained no evidence for an effect of cyclic AMP analogous to its effect on catabolite repression, but it may not have penetrated the organisms. The correlation between content of the internal membrane network described by Oppenheim & Marcus (1970a) and nitrogenase content extended to partially repressed populations in steady states, in which all members had a partial complement of membranes; we have drawn attention elsewhere to the fact that this information could only have been obtained by chemostat techniques (Hill *et al.* 1972). In contrast to the preliminary reports of Oppenheim & Marcus (1970b) and ourselves, we could obtain no consistent reflexion of the membrane contents of organisms in their phospholipid contents,

when carefully compared at similar population densities and growth rates. We are obliged to attribute our earlier findings to analytical error. The precise function of the membranes remains obscure.

The immediate inhibitory effect of ammonium ions on nitrogenase activity of derepressed populations is unconnected with regulation of enzyme synthesis since it did not occur with nitrate; it was not observed with cell-free nitrogenase preparations from *Azotobacter chroococcum* (Dr M. Kelly, unpublished) nor from *A. vinelandii* (see Introduction).

Assimilation of fixed N. The free amino acid pools are quantitatively typical of those from Gram-negative bacteria (Tempest, Meers & Brown, 1970) with glutamate and NH_3 , the predominant constituents, normally present in approximately equimolar concentrations. The free ammonia pool increased only during repression of nitrogenase synthesis. Yet there is likely to be an appreciable concentration of free repressor in N_2 -fixing organisms because extreme derepression of nitrogenase synthesis can be observed in ammonia-limited populations grown under argon (Dalton & Postgate, 1969*b*); Gorini & Maas (1957) associated high levels of production of repressible enzymes with very low intracellular concentrations of repressor. Either aspartate or glutamate could be the true repressor because the pool levels of these increased several-fold (Table 2) respectively. Since they do not enter the organism, they would be ineffective as repressors, or as nitrogen sources, when added to media for growth tests. Parejko & Wilson (1970) concluded that, of the common organic nitrogen compounds (except urea), only glutamine had any repressive effect on nitrogenase synthesis in *Klebsiella pneumoniae*. Yoch & Pengra (1966) measured the free amino acid pools of *K. pneumoniae* in batch cultures (i) when the organisms had just exhausted a limiting NH_4^+ -supply, and (ii) when nitrogenase had just been synthesized. At (i) aspartate, glutamate, and ammonia were in the range 3 to 5 mM; at (ii) the only major difference was that aspartate had dropped to near zero; aspartate, however, had no repressive effect on nitrogenase synthesis even though it was assimilated by the organisms. Like St John & Brill (1972), we obtained no evidence for repression by Sorger's (1968) ammonia analogues.

Nagatani, Shimazu & Valentine (1971) suggested that nitrogen fixation and ammonia consumption of *Klebsiella pneumoniae* followed different assimilatory pathways characterized by the use of GOGAT and GDH respectively. Their experiments, in which pronounced differences in contents of these enzymes were shown, were performed with batch cultures, with which the parameters of growth rate and nutritional status were undoubtedly different. Meers *et al.* (1970) had earlier shown that the contents of GOGAT and GDH in *K. aerogenes* (syn. *Aerobacter aerogenes*) were very dependent on growth rate and nutritional status and it is likely that the observations of Valentine and his colleagues were simply a reflexion of this fact. With *Azotobacter chroococcum*, examined at strictly comparable growth rates ($D = 0.20 \text{ h}^{-1}$) and nutritional status (SO_4^{2-} -limitation), we observed no significant difference in assimilating enzymes between nitrogen-fixing organisms and those assimilating ammonia or nitrate.

We are grateful to Dr J. Hall, School of Biological Sciences, University of Sussex, for allowing us to use the electron microscope, and to Miss C. David for helping to operate it and to prepare specimens. We also thank Dr M. Wallis and Mr D. Watson of the above Department for operating the amino acid analyser and Dr M. Kelly, lately of our research Unit, for providing specimens of nitrogenase proteins from *Azotobacter chroococcum*.

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