Biochem. J. (1984) **224**, 853–862
Printed in Great Britain

Characteristics of N_2 fixation in Mo-limited batch and continuous cultures of *Azotobacter vinelandii*

Robert R. EADY and Robert L. ROBSON
Agricultural and Food Research Council Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.

(*Received 4 June 1984/Accepted 31 August 1984*)

Steady-state chemostat cultures of *Azotobacter vinelandii* were established in a simple defined medium that had been chemically purified to minimize Mo and that contained no utilizable combined N source. Growth was dependent on N_2 fixation, the limiting nutrient being the Mo contaminating the system. The Mo content of the organisms was at least 100-fold lower than that of Mo-sufficient cultures, and they lacked the characteristic $g = 3.7$ e.p.r. feature of the MoFe-protein of nitrogenase. A characteristic of nitrogenase activity *in vivo* in Mo-limited populations was a disproportionately low activity for acetylene reduction, which was 0.3 to 0.1 of that expected from the rate of N_2 reduction. Acetylene was also a poor substrate in comparison with protons as a substrate for nitrogenase, and did not markedly inhibit H_2 evolution, in contrast with Mo-sufficient populations. In batch cultures in similar medium or 'spent' chemostat medium inoculated with Mo-limited organisms, the addition of Mo elicited a biphasic increased growth response at concentrations as low as 2.5 nM, provided that sufficient Fe was supplied. In this system V did not substitute for Mo, and Mo-deficient cultures ceased growth at a 25-fold lower population density compared with cultures supplemented with Mo. Nitrogenase component proteins could not be unequivocally detected by visual inspection of fractionated crude extracts of Mo-limited organisms. $^{35}SO_4^{2-}$-pulse-labelling studies also showed that the rate of synthesis of the MoFe-protein component of nitrogenase was too low to be quantified. However, the Fe-protein of nitrogenase was apparently synthesized at high rates. The discussion includes an evaluation of the possibility that *A. vinelandii* possesses an Mo-independent N_2-fixation system.

It is generally accepted that Mo is an essential trace metal required for biological N_2 fixation. Early nutritional studies (Bortels, 1930) implicating Mo in diazotrophy have been rationalized by the identification of an essential Mo-containing cofactor (FeMoco) (Shah & Brill, 1977) in one of the component proteins that comprise nitrogenase, the enzyme responsible for biological N_2 fixation. FeMoco is highly likely to be involved in N_2 binding (Hawkes *et al.*, 1984). In addition to its role in catalysis, Mo is involved in regulation of nitrogenase synthesis in a number of organisms (Eady *et al.*, 1982).

However, in the case of *Azotobacter vinelandii* it has been shown that some mutant strains presumed to be defective in nitrogenase structural genes are unable to grow on N_2 (Nif^-) in the presence of Mo, but are capable of growing on N_2 in the absence of added Mo, and it has been proposed that this organism has an alternative system for N_2 fixation that may be Mo-independent (see Bishop *et al.*, 1980, 1982; Riddle *et al.*, 1982). This suggestion also finds support in the many reports (see Esposito & Wilson, 1956; Keeler & Varner, 1957; Nagatani & Brill, 1974) that describe slow N_2-dependent growth of *A. vinelandii* on media to which no Mo was added. In addition, under conditions of Mo-deprivation V stimulated growth of *A. vinelandii*, and it was proposed that a functional nitrogenase was formed in which V replaced Mo (Burns *et al.*, 1971), although this interpretation has been questioned (Benneman *et al.*, 1973).

In order to clarify the role of Mo in N_2 fixation by *Azotobacter* we established N_2-fixing continuous cultures growing on medium chemically

purified to minimize traces of Mo. We report here that, in such cultures, growth limitation can be ascribed to residual Mo, which is required for diazotrophy. A novel pattern of substrate reduction by nitrogenase is exhibited by such Mo-limited steady-state cultures. Spent chemostat medium was used as a system freed microbiologically from Mo to show that Mo was essential for diazotrophy, and that V did not substitute for Mo.

Materials and methods

Maintenance and growth of the organism

Stock cultures of *Azotobacter vinelandii* UW *rif*^{r} were maintained and subcultured at monthly intervals on agar slopes of RM, which contained per litre of distilled water: K_2HPO_4, 0.64g; KH_2PO_4, 0.16g; Na_2SO_4, 0.142g; $MgCl_2,6H_2O$, 0.1g; $CaCl_2,2H_2O$, 3.0mg; Na_2MoO_4, 2.0mg; $FeSO_4,2H_2O$, 5mg; sucrose, 10g; BactoTryptone (Difco), 2g; yeast extract, 1g. Chemostat medium had the following composition (g/l): glucose, 20; KH_2PO_4, 0.8; $MgCl_2,6H_2O$, 0.203; NaCl, 0.2; $CaCl_2,2H_2O$, 0.0074; Na_2SO_4, 0.0028. The phosphate and bulk media were autoclaved separately, mixed when cool, and then a filter-sterilized solution of ferric nitrilotriacetate was added to 30 μM.

Purification of media

Medium was routinely prepared in 20-litre batches, and purified to remove trace Mo contamination as follows. The bulk medium was dissolved in 20 litres of double-glass-distilled water contained in the chemostat reservoir vessel, and the phosphate was dissolved in 0.5 litre of distilled water and treated separately. The medium was adjusted to pH3.5 with 5M-HCl, and 4g of 8-hydroxyquinoline was added. Approx. 500ml of dichloromethane was added, and the mixture was stirred vigorously with a Teflon-coated stirring bar to disperse droplets of dichloromethane throughout the aqueous phase. After 2h stirring, the dichloromethane was allowed to settle and was then siphoned off with a glass tube. More dichloromethane was added, and the extraction procedure was repeated six times. The pH was then adjusted to 7.2 with solid NaOH, and the excess of 8-hydroxyquinoline was removed by repeated extraction with dichloromethane until the medium was colourless. Essentially the same procedure was used to purify the phosphate solution, except that the initial pH adjustment was not necessary and 150ml of dichloromethane was used. The amount of 8-hydroxyquinoline was decreased to 0.5g and after the first extraction an additional 0.5g was added before proceeding. After six extractions the pH was adjusted to pH7.2 with solid NaOH, and the excess of 8-hydroxyquinoline was removed by two extractions with dichloromethane.

The ferric nitrilotriacetate solution was not purified but was prepared as a concentrate in 4ml of double-glass-distilled water.

Chemostat

The chemostat vessel was all-glass with a Teflon-coated magnetic stirring bar (Baker, 1968), and the culture volume was approx. 180ml. Medium was supplied by a peristaltic pump operating on a silicone-rubber tube. The headspace was accessible for sampling through a rubber closure and a sterile 0.4 μm-pore size filter assembly, which allowed the sterile removal or addition of gas samples by syringe; the gas headspace was 475ml, calibrated by injecting a known volume of methane and measuring the dilution by gas chromatography. The gas supply (air unless otherwise stated) to the culture was purified by passage through 0.5M-H_2SO_4, 1M-NaOH and water to trap possible volatile compounds. Cultures were maintained at 30°C.

Cleaning of glassware

Wherever possible sterile plastic pipettes and containers were used for the handling and growth of cultures or media. Glassware, including the chemostat vessel and associated connectors, was cleaned by soaking for 48h in 10% (v/v) Decon containing 50mM-EDTA and exhaustively rinsed with double-glass-distilled water before use.

Measurement of growth, viability, yield and N analyses

Growth of batch cultures was followed turbidimetrically with a Klett–Summerson photoelectronic colorimeter (Klett Mfg. Co., New York, NY, U.S.A.) with a green filter. For routine measurement of turbidity of chemostat cultures an EEL spectrophotometer (EEL Ltd., Halstead, Essex, U.K.) was used. Viable organisms were determined by plating serial dilutions of culture samples on to RM agar. Dilutions were made in PEM, which was 5mM-potassium phosphate buffer, pH7.0, containing 0.1mM-EDTA and 0.5mM-$MgCl_2$ (Robson *et al.*, 1984*a*). Colonies were grown at 30°C. Results are expressed as no. of colony-forming units (c.f.u.). For dry weights, a volume of culture (containing approx. 1mg dry wt.) was removed from the chemostat, and the organisms were collected by filtration on a cellulose nitrate filter (0.45 μm pore size, 4.7cm diam.). The cells were washed on the filter twice with 10ml of 3mM-NaCl. The filter was transferred to a plastic Universal bottle and the cells were removed from the filter by repeated washing of the filter surface with 1ml of 3mM-NaCl. The resuspended cells

were recovered by centrifuging at 200*g* for 10min, and the liquid was quantitatively transferred to an Eppendorf centrifuge tube. Cells were collected by centrifugation, 900μl of the supernatant was removed and the pellet was resuspended in the remaining supernatant before transfer to a tared combustible aluminium boat, which was then dried at 75°C to constant weight. Weighed samples were analysed for total N by combustion at 960°C in a Perkin–Elmer 240b elemental analyser. ^{15}N was determined by mass spectrometry after digestion of the dried samples.

Assay of nitrogenase

Nitrogenase activity of whole cells was assayed *in situ* from either the rate of reduction of added acetylene or from the rate of evolution of H_2 from the culture. The chemostat vessel was clamped off, and the air and medium supply stopped. Acetylene (60ml) and O_2 (16ml) were injected into the gas headspace, and after 1min the pressure was equilibrated by release of the excess gas pressure. At intervals gas samples (0.5ml) were removed by syringe and analysed by gas chromatography for H_2, O_2 and N_2 by using thermal-conductivity detection or by flame ionization detection for acetylene. The time course was usually measured for 30–45min. When longer assay times were used the O_2 content of the gas headspace was maintained by pumping O_2 into the vessel at a rate sufficient to compensate for the O_2-uptake rate of the culture.

Assay of hydrogenase

Hydrogenase activity was measured on samples removed from the chemostat by the tritium-exchange method as described by Partridge & Yates (1982).

Measurement of nitrogenase synthesis

Samples removed from the chemostat were pulse-labelled with $^{35}SO_4^{2-}$ and the radioactivity incorporated into nitrogenase polypeptides was determined essentially as described previously (Robson, 1979; Eady *et al.*, 1978).

Mo analysis

Mo was determined colorimetrically on wet-ashed samples with toluene-3,4-dithiol as described by Clarke & Axley (1955). Recovery of added Mo (10ng) to wet-ashed samples of organisms was 95%.

E.p.r. spectra

Samples were removed from the chemostat and the low-temperature e.p.r. spectra of the organisms were recorded as described by Davis *et al.* (1972).

Results

Continuous culture of A. vinelandii on Mo-deficient medium

A continuous culture of *A. vinelandii* UW *rif*^{r} was established on purified 'N-free' medium containing no added Mo. As a preliminary to studying the involvement of Mo in N_2-fixation, it was necessary to establish that growth under these conditions was dependent on dinitrogen as N source, and not due to fixed N in the medium or the inflowing air. This was demonstrated in two ways.

Firstly, when the inflowing air supplied to a steady state was changed to an argon/O_2 (21:79) mixture the population density decreased with kinetics very close to the theoretical 'wash-out' rate for non-growing organisms (Fig. 1). The displacement of the observed decay curve from the theoretical by approx. 0.4 replacement time may result from the time taken to dilute out the N_2, and some division of N-starved organisms. After 5.5 replacement times, when the experiment was

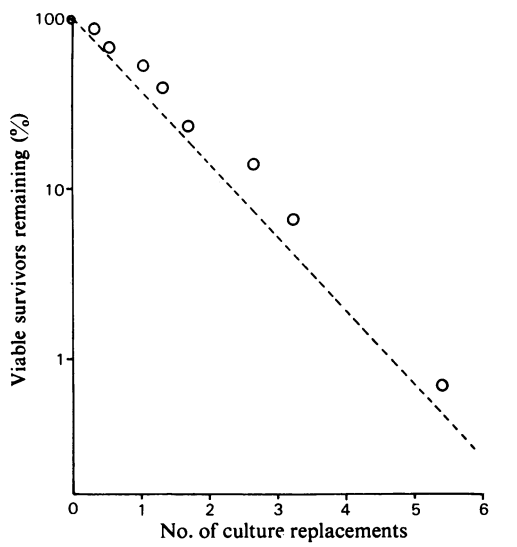

Fig. 1. *Dependence of N_2 as N source for growth of Mo-limited chemostat culture of A. vinelandii*

A steady-state chemostat of *A. vinelandii* ($D = 0.1$) was established under air in chemically purified Mo-deficient medium containing no added utilizable combined N source. At time zero the air passing over the culture was replaced with an argon/O_2 (4:1) mixture and the effect on the population was determined by measuring viable counts expressed as c.f.u. in culture samples withdrawn at timed intervals. The broken line indicates the theoretical dilution of a non-dividing though viable population. ○ indicates viable organisms expressed as percentage of initial population at time zero (3.69×10^7 c.f.u./ml).

terminated, only 0.7% of the initial population remained. The absence of deviation at this point suggests that this residual population was not dividing in the absence of N_2. In the 50h following the N_2-to-argon shift there was no evidence for death of the population occurring.

N_2 fixation was also established by ^{15}N-enrichment experiments *in situ* in the chemostat as described in the Materials and methods section. After 5h incubation under air containing $^{15}N_2$ (10.3% N_2) the N content of the culture increased from 8.9 μg/ml to 11.43 μg/ml. Analysis of the isotopic composition of the cells at the end of the experiment showed a 3.64 atom% ^{15}N excess. These data show that there was reasonable agreement between the calculated rate of N_2 fixation as determined by ^{15}N enrichment and the observed increment in N content.

These data indicate that, when grown on chemically purified Mo-deficient media, chemostat cultures of *A. vinelandii* are able to utilize N_2 as N source.

Evidence for Mo as the limiting nutrient in the purified medium

Two approaches were used to determine the limiting nutrient in purified media. Firstly, organisms were removed from the chemostat and grown as batch cultures with various additives, to determine their effect on growth. Secondly, the effect on the yield of organisms in the chemostat, consequent on supplementation of the chemostat medium with Mo, was measured.

Populations removed from the chemostat grown at a dilution rate (D) of 0.1 h^{-1} continued to grow as batch cultures, but showed linear growth kinetics with a specific growth rate constant (μ) of approx. 0.0714 h^{-1}. Growth was stimulated markedly by added Mo, provided that sufficient Fe was present (Fig. 2). Deficiency of Fe, which occurred with some batches of medium in which the chelating agent nitrilotriacetic acid was not added, was characterized by a yellow-green fluorescent coloration. Such cultures did not respond maximally to added Mo unless additional Fe was supplied, an observation that confirms earlier reports of the synergistic stimulatory effects of Fe and Mo as growth-limiting nutrients for *Azotobacter* (see Rubenchik, 1963).

In later experiments Fe limitation was prevented by adding nitrilotriacetic acid, at a concentration (30 μM) equivalent to that of Fe in the chemostat medium. Batch cultures derived from such steady states showed no yellow-green coloration, and the addition of Mo alone was sufficient to stimulate growth. Mo was effective at very low concentrations (2.5nM) and produced a biphasic response, a small immediate increase in growth

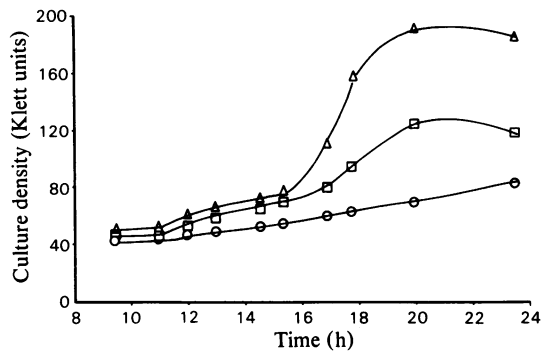

Fig. 2. *Effect of MoO_4^{2-} and Fe^{3+} on diazotrophic growth of A. vinelandii in Mo-deficient medium*
At time zero, 10ml portions of chemically purified Mo-deficient 'chemostat medium' were inoculated to an initial density of 25 Klett units with *A. vinelandii* grown in chemostat culture ($D = 0.1$) in similar medium. Cultures were incubated aerobically at 30°C. At 10h populations had reached similar densities and the following additions were made: △, Na_2MoO_4 and ferric nitrilotriacetate (10nM each); □, Na_2MoO_4 (10nM); ○, no addition. For further details see the text.

rate followed 6h later by a rapid increase (Fig. 3). Fe (30 μM), Zn (7.3 μM), Cu (400nM), Co (500nM), Mn (330nM), Ni (660nM), SO_4^{2-} (500nM), ReO_4^- (3.3nM), BO_3^{3-} (6.6 μM), WO_4^{2-} (6.6 μM) and V_2O_5 (50nM) were without effect on growth. No addition of trace metals was required for good growth with NH_4^+ as N source.

The addition of increasing concentrations of Mo to the chemostat medium in the range 1.25–12.5nM resulted in a number of steady states being obtained that initially showed an increase in dry weight and cell numbers with increasing Mo concentrations (Fig. 4*a*). The yield of organisms in unsupplemented media would be consistent with a residual Mo content of 3.5nM. Whether this represents unextracted Mo or release of bound Mo from the glass reservoir or chemostat vessel is not known.

All these observations indicate that the limiting nutrient in the purified medium was residual Mo. It is clear that *A. vinelandii* must possess an Mo-uptake system of very high affinity that can allow growth on N_2 at Mo contents that are at least 1000-fold lower than that in normal medium.

Evidence for intrinsic N limitation

The rate of N_2 fixation by chemostat cultures in the steady state was determined from the N content of the culture and the dilution rate. Table 1 shows the data for steady-state cultures growing in Mo-deficient medium. As the growth rate was

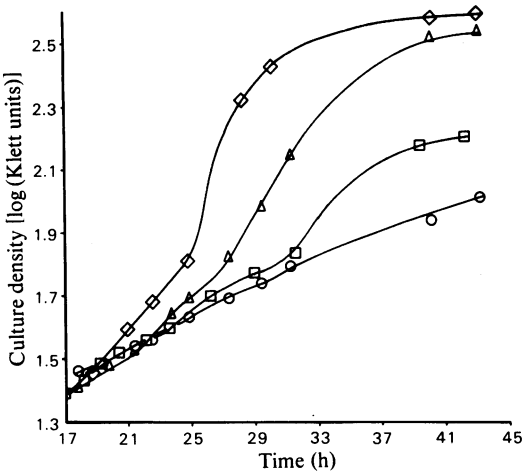

Fig. 3. *Influence of MoO_4^{2-} concentration on diazotrophic growth of A. vinelandii in Mo-deficient 'spent chemostat medium'*

At time zero, 20ml portions of 'spent chemostat medium' supplemented with Na_2SO_4 (to 50μM) were inoculated with *A. vinelandii* that had been grown diazotrophically in chemostat culture under Mo limitation. Cultures were incubated aerobically at 30°C. At 18h Na_2MoO_4 was added to the following concentrations: ○, no addition; □, 5nM; △, 10nM; ◇, 50nM. For further details see the text.

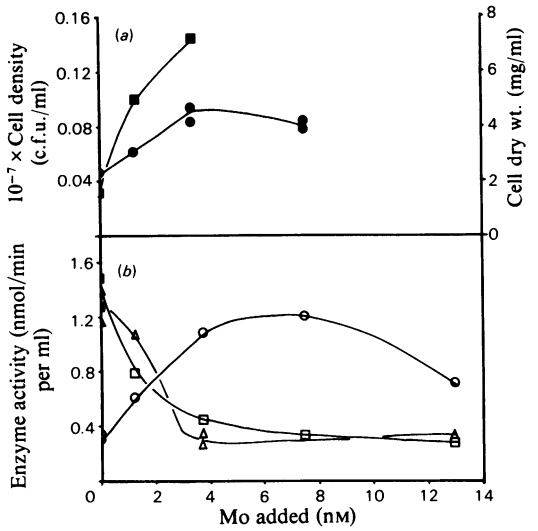

Fig. 4. *Growth and nitrogenase activity in A. vinelandii grown in Mo-limited steady states established at different initial Mo concentrations*

Chemically purified chemostat medium was initially supplemented with Na_2MoO_4 to the concentrations given. Populations of *A. vinelandii* were grown at a dilution rate of 0.1. The effects of increased initial Mo concentration were assessed either by (*a*) determination of growth cell yield measured either as cell dry weight (●) or viable organisms (as c.f.u.) (■), or (*b*) by assaying whole-cell nitrogenase activity *in situ* by determining: acetylene reduction in air containing acetylene (10%, v/v) (○); H_2 evolution under air (△); H_2 evolution in an atmosphere of 10% (v/v) acetylene in air (□). Activities are expressed in terms of unit volume of culture.

increased over the range $D = 0.089$ to 0.205 the rate of N_2 fixation per unit volume of culture remained approximately the same although the population density decreased 7-fold. Since the protein content also decreased 3-fold, the specific activity for N_2 fixation increased with increasing D.

Pattern of substrate reduction by nitrogenase in chemostat cultures

Substantial rates of H_2 evolution were exhibited by these chemostat cultures (Table 1). In one steady state H_2 was evolved at a rate of 0.25nmol/min per ml of culture despite the presence of an uptake hydrogenase activity, measured in samples removed from the chemostat, of 84nmol/min per ml of culture. This uptake activity is surprising, because (i) the medium contained no added Ni, a metal that is essential for activity in *Azotobacter chroococcum*, and (ii) nitrilotriacetic acid was present; it is an inhibitor of uptake hydrogenase in this organism (Partridge & Yates, 1982).

An unusual characteristic of these cultures was the finding that H_2 evolution was not inhibited by acetylene. Also, the rates of acetylene reduction measured *in situ* were low: at most one-third, but in two steady states one-tenth, of the rate of N_2 fixation (Table 1). A comparison of the rates of reduction of acetylene in all Mo-limited steady-state cultures showed that, at 10kPa (0.1atm), acetylene was a relatively poor substrate compared with either H^+ or N_2. The apparent K_m for acetylene determined *in situ* was 3.7kPa (0.037atm), a value that is not significantly different from that of 5kPa (0.05atm) determined for Mo-sufficient chemostat cultures of *A. chroococcum* (Walker, 1979), or the 4kPa (0.04atm) determined for batch cultures of *A. vinelandii* (Davis & Wang, 1980).

These patterns of substrate reduction by nitrogenase are in marked contrast to Mo-sufficient cultures, where the rate of acetylene reduction is relatively greater, and where acetylene effectively competes with H^+ and inhibits H_2 evolution (Table 1).

The data of Fig. 4(*b*) show that, as the Mo concentration in the chemostat medium is increased, the pattern of substrate reduction tends to be that found for Mo-sufficient populations. As the

Table 1. *Experimental parameters and patterns of substrate reduction of nitrogenase in chemostat cultures of A. vinelandii UW, grown under Mo limitation and Mo sufficiency*

The rate of N_2 fixation was calculated from the N content of the organisms and D for different steady states; H^+ is the rate of H_2 evolved under air or H_2 evolution concomitant with acetylene reduction. Total electron flux was calculated by the summation of the specific activities for H_2 and ethylene formation or multiplication of the N_2-fixation rate by 3. All three batches of media contained 30 μM-nitrilotriacetic acid and 30 μM-Fe^{3+}.

	Medium batch	D (h^{-1})	A_{650}	Concn. of cells (no./ml)	Dry wt. (μg/ml)	N content (μg/ml)	Protein content (μg/ml)	Rates of substrate reduction (nmol/min per ml of culture)				Specific activity (nmol/min per mg of protein)				Total electron flow (electron pairs)		Ratio
								N_2	H^+	H^+ under C_2H_2	H^+ under C_2H_2	N_2	H^+	H^+ under C_2H_2	H^+ under C_2H_2	$H_2+C_2H_4$	$N_2\times3$	$N_2\times3/C_2H_4$
Mo-sufficient	A	0.1	52	2.4×10^9	940	112.7	670	6.6	1.5	22.1	1.6	9.85	2.2	33	2.38	35.38	29.55	0.895
Mo-limited	A	0.1	36	5.95×10^8	505	60.9	300	3.6	5.0	1.16	7.3	12	16.6	3.86	24.3	28.16	36	9.32
	B	0.108	32	—	430	57.48	—	3.4	0.32	1.19	2.8	—	—	—	—	—	—	—
	B	0.1	54	—	487	69.21	320	4.1	—	1.78	1.94	12.8	—	5.56	6	11.56	38.4	6.9
	C	0.089	8.1	1.1×10^8	74	9.9	37	0.53	0.56	0.77	2.90	14.3	15.1	20.8	78.3	99.3	42.9	2.06
	C	0.155	3.9	4.6×10^7	39	5.89	20	0.55	—	0.60	2.34	27.5	—	30	117	147	82.5	2.75
	C	0.172	3.0	2.2×10^7	34.4	4.58	17	0.47	—	—	—	—	—	—	—	—	—	—
	C	0.205	1.5	1.5×10^7	23.4	3.213	12	0.39	—	0.32	0.99	32.5	—	26.6	82.5	109.1	97.5	3.66

rate of H_2 evolution decreased the rate of acetylene reduction increased, but the total electron flux to these alternative substrates remained approximately constant. Over this range of Mo concentrations, the N_2-fixation rate increased, and H_2 evolution under air decreased. This may indicate that as the Mo concentration is increased N_2 can compete more effectively with H^+. Supplementation of a Mo-limited chemostat culture having a steady-state population of 5.9×10^8 c.f.u./ml with the normal Mo concentration in media for *Azotobacter* (5 μM) resulted in the attainment of a new steady state with a 4-fold increase in population density (Table 1). The limiting nutrient under these conditions was apparently glucose, since it was not detectable in the culture supernatant. The substrate-reduction pattern characteristic of Mo-limited steady state changed when Mo limitation was removed. The specific activities changed from 24.3 nmol of H_2 and 4 nmol of ethylene produced/min per mg of protein to 3.4 nmol of H_2 and 49.2 nmol of ethylene produced/min per mg of protein. The populations still evolved H_2 at a rate of 2.7 nmol/min per mg of protein under air in the absence of acetylene.

In Mo-limited steady states the rates of acetylene reduction and H_2 evolution became non-linear after 45 min, but the duration of the linear phase varied from batch to batch of medium. In some steady states, when the O_2 concentration of the gas phase was maintained, the rates were linear for up to 7 h, but more usually H_2 evolution ceased after 1 h. Non-linearity was also observed when nitrogenase activity was measured, both by H_2 evolution and acetylene reduction, in samples removed from the chemostat and assayed under different partial pressures of O_2.

Steady-state concentrations and rates of synthesis of nitrogenase components

Concentrations of nitrogenase components in Mo-limited steady states were determined by examining stained polypeptides fractionated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Bands corresponding to the MoFe-protein component of nitrogenase (Av1) were not discernible, but a polypeptide corresponding to the Fe-protein component of nitrogenase (Av2) was seen, although it was present at low concentrations when compared with Mo-sufficient extracts. In order to examine whether these steady-state concentrations were a reflection of the rates of synthesis of components, populations were first pulse-labelled with $^{35}SO_4^{2-}$ before electrophoresis and the gel was autoradiographed. No clear evidence for synthesis of the polypeptides of Av1 was obtained, although a polypeptide corresponding in size to that of Av2 was prominently labelled.

These results are consistent with high rates of Av2 turnover occurring under Mo limitation, and also indicate that synthesis of Av1 and Av2 can be regulated independently under these conditions. An involvement of Mo in regulation of the synthesis of nitrogenase structural polypeptides in *A. vinelandii* is similar to the involvement of Mo in *Klebsiella pneumoniae* and *Clostridium pasteurianum* (see Eady *et al.*, 1982).

Mo content of Mo-limited and Mo-sufficient continuous cultures

Organisms were removed from a Mo-limited continuous culture and analysed for Mo as described in the Materials and methods section. The Mo content found was at the limit of detection but was less than 80pmol of Mo/mg of protein. It was established that the recovery of added Mo to the wet-ashed material was 95%, indicating that the low values obtained were not due to inhibition of the assay procedure for Mo. In agreement with the low Mo content, low-temperature e.p.r. spectroscopy of intact organisms failed to detect the $g = 3.7$ feature attributed to the FeMoco of Av1 (results not presented).

In contrast, organisms from a Mo-sufficient continuous culture contained 7.7nmol of Mo/mg of protein, equivalent to 2.2μmol of Mo in 10^{12} cells, a value that agrees well with those reported by Pienkos & Brill (1981) for *A. vinelandii*. The e.p.r. spectrum of Mo-sufficient organisms showed the characteristic e.p.r. feature of FeMoco.

Batch culture in spent chemostat medium

The ability of *A. vinelandii* to achieve steady states in Mo-limited chemostat culture suggests that the chemically extracted medium still contained significant concentrations of Mo (approx. 3.5nM). During growth the organisms should remove most of the Mo from the medium, and this ability was used to decrease the Mo content of the medium further, for use in experiments in which outgrowth was measured by determination of viable cell numbers.

To minimize Mo input from the organisms themselves, low densities (approx. 6×10^6c.f.u./ml) of organisms from a Mo-limited chemostat steady state were inoculated into filter-sterilized spent medium supplemented with Na_2SO_4 (50μM). This supported the growth to 5×10^8c.f.u./ml with NH_4^+ as a source of fixed N, and also on N_2 provided that Mo (10nM) was added. No significant growth occurred during 50h under argon/O_2 (87:21) with no added Mo, indicating that no significant utilizable combined N was available in the medium. In media not supplemented with Mo, growth under N_2 stopped at 2×10^7c.f.u./ml, a 25-fold lower population density than with NH_4^+. In the absence of added Mo under N_2 a marked decrease in viability was observed 20h after growth ceased. These data show that *A. vinelandii* UW *rif*^{r} has an absolute requirement for Mo when growing on N_2. The apparent Mo-independent growth observed initially can be attributed to the ability of this organism to scavenge sufficient Mo to allow only limited growth. Fig. 5 also shows that V added at 50nM did not significantly affect the growth of Mo-deficient cultures.

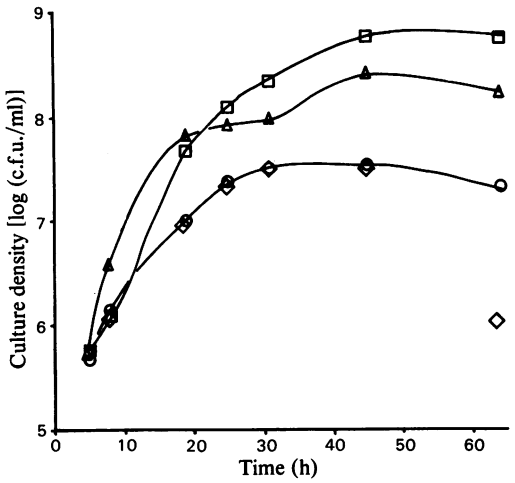

Fig. 5. *Comparison of effects of Mo and V addition on diazotrophic growth of A. vinelandii in 'spent Mo-deficient chemostat medium'*

At time zero, 3ml portions of spent chemostat medium supplemented with Na_2SO_4 (to 50μM) from an Mo-limited chemostat were inoculated to an initial density of 6×10^6c.f.u./ml with *A. vinelandii* from an Mo-limited chemostat culture ($D = 0.1$). Cultures were incubated aerobically at 30°C. Growth was determined by measuring increase in the number of viable organisms (as c.f.u.). Additions were: □, Na_2MoO_4 (to 10nM); △, NH_4Cl (to 20mM) plus V_2O_5 (to 50nM); ○, V_2O_5 (to 50nM); ◇, no addition.

Discussion

The establishment of Mo-limited N_2-fixing chemostat cultures of *A. vinelandii* UW *rif*^{r} is not consistent with the possibility that this organism possesses an Mo-independent alternative nitrogenase system that functions under our conditions. Our finding that diazotrophic growth of *A. vinelandii* UW *rif*^{r} in batch culture on biologically scavenged Mo-free media ceases at a population density 25-fold lower than the same media will support when supplemented with Mo also argues against the

existence of an Mo-independent route for N_2 fixation. Under conditions of acute Mo deprivation in batch culture, linear growth was observed, consistent with an intrinsic growth limitation. Previous reports (Benneman *et al.*, 1973; Nagatani & Brill, 1974; Bishop *et al.*, 1982; Riddle *et al.*, 1982) have shown that diazotrophic growth of *Azotobacter* species in medium with no added Mo is slow compared with growth in the presence of Mo, and published growth data do not conform to exponential growth. Our data indicate that the limiting factor is the Mo required for diazotrophy, since during cell division the Mo present initially is diluted, eventually to the extent that organisms are unable to fix sufficient N_2 to allow further growth.

Chemostat yield data predicted an effective residual Mo contamination in media of approx. 3.5nM, although this varied from batch to batch of chemically purified medium. Since the Mo content of organisms derived from an Mo-limited continuous culture was less than 80pmol/mg of protein (corresponding to less than 24nM-Mo in the culture), the question arises as to whether this residual Mo is sufficient to account for the rate of N_2 fixation by conventional nitrogenase. Assuming that Av1 has a specific activity of 700nmol of N_2 reduced/min per mg, and contains 2 atoms of Mo in a molecule of 240000Da, then a fixation rate of 0.084nmol of N_2 fixed/pg-atom of Mo can be expected. From the data for N_2-fixation rates in Table 1, this calculation suggests an effective contamination of Mo within the range 6.4–48.8nM, depending on the batch of medium. Considering the inherent uncertainties in (*a*) the assumption of the specific activity of Av1 *in vivo* and (*b*) how much of the Mo added to the chemostat medium is available to the organisms, these values are in fair agreement with that of 3.5nM derived from the chemostat yield data and the Mo content of the organisms. The estimated Mo content of the medium is thus sufficient to account for the N_2-fixation rates.

In *A. vinelandii* Mo is involved in maintaining normal concentrations of Av1 (Nagatani & Brill, 1974). These workers reported that extracts of *A. vinelandii* de-repressed under Mo deprivation had nearly normal concentrations of active Av2 but only 4% of antigenic cross-reacting material to anti-Av1. Such data, which suggest a role for Mo in regulating the expression of nitrogenase genes, are substantiated by our pulse-labelling data, which also suggest a relatively high rate of synthesis of Av2 (approx. 4.5% of total protein synthesis) under Mo deprivation. Synthesis of Av1, if occurring, was insufficient to allow quantification. The steady-state protein profile showed Av2 to be present at lower concentrations than would be expected from its rate of synthesis. In the absence of normal concentrations of Av1, Av2 is apparently turned over during growth.

Mo-limited growth of *A. vinelandii* causes an altered substrate specificity, in particular a high rate of H^+ reduction in the presence of the reducible substrate acetylene. Under air the proportion of electrons appearing in H_2 compared with that utilized for N_2 fixation was as low as 33%. This is close to the theoretical minimum of 25%, which would be predicted if N_2 binds to nitrogenase by displacement of H_2 (see Thorneley & Lowe, 1984).

In most diazotrophs growing under conditions of Mo sufficiency, high rates of synthesis of nitrogenase components are observed (Eady *et al.*, 1978; Robson, 1979), which could lead to high steady-state concentrations *in vivo*. From the data in the present paper it is clear that, in *A. vinelandii* at least, high concentrations of nitrogenase are not a prerequisite to obtain adequate rates of N_2 fixation, since the specific activity for N_2 *in vivo* is almost independent of the Mo status (Table 1). In the case of Mo-deprived *K. pneumoniae* it has been shown that addition of Mo restored 60% of the acetylene-reducing activity of organisms that had only 3.6% of the normal amount of cross-reacting material to anti-Kp1 (Kahn *et al.*, 1982). It is apparent that in these organisms the nitrogenase activity measured *in vivo* is higher than would be expected from their nitrogenase contents.

The altered pattern of substrate reduction that we observed may be relevant to the interpretation of acetylene-reduction data collected to estimate N_2-fixation in the field. The contribution of *Azotobacter* to this process in Mo-deficient soils could be considerably underestimated on two accounts. Firstly, the rates of acetylene reduction are often non-linear after short (30min) incubation times. Secondly, the acetylene-reduction rate significantly underestimates the actual rate of N_2 fixation (Table 1), if a value of 3 was assumed for the acetylene/N_2 reduction ratio. These factors may not be restricted to *Azotobacter*, and could account for differences in the estimate of N_2-fixation rates when $^{15}N_2$-reduction and acetylene-reduction assays are compared (Hardy *et al.*, 1973).

The observation that some Nif^- mutants of *A. vinelandii* undergo phenotypic reversion when deprived of Mo has led to the suggestion that this organism possesses an alternative nitrogenase system (Bishop *et al.*, 1980, 1982). When analysed by two-dimensional gel electrophoresis, extracts of these mutants did not show the polypeptides of conventional Av1, but instead four new NH_4^+-repressible proteins were found. The new proteins were also present in the wild-type grown under Mo deprivation.

The ability of the revertants to grow in the

presence of normally inhibitory concentrations of WO_4^{2-} (Bishop & Hetherington, 1980) and the altered protein pattern seen on two-dimensional electrophoresis in these, and also in WO_4^{2-}-resistant mutants (Riddle *et al.*, 1982), has provided support for the existence of an alternative system. Our data are consistent with the operation of a system that is adapted for Mo limitation but in which Mo is still necessary for nitrogenase activity. The Mo status of the organism may regulate the extent of expression of components that are necessary to allow N_2 fixation when only low concentrations of Av1 are present.

The lack of growth response to added V that we observe in biologically scavenged medium suggests that V is incapable of replacing Mo to form a functional nitrogenase. Benneman *et al.* (1973) reported that in the presence of V the Mo content of Mo-starved organisms increased by 50%, and concluded that V exerted a sparing effect permitting the organisms to mobilize the limited Mo available more efficiently. Our results show that V does not fulfil this role in medium with the very low concentrations of residual Mo in biologically scavenged medium. It may be noted that, when V stimulated growth of *A. vinelandii* in the work of Benneman *et al.* (1973), the doubling time was 6h compared with 3.3h for Mo-sufficient cultures, but the acetylene-reduction activity was only 10% that of control cultures. Although they did not report data on the rate of N_2 fixation under their conditions, it would appear that in 'V-spared' organisms the rate of acetylene reduction underestimated the rate of N_2 fixation.

Biochemical complementation of nitrogenase activity in extracts showed that two mutant strains that lacked Av2 activity when de-repressed under Mo-sufficient conditions regained activity when grown under Mo deficiency. These observations were interpreted as indicating a different Av2 being present in the mutants (Premakumar *et al.*, 1984). This suggestion finds some support in the observation of multiple copies of *nifH*-like sequences in *Anabaena* 7120 (Rice *et al.*, 1982), *Calothrix* (Kallas *et al.*, 1983), *Rhodopseudomonas capsulata* (Scolnik & Haselkorn, 1984) and *A. chroococcum* (Robson *et al.*, 1984*b*). In the case of *R. capsulata* it has been shown that mutations in the functional *nifH* resulting in a Nif^- phenotype can be corrected by activation of another copy of *nifH* (Scolnik & Haselkorn, 1984). These alternative genes, which are apparently silent normally, may be activated by a suitable nutritional trigger (e.g. Mo limitation).

We thank Professor J. R. Postgate and Dr. P. E. Bishop for critical reading of the manuscript, Mrs. Marie Hawkins and Mr. Paul Woodley for their skilled technical assistance, Miss B. Scutt for preparation of the typescript, Dr. R. L. Richards for ^{15}N analyses, Mr. C. Macdonald for N analyses and Dr. D. J. Lowe for e.p.r. measurements.

References

Baker, K. (1968) *Lab. Pract.* **17**, 817–824
Benneman, J. R., Smith, G. M., Kastel, P. J. & McKenna, C. E. (1973) *FEBS Lett.* **29**, 219–221
Bishop, P. E. & Hetherington, D. R. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7342–7346
Bishop, P. E., Jarlenski, D.-M. L. & Hetherington, D. R. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7342–7346
Bishop, P. E., Jarlenski, D.-M. L. & Hetherington, D. R. (1982) *J. Bacteriol.* **150**, 1244–1251
Bortels, H. (1930) *Arch. Mikrobiol.* **1**, 333–342
Burns, R. C., Fuchsman, W. H. & Hardy, R. W. F. (1971) *Biochem. Biophys. Res. Commun.* **42**, 353–358
Clarke, L. & Axley, J. (1955) *Anal. Chem.* **27**, 2000–2003
Davis, L. C. & Wang, Y.-L. (1980) *J. Bacteriol.* **141**, 1230–1238
Davis, L. C., Shah, V. K., Brill, W. J. & Orme-Johnson, W. H. (1972) *Biochim. Biophys. Acta* **256**, 512–523
Eady, R. R., Issack, R., Kennedy, C., Postgate, J. R. & Ratcliffe, H. D. (1978) *J. Gen. Microbiol.* **104**, 277–285
Eady, R. R., Kahn, D. & Buchanan-Wollaston, V. (1982) *Isr. J. Bot.* **31**, 45–60
Esposito, R. G. & Wilson, P. W. (1956) *Proc. Soc. Exp. Biol. Med.* **93**, 564–567
Hardy, R. W. F., Burns, R. C. & Holsten, R. D. (1973) *Soil Biol. Biochem.* **5**, 47–81
Hawkes, T. R., McLean, P. A. & Smith, B. E. (1984) *Biochem. J.* **217**, 317–321
Kahn, D., Hawkins, M. & Eady, R. R. (1982) *J. Gen. Microbiol.* **128**, 779–787
Kallas, T., Rebier, M. C., Rippka, R. & Tandeau de Marsac, N. (1983) *J. Bacteriol.* **155**, 427–431
Keeler, R. F. & Varner, J. E. (1957) *Arch. Biochem. Biophys.* **70**, 585–590
Nagatani, H. H. & Brill, W. J. (1974) *Biochim. Biophys. Acta* **362**, 160–166
Partridge, C. D. P. & Yates, M. G. (1982) *Biochem. J.* **204**, 339–344
Pienkos, P. T. & Brill, W. J. (1981) *J. Bacteriol.* **145**, 743–751
Premakumar, R., Lemos, E. M. & Bishop, P. E. (1984) *Biochim. Biophys. Acta* **797**, 64–71
Rice, D., Mazur, B. J. & Haselkorn, R. (1982) *J. Biol. Chem.* **257**, 13157–13163
Riddle, G. D., Simonson, J. G., Hales, B. J. & Braymer, H. D. (1982) *J. Bacteriol.* **152**, 72–80
Robson, R. (1979) *FEMS Microbiol. Lett.* **5**, 259–262
Robson, R., Chesshyre, J., Wheeler, C., Jones, R., Woodley, P. & Postgate, J. R. (1984*a*) *J. Gen. Microbiol.* **130**, 1603–1612
Robson, R., Jones, R., Kennedy, C. K., Drummond, M., Ramos, J., Woodley, P. R., Wheeler, C., Chesshyre, J. & Postgate, J. R. (1984*b*) in *Advances in Nitrogen Fixation Research* (Veeger, C. & Newton, W. E., eds.), pp. 643–651, Nijhoff/Junk, The Hague

Rubenchik, L. I. (1963) *Azotobacter and Use in Agriculture*, National Science Foundation, Washington

Scolnik, P. A. & Haselkorn, R. (1984) *Nature* (*London*) **307**, 289–292

Shah, V. K. & Brill, W. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3241–3253

Thorneley, R. N. F. & Lowe, D. J. (1984) in *Metal Ions in Biology* (Spiro, T., ed.), John Wiley and Sons, New York, in the press

Walker, C. C. (1979) D.Phil. Thesis, University of Sussex