New Phytol. (1991), **118**, 303–308

An alternative nitrogenase is not expressed in molybdenum-deficient legume root nodules

BY M. J. DILWORTH AND J. F. LONERAGAN

Nitrogen Fixation Research Group, School of Biological and Environmental Sciences, Murdoch University, Murdoch, Western Australia 6150

(Received 31 October 1990; accepted 20 February 1991)

SUMMARY

Three legume root nodule bacteria systems (*Medicago polymorpha* L.–*Rhizobium meliloti*, *Ornithopus sativus* Brot.–*Bradyrhizobium lupini* and *Trifolium subterraneum* L.– *Rhizobium leguminosarum* bv. *trifolii*) were grown in solution culture under conditions likely to lead to the production of alternative nitrogenases (molybdenum-deficient, or molybdenum-deficient but supplemented with vanadium). Addition of 1 μM molybdenum produced significant responses in both nodule and top weights while 2 μM vanadium did not. Ethane, which is produced as well as ethylene when acetylene is reduced by vanadium nitrogenase or nitrogenase-3 from *Azotobacter*, was not found in significant amounts during assays of acetylene reduction in either molybdenum-deficient or molybdenum-deficient, vanadium-supplemented treatments, suggesting that no non-molybdenum nitrogenase was produced by these root nodule bacteria.

Key words: Alternative nitrogenase, legume nodules, molybdenum deficiency, vanadium.

INTRODUCTION

The involvement of molybdenum with the process of N_2 fixation in *Azotobacter* has been known since the work of Bortels (1930), and was given biochemical meaning by the isolation of the molybdenum iron protein (MoFe protein) of nitrogenases from a variety of bacteria (see Miller, 1990). Although early studies also indicated a role for vanadium in N_2 fixation in *Azotobacter* (Burk, 1934; Bortels, 1936), a biochemical explanation was not forthcoming for 50 years. Genetic studies with Nif$^-$ mutants of *Azotobacter vinelandii* showed that some could fix N_2 only under Mo-deficient conditions (Bishop, Jarlenski & Hetherington, 1980). Unequivocal proof of the existence of a non-molybdenum nitrogenase system came with the construction of strains of both *Azotobacter chroococcum* and *A. vinelandii* which had had the structural genes for Mo nitrogenase (*nifHDK*) specifically deleted. Such strains grew on N_2, but only if Mo was omitted from the medium (Bishop *et al.*, 1986*a*; Robson, 1986). The conventional acetylene reduction assay for nitrogenase activity gave misleadingly low estimates of N_2 fixation in cultures of one such mutant (*A. vinelandii* CA11) (Bishop, Hawkins & Eady, 1986*b*).

The isolation of vanadium nitrogenases from both *A. chroococcum* (Robson *et al.*, 1986) and *A. vinelandii* (Hales *et al.*, 1986) allowed the properties of these enzymes to be studied. Among their characteristics is the ability to reduce acetylene (ethyne) not only to ethylene (ethene) but also to ethane (Dilworth *et al.*, 1987), a reaction not catalyzed by Mo nitrogenases and thereby offering a possible differential assay for the V nitrogenase *in vivo*. Molybdenum-deprived, V-supplemented cultures of *A. chroococcum*, *A. vinelandii* and *Clostridium pasteurianum* produced ethane during acetylene reduction assays, consistent with the possession of V-nitrogenase. Mutants of *A. vinelandii* lacking structural genes for both the Mo and V nitrogenases are now known also to produce ethane from acetylene (Pau, Mitchenall & Robson, 1989). This comes about because in *A. vinelandii* there is a third nitrogenase system, the metal status of which is not yet clear, but which produces ethane from acetylene (Chisnell, Premakumar & Bishop, 1988; Pau *et al.*, 1989).

The distribution of V nitrogenases in diazotrophic bacteria is not yet clear. Their presence is confirmed for *A. chroococcum* (Robson *et al.*, 1986) and *A. vinelandii* (Hales *et al.*, 1986) and presumptive for *Anabaena* (Kentemich *et al.*, 1988) and *C. pasteurianum* (Dilworth *et al.*, 1987). The nitrogenase-3 system (the non-Mo, non-V system) is known only

Table 1. *Plant cultivars and root nodule bacterial strains*

Legume	Cultivar	Root nodule bacteria
Medicago polymorpha L.	Serena	*Rhizobium meliloti* WSM419: J. Howieson, W. Australian Department of Agriculture
Ornithopus sativus Brot.	Pitman	*Bradyrhizobium lupini* WU425: Institute of Agriculture, University of W. Australia
Trifolium subterraneum L.	Seaton Park	*Rhizobium leguminosarum* bv. *trifolii* WU95 W. Australian Department of Agriculture

from *A. vinelandii* (Bishop *et al.*, 1988; Pau *et al.*, 1989).

In legume root nodule bacteria, it is not clear whether the V nitrogenase or nitrogenase-3 are present. Other bacteria carrying the V nitrogenase carry a gene for the Fe protein of the V nitrogenase (*vnfH*) with substantial homology to the *nifH* gene, which specified the Fe protein of the Mo nitrogenase. The detection in bacteria of multiple genes hybridizing to *nifH* probes might therefore be evidence for the occurrence of alternative nitrogenase systems, but such data require other confirmation. It is known that *Rhizobium leguminosarum* bv. *phaseoli* does indeed carry multiple genes reacting with *nifH* probes (Quinto *et al.*, 1985). Although limited ethane formation from acetylene has been reported for Mo-deficient cultures of *Azorhizobium caulinodans* ORS571, together with a weak hybridization between its DNA and a *vnfDK* probe from *A. chroococcum* (Chan *et al.*, 1988), the significance of these findings has been questioned (Robson, 1990). Moreover, there is no report of the expression of an alternative nitrogenase in a functioning legume nodule system.

The expression of alternative nitrogenases in *Azotobacter* depends on the absence of repressive concentrations of Mo (Robson, 1990). We reasoned that if either of the alternative nitrogenases occurred in root nodule bacteria, and were ever significant for N_2 fixation in legume nodules, they should be expressed there under Mo-deficiency. Three different legumes have therefore been grown under Mo-deficient conditions in solution culture, with or without added V, and the presence of alternative nitrogenases studied by testing whether ethane was generated during acetylene reduction assays. The legumes were selected to cover two different species of *Rhizobium* and one of *Bradyrhizobium*.

MATERIALS AND METHODS

Plant species and bacterial strains

The plant species and cultivars, and the strains and sources of root nodule bacteria used to nodulate them, are given in Table 1.

Growth of bacteria

Rhizobium meliloti WSM419 was grown in JMM broth of the composition given by O'Hara *et al.* (1989), to which neither Mo nor V had been added. *Rhizobium leguminosarum* bv. *trifolii* WU95 was grown in the liquid minimal medium of Brown & Dilworth (1975) using 10 mM glucose as carbon source, 3 mM NH_4Cl as nitrogen source and 0·4 mM phosphate; sodium molybdate was omitted from the minor element mixture and no V was added. Cells of these two organisms were used directly as inocula. *Bradyrhizobium lupini* WU425 was grown on solidified JMM medium (O'Hara *et al.*, 1989) with no added Mo or V. For use as inoculum, the cells were washed off into sterile deionized water.

Seedling growth and inoculation

Seeds of *Ornithopus sativus* Brot. (serradella) were dehulled before use; they and the others were lightly scarified to aid germination. Seeds were placed on a continuously moistened layer of gauze on a stainless steel grid over deionized water for 1 d, and then over half-strength nutrient solution containing no added Mo or V. It was, however, necessary to add 0·1 μg l^{-1} Mo in the case of serradella as nodulation did not occur in its absence. Appropriate root nodule bacteria were added to the half-strength nutrient solution on the second day; plants were transferred to 5 l solution cultures when the radicles were approximately 10 mm long. Seedlings were inserted through 3 mm diameter grommets in holes in a black polythene disc covering the solutions, which were aerated continuously and held at 22 °C in root cooling tanks in the glasshouse.

Nutrient solutions

The basal nutrient solution was modified from that of Delwiche, Johnson & Reisenauer (1961) and initially contained 0·3 g $CaCO_3$ per 5 l of solution of the following composition (μM): K_2SO_4 (2500), $MgSO_4$ (1000), KH_2PO_4 (250), FeEDDHA (30), H_3BO_3 (15), NaCl (10), $ZnSO_4$ (2·5), $MnSO_4$ (1), $CuSO_4$ (0·5) and $CoSO_4$ (0·2). Molybdenum was

removed from all stock macronutrient solutions by adjusting their pH to 5 and passing them through a column of 8-hydroxyquinoline bonded to controlled porosity glass beads (Eskew, Welch & Cary, 1984). Molybdenum was added as sodium molybdate to a concentration of 1 μM and vanadium as ammonium vanadate at 2 μM to allow for adequate vanadium uptake.

The experiment had four treatments: 0 (−Mo −V), +Mo, +V and +Mo+V, with two replicates of each treatment.

Growth and harvest of plants

Pots of *Medicago polymorpha* L. initially contained 14 plants, subsequently thinned to 10; for serradella, 20 plants were thinned to 15 and the corresponding numbers for subterranean clover were 14 and 9. Plants were grown until there were clear differences in plant colour and size before harvest; the growth periods after transfer to solution cultures were: 40 d for *M. polymorpha*, 42 d for serradella and 42 d for subterranean clover.

Acetylene reduction assays

Harvested plants were freed from the plastic grommets, the roots blotted dry with paper towelling, and whole plants assayed for acetylene reduction as described by Trinick, Dilworth & Grounds (1976). Acetylene, ethylene and ethane were separated by gas chromatography on an alumina column as described by Dilworth, Eady & Eldridge (1988). Ethane contamination in the acetylene was extremely low but was nevertheless subtracted from any detected in the samples. To allow determination of specific acetylene reduction activity, all nodules were picked from *M. polymorpha* and serradella; for subterranean clover sufficient representative samples were taken only for Mo and V determinations. The enormous number of very small nodules formed by the clover in the nil Mo treatments made it impossible to measure nodule weight.

Determination of molybdenum and vanadium in nodules

Nodules were dried at 80 °C and 0·2–0·5 g samples were wet-ashed in a mixture of concentrated nitric acid, 70% (w/v) perchloric acid and concentrated sulphuric acid (10:2:2 by volume). Digestion was initially at 75 °C until the digest was clear and N_2O_4 evolution had ceased (30–45 min), then at 160 °C until white fumes appeared, and finally at 215 °C until the digest had turned from yellow to colourless. The digest was cooled, transferred to a 100 ml separating funnel marked for a 25 ml volume, and diluted to the mark with double deionized water. Following addition of 0·5 ml of 0·1% (w/v) ceric sulphate in 0·5 M H_2SO_4, the mixture was shaken well, and allowed to stand for 10 min for oxidation of the vanadium to the 5+ oxidation state (Ryan, 1960; Priyadashini & Tandon, 1961). Concentrated HCl (10 ml) was added, followed immediately by 2 ml of 0·1% (w/v) *N*-benzoyl-*N*-phenylhydroxylamine in 4-methylpentan-2-one saturated with water at pH 2. After 1 min timed shaking, the mixture was allowed to separate and the aqueous layer discarded. The organic phase was allowed to settle to release residues of the aqueous phase, which were also discarded before the organic phase was transferred to a 1·5 ml Eppendorf tube.

Reference materials and reagent blanks were carried through the whole digestion and extraction procedure.

Molybdenum and vanadium were determined by atomic absorption spectrophotometry using a hollow graphite anode and pyrolytic-coated tubes (Buchet, Knepper & Lauwerys, 1982; Manning & Slavin, 1985), using 313·3 and 318·3 nm for Mo and V, respectively.

RESULTS AND DISCUSSION

Molybdenum and vanadium concentrations in nodules

When vanadium was not added to the nutrient solution, the concentrations of vanadium in the three types of legume root nodules were all extremely low but showed wide variation among species (Table 2). Since the initial seed was not analysed, it is not possible to say whether this variation was as a consequence of seed vanadium levels or major differences in uptake capability.

In the absence of added molybdenum, molybdenum concentrations were ten to one hundred times higher than those of vanadium in nodules of plants not receiving vanadium. The concentrations of molybdenum were also much more uniform among species. In all three species they were somewhat higher than the critical concentration of 1·3 mg kg^{-1} in nodules suggested for diagnosis of molybdenum deficiency in soybean (Chotechaungmanirat, 1988).

Addition of vanadium resulted in 35-fold (serradella) to 1600-fold increases in nodule vanadium concentration, with the lowest absolute concentration in *M. polymorpha*. In subterranean clover and *M. polymorpha*, addition of vanadium had no effect on molybdenum concentrations, but vanadium addition increased the concentration of molybdenum in serradella nodules. Addition of molybdenum alone produced very large increases in nodule molybdenum concentrations (Table 2); only in *M. polymorpha* did molybdenum addition depress the already low concentrations of vanadium.

When both molybdenum and vanadium were applied, concentrations of both increased in the nodules (Table 2). For *M. polymorpha*, the final molybdenum concentration was unaltered from that

Table 2. *Molybdenum and vanadium concentrations (mg kg^{-1}) in nodules of Serena medic, serradella and subterranean clover*

	Medicago polymorpha		*Ornithopus sativus*		*Trifolium subterraneum*	
Treatment	Mo	V	Mo	V	Mo	V
−Mo−V	2·9	0·44	2·6	1·4	2·6	0·03
	3·3	0·13	4·4	1·9	2·4	0·02
−Mo+V	5·5	12·8	12·9	78	2·0	41
	3·4	13·3	6·9	41	3·0	39
+Mo−V	337	0·02	1009	1.4	70	0·02
	400	0·05	897	1·0	80	0·01
+Mo+V	323	8·5	806	47	55	20
	427	5·3	760	38	47	22

Table 3. *Effects of molybdenum and vanadium treatments on* Medicago polymorpha *cv. Serena*

Treatment	Top fresh weight (g plant^{-1})	Nodule fresh weight (mg plant^{-1})	Acetylene reducing activity (nmol C_2H_4 min^{-1} plant^{-1})	Acetylene reducing activity [nmol C_2H_4 min^{-1} (g f. wt)$^{-1}$]	Acetylene reducing activity (pmol C_2H_6 min^{-1} plant^{-1})
−Mo−V	0·89	91	24	268	0·0002
	1·09	100	23	229	
−Mo+V	0·86	64	19	305	0·0006
	0·83	86	17	198	
+Mo−V	1·54	135	90	667	0·0008
	1·57	149	90	606	
+Mo+V	1·53	142	75	523	0·0006
	1·57	135	68	507	

Table 4. *Effects of molybdenum and vanadium treatments on* Ornithopus sativus *cv. Pitman*

Treatment	Top fresh weight (g plant^{-1})	Nodule fresh weight (mg plant^{-1})	Acetylene reducing activity (nmol C_2H_4 min^{-1} plant^{-1})	Acetylene reducing activity [nmol C_2H_4 min^{-1} (g f. wt)$^{-1}$]	Acetylene reducing activity (pmol C_2H_6 min^{-1} plant^{-1})
−Mo−V	0·84	115	46	397	0
	0·77	112	31	272	0
−Mo+V	0·70	90	40	441	0
	0·72	80	34	428	0
+Mo−V	1·61	182	47	256	0
	1·59	226	65	289	0
+Mo+V	1·52	208	72	348	0
	1·46	196	70	356	0

produced by molybdenum alone, but the vanadium concentration achieved was only half that for vanadium alone. For serradella, both vanadium and molybdenum concentrations were slightly decreased, but for subterranean clover the decreases were considerably greater.

With the appropriate additions, the overall concentrations of molybdenum and vanadium in nodules were considerable, though it is not possible to conclude that adequate supplies of vanadium were reaching the bacteroids within their peri-bacteroid membranes. However, if vanadium is not available to the bacteroids when present at these concentrations in the nodule, it is unlikely that vanadium would ever be significant for bacteroid nitrogen fixation.

Table 5. *Effects of molybdenum and vanadium treatments on* Trifolium subterraneum *cv. Seaton Park*

Treatment	Top fresh weight (g plant^{-1}	Acetylene reducing activity (nmol C_2H_4 min^{-1} plant^{-1})	Acetylene reducing activity (pmol C_2H_6 min^{-1} plant^{-1})
−Mo−V	0·85	14	0·013
	1·20	28	0·025
−Mo+V	1·10	17	0·016
	1·31	26	0·028
+Mo−V	3·16	66	0·017
	2·66	46	0·007
+Mo+V	3·08	65	0·017
	3·31	73	0·021

Response of plants to molybdenum and vanadium

Molybdenum-deficient plants were generally lighter green, smaller and carried nodules that tended to be white or green, while those on Mo-treated plants were pink. Nodule numbers were greater on Mo-deficient plants than on Mo-supplemented plants except for serradella where the numbers were similar.

In none of the three cases did the addition of vanadium increase top weight in Mo-deficient plants (Tables 3–5). Molybdenum produced significant increases in top weight in all three, and the increases occurred whether vanadium was added or not. Nodule weights were also increased by molybdenum but not by vanadium for all three species (Tables 3–5).

Acetylene reduction activities

Only molybdenum addition increased the acetylene reduction activity per plant for all species; vanadium addition was ineffective (Tables 3–5). Top fresh weight was strongly correlated with ethylene production per plant for all three species (data not shown); nodule weight was also strongly correlated with it in *M. polymorpha* and serradella.

Ethane concentrations in the acetylene reduction assays were barely above the limit of detection; and the ratio of C_2H_6/C_2H_4 was extremely low, and always less than $1{\cdot}1 \times 10^{-6}$. For comparison, cultures of Mo-deficient, V-supplemented *A. chroococcum*, *A. vinelandii* or *C. pasteurianum* showed ratios of C_2H_6/C_2H_4 of $0{\cdot}5\text{–}25 \times 10^{-2}$ (Dilworth *et al.*, 1987). Vanadium addition did not increase the ratio of C_2H_6/C_2H_4 as would be expected under molybdenum deficiency.

If molybdenum deficiency had resulted in the derepression of either a V nitrogenase or a nitrogenase-3 system, significant ethane production would have been expected. Since the nodules were all clearly Mo-deficient, as indicated by the top weight response to molybdenum addition, it appears most unlikely that alternative nitrogenases play any significant role in nitrogen fixation in these nodule systems in the field, even if the genes for them were to occur in the root nodule bacteria.

ACKNOWLEDGEMENTS

We acknowledge the skilled technical assistance of Yvonne Haynes and Janet Wroth with plant growth and acetylene reduction assays, and of Derek Plaskett for the development of the analytical method for molybdenum and vanadium in the same plant material.

REFERENCES

Bishop, P. E., Hawkins, M. E. & Eady, R. R. (1986*b*). Nitrogen fixation in molybdenum-deficient continuous cultures by strains of *Azotobacter vinelandii* carrying a deletion of the structural genes for nitrogenase (*nifHDK*). Biochemical Journal **238**, 437–442.

Bishop, P. E., Jarlenski, D. M. L. & Hetherington, D. R. (1980). Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. *Proceedings of the National Academy of Sciences of the USA* **77**, 7342–7346.

Bishop, P. E., Premakumar, R., Dean, D. R., Jacobson, M. R., Chisnell, J., Rizzo, T. M. & Kopezynski, T. (1986*a*). Nitrogen fixation by *Azotobacter vinelandii* strains having deletions in structural genes for nitrogenase. *Science* **232**, 92–94.

Bishop, P. E., Premakumar, R., Joerger, R. D., Jacobson, M. R., Dalton, D. A., Chisnell, J. R. & Wolfinger, E. O. (1988). Alternative nitrogen fixation systems in *Azotobacter vinelandii*. In: *Nitrogen Fixation: Hundred Years After* (Ed. by H. Bothe, F. J. de Bruijn & W. E. Newton), pp. 71–79. Gustav Fischer, Stuttgart.

Bortels, H. (1930). Molybdän als Katalysattor bei biologischen Stickstoffbindung. *Archiv für Mikrobiologie* **1**, 333–342.

Bortels, H. (1936). Weitere Untersuchungen über die Bedeutung von Molybdän, Vanadium, Wolfram und andere Erdaschenstoffe für stickstoffbindene und andere Mikroorganismen. *Zentralblatt für Bakteriologie und Parasitenkunde Abteil* II **95**, 193–218.

Brown, C. M. & Dilworth, M. J. (1975). Ammonia assimilation by *Rhizobium* cultures and bacteroids. *Journal of General Microbiology* **86**, 39–48.

Buchet, J.-P., Knepper, E. & Lauwerys, R. (1982). Determination of vanadium in urine by electrothermal atomic absorption spectrometry. *Analytica Chimica Acta* **136**, 243–248.

Burk, D. (1934). Azotase and nitrogenase in *Azotobacter*. *Ergebnisse Enzymforschung* **3**, 23–36.

CHAN, Y.-K., PAU, R., ROBSON, R. L. & MILLER, R. W. (1988). Expression of unconventional nitrogenase reactions in several nitrogen-fixing bacteria. In: *Nitrogen Fixation : Hundred Years After* (Ed. by H. Bothe, F. J. de Bruijn & W. E. Newton), p. 88. Gustav Fischer, Stuttgart.

CHISNELL, J. R., PREMAKUMAR, R. & BISHOP, P. E. (1988). Purification of a second alternative nitrogenase from a *nifHDK* deletion strain of *Azotobacter vinelandii*. *Journal of Bacteriology* **170**, 27–33.

CHOTECHAUNGMANIRAT, S. (1988). *Diagnosis of molybdenum deficiency in soybean* (Glycine max L. *Merr.*) *by plant analysis*. Master of Agricultural Science Thesis, University of Western Australia, Perth, Western Australia.

DELWICHE, C. C., JOHNSON, C. M. & REISENAUER, H. M. (1961). Influence of cobalt on nitrogen fixation by *Medicago*. *Plant Physiology* **36**, 73–78.

DILWORTH, M. J., EADY, R. R. & ELDRIDGE, M. E. (1988). The vanadium nitrogenase of *Azotobacter chroococcum*. Reduction of acetylene and ethylene to ethane. *Biochemical Journal* **249**, 745–751.

DILWORTH, M. J., EADY, R. R., ROBSON, R. L. & MILLER, R. W. (1987). Ethane formation from acetylene as a potential test for vanadium nitrogenase *in vivo*. *Nature* **327**, 167–168.

ESKEW, D. L., WELCH, R. M. & CARY, E. E. (1984). A simple plant nutrient solution purification method for the effective removal of trace metals using controlled pore glass 8-hydroxyquinoline chelation column chromatography. *Plant Physiology* **76**, 103–105.

HALES, B. J., CASE, E. E., MORNINGSTAR, J. E., DZEDA, M. F. & MAUTERER, L. A. (1986). Isolation of a new vanadium-containing nitrogenase from *Azotobacter vinelandii*. *Biochemistry* **25**, 7251–7255.

KENTEMICH, T., DANNENBERG, G., HUNDESHAGEN, B. & BOTHE, H. (1988). Evidence for the occurrence of the alternative vanadium-containing nitrogenase in the cyanobacterium *Anabaena variabilis*. *FEMS Microbiology Letters* **51**, 19–24.

MANNING, D. C. & SLAVIN, W. (1985). Factors affecting the atomization of vanadium in graphite furnace AAS. *Spectrochimica Acta* **40B**, 461–473.

MILLER, R. W. (1990). Molybdenum nitrogenase. In: *Biology and Biochemistry of Nitrogen Fixation* (Ed. by M. J. Dilworth & A. R. Glenn), pp. 9–36. Elsevier, Amsterdam.

O'HARA, G. W., GOSS, T. J., DILWORTH, M. J. & GLENN, A. R. (1989). Maintenance of intracellular pH and acid tolerance in *Rhizobium meliloti*. *Applied and Environmental Microbiology* **55**, 1870–1876.

PAU, R. N., MITCHENALL, L. A. & ROBSON, R. L. (1989). Genetic evidence for an *Azotobacter vinelandii* nitrogenase lacking molybdenum and vanadium. *Journal of Bacteriology* **171**, 124–129.

PRIYADASHINI, U. & TANDON, S. G. (1961). Spectrophotometric determination of vanadium (V) with *N*-benzoyl-*N*-phenylhydroxylamine. *Analytical Chemistry* **33**, 435–438.

QUINTO, C., DE LA VEGA, H., FLORES, M., LEEMANS, J., CEVALLOS, M. A., PARDO, M. A., ASPIROZ, R., GIRARD, M. L., CALVA, E. & PALACIOS, R. (1985). Nitrogenase reductase: a functional multigene family in *Rhizobium phaseoli*. *Proceedings of the National Academy of Sciences of the USA* **82**, 1170–1174.

ROBSON, R. L. (1986). Nitrogen fixation in strains of *Azotobacter chroococcum* bearing deletions of a cluster of genes coding for nitrogenase. *Archives of Microbiology* **146**, 74–79.

ROBSON, R. L. (1990). The genetics of the alternative nitrogenases. In: *Biology and Biochemistry of Nitrogen Fixation* (Ed. by M. J. Dilworth & A. R. Glenn), pp. 142–161. Elsevier, Amsterdam.

ROBSON, R. L., EADY, R. R., RICHARDSON, T. H., MILLER, R. W., HAWKINS, M. & POSTGATE, J. R. (1986). The alternative nitrogenase of *Azotobacter chroococcum* is a vanadium enzyme. *Nature* **322**, 388.

RYAN, D. E. (1960). The colorimetric determination of vanadium with benzoylphenyl-hydroxylamine. *Analyst* **85**, 569–574.

TRINICK, M. J., DILWORTH, M. J. & GROUNDS, M. (1976). Factors affecting the reduction of acetylene by root nodules of *Lupinus* species. *New Phytologist* **77**, 359–370.