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COBALT AND NITROGEN FIXATION IN *LUPINUS ANGUSTIFOLIUS* L. II. NODULE FORMATION AND FUNCTION

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SUMMARY

The effects of cobalt deficiency on nodule formation and function in sweet lupin (*Lupinus angustifolius* L. cv. Unicrop) were studied in cobalt-deficient Lancelin sand in the glasshouse.

Bacteroid densities in cobalt-deficient nodules were lower than in normal nodules. Recovery from cobalt deficiency in inoculated treatments was associated with increases in bacteroid density and cobalt accumulation in lateral nodules. Such changes did not occur in treatments infected with rhizobia from the soil.

Acetylene-reducing activity of cobalt-deficient plants was not initiated until plants were nearly 6 weeks old, at which time cobalt-treated plants were at their peak of activity. Specific activities of cobalt-deficient nodules remained very low even when nitrogenase did develop. Their large mass of nodules allowed cobalt-deficient plants to reach 20 to 50 % of the normal activity per plant, but specific activities were only 5 to 13 % of peak activities in cobalt-treated nodules.

Nodule bacteroid content and leghaemoglobin content were linearly related to cobalamin content, each with a single relationship. Plotting acetylene-reducing activity against cobalamin content or leghaemoglobin content generated two different linear response curves in each case; the slopes of the lines were different, depending on the presence or absence of cobalt. It is suggested that there may be a function in N_2 fixation in legume nodules for a non-cobalamin form of cobalt.

INTRODUCTION

Field responses of sweet lupins (*Lupinus angustifolius* L.) to cobalt have been reported for coarse-textured soils in south-western Australia (Gladstones, Loneragan and Goodchild, 1977; Chatel *et al.*, 1978). Cobalt addition caused responses in dry matter production, nodule distribution between tap and lateral roots, and in leghaemoglobin concentration (Gladstones *et al.*, 1977). Nitrogen concentration was shown to increase or decrease after cobalt treatment, depending on the time of harvest (Gladstones *et al.*, 1977; Chatel *et al.*, 1978), but nitrogen content was increased by cobalt in the experiments of both Gladstones and Chatel and their colleagues. Bacteroid numbers

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per g of nodule tissue were lowered in cobalt-deficient plants, but overall bacteroid length was greater (Chatel *et al.*, 1978). An interaction between cobalt and seed inoculation was also noted under field conditions, such that inoculation alleviated cobalt deficiency (Chatel *et al.*, 1978). Cobalt distribution studies in normal and cobalt-deficient lupins have shown that cobalt is preferentially directed towards nodules in cobalt deficiency (Robson, Dilworth and Chatel, 1979).

It has been shown that cobalt is essential for normal growth of *Rhizobium* species (Lowe, Evans and Ahmed, 1960; Lowe and Evans, 1962; Cowles, Evans and Russell, 1969) and that its effect is mediated by vitamin B_{12} and its coenzyme forms (Kliewer and Evans, 1962a, b, 1963a, b; Cowles and Evans, 1968). While it seems highly probable that the effects of cobalt on leguminous nitrogen fixation are mediated by its effects on rhizobial growth and cobalamin production these effects have not been much studied in cobalt-deficient plants.

Three specific cobalamin-dependent enzyme systems are known in *Rhizobium*.

(1) Methylmalonyl-coenzyme A mutase, involved in isomerization of succinyl-coenzyme A to methylmalonyl-coenzyme A, and therefore in propeionate-succinate interconversions in *Rhizobium meliloti* and *R. japonicum* (De Hertogh, Mayeux and Evans, 1964). Since propionate is known to be incorporated into haem by bacteriods of *R. japonicum* (Jackson and Evans, 1966) and since bacteroid haem synthesis is apparently required for leghaemoglobin production (Cutting and Schulman, 1969; Godfrey and Dilworth, 1971; Nadler and Avissar, 1977), cobalt deficiency and decreased nodule leghaemoglobin concentration (Ahmed and Evans, 1961; Kliewer and Evans, 1963b; Gladstones *et al.*, 1977) might be expected to be correlated.

(2) Ribonucleotide reductase, involved in reduction of ribonucleotides to deoxy-ribonucleotides, and therefore in DNA synthesis (Cowles and Evans, 1968; Cowles *et al.*, 1969). Cobalt deficiency might therefore be expected to result in defective DNA synthesis and division in rhizobia, and an increase in cell size. Such increases have been recorded for laboratory cultures of *R. meliloti* (Cowles *et al.*, 1969; Evans, Russell and Johnson, 1965) and for bacteroids of *R. lupini* (Chatel *et al.*, 1978).

(3) Methionine synthetase, involved in methyl group transfer from [N^5]methyltetra-hydrofolic acid to homocysteine to produce methionine (Sato, Inukai and Shimizu, 1974; Inukai, Sato and Shimizu, 1977). Cobalt deficiency might therefore be expected to impair growth by lowering the rate of protein synthesis because of limiting methionine concentration. There is some indication that the level of cobalt required for normal methionine synthesis in *R. meliloti* is higher than that required for normal methylmalonyl-coenzyme A mutase or ribonucleotide reductase function because methionine alone would allow growth in cobalt-deficient media (Inukai *et al.*, 1977).

Rhizobial multiplication outside the root, or during infection and nodule formation is therefore likely to be defective in cobalt deficiency, resulting in lowered or delayed nodulation and N_2 fixation activity, and lowered leghaemoglobin concentration in nodules.

The current experiments were designed to follow the time course of cobalt responses in lupins, to determine how and where cobalt exerted its effects, and to ask whether the responses observed were all mediated through the effect of cobalt on cobalamin production in lupin rhizobia.

Materials and Methods

Plant growth

The design of the experiment was a factorial of cobalt application (nil, or 0·9 mg $CoSO_4.7H_2O$ pot^{-1}) and inoculation (not inoculated, or inoculated with *R. lupini* WU 425) with 20 replicates of each treatment. Details of fertilizer purification and application, and inoculation are given in a preceding paper (Robson *et al.*, 1979).

Low-cobalt seeds of *L. angustifolius* cv. Uniharvest were pre-germinated and sown in 5·8 kg pots of Lancelin sand (Chatel *et al.*, 1978). Pots were maintained in root-cooling tanks at 25 °C throughout the experiment; the higher temperature accentuated the effects of cobalt deficiency (McGrath, Robson and Dilworth, unpublished results).

Plants were harvested at 2, 4, 6, 8 and 11 weeks.

Measurements

Plant parts were separated for determination of wet weight, dry weight, nitrogen content and cobalt content.

Nodulation was assessed in two classes: crown nodulation (on the top 10 cm of the main root) or lateral nodulation (on lateral roots or below 10 cm on the main root).

Acetylene reduction was measured by the method of Trinick, Dilworth and Grounds (1976) at least 4 h after sunrise.

Bacteroid numbers were assessed as described previously (Chatel *et al.*, 1978).

For vitamin B_{12} assay, whole nodule homogenates in 0·1 M potassium phosphate buffer (pH 6·8) were autoclaved in the presence of cyanide. Cyanocobalamin was measured by the method of Raven and Barkham (1973), which uses the dilution of intrinsic factor binding of radioactive cyanocobalamin to determine cobalamin in unknown samples.

Leghaemoglobin was measured as the pyridine haemochromogen (Paul, Theorell and Åkeson, 1953) on supernatants from bacteroid isolation after they had been passed through columns of Sephadex G-75 to purify partially the leghaemoglobin fraction (Coventry and Dilworth, 1977).

Cobalt was measured by atomic absorption methods after solvent extraction of acid-digested material (Simmons, 1975) and nitrogen by Kjeldahl digestion (McKenzie and Wallace, 1954).

Results

Nodulation

Semi-logarithmic plots of the increase of nodule weight with time are presented in Figures 1 and 2.

In the inoculated treatments, cobalt clearly increased the wet weight of crown nodules at all times [Fig. 1(a)] with a ratio between cobalt-fertilized and -deficient plants of about 4 at the 6-week harvest. The effect of cobalt on lateral root nodule weight was entirely different; in this case [Fig. 2(a)], the treatment not given cobalt had 9 to 10 times the lateral nodule weight of the cobalt-fertilized pots. Cobalt deficiency appeared to decrease crown nodulation and either that or the cobalt deficiency itself then led to a large compensatory production of lateral root nodules.

In Figure 1 it is also obvious that increase in mass of crown nodules virtually stopped at 6 weeks in all treatments. The relative importance of crown and lateral nodulation is clearly shown in Figure 3 where the proportion of total nodule weight

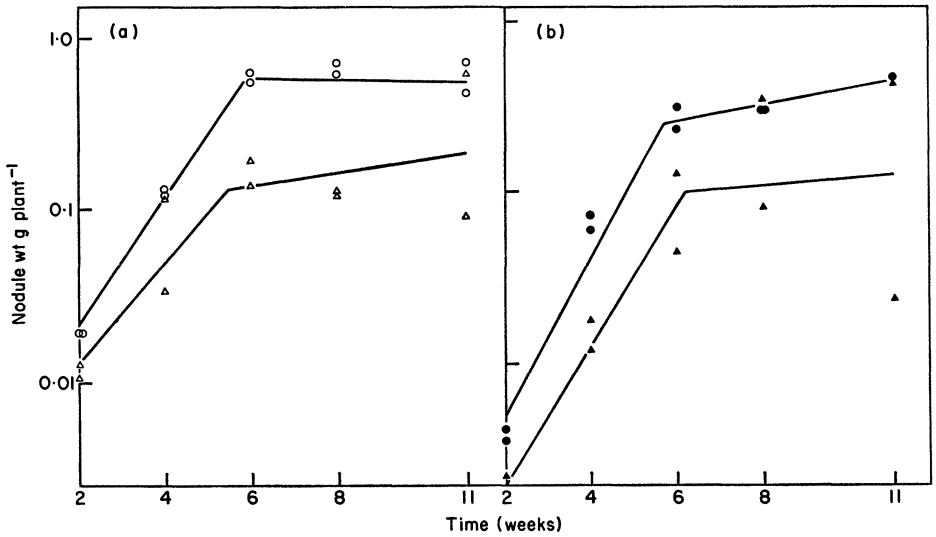

Fig. 1. Wet weight of crown nodules on sweet lupins as a function of time. Each pot contained 8 plants. (a) Inoculated with *Rhizobium lupini* WU 425; ○, plus cobalt; △, minus cobalt. (b) Not inoculated with WU 425: ●, plus cobalt; ▲, minus cobalt.

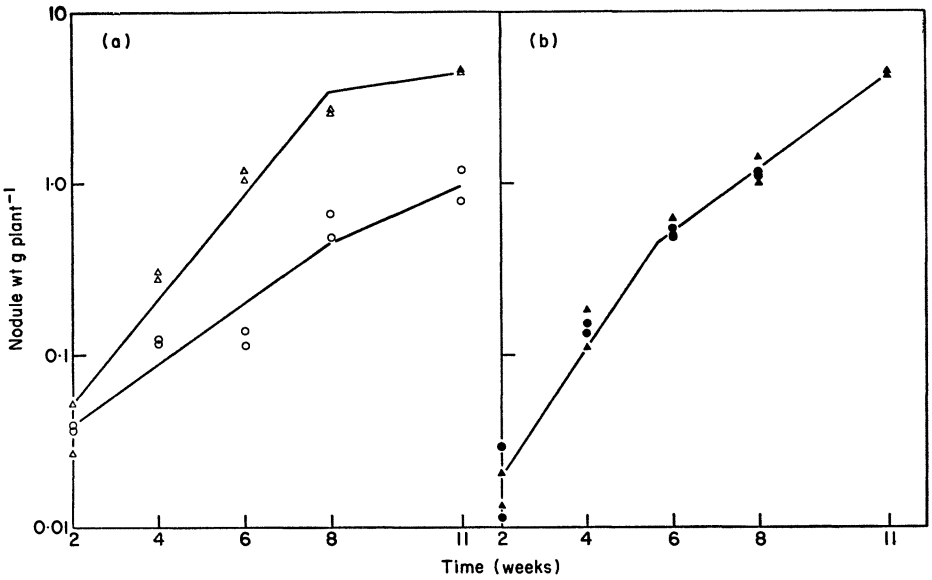

Fig. 2. Wet weight of lateral root nodules on sweet lupins as a function of time. (a) Inoculated; ○, plus cobalt; △, minus cobalt. (b) Not inoculated; ●, plus cobalt; ▲, minus cobalt.

contributed by crown nodules is plotted against time. In both plus-cobalt treatments, there was an increase in proportion of nodules on the crown up to 6 weeks; the effect was much more pronounced with inoculation than without. Relative importance of crown nodulation was always low in the absence of cobalt, and it declined with time irrespective of inoculation.

In the uninoculated treatments, where nodules were formed by *R. lupini* strains

already in the soil, the effect of cobalt deficiency on crown nodule formation [(Fig. 1(b))] was qualitatively similar to that in treatments inoculated with WU 425, but lateral root nodulation was not affected by cobalt deficiency [Fig. 2(b)], possibly because both represent maximum lateral nodule development.

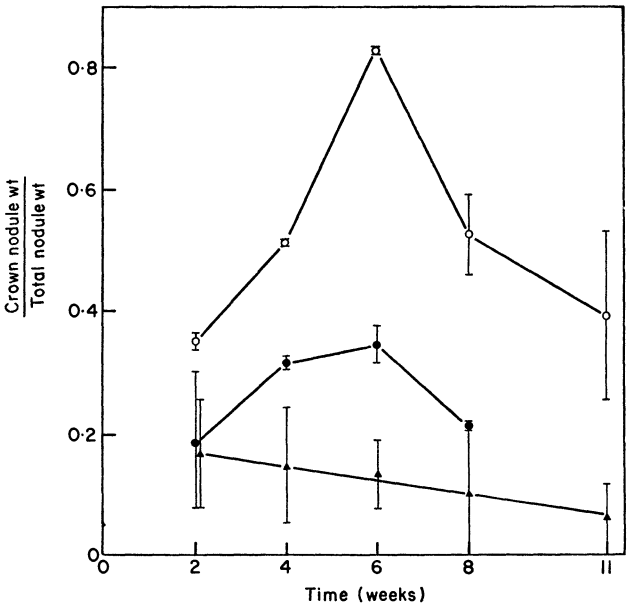

Fig. 3. Crown nodule weight as a fraction of total nodule weight as a function of time. Mean of 2 to 4 estimates, with bars representing standard deviation. ○, Plus cobalt, inoculated; ●, plus cobalt, not inoculated; ▲, minus cobalt, inoculated or not inoculated.

Table 1. *The identity of* Rhizobium lupini *isolated from sweet lupins grown in Lancelin soil in pots*

Treatment	Identity	Nodule position on root		
		Tap root	Lateral root	Total
Inoculated + cobalt	Like WU 425	11	11	22
	Unknown	2	2	4
Inoculated − cobalt	Like WU 425	8	10	18
	Unknown	3	5	8

In earlier pot experiments (Chatel *et al.*, 1978) it was shown (Table 1) that when WU 425 was added as the inoculant strain, it formed the majority of both crown and lateral nodules. It therefore appears that the difference in response to cobalt between treatments with inoculant WU 425 and those with naturally occurring soil rhizobia must lie in the quality of the lateral nodules formed by WU 425 compared with the other strains.

Nodule cobalt concentrations

Cobalt fertilization clearly increased nodule cobalt concentrations whether inoculant WU 425 was added or not (Table 2). Cobalt accumulated during growth in both crown and lateral nodules in all treatments except that missing both cobalt and inoculation. With the possible exception of those in the latter, lateral nodules had considerably higher cobalt concentrations than crown nodules. In the absence of added cobalt, inoculation with WU 425 was associated at the 11-week harvest with a cobalt accumulation in both crown and lateral nodules, which did not occur without

Table 2. *Mean cobalt concentrations (ng g dry wt^{-1}) in nodules and roots as a function of time*

Cobalt	+			+		
Inoculation	+			−		
	Nodules			Nodules		
	C*	L*	R*	C	L	R
4 weeks†	116	232	865	125	199	710
6 weeks†	105	221	856	167	256	1080
11 weeks‡	267	412	595	326	483	370
Cobalt	−			−		
Inoculation	+			−		
	Nodules			Nodules		
	C*	L*	R*	C	L	R
4 weeks†	34	90	148	60	52	142
6 weeks†	45	64	181	38	62	165
11 weeks‡	95	198	120	45	61	153

* C, Crown; L, lateral; R, roots.
† Mean of two replicate analyses.
‡ One analysis only.

inoculation. If cobalt has to accumulate to a critical level before N_2 fixation is initiated, this apparent ability of nodules on inoculated plants to accumulate cobalt may explain how inoculation overcomes cobalt deficiency. Despite very large increases in lateral nodule cobalt content in cobalt-deficient uninoculated plants, the very rate of nodule proliferation appears to prevent any improvement in cobalt concentration.

Nitrogen fixation and plant growth

The variation of plant nitrogen content with time is shown in Figure 4. There was little difference between the two cobalt-fertilized treatments, but cobalt deficiency clearly delayed increases in plant nitrogen until about 6 weeks. Inoculation with WU 425 allowed a greater increase in nitrogen content than occurred with rhizobia already present in the soil. This effect cannot be due to cobalt added with the inoculum; amounts present in it (0·06 ng cm^{-3}) were inadequate to explain the increased cobalt content of inoculated plants.

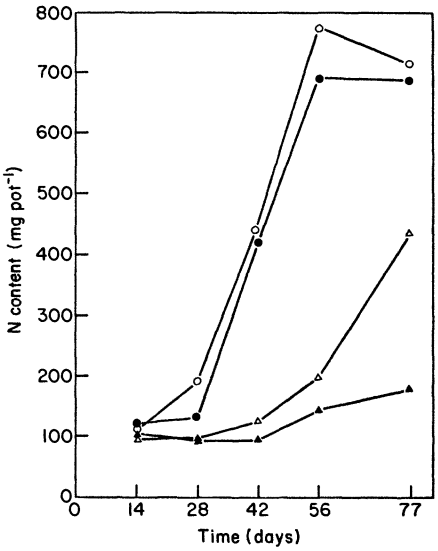

Fig. 4. Nitrogen contents of treatments (8 plants per pot) as a function of time. ○, Plus cobalt, inoculated; ●, plus cobalt, not inoculated; △, minus cobalt, inoculated; ▲, minus cobalt, not inoculated.

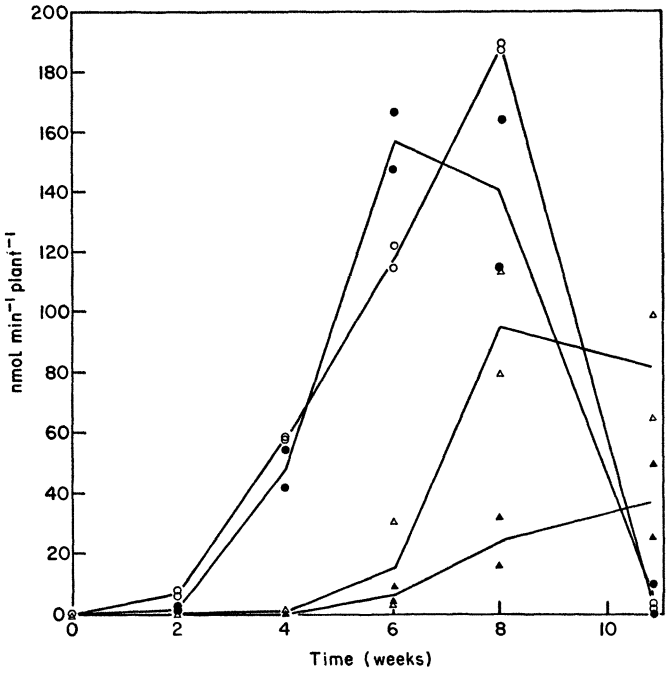

Fig. 5. Acetylene-reducing activity per plant as a function of time. ○, Plus cobalt, inoculated; ●, plus cobalt, not inoculated; △, minus cobalt, inoculated; ▲, minus cobalt, not inoculated.

Acetylene reduction activity per plant varied with time as shown in Figure 5. The activity in cobalt-treated plants declined rapidly after flowering began at about 6 weeks, as in other studies (Farrington *et al.*, 1977) and there was little difference between inoculated and uninoculated treatments. Cobalt-deficient plants inoculated with WU 425 eventually reached 50% of the activity shown by normal plants but cobalt-deficient uninoculated plants never exceeded 20 to 25%. Both types of cobalt-deficient lupin showed delayed flower initiation compared to the cobalt-fertilized plants. The rise in acetylene reduction activity after 6 weeks in the cobalt-deficient inoculated treatment appeared to coincide with the increase in nodule cobalt concentration (Table 2). A similar late development of nodule function has been reported for lucerne (Delwiche, Johnson and Reisenauer, 1961).

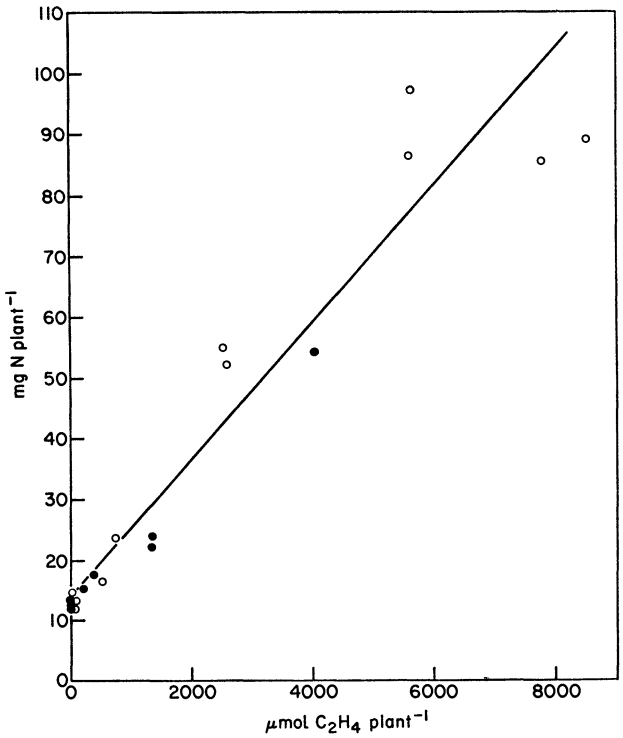

Fig. 6. Relationship between cumulative acetylene reduction at various times and plant nitrogen content. ○, Plus cobalt; ●, minus cobalt.

Cumulative acetylene reduction was calculated by integrating the areas under the curves in Figure 5, assuming that diurnal fluctuation in activity is slight in this species at constant temperature (Trinick *et al.*, 1976). Plant nitrogen content was very strongly correlated ($r = 0{\cdot}97$, $P < 0{\cdot}001$) with cumulative acetylene reduction (Figure 6); cobalt-fertilized and cobalt-deficient plants fitted the same curve, so that there was no indication of a difference in the acetylene:N_2 ratio caused by cobalt deficiency.

Bacteroid numbers

Bacteroid numbers per unit wet weight of nodules were decreased by cobalt deficiency (Table 3), as reported for the field situation (Chatel *et al.*, 1978). In the

inoculated treatments, the bacteroid numbers in crown and lateral nodules were followed separately after the trends of Figures 1 and 2 were recognized. In the inoculated plus cobalt treatment, there was no difference between crown and lateral nodules in bacteroid concentration up to week 11, after which crown nodule senescence became obvious. In the inoculated minus cobalt treatment, however, lateral nodules contained 3 to 4 times the bacteriod density found in crown nodules (Table 4) from 6 to 11 weeks.

Table 3. *Numbers of bacteroids* ($\times 10^{-9}$) *per g fresh wt of nodules (means of two determinations)*

	Time (weeks)				
	2	4	6	8	11
Inoculated + cobalt	22·7	32·1	27·3	35·7	15·0
Inoculated − cobalt	8·0	13·4	15·0	14·6	10·7
Not inoculated + cobalt	23·7	30·4	23·5	20·5	8·3
Not inoculated − cobalt	14·3	14·9	18·6	17·2	5·6

Significance levels: cobalt, $P < 0{\cdot}001$; time, $P < 0{\cdot}05$.

Table 4. *Numbers of bacteroids* ($\times 10^{-9}$) *per g fresh wt of nodules in the inoculated minus cobalt treatment (means of two determinations)*

	Time (weeks)		
	6	8	11
Crown	4·8	3·5	4·2
Lateral	16·6	15·1	11·3

Significance level: nodule location, $P < 0{\cdot}001$.

Vitamin B_{12}

Since other workers (Kliewer and Evans, 1963b; Wilson and Hallsworth, 1965b) have shown that vitamin B_{12} materials are found in both plant and bacteroid fractions of nodule homogenates, probably indicating leakage from the bacteroids, no attempt was made to partition the nodules.

Nodule cobalamin levels were lower in cobalt-deficient lupin nodules than in cobalt-normal ones (Table 5), in agreement with earlier results (Ahmed and Evans, 1961; Kliewer and Evans, 1963b; Wilson and Hallsworth, 1965b) with other legume root nodules. Nodules on plants inoculated with WU 425 had higher cobalamin contents than those caused by rhizobia already present in the soil.

Cobalamin concentrations measured in lupin nodules were comparable to those reported for soybean nodules (Ahmed and Evans, 1961) but much lower than those reported for other legumes (Kliewer and Evans, 1962a, b; Wilson and Hallsworth, 1965b). They were consistent with the nodule cobalt concentrations recorded in Table 2. Values from dimethylbenzimidazolyl cobamide coenzyme (DMBC) assays (Kliewer and Evans, 1962a, b) for a variety of legume nodules fell in the range 18 to 64 nmol g fresh wt^{-1}; the corresponding cobalt concentrations would have been 1060 to 3780 ng cobalt g fresh wt^{-1}. In lupin nodules, values for cobalt concentration did

not exceed 20 ng g fresh wt^{-1} over the first 6 weeks. Values for cobalamin concentration could not therefore reach the levels found for other nodule systems.

Cobalamin concentrations reported for subterranean clover nodules ranged from 30 μg g fresh wt^{-1} in cobalt deficiency to 79 μg g fresh wt^{-1} with 1 μM cobalt in the nutrient solution (Wilson and Hallsworth, 1965b). These cobalamin concentrations correspond to cobalt concentrations of 1300 to 3440 ng g fresh wt^{-1} of nodules; since cobalt concentrations in lupin nodules were nowhere near these levels, similar concen-

Table 5. *Cobalamin concentration (ng g fresh wt^{-1}) in nodules of* L. angustifolius

	Nodule age (weeks)							
	2		4		6		11	
	Inoc.	Not inoc.	Inoc.	Not inoc.	Inoc.	Not inoc.	Inoc.	Not inoc.
Plus cobalt	30·2	28·8	38·5	30·9	28·3	13·3	36·9	21·7
Minus cobalt	16·5	15·5	12·0	10·3	5·9	4·7	8·2	4·7

Significance levels: cobalt, $P < 0{\cdot}001$; inoculation, $P < 0{\cdot}05$; time, $P < 0{\cdot}05$.

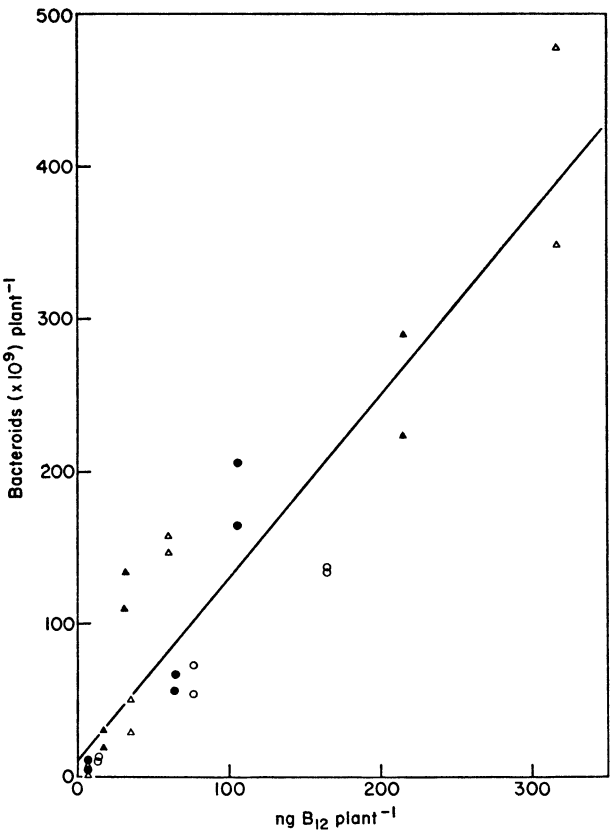

Fig. 7. Relationship between nodule cobalamin content and total bacteroid number per plant. Data shown are for the various treatments at 2, 4, 6 and 11 weeks. ○, Plus cobalt, inoculated; ●, plus cobalt, not inoculated. △, minus cobalt, inoculated; ▲, minus cobalt, not inoculated.

trations of cobalamin to those reported by Wilson and Hallsworth (1965b) could not be expected. The explanation for the markedly lower cobalt and cobalamin concentrations in lupin nodules remains unclear; it is unlikely to be due to inefficient extraction or conversion to cyanocobalamin under the conditions used.

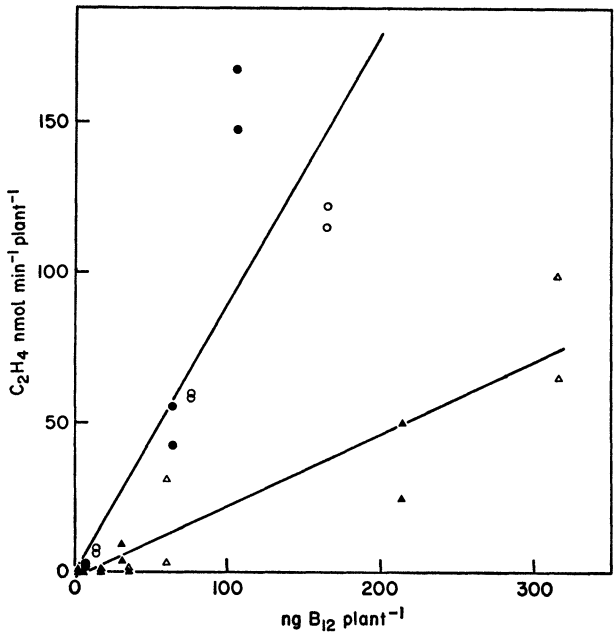

Fig. 8. Relationships between acetylene reduction activity per plant and cobalamin content of nodules. Both regression lines are significant ($P < 0{\cdot}01$); the difference in slopes and intercepts are also significant ($P < 0{\cdot}001$). ○, ●, Plus cobalt; △, ▲, minus cobalt.

Only a relatively small proportion (less than 12%) of total nodule cobalt was present as cobalamin, even in cobalt deficiency, and the proportion was lower in cobalt-treated plants. This raises some questions about the chemical form and location of the major fraction, and why it was not available for cobalamin formation. In root nodules of cobalt-deficient non-legumes the proportion of cobalt found as cobalamin was also low (2%) and that in normal nodules even lower (0·3%) according to Hewitt and Bond (1966).

The vitamin B_{12} content of the nodules and the number of bacteroids per plant were strongly correlated ($r = 0{\cdot}92$, $P < 0{\cdot}001$), as shown in Figure 7. The conversion of cobalt into vitamin B_{12} by the bacteroids may therefore limit the rate of production of the bacteroids.

Nitrogen fixation–vitamin B_{12} relationship

Since cobalamin content apppeared to determine bacteroid content of nodules and since acetylene reduction is a bacteroid property, a single relationship between acetylene-reducing activity and cobalamin content would have been expected. The actual results are shown in Figure 8; there were clearly two response curves for nitrogen fixation versus cobalamin content, depending on the cobalt status of the plants. The two regression lines have different slopes ($P < 0{\cdot}001$) and cannot be due to

different bacteroid contents, since Figure 7 shows that there is a single relationship between bacteroid numbers and cobalamin content in lupin nodules.

As expected, a plot of acetylene-reducing activity per plant versus bacteroid numbers per plant also generated two lines of different slope, depending on the cobalt status of the plants. Hence, although cobalamin content appeared to dictate the number of bacteroids per plant, the bacteroids still varied considerably in nitrogen-fixing ability, depending on the cobalt status of the nodules.

Leghaemoglobin and vitamin B_{12}

Since decreases in leghaemoglobin content and concentration have been shown in the nodules of cobalt deficient legumes (Ahmed and Evans, 1961; Wilson and Hallsworth, 1965a; Manorik and Lisova, 1969; Gladstones *et al.*, 1977), the relation-

Table 6. *Leghaemoglobin concentrations (mg g fresh wt^{-1}) in nodules of cobalt-deficient and normal lupins*

	Plus cobalt		Minus cobalt	
Weeks	Inoculated	Not inoculated	Inoculated	Not inoculated
4	—	1·14	—	0·62
6	1·91	1·16	0·71	0·76
8	1·82, 1·73	1·54, 1·59	1·28, 1·22	0·79, 1·07
Mean	1·57		0·92	

ships between cobalt, leghaemoglobin, cobalamin and acetylene reduction activity per plant were also checked. Cobalt deficiency resulted in lowered leghaemoglobin concentration in nodules ($P < 0{\cdot}001$) at the 4-, 6- and 8-week harvests (Table 6). Nodule senescence in the plus cobalt treatments was obvious at the week-11 harvest and was associated with loss of leghaemoglobin. For this reason, leghaemoglobin values from the plus cobalt treatments were not used after 8 weeks.

Several investigators have shown correlations between N_2 fixation and leghaemoglobin concentration in legume nodules (Virtanen, Erkama and Linkola, 1947; Jordan and Garrard, 1951; Graham and Parker, 1963), so that a single response curve would be expected. Low cobalt would be expected to be associated with low leghaemoglobin concentration and therefore low N_2 (C_2H_2) fixation. However, when plus and minus cobalt treatments were compared over the course of the present experiment, the data of Figure 9 were obtained. Both regression lines have an associated probability less than 0·01 and the slopes were significantly different ($P < 0{\cdot}01$). Leghaemoglobin at a particular level apppeared to be less 'effective' in promoting N_2 fixation if cobalt were deficient.

When the relationship between cobalamin content and leghaemoglobin content was examined, the data shown in Figure 10 were observed. The relationship between cobalamin and leghaemoglobin contents had a correlation coefficient of 0·91 ($P < 0{\cdot}001$). The data imply that while cobalamin content determines leghaemoglobin content, probably via its effect on bacteroid propionate–succinate interconversions, there is a difference between plus and minus cobalt treatments in the usefulness of that leghaemoglobin for N_2 fixation.

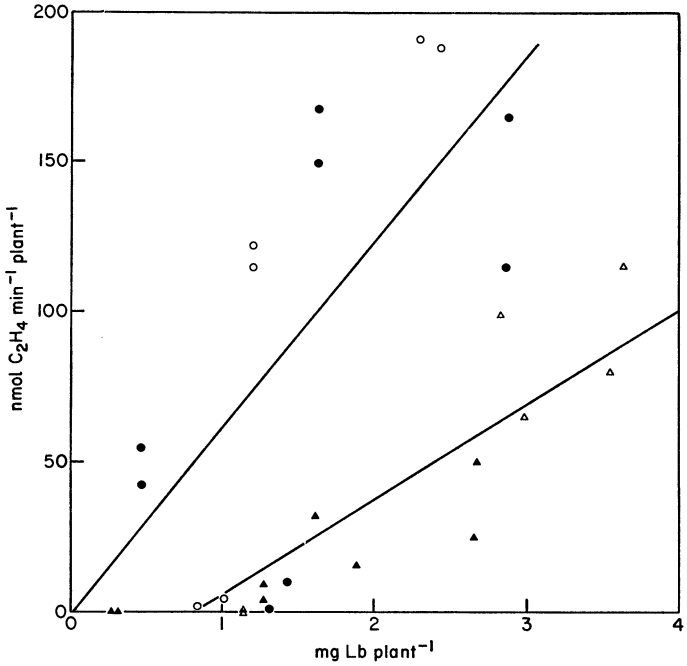

Fig. 9. Relationships between leghaemoglobin content of lupin nodules and acetylene reduction activity per plant. Both regression lines are significant ($P < 0{\cdot}01$); the slopes and intercepts are significantly different ($P < 0{\cdot}01$). ○, ●, plus cobalt; △, ▲, minus cobalt.

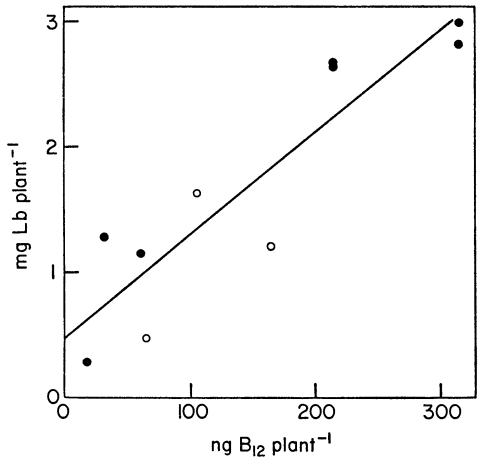

Fig. 10. Relationship between cobalamin and leghaemoglobin contents of lupin nodules. Regression significant ($P < 0{\cdot}001$). ○, Plus cobalt; ●, minus cobalt.

Discussion

The responses of lupins to soil application of cobalt to cobalt-deficient plants were quite similar to those of other legumes – increased nitrogen content, cobalt concentration, leghaemoglobin concentration and cobalamin concentration. In addition nodule distribution within the root system was observed to be sensitive to cobalt.

Crown (early) nodule development was considerably reduced by cobalt deficiency, with or without inoculation. The possible reasons for the decrease in crown nodule development include:

(a) less multiplication of nodule bacteria in the rhizosphere due to lack of cobalt;
(b) a similar number of nodule initiations with or without cobalt, but lowered development rates of nodules thereafter due to cobalt deficiency;
(c) a reduction by cobalt deficiency of the number of nodule initiations but a normal rate of growth thereafter.

The first possibility has never been examined experimentally but the cobalt concentration in soil has been reported to be far in excess of that found in subterranean clover growing on it (Ozanne, Greenwood and Shaw, 1963). Hence rhizobial multiplication in soil or at the soil–plant interface may not be restricted by cobalt deficiency as severely as subsequent multiplication within the plant. However, if cobalt availability were very low this conclusion might well be invalid. Since lupin seed of high cobalt concentration produces plants which do not show cobalt deficiency on Lancelin soil (Mead and Robson, unpublished), external cobalt is probably not important in rhizosphere multiplication of *Rhizobium lupini*.

The second possibility is not borne out by Figures 1 and 2, where the logarithmic rates of increase are virtually identical with or without cobalt. This lends some support to the idea that less early nodules may be initiated in cobalt-deficient plants. Further experiments on this point are being undertaken.

In cobalt-deficient plants inoculated with the highly effective commercial strain WU 425, there was a compensatory increase in lateral root nodule development. In plants dependent on rhizobia already present in the soil this compensation did not occur; plus and minus cobalt treatments had similar lateral root nodule masses. Total lateral root nodule mass was essentially similar in cobalt-deficient plants whether or not they were inoculated [Figs 1(b) and 2(b)].

The specific nitrogen-fixing activity of these lateral root nodules was almost certainly very low. The maximum specific activities achieved in the minus cobalt treatments were (in nmoles min^{-1} g fresh wt^{-1}): inoculated, 31; uninoculated, 13, compared with a peak value of 238 for the plus cobalt inoculated treatment. Since the minus cobalt treatments have 77 to 95% of their nodule weight as lateral nodules, it follows that the lateral root nodule activity was almost certainly low.

The mechanism that makes lateral nodulation of cobalt-deficient plants effective where crown nodulation is ineffective seems to be associated with cobalt accumulation. Lateral nodules on inoculated plants showed an apparent increase in cobalt concentration between 6 and 11 weeks; nitrogen fixation activity increased over the same time period in the inoculated treatment. Where there was no increase in cobalt concentration in lateral nodules of uninoculated cobalt-deficient plants, there was only slight nitrogen fixation activity.

It appears likely that below a critical cobalt concentration in the nodule normal development of nitrogenase activity does not occur. Inoculation of cobalt-deficient plants could therefore be seen as producing lateral root nodules able to concentrate cobalt to the level allowing at least partial nitrogenase activity to occur. The inoculation effect in overcoming cobalt deficiency may therefore be variable with rhizobial strain.

Inoculation was also associated with higher cobalamin concentration (Table 5) and

higher bacteroid density in lateral nodules of minus cobalt plants (Table 4). From these observations it would also appear that crown nodules on minus cobalt plants are virtually ineffective.

The interrelations between cobalamin content, bacteroid content and $N_2(C_2H_2)$ fixation appear to be more complex than expected. Bacteroid numbers and leghaemoglobin content were apparently directly dependent on cobalamin content, but the N_2-fixing activity of the bacteroids appeared to be affected by cobalt status as well as cobalamin content. Thus the 'effectiveness' of bacteroids or leghaemoglobin in N_2 fixation showed an influence of cobalt even after the effect of variation due to cobalamin content had been removed.

The simplest explanation would be that in the bacteroids there is a cobalt-dependent process required for nitrogenase activity which is not a cobalamin-mediated one. Cobalt deficiency would then have two effects – one mediated via the cobalamin dependent processes discussed earlier, and the other operating via some other cobalt compound not measured as cobalamin. Since the plus- and minus-cobalt bacteroids are non-equivalent in nitrogenase function, this other form of cobalt is more likely to be in the bacteroids than in the plant fraction, and cannot be operating through effects on leghaemoglobin production.

There is as yet no firm evidence for any such compound or a function for it. The non-cobalamin cobalt compound reported in non-nodulated *Trifolium subterraneum* (Wilson and Nicholas, 1967) and the cobalt fraction which was neither Co^{2+} nor cobalamin in *T. subterraneum* nodules (Wilson and Hallsworth, 1965b) seem likely to have been cobalt complexes with amino or organic acids. That no amino acid was found associated with the ^{60}Co used by Wilson and Hallsworth (1965b) probably only meant that the amount found with ^{60}Co was too small to be measured as amino acid. Acceptance of any suggestion for a further role for non-cobalamin cobalt in nodules will require demonstration both of the chemical nature of such cobalt and how it might function in relation to N_2 fixation.

In *Rhizobium* the cobalt concentration required for growth on nitrate has been reported to be higher than that for growth on ammonia (Kliewer and Evans, 1963b; Nicholas, Maruyama and Fisher, 1962). Similarly, cobalt has been reported as being required for growth of *Azotobacter vinelandii* on N_2 (Kliewer and Evans, 1963b; Nicholas, Kobayashi and Wilson, 1962) but not on ammonia (Kliewer and Evans, 1963b). No satisfactory explanation of these effects has yet been put forward; since cobalt concentrations in the different types of cells were not measured, it remains possible that ammonia facilitated the uptake of cobalt, or that ammonia leached cobalt from the vessels used (Evans *et al.*, 1965).

Cobalt at 50 μM largely replaced the nickel requirement for urease production (Polacco, 1977) in *Lemna paucicostata* (Gordon, Schwemmer and Hillman, 1978). The suggestion that cobalt acted by releasing otherwise chelated essential metal (in that case nickel) might also apply to the present study. However, since there was a clear response to cobalt when nodules increased from 0·05 to 0·2 μM, this seems an unlikely mechanism for the non-cobalamin effect.

An effect of cobalt separate from its function in cobalamin need not be directly on nitrogenase – indeed, there is no evidence that metals other than molybdenum and iron are required. However, cobalt would have to operate on some pathway affecting N_2 fixation – electron supply, energy supply or possibly an uptake hydrogenase system (Dixon, 1972; McCrae, Hanus and Evans, 1978). Since only very small

concentrations of cobalt are likely to be involved in such a process, they could very easily be overlooked.

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