NITROGEN FIXATION IN LEGUMINOUS PLANTS. III.

THE IMPORTANCE OF MOLYBDENUM IN SYMBIOTIC NITROGEN FIXATION.

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Introduction.

The process of nitrogen fixation by *Azotobacter* is activated by molybdenum, which functions as a specific catalyst, replaceable only by vanadium (Wilson, 1940). A similar effect of molybdenum on the symbiotic nitrogen fixation by leguminous plants and root-nodule bacteria might be expected, particularly since Wyss *et al.* (1941) have brought forward strong evidence to show that the biochemical mechanism of nitrogen fixation is essentially the same in legumes and in *Azotobacter*. It has also been shown conclusively by Arnon and Stout (1939) and by Piper (1940) that molybdenum is one of the several "minor" elements required by higher plants. Its precise function in these is unknown, but doses as small as 0·01–0·02 mgm. per litre seem sufficient for normal growth of oats and tomatoes in water culture. Concentrations higher than these minute amounts might be optimal for leguminous plants, if molybdenum fulfils a specific function in the process of nitrogen fixation besides being necessary for growth in general. There is some actual evidence that legumes have a particularly high demand for molybdenum. Ter Meulen (1930) and Bertrand (1940*a*) found that leguminous materials, and especially the seeds, were as a whole considerably richer in molybdenum than other vegetable matter. Bertrand (1940*c*) also showed that root-nodules of various legumes generally contained more molybdenum than the roots themselves, and these again more than the tops. On the other hand, Dingwall *et al.* (1934) failed to detect molybdenum in the tops of legumes from all except one locality in Canada, and Konishi and Tsuge (1936) found that it was often absent from the roots of some legumes and could not always be detected in the nodules.

Direct experiments on the influence of molybdenum compounds on the growth of legumes in nitrogen-deficient media were first made by Bortels (1937); greenhouse experiments with peas, soy-beans and red clover grown in sand gave somewhat irregular results, but field trials with lucerne showed that both molybdenum and vanadium had favourable effects which in some cases appear significant, as discussed by Wilson (1940). Obraztsova *et al.* (1937) state that the yields of peas and lupins in both pot and field trials were increased by small doses of molybdenum, but these results, too, seem erratic, and were not proved to be statistically valid. These authors, as well as Bortels, point out that harmful effects may result from over-dosage. Kronberger and Lehner (1938) found in one case a favourable effect of molybdenum on red clover in sand culture, but the results were not consistent. Bertrand (1940*b*) grew peas in liquid medium of very low molybdenum content (4 γ/l.), and found that a dose of 40 γ/l. increased the yield of dry matter by about 50%, while 400 γ/l. again caused a decrease. His figures show that the growth of the plants must have been rather poor, and the agreement between replicates was not stated. Bobko and Savvina (1940) observed a small but significant stimulating effect of 0·05–0·10 p.p.m. molybdenum on peas in water culture, but not in sand or soil. They also state that no nodules were formed in molybdenum-free water or sand cultures, and that nodules were formed in largest numbers in sand with 0·01–0·1 p.p.m. molybdenum. This statement is difficult to reconcile with the apparently very good growth of the plants in the Mo-free media.

Anderson (1942) saw a great response of mixed grasses and clover to molybdenum in field trials on a South Australian soil; pot experiments showed that the yield of lucerne was increased approximately 100% by a small dose of sodium molybdate. This is unquestionably significant, but since the effect was also observed in grasses, it remains uncertain whether the treatment had exerted a specific stimulating influence on nitrogen fixation or whether it had merely corrected a general deficiency of molybdenum.

All these experiments, except the few water-culture tests of Bertrand (1940*b*), seem to have been carried out in media where the amount of molybdenum present as impurity in the various constituents was not analytically checked. Bortels (1937), indeed, states that a Mo-free sand was used, but gives no analytical figures. We have therefore made a number of experiments in media of known molybdenum content in order to arrive at a closer understanding of the importance of this element for the nutrition of leguminous plants.

Experimental.

Methods.—Annual legumes with big seeds, such as peas, lupins or soy-beans, would appear less suitable for experiments of this kind, on account of the considerable reserves of molybdenum that may be carried by the seed. We have therefore worked only with small-seeded plants, viz., lucerne (*Medicago sativa*) and clovers, mostly white clover (*Trifolium repens*). Since we did not primarily aim at demonstrating the theoretical necessity of molybdenum for green plants, we have not attempted to prepare an absolutely Mo-free medium; this is exceedingly difficult, if not impossible (cf. Bertrand, 1940*b*; Piper, 1940). Instead, we have used basal media of low but analytically defined molybdenum content, and have tested whether any extra supply of molybdenum, in addition to the traces present in the control medium plus the seed, resulted in a stronger nitrogen fixation. The vanadium content of the media was also determined. Plants were grown under greenhouse conditions, partly in sterile agar medium, partly in sand, and inoculated with effective strains of the corresponding root-nodule bacteria. Nitrogen was determined by the Kjeldahl method, with selenium as a catalyst.

Molybdenum in sands and soils was extracted and estimated by the method of Stanfield (1935), except that ether was used as an extractant and the concentrations of reagents modified according to the findings of Hurd and Allen (1935). Plant materials were ashed wet (no muffle furnace being available), the ash was fused with sodium carbonate, and the melt treated and molybdenum estimated by the method of Sandell (1936), which was modified slightly: a blank containing a known amount of molybdenum and an amount of sodium carbonate equal to that used in the fusion of the unknown was treated similarly to the unknown, and the ether-extracts compared in a colorimeter with micro-cups. The following quantities of molybdenum were found in the samples of seed used:

	Medicago sativa.	*Trifolium repens.*	*T. subterraneum.*
P.p.m.	2·5	0·62	2·9
γ per seed	0·0055	(<0·001)	0·020

For the estimation of vanadium, plant materials were ashed by ignition, the ash fused with sodium carbonate, and vanadium then determined by the method of Sandell (1936). Sands were powdered and fused with sodium carbonate and the estimations carried out in the same way.

Cultures in Agar.—The medium was the same as previously used (Jensen, 1942): $CaHPO_4$ 0·1%, $MgSO_4$ 0·02%, K_2HPO_4 0·02%, NaCl 0·02%, $FeCl_3$ 0·01%, washed agar 0·6%; pH 6·5–6·6. The analysis showed a content of 1·4 γ molybdenum and 5·0 γ vanadium per litre of medium. Cultures of *Azotobacter chroococcum* in 25-ml. portions of this medium with addition of 0·1% $CaCO_3$ and 2·0% glucose or mannite fixed 1·8–2·0 mgm. N in 2–3 weeks at 28–30°C.; when 0·0001 or 0·002% Na_2MoO_4 was added, the fixation was increased to 4·2–6·6 mgm. The basal medium is thus clearly deficient in molybdenum (plus vanadium) towards *Azotobacter*, even allowing for the possibility that further small amounts of the two metals may have been introduced with the calcium carbonate and the sugar.

The following experiments were set up: (1) *Trifolium glomeratum*, 3 plants and 40 ml. medium in test-tubes, grown for 104 days. (2) *T. repens*, 6 plants and 120 ml. medium in 1-pint bottles, 125 days. (3) *Medicago sativa*, 2 plants and 20 ml. medium in test-tubes, 104 days. (4) *M. sativa*, 4 plants and 125 ml. medium in 1-pint bottles, 191 days. (5) *M. sativa*, 5 plants and 150 ml. medium in 1-pint bottles, 196 days. Doses of molybdenum, numbers of replicates, and means and standard deviations of nitrogen content of the cultures at the end of the experiments are shown in Table 1.

TABLE 1.
Influence of Molybdenum on Nitrogen Fixation in Agar Cultures.

Plant.	Addition to Medium.	Inoculation.	Replicates No.	Total N, mgm. per Culture.		Conc. of Mo, γ/plant.
				Mean.	S.D.	
1.	Nothing.	−	6	0·39	±0·068	0·03
Trifolium	Nothing.	+	6	1·80	±0·159	0·03
glomeratum.	0·001% Na_2MoO_4.	+	6	1·66	±0·193	93·23
2.	Nothing.	+	5	5·84	±0·243	0·03
Trifolium repens.	0·0001% Na_2MoO_4.	+	4	5·61	±0·241	7·83
3.	Nothing.	−	6	0·47	±0·047	0·03
Medicago	Nothing.	+	6	1·14	±0·128	0·03
sativa.	0·001% Na_2MoO_4.	+	6	0·88	±0·106	46·63
4.	Nothing.	+	3	7·32	±0·784	0·04
Medicago						
sativa.	0·0001% Na_2MoO_4.	+	4	8·55	±0·653	14·44
5.	Nothing.	−	2	1·65	±0·07	0·048
Medicago	Nothing.	+	6	10·8	±1·14	0·048
sativa.	0·000044% Na_2MoO_4.	+	6	11·1	±0·58	6·048
	0·000020% Na_2VO_3.	+	4	10·7	±1·13	0·048

In no case is the gain of nitrogen significantly increased by the extra supply of molybdenum. The only significant effect is an inhibition of lucerne by 0·001% Na_2MoO_4 (Exp. 3). If we compare the two sets of inoculated plants, the *t*-test (Fisher, 1936) shows that $n = 11$, $t = 3{\cdot}689$, $P < 0{\cdot}01$. Vanadium, too, is without effect (Exp. 5). Although the actual quantities of fixed nitrogen are small, they appear considerable in proportion to the small volumes of medium used; thus, the minute amounts of 0·03–0·05 γ Mo per plant, provided by medium plus seed, are sufficient under these conditions, where as much as 37,000 parts of N have been fixed for every part of molybdenum available (Exp. 5), and this even under the assumption that all the molybdenum is present in an assimilable form. The rate of plant growth, however, is always slow in agar cultures, and this circumstance may have prevented the extra doses of molybdenum from showing their stimulating effect. Other experiments were therefore performed under conditions allowing more rapid growth and representing more natural surroundings.

Cultures in Sand.—The medium was a coarse, almost nitrogen-free river sand, of pH 7·3, and by analysis found to contain 0·0046 p.p.m. Mo and 10 p.p.m. V (including the nutrients added at the start of the main experiment); it was thus poorer in molybdenum than certain Californian soils which van Niel (1935) found deficient in molybdenum towards *Azotobacter*. The plants were grown in glazed earthenware pots of 6 in. diameter. Two smaller experiments were carried out with white and subterranean clover in pots with 2·6 kgm. sand and 500 ml. nutrient solution corresponding to the agar medium, besides traces of Cu, Mn, Zn (as sulphates) and B (as sodium biborate), and doses of 0·5 and 2·5 mgm. Na_2MoO_4 per kgm. sand. After germination, 10 plants of white clover and 6 of subterranean clover were left per pot and allowed to grow for 92 and 87 days respectively. As shown in Table 2, molybdenum has no significant effect on yield or N-percentage of either plant; the apparent decrease in yield of white clover with the higher dose does not reach significance (P: 0·2–0·1).

TABLE 2.
Influence of Molybdenum on Yield of Clover in Sand Culture.

Plant.	Dose of Na_2MoO_4, p.p.m.	Replicates No.	Dry Matter, gm. per Pot.		Percentage N in Dry Matter.
			Mean.	S.D.	
White clover. (92 days.)	0	5	1·61	±0·225	2·96
	0·5	6	1·64	±0·254	3·01
	2·5	6	1·34	±0·225	3·00
Subterranean clover. (87 days.)	0	3	2·97	±0·129	3·11
	0·5	3	2·75	±0·710	3·05
	2·5	4	2·62	±0·495	2·97

Lucerne was the subject in the main experiment, where we followed the principle of Bortels (1937) in taking successive cuts of the crop, so as gradually to exhaust the control medium of molybdenum; this procedure should also serve to reduce the amount of molybdenum stored away in the roots, since periodical clipping of lucerne encourages the development of the tops at the expense of the root system (Thornton and Nicol, 1934). The medium consisted of 3 kgm. sand, to which at the outset of the experiment were added 500 ml. of Crone's solution, besides extra 0·2 gm. $CaHPO_4$, Mn, Cu, Zn and B as above, and doses of 1·0 and 5·0 mgm. Na_2MoO_4 per kgm. sand. The first crop was sown on 11th March, 1941, and 7 plants per pot were left after germination. The tops were clipped after 136, 178, 223 and 268 days, and the plants were finally harvested and the roots collected after 300 days. During the period of growth, each pot was given additional doses of 2 × 0·1 gm. K_2HPO_4, 2 × 0·1 gm. $CaCl_2$, 0·1 gm. $MgSO_4$, and 2 × 0·05 gm. $FeCl_3$. The plants were watered with tap-water, which was found to contain only 0·055 γ Mo per litre (0·0011 mgm. Mo in evaporation-residue of 20 l.). The results are seen in Table 3.

TABLE 3.
Influence of Molybdenum on Yield of Lucerne in Sand Culture. (First Crop ; Six Replicate Pots, except in the Series – Mo, from which One Pot was omitted owing to Bad Germination, leaving only Six Plants.)

Yield and Composition of Crop.		−Mo (Control).		1 p.p.m. Na_2MoO_4.		5 p.p.m. Na_2MoO_4.	
		Mean.	S.D.	Mean.	S.D.	Mean.	S.D.
Dry Matter, gm. per Pot.	Tops.	7·71	±0·661	7·73	±1·375	7·47	±1·146
	Roots.	1·33	±0·168	1·34	±0·239	1·28	±0·228
Percentage N in Dry Matter.	Tops.	3·70		3·59		3·70	
	Roots.	1·86		1·67		1·76	
Mo in Dry Matter, p.p.m.	Tops.	0·64		6·0		21·0	
	Roots.	8·1		12·6		73·0	

Neither the yields of dry matter nor the percentages of nitrogen are influenced by the addition of molybdenum, of which the small amount in the control medium, corresponding to approximately 2 γ per plant, has thus been sufficient under these conditions. The analysis of the crop shows that nearly 20,000 parts of nitrogen have been fixed per part of molybdenum assimilated by the control plants, and that the roots are much richer in molybdenum than the tops (cf. Bertrand, 1940*c*), especially in the control plants. A simple calculation shows that the sand in the control pots should now be theoretically exhausted of molybdenum: each pot contained at the start 13·8 γ Mo, while an average of 15·7 γ was recovered in the crop. These figures agree within the limits of error to be expected. On 16th February, 1942, a second crop of lucerne was sown in the same pots, each of which was given the following fertilizer mixture: $CaHPO_4$ 0·6 gm., KCl 0·1 gm., $MgSO_4$ 0·05 gm., $FeCl_3$ 0·05 gm., $MnSO_4$, $ZnSO_4$ and $CuSO_4$, 2 mgm. each. Eight plants were left per pot, and this time distilled water was used for watering. The tops were clipped after 124 and 175 days, and the experiment was terminated after 242 days. Table 4 shows the results.

TABLE 4.

Influence of Molybdenum on Yield of Lucerne in Sand Culture. (Second Crop; Six Replicates in Each Treatment.)

Yield and Composition of Crop.		−Mo.		1 p.p.m. Na_2MoO_4.		5 p.p.m. Na_2MoO_4.	
		Mean.	S.D.	Mean.	S.D.	Mean.	S.D.
Dry Matter, gm. per Pot.	Tops, cuts 1+2.	2·10	±0·334	2·48	±0·659	2·51	±0·287
	Tops, 3rd cut.	3·41	±0·321	4·13	±0·452	4·63	±0·365
	Roots.	2·34	±0·334	2·32	±0·299	2·18	±0·208
Percentage N in Dry Matter.	Tops.	3·51		3·78		3·71	
	Roots.	2·43		2·84		2·93	
Mo in Dry Matter, p.p.m.	Tops.	0·38		2·6		9·6	
	Roots.	0·37		5·3		13·7	
V in Dry Matter of Roots, p.p.m.		0·9		1·0		1·1	

The slight increases due to molybdenum in the two first cuts are hardly significant, as shown by the *t*-test:

Dose.	Increase, gm.	*n.*	*t.*	*P.*	
1 Mo	0·38	10	1·261	0·3 –0·2	(not significant)
5 Mo	0·41	10	2·231	0·05–0·02	(doubtful)

The final harvest, however, shows a clearly significant response to molybdenum in the weight of the tops:

Dose.	Increase, gm.	*n.*	*t.*	*P.*	
1 Mo	0·72	10	3·182	<0·01	(significant)
5 Mo	1·22	10	6·122	<0·01	(significant)

The dry weight of the roots is not significantly affected, but the percentage of nitrogen is appreciably lower in the roots of the molybdenum-deficient plants, and there is some indication of a similar effect on the nitrogen content of the tops. The analytical data also show that even from the theoretically molybdenum-free sand the plants have been able to assimilate an average of nearly 3 γ Mo per pot, probably derived from impurities in the nutrient salts and the water. This has still sufficed for the fixation of more than 80,000 parts of nitrogen per part of molybdenum. Since the response to extra supply of molybdenum is only moderate (20–30% in total weight of tops), this ratio does not appear to be greatly sub-optimal. It is therefore not surprising that no effect of molybdenum was observed in the first crop of lucerne, in the considerably smaller clover crops, or in the agar cultures. We thus find clear evidence that a certain, very small but not infinitestimal, supply of molybdenum is necessary for optimal growth of lucerne, and, as also shown by the data in Table 4, there is no indication of a compensatory assimilation of vanadium from the molybdenum-deficient sand. So far, it remains uncertain whether molybdenum acts specifically on nitrogen fixation, although this is suggested by the fact that not only the total yield but also the N-percentage is depressed by Mo-deficiency. That the better growth in the presence of molybdenum should be due to stimulated non-symbiotic nitrogen fixation by *Azotobacter* may be regarded as more than unlikely, in view of results recently found by one of us (Jensen, 1942).

Distribution of Molybdenum in Roots and Nodules.—As already mentioned, more molybdenum was generally found in the root system than in the tops, but insufficient material was available for separate analyses of roots and nodules. Some such determinations were therefore made in other materials available in larger quantity: *Trifolium subterraneum* and *Medicago arabica* growing wild, lucerne grown under greenhouse conditions in an acid soil with and without addition of 0·2% lime to produce alkaline reaction, and a field crop of soy-beans (*Glycine hispida*).* The lucerne soil was analysed

* We are indebted to Mr. R. J. Swaby, M.Agr.Sc., Department of Agriculture, N.S.W., for the soy-bean nodules.

for molybdenum after removal of the plants, and was found to contain 0·04 p.p.m. Mo. The lime contained 0·1 p.p.m. Mo; only an insignificant extra amount had thus been introduced in the alkaline soil. In the material of *M. arabica*, ash and vanadium were also determined. The following results were found:

	In Dry Matter of: Roots.	Nodules.
T. subterraneum: Mo, p.p.m.	1·8	19
M. sativa, soil pH 4·9-5·4: Mo, p.p.m.	0·6	3·2
,, ,, ,, ,, 7·5-8·0: Mo, p.p.m.	1·3	10
M. arabica: Mo, p.p.m.	3·0	20
,, ,, : V, p.p.m.	3·2	4·0
,, ,, : Ash, %	8·2	7·7
Glycine hispida: Mo, p.p.m.	0·4	5·9

The molybdenum content of the nodules is thus 5 to 15 times as high as that of the root-substance itself. This agrees in principle with the findings of Bertrand (1940*c*), whose paper was not seen by us until most of our experimental work had been finished; the difference, however, is far more striking in our data than in those of Bertrand, whose plant materials were derived from soils very rich in molybdenum (49–69 p.p.m.). In *M. arabica*, the difference in molybdenum content of nodules and roots is much the same whether it is calculated on the basis of dry matter or of ash (unlike Bertrand's findings in several cases), and vanadium shows no corresponding accumulation in the nodules. The soy-bean nodules were big enough to allow an approximate separation into cortex and bacterial tissue, which accounted for 41 and 59 per cent., respectively, of the total dry weight of the nodules. The bacterial tissue proved to be significantly richer in molybdenum, containing 6·9 p.p.m. Mo against 4·5 p.p.m. in the cortical portion.

Determination of molybdenum was also carried out in plants nourished with free and combined nitrogen. Lucerne was grown for 15 weeks in sand containing 0·02 p.p.m. Mo and 2·5 p.p.m. V, besides the usual mineral nutrients. Some pots were sown with seed inoculated with *Rhizobium Meliloti*, others with uninoculated seed but provided with ammonium nitrate; no nodules were found on the roots of these plants at the time of harvesting. Result:

Source of Nitrogen:	Mo, p.p.m., in Dry Matter of: Tops.	Roots.	Nodules.
Free Nitrogen	0·8	1·5	10·3
NH_4NO_3	0·30	0·45	—

The plants fixing free nitrogen show quite the same distribution of molybdenum as found before, and have taken up much more molybdenum than the plants provided with combined nitrogen; this difference is particularly marked in the root system. The remarkable difference in molybdenum content of lucerne from acid and alkaline soil (see above) might suggest that in the acid soil a larger proportion of the plants' supply of nitrogen is represented by combined nitrogen derived from the soil. The fact that legumes are not invariably richer in molybdenum than non-legumes (Bertrand, 1940*a*) might also be due, not only to varying molybdenum content of the soil (Bertrand, 1940*b*), but also to variations in the proportion of free and combined nitrogen assimilated by the legumes.

General Conclusions.

The results show clearly that reduction of the molybdenum content of the medium below a certain minimum, results in a decreased production of organic matter, and the marked accumulation of molybdenum in the nodules, as well as the stronger assimilation of molybdenum by plants dependent on free nitrogen, gives strong evidence that this element has a specific influence on the process of nitrogen fixation, besides being needed for the general metabolism. Also in this respect the biochemical mechanism of nitrogen fixation in legumes thus seems analogous to that in *Azotobacter*, where the assimilation of combined nitrogen is reported to show optimal stimulation by a concentration of molybdenum lower than that which is optimal for fixation of free nitrogen (Burk and Horner, cit. after Wilson, 1940). The margin of difference between the actual amounts of molybdenum needed for these two purposes seems to be very small, since further supply of molybdenum was without effect when the medium provided merely one part

of assimilable molybdenum per 20,000 parts of fixed nitrogen (Table 3), and the response was only moderate when the ratio was widened to 1:80,000 (Table 4). It therefore seems unlikely that direct increases in crop yield could be expected from the use of molybdenum salts as fertilizers, except on special soils like the one studied by Anderson (1942), which, to judge by the results in Tables 3 and 4, must have been extremely poor in assimilable molybdenum; but where heavy crops of legumes are regularly carried away from the land there would, even if most of the assimilated molybdenum remains in the roots and consequently in the soil, seem to be a good reason to provide against exhaustion of this and other "minor" elements by the use of fertilizers that contain these elements in sufficient quantity. Also the possibility is not excluded that higher concentrations of molybdenum than found by us might be optimal for other plants and under different conditions of growth (cf. Bortels, 1937).

The fact that Mo-deficient plants show no increased uptake of vanadium (Table 4) and that no accumulation of this element is noticeable in the nodules, suggests that vanadium is not capable of replacing molybdenum, such as it is to some extent in *Azotobacter*. In agreement herewith, Konishi and Tsuge (1936) found that vanadium was only rarely present in leguminous plants, even those containing no molybdenum. Further investigations, however, are needed on this point.

It also remains an open question whether molybdenum is absolutely essential or merely beneficial for plant growth and symbiotic nitrogen fixation. The morbid symptoms observed by Arnon and Stout (1939) and Piper (1940) speak for the first, but the occasional findings of Mo-free, nodule-bearing plants by Konishi and Tsuge (1936), as well as the results of Dingwall *et al.* (1934), for the second possibility. In this connection it is also worth noticing the observations of Bortels (1939) and Horner *et al.* (1942) on fairly strong nitrogen fixation by some strains of *Az. vinelandii* in (absolutely?) Mo- and V-free medium with suitable concentrations of iron.

Summary.

Nitrogen fixation by lucerne and white clover in agar culture was not stimulated by additions of molybdenum in quantities exceeding 0·03–0·05 γ per plant. As much as 37,000 parts of nitrogen could be fixed per part of molybdenum present.

Lucerne grown in sand showed a relatively small but significant response to molybdenum when the medium provided only one part of assimilable molybdenum per 80,000 parts of nitrogen fixed. At a Mo/N ratio of 1:20,000, further addition of molybdenum had no effect.

Root-nodules from leguminous plants grown in soil or sand of low molybdenum content were found to be 5 to 15 times richer in molybdenum than the actual roots, which again were generally richer in molybdenum than the tops. Lucerne plants took up more molybdenum when fixing free nitrogen than when utilizing combined nitrogen.

The results indicate that molybdenum stimulates the process of symbiotic nitrogen fixation, besides presumably being required for the general metabolism. Vanadium does not seem capable of replacing molybdenum.

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