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IMPORTANCE OF MOLYBDENUM IN THE NITROGEN METABOLISM OF MICROORGANISMS AND HIGHER PLANTS

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Introduction. The biological importance of molybdenum was first demonstrated by Bortels[10]) in 1930 in experiments with the free-living nitrogen-fixing bacterium *Azotobacter chroococcum*. In a synthetic medium without combined nitrogen this organism grew poorly, unless small amounts of molybdenum were added to the nutrient solution. Apparently this element is indispensable for the assimilation of the gaseous nitrogen. Other investigators have since confirmed this observation (Burk and Lineweaver[14]), Birch-Hirschfeld[8]), Schröder[27]), Kluyver and Reenen[20]), Krzemieniewsky and Kovats[21]), Burema and Wieringa[17]).

Not only *Azotobacter* but *Clostridium pasteurianum* as well requires traces of molybdenum when growing without combined nitrogen[11]).

As for the nitrogen fixation by leguminous plants there is some evidence that, in this process too, molybdenum is an important element (Bortels[12]), Bertrand[7]), Bobko and Savvina[9])). These authors, however, merely reported a more or less stimulating effect of molybdenum on the growth of leguminous plants and Hoagland[18]), reviewing the literature on this subject, came to the conclusion that „the few experiments now on record certainly do not provide decisive, or even very strong, evidence, that molybdenum is indispensable for fixation of nitrogen by leguminous plants".

In Australia and New Zealand a striking response to molybdenum was found with alfalfa and subterranean clover growing in

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acid ironstone soils (personal communications of Miss Margaret Thomas, Adelaïde, South Australia and Dr. A. C. S. Wright, Wellington, New Zealand). See also Anderson[2]) and Jensen and Betty[19]), cited by Hoagland[18]), and Anderson *et al.*[3]). The fact that the legumes gave only insignificant responses to molybdenum when sufficient nitrate or ammonium nitrogen was provided, whereas grasses grown alone on the same soil responded to combined nitrogen but not to molybdenum, shows that molybdenum stimulated the fixation of gaseous nitrogen by the leguminous plants growing on these soils.

In 1936 Steinberg[28]) showed that *Aspergillus niger* requires small amounts of molybdenum when grown in a medium with nitrate as the sole source of nitrogen. With ammonium nitrogen the response to molybdenum was found to be considerably less.

The essentiality of traces of molybdenum for the normal development of higher plants was first demonstrated by Arnon and Stout[6]) in 1939 in waterculture experiments with tomato plants. Grown in a rigidly purified nutrient solution, containing all of the nutrient elements regarded as essential, (N, P, K, Ca, Mg, S, Fe, B, Mn, Z and Cu), characteristic deficiency symptoms became apparent in a few weeks. The lower leaves developed a mottling and in later stages necrosis at the margins and an involution of the laminae was shown. Almost all of the blossoms abcissed without setting fruit. With 10 γ of molybdenum added to 1 l of nutrient solution normal growth was obtained. Of 19 other elements tested, (Ti, V, Cr, W, Co, Ni, Al, As, Cd, Sr, Mg, Pb, Li, Rb, Br, I, F, Se and Be), none was capable of replacing molybdenum. No details concerning the composition of the nutrient solution and the nitrogen compound used in these experiments were given. In two other series of experiments with barley [4]) and with lettuce and asparagus [5]) respectively, some evidence was obtained that growth improved after the addition of molybdenum. In the first-mentioned experiments this happened when the nitrogen was present as an ammonium salt.

Hoagland[18]) in culture solution experiments with plum seedlings observed qualitatively a response to molybdenum. The leaves of the molybdenum-deficient plants were dwarfed; some developed a diffuse mottling and many showed light brown areas of dead tissue at the tips and margins.

P i p e r [25]) found that molybdenum was essential for the normal growth of oat plants. His nutrient solutions were prepared with specially purified nutrient salts. In the absence of molybdenum necrotic areas appeared on the leaves. At first these leaves turned backwards in a sharp but smooth bend, but later a kink developed at the collapsed area and the midportion of the leaf dried out to a light reddish-brown colour. The yields of grain but not total yield were influenced by molybdenum.

W a r i n g t o n [30]) observed a marked beneficial effect of the addition of small amounts of molybdenum on both yield and appearance of lettuce grown in nutrient solutions.

In the authors previous experiments [23]), [24]) on the influence of copper on the growth of *Aspergillus niger* a comparison was made of two methods of purification of the nutrient medium: adsorption on charcoal and recrystallization of the nutrient salts and glucose. Upon addition of copper impaired growth of *Aspergillus* was still obtained in the medium purified by recrystallization. Obviously, the medium purified with charcoal, though free from copper, contained some substance which was removed by recrystallization of the nutrient salts and the sugar. The results of S t e i n b e r g [29]) suggested that molybdenum deficiency was the cause of the poor growth of *Aspergillus* in the medium purified by recrystallization. Another set of experiments was therefore started with recrystallized salts, this time with a trace of molybdenum added to some of the cultures. When molybdenum was added a normal development of *Aspergillus niger* took place, thus confirming the results of S t e i n b e r g that molybdenum is an indispensable element for this organism.

The purpose of the present investigation was to study the biological significance of molybdenum for both microorganisms and higher plants with special reference to the effect of varying concentrations on growth and nitrogen metabolism.

Experimental methods. Culture solutions were used in the experiments with microorganisms as well as in those with higher plants. In preparing these solutions water distilled in glassware was used throughout. Salts free or practically free from molybdenum were selected in the preparation of the nutrient media. A biological assay, described below, was employed to test the different brands of salts for molybdenum. This method appeared to be of

great value in preparing solutions practically free from molybdenum.

The culture solutions employed for the experiments with tomato, barley, oat and pea plants were generally not aerated in order to prevent any contamination with traces of molybdenum. An improved air supply, especially important in the experiments with peas, was obtained by only half filling the culture vessels with nutrient solution.

Experiments with Aspergillus niger. a. Effect of molybdenum concentration on growth. The following nutrient solution was used for growing *Aspergillus*:

H_2O	1 l	$Na_2CO_3.10H_2O$ . .	0.25 mg
Glucose	50 g	$FeCl_3.6H_2O$	3.00 mg
KNO_3	5 g	$MnSO_4.4H_2O$ . . .	0.30 mg
K_2HPO_4	0.6 g	$ZnSO_4.7H_2O$ . . .	2.00 mg
$MgSO_4.7H_2O$	0.6 g	$CuSO_4.5H_2O$ . . .	0.60 mg
$CaSO_4.2H_2O$	0.5 g		

50 cc portions of this solution were pipetted into 500 cc Erlenmeyer flasks, autoclaved for 10 minutes at 105°C, inoculated with a spore suspension of *Aspergillus*, strain M, and incubated for 4 days at 31°C. Molybdenum was added in increasing amounts. The basic source of nitrogen was nitrate but some flasks were provided with an additional quantity of $(NH_4)_2SO_4$ *). Table 1 gives the yields of dry mycelium.

TABLE I

Effect of increasing amounts of molybdenum on the growth of *Aspergillus niger*

$Na_2MoO_4.2H_2O$ added per flask γ	Yield †) (dry weight) g
0	0.187
0 §)	0.765
0.01	0.562
0.05	0.901
0.10	0.946
1.00	0.951
10.00	1.020
10.00 §)	1.148

§) 25 mg of $(NH_4)_2SO_4$ per flask added.
†) average of duplicate values.

*) This salt was recrystallized two times from glass distilled water.

From these data it is seen that the addition of very small amounts of molybdenum permitted good development of *Aspergillus*. Ammonium nitrogen had a marked effect on the molybdenum requirement of the fungus, as was observed by Steinberg [29]).

Since it was intended to use the growth rate curve of *Aspergillus* for the biological estimation of small amounts of molybdenum, the above-mentioned experiment was repeated with quantities of Na_2Mo_4O smaller than 0.01 γ. Some cultures with traces of ammonium nitrogen were included to determine whether molybdenum could be estimated by *Aspergillus* in materials containing some ammonia, as for instance, soil †). The results of this experiment are given in table II.

TABLE II

Effect of increasing amounts of molybdenum on the yield of *Aspergillus niger*

$Na_2MoO_4.2H_2O$ per 50 cc of nutrient sol. γ	NH_4–N per 50 cc of nutrient sol. γ	yield *), dry weight g	sporulation	appearance of mycelium
0	0	0.165	0	entirely mucous
0	20	0.171	0	,, ,,
0	200	0.211	0	,, ,,
0.001	0	0.253	0	,, ,,
0.0025	0	0.294	0	partially ,,
0.005	0	0.450	0	,, ,,
0.010	0	0.558	0	,, ,,
0.025	0	0.797	scant	normal
0.050	0	0.868	normal	normal
0.100	0	0.900	normal	normal

*) average of duplicate values.

The data of table II are plotted in fig. 1. This curve as well as the difference in sporulation with different amounts of molybdenum may be used for the biological determination of minute quantities of molybdenum in various materials, as will be described below.

b. Effect of nitrate and ammonium nitrogen on the molybdenum requirement of *Aspergillus niger*. In order to elucidate the influence of ammonium nitrogen on the molybdenum requirement a series of experiments was undertaken to test the following possibilities:

†) Vanadium which partly can replace molybdenum in the fixation of gaseous nitrogen by *Azotobacter* had no influence on the growth of *Aspergillus niger* when added in an amount of 0.1 γ $NaVO_3$ per 50 cc of molybdenum-free nutrient solution.

1. Ammonium salts favour, whereas nitrates depress the absorption of the trace of molybdenum unavoidably present in every nutrient medium as an impurity. If this assumption were correct then cultures with $(NH_4)_2SO_4 + KNO_3$ should have made less growth than those with the ammonium salt only. No effect was observed, however, from the addition of potassium nitrate.

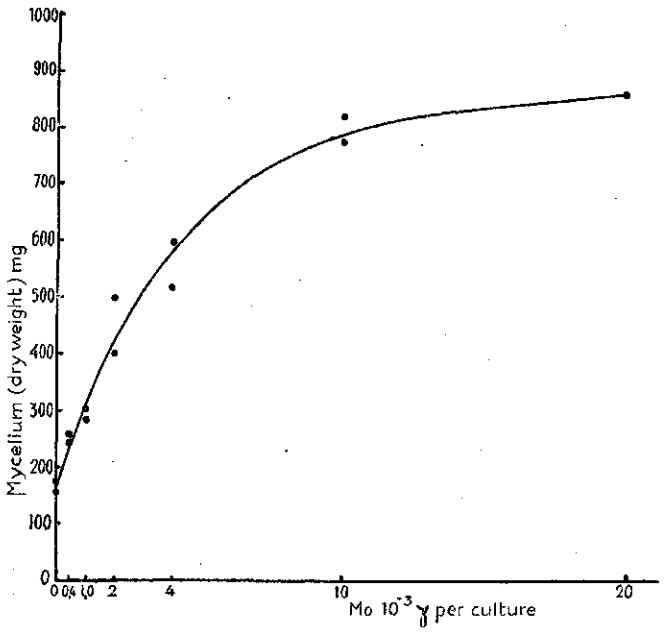

Fig. 1. Effect of molybdenum on the growth of *Aspergillus niger* in a nutrient solution with nitrate nitrogen.

2. The less acid reaction of the nutrient medium following the absorption of nitrate nitrogen depresses the absorption of molybdenum. 1 γ of Na_2MoO_4 was added to two-day and three-day old molybdenum-deficient nitrate cultures. Although the growth of these mycelia was poor and their appearance mucous, a response to molybdenum was seen twelve hours after the addition of this element. 30 hours later the appearance of these cultures was quite normal with an abundant formation of black spores. Thus the reaction of the nitrate-containing medium was no hindrance to the absorption of newly introduced molybdenum.

3. The ammonium salts are less pure than the nitrates. To test

this possibility an excess of molybdenum-free sodium carbonate was added to a solution of the ammonium salt. The ammonium was distilled and absorbed in a solution of molybdenum-free KH_2PO_4. The residue, which should have contained the impurities, and the distillate were separately tested for molybdenum with *Aspergillus*. Table III contains the results.

TABLE III

Effect of distilled ammonium nitrogen and residue of ammonium sulfate on the growth of *Aspergillus niger* in a molybdenum-deficient nitrate medium

Nitrogen source	Addition	$Na_2MoO_4.2H_2O$ added, γ	yield *) of *Aspergillus*, g
KNO_3	Control	0	0.134
,,	$KH_2PO_4 + Na_2CO_3$	0	0.241
,,	$KH_2PO_4 + Na_2CO_3$	1	0.892
,,	residue of distilled $(NH_4)_2SO_4$ . .	0	0.175
,,	,, ,, $(NH_4)_2SO_4$ . .	1	0.788
,,	distilled NH_3, absorbed in KH_2PO_4	0	0.786
,,	,, NH_3, ,, KH_2PO_4	1	0.855
$(NH_4)_2SO_4$	Control	0	0.810
,,	Control	1	0.900

*) average of duplicate values

These data clearly show that the beneficial effect of the ammonium salt has to be attributed to the ammonium nitrogen as such and not to a molybdenum impurity. This could be demonstrated in another way. *Aspergillus niger* was grown in a series of ammonium and nitrate media with and without molybdenum. At the conclusion of the growing period the mycelia were weighed, ashed and the ash added to the nutrient solution of a new series of molybdenum-deficient cultures supplied with nitrate nitrogen only. The results are given in table IV.

TABLE IV

Effect of adding ashed mycelia to molybdenum-deficient *Aspergillus* cultures supplied with nitrate nitrogen only

History of ashed mycelia		Dry weight (g) of *Aspergillus* *), supplied with ash
KNO_3, no Mo,	Dr. wt. *) 0.351 g	0.297
KNO_3, 1 γ of Na_2MoO_4 per cult.	,, ,, 0.776 g	0.797
$(NH_4)_2SO_4$, no Mo	,, ,, 0.778 g	0.265
$(NH_4)_2SO_4$, 1 γ of Na_2MoO_4 per cult.	,, ,, 0.771 g	0.814
distilled NH_4, no Mo	,, ,, 0.786 g	0.276
distilled NH_4, 1 γ of Na_2MoO_4 per cult.	,, ,, 0.855 g	0.751

*) average of duplicate values.

It can be seen that the ash of the nitrate and ammonium mycelia gave similarly poor growth of *Aspergillus* in a molybdenum-free nitrate medium, thus indicating that the heavier yields of the ammonium mycelia contained as little molybdenum as the nitrate ones.

The conclusion which must be drawn from all of these data is, that in a medium with nitrate nitrogen *Aspergillus* requires a higher molybdenum supply than in a solution with an ammonium salt.

The question arises to what extent the poor growth of the fungus in the molybdenum-deficient nitrate medium is caused by an insufficient reduction of the nitrate to ammonia, i.e. by a lack of available nitrogen. Steinberg [29]), who also discusses this question, is of the opinion that nitrogen and molybdenum deficiency are quite similar. However, molybdenum-deficient mycelia are mucous and free of spores. On the other hand mycelia with an insufficient nitrate supply do not show this mucous type of growth and develop relatively abundant spores.

More details concerning the influence of molybdenum on nitrate reduction will be recorded in the paragraphs on the experiments with denitrifying bacteria and higher plants.

c. Estimation of molybdenum with *Aspergillus niger*. The fact that increasing amounts of molybdenum induce increasing yields of mycelium and sporulation of *Aspergillus niger* suggests the use of this organism as a biological test for the estimation of molybdenum. To determine plant-available molybdenum in soil, small amounts of air dried soil were added to 50 cc portions of molybdenum-free nutrient solution in 500 cc Erlenmeyer flasks, previously sterilized for 5 minutes at 105°C. After inoculation with a suspension of *Aspergillus* spores and incubation at 31°C for four days the amount of available molybdenum was estimated by comparing the yield and the spore formation of the culture with a set of standards grown with known amounts of molybdenum.

In one experiment three soils of different fertility were compared: a clay soil, a garden soil, both of high fertility, and a poor, sandy soil, low in humus. In these cases 500 mg of air-dried soil were added to each flask. This amount appeared to be too high: normal yields and spore formation were obtained, showing that the content of available molybdenum was higher than 0.025 γ in 500 mg of soil.

In a second experiment three different soils were compared in por-

tions of 100 mg of air-dried soil per flask. Amounts of 0.015, 0.02, and 0.10 γ per gram of soil were found *).

For the estimation of total molybdenum in plant materials 10 mg of dried, ground tissue were ashed and the ash used in the *Aspergillus* test. Table XVI contains some results.

Experiments with denitrifying bacteria. If traces of molybdenum are essential for the reduction of nitrate, this element should prove indispensable for the denitrifying bacteria, which when grown anaerobically depend on nitrates for the oxygen used in oxidizing energy-yielding substrates. This assumption was tested as follows. A denitrifying bacterium, strain S, was isolated from a garden soil, and grown in a synthetic medium of the following composition:

H_2O	1 l		
Glucose	2.5 g	$FeCl_3.6H_2O$	0.5 mg
KNO_3	1.0 g	$MnSO_4.4H_2O$	0.1 mg
K_2HPO_4	1.0 g	$ZnSO_4.7H_2O$	0.1 mg
$MgSO_4.7H_2O$	0.5 g	$CuSO_4.5H_2O$	0.1 mg
$Na_2CO_3.10H_2O$	0.2 mg		

An extract of ground *Aspergillus* mycelia grown in a molybdenum-free nutrient solution was also added.

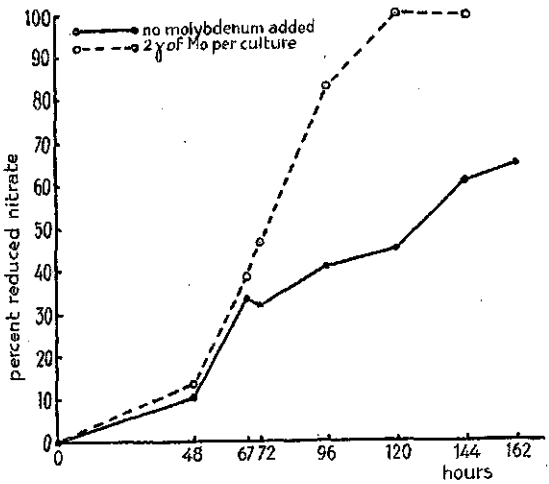

Fig. 2. Effect of molybdenum on denitrification.

*) Ter Meulen [22]) determined molybdenum in many plant tissues and in a number of soils.

The bacteria were incubated at 25°C in 50 cc stoppered bottles, without and with 5 γ of $Na_2MoO_4.2H_2O$ added. After different periods of incubation one bottle with and one without molybdenum were analysed for nitrate according to the xylenol-method [1]).

The results are shown in fig. 2. It is seen that with molybdenum denitrification proceeded more intensively than in the absence of this element.

In a second series of experiments four strains of denitrifying bacteria were used. Extract of molybdenum-free *Aspergillus* mycelia was added to one set of bottles only. Nitrate and nitrite †) determinations were carried out after five and ten days of incubation at 25°C respectively. Tables V and VI contain the results of these experiments.

TABLE V

Effect of molybdenum on denitrification (incubation time 5 days)

Bact. strain	$Na_2MoO_4.2H_2O$ per bottle, γ	Extract of *Asp. niger* added	After five days incubation at 25°	
			NO_3–N disappeared *), mg	NO_2–N per bottle, mg
W 1	0	0	2.7	1.3
,,	0	+	1.7	1.7
,,	5	0	9.6	7.9
,,	5	+	9.3	8.7
S 1	0	0	0.0	0.0
,,	5	0	12.6	6.0
S 2	0	0	0.0	0.0
,,	0	+	0.2	0.0
,,	5	0	10.8	5.0
,,	5	+	9.8	9.4
W 2	0	0	0.7	0.0
,,	0	+	2.3	0.0
,,	5	0	6.2	2.7
,,	5	+	9.2	7.8

*) Initial nitrate concentration: 13.1 mg N per bottle (50 cc).

These data reveal quite clearly that molybdenum is indispensable for the denitrification process. With two strains of bacteria, S 1 and S 2, no growth at all was obtained in the absence of molybdenum. Obviously the nitrate could not be used as an oxygen supply. Strain W 1, which only reduces nitrate to nitrite, showed an insignificant reduction of nitrate when molybdenum was omitted

†) According to the Griess–Romijn-method (26).

TABLE VI

Effect of molybdenum on denitrification †) (incubation time 10 days)

Bact. strain	$Na_2MoO_4.2H_2O$ per bottle, γ	After 10 days incubation at 25°C	
		NO_3–N disappeared *), mg	NO_2–N per bottle, mg
W 1	0	1.4	1.7
,,	5	9.3	8.5
S 1	0	4.5	0.0
,,	5	13.1	0.0
S 2	0	0.0	0.0
,,	5	10.2	8.1
W 2	0	0.0	0.0
,,	5	8.0	7.4

†) No extract of *Aspergillus* was added to these cultures.
*) Initial nitrate concentration: 13.1 mg N per bottle (50 cc).

from the nutrient solution. With this strain, as well as with strain W 2, the effect of molybdenum was very conspicuous.

In a preliminary experiment with strain S 1 no effect of molybdenum on the *oxidation-reduction potentials* of denitrifying cultures was observed during the first four days of incubation. After that the cultures with molybdenum had a lower E_h than those deficient in this element. From nitrate determinations it was concluded that these differences were brought about by the fact that in the + Mo-cultures the nitrate had been consumed whereas in the absence of molybdenum this salt was still present.

Experiments with higher plants. In the years 1942 and 1943 water-culture experiments were carried out with tomato, barley and oat plants. In the barley and tomato experiments of 1942 a nutrient solution of the following composition was used

H_2O	1 l	$Na_2CO_3.10H_2O$	1.25 mg
KNO_3	0.35 g	$FeCl_3.6H_2O$	25.— mg
$(NH_4)_2SO_4$	0.15 g	$MnSO_4.4H_2O$	1.— mg
K_2HPO_4	0.125 g	$ZnSO_4.7H_2O$	0.25 mg
KH_2PO_4	0.200 g	$CuSO_4.5H_2O$	0.25 mg
$MgSO_4.7H_2O$	0.25 g	H_3BO_3	0.25 mg
$CaSO_4.2H_2O$	0.25 g		

Only those salts were applied, which in experiments with *Aspergillus* were found to be practically free from molybdenum. The seeds were germinated in quartz sand, previously treated with nitric acid.

a. Experiments with tomato plants. Two plants were placed in each glass jar containing 1.21 of culture solution. When molybdenum was omitted from this solution very pronounced deficiency symptoms were observed. First a mottling of the leaves occurred which after some time changed to an almost yellow shade. The growth of the whole plant decreased and soon stopped entirely. Necrotic spots appeared in the yellow tissue, especially along the margins of the upper part of the leaflets and many older leaves

Fig. 3. Effect of molybdenum on tomato plants (left without, right with 100 γ of Na_2MoO_4 per culture).

died. In many cases a characteristic involution of the leaflets occurred (see fig. 3). The plants were harvested at an early stage. The yields are given in table VII.

TABLE VII

Effect of molybdenum on the yield of tomato plants in a nutrient solution

$Na_2MoO_4 . 2H_2O$ per culture, γ	Leaves + stem dry weight, g	Roots, dry weight, g	Remarks
0	6.45	0.77	pronounced deficiency symptoms
0	4.77	0.60	,, ,, ,,
0 *)	13.40	2.37	light ,, ,,
,, *)	13.12	2.32	,, ,, ,,
100 †)	18.37	3.03	normal plants

*) probably contaminated with traces of molybdenum.

†) added after every change of the culture solution.

In 1943 another experiment with tomato plants was carried out. The nutrient solution used, differed only slightly from the previous one: KH_2PO_4 and K_2HPO_4 were added in amounts of 0.25 and 0.07 g per l respectively, whereas nitrogen was supplied as KNO_3 only. The first symptoms of molybdenum deficiency were observed after 3 weeks. Two months later when the greater part of the lower half of the plants had died down, they were harvested, dried and weighed (table VIII).

TABLE VIII

Effect of molybdenum on the yield of tomato plants			
$Na_2MoO_4.2H_2O$ per culture, γ	Leaves + stems dry weight, g	Roots, dry weight, g	Remarks
0	5.85	0.70	no fruits formed
0	7.42	1.01	,, ,, ,,
0	10.67	1.89	probably some contamination
0	10.90	1.90	with molybdenum; fruit formation rather good
100 *)	20.60	3.93	normal fruit-setting
100 *)	20.25	2.65	,, ,, ,,

*) added after every change of the culture solution.

b. Experiments with barley. In barley molybdenum deficiency symptoms appeared when the plants were about four weeks old. The leaves became light green in colour and in the youngest ones a chlorotic striation was seen. With 100 γ of Na_2MoO_4 added per jar the plants had a dark green colour. Some weeks later in the absence of molybdenum the ends of the leaves had died. The ears emerged slowly and showed a yellowish-green shade. The seed production was poor (table IX). The yield of straw was somewhat higher in the absence of molybdenum, due to the emergence of young green shoots.

TABLE IX

Effect of molybdenum on the yield of barley in nutrient solution *)						
$Na_2MoO_4.2H_2O$ per culture, γ	Grains		Straw, dry weight, g	Roots, dry weight, g	Number of ears per jar.	
	dry weight g	number			normal	green
0	1.79 ± 0.46	52 ± 12	10.10 ± 0.74	1.36 ± 0.08	6 ± 0.5	9 ± 1.6
100 †)	5.77 ± 0.38	135 ± 8	8.42 ± 0.86	1.49 ± 0.05	8 ± 0.7	3 ± 1.1

*) average values of four cultures.

†) added after every change of the culture solution.

c. Experiments with oats (1943). In this experiment a nutrient solution of a similar composition as in the experiment with tomato plants in the same year was employed.

In the absence of molybdenum the ripening of the plants was much retarded. The emergence of young green shoots was again observed. The results are presented in table X.

TABLE X

Effect of molybdenum on the yield of oats in nutrient solution *)

$Na_2MoO_4 . 2H_2O$ per culture, γ	Grains		Straw, dry weight, g	Roots, dry weight, g
	dry weight, g	number		
0	3.54 ± 0.44	137 ± 13	12.22 ± 0.78	1.36 ± 0.10
100 †)	5.98 ± 0.45	240 ± 26	12.82 ± 0.66	1.36 ± 0.74

*) average value of four, (0 Mo), and three cultures, (+ Mo), respectively.
†) added after every change of the culture solution.

d. Importance of molybdenum in the nitrate reduction of green plants. In order to know whether or not molybdenum is essential for the nitrate reduction in green plants, the following experiments with barley and tomato plants were carried out. Young plants of both species were transplanted into glass jars containing a nutrient solution without molybdenum and with a moderate supply of nitrate nitrogen. The experiment with barley plants consisted of two series of eight jars each, one series without and one with 100 γ of Na_2MoO_4 per jar. After two weeks the nutrient solution was replaced by a solution without nitrogen. Nine days later when the nitrate in the plants was depleted, 0.75 g of potassium nitrate was added to each jar. After six days all of the plants were harvested and analysed for protein *) and nitrate nitrogen. The plants without molybdenum had a somewhat pale green colour, while those provided with traces of this element were dark green. The results of the analyses are given in table XI.

These data show that molybdenum-deficient plants contain more nitrate and less protein nitrogen than those provided with this element, This fact was more clearly shown in an experiment with tomato plants which was carried out in August and September 1944. As in the experiment with barley, the tomato plants were first

*) The proteins were precipitated in the ground fresh tissue with trichloroacetic acid.

grown in a nutrient solution with a moderate supply of nitrate and without molybdenum. When the plants had grown for about five weeks in this medium the first symptom of molybdenum deficiency, a slight mottling, was observed. In addition slight symptoms of nitrogen deficiency became apparent. Of the fourteen jars used, each

TABLE XI

Effect of molybdenum on the nitrate reduction in barley plants

$Na_2MoO_4.2H_2O$ added, γ	Leaves + stems			Roots	
	fresh weight g	protein-N mg	nitrate-N mg	fresh weight g	nitrate-N mg
0 *)	20.0 ± 0.60	62.4 ± 1.96	16.8 ± 0.58	9.6 ± 0.91	4.3 ± 0.48
200 †)	21.7 ± 0.40	71.5 ± 1.73	11.3 ± 0.83	7.9 ± 0.18	3.2 ± 0.16

*) average values of six separately analysed cultures.
†) ,, ,, ,, eight ,, ,, ,,

containing one tomato plant, two were harvested and analysed for different nitrogen fractions. The other twelve were divided into two series of plants resembling each other as much as possible. The nutrient solution of all of these cultures was changed. The nitrate concentration was raised to 1 g of potassium nitrate per culture. One group of plants received 200 γ of $Na_2MoO_4.2H_2O$ per jar, the other group remained without molybdenum. Three days later, when the first colour differences between the two groups of plants were observed, they were harvested and the fresh leaves, stems and roots analysed for protein, soluble organic nitrogen and nitrate (table XII).

The results of this experiment clearly show that molybdenum is indispensable for the nitrate reduction in tomato plants. In the absence of this element nitrate is accumulated in the leaves. Soluble organic nitrogen compounds and proteins are practically not formed. In the stems the nitrate differences are less pronounced, whereas in the roots practically no differences are shown. Apparently in tomato plants the nitrate reduction takes place mainly in the leaves and the stems.

In order to prove that molybdenum is essential for the first step of the nitrate assimilation, i.e. the reduction of nitrate to ammonia, an experiment was carried out in which molybdenum- and nitrogen-deficient tomato plants were provided with nitrate and with ammonium sulphate respectively. Some weeks after the addition of the different nitrogen compounds great differences between the plants

TABLE XII

Effect of molybdenum on the nitrate reduction in tomato plants

$Na_2MoO_4.2H_2O$ added, γ	Leaves				Stems				Roots			
	Fresh weight, g	Protein-N, mg	Sol.org. N, mg	NO_3–N mg	Fresh weight, g	Protein-N, mg	Sol. org. N, mg	NO_3–N mg	Fresh weight, g	Protein-N, mg	Sol. org. N, mg	NO_3–N mg
0 *)	11.30 †)	15.5	3.85	0.00	—	8.09	4.09	0.00	2.75	6.23	2.52	0.00
0 *)	11.72 †)	14.7	4.58	0.00	—	7.41	3.93	0.00	2.74	—	—	—
0 **)	6.08 ± 0.41	15.8 ± 0.58	5.42 ± 0.28	4.19 ± 0.48	7.54 ± 0.82	6.74 ± 0.80	3.79 ± 0.42	3.53 ± 0.36	3.34 ± 0.88	5.26 ± 0.57	2.62 ± 0.35	1.41 ± 0.46
200 **)	6.10 ± 0.26	19.1 ± 0.93	5.65 ± 0.26	0.61 ± 0.18	8.61 ± 0.71	8.63 ± 1.16	6.20 ± 0.66	2.70 ± 0.30	3.61 ± 0.15	4.92 ± 0.41	3.12 ± 0.55	1.32 ± 0.18

*) plants harvested before adding the nitrate.

†) fresh weight of leaves + stem.

**) average values of six seperately analysed plants.

were found. Those provided with nitrate showed yellow-green leaves with upwards curling margins. The addition of ammonium nitrogen, however, had resulted in the formation of dark green leaves, indicating an intensive nitrogen assimilation. The plants were harvested and analysed for some nitrogen fractions (table XIII).

TABLE XIII

Effect of molybdenum on the assimilation of nitrate and ammonium nitrogen

N-compound added	$Na_2MoO_4.2H_2O$ γ per jar	Leaves				Stems				Roots
		Fresh weight g	Protein-N *)	Sol. org. N *)	NO_3-N *)	Fresh weight g	Protein-N *)	Sol.-org. N *)	NO_3-N *)	Fresh weight g
KNO_3	0	19.38	— †)	7.9	13.77	27.50	6.3	4.6	9.58	6.60
,,	0	16.47	— †)	7.7	14.19	24.21	7.4	4.7	9.57	6.50
,,	200	17.75	21.7	9.9	0.41	33.35	9.1	5.4	3.61	13.55
,,	200	17.75	21.6	10.6	0.49	29.30	9.1	6.0	3.49	10.50
$(NH_4)_2SO_4$	0	10.50	31.5	10.6	1.34	15.35	10.7	10.7	2.60	5.40
,,	0	10.26	32.0	10.5	1.08	18.90	10.2	10.7	2.60	5.65

*) mg per 10 g of fresh weight.
†) both samples lost.

Although the total growth of the ammonium plants was less than that of the nitrate plants the table shows the following:

1. In the absence of molybdenum the nitrate content of the leaves is more than thirty times and that of the stems almost three times higher than that of the plants supplied with this element.
2. The content of the organic nitrogen fractions in leaves as well as in stems of the ammonia plants without molybdenum is even higher than that of the nitrate plants with molybdenum added.

This clearly shows that without molybdenum formation of organic nitrogen compounds from ammonia can take place.

Although it is possible that molybdenum is required for some other reaction in the plant life, one of the main functions is that of catalysing the nitrate reduction. This is not only true for the green plant but also for such microorganisms as *Aspergillus niger* and denitrifying bacteria.

The importance of molybdenum in the fixation of gaseous nitrogen. a. Experiments with *Azotobacter chroococcum.* The question whether molybdenum is an essential element for the growth of *Azotobacter* or for the nitrogen fixation of this organism only,

is dealt with by some authors. Birch–Hirschfeld[8]), cultivating *Azotobacter* in an oxygen-hydrogen atmosphere with nitrate as a nitrogen source, could not observe a respond to molybdenum. Burk[15]) first came to the conclusion that molybdenum is required for the N_2-fixation only; in media with nitrate, nitrite and asparagine no favourable effect of the addition of this element could be found. In a later report, however, this same author[16]) contends that molybdenum stimulates the growth of *Azotobacter*, when the nitrogen is added to the nutrient media as nitrate or asparagine.

Bortels[13]) found a highly stimulating effect of traces of molybdenum on *Azotobacter chroococcum* when this organism was grown in a nitrate medium. The concentration of molybdenum essential for optimal growth was found to be much smaller than when grown without combined nitrogen. With ammonium sulphate and asparagine a beneficial effect of molybdenum was also observed. In the absence of molybdenum far better results were obtained with ammonium than with nitrate nitrogen.

Burema and Wieringa[17]) also found that *Azotobacter chroococcum* requires less molybdenum in a nitrate medium than when grown without combined nitrogen.

The relation between molybdenum and nitrogen nutrition was investigated in the following experiment.

Azotobacter was grown in a medium of the following composition:

H_2O	1 l	$Na_2CO_3.10H_2O$	5 mg
Glucose	40 g	$FeCl_3.6H_2O$	25 mg
K_2HPO_4	2 g	$MnSO_4.4H_2O$	2 mg
$MgSO_4.7H_2O$	1 g	$ZnSO_4.6H_2O$	5 mg
$CaCO_3$	4 g	$CuSO_4.5H_2O$	4 mg

Erlenmeyer flasks of 100 cc capacity, containing 15 cc of nutrient solution, served as culture vessels. One set of flasks was supplied with 10 mg of nitrogen per flask as potassium nitrate, another set with 10 mg of nitrogen as ammonium sulphate, whereas a third one was given no combined nitrogen. No precautions were taken to exclude the N_2 of the air from the nitrate and ammonium cultures, as it was expected that no fixation of gaseous nitrogen would occur when combined nitrogen was present.

Molybdenum was given in amounts of 0, 0.05, 0.1, 0.2, 0.5, 1, 2,

5, 10 and 20 γ of $Na_2MoO_4.2H_2O$ per flask. Each flask was inoculated with five drops of a suspension of *Azotobacter chroococcum*. After eight days of incubation at 25°C differences were seen between the three sets of cultures. Without combined nitrogen practically no growth occurred in flasks with a molybdenum concentration lower than 0.5 γ. With nitrate a great response to molybdenum could be

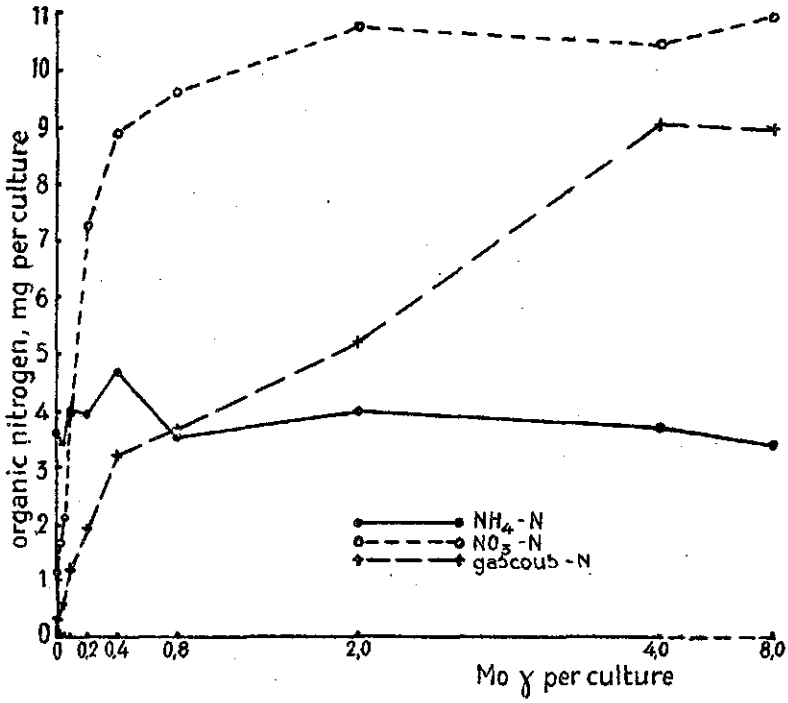

Fig. 4. Effect of molybdenum on the assimilation of different N-compounds by *Azotobacter chroococcum*.

observed, though considerably smaller amounts were needed to reach a normal growth. With ammonium sulphate, however, no influence of the addition of molybdenum could be seen. A somewhat poor growth occurred in all the cultures of this series. Microscopically many *Azotobacter* cells could be seen in the molybdenum-free nutrient solution with ammonium nitrogen; in the nitrate and nitrogen-free solutions, however, only few could be discovered.

Bacterial growth was measured by determining the total organic nitrogen in the cultures. In the set without combined nitrogen the

ordinary Kjeldahl method was used. In the ammonia cultures, however, the ammonia present had to be driven out in vacuo after addition of a boric acid buffer and in the nitrate series the nitrate had to be removed by reducing with $FeCl_2$ and HCl before the organic nitrogen could be determined. The results of this experiment are graphically represented in fig. 4. It is seen that in a medium without combined nitrogen *Azotobacter chroococcum* requires considerably more molybdenum than in a solution with nitrate *). With ammonium sulphate added no response to molybdenum could be observed. This is in good agreement with the results of the abovementioned experiment with tomato plants according to which the assimilation of nitrate requires traces of molybdenum whereas ammonium compounds can be assimilated in the absence of molybdenum.

The relatively poor growth of *Azotobacter* in the NH_4-solution was possibly caused by an insufficient phosphorus supply. This was concluded from a similar experiment with increasing amounts of phosphate in which solutions with ammonium nitrogen needed about 10 times as much phosphate as those with nitrate and gaseous N_2.

The fact that nitrate assimilation requires less molybdenum than the fixation of N_2 from the atmosphere shows that the molybdenum-requiring reactions involved in nitrate and N_2-assimilation are not identical.

b. Experiments with symbiotic nitrogen-fixing bacteria. In order to know whether or not molybdenum is indispensable for the nitrogen fixation by the nodules of leguminous plants some experiments with peas were carried out.

In preliminary experiments in 1941 and 1942 much evidence was obtained that in the absence of molybdenum the fixation of nitrogen by the nodules of peas was greatly curtailed. Heavy symptoms of nitrogen deficiency were observed. The plants died at an early stage and the yields of seeds and straw were low. Nitrogen determinations in these first experiments, however, were not carried out. In order to confirm these results a third experiment was started in July 1942.

*) That in a medium with nitrate and ammonium nitrogen respectively *Azotobacter* is assimilating these nitrogen compounds and not the N_2 from the air was shown by ammonium and nitrate determinations in the culture solutions.

Pea seeds were germinated in purified quartz sand and on July 7 transplanted into Weck glass jars containing 1.4 l of the following nutrient solution:

H_2O	1 l	$Na_2CO_3.10H_2O$	1.25 mg
KNO_3	0.2 g	$FeCl_3.6H_2O$	25.0 mg
K_2HPO_4	0.125 g	$MnSO_4.4H_2O$	1.0 mg
KH_2PO_4	0.186 g	$ZnSO_4.7H_2O$	0.25 mg
$MgSO_4.7H_2O$	0.250 g	$CuSO_4.5H_2O$	0.25 mg
$CaSO_4.2H_2O$	0.250 g	H_3BO_3	0.25 mg

Of the 16 cultures used, 8 were provided with 100 γ of $Na_2MoO_4.2H_2O$ per jar. All the cultures were inoculated with a suspension of *Rhizobium*. The culture solution was changed three times. No nitrogen was given to the renewed solution, however. All of the other constituents, including molybdenum, were added in concentrations two times as high as those of the medium used initially.

At first no differences between the plants of both series were observed. In both cases many nodules had been formed. In the second part of August, however, the first symptoms of nitrogen deficiency were seen in the absence of molybdenum: a yellow green colour of the leaves and a dying back of the lower ones. These deficiency symptoms progressively worsened (figure 5 and 6). In the presence of molybdenum normal plants were obtained with dark green leaves, indicating an abundant nitrogen supply. The nodules of these plants showed a somewhat pink shade as contrasted with a more pale yellow-brown colour in the absence of molybdenum. The molybdenum-deficient plants died at a much earlier stage than those growing in a complete culture solution. Yields of peas, straw and roots as well as their total nitrogen content are given in table XIV.

TABLE XIV

Effect of molybdenum on the yield and nitrogen fixation of pea plants

$Na_2MoO_4.2H_2O$ per culture, γ	Seeds		Leaves + stems		Roots	
	dry wt. †), g	nitrogen †) mg	dry wt. †), g	nitrogen †) mg	dry wt. †), g	nitrogen †) mg
0	2.35 ± 0.42	77.5 ± 12.4	4.82 ± 0.25	97.5 ± 8.7	1.01 ± 0.04	27.6 ± 1.0
200 *)	5.57 ± 0.34	170.3 ± 12.9	5.92 ± 0.28	114.3 ± 7.4	1.05 ± 0.03	30.2 ± 1.2

*) added after every change of the culture solution.

†) average values of eight separately analysed cultures.

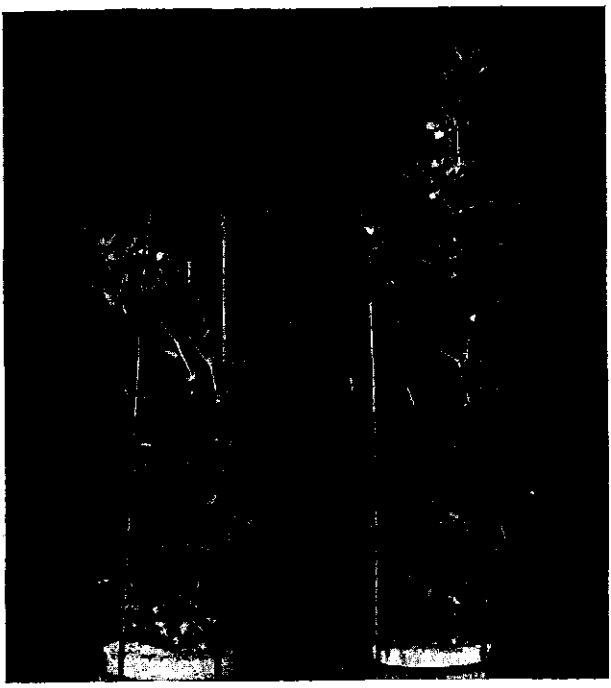

Fig. 5.

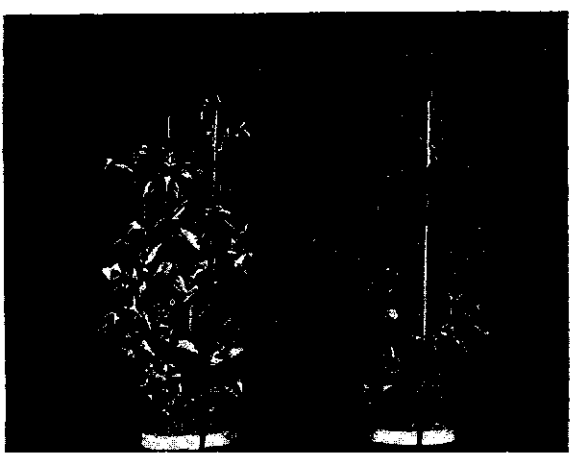

Fig. 6.

Fig. 5/6. Effect of molybdenum on pea plants (left without molybdenum, right with 200 γ of Na_2MoO_4 per culture).
The plants in fig. 6 photographed some weeks after those in fig. 5.

From these results it is concluded that pea plants grown with traces of molybdenum have fixed considerably more nitrogen than those with an inadequate molybdenum supply. These nitrogen data, however, may not have been fully representative due to the fact that in the absence of molybdenum the plants died at an earlier date. In order to acquire a more clear picture of the influence of molybdenum upon the nitrogen-fixing-capacity of the nodules, the plants should have been analysed at a time when differences in colour but not in development were observed.

In 1943 a new experiment with pea plants was started on May 27. Six weeks later the first differences in colour between the two series of plants were observed. In both cases nodules had been formed abundantly. Two cultures, grown in the absence of molybdenum, showing a somewhat light green colour of the leaves, and two with a normal molybdenum supply, showing dark green leaves, were harvested and analysed for total nitrogen. The data are given in table XV.

TABLE XV

Effect of molybdenum on the nitrogen fixation by the nodules of pea plants

$Na_2MoO_4.2H_2O$ per culture, γ	Leaves + stems			Roots		
	dry weight, g	nitrogen mg	nitrogen %dry.wt.	dry weight, g	nitrogen mg	nitrogen % dry.wt.
0	4.15	84.9	2.05	1.78	40.4	2.27
0	4.07	79.0	1.94	1.50	32.9	2.19
200	3.22	108.6	3.37	1.35	42.9	3.18
200	4.20	159.9	3.81	1.60	51.9	3.22

These results very clearly show that the fixation of gaseous nitrogen by the nodules of pea plants is reduced when molybdenum has been omitted from the culture solution. Due to this poor nitrogen supply the leaves turn light green and after some time the growth of the plants is stopped.

The view that molybdenum is acting as a catalyst in the fixation of nitrogen in the nodules of legumes is in good agreement with the high molybdenum content found in these tissues. For the molybdenum determinations the *Aspergillus* assay as described on page 101 was employed. Ten or five mg of dried and ground tissue were ashed at 500–600°C and the ash added to molybdenum-free solutions of *Aspergillus*. Table XVI contains the results of this experiment

TAVLE XVI

Molybdenum content of some plant tissues	
Tissue	Molybdenum content p.p.m.
Pea leaves	0.3
Pea roots	2
Pea nodules	4–8
Pasture gras	0.7
Red clover, leaves	2

Molybdenum with reference to agriculture. In large areas in South Australia and New Zealand molybdenum applications are essential for a normal growth of legumes in pastures. Apparently molybdenum stimulates the nitrogen fixation of these plants and this again increases the nitrogen supply of the grasses.

It is highly improbable that an occurrence of molybdenum deficiency will be limited to these Australian soils. It may be expected that in the future in other parts of the world molybdenum-deficient soils will be discovered. As the above-mentioned soils are rather acid and rich in ironstone, a beneficial effect of applying small amounts of molybdenum may be expected in growing leguminous crops in acid soils, which are rich in iron. So far, no response to molybdenum in Dutch soils has been found.

Summary

The effect of molybdenum on the growth of microorganisms and higher plants and on some well-defined biochemical reactions was investigated.

In good agreement with the results of S t e i n b e r g it could be shown that *Aspergillus niger* requires small amounts of molybdenum when growing in a culture solution supplied with nitrate nitrogen. With ammonium sulphate as a source of nitrogen the response of the fungus to molybdenum is much smaller. It was shown that this different response of *Aspergillus* to molybdenum was not brought about by a difference in purity of both nitrogen compounds used, nor by a difference in absorption of the molybdenum impurity, but by a considerably higher requirement of molybdenum in a medium with nitrate nitrogen.

The growth-rate curve and the increasing sporulation of *Aspergillus niger* with increasing amounts of molybdenum were used in estimating very small amounts of this element in various materials.

In experiments with 4 strains of denitrifying bacteria the essentiality of molybdenum for the denitrification process was demonstrated. With two strains no growth at all was observed in the absence of molybdenum, with the other two an insignificant denitrification occurred.

An influence of molybdenum on the oxydation-reduction potentials of denitrifying cultures could be seen only when the nitrate in the cultures with molybdenum added was depleted but still present in the o-Mo-cultures.

In culture solution experiments with tomato, barley and oat plants the effect of traces of molybdenum on the growth of these plants was investigated. In good agreement with the results of the experiments with *Aspergillus* and denitrifying bacteria it could be shown that in the green plant as in these microorganisms molybdenum is acting as a catalyst in nitrate reduction.

In experiments with *Azotobacter chroococcum* and leguminous plants the effect of molybdenum on the fixation of gaseous N_2 was studied. When *Azotobacter* was grown in culture solutions without combined nitrogen considerably more molybdenum was required than in media with nitrate nitrogen. With ammonium sulphate as a nitrogen source no response to molybdenum could be observed at all. From these results it was concluded that in *Azotobacter* the molybdenum-requiring reactions involved in nitrate and N_2-assimilation are not similar.

In culture solutions with pea plants the effect of molybdenum on the nitrogen fixation of the nodules was investigated. In the absence of molybdenum as well as in a complete nutrient medium many nodules were formed. In the former case, however, the nitrogen fixation of the nodules was greatly curtailed, resulting in a poor nitrogen supply to the plants. Pronounced nitrogen-deficiency symptoms were shown and the plants died at an early stage. Apparently, in symbiotic as well as in free-living nitrogen-fixing organisms molybdenum is acting as a catalyst in nitrogen fixation.

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