27 Thompson, J. F. and Steward, F. C., Investigations on nitrogen compounds and nitrogen metabolism in plants. II. Variables in two-directional paper chromatography of nitrogen compounds: A quantitative procedure. Plant Physiol. **26**, 421–440 (1951).

28 Thompson, J. F., Zacharius, R. M., and Steward, F. C., Investigations on nitrogen compounds and nitrogen metabolism in plants. I. The reaction of nitrogen compounds with ninhydrin on paper; A quantitative procedure. Plant Physiol. **26**, 375–397 (1951).

29 Wieringa, K. T. and Bakhuis, J. A., Chromatography as a means of selecting effective strains of Rhizobia. Plant and Soil **8**, 254–262 (1957).

30 Zelitch, I., Rosenblum, E. D., Burris, R. H., and Wilson, P. W., Isolation of the key intermediate in biological nitrogen fixation by Clostridium. J. Biol. Chem. **191**, 295–298 (1958).

31 Zelitch, I., Wilson, P. W., and Burris, R. H., The amino acid composition and distribution of N^{15} in soybean root nodules supplied N^{15}-enriched N_2. Plant Physiol. **27**, 1–8 (1952).

27 Thompson, J. F. and Steward, F. C., Investigations on nitrogen compounds and nitrogen metabolism in plants. II. Variables in two-directional paper chromatography of nitrogen compounds: A quantitative procedure. Plant Physiol. **26**, 421–440 (1951).

28 Thompson, J. F., Zacharius, R. M., and Steward, F. C., Investigations on nitrogen compounds and nitrogen metabolism in plants. I. The reaction of nitrogen compounds with ninhydrin on paper; A quantitative procedure. Plant Physiol. **26**, 375–397 (1951).

29 Wieringa, K. T. and Bakhuis, J. A., Chromatography as a means of selecting effective strains of Rhizobia. Plant and Soil **8**, 254–262 (1957).

30 Zelitch, I., Rosenblum, E. D., Burris, R. H., and Wilson, P. W., Isolation of the key intermediate in biological nitrogen fixation by Clostridium. J. Biol. Chem. **191**, 295–298 (1958).

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MOLYBDENUM IN SYMBIOTIC NITROGEN FIXATION AND IN NITRATE ASSIMILATION *

by E. G. MULDER, K. BAKEMA **, and W. L. VAN VEEN

Laboratory of Microbiology, Agricultural University,
Wageningen, the Netherlands.

INTRODUCTION

The essentiality of molybdenum as a catalyst in nitrogen fixation by free-living micro-organisms has been demonstrated by several workers [7] [9] [10] [13] [16] [22] following the discovery by Bortels [8] in 1930 that this element is a micronutrient for *Azotobacter chroococcum*. That molybdenum is also of major importance in symbiotic nitrogen fixation was shown by the senior author in culture-solution experiments with pea plants [16]. In the absence of added molybdenum the nodules were somewhat smaller than those of normal plants. They had not the normal pink shade, but were of a pale yellow-brown colour. Their nitrogen-fixing capacity was very poor as was concluded from nitrogen analyses and from the light-green colour of the molybdenum-deficient plants as contrasted to the dark green of molybdenum-treated plants. These experiments confirmed earlier findings of Anderson [3] [4] that the addition of small amounts of molybdenum to certain Australian soils had a remarkable effect on nitrogen fixation by clover and lucerne plants.

The essentiality of molybdenum for nitrate assimilation was demonstrated by experiments with *Aspergillus niger* [16] [21] [25] [26], *Neurospora crassa* [21], algae [5] [12] and higher plants [16] [17]. Denitrifying bacteria require also molybdenum when growing under anaerobic conditions in the presence of nitrate [16].

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** Institute for Soil Fertility, Groningen.

The mechanisms of nitrogen fixation, symbiotic as well as non-symbiotic, have not so far been cleared up. As a consequence the precise function of molybdenum in these processes is still obscure. Shug et al. [24] have found that hydrogenase requires molybdenum for the reduction of cytochrome c or other one-electron-accepting compounds but not for the reduction of two-electron acceptors like methylene blue. Hydrogenase is postulated by some authors to play a specific role in the reduction of N_2 to ammonia. This is concluded from the fact that nitrogen-fixing organisms contain relatively large amounts of hydrogenase, while factors which inhibit nitrogen fixation, as nitrate, also inhibit the formation of hydrogenase [11] [14] [23]. It must be stated, however, that no hydrogenase has been detected in Rhizobium cells when grown in the laboratory or when taken directly from the nodule (Shug et al.[23]), while mutants of *Azotobacter vinelandii* which are unable to fix nitrogen contain 50 per cent of the hydrogenase activity of the active nitrogen fixers [1]. Both these observations are not consistent with the essentiality of hydrogenase in nitrogen fixation. In experiments with Azotobacter and Clostridium, Nicholas [20] found that molybdenum deficiency decreased nitrogen fixation but it did not markedly reduce hydrogenase activity.

That nitrogen fixation is a reductive process was shown by investigations with *Clostridium pasteurianum* [30], *Azotobacter vinelandii* [1] [19] and with nodules from soybean plants [31]. The cultures were exposed for a short period of time to an N^{15}_2-containing gas mixture and subsequently the distribution of N^{15} among various N-compounds of the cells and the culture solution was estimated. Evidence was obtained that the first detectable compound after fixation is ammonia from which subsequently glutamic acid is formed.

In order to secure information upon the sequence of compounds during the assimilation of N^{15}_2, the cultures must be killed a short time after their exposure to N^{15}_2. The nitrogenous compounds, preferably those not yet incorporated in cellular proteins, must be isolated and analysed for N^{15}. Since these fractions generally are present in small amounts, their isolation is extremely difficult.

The purpose of the present investigation was to study the mechanism of symbiotic nitrogen fixation as well as that of nitrate assimilation by using molybdenum-deficient plants to which small

amounts of sodium molybdate are added. Since nitrogen-fixation reactions have practically come to a standstill in molybdenum-deficient nodules, their content of non-protein nitrogenous compounds is very low. Addition of molybdate restores nitrogen fixation so that by estimating various nitrogenous fractions after appropriate periods of time, the sequence of reactions in nitrogen fixation may be studied. Isolation of these compounds as in the case of N^{15}-tests is superfluous.

In the present work a study was made of the synthesis of amino acids in the nodules of molybdenum-deficient lucerne and clover plants when small amounts of molybdate were added to their nutrient medium. In similar experiments the synthesis of amino acids from accumulated nitrate was investigated in molybdenum-deficient cauliflower, spinach, and tomato plants after the addition of molybdate.

<div align="center">EXPERIMENTAL</div>

Cultivation of plants

Lucerne, red clover, cauliflower, spinach, and tomato plants were grown in Neubauer jars containing 0.5 or 1 kg of molybdenum-deficient soil. In the case of leguminous plants and spinach approximately 20 plants were cultivated in small jars and 30 in large jars; with cauliflower and tomato the number of plants per pot was about one-half and one-third, respectively, of that with spinach. The basic dressing given to these pots consisted of 0.6 g K_2HPO_4 and 0.1 g $MgSO_4.7H_2O$ per kg of soil. Some of the pots received 5 mg $Na_2MoO_4.2H_2O$ per kg of soil. In order to eliminate a possible sodium effect of the molybdate 3.0 mg $Na_2CO_3.10H_2O$ was added to the control pots. In the case of leguminous plants the soil had to be inoculated with a suspension of the appropriate Rhizobium species.

When the molybdenum-deficient lucerne and clover plants had been cultivated for approximately 8 to 9 weeks, 5 mg $Na_2MoO_4.2H_2O$ in 10 ml glass-distilled water was added per kg of soil. This solution was washed into the soil with a further 20 ml H_2O. Control pots received distilled water only.

The plants were incubated in a greenhouse at 20 to 25°C under natural light conditions and after periods of time varying from 5 hours to 10 days the nodules from 5 pots were collected after careful washing of the roots on a sieve.

The cauliflower, spinach, and tomato plants which were used for studying the effect of molybdenum on nitrate assimilation received in addition to the basic dressing mentioned above 1.5 g KNO_3 per pot containing 1 kg of soil. In order to produce plants of different deficiency levels one series of pots was left without molybdenum, whilst a second series was dressed with 50 µg, and a third with 2.5 mg $Na_2MoO_4.2H_2O$ per pot. After 4 to 5 weeks, 2.5 mg $Na_2MoO_4.2H_2O$ dissolved in 10 ml H_2O

was added to some of the pots with molybdenum-deficient plants and washed into the soil as described above. After different periods of time, plants were harvested and used for various tests.

Analytical methods

Preparation of the samples. In the case of nodules, 1 to 4 g of fresh tissue was ground in a chilled mortar and treated with 200 ml of 80% ethanol to precipitate the protein. The latter was separated by filtration and the filtrate concentrated at 35°C to approximately 1 ml. The concentrate was made up to 5 or 6 ml with distilled water and the free amino acids estimated by paper chromatography.

In the case of cauliflower, spinach, and tomato, average samples of all the laminae – excepting those which showed symptoms of necrosis – were taken from a number of pots. Ten to twenty grams of cut leaf tissue were ground in a porcelain mortar and transferred with 200 ml H_2O to Erlenmeyer flasks.

These were heated in a water-bath at 90°C for 5 minutes. After rapid cooling the samples were kept for a few days at 3°C. The precipitated protein was separated by filtration on a Büchner funnel and washed three times with distilled water. The filtrates, which contained the free amino acids, were concentrated *in vacuo* at 35°C to 10 ml.

Estimation of amino acids by quantitative paper chromatography. The method used, which is fully described elsewhere [18] consists of a two-directional descending procedure with phenol–water and collidine–lutidine–water as the moving phases. Development of the chromatograms took place in a special cabinet in which they were heated under ethanol-saturated anaerobic conditions so that an optimal colour intensity was obtained [18] [27] [28]. After the papers had been dried, the coloured spots were cut out, extracted with 10 ml 50% ethanol and the intensity of the colour measured in a spectrophotometer.

Protein and soluble non-protein and nitrate nitrogen were determined in the samples used for amino-acid determination or in separate samples of 1 to 10 g of ground fresh plant tissue. In the latter case the macerates were transferred with distilled H_2O to 100-ml Erlenmeyer flasks, heated for 15 minutes at 90°C and after cooling treated with 12.5 ml of a 20% solution of trichloro-acetic acid. Subsequently they were filtered on a Büchner funnel, and the precipitates washed several times with a 1% solution of trichloro-acetic acid. Residues as well as filtrates were analysed for nitrogen according to the Kjeldahl-Lauro method. Nitrate was removed from the filtrates by diluting 10 to 20 ml with 50 ml of distilled H_2O and heating the mixture with 5 ml of concentrated H_2SO_4 and 2 g of selenium catalyst. During the concentration of this solution, nitrate was quantitatively decomposed and removed [15]. Nitrate was estimated in a separate aliquot according to the xylenol method [2].

RESULTS

1. *Synthesis of amino acids in nodules of molybdenum-deficient lucerne and clover plants after addition of molybdate*

a) First experiment with lucerne plants (14 to 28 September 1954). Nodules were collected 3, 6, 8, 10, and 14 days after application of 5 mg of $Na_2MoO_4 \cdot 2H_2O$ to lucerne plants with pronounced symptoms of molybdenum deficiency. Control plants, which had received water only, were harvested at 0 and 14 days – plants from soils treated with molybdenum before sowing the lucerne seeds, at 14 days. At each harvest time the nodules of three replicate pots were collected.

Three days after the addition of molybdate to molybdenum-deficient lucerne plants considerable increases in the α-alanine, glutamic acid, γ-aminobutyric acid, and asparagine contents were found (Table I). The protein content was also higher. At six days the

TABLE I

Effect of the addition of molybdate to molybdenum-deficient lucerne plants on the content of free amino acids in their root nodules (mg amino acids per g dry matter) *.								
Plants	Molydenum-deficient							Normal
Treatment.	No Mo added		5 mg $Na_2MoO_4.2H_2O$ added					(Mo before sowing only)
Days after treatment . .	0	14	3	6	8	10	14	14
Amino compound **								
Glycine	0.1	tr	0.2	0.3	tr	tr	tr	tr
α-Alanine	0.7	0.6	2.0	2.7	1.9	1.4	1.2	1.1
Serine	0.3	tr	0.4	0.7	0.3	0.7	tr	0.3
Valine	0.2	0.2	0.4	0.6	0.5	0.5	tr	0.3
Leucines	0.3	0.3	0.4	0.5	tr	0.7	tr	0.4
Proline	tr	tr	tr	tr	tr	tr	1.7	tr
Aspartic acid	0.3	tr	0.4	0.6	0.8	0.7	0.5	0.5
Glutamic acid	4.0	3.2	5.4	6.8	5.6	4.9	4.1	4.3
Arginine	tr	tr	tr	tr	0.6	tr	0.5	1.5
γ-Aminobutyric acid	0.6	0 3	1.3	1.9	1.4	1.1	2.0	1.4
Asparagine	18.4	14.1	22.4	54.3	48.5	63.8	64.7	49.2
Glutamine	0.5	tr	0.5	1.4	0.6	tr	tr	0.4
Soluble non-protein N †	9.5	9.1	10.5	19.8	23.5	19.5	22.6	21.3
Protein-N †	37.6	34.8	45.6	52.8	50.5	—	47.7	52.0

* Averages of quadruplicate values

** Cystine + cysteine, and threonine were found in traces. Methionine + methionine sulphoxide, tyrosine, and lysine were absent.

Phenylalanine, tryptophane, and histidine were not estimated.

† mg per g dry matter.

free amino acids were only slightly higher than at the previous harvest but by contrast asparagine had more than doubled. This rise of asparagine content continued until the tenth day after molybdenum treatment. α-Alanine and glutamic acid reached their maximal values six days after molybdenum treatment. Although the results of this experiment provide evidence that glutamic acid and perhaps α-alanine are formed first whereas asparagine may be considered as a secondary product, it is clear that more conclusive evidence might have been obtained if the nodules had been harvested at shorter time-intervals. Therefore in a second experiment with lucerne plants, the nodules were harvested at 5, 24, and 48 hours after molybdenum treatment.

b) Second experiment with lucerne plants (26 to 28 July 1955). The results of this experiment are recorded in Table II. The protein content as well as the soluble non-protein nitrogen and asparagine contents of the molybdenum-deficient nodules were considerably lower than in the first experiment. Five hours after

TABLE II

Effect of the addition of molybdate to molybdenum-deficient lucerne plants on the content of free amino acids in their root nodules (mg amino acid per g dry matter) *				
Plants	Molybdenum-deficient			Normal
Treatment	No Mo added	5 mg $Na_2MoO_4.2H_2O$ added		(Mo before sowing only)
Hours after treatment . . .	0	5	24	48	24
Amino compound **					
Glycine	tr	0.1	0.2	0.2	tr
α-Alanine	0.9	1.2	2.2	2.4	1.2
Serine	tr	0.3	0.4	0.5	tr
Valine	tr	tr	0.3	0.4	0.3
Leucines	tr	tr	0.5	0.7	0.3
Aspartic acid	tr	0.6	0.5	0.8	1.5
Glutamic acid	2.7	4.7	5.0	7.5	4.6
γ-Aminobutyric acid	0.5	1.2	1.4	2.0	1.1
Asparagine	10.4	9.2	10.7	17.1	55.4
Soluble non-protein-N †	3.2	4.4	5.8	8.5	22.9
Protein-N †	32.2	32.2	38.3	43.5	56.8

* Averages of quadruplicate values.

** Threonine, methionine + methionine sulphoxide, tyrosine, proline, arginine, and glutamine were found in traces only; cystine + cysteine, and lysine were absent; phenylalanine, tryptophane, and histidine were not estimated.

† mg per g dry matter.

application of molybdate to Mo-deficient plants, the glutamic-acid content had increased from 2.7 to 4.7 mg per g of dry nodule tissue. α-Alanine was also slightly higher; probably it is formed by trans-aminase activity from glutamic acid. The same may be the case with γ-aminobutyric acid which is a small but consistent constituent of the soluble non-protein fraction. Asparagine had not increased after 5 and 24 hours, and only slightly after 48 hours. This demonstrates clearly that asparagine does not belong to the primary reaction products in nitrogen fixation of lucerne plants.

In a subsequent experiment with white clover, which showed less pronounced symptoms of Mo-deficiency than the lucerne plants, harvests of nodules were made 28, 47, and 71 hours after addition of molybdate to molybdenum-deficient plants. As with the molyb-denum-deficient lucerne plants, α-alanine, aspartic acid, glutamic acid, γ-aminobutyric acid, and particularly asparagine were the only amino compounds which occurred to any extent in the free state in the clover nodules. Twenty-eight hours after the addition of molyb-denum to molybdenum-deficient plants an increase had occurred in α-alanine, γ-aminobutyric acid and asparagine; the glutamic acid content had hardly increased. After 47 hours, however, the glutamic-acid concentration had changed from 2.4 to 3.2, and the asparagine concentration from 7.4 to 28.7 mg per g of dry tissue.

2. *Synthesis of amino acids from nitrate in molybdenum-deficient cauliflower, spinach, and tomato plants after addition of molybdate*

a) Experiment with cauliflower plants (7 to 9 July 1955). Three types of plants were used in this experiment, viz plants grown without added molybdenum, and plants dressed either with 50 μg or with 2.5 mg of $Na_2MoO_4.2H_2O$. Plants of the first type were small and showed severe symtoms of molybdenum deficiency, those of the second type showed moderate symptoms of Mo-deficiency whereas those receiving a dressing of 2.5 mg $Na_2MoO_4.2H_2O$ were well developed and had dark-green leaves.

Free amino acids in the leaves were determined at 18 and 42 hours after the addition of 2.5 mg $Na_2MoO_4.2H_2O$ to plants of the first two types. Control plants of all three types treated with water only were also analysed (see Table III). It will be seen from these data that 17 hours after the application of molybdate to molybdenum-deficient cauliflower plants the content of the following amino compounds of

TABLE III

Effect of the addition of molybdate to cauliflower plants with different molybdenum deficiencies on the content of free amino acids in their leaves (mg amino acid per g dry matter). *

Amino compound **	Basic dressing only		Basic dressing				+ 2.5 mg Na$_2$MoO$_4$. 2H$_2$O
			+ 50 μg Na$_2$MoO$_4$.2H$_2$O				
	17 h after addition of		18 h after addition of		42 h after addition of		18 h after addition of
	H$_2$O	Mo	H$_2$O	Mo	H$_2$O	Mo	H$_2$O
Glycine	0.5	3.6	0.3	0.9	1.2	3.1	0.2
α-Alanine	1.0	2.2	0.7	1.4	1.0	1.3	1.2
Serine	0.6	1.8	1.0	1.1	1.4	1.3	1.0
Threonine†	1.0	1.1	0.4	0.4	0.6	0.7	0.4
Aspartic acid	1.0	3.2	2.1	2.3	2.0	2.5	1.4
Glutamic acid	3.1	5.0	4.1	4.1	5.1	5.7	3.2
γ-Aminobutyric acid	0.4	0.4	0.9	1.5	0.9	1.0	1.9
Glutamine	1.8	7.5	1.4	2.4	2.0	4.3	1.9
Soluble non-protein-N ††	9.9	12.7	9.8	11.0	10.3	11.0	7.7
Protein-N ††	32.0	34.1	38.4	41.6	36.8	40.2	41.1
NO$_3$-N ††	29.8	20.1	12.6	11.3	7.3	7.1	1.5

* Averages of quadruplicate values.

** Valine, methionine + methionine sulphoxide, leucines, proline, and lysine were found in traces only; cystine + cysteine, tyrosine, arginine, and asparagine were absent; phenylalanine, tryptophane, and histidine were not estimated. Close to glutamine a strongly coloured spot of an unknown amino compound was found.

† The spot of threonine on the chromatogram may have consisted partly of homoserine (cf Ref. [6] [20]).

†† mg per g of dry tissue.

the non-protein fraction had risen enormously: glycine, α-alanine, serine, aspartic acid, glutamic acid and glutamine. In the plants with slight deficiency symptoms a pronounced increase due to molybdenum treatment was found only with glycine and glutamine. The very high content of free glycine in these plants after addition of molybdenum is very remarkable, especially since the normal plants, which had received an adequate supply of molybdenum during their entire development, contained very little. The normal plants were also relatively low in glutamine and high in γ-aminobutyric acid. This is in agreement with the fact that glutamine may be considered as a storage compound for excessive amounts of ammonia which, in the case of normal plants, had been used up for

protein synthesis. The high content of γ-aminobutyric acid may have been due to the fact that it is not built into protein.

b) Experiments with spinach plants (July and August 1955). As in the experiment with cauliflower, three types of plants were used in this case, viz. those with severe symptoms of molybdenum deficiency, those with slight symptoms, and healthy plants. Analyses were carried out in samples harvested approximately 8 and 25 hours after addition of molybdate. The results accord with those of the previous experiment in respect of the response to molybdenum of the amino acids of the non-protein fraction. Glycine, however, which was formed in large amounts in molybdenum-deficient cauliflower plants upon addition of molybdate, was not found – or occurred in traces only – under similar conditions in spinach plants. In as little as 8 hours the addition of molybdate to the soil in which the Mo-deficient spinach plants were growing caused great increases (between 30 and 250 per cent) in the contents of 7 amino compounds. Hence no conclusions can be drawn as to the sequence of formation of these compounds. A second experiment with molybdenum-deficient spinach plants was therefore carried out in which the amino acids were assayed 2, 4, 6, and 24 hours after the addition of molybdate to deficient plants (Table IV).

It will be seen that 2 hours after molybdenum treatment the contents of free glutamic acid and glutamine showed the most pronounced rises. Serine, α-alanine, and aspartic acid had only slightly increased. Four hours after molybdenum application, the increase in content of free serine, α-alanine, aspartic acid, glutamic acid and particularly glutamine had continued. Twenty-four hours after molybdenum treatment glutamine had increased enormously. The amino acid aspartic acid now showed the highest rise, and in addition protein synthesis had also started. When a comparison is made between molybdenum-deficient plants 24 hours after treatment with molybdenum and those grown with adequate amounts of molybdenum during their entire development, it is seen that the protein content of the latter plants is considerably higher whereas their content of the free amino compounds, including glutamine, is much lower.

c) Experiment with tomato plants (August 1954). In this experiment a comparison was made between the synthesis of amino acids in molybdenum-deficient plants dressed with molybdate and

TABLE IV

Effect of the addition of molybdate to molybdenum-deficient spinach plants on the content of free amino acids in their leaves (mg amino acid per g dry matter) *										
	Plants with basic dressing only								Normal plants (Mo before sowing)	
Amino compound **	2 h after addition of		4 h after addition of		6 h after addition of		24 h after addition of			
									1 h	25 h
	H₂O	Mo	H₂O	Mo	H₂O	Mo	H₂O	Mo	(H₂O)	(H₂O)
Serine †	0.6	0.9	0.5	1.4	0.5	1.9	0.8	1.9	0.7	0.4
α-Alanine	0.5	0.7	0.7	1.4	0.9	1.7	0.9	2.2	0.5	0.4
Threonine ††	0.3	0.3	0.3	0.4	0.3	0.5	0.5	0.5	0.4	0.3
Valine	0.2	0.3	0.2	0.3	0.3	0.3	0.1	0.1	0.3	0.2
Leucines	0.3	0.2	0.3	0.4	0.3	0.5	0.2	0.8	0.2	0.1
Aspartic acid	1.5	1.7	1.4	3.2	1.7	3.4	1.1	6.4	2.7	2.2
Glutamic acid	4.7	5.7	5.4	7.0	5.6	7.0	4.8	8.0	5.8	3.4
γ-Aminobutyric acid	0.8	0.6	0.7	1.3	1.0	1.0	1.2	1.6	0.6	0.8
Glutamine	0.4	1.1	0.4	3.2	0.6	5.6	0.9	10.1	1.4	0.8
Soluble non-protein-N ++	11.4	9.3	8.9	10.6	9.2	10.4	8.3	12.9	8.8	7.5
Protein-N ++	27.7	28.0	26.7	26.1	26.6	26.3	28.0	30.0	43.0	41.8
NO₃-N++	28.8	30.7	37.4	31.9	36.1	31.0	30.6	23.9	2.8	2.9

* Averages of quadruplicate values.

** Proline was found in traces only; cystine + cysteine, methionine + methionine sulphoxide, tyrosine, arginine, lysine, and asparagine were absent; phenylalanine, tryptophane, and histidine were not determined.

† Serine was contaminated with a trace of glycine.

†† The spot of threonine on the chromatogram may have consisted partly of homo-serine (cf Ref. [6] [29]).

++ mg per g of dry tissue.

that in nitrogen-deficient plants dressed with nitrate. For this purpose the plants were grown in a molybdenum-deficient soil, in one case in the absence of added molybdenum but supplied with nitrate and in the second in the presence of added molybdenum but without nitrate. Amino-acid analyses were carried out in samples harvested 2, 3, 6, and 8 days after the addition of molybdate to the molybdenum-deficient plants and of nitrate to the nitrogen-deficient plants. Although the first harvests were made relatively late, some interesting conclusions can be drawn from the amino-acid analyses (Table V). As with the cauliflower and spinach plants, asparagine was not found among the free amino compounds in tomato leaves. Glutamine showed the most striking rise two days after the application of molybdenum to molybdenum-deficient plants and of

TABLE V

Effect of the addition of molybdate to molybdenum-deficient and of nitrate to nitrogen-deficient tomato plants on the content of free amino acids in their leaves (mg amino acid per g dry matter). *										
Plants	Mo-deficient					Nitrogen-deficient				
Treatment. . . .	No Mo added	5 mg $Na_2MoO_4 . 2H_2O$ added				No KNO_3 added	1.5 g KNO_3 added			
Days after treatment	1	2	3	6	8	1	2	3	6	8
Amino compound **										
Glycine	tr	tr	0.9	1.5	1.7	tr	tr	0.2	0.3	0.8
α-Alanine	0.4	2.8	1.0	1.1	1.2	0.1	0.5	0.6	0.9	1.3
Serine	tr	tr	0.7	0.4	0.6	tr	0.1	0.2	0.2	0.4
Threonine	tr	1.2	0.4	0.6	0.5	tr	tr	0.1	tr	0.4
Valine	tr	0.3	tr	0.3	0.2	tr	tr	tr	tr	0.2
Leucines	tr	0.5	tr	0.5	0.4	0.0	tr	tr	tr	0.2
Aspartic acid	0.7	1.4	0.9	0.9	0.5	0.2	0.5	0.7	0.6	0.5
Glutamic acid	1.3	3.2	1.9	0.8	0.6	0.2	0.2	0.6	0.5	0.5
Arginine	tr	2.2	tr	1.2	1.1	0.0	0.0	0.4	tr	0.0
γ-Aminobutyric acid	1.9	3.6	3.6	3.9	4.1	0.5	1.2	2.0	3.2	3.8
Glutamine	tr	6.6	1.9	3.6	3.3	tr	4.0	4.0	1.6	2.0

* Averages of quadruplicate values.

** Proline and asparagine were found in traces. Cystine + cysteine, tyrosine, methionine + methionine sulphoxide and lysine were absent. Phenylalanine, tryptophane, histidine, soluble non-protein-N, protein-N, and NO_3-N were not estimated.

nitrate to nitrogen-deficient plants. In subsequent harvests its content decreased, apparently because it was used for protein synthesis. Glutamic and aspartic acids showed similar trends. γ-Aminobutyric acid, however, increased constantly, so that eight days after the addition of molybdate and nitrate this amino compound occurred in the highest amount. When a comparison is made between the molybdenum-deficient and the nitrogen-deficient plants it is seen that the latter were lower in free amino compounds than the former, and that the rise in content of these compounds was somewhat less upon the addition of nitrate than on the addition of molybdenum. A close resemblance may be observed, however, between the responses to molybdenum and nitrogen.

DISCUSSION

To study the mechanism of nitrogen fixation, experiments with labelled nitrogen have been carried out [1] [19] [30] [31]. In these experi-

ments isolation and subsequent analysis for N^{15} of the nitrogenous compounds, involved in these reactions, are essential procedures. Isolation of adequate quantities of these compounds to enable testing for N^{15} is not an easy task, however.

The method described in the present paper enables the study of the mechanism of nitrogen fixation and of nitrate assimilation without isolation of the nitrogenous compounds. As a result of this the estimation of the formation of much smaller quantities of these compounds is possible than in the case of the isotopic technique.

The procedure depends on the addition of small amounts of molybdate to molybdenum-deficient leguminous plants. The latter are provided with normal or even than normal numbers of nodules which fix the atmospheric nitrogen inadequately or not at all, however. The enzymes required for N_2-fixation presumably are present in approximately normal amounts; their inactivity as far as nitrogen fixation is concerned apparently depends on the lack of molybdenum only. Addition of molybdate to the molybdenum-deficient plants restores the activity of the nitrogen-fixing enzymes within some hours (cf Table II). Estimation of free amino acids by chromatographic methods in the nodules, different periods of time after the addition of molybdenum, enables the study of the sequence of reactions in nitrogen fixation. Similarly the effect of added molybdenum on other metabolic processes may be studied.

In the present investigation amino-acid analyses have been carried out in two experiments with nodules from molybdenum-deficient lucerne plants and in one with clover nodules. These analyses showed that the number of amino acids occurring in the free state in these nodules is restricted. In molybdenum-deficient lucerne, glutamic acid was the only amino acid which occurred in the nodules in amounts higher than 1 mg per g of dry matter; in addition relatively large amounts of aspargine were found. Addition of molybdate brought about a rise in the contents of free glutamic acid, α-alanine, γ-aminobutyric acid, and asparagine. A number of other amino acids including glycine, serine, valine, the leucines, and aspartic acid were also slightly increased. In the second experiment with lucerne nodules the first analyses were carried out 5 hours after the addition of molybdate. During the first few hours glutamic-acid content showed the most pronounced rise. This may indicate that glutamic dehydrogenase is the most important enzyme in

synthesizing amino acids from fixed nitrogen in lucerne nodules, a conclusion which confirms the results of Allison and Burris [1] obtained with labelled N_2 in *Azotobacter vinelandii*. The fact that the contents of α-alanine, aspartic acid, and γ-aminobutyric acid had also increased slightly a few hours after addition of molybdate, indicates the existence either of a very active transaminase system or, in addition to glutamic dehydrogenase, of enzyme systems which catalyze the direct synthesis of α-alanine and aspartic acid. The increase of the asparagine content started relatively late but continued to very high values ten days after the addition of molybdate. This shows that asparagine must be considered as a secondary assimilation product. Definite proof of the existence in nodules of leguminous plants of more than one primary amino-acid-synthesizing system awaits the application of appropriate inhibitors for eliminating transaminase activity.

The experiments with molybdenum-deficient cauliflower, spinach, and tomato plants have shown that synthesis of amino acids from accumulated nitrate started within a few hours after the addition of molybdate to the soil in which the plants were growing. Consequently, no conclusions concerning the sequence of formation of amino acids can be drawn from the experiment with cauliflower plants in which the first analyses were carried out 17 hours after the addition of molybdate (Table III). Responses of molybdenum-deficient cauliflower leaves to added molybdate differed from those of nodules of molybdenum-deficient lucerne and clover plants. Although glutamic-acid content had increased considerably, the rise of glutamine was more striking. Apparently the amide was formed readily from glutamic acid. Other amino acids which had increased considerably were glycine, α-alanine, serine, and aspartic acid. The rise of glycine content to very high levels is very remarkable, since this amino acid was practically absent in normal cauliflower leaves.

The experiment with spinach leaves provided evidence that glutamic acid is the primary amino acid to be synthesized. This may be concluded from the fact that two hours after the addition of molybdate to Mo-deficient plants, glutamic acid and particularly glutamine had increased considerably, but aspartic acid and α-alanine had increased only slightly.

SUMMARY

A study was made of the sequence in which amino acids are formed during nitrogen fixation by root nodules of lucerne and clover plants, and during nitrate assimilation by cauliflower, spinach, and tomato plants. For this investigation, use was made of the fact that both nitrogen fixation and nitrate reduction require molybdenum as an essential element. In the absence of adequate amounts of molybdenum, leguminous plants are well supplied with nodules but the latter are unable to fix atmospheric nitrogen. As a result of this, the content of free amino acids in molybdenum-deficient nodules is very low. By adding small amounts of molybdate to Mo-deficient plants and estimating the amino acids in the nodules different periods of time after the application of molybdenum, information was gained concerning the synthesis of amino acids. The same principle was used for studying nitrate assimilation in cauliflower, spinach and tomato plants.

Some hours after the application of molybdate to the soil in which Mo-deficient lucerne plants were growing, the content of free glutamic acid showed a pronounced rise. The contents of α-alanine, aspartic acid, and γ-aminobutyric acid had increased slightly. The synthesis of asparagine, however, which is the most abundant amino compound in normal nodules, started more than 24 h after the application of molybdenum.

In the case of nitrate assimilation, response to added molybdenum was observed two hours after the addition of molybdate to the soil in which molybdenum-deficient spinach plants were growing. The contents of glutamic acid and glutamine showed the most pronounced increases; those of aspartic acid and α-alanine were only slightly increased. Asparagine was not found among the free amino compounds in leaves of cauliflower, spinach and tomato plants.

REFERENCES

1 Allison, R. M. and Burris, R. H., Kinetics of fixation of nitrogen by *Azotobacter vinelandii*. J. Biol. Chem. **224**, 351–364 (1957).

2 Alten, F., Wandrowsky, B., and Hille, E., Die Bestimmung des Nitratstickstoffs in Pflanzensubstanzen als Nitroxylenol. Bodenk. u. Pflanzenernähr. **1**, 340–348 (1936)

3 Anderson, A. J., Molybdenum deficiency on a South Australian ironstone soil. J. Australian Inst. Agr. Sci. **8**, 73–75 (1942).

4 Anderson, A. J., Thomas, M. P., and Oertel, A. C., Plant responses to molybdenum as a fertilizer. Council Sci. Ind. Research Commonwealth Australia Bull. **198** (1946).

5 Arnon, D. J., Ichioka, P. S., Wessel, G., Fujiwara, A., and Woolley, J. T., Molybdenum in relation to nitrogen metabolism. I. Assimilation of nitrate nitrogen by Scenedesmus. Physiol. Plantarum **8**, 538–551 (1955).

6 Bakhuis, J. A., Homoserine in bleeding sap of pea plants. Nature (London) **180**, 713 (1957).

7 Birch-Hirschfeld, L., Über den Einfluss von Molybdän und Bodenextraktstoffen auf die N-Bindung von *Azotobacter chroococcum*. Arch. Mikrobiol. **3**, 341–361 (1932).

8 Bortels, H., Molybdän als Katalysator bei der biologischen Stickstoffbindung. Arch. Mikrobiol. **1**, 333–342 (1930).

9 Burema, S. J. and Wieringa, K. T., Molybdenum as a growth factor of *Azotobacter chroococcum*. Antonie van Leeuwenhoek **8**, 123–133 (1942).

10 Burk, D. and Lineweaver, H., The influence of calcium and strontium upon the catalysis of nitrogen fixation by Azotobacter. Arch. Mikrobiol. **2**, 155–186 (1931).

11 Green, M. and Wilson, P. W., Hydrogenase and nitrogenase in Azotobacter. J. Bacteriol. **65**, 511–517 (1953).

12 Ichioka, P. S. and Arnon, D. J., Molybdenum in relation to nitrogen metabolism. II. Assimilation of ammonia and urea without molybdenum by Scenedesmus. Physiol. Plantarum **8**, 552–560 (1955).

13 Kluyver, A. J. and Reenen, W. J. van, Über *Azotobacter agilis* Beijerinck. Arch. Mikrobiol. **4**, 280–300 (1933).

14 Lee, S. B. and Wilson, P. W., Hydrogenase and nitrogen fixation by Azotobacter. J. Biol. Chem. **151**, 377–385 (1943).

15 Man, Th. J. de, De samenstelling van het graseiwit. Landbouwk. Tijdschr. **54**, 739–748 (1942).

16 Mulder, E. G., Importance of molybdenum in the nitrogen metabolism of microorganisms and higher plants. Plant and Soil **1**, 94-119 (1948).

17 Mulder, E. G., Molybdenum in relation to growth of higher plants and microorganisms. Plant and Soil **5**, 368–415 (1954).

18 Mulder, E. G. and Bakema, K., Effect of the nitrogen, phosphorus, potassium and magnesium nutrition of potato plants on the content of free amino-acids and on the amino-acid composition of the protein of the tubers. Plant and Soil **7**, 135–166 (1956).

19 Newton, J. W., Wilson, P. W., and Burris, R. H., Direct demonstration of ammonia as an intermediate in nitrogen fixation by Azotobacter. J. Biol. Chem. **204**, 445–451 (1953).

20 Nicholas, D. J. D., The function of trace metals in the nitrogen metabolism of plants. Ann. Botany **21**, 587–598 (1957).

21 Nicholas, D. J. D. and Nason, A., Molybdenum and nitrate reductase. II. Molybdenum as a constituent of nitrate reductase. J. Biol. Chem. **207**, 353–360 (1954).

22 Schröder, M., Die Assimilation des Luftstickstoffs durch einige Bakterien. Zentrbl. Bakteriol. Parasitenk. II **85**, 177–212 (1931–1932).

23 Shug, A. L., Hamilton, P. B., and Wilson, P. W., Hydrogenase and nitrogen fixation. *In:* Inorganic Nitrogen Metabolism, p. 344–360; ed. by W. D. McElroy and Bentley Glass; Hopkins Press, Baltimore, U.S.A. 1956.

24 Shug, A. L., Wilson, P. W., Green, D. E., and Mahler, H. R., The role of molybdenum and flavin in hydrogenase. J. Am. Chem. Soc. **76**, 3355–3356 (1954).

25 Steinberg, R. A., Relation of accessory growth substance to heavy metals including molybdenum, in the nutrition of *Aspergillus niger*. J. Agr. Research **52**, 439–448 (1936).

26 Steinberg, R. A., Role of molybdenum in the utilization of ammonium and nitrate nitrogen by *Aspergillus niger*. J. Agr. Research **55**, 891–902 (1937).

27 Thompson, J. F. and Steward, F. C., Investigations on nitrogen compounds and nitrogen metabolism in plants. II. Variables in two-directional paper chromatography of nitrogen compounds: A quantitative procedure. Plant Physiol. **26**, 421–440 (1951).

28 Thompson, J. F., Zacharius, R. M., and Steward, F. C., Investigations on nitrogen compounds and nitrogen metabolism in plants. I. The reaction of nitrogen compounds with ninhydrin on paper; A quantitative procedure. Plant Physiol. **26**, 375–397 (1951).

29 Wieringa, K. T. and Bakhuis, J. A., Chromatography as a means of selecting effective strains of Rhizobia. Plant and Soil **8**, 254–262 (1957).

30 Zelitch, I., Rosenblum, E. D., Burris, R. H., and Wilson, P. W., Isolation of the key intermediate in biological nitrogen fixation by Clostridium. J. Biol. Chem. **191**, 295–298 (1958).

31 Zelitch, I., Wilson, P. W., and Burris, R. H., The amino acid composition and distribution of N^{15} in soybean root nodules supplied N^{15}-enriched N_2. Plant Physiol. **27**, 1–8 (1952).

MOLYBDENUM IN SYMBIOTIC NITROGEN FIXATION AND IN NITRATE ASSIMILATION *

by E. G. MULDER, K. BAKEMA **, and W. L. VAN VEEN

Laboratory of Microbiology, Agricultural University,
Wageningen, the Netherlands.

INTRODUCTION

The essentiality of molybdenum as a catalyst in nitrogen fixation by free-living micro-organisms has been demonstrated by several workers [7] [9] [10] [13] [16] [22] following the discovery by Bortels [8] in 1930 that this element is a micronutrient for *Azotobacter chroococcum*. That molybdenum is also of major importance in symbiotic nitrogen fixation was shown by the senior author in culture-solution experiments with pea plants [16]. In the absence of added molybdenum the nodules were somewhat smaller than those of normal plants. They had not the normal pink shade, but were of a pale yellow-brown colour. Their nitrogen-fixing capacity was very poor as was concluded from nitrogen analyses and from the light-green colour of the molybdenum-deficient plants as contrasted to the dark green of molybdenum-treated plants. These experiments confirmed earlier findings of Anderson [3] [4] that the addition of small amounts of molybdenum to certain Australian soils had a remarkable effect on nitrogen fixation by clover and lucerne plants.

The essentiality of molybdenum for nitrate assimilation was demonstrated by experiments with *Aspergillus niger* [16] [21] [25] [26], *Neurospora crassa* [21], algae [5] [12] and higher plants [16] [17]. Denitrifying bacteria require also molybdenum when growing under anaerobic conditions in the presence of nitrate [16].

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** Institute for Soil Fertility, Groningen.