THE EFFECT OF MINERAL NUTRITION ON THE CONTENT OF FREE AMINO ACIDS AND AMIDES IN TOMATO PLANTS

II. A STUDY OF THE EFFECT OF MOLYBDENUM NUTRITION

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Summary

Tomato plants which had been cultured in the absence of molybdenum were provided with molybdate, and the consequent changes in the relative amounts of the free amino acids and amides were followed over an experimental period of 4 days. The technique of quantitative paper chromatography was used to estimate the individual free amino acids and amides, and the results are expressed on a dry weight basis.

Changes in the concentrations of many of the individual amino acids occurred within 4 hr of applying molybdenum. Large increases in the concentration of aspartic acid, glutamic acid, glycine, glutamine, and asparagine occurred initially with molybdenum treatment, but were followed by steady declines. A different pattern of change was shown by the two compounds β-alanine and γ-aminobutyric acid, both of which decreased in concentration soon after molybdenum was applied.

The results of this investigation are discussed in relation to the pathway by which the products of nitrate reduction are incorporated into proteins.

I. INTRODUCTION

Spencer and Wood (1954) have shown that the provision of molybdenum to plants deficient in this element promotes rapid changes in the concentrations of a variety of nitrogenous compounds. In terms of concentration, nitrate nitrogen decreases and nitrite, ammonia, amino acid and amide nitrogen fluctuate during a 4-day period after treatment. The present investigation has been designed to amplify that of Spencer and Wood with respect to the amino acid and amide fractions. The same general experimental approach has been used, but whereas in the earlier investigation only total amino nitrogen and total amide nitrogen were measured, in this an attempt has been made to observe changes with individual compounds.

Tomato plants cultured in the absence of molybdenum were provided with molybdate, and the consequent changes in the concentrations of the individual amino acids and amides were measured. In the first phase of the investigation an experimental period of 4 days was adopted. The results of this experiment, however, showed that important changes occurred during the first 24 hr after the addition of molybdenum, and in a later phase, therefore, an experimental period of less than 24 hr was incorporated in the design with frequent samples during this period.

This investigation, like that of Spencer and Wood, is intended as a contribution to the elucidation of the nutritional effect of molybdenum. It may be noted, however,

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that since Nicholas, Nason, and McElroy (1953) have shown that molybdenum is required for the activity of nitrate reductase, the present series of observations are also of some significance in relation to the mechanism of nitrate incorporation.

II. Methods

The culture of tomato plants deficient in molybdenum, and the analysis of extracts of the experimental plants for free amino acids and amides were carried out as described by Possingham (1956). In this investigation the plants were grown in an unheated glass-house and they were kept in the glass-house throughout the experimental period.

III. Experimental and Results

As indicated above the investigation involved two experimental series. The details of the experimental design and the results are given separately for each series.

(*a*) *Experiment* I

Twenty-seven pots, each of which held eight molybdenum-deficient plants, provided the material for this experiment. The experimental period commenced when the plants first clearly showed the symptoms of molybdenum deficiency. This was 2 weeks after the seedlings were transferred to culture solutions deficient in molybdenum, and 5 weeks from the commencement of germination.

The experiment involved an unreplicated design in which the set of deficient seedlings was divided into two groups. One group, the untreated series, was not treated further, but to the second, the treated series, a solution of sodium molybdate was added at the commencement of the experimental period. The volume added to each 3-l. pot gave 0·0151 g of the salt per pot which was equivalent to a molybdenum concentration of 2 p.p.m. A random sample of three pots was taken at the beginning of the experimental period, and this is termed the zero time harvest. Subsequently random samples of three pots were taken from each series at intervals of 24 hr over the next 4 days. The sample of three pots comprised 24 plants, and the shoots were removed by cutting through the stem immediately above the cotyledons. From each bulked sample of 24 shoots, six were taken for the determination of dry weights, the remaining 18 shoots being used for the determination of amino acids and amides.

In this and in the subsequent experiment the results of the amino acid analyses are expressed as concentrations in μg of amino acid or amide per mg dry weight. The results for experiment I are given in Table 1, from which it is evident that molybdenum treatment promoted considerable changes.

Statistical assessment of the significance of the many concentration drifts required logarithmic transformation of the values. The data were first examined to ascertain whether the observed increases in the individual amino acid and amide concentrations after molybdenum treatment were contributed to significantly by downward time drifts in the untreated series. Serine, alone of all the constituents, showed a significant downward trend between zero time and 96 hr. Although there is some indication that this is true for other constituents, the trends are erratic in the untreated control plants. Accordingly the five observations on the untreated

TABLE 1

EXPERIMENT I: CHANGES IN DRY WEIGHT (MG) AND CONCENTRATION OF AMINO ACIDS AND AMIDES (μG PER MG DRY WEIGHT) OF MOLYBDENUM-DEFICIENT PLANTS FOLLOWING TREATMENT WITH MOLYBDATE

Amino Acid or Amide	Treatment	Length of Time after Molybdate Addition (hr)				
		Zero Time	24	48	72	96
Aspartic acid	−Mo	0·170	0·234	0·098	0·147	0·114
	+Mo		2·931	1·989	1·827	1·363
Glutamic acid	−Mo	2·950	2·518	3·076	2·083	1·856
	+Mo		12·789	9·185	7·824	5·069
Asparagine	−Mo	0·108	0·144	0·108	0·154	0·292
	+Mo		0·987	1·239	0·662	0·606
Glutamine	−Mo	0·309	0·248	0·160	0·143	0·671
	+Mo		1·852	1·743	3·067	2·120
Citrulline	−Mo	0·295	0·238	0·155	0·132	0·447
	+Mo		0·539	0·287	0·256	0·236
Arginine	−Mo	0·721	0·646	0·222	0·268	0·368
	+Mo		0·559	0·407	0·150	0·101
"Under arginine"*	−Mo	0·515	0·298	0·155	0·222	0·251
	+Mo		0·809	0·297	0·022	0·101
Serine	−Mo	2·548	1·490	1·301	1·138	0·825
	+Mo		2·577	1·932	1·489	1·514
Glycine	−Mo	0·319	0·189	0·129	0·114	0·206
	+Mo		1·617	1·843	0·800	0·943
Threonine	−Mo	0·273	0·323	0·279	0·199	0·206
	+Mo		0·770	0·687	0·645	0·808
α-Alanine	−Mo	1·014	1·082	0·806	0·934	0·591
	+Mo		2·003	1·656	1·022	1·649
β-Alanine	−Mo	0·113	0·174	0·155	0·154	0·109
	+Mo		Absent	Absent	Absent	Absent
γ-Aminobutyric acid	−Mo	0·515	1·058	1·094	1·300	0·653
	+Mo		Absent	Absent	0·022	0·303
Ethanolamine	−Mo	0·257	0·263	0·222	0·137	0·251
	+Mo		0·086	0·074	0·044	0·075
Phenylalanine	−Mo	0·196	0·234	0·186	0·069	0·206
	+Mo		0·068	0·091	0·130	0·124
Valine	−Mo	0·294	0·308	0·114	0·154	0·150
	+Mo		0·077	0·062	0·133	0·163
Leucine	−Mo	0·232	0·462	0·186	0·205	0·251
	+Mo		0·135	0·142	0·145	0·172
Proline	−Mo	0·772	0·845	1·363	0·821	0·413
	+Mo		1·348	1·411	1·022	0·959
Total amino acids	−Mo	12·714	10·670	9·541	8·077	6·897
	+Mo		26·374	20·034	15·531	13·580
Total amides	−Mo	0·417	0·392	0·269	0·297	0·963
	+Mo		2·839	2·982	3·729	2·726
Dry weight per plant (mg)	−Mo	10·79	14·38	20·77	20·38	23·06
	+Mo		14·42	22·43	24·99	33·00

* See Part I of this series (Possingham 1956).

plants were used to estimate the variance of the mean of the untreated series, against which the various molybdenum treatments were compared. As only a single observation could be made at each harvest, owing to the complexity of amino acid analysis, it was necessary to assume that the variance of the untreated series would also apply at each harvest of the treated series. Thus the significance of the changes in each constituent after 24 hr molybdenum treatment was assessed. In addition an assessment of the changes in each constituent of the molybdenum-treated plants

TABLE 2

EXPERIMENT I: STATISTICAL SUMMARY OF THE RESULTS

The results are the transformed values of the concentrations of amino acids and amides (transformation log (1000x))†

Amino Acid or Amide	−Mo (mean of −Mo treatments)	+Mo (24 hr)	Difference	Regression (+Mo 24 hr to +Mo 96 hr)
Aspartic acid	2·163	3·467	1·304***	−*
Glutamic acid	3·389	4·106	0·717***	−*
Asparagine	2·175	2·994	0·819**	−*
Glutamine	2·414	3·268	0·854**	+*
Citrulline	2·362	2·732	0·370	−
Arginine	2·602	2·747	0·145	−*
"Under arginine"	2·424	2·908	0·484	−
Serine	3·133	3·411	0·278*	−
Glycine	2·252	3·209	0·957**	−
Threonine	2·401	2·886	0·485**	+
α-Alanine	2·938	3·302	0·364	−
β-Alanine	2·142	Absent	−( )***	
γ-Aminobutyric acid	2·941	Absent	−( )***	+***
Ethanolamine	2·343	1·934	−0·409*	−
Phenylalanine	2·217	1·833	−0·384*	+
Valine	2·276	1·886	−0·390	+
Leucine	2·402	2·130	−0·272*	+
Proline	2·896	3·130	+0·234	−

* Significant at $P < 0.05$. ** Significant at $P < 0.01$. *** Significant at $P < 0.001$.

† In this table, as also in Table 4, the estimations of amino acid content, reported here to three or four decimal places for convenience of statistical analysis, are probably reliable to within 1 per cent. The statistical treatment of the data has been explained on pp. 41 and 46 of this paper.

over the period from 24 to 96 hr was made by extracting the regressions on time. A summary of these changes with the statistical significance of each is given in Table 2.

It is evident from Tables 1 and 2 that molybdenum treatment enhanced dry weight and increased the concentrations of the amino acid and amide fractions. The total amino acid fraction reached a peak value 24 hr and the amide fraction 72 hr after the beginning of the experimental period.

TABLE 3

EXPERIMENT II: CHANGES IN DRY WEIGHT (MG) AND CONCENTRATION OF AMINO ACIDS AND AMIDES (μG PER MG DRY WEIGHT) OF MOLYBDENUM-DEFICIENT PLANTS FOLLOWING TREATMENT WITH MOLYBDATE

Amino Acid or Amide	Treatment	Length of Time after Molybdate Addition (hr)					
		Zero Time	½	1	2	4	8
Aspartic acid	−Mo	0·154				0·085	0·099
	+Mo		0·127	0·098	0·393	1·388	2·240
Glutamic acid	−Mo	2·761				1·862	1·987
	+Mo		2·200	1·903	3·037	5·111	6·505
Asparagine	−Mo	0·136				0·102	0·084
	+Mo		0·154	0·121	0·201	0·463	0·697
Glutamine	−Mo	0·289				0·187	0·265
	+Mo		0·260	0·195	0·262	1·545	5·089
Citrulline	−Mo	0·234				0·136	0·084
	+Mo		0·232	0·144	0·178	0·296	0·331
Arginine	−Mo	0·318				0·267	0·298
	+Mo		0·382	0·334	0·277	0·414	0·408
"Under arginine"	−Mo	0·222				0·183	0·091
	+Mo		0·247	0·196	0·174	0·310	0·298
Serine	−Mo	1·147				0·964	1·065
	+Mo		0·920	0·848	1·337	1·873	2·618
Glycine	−Mo	0·121				0·082	0·104
	+Mo		0·116	0·105	0·147	0·293	0·498
Threonine	−Mo	0·208				0·144	0·191
	+Mo		0·233	0·165	0·230	0·358	0·448
α-Alanine	−Mo	0·865				0·569	0·755
	+Mo		0·577	0·687	0·841	1·199	1·693
β-Alanine	−Mo	0·104				0·056	0·086
	+Mo		0·108	0·087	0·056	Absent	Absent
γ-Aminobutyric acid	−Mo	0·995				0·773	0·844
	+Mo		0·871	0·724	0·663	0·310	0·212
Ethanolamine	−Mo	0·212				0·103	0·184
	+Mo		0·275	0·158	0·211	0·099	0·187
Phenylalanine	−Mo	0·172				0·082	0·098
	+Mo		0·163	0·102	0·082	0·103	0·074
Valine	−Mo	0·201				0·159	0·197
	+Mo		0·289	0·152	0·303	0·154	0·105
Leucine	−Mo	0·310				0·188	0·218
	+Mo		0·298	0·312	0·222	0·268	0·122
Proline	−Mo	1·040				0·774	0·924
	+Mo		0·732	0·696	1·152	1·032	1·244
Total amino acids	−Mo	9·064				6·427	7·225
	+Mo		7·770	6·681	9·303	13·208	16·983
Total amides	−Mo	0·425				0·289	0·349
	+Mo		0·414	0·316	0·463	2·008	5·786
Dry weight per plant (mg)	−Mo	31·98				36·62	37·74
	+Mo		36·45	38·32	31·83	39·62	33·48

With respect to the individual constituents, the largest changes were recorded with aspartic acid, glutamic acid, and glycine. The maximum concentrations of both aspartic and glutamic acid were found at 24 hr, when in the treated plants the concentration of aspartic acid was 10 times greater, and that of glutamic acid five times greater, than it was in the untreated plants. After 24 hr the concentrations of both these acids in the treated plants decreased steadily with time. Glycine was at a maximum at 48 hr, when its concentration in the treated plants was approximately 10 times that of the untreated plants. Thereafter this compound also decreased in concentration.

Serine and γ-alanine showed similar although smaller changes, and with both these compounds the highest concentrations were found at 24 hr. Threonine increased rapidly in the first 24 hr and continued to increase slightly over the succeeding 72 hr. β-alanine and γ-aminobutyric acid, although they were present in the untreated molybdenum-deficient plants, disappeared during the first 24 hr after treatment. Although γ-aminobutyric acid was detected in the later phases of the experimental period in the treated plants, β-alanine did not reappear and this substance may therefore be taken as characteristic of the deficient tissue. Ethanolamine, phenylalanine, and leucine decreased as a result of treatment during the first 24 hr. During the final 72 hr ethanolamine continued to decrease while phenylalanine and leucine tended to increase.

With respect to the amides asparagine and glutamine the concentrations of both at first increased and then decreased after treatment. In the case of asparagine a peak value was reached at 48 hr and with glutamine at 72 hr.

(*b*) *Experiment* II

Sixteen pots of molybdenum-deficient tomato plants were used in this experiment. The plants were harvested 3 weeks after planting out the seedlings and 6 weeks from the commencement of germination. This experiment was similar to experiment I; it involved an unreplicated design in which the deficient seedlings were divided into two groups. One group was not treated further, but sodium molybdate was added to the second group at the beginning of the experimental period. The untreated group was sampled at the start of the experimental period (zero time) and after 4 and 8 hr. The treated group was sampled at $\frac{1}{2}$, 1, 2, 4, and 8 hr after zero time. Each sample involved two pots or 16 shoots and, as before, separate samples were taken for determination of dry weight and for the determination of amino acids.

The results of both the amino acid analyses and the dry weight estimations are given in Table 3. The dry weight yields of the plants used in this experiment were higher than those recorded in experiment I. The differences were not large and were a result of the greater age of the experimental plants. Approximately the same range and concentrations of amino acids and amides were found in untreated plants at zero time in this experiment as in experiment I.

For statistical analysis the actual concentration values were transformed to logarithms. A summary of this analysis is given in Table 4.

There is a similar difficulty here as in the previous experiment in attempting to form an estimate of error. It was assumed that the variation between zero time, 4, and 8 hr in the absence of molybdenum, and the ½- and 1-hr treatments with molybdenum, could be ascribed to chance variation between groups of plants. This assumption would tend to give an estimate of variance in excess of the replicate variation if in fact there are real differences between these increases. It was necessary to assume that this variance would also apply to the constituents present at 8 hr in the molybdenum-treated plants.

TABLE 4

EXPERIMENT II: STATISTICAL SUMMARY OF THE RESULTS

The results are the transformed values of the concentrations of amino acids or amides (transformation log (1000x))

Amino Acid or Amide	−Mo (mean of 5 values)†	+Mo (8 hr)	Difference
Aspartic acid	2·0416	3·350	1·3084***
Glutamic acid	3·3260	3·813	0·4870**
Asparagine	2·0676	2·843	0·7754**
Glutamine	2·3722	3·707	1·3348***
Citrulline	2·2200	2·520	0·3000
Arginine	2·5018	2·611	0·1092
"Under arginine"	2·2504	2·474	0·2236
Serine	2·9952	3·418	0·4230***
Glycine	2·0198	2·697	0·6772***
Threonine	2·2682	2·651	0·3828*
α-Alanine	2·8336	3·229	0·3954**
β-Alanine	1·9344	Absent	−( )***
γ-Aminobutyric acid	2·9224	2·326	−0·5964***
Ethanolamine	2·2484	2·272	0·0236
Phenylalanine	2·0724	1·869	−0·2034
Valine	2·2882	2·021	−0·2861
Leucine	2·4142	2·086	−0·4056*
Proline	2·9160	3·095	0·1790

* Significant at $P < 0.05$. ** Significant at $P < 0.01$. *** Significant at $P < 0.001$.

† Mean of the three −Mo harvests plus the ½- and 1-hr harvests of the molybdenum-treated series.

In the comparison between the untreated plants and those treated with molybdenum for 8 hr the important group of compounds aspartic acid, glutamic acid, asparagine, and glutamine all showed significant increases in concentration in the treated plants. The changes in the cases of aspartic acid, glutamic acid, and asparagine had begun probably between 1 and 2 hr after the molybdenum was applied while the increase in glutamine concentration was initiated between 2 and 4 hr after zero time. Serine, glycine, threonine and α-alanine all increased in concentration in the molybdenum-treated plants. The changes started between 1 and 2 hr after treatment in the cases of serine and glycine and between 2 and 4 hr with threonine and α-alanine.

Three other significant changes had taken place in 8 hr in the molybdenum-treated plants. They were all decreases in concentration and involved the compounds β-alanine, γ-aminobutyric acid, and leucine. The concentration of β-alanine decreased between 2 and 4 hr to the extent that this constituent was not detectable in the 4-hr sample of the treated plants. The downward trend in the concentration of γ-aminobutyric acid commenced between 2 and 4 hr, while that of leucine started between 4 and 8 hr after treatment.

IV. Discussion

The present series of observations are restricted to the effects of molybdenum on the amino acids and amides. It is clear however that the significance of these effects can be evaluated only by reference to the accompanying changes in the nitrate and protein fractions. Measurements of these have been made by Spencer and Wood (1954) whose results are immediately comparable with those of this investigation since they were made with a basically similar experimental approach.

Changes in the amino acid and amide fraction may be considered in terms of a system in which nitrate is reduced to ammonia, the ammonia reacts with a keto acid to give an amino acid, and this in turn is finally incorporated into a protein. In this sequence the position may be complicated by transamination reactions in which the products of nitrate reduction are transferred from one carbon skeleton to another, and by amidations in which ammonia combines with a dicarboxylic acid to give an amide. These, however, do not affect the situation that a basic sequence is probably involved in which the concentrations of the amino acids considered as intermediates are likely to be determined by the rate of nitrate reduction, on the one hand, and by the rate of incorporation into protein on the other, but modified by transamination rates.

Nicholas, Nason, and McElroy (1953) have shown that molybdenum is required in the nitrate reductase system. Spencer and Wood (1954) have shown that nitrate accumulates in the absence of molybdenum. These workers, however, have also shown that, when molybdenum is added, nitrate is reduced with a consequent increase in total amino and amide nitrogen. In experiments I and II of this investigation an increase in total amino acids and amides has also been demonstrated. In experiment II the increase occurred within 8 hr, and, during this time, it is unlikely that a large quantity of protein was synthesized, and the data therefore suggest that the initial accumulation of amino acids and amides results from the rapid acceleration in the reduction of nitrate.

During this first phase of increase in the total amino acid level, it is significant that the greatest increases are recorded with aspartic and glutamic acids and with glycine. The rapid increase in the dicarboxylic acids is no doubt a consequence of the reaction of ammonia with oxaloacetic and α-ketoglutaric acids; and in this connection it is significant that Vickery *et al.* (1940) have shown that when ammonia carrying isotopic nitrogen is fed to tobacco plants the most rapid and extensive incorporation is observed with glutamic and aspartic acids.

The significance of the rapid increase in glycine cannot be evaluated from the present series of data. It is known that, in the animal body, glycine may be

formed by a degradation of serine (Shemin 1946); but the data of experiment II do not indicate a decrease in serine corresponding to an increase in glycine. Weinhouse (1955) has shown that glycine can also be formed in transamination reactions involving glyoxylic acid and a whole range of amino acids. It is possible that in molybdenum-deficient plants an accumulation of glyoxylic acid, or its precursor, glycolic acid, may occur, and this may be the basis of the rapid increase in this amino acid. For this interpretation however, no evidence is available.

Several amino acids increased slightly during the initial phase, and these increases are no doubt due to secondary reactions consequent on the formation of glutamic and aspartic acids.

Some amino acids decrease in concentration from the beginning of the experimental period and most tend to decrease during the later phases. The data of Spencer and Wood (1954) indicate that the decreases occur during a phase of protein accumulation, and many of the decreases must be attributed to incorporation into protein. This is probably also the reason for the final decrease in amide concentration, since Damoradan, Joaback, and Chibnall (1932) and Damoradan (1932) have shown that asparagine and glutamine are constituents of some plant proteins.

Some of the decreases may be due to other reactions. The dicarboxylic amino acids decrease after the initial rise during the first 24 hr. It is significant, however, that the concentration of the amides continues to increase. This suggests that at least part of the decrease in the concentration of glutamic and aspartic acids is due to a conversion of these compounds to the corresponding amides by reactions such as those described by Elliott (1951) and by Webster (1955).

The decreases in the concentrations of β-alanine and γ-aminobutyric acid are also probably not due to direct incorporation. Steward, Thompson, and Dent (1949) have recorded a similar dispersal of one of these compounds, γ-aminobutyric acid, during a phase of rapid protein synthesis, but it is significant that neither of these compounds has been identified in protein hydrolysates. It may be that they are consumed in reactions that a company protein synthesis, since Miettinen and Virtanen (1953) have shown that γ-aminobutyric acid can transaminate with α-ketoglutarate, and Steward and Pollard (1956) have provided evidence showing that γ-aminobutyric acid may be readily converted to glutamic acid.

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VI. References

Damoradan, M. (1932).—*Biochem. J.* **26**: 235–47.
Damoradan, M., Joaback, G., and Chibnall, A. C. (1932).—*Biochem. J.* **26**: 1704–13.
Elliott, W. H. (1951).—*Biochem. J.* **49**: 106–12.
Miettinen, J. K., and Virtanen, A. I. (1953).—*Acta Chem. Scand.* **7**: 1243–6.
Nicholas, D. J. D., Nason, A., and McElroy, W. D. (1953).—*Nature* **172**: 34.
Possingham, J. V. (1956).—*Aust. J. Biol. Sci.* **9**: 539–51.
Shemin, D. (1946).—*J. Biol. Chem.* **162**: 297–305.

SPENCER, D., and WOOD, J. G. (1954).—*Aust. J. Biol. Sci.* **7**: 425–34.

STEWARD, F. C., and POLLARD, J. K. (1956).—In "Inorganic Nitrogen Metabolism." pp. 377–407. (Ed. W. D. McElroy and B. Glass.) (Johns Hopkins Press: Baltimore.)

STEWARD, F. C., THOMPSON, J. F., and DENT, C. E. (1949).—*Science* **110**: 439–40.

VICKERY, H. B., PUCKER, G. W., SCHOENHEIMER, R., and RITTENBERG, D. (1940).—*J. Biol. Chem.* **135**: 531–9.

WEBSTER, G. C. (1955).—*Annu. Rev. Pl. Physiol.* **6**: 43–70.

WEINHOUSE, S. (1955).—In "Symposium on Amino Acid Metabolism." pp. 637–57. (Ed. W. D. McElroy and B. Glass.) (Johns Hopkins Press: Baltimore.)