

# Growth-Modifying & Antimetabolite Effects of Amino Acids on Chrysanthemum<sup>1, 2</sup>

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The question of the significance of free amino acids in soil or other cultural media has received attention in recent years in the fields of plant physiology (1, 3, 7, 13, 14, 15, 16, 17), soil microbiology (4, 5, 11, 16) and soil chemistry (9, 10, 12). Although it originally appeared that free amino acids were not to be found in soils, later reports (2, 10) indicated they could be extracted with aqueous and alcoholic solutions and determinations made with chromatographic methods. The quantities of amino acids found were quite variable. Amino acids added to soil were quite rapidly transformed, and usually undetectable in extracts made after 72 to 96 hours of incubation (11, 16). D-Amino acids and L-alloisoleucine appeared more resistant to change than other amino acids studied (16).

Reports indicate that amino acids are absorbed by plants (3, 13, 14) and translocated with great speed in both phloem and xylem (8). Inhibitors of respiratory processes as well as low temperatures applied to fibrovascular tissue considerably retard translocation.

The potential applications of research with the so-called unnatural (D-) isomers was pointed out (6, 17) in connection with the natural occurrences of D-amino acids in antibiotics and parts of microorganisms. More recently, Ikawa, et al. (6) reported the finding of D-phenylalanine, D-allothreonine and D-alloisoleucine<sup>3</sup> in peptido-lipids of bacterial origin.

The demonstrated effectiveness of amino acids applied to root zones of plants in producing changes in morphology and growth rate (1, 13, 17) suggests a need for further research along these lines. Steinberg (13) found that frenching of tobacco could be simulated by furnishing leucine, isoleucine, or alloisoleucine to tobacco plants growing in sterile cultures. He reported that relatively large amounts of these amino acids were required under non-sterile conditions to produce frenching symptoms. Woltz and Jackson (15, 16, 17) found that symptoms of

yellow strapleaf of chrysanthemum could be produced by small amounts of certain isomers of leucine, isoleucine, and alloisoleucine applied to the root zones of test plants growing in sterilized and non-sterilized media. Fifteen of twenty-two test plants developed syndromes similar to those of frenching and yellow strapleaf when a mixture of alloisoleucine and isoleucine isomers was applied to the root zones.

Methionine applied to the root zones of chrysanthemum plants produced a physiological disorder given the name methionosis (17). Two of the twenty-two plants exhibited morphological changes due to methionine.

A consideration of the information about the effects of externally applied amino acids on plant growth in the light of certain antimetabolite phenomena discussed by Woolley (18) led to the hypothesis that the growth-modifying effects observed might be largely antimetabolite effects. Natural (L-) amino acids could act as antimetabolites because of their structural similarity to other natural amino acids. Unnatural amino acids (D-form & others), due to their structural similarity to specific natural amino acids, could function as antimetabolites by being incorporated into peptides and blocking further synthesis due to the failure to completely fit the pattern of the natural amino acid.

Experiments were planned to examine further the effects of various natural and synthetic amino acids on the growth rate and morphology of chrysanthemum plants. Chrysanthemum was selected for these studies because of its sensitivity to isomers of leucine, isoleucine, and methionine (17). The types of cultural media, amounts of amino acids applied, and timing of application relative to the development of new growth of axillary shoots were chosen to enhance growth effects so the effects might be recorded and evaluated. Interactions of related amino acids were explored to learn the degree to which the results would fit metabolite-antimetabolite relationships.

## Methods

Clones of Iceberg variety chrysanthemum were grown in 4-inch plastic pots containing partially sterilized growing media. Methyl bromide-treated sandy soil was used as the growing medium for Experiment I; expanded vermiculite was used in Ex-

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<sup>3</sup> The occurrence of D-alloisoleucine was noted in a personal communication from M. Ikawa, April 25, 1961.

periment II; and a 50:50 mixture by volume of expanded vermiculite and washed quartz sand was used in the other experiments. Triplicate single plant cultures were used as experimental units. Each plant received 25 ml weekly of a complete nutrient solution containing 600 ppm nitrogen, 220 ppm phosphorus, 420 ppm potassium, 220 ppm calcium, 100 ppm magnesium, and traces of the minor elements. Plants were watered as required and were illuminated nightly with a minimum of 25 ft-c hours of light to prevent flower bud initiation. Soluble amino acids were dissolved in 25 ml of water for each plant and applied to the surface of the growing media around the base of the plant. Poorly soluble amino acids were worked into the surface and watered in with 25 ml of water. Immediately before and after application of amino acid solutions, special care was taken to avoid over- or under-watering that might interfere with amino acid uptake.

At the time amino acids were first applied, the terminal growing point of each plant was removed to cause axillary buds to develop. The length of the uppermost new shoot was measured regularly as an

index of the growth-modifying capacity of an amino acid. In Experiment I the entire amounts of the natural (L-) amino acids were applied to the soil surface in a single application. The total amounts for Experiment II were divided, half being applied initially and the remainder a week later. The growing points were removed at the time of the first application. A time differential was employed in Experiment III in applying combinations of alloisoleucine and other amino acids. Single applications and the first of two applications were made the 1st day, at which time the terminal growing points were removed from all plants. A day later the second applications were made to appropriate plants. The same sequence of timing was employed in Experiment V when the order of application of norleucine and methionine was varied.

## Results

Experiment I. Effects of Natural (L-) Amino Acids. The first four amino acids listed in table I produced growth-modifying effects that were correlated with significant suppression of shoot elongation. L-Isoleucine caused the development of yellow strapleaf symptoms: namely, strap-shaped, hooked

**Table I**

Effect of Natural (L-) Amino Acids Upon Height of Upper, New *Chrysanthemum* Shoots

Chemical applied	mg/plant	Height* at indicated days after treatment		
		7	14	21
Control	...	96	94	103
Control with ammonium nitrate	90	100	100	100
<i>Morphological changes</i>				
L-Isoleucine	300	47	63	92
L-Leucine	300	51	66	80
L-Methionine	300	22	32	58
L-Valine	300	33	57	86
<i>No morphological changes</i>				
L-Alanine	300	76	88	94
L-Arginine	300	111	93	98
L-Asparagine	300	102	103	110
L-Aspartic acid	300	100	85	92
L-Cysteine	300	91	89	95
L-Cystine	300	93	84	95
L-Glutamic acid	300	133	102	109
L-Glycine	300	82	90	103
L-Histidine	300	78	89	99
L-Lysine	300	76	90	102
L-Phenylalanine	300	85	90	96
L-Proline	300	96	89	99
L-Serine	300	120	94	103
L-Threonine	300	91	93	95
L-Tryptophane	300	80	85	102
L-Tyrosine	300	93	88	100
LSD, 5% level of significance		40	17	13

\* Height of the upper new shoot was expressed as percentage of the values for the control plants that received ammonium nitrate. Italicized values differ significantly from control values.

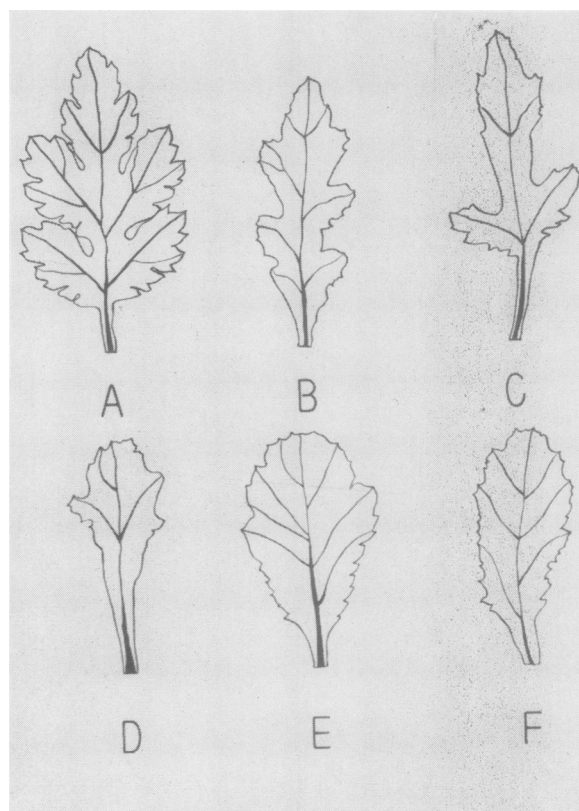


Fig. 1. Traced leaf patterns showing the effect on leaf shape and prominent venation produced by amino acids applied to the root zone. A, control; B, DL-alloisoleucine; C, DL-norleucine; D, DL-methionine; E, DL-norvaline; and F, DL-methionine.

leaves with green netting. L-leucine and L-valine caused the development of strap-shaped leaves with relatively little chlorosis or green netting. L-Me-

thionine resulted in leaves with entire margins, prominent midribs and the loss of the prominent lobes. The 16 other treatments, listed as the second group in table I, had no significant growth-modifying effects.

Table II

Effect of Racemic Mixtures of Amino Acids  
Upon Height of Upper, New Shoots &  
Weight of Chrysanthemum Plants

Amino acid applied	Mg/plant	Height*, days after treatment			Fr wt
		17	24	31	
<i>Morphological changes</i>					
DL-Alloisoleucine	2.5	148	115	108	98
DL-Alloisoleucine	5	78	89	98	91
DL-Alloisoleucine	10	45	56	53	88
DL-Alloisoleucine	20	23	26	33	94
DL-Norleucine	10	99	130	105	91
DL-Norleucine	20	66	73	84	83
DL-Norleucine	40	40	56	68	95
DL-Norleucine	80	45	38	52	84
DL-Ethionine	4	32	41	56	81
DL-Ethionine	16**	...	...	...	...
DL-Methionine	20	83	101	97	107
DL-Methionine	40	56	59	73	103
DL-Methionine	80	25	25	36	92
DL-Methionine	160	6	3	3	77
DL-Valine	80	77	90	99	103
DL-Valine	160	44	71	86	95
DL-Valine	320	17	45	81	104
DL-Valine	640	15	15	57	87
DL-Isovaline	20	80	96	104	96
DL-Isovaline	40	77	82	87	84
DL-Isovaline	80	51	51	62	92
DL-Isovaline	160	23	28	38	77
DL-Norvaline	20	82	97	111	102
DL-Norvaline	40	40	83	94	91
DL-Norvaline	80	24	42	63	83
DL-Norvaline	160	15	22	35	77
<i>No Morphological Changes</i>					
DL-Alpha alanine	320	62	78	81	88
DL-Asparagine	320	95	100	97	83
DL-Aspartic acid	320	105	90	94	83
Gamma amino butyric acid	320	97	93	84	86
DL-Glutamic acid	320	34	89	96	85
DL-Glutamine	320	116	108	101	83
DL-Histidine	320	68	90	81	84
DL-Lysine	320	76	95	94	81
DL-Phenylalanine	320	94	83	87	79
DL-Serine	320	105	105	105	86
DL-Tryptophane	320	72	84	88	96
DL-Tyrosine	160	102	107	95	80
LSD, 5% Level of significance		42	31	28	18

\* Height and weight were expressed as percentage of control values calculated from a regression curve plotting growth response from increments of ammonium nitrate applied. Control values were selected to represent the same amount of nitrogen furnished by the amino acid treatments. Italicized values differ significantly from control values.

\*\* All plants killed by this amount of ethionine.

Experiment II. Effects of Racemic Mixtures of Amino Acids. Amino acids causing growth modification are listed in the first group in table II. DL-alloisoleucine produced typical yellow strapleaf symptoms (see fig. 1,B) and caused growth retardation proportional to the amounts applied. New growth developing after the removal of the terminal growing point emerged yellow, with hooked leaf tips. As growth proceeded, the green netting pattern produced by green veins became apparent. The effect of DL-alloisoleucine appeared rapidly, causing a noticeable yellowing of the older leaves 3 days after the chemical was applied to the root zone.

DL-ethionine (16 mg) caused the death of all plants. Necrotic areas developed in 4 days. The necrosis followed the vascular bundles up the stem and through the leaf veins. The last portion of the leaf to die was the margin. Roots appeared unharmed by this amount of DL-ethionine. Growth modification, significant retardation of shoot elongation, and significant reduction in fresh weight were caused by 4 mg of DL-ethionine. DL-Ethionine caused the development of strap-shaped leaves (fig 1,D).

DL-Norleucine caused growth modification and retardation. Leaves formed after the removal of the terminal growing point had a disorganized, random development of veinlets. The leaf surface was rough and mottled with an appearance suggesting the development of scar tissue; microscopic examination revealed the presence of many small necrotic areas. Leaves were imperfectly formed (fig 1,C) and expanded very slowly.

DL-Methionine caused severe growth retardation and a change in leaf pattern (fig 1,F). The effect on leaf pattern was the same as that described for L-methionine (experiment I). A reduction in fresh weight of plant tops resulted from 160 mg applied to each plant.

DL-Valine lowered the rate of shoot elongation and caused the development of strap-shaped leaves without a prominent green-netting pattern of leaves. Leaves were only slightly chlorotic.

DL-Isovaline had a more pronounced effect than DL-valine in growth retardation and caused a significant depression in fresh weight when 160 mg was applied to each plant. The growth that developed after removing the terminal point was uniformly chlorotic, with little or no green netting of veins. Leaves were strap shaped but expanded laterally more than the leaves of plants affected with the same degree of retardation of shoot elongation due to DL-alloisoleucine.

DL-Norvaline produced the same leaf symptoms as methionine (compare E with F, fig 1). This amino acid repressed elongation of the upper new shoot as well as yield of fresh plant material. The yield re-

**Table III**

Partial Prevention of Ethionine Toxicity to Chrysanthemums by Simultaneous Application of Methionine

DL-Ethionine mg*	DL-Methionine mg*	Height** of uppermost new shoots Weeks after application			Fr wt** of plant tops g	Browning*** of leaf veins	Cupping*** of leaves
		1	2	3			
0	0	100	100	100	100	0	0
2	0	48	78	89	90	+	+
4	0	44	55	67	92	++	++
8	0	16	28	42	79	+++	+++
2	4	72	78	82	110	0	0
4	4	24	44	60	92	+	0
8	4	12	22	44	89	++	0
2	8	120	82	81	85	0	0
4	8	64	79	85	95	0	0
8	8	56	33	38	74	+	0
2	16	76	94	89	98	0	0
4	16	80	68	86	90	0	0
8	16	72	65	62	82	0	0
0	4	84	90	83	97	0	0
0	8	76	82	73	89	0	0
0	16	60	64	67	87	0	0
LSD, 5 % Level of significance		32	29	18	18		

\* Amount applied to each plant.

\*\* Percentage of the control.

\*\*\* 0 = none; + = slight; ++ = moderate; +++ = severe.

duction occurred only when 160 mg was applied to each plant.

The second part of table II includes amino acids that caused no significant morphological or growth changes. DL-Glutamic acid caused an initial growth retardation of the upper, new shoots. DL-Lysine and DL-tyrosine applications were associated with decreased growth on the fresh weight basis.

Experiment III. Alloisoleucine as an Antimetabolite. This experiment was carried out to determine if DL-alloisoleucine effects in producing symptoms of yellow strapleaf (YSL) could be prevented to any degree by administering several amino acids

simultaneously or before or after as metabolites or as simple antagonists of the very effective alloisoleucine.

It is apparent (fig 2) that DL-leucine and DL-valine are especially effective in promoting nearly normal elongation of new shoots. The plants that received DL-alloisoleucine and DL-leucine had typical YSL symptoms initially, but with the passing of time normal plant characteristics developed in the newer growth. DL-Norleucine and DL-norvaline similarly gave partial correction of the YSL condition. DL-Methionine and DL-isovaline showed very little, if any, benefit. Of the amino acids exerting a favorable influence, only DL-valine showed much benefit

**Table IV**

Competitive Relationship Between Methionine &amp; Norleucine

DL-Methionine mg*	DL-Norleucine mg*	Height** of uppermost new shoots Weeks after application					Fr wt** of plant tops g	Midrib browning†	Petiole browning†	Stem browning†
		1	2	3	4	5				
0	0	100	100	100	100	100	0	0	0	
0	150	25	4	3	15	24	80	+	+	++
131	0	25	13	30	81	90	79	+++	0	0
131	150***	25	8	11	38	45	96	0	Tr	0
131	150	25	8	14	46	54	96	0	Tr	0
131***	150	29	9	13	31	48	101	0	+	0
LSD, 5 % Level of Significance		11	7	11	18	18	10			

\* Applied to each plant.

\*\* Percentage of the control.

\*\*\* Applied 24 hrs. before other Amino Acid.

† = none; Tr = trace; + = slight; ++ = moderate; +++ = severe.

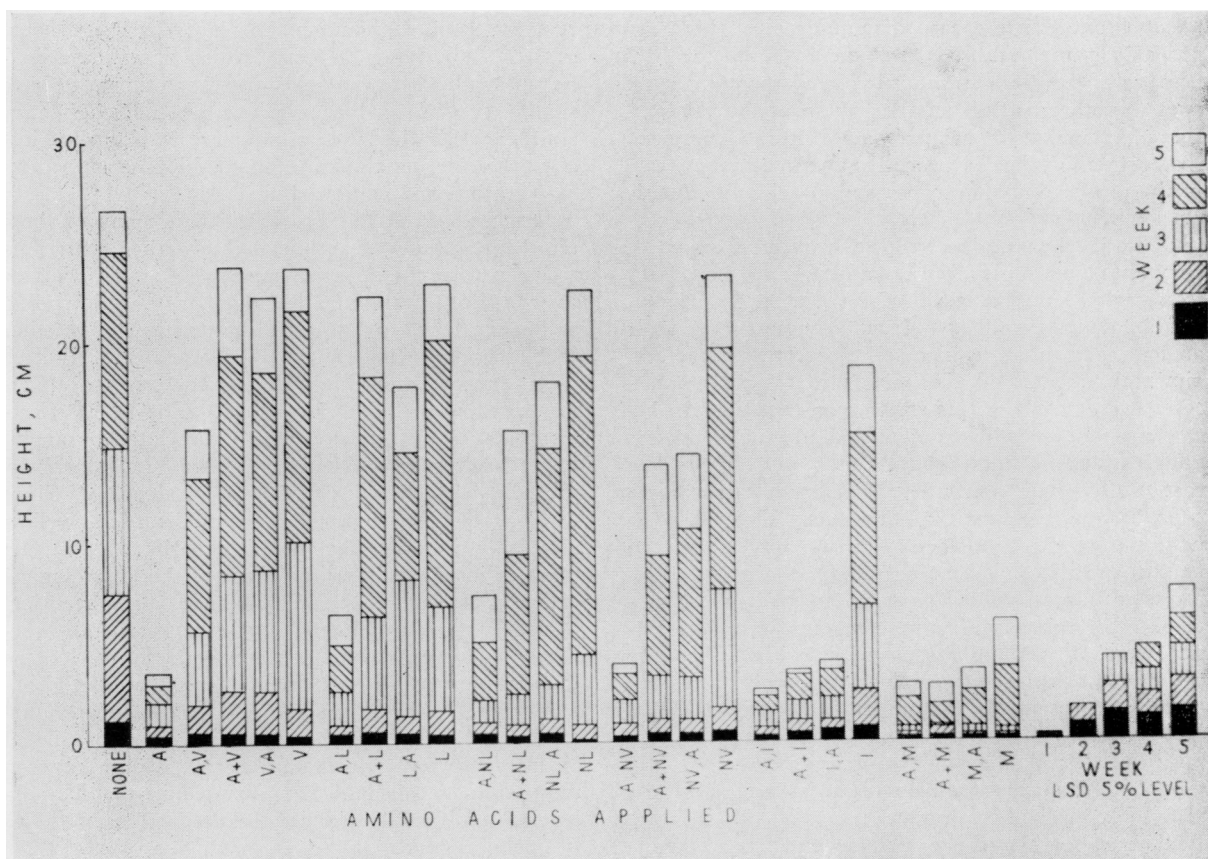


Fig. 2. Height of uppermost new shoots of chrysanthemum plants showing the effects of several amino acids when applied to the root zones singly or in combination with DL-alloisoleucine. Symbols for the amino acids are the following: A = DL-alloisoleucine; I = DL-isovaline; L = DL-leucine; M = DL-methionine; NV = DL-norvaline; and V = DL-valine. Single amino acids, combinations joined by a plus sign and the first amino acid of the combinations joined by commas were all applied the same day. The second listed amino acids of the combination joined by commas were applied 24 hours later. One mmole each of the indicated amino acids was applied to each of triplicate plants, except in the case of DL-alloisoleucine 0.154 mmole was applied.

when applied 24 hours after DL-alloisoleucine.

Experiment IV. Ethionine as an Antimetabolite. The toxic effects of small amounts of DL-ethionine to chrysanthemum (tables II & III) were largely prevented by the simultaneous application of DL-methionine (table III). The effects included growth retardation and browning of veins and convex margins on older leaves. The molecular ratio of DL-methionine to DL-ethionine necessary for the reversal appears to be about 2:1. Larger ratios employed in preliminary experiments were of no greater benefit. The amount of DL-methionine required to overcome DL-ethionine toxicity is not of sufficient magnitude to cause appreciable growth modification per se.

Experiment V. Norleucine as an Antimetabolite. DL-Norleucine (table IV) retarded shoot elongation, reduced production of fresh weight of plant tissue, and caused midrib browning of the older leaves and scar tissue in the new leaves. DL-Methionine retarded shoot elongation more than DL-norleucine but caused much less midrib browning.

DL-Methionine, however, caused a petiole and stem browning. These effects were easily identifiable in all three replicate series. The interactions between DL-norleucine and DL-methionine were equally pronounced. DL-Norleucine reversed to a large degree the growth-retarding property of DL-methionine. Each of the two amino acids together nullified the midrib browning characteristics of the other. DL-Methionine applied simultaneously or in advance reduced petiole browning noticeably but failed to do so when applied 24 hours after DL-norleucine. DL-Norleucine prevented stem browning by DL-methionine regardless of timing of application.

### Discussion

The data reported indicate that amino acids and their analogs applied to the roots of chrysanthemum plants frequently may act as antimetabolites in upsetting normal metabolism. Woolley (18) defines antimetabolites as "structural analogs antagonistic to

metabolites." In the case of amino acids, there are probably many natural amino acids antimetabolites because of the close structural resemblance of certain ones for others. Still another phase of the relationship may include a balance factor if it is assumed that protein synthesis proceeds at an optimum rate in the presence of the proper amino acids in suitable proportions.

The problem of the yellow strapleaf disorder of chrysanthemum (15, 16, 17) appears to be closer to a partial solution because of the reported demonstration that DL-valine, DL-leucine and some of their analogs tend to act as metabolites correcting the detrimental effects of alloisoleucine. Racemic mixtures were used in this and other experiments because of the usually greater stability of D-isomers under attack by microorganisms as well as the low cost of racemic mixtures as chemotherapeutants. Further experiments are, however, planned with natural isomers to be sure of full applicability to natural processes. A major part of the problem remains in natural yellow strapleaf to apply these metabolites or related, effective compounds as therapeutic measures. If the response is positive, then the theory that soil microorganisms produce a causal chemical, presumably an amino acid antimetabolite, will have additional support. Work is now in progress to isolate such causal antimetabolites and the microorganisms producing them as well as to test metabolite chemotherapeutants.

The correction of the adverse effects of DL-ethionine by DL-methionine follows the typical pattern of a metabolite-antimetabolite relationship. That the relationship will not always be simple is shown by the competitive effects of DL-methionine for DL-norleucine and vice versa. It is thought that in this case methionine may be functioning as an antimetabolite which interferes with normal amino acid metabolism, especially in view of the large amounts of methionine applied in the norleucine-methionine experiment. The fact that norleucine reduces the toxicity of methionine is believed due to the fact that it is a structural analog even though not a metabolite. Similar relationships were shown for alloisoleucine on the one hand and norleucine and norvaline on the other (fig 1). The latter three amino acids are not known to be metabolites in plants; however, they follow the pattern of metabolite-antimetabolite relationships at least superficially. The condition may simply be that a much less toxic antimetabolite may antagonize the role of the more toxic chemical by mass action in the plant.

The possible antagonism of one amino acid on the uptake by plants of another amino acid needs to be clarified, however, it is considered that the greater part of the interactions reported here occur within the plant rather than in the rhizosphere.

### Summary

Four of twenty natural amino acids (L-isoleucine, L-leucine, L-methionine & L-valine) applied to the

root zones of chrysanthemum plants had significant growth modifying effects which are described. Seven synthetic amino acids also were shown to have similar growth modifying properties. The effective amino acids in the latter group were DL-alloisoleucine, DL-norleucine, DL-ethionine, DL-methionine, DL-valine, DL-isovaline, and DL-norvaline.

Proceeding on the hypothesis that many of these effects might be of antimetabolite rather than hormonal nature, the apparent metabolite amino acids or their analogs were applied together with offending amino acids to obtain information as to whether such a relationship existed. In practically all such cases, growth was improved and the adverse effects prevented or reduced in severity. The toxic effects of DL-alloisoleucine were greatly alleviated by applying DL-valine or DL-leucine, either simultaneously, preceding or following the DL-alloisoleucine. Since DL-alloisoleucine reproduces the disorder, yellow strapleaf of chrysanthemum, it is believed this fact may aid in determining the cause of the physiological disorder as well as superior methods of prevention.

The very toxic effects of DL-ethionine were practically prevented by DL-methionine. DL-Norleucine toxicity although less severe was counteracted by DL-methionine. The amounts of DL-methionine required for this purpose were of sufficient magnitude to be toxic per se. A competitive relationship between these amino acids was demonstrated in that DL-norleucine significantly reduced the toxic effects peculiar to DL-methionine.

### Literature Cited

1. ANDRUS, L. J. & J. H. QUASTEL. 1947. Toxic effects of amino acids on seedling growth. *Nature* 160: 222-223.
2. DADD, C. C., L. FOWDEN, & W. H. PEARSALL. 1953. An investigation of the free amino acids in organic soil types using paper chromatography. *J. Soil Sci.* 4: 69-71.
3. GHOSH, B. P. & R. H. BURRIS. 1950. Utilization of nitrogenous compounds by plants. *Soil Sci.* 70: 187-203.
4. GREENWOOD, D. J. & H. LEES. 1956. Studies on the decomposition of amino acids in soil. I. A preliminary survey of techniques. *Plant & Soil* 7: 253-268.
5. GREENWOOD, D. J. & H. LEES. 1960. Studies on the decomposition of amino acids in soil. II. The anaerobic metabolism. *Plant & Soil* 12: 69-80.
6. IKAWA, M., E. E. SNELL, & E. LEDERER. 1960. Occurrence of D-phenylalanine, D-allothreonine, & other D-amino acids in peptido-lipids of bacterial origin. *Nature* 188: 558-560.
7. KATZNELSON, H., J. W. ROUATT, & T. M. B. PAYNE. 1955. The liberation of amino acids & reducing compounds by plant roots. *Plant & Soil* 7: 35-48.
8. KURSANOV, A. L. 1961. The transport of organic substances in plants. *Endeavour* 20: 19-25.
9. PAUL, E. A. & E. L. SCHMIDT. 1960. Extraction of free amino acids from soil. *Soil Sci. Soc. Am. Proc.* 24: 195-198.

10. PUTNAM, H. D. & E. L. SCHMIDT. 1959. Studies on the free amino acid fractions of soil. *Soil Sci.* 87: 22-27.
11. SCHMIDT, E. L., H. D. PUTNAM, & E. A. PAUL. 1960. Behavior of free amino acids in soil. *Soil Sci. Soc. Am. Proc.* 24: 107-109.
12. SIMONART, P. & F. PEETER. 1954. Acides amines libres dans l'humus. *Trans. Internat. Congr. Soil Sci.*, 5th Congr. 3: 132-135.
13. STEINBERG, R. A. 1952. Frenching symptoms produced in *Nicotiana tabacum* and *Nicotiana rustica* with optical isomers of isoleucine & leucine & *Bacillus cereus* toxin. *Plant Physiol.* 27: 302-308.
14. STEINBERG, R. A. 1956. Production & prevention of frenching of tobacco grown in the greenhouse. *Plant & Soil* 7: 281-289.
15. WOLTZ, S. S. & C. R. JACKSON. 1960. Yellow strapleaf of chrysanthemums. *Fla. Agr. Expt. Sta. Sunshine State Agr. Res. Rept.* 5: 12-13.
16. WOLTZ, S. S. & C. R. JACKSON. 1960. Relationship of amino acids to yellow strapleaf of chrysanthemums & similar disorders. *Proc. Fla. State Hort. Soc.* 73: 381-384.
17. WOLTZ, S. S. & C. R. JACKSON. 1961. Production of yellow strapleaf of chrysanthemum & similar disorders by amino acid treatment. *Plant Physiol.* 36: 197-201.
18. WOOLLEY, D. W. 1952. *A Study of Antimetabolites.* John Wiley & Sons, New York. Pp. 269.

## Cobamide Coenzyme Contents of Soybean Nodules & Nitrogen Fixing Bacteria in Relation to Physiological Conditions<sup>1, 2</sup>

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During the last 2 years results of experiments were reported that showed cobalt is essential for the growth of soybeans (1, 2) and alfalfa (6) under conditions where these legumes must use atmospheric nitrogen. Cobalt is required also for *Rhizobium meliloti* and strikingly stimulates the growth of *R. japonicum*, *R. leguminosarum*, *R. trifolii*, and *R. phaseoli* when these organisms are cultured in purified media containing ammonium and nitrate nitrogen (13, 14). Twelve elements not considered essential for bacteria or higher plants lacked the capacity to replace the stimulatory effect of cobalt (13).

Recently it was found that *R. meliloti* and the nodules from several legumes and from alder contain one or more of the cobamide coenzyme(s) (10). The coenzyme in *R. meliloti* (11) has been isolated and identified as the 5,6-dimethylbenzimidazole derivative (vitamin B<sub>12</sub> coenzyme). Further investigations have provided evidence that the B<sub>12</sub> coenzyme content of *R. meliloti* cells is positively correlated with the cobalt concentration of the culture medium (12).

This paper describes several physiological experiments designed to determine any relationships between cobamide coenzyme contents of nitrogen fixing organisms and certain factors reported to influence the nitrogen fixing process. Factors considered in these investigations include: age of leguminous species; species and strains of rhizobia, and cobalt content and source of nitrogen in the media used to culture rhizobia and azotobacter.

### Materials & Methods

**Culture Methods:** Soybean seeds (*Glycine max* Merr., 'Chippewa'), inoculated with a commercial source of *R. japonicum*, were germinated in 2-gallon vessels filled with Perlite and were cultured in a greenhouse in these containers during the spring of 1962. Each vessel contained eight plants and was supplied every other day with about 500 ml of an unpurified nitrogen-free nutrient solution identical with that described previously (1), with the exception that cobalt in the form of CoCl<sub>2</sub> · 6H<sub>2</sub>O, was added at a concentration of 0.05 ppm. Cultures were flushed with water on those days when the nutrient solution was not applied. The plants were supplied with approximately 500 ft-c of fluorescent light for 14 hours a day in addition to normal sun light in the greenhouse. The experiment (table I) was arrang-

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