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DIVISION III—SOIL MICROBIOLOGY

Formation of Free Amino Acids in Rhizosphere and Nonrhizosphere Soil[1]

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ABSTRACT

Untreated samples of nonrhizosphere and soybean rhizosphere soils each contained about 15 identified free amino acids totaling 2 to 4 μg. per g. of soil; lysine was the most prevalent amino acid in each preparation. Numerous additional unidentified compounds occurred at concentrations estimated as 0.1 to 0.5 μg. per g. Treatment with glucose and potassium nitrate increased the amount of free amino acids to about 100 μg. per g. after 3 days. Concentrations declined after 3 days but still were 4 to 5 times that of the untreated control after 2 weeks' incubation. Glutamic acid was the dominant amino acid in all treated soils. Rhizosphere soil did not differ quantitatively from nonrhizosphere in samples treated with glucose, although a greater variety of ninhydrin reacting compounds was encountered in rhizosphere soil. Treated soils incubated at 20% field moisture capacity differed little in free amino acids from those held at 30%. The features of the free amino acid fraction are discussed.

Characterization of the free amino acids that occur in soils must rely on effective extraction procedures. The few attempts to study the quantities of free amino acids in soil used ethanol as the extractant (6, 7), but the low efficiency of ethanol extraction was obvious when recovery of added amino acids was attempted (7). A study of extraction procedures by Paul and Schmidt (5) demonstrated the marked superiority of some ionic extractants over ethanol, and led to the adoption of a technique based on the use of NH_4OAc for removal of free amino acids from soil. The present report is concerned with a quantitative characterization of the free amino acids present in preparations obtained by NH_4OAc extraction of rhizosphere and nonrhizosphere soils as influenced by conditions of incubation.

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EXPERIMENTAL PROCEDURE

The soil used throughout was a Waukegan silt loam collected from the University experimental plots at St. Paul, Minn. Rhizosphere samples were taken as that portion of the soil that adhered closely to the roots of mature soybeans, but could be shaken free of the root system. Soils were never allowed to air dry, and were stored in the frozen state prior to extraction. Nonrhizosphere samples were collected between soybean rows. Extraction of the soil, concentration and preparation of the extract, resolution of the extract by elution chromatography and identification of the resultant ninhydrin reacting compounds were carried out as described previously (5). An extractant/soil (vol./wt.) ratio of 10:1 was applied to 250 g. of untreated soil, and to 50 g. of amended soils. Soil treatments consisted of an amendment with 1.0% glucose and 0.3% potassium nitrate, followed by incubation at 28° C. and 80 to 85% humidity. Incubated soils were adjusted to either 20 or 30% moisture, corresponding to 60 or 90%, respectively, of the field capacity.

RESULTS

Analyses of Untreated Soils

The free amino acids and the concentrations of each per gram of untreated rhizosphere and nonrhizosphere soil are listed in table 1. At the low concentrations encountered, certain compounds were difficult to delineate as separate symmetrical peaks and only those readily identified were included in the data. Lysine was the amino acid present in largest amounts in each of the soils. In addition to those listed in table 1, numerous unidentified compounds emerged over the complete range of effluent volume. These results suggest that a wide variety of

Table 1—Amino acids in untreated rhizosphere and nonrhizosphere soil.

Amino acid	Nonrhizosphere μg./g.	Rhizosphere μg./g.
Aspartic acid	0.20	0.59
Threonine	0.20	0.19
Serine	0.16	0.09
Glutamic acid	0.31	0.32
Glycine	0.21	0.32
Alanine	0.18	0.45
Valine	0.20	*
Isoleucine	0.12	0.32
Leucine	0.13	*
Beta alanine	0.09	0.07
Tyrosine	0.27	0.46
Phenylalanine	0.12	0.18
Gamma amino butyric acid	0.08	*
Lysine	0.52	0.99
Histidine	0.33	0.41

* Added as markers, probably present in concentrations similar to adjacent compounds.

ninhydrin reacting substances were present in the untreated soils at concentrations of 0.1 to 0.5 μg. per g.

Little difference was apparent between the unamended samples of rhizosphere and nonrhizosphere soil. At such low concentrations and with many small elution peaks represented on the chromatograms, calculations were necessarily subject to considerable error. It is clear that the two samples were essentially the same qualitatively and that any quantitative differences between them were slight.

Free Amino Acid Content of Amended Soils

Treatment of soil with glucose and nitrate N was shown in some previous work to greatly increase concentrations of free amino acids over those present in the untreated soil (5, 6). Further data on this effect are presented in table 2. Soils were treated with 1% glucose and 0.3% potassium nitrate, and were incubated for 3 days.

As expected, the amended nonrhizosphere soil contained a greater variety of amino acids than the control (table 1), and showed a 10- to 50-fold increase in concentration for most compounds common to both soils. Except for leucine and tyrosine only minor differences were noted in results between the duplicate samples studied at the 20% moisture level. The sample held at more saturated conditions was similar qualitatively to that incubated at 20% moisture, but the total quantity of amino acids was somewhat larger in the former; 124 μg. per g. at 30% moisture and about 80 μg. per g. at 20%. Aspartic acid, proline, glutamic acid, and arginine accounted for most of the increase. Beta aminoisobuytric acid, tyrosine, and phenylalanine formed a single large peak of 0.22 μmoles, equivalent to 36 μg. of amino acid at an assumed molecular weight of 181.

Table 2—Amino acid content of amended rhizosphere and nonrhizosphere soil incubated for 3 days at 20 and 30% moisture levels.

	Rhizosphere			Nonrhizosphere		
	20% moisture		30% moisture	20% moisture		30% moisture
	1 μg./g.	2 μg./g.	μg./g.	1 μg./g.	2 μg./g.	μg./g.
Methinonine sulfoxide	0.68	0.93	0.84	4.30	--	2.15
Aspartic acid	5.26	4.92	5.04	9.48*	8.16	12.48
Threonine	6.84	2.02	2.91		3.09	--
Serine	1.65	0.34	3.15	3.44	3.17	4.60
Proline	4.19	3.68	--	5.89	5.38	8.18
Glutamic acid	15.15	37.21	7.49	12.01	13.55	21.30
Glycine	3.26	1.74	2.57	2.12	1.84	2.83
Alanine	6.72	6.31	4.17	8.19	9.44	10.71
Valine	4.12	3.19	2.90	4.25	4.26	4.53
Cystine	1.29	11.25	--	--	--	--
Methionine	2.46	1.87	2.60	0.64	1.74	2.38
Isoleucine	2.80	1.78	3.68	2.35	4.80	3.75
Leucine	15.90	12.61	8.18	3.14	13.16	5.24
Beta alanine	2.83	1.25	--	--	--	--
Tyrosine	3.17	5.00	2.75	14.71	1.70	36.34†
Phenylalanine	2.65	2.86	1.64	2.24	3.14	
Gamma amino buytric acid	1.06	2.14	4.65	1.81	0.50	1.21
Lysine	1.25	2.76	1.41	2.22	1.98	2.05
Histidine	1.04	0.98	1.19	1.38	2.92	1.14
Arginine	2.78	3.56	3.29	1.81	2.94	5.11
Total	85.10	106.40	58.46	79.98	81.77	124.00

* Aspartic acid plus threonine. † Tyrosine and phenylalanine plus one unknown compound.

Results obtained for the rhizosphere soil samples are presented also in table 2. The duplicate rhizosphere samples analyzed at 20% moisture varied considerably with respect to threonine, serine, glutamic acid and cystine concentrations. Such variability between samples precludes quantitative comparison between rhizosphere and nonrhizosphere soil; qualitatively, however, it may be noted that two amino acids, beta alanine and cystine were present only in rhizosphere soils. Incubation of rhizosphere soil at 30% moisture, unlike the comparable nonrhizosphere soil, resulted in fewer amino acids and generally lower concentrations of amino acids. Total identifiable amino acids in this sample amounted to 58.5 μg. per g. of soil, substantially less than any of the other rhizosphere or nonrhizosphere samples.

In addition to the compounds listed in tables 2 and 3, all soil samples contained numerous ninhydrin reacting compounds identified only tentatively, if at all. Two representative chromatograms are shown in figure 1 to illustrate the occurrence and position of the poorly characterized peaks in relation to those identified.

Among the early peaks, those found at 28 to 36 ml. and 44 to 50 ml. effluent volume were especially interesting in that they occurred in all samples. Cysteic acid, urea, and taurine are known to emerge in this range, but paper chromatograms of the unknown peaks were uninformative. Hydroxyproline, which was not encountered in previous work, was found in one preparation as a distinctive, identifiable peak at 82 to 84 ml. effluent and 0.41 μg. per g. concentration (amended nonrhizosphere soil, not shown in figure 1). An unknown sometimes emerged just after serine as in the lower chromatogram of figure 1; the amides, glutamine and asparagine, could have occupied this position but their Rf values did not correspond to that of the unknown. Another compound, restricted to rhizosphere preparations, overlapped into glycine at effluent volumes 227 to 240 ml. (figure 1). Although the compound was found at about 0.015 μmoles leucine equivalents, and gave well-defined Rf values of 0.80 with phenol and 0.63 with butanol-acetic acid, it was not similar to amino acids known to be resolved in this region.

The range of effluent volumes in which the neutral amino acids separate was characterized by numerous small unidentified peaks associated with amended rhizosphere samples. As can be seen in the lower portion of figure

Table 3—Changes in the amino acid composition of an amended nonrhizosphere soil in the course of incubation.

Effluent, ml.	Compound	Ninhydrin reacting compounds per gram of soil				
		0 day	3 day	5 day	7 day	14 day
		μg.	μg.	μg.	μg.	μg.
84-96	Methionine sulfoxide	0.09	0.20	1.11	0.09	0.32
100-110	Aspartic acid	0.12	2.37	0.65	0.88	0.52
112-118	Threonine	--	0.23	--	--	0.41
120-122	Serine	0.07	1.03	1.03	0.80	0.45
170-194	Glutamic acid	0.16	18.41	22.90	7.43	3.98
218-226	Glycine	0.16	1.82	1.04	0.25	0.41
228-240	Alanine	--	12.23	1.98	1.69	0.60
284-306	Valine	0.01	4.46	1.69	0.21	0.43
400-414	Methionine	0.01	2.27	0.88	0.34	0.22
416-424	Isoleucine	0.08	3.97	1.23	0.33	0.32
426-432	Leucine	0.14	5.40	1.82	0.56	0.33
433-447	Beta alanine	0.11	4.16	--	--	--
462-470	Tyrosine and Beta-amino Isobuytric acid	0.14	2.35	1.32	0.16	--
472-480	Phenylalanine	0.15	3.25	1.20	0.25	--
510-520	Gamma amino butyric acid	0.05	0.70	0.02	0.16	--
658-668	Lysine	0.33	1.99	1.35	0.21	--
678-690	Histidine	0.18	1.29	1.61	0.37	0.87
778-808	Arginine	--	4.29	2.00	--	0.41
	Total	1.79	72.42	41.83	13.73	9.27

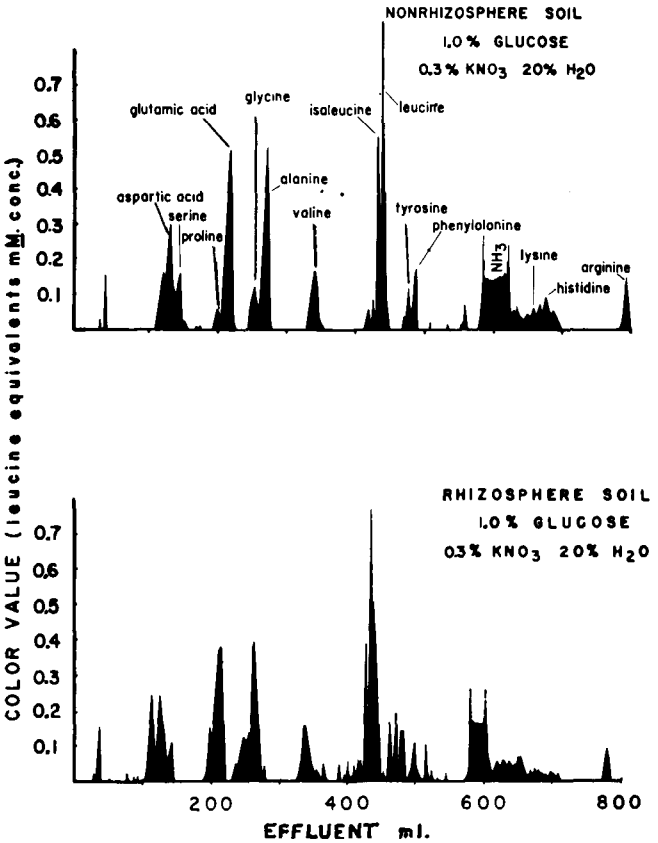

Figure 1—The distribution of ninhydrin reacting compounds in representative extracts of amended rhizosphere and nonrhizosphere soil.

1, four compounds emerged between valine at 326 to 348 ml. and methionine at 404 to 412 ml. One of these substances was cystine; those not identified may have included cystathionine and diaminopimelic acid. Several poorly resolved components emerged as overlapping peaks after ammonia in both rhizosphere and nonrhizosphere samples.

Changes in the Free Amino Acid Fraction During Incubation

As noted above, soils examined after 3 days' incubation subsequent to the addition of available energy and inorganic nitrogen showed large increases in free amino acids over untreated soil. Free amino acids present at this time represent the net of microbial synthesis over microbial destruction at, or near, the peak of microbiological activity. Additional determinations were made to characterize the free amino fraction as microbiological activity declined during more prolonged incubation; the results of these determinations are reported in table 3.

Data for the 0-day and 3-day samples were in good general agreement with those of comparable untreated and amended nonrhizosphere soil listed in tables 1 and 2, despite different collection dates of the soil. Once again lysine was the most abundant component found in the untreated soil. Treatment with glucose and potassium nitrate, as before, greatly increased the concentrations of free amino acids present, with the maximal total concentration found after 3 days' incubation. At 3 days the majority of the amino acids were present at concentrations of 1 to 5 μg. per g. of soil, but glutamic acid and alanine were much higher at 18.41 and 12.23 μg. per g., respectively.

A gradual decline in free amino acid concentrations was evident for samples incubated longer than 3 days. After 5 days, glutamic acid was recorded at its highest concentration, but the other amino acids had declined to $<$ 2 μg. per g. while threonine, beta alanine, and several small unidentified peaks were no longer present in measurable amounts. Effects of the treatment were still evident after 2 weeks' incubation, for the glutamic acid concentration was substantial, and the total quantity of amino acids was 4 to 5 times that of the control soil. Numerous unknown peaks similar to those mentioned previously were noted in each preparation, so that the amino acids listed in table 3 comprise only the major part of the ninhydrin reacting substances present.

DISCUSSION

The development of effective extraction procedures, and the application of gradient elution chromatography for analysis, have made feasible the quantitative study of free amino acids in soil. Data presented in this report, together with those obtained in related studies (5, 6, 7), permit a reasonable characterization of the free amino acid fraction of soil.

It is clear that free amino acids do exist in soil. These compounds are free in the sense that they occur as individual amino acids, uncombined into peptides or other polymers; however, the amino acids do become adsorbed to soil surfaces. Adsorption to soil is a factor of prime importance in the extraction of the free amino acids. Whereas water and ethanol are notably inefficient as extractants, use of neutral NH_4OAc will give 60 to 100% recovery of most of the neutral and acidic amino compounds, and 30 to 50% recovery of the basic ones.

The size and nature of the free amino acid fraction appears to be closely related to the level of microbial activity in the soil. At the normally low levels of microbial respiration imposed by the absence of readily available substrate, field soils contain only small quantities of free amino acids. About 15 identifiable amino acids were encountered in such soils (tables 1 and 3) in concentrations of 0.05 to 0.5 μg. per g. of soil. Quantities in this range are difficult to measure accurately, but such data indicate that a variety of amino acids aggregating at least a few parts per million are probably a consistent feature of soil, even during periods of reduced microbial activity. Intensive microbial activity, as engendered by the addition to soil of glucose and inorganic nitrogen, will result in the temporary accumulation of high concentrations of free amino acids. As noted previously (5), and as evident here in the data of tables 2 and 3, concentrations of about 100 ppm. are present at a time near the peak of microbiological activity.

A highly important feature of the free amino acid fraction of soil is its dynamic nature. The bulk of the free amino acids found in soil probably are excretory or autolytic products of microorganisms. Some amino acids may be introduced to rhizosphere sites by root excretions or to the soil generally, as a consequence of the addition of residues. Irrespective of origin, and despite adsorption to soil particles, the amino acids appear to be subject to rapid destruction by microorganisms. Even large concentrations added to soil do not persist for more than 96 hours under aerobic circumstances (7). The fraction extracted and examined at any one time then, is considered to represent the equilibrium state between microbial synthesis and microbial destruction.

The data in table 3 suggest that both synthesis and destruction of amino acids continues in the soil for some time after the intial substrate has disappeared. At 3 days, the time of maximum accumulation of amino acids, it is unlikely that any of the added glucose remained. The concentration of amino acids 2 weeks after treatment,

although much lower than at 3 days, was still 4 to 5 times that of the control soil. Those amino acids present at the 3-day incubation time probably persisted only a few days; however, release of additional amino acids continued as cells lysed and as new cells were formed on the available remains of the old. Glutamic acid is of special significance to the free amino acid fraction, as it was found consistently at high concentrations in the treated soils, and accounted for nearly half of the total amino acid fraction present after 2 weeks.

Numerous properties of the free amino acid fraction remain to be clarified. Attempts to examine the influence of the microbial flora through a comparison of rhizosphere and nonrhizosphere soils indicated some qualitative but no consistent quantitative differences. The populations of the rhizosphere samples responded to treatment with glucose and potassium nitrate by accumulating a greater variety of amino compounds than did the nonrhizosphere population. Rhizosphere soils, as sites of high microbial activity, merit greater attention in relation to amino acid occurrence. The influence of the moisture level of the soil was examined briefly since some unpublished analyses of $Ba(OH)_2$ extracts had suggested that both qualitative and quantitative differences in free amino acids were associated with soil moisture content. These results were not confirmed in the present study and soil moisture effects were apparent only as changes in the concentrations of individual amino acids. Probable effects due to soil type, the nature and rate of residues added to soil as microbiological substrate, and the significance of inorganic nitrogen, are all factors that have not been examined in relation to the character of the free amino acid fraction.

Although temporary, accumulations of free amino acids of the magnitude observed under conditions of high microbial activity might well influence microbial nutrition, plant nutrition, and soil organic matter formation. The amino acid dependent segment of the microflora described by Lochhead and his associates (1, 2, 3, 8) would be markedly influenced by the nature of the free amino acid fraction in both rhizosphere and nonrhizosphere sites. Moreover, the amino acids released by a rhizosphere microflora may be of consequence to the nitrogen nutrition of the plant root, for there is ample evidence that a variety of plants can utilize intact amino acids (4).

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