

DIVISION III—SOIL MICROBIOLOGY

Extraction of Free Amino Acids from Soil¹

E. A. PAUL AND E. L. SCHMIDT²

ABSTRACT

Alternatives to extraction with ethanol were examined in order to obtain more effective removal of free amino acids from soil. $\text{Ba}(\text{OH})_2$ was a promising extractant and proved to be very effective in recovering a mixture of 17 amino acids that had been added to soil. Resolution of the extract by elution chromatography was followed by colorimetric analysis; 73 to 121% of the acidic and neutral components of the mixture and 36 to 41% of the basic amino acids were recovered. A second extraction procedure, based on the use of NH_4OAc , was developed to avoid any slight hydrolysis of amino acid polymers that may have occurred during preparation of $\text{Ba}(\text{OH})_2$ extracts. The NH_4OAc extraction procedure was 31 to 83% effective in the recovery of added amino acids from soil. When both extraction techniques were applied in preliminary trials to characterize the free amino acids fraction of a soil, the NH_4OAc extraction was considered the better. Both NH_4OAc and $\text{Ba}(\text{OH})_2$ extractions yielded many more kinds of free amino acids in concentrations 5 to 25 times greater than that reported earlier in comparable studies using ethanol extraction.

INFORMATION on free amino acids in soil has been confined largely to the study of extracts obtained by the use of water and ethanol, the standard agents for extraction of the free amino acids in biological materials. Most studies were qualitative in nature and indicated that amino acids were either absent from such extracts or present in trace amounts (3, 4, 10, 13). Quantitative estimates of free amino acids in soil were reported only by Putnam and Schmidt (11) who analyzed a few ethanol extracts. They reported 10 amino acids in concentrations to 31 μg . per kg. of unamended soil and 11 amino acids ranging to 387 μg . per kg. of the soil amended with glucose and sodium nitrate. Evidence for the adsorption of basic amino acids was presented and it seemed likely that more effective extraction procedures would have changed materially both the qualitative and quantitative features of the fraction recovered (11). Later work (12) showed that 80% ethanol was notably inefficient in extracting amino acids that had been added to soil.

Experimental Procedure

Waukegan silt loam, collected from the top 6 inches of experimental plots, University of Minnesota, St. Paul, was used in all experiments. The moist soil was passed through a 8-mesh sieve and 1 kg. quantities were packaged in polyethylene

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²Formerly Research Assistant in Soils, University of Minnesota, now Assistant Professor, Department of Soil Science, University of Saskatchewan, Saskatoon, Can., and Associate Professor, jointly, Department of Soils and Department of Bacteriology, University of Minnesota, respectively.

bags and frozen. Prior to use the soil was thawed, the moisture content determined and all subsample weights corrected to a moisture-free basis. Mixtures of soil and amino acid were always equilibrated at 5° C. for 1 hour.

A variety of extractants was used initially to test the efficiency of each in recovering 2.5 mg. of arginine, leucine, or aspartic acid added individually to 25 g. of soil in that amount of water required to bring the soil to its water-holding capacity at a tension of 40 cm. of mercury. Duplicate samples were leached with 250 ml. of extractant and leachates were analyzed colorimetrically (7). Untreated soil leachates served as controls.

Some relationships of leachate volume to amino acid recovery were examined for the extractants 80% ethanol, 0.1N barium hydroxide ($\text{Ba}(\text{OH})_2$), and water, and the same amino acids, arginine, leucine and aspartic acid. Each extractant was characterized according to the amount of amino acid recovered in successive 15-ml. fractions of leachate. Corresponding fractions of leachate taken from untreated soil were developed with ninhydrin as controls.

The applicability of $\text{Ba}(\text{OH})_2$ extraction was then tested for ability to recover 17 amino acids that had been added to the soil. Five hundred grams of soil in a Buchner funnel were leached with 5 liters of 0.1N $\text{Ba}(\text{OH})_2$. The leachate was treated with hot 1.0N H_2SO_4 to precipitate the $\text{Ba}(\text{OH})_2$, and after filtering, the filtrate was evaporated to dryness in vacuo at 12° to 15° C. The residue was taken up in 10 ml. of pH 2.2 sodium citrate buffer, and an aliquot was analyzed for amino acids by ion exchange chromatography. Comparable extraction of untreated soil accounted for amino compounds of natural occurrence.

Ammonium acetate (NH_4OAc) extraction was tested similarly with 19 added amino acids as an alternative to use of $\text{Ba}(\text{OH})_2$. Five liters of 0.5N NH_4OAc pH 6.8 was used to extract 500 g. of control soil or amino acid treated soil. The leachate was reduced to dryness by vacuum distillation at 12° to 15° C., and removal of the NH_4OAc was accomplished by a sublimation procedure similar to that of Hirs et al. (6). As the residue following sublimation contained too many salts for analysis by elution chromatography, the amino acids were extracted from the residue with three washings of 80% ethanol, using a total of 75 ml. Each of the washings was centrifuged, supernatants were pooled and again evaporated to dryness. The final residue, strongly buffered at pH 4.5 was adjusted to pH 2.2 with HCl and made to a final volume of 15 ml.

A final experiment compared the usefulness of the $\text{Ba}(\text{OH})_2$ and NH_4OAc procedures in the characterization of the free amino acid content of soil at a high level of microbial activity. Soil was amended with 1% glucose and 0.3% KNO_3 . After 3 days' incubation, 100-g. samples were extracted; extractant volumes were reduced throughout in accordance with the small soil sample.

The Moore and Stein (8) procedure was used for resolution of the amino acids in the soil extracts. A 1-ml. aliquot of the $\text{Ba}(\text{OH})_2$ extract was added to the previously standardized 150-cm. column of Dowex 50-X4 ion-exchange resin. Amino compounds in a 1.5-ml. aliquot obtained from the NH_4OAc extraction were resolved on Dowex 50-X8 in a particle size of minus 400-mesh, when it was found that the higher cross-linkage and finer mesh resin resulted in improved resolution.

Fractions containing 1 ml. of eluate from the ion-exchange column were collected and alternate tubes were analyzed by the ninhydrin procedure (8). The undeveloped fractions in the area of an individual peak on the elution diagram were combined and desalted with ion-exchange resins (5) in preparation for qualitative paper chromatography. Identification of ninhydrin reacting compounds was accomplished by comparison of the elution diagrams for the amino compounds obtained from the soil with those obtained with known amino acids. The identification was confirmed by the utilization of two sets of one dimensional paper chromatographs using 80% phenol and butanol acetic acid as the developing solvents. Individual spot tests, where applicable, were also utilized: alpha-naphthol hypochlorite reagent for arginine, platinum iodide for

Table 1—Effectiveness of various reagents in the recovery of 2.5 mg. of arginine added to 25 g. of soil.

Leaching solution	Recovery %*
Ethanol, 80%	0
Pyridine-acetic acid-water-ethanol (100-70-30-80)	1.6
Sodium acetate, 0.1 N in ethanol	26.6
Acetic acid, 0.1 N in 80% ethanol	52.6
Sodium hydroxide, 0.1 N in 80% ethanol	65.2
Sodium hydroxide, 0.25 N	78.5
Barium hydroxide, 0.5 N	97.5
Barium nitrate, 0.5 N	103.0
Barium hydroxide, 0.25 N	100.8
Barium nitrate, 0.25 N	104.9

* Average of duplicate samples

methionine, p-dimethyl amino benzaldehyde (Ehrlich's reagent) for tryptophan, Morgans and Elson's reagent for amino sugars, and diazotized sulfanilamide for histidine and tyrosine (1). The presence of phenylalanine was verified with NaHCO_3 (9).

RESULTS AND DISCUSSION

Effectiveness of Various Extracting Reagents

The data in tables 1 and 2 suggest some of the requirements for extraction of free amino acids from soil. Leaching with ethanol or various ethanolic solutions did not recover arginine from the soil, whereas the aqueous solutions $\text{Ba}(\text{OH})_2$, $\text{Ba}(\text{NO}_3)_2$, or NaOH , effectively recovered the added arginine. The ionic solutions were also effective extracting agents for the acidic amino acid, aspartic acid, and the neutral amino acid, leucine, as shown in table 2. $\text{Ba}(\text{OH})_2$ in particular was of special interest for the bulk of the salts present in such an extract could be removed conveniently by precipitation with acid.

A further comparison of the usual extractants, water and ethanol, with $\text{Ba}(\text{OH})_2$ showed that the effectiveness of each extractant varied with the test amino acid and with the soil/leachate ratio used. It can be seen from the data summarized in figure 1 that $\text{Ba}(\text{OH})_2$ was clearly superior to ethanol in the recovery of each of the three amino acids used. Water alone was as good or slightly better than $\text{Ba}(\text{OH})_2$ in the extraction of aspartic acid and leucine, but was notably ineffective in removing arginine from soil. The basic amino acid, arginine, responded quite differently than did aspartic acid and leucine to an effective extractant. Maximum recovery of both leucine and aspartic acid was achieved at about 60 ml. of leachate volume, or a ratio of about 2.5 to 3 volumes of leachate per unit weight of soil, while the added arginine was extractable only with the $\text{Ba}(\text{OH})_2$ and then, but slowly, in approximately equal increments to the maximum at a very high leachate/soil ratio.

The results summarized in figure 1 are in agreement with earlier studies by Putnam and Schmidt (11) which indicated that retention of amino acids varied with their isoelectric properties, and with subsequent studies in which extraction of soil with ethanol removed only 5 to

Table 2—Recovery of 2.5 mg. of aspartic acid and leucine added to 25 g. of soil.

Leaching solution	Recovery, %	
	Aspartic acid	Leucine
Barium hydroxide, 0.25 N	96.2	93.1
Barium nitrate, 0.25 N	80.0	62.1
Sodium acetate, 0.25 N	104.5	96.7

50% of added acidic or neutral amino acids and none of the basic ones (12). Water has also been tried as an extractant for the qualitative examination of free amino acids in soils (2, 10). According to figure 1, water should be capable of extracting the acidic and neutral amino acids, if used at a leachate/soil volume ratio of at least 3:1, but is not likely to remove the basic amino acids strongly adsorbed to the colloidal fraction of the soil, nor is it likely to be an effective extractant of the neutral amino acids present in strongly acid soils.

The necessary requisites for an efficient leaching agent include not only the ability to replace adsorbed amino acids but also properties that allow for subsequent removal of the large concentrations of replacing agent used. An ionic solution such as $\text{Ba}(\text{OH})_2$ is one of a number of compounds which meets the requirements. A divalent ion, barium has a high replacing power and tends to flocculate rather than peptize organic matter. Barium salts of humic acids, sulfate and carbonate do not interfere as they tend to be insoluble, and a leachate is readily freed of the extracting ions by neutralization of the base. A disadvantage of this technique is the possibility that the alkalinity of the $\text{Ba}(\text{OH})_2$ might result in hydrolysis of peptide bonds or racemization of individual amino acids.

Recovery of a Mixture of Amino Acids Added to Soil

The efficacy of the $\text{Ba}(\text{OH})_2$ extraction procedure was tested with a large quantity of soil to which a wide range of amino acids had been added. Results are shown in table 3. Except for the basic amino acids, lysine, histidine and arginine, concentrations as low as 1 to 3 ppm. of amino acids in soil could be determined accurately by the use of $\text{Ba}(\text{OH})_2$ in conjunction with gradient elution chromatography. The sensitivity of the procedure permitted detection of numerous free amino acids in the untreated soil (center column, table 3). Certain amino acids were present naturally in amounts up to 14% of that added to the soil and were comparable quantitatively to that found previously only in soil amended with glucose (11).

As noted in the final column of table 3, added acidic and neutral amino acids were generally recovered very effectively by the $\text{Ba}(\text{OH})_2$ extraction procedure. The recoveries of several amino acids, and of glutamic acid in particular, were unaccountably high. One of a number of possible explanations for the high recovery, but one

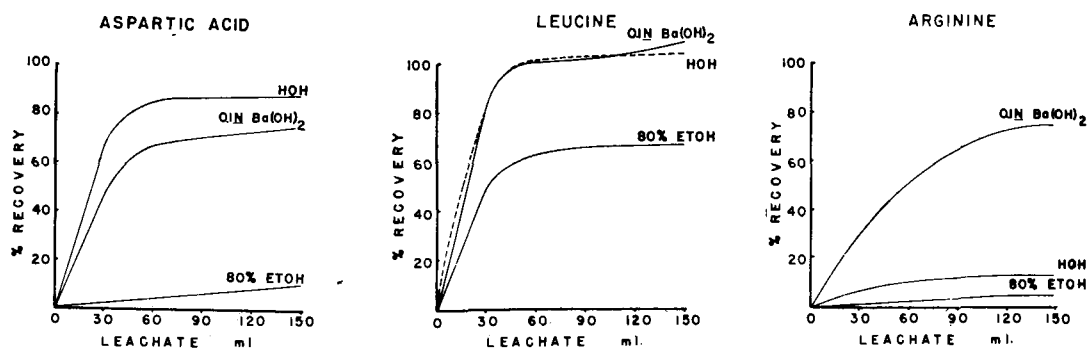


Figure 1—Effect of leachate volume on amino acid extraction. Recovery of 2.5 mg. each of aspartic acid, leucine, and arginine added to 25 g. of soil, in successive 15-ml. increments of water, ethanol, or $\text{Ba}(\text{OH})_2$.

Table 3—Recoveries of 1.5 mg. of each of 17 amino acids added as a mixture to 500 g. of Waukegan silt loam, with 0.1N Ba(OH)₂ as the extractant, and extraction of untreated soil as the control (column 2).

Amino acid	Total recovery %	Amount from soil %	Recovery of added amino acids %
Aspartic acid	77	4.2	73
Threonine	95	-	95
Serine	114	13.0	101
Proline	96	-	96
Glutamic acid	135	14.0	121
Glycine	102	11.0	91
Alanine	114	5.0	109
Valine	95	-	95
Isoleucine	114	12.0	102
Leucine	110	2.1	108
Beta alanine	86	-	86
Tyrosine	72	10.0	62
Phenylalanine	105	1.6	103
Gamma amino-butyric acid	91	-	91
Lysine	38	1.7	36
Histidine	44	3.2	41
Arginine	51	14.0	37

that was of special concern throughout, was that mildly hydrolytic conditions may have developed to varying degrees in different Ba(OH)₂ preparations.

Although there was little indication that hydrolysis of soil constituents actually was a factor, reagents other than Ba(OH)₂ were considered in an effort to avoid the effects of strongly alkaline conditions. NH₄OAc seemed especially promising as an extractant, in view of the reaction at which it could be used (pH 6.8), and the possibility of its removal from the final extract by sublimation (6). In practice, sublimation of the concentrated extract left a residue of salts greater than that resulting from the Ba(OH)₂ procedure and necessitated an additional step to take up the amino acids from interfering salts with alcohol. The more extensive sample preparation was considered justifiable, however, in order to avoid the hydrolytic conditions encountered with Ba(OH)₂.

The effectiveness of the NH₄OAc procedure in the recovery from soil of 19 added amino acids is reported in table 4. Results are the average of duplicate determinations which had a coefficient of variation of 10%. Comparison of these data with the recoveries shown in table 3 indicates that the Ba(OH)₂ procedure resulted in generally higher recoveries than did the NH₄OAc. Although excessively high amounts of several amino acids were encountered in the Ba(OH)₂ preparation, only tryptophan recovery was high with NH₄OAc. The two extraction techniques were about equally effective for the basic amino acids as each recovered only about 1/3 to 1/2 of the added arginine, lysine, and histidine. The amino acids that occurred naturally in the control soils are reported in the second column of tables 3 and 4; these were

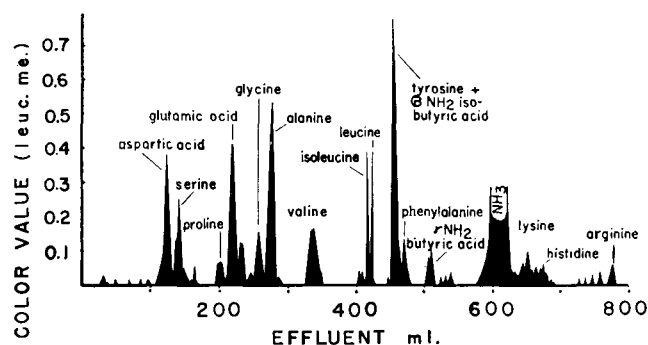


Figure 3—Gradient elution chromatogram showing ninhydrin reacting compounds present in a 0.5N NH₄OAc extract of soil amended with glucose and KNO₃.

Table 4—Recoveries of 1.5 mg. of each of 19 amino acids added as a mixture to 500 g. of Waukegan silt loam, with 0.5N NH₄OAc as the extractant, and extraction of untreated soil as the control (column 2).

Amino acid	Total recovery %	Amount from soil %	Recovery of added amino acids %
Aspartic acid	79	9.2	70
Threonine	47	5.7	41
Serine	66	4.9	51
Asparagine	66	-	66
Proline	69	-	69
Glutamic acid	66	13.0	53
Glycine	59	7.8	51
Alanine	66	6.4	60
Valine	76	4.8	71
Methionine	63	-	63
Isoleucine	87	4.2	83
Leucine	63	6.0	57
Tyrosine	62	12.0	50
Phenylalanine	69	4.2	65
Gamma amino-butyric acid	50	2.1	48
Lysine	71	22.3	49
Histidine	63	15.4	48
Tryptophan	106	-	106
Arginine	31	-	31

extracted as well, if not better, by NH₄OAc than by Ba(OH)₂.

Application to Analysis of Amended Soil

In further trials application was made of the improved extraction techniques to characterize the free amino acid fraction of the Waukegan soil at a high level of microbial activity. In earlier work with the same soil (11) it was found that amendment with glucose and sodium nitrate greatly increased over the control soil the variety and amount of free amino acids that could be detected in ethanol extracts. Analysis of extracts prepared with Ba(OH)₂ and NH₄OAc from soils similarly amended and incubated 3 days are shown in figures 2 and 3.

Each of the extraction techniques resulted in a preparation that yielded a wide range of amino acids. In addition to the numerous, well-defined peaks that could be identified, unidentified peaks of ninhydrin reacting substances occurred commonly. The NH₄OAc procedure (figure 3) gave a more satisfactory preparation than the Ba(OH)₂ (figure 2) with regard to the number of identifiable peaks and the estimated total amount of ninhydrin reacting substances present. Most of the amino nitrogen extracted by the NH₄OAc was found as aspartic acid, threonine, serine, proline, glutamic acid, glycine, alanine, methionine, isoleucine, leucine, beta alanine, tyrosine, phenylalanine, gamma amino butyric acid, lysine, histidine and arginine. The NH₄OAc procedure showed a somewhat lower recovery of known amino acids added to soils; however, the effectiveness of the NH₄OAc procedure in the removal of naturally occurring amino compounds from both unamended and amended soil, together with the mild, nonhydrolytic conditions the technique allows, make this method the choice over the Ba(OH)₂ method.

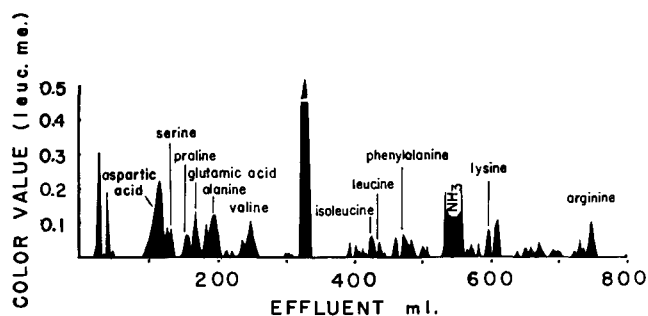


Figure 2—Gradient elution chromatogram showing ninhydrin reacting compounds present in a 0.1N Ba(OH)₂ extract of soil amended with glucose and KNO₃.

A subsequent paper will deal in detail with quantitative aspects of the occurrence of free amino acids in NH_4OAc preparations of amended soils. However, some approximations are in order to emphasize that both the NH_4OAc and the $\text{Ba}(\text{OH})_2$ procedures are much superior to ethanol extraction. The chromatograms shown in figures 2 and 3 represent the amino compounds found in portions of the extract equivalent to 50 g. of soil; the peaks, then, represent concentrations of from 0.5 to 10 μg . amino acid per g. of soil. None of the 11 amino acids extracted by Putnam and Schmidt (11) from a sample of the same soil under comparable conditions with alcohol exceed 0.4 μg . per g. of soil and most were < 0.1 μg . per g. Amino acids together with the unknown peaks in figure 3 could amount to roughly 100 μg . of amino acid or about 16 μg . of amino nitrogen per g. of amended soil. Concentrations of nitrogen of this magnitude emphasize the need for further examination and characterization of the free amino acid fraction of soils, particularly in those sites of high microbial activity.

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