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Amino Acids in Soil

I. Water-soluble Acids

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Free amino acids in the H-layer of a pine forest soil profile from Western Norway have been studied, by a partition chromatography technique with circular filter papers as the stationary phase. The soil samples were first extracted with ether and then (after drying) with cold water, and the water extracts were concentrated *in vacuo*.

25 ninhydrin-positive spots were observed. Of these 22 were identified and 14 were quantitatively determined.

In 1910 Scheiner and Shorey [1] isolated many simple organic substances, including arginine and histidine, from a "fulvic acid" fraction of soil, and Shorey [2] later reported isolation of lysine from the organic fraction of a few of the soils examined. As a 2 % sodium hydroxide solution was used in their extractions, it is likely that the amino acids they reported were derived from the hydrolysis of polymerized amino compounds.

Studies of concentrated extracts have later confirmed the existence of free amino acids in soil. Payne *et al.*[3] noted that aqueous soil extracts concentrated by freeze-drying yielded ninhydrin-positive spots on paper.

Recently some quantitative measurements of free amino acids in soil have been reported by Putnam and Schmidt.[4] Their figures vary within wide limits, and not much information is given about the actual amounts.

In one of our earlier studies on sugars in a cold water extract of a pine forest soil from Western Norway,[5] several ninhydrin-positive spots were observed on paper chromatograms. In the present work an attempt has been made to determine, qualitatively and quantitatively, the free amino acids present in soil organic matter, by use of the circular paper chromatographic technique.

EXPERIMENTAL

Soil sample. The soil sample for the analysis was taken in May 1955 from the H-layer in a pine forest soil profile from Sandanesset, Söfteland, south of Bergen. This locality was exactly the same as that described by Alvsaker,[6] where the profile examined was found to be typical for such soils from Western Norway. In the present profile no distinct borderline could be observed neither between the two F-layers nor between the two A-layers. The combined F-layers were 3 cm thick, the H-layer 3 cm, and the combined A-layers 4–5 cm.

Extractions. 400 g samples of air-dry soil were first extracted with ether and then with water, both extractions carried out at room temperature (20°C). The water extracts were filtered and concentrated *in vacuo*.

Paper. Whatman No. 1 filter paper was used. The circular filter papers had diameters from 26.5 cm to 35 cm. For quantitative estimations, the most useful size was found to be 30–33 cm in diameter. All the papers were used as such without any treatment, except when cresol was used as developing solvent.[11]

Pipettes. Micro pipettes were prepared and calibrated according to Anderson.[7] The volumes were from 2.5 µl to 6 µl.

Chromatographic chambers. Chromatographic chambers were constructed as described by Juvvik and Michelsen,[8] with diameters varying from 24 cm to 35 cm.

Standard solutions. Stock standard solutions of amino acids (Eastman) were prepared in concentrations of 0.1 % in 10 % isopropyl alcohol. In the case of difficultly soluble amino acids a drop of concentrated hydrochloric acid was added. Standard mixtures of amino acid solutions containing 10–12 different amino acids were prepared in the same way and at the same concentrations as mentioned above.

Solvents. Successful chromatographic separation is very dependent upon choice of solvents. About 20 different solvents (butyl alcohol, *tert.*-butyl alcohol, iso-butyl alcohol, phenol, benzyl alcohol, amyl alcohol, methyl ethyl ketone, *m*-cresol, and others) have been tested in the present study, but most of them were not found useful at all. The solvents found especially suitable are listed in Table 1. This table also shows the separation in each solvent system. The amino acids are denoted by the three first letters of their names with the appendage "A" for the acid ones.

Reagents. Ninhydrin reagent: 0.5 % ninhydrin dissolved in 95 % acetone. The other reagents were prepared as described in the literature.[15-21]

PROCEDURE

The technique used was essentially the same as that described by Giri and Rao.[14] The solution to be analysed was spotted on a circle (4 cm diameter) drawn around the centre of a circular filter paper. Usually the circle was divided into four equal parts, and on two of the arcs the solutions to be analysed were spotted, and on the two others solutions of known amino acids for comparison and identification. The spotted arcs were not allowed to become more than about 2 mm broad. When it was necessary to apply the solution to the arcs several times, the paper was dried at room temperature before each application. Such mixed chromatograms were often made into twelve sectors with solutions of known and unknown amino acids spotted alternatively on the arcs.

Usually 5–10 µl of standard solution containing 5–10 µg of each amino acid was applied to each sector. It was not necessary to saturate the atmosphere inside the chromatographic chamber with the vapour of the solvent before the development of the chromatograms. The speed of progress of the solvent front was controlled by varying the broadness of the wick. Smaller wicks gave lower speed and often better separation and more distinct bands. The time necessary for running the chromatograms varied for the different solvents; with a suitable wick the time for the large size chromatograms (35 cm) was about 16–18 h.

As amino acids having very nearly the same R_F values rarely separate into distinct bands, Giri's [14] multiple development technique was used. After the first development the chromatogram was dried at room temperature and developed once more with the same solvent, until the solvent boundary occupied the same position as before, and then dried again. The same process could be repeated, if necessary, several times.

The chromatogram was air-dried and the amino acid positions made visible by means of suitable colour reagents. The paper was placed on a clean, dry glass plate and the reagents were usually applied by means of cotton wool. In this way the reagents became more homogeneously distributed than by spraying. Water-containing reagents must be applied to the paper by spraying since otherwise the water-soluble amino acids would be displaced from their respective positions. Finally the chromatogram was dried under the special conditions described for each reagent.

Qualitative estimation

On concentrating the water extracts a brownish precipitate occurred which could not be completely removed neither by successive filterings nor by centrifugations. Small amounts of this precipitate followed the solution to the paper, but it had little or no movement in most of the developing solvents and gave no colour reaction with ninhydrin.

As the reproducibility of R_F values can not be completely relied upon, the identification of amino acids must be based on their movement in relation to standard amino acids run on the same paper, and on specific tests.

To get some information about the amino acid concentration in the unknown solution, mixed chromatograms were run containing different quantities of unknown solution and standard solution side by side.

The relative rates of movement of the amino acids were always found to be constant in the same solvent and, with some exceptions, also from one solvent to another. Often amino acids having nearly the same R_F values could be well separated in one solvent, but would overlap in another. For estimation of the relative positions of the amino acids on the chromatogram for each solvent, mixed chromatograms were run for all solvents and standard solutions with one amino acid in each sector. Thereafter mixed standard solutions could be used.

An attempt was made to find a combination of the most useful solvents in such a way that there should be at least three solvents, as different in composition as possible, which

Table 1. Qualitative estimation of amino acids.

No. of solvent	Composition of the solvent (v/v)	No. of runs	Amino acids separated
I	BuOH:HAc:H_2O (4:1:1)[9]*	2	Oct A, Leu (Ileu), Phe, Val, Met, (But A), EtAm, Try, Tyr, Pro, β-Ala, α-Ala, Thr, Glu A, Gly, Ser, Asp A, Glu, Tau, Asp, Cys.
II	Water-saturated PhOH [10]	2	β-Ala, α-Ala, Thr, Glu A, Gly, Ser, Asp A.
III	PhOH:BuOH:HAc:H_2O (20:20:8:40, upper layer)[11]	3	Oct A, Phe, Val, But A, Met, EtAm, Try, Tyr, Pro, β-Ala, α-Ala, Thr, Glu A, Gly, Ser, Asp A, Glu, Tau.
IV	*m*-Cresol saturated with 8.4 pH buffer. The paper buffered at pH = 8.4 [11]	2	Phe, Val, Met, Pro, Hpro.
V	MeCOEt:Py:H_2O (70:15:15)[12]	3	Oct A, Leu, Ileu.
VI	*t*-BuOH:MeCOEt:H_2O:HCOOH (160:160:39:1)[13]	3	Leu, Ileu, Phe, Val, But A, Met, EtAm, Try, Tyr, Pro, α-Ala.

* The upper layer of butanol-acetic acid-water mixture, 40:10:50 by volume, has widely been used as developing solvent for amino acid separation. The same components in mixture 4:1:1 seem to give greater R_F values and better separation and have therefore been used in the present work.

gave the same movement of an unknown as that of the standard amino acid it was expected to be identical with. Only the solvents listed in Table 1 were used in the final estimation of the unknown solution.

After development with BuOH:HAc:H_2O and colour development with ninhydrin about 20 violet and red-violet ellipses were observed (Table 1). These acids may be divided into three groups named I, II, and III from the solvent boundary to the centre of the paper, as follows:

Group I. This showed 10 bands. The innermost was yellowish and a weak red one gradually overlapped. Then followed a weak red one, three red ones, one blue-gray and three red. The outermost was very weak.

Group II. 6 bands were visible. The colour varied from red to blue-violet. The separation was incomplete.

Group III. The bands showed relatively little movement. There were two red-violet bands innermost and then two very weak ones. The groupings and separation are shown in Fig. 1.

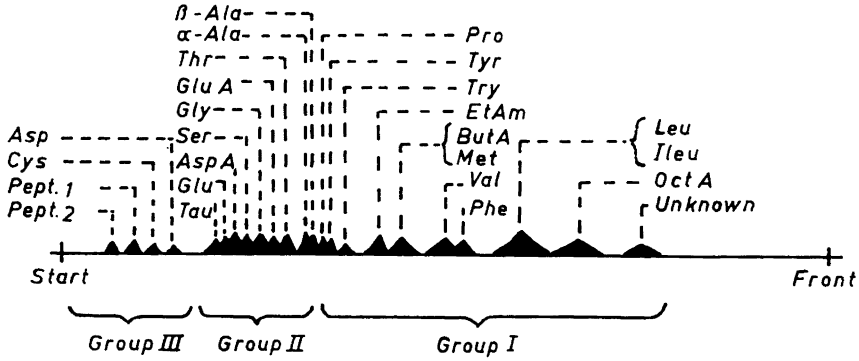

Fig. 1. The separation of the amino acids in the unknown solution developed with BuOH:HAc:H_2O (4:1:1). The distance is from the start point to the middle of the band. The broadness of the bands and degree of overlapping are shown.

For further identification multi-sector chromatograms were run with known and unknown solutions in different solvents and specific colour tests applied.

The outermost band in group I could not be identified by comparison with any standard amino acid and only trace of it was present. MeCOEt:Py:H_2O (70:15:15) was the only solvent which gave distinct separation of Leu and Ileu.

The two outermost bands in group III were very weak and their R_F values were found to be identical with Asp (the outermost one) and Cys. The two other bands did not correspond with any standard amino acids and were believed to be due to peptides. The bands were weak and to obtain some information whether they were due to peptides or not the following procedure was used:

The solution was spotted on the paper and the chromatogram was developed two times with the system BuOH:HAc:H_2O. Four small sectors were cut out and colour developed with ninhydrin. The ellipses for the two bands concerned were constructed, the ellipses cut off and eluted with water, and the solutions made 5 M with respect to formic acid. The test tubes containing the solutions were sealed and the contents hydrolyzed for 7 h on a boiling water bath. The hydrolyzates were evaporated to dryness *in vacuo* and the residues dissolved in a few drops of 10 % isopropanol. Chromatographic analysis showed that the assumption of the existence of peptides was probably correct. The hydrolyzate of the outermost band contained two weak zones identified as Asp A and α-Ala, and the other hydrolyzate contained three zones: Asp A, α-Ala (weak) and EtAm. The fact that one of the bands in the hydrolyzate of the innermost peptide was identical with one of the bands in group I led to the identification of EtAm. From cultures

Table 2. Colour reagents and colour reactions for amino acids.

Group No.	Amino acids identified	1. Ninhydrin	2. N-CN[15]	3. Isatin[16]	4. Chlor-amine T *p*-DMAB[17]	5. Nesslers reagent HIO_4[18]	6. PhOH NaOCl[19]	7. *p*-Anis-idine Amyl-nitrite NH_3[20]	8. HIO_4 Coll. AmAc Hac Acetyl-acetone[21]	9. HIO_4 Coll. Na-nitropr. Piperidine[21]
	Unknown	red	—	—	—	—	—	—	—	—
	Oct A	red	red-viol.							
			r.-br. ring	—	—	—	—	—	—	—
	Leu	red	red-viol.							
			r.-br. ring	—	—	—	—	—	—	—
I	Ileu	red	red-viol.	—	—	—	—	—	—	—
	Phe	blue-gray	green-blue	—	—	—	—	—	—	—
	Val	red	red-viol.	—	—	—	—	—	—	—
	But A	red	violet	—	—	—	—	—	—	—
	Met	red	gray							
			br. ring	—	—	—	—	—	—	—
	EtAm	red	violet	—	—	—	—	—	—	—
	Try	red	brown	—	—	—	pink	—	—	—
	Tyr	red	pink	—	—	—	—	orange	—	—
	Pro	yellow	gray-brown	blue	yellow	—	—	—	—	—
	β-Ala	blue-viol.	blue-viol.	—	—	—	—	—	—	—
	α-Ala	red-viol.	violet	—	—	—	—	—	—	—
	Thr	gray-blue	brown-viol.	—	—	brown	—	—	—	gray-blue
	Glu A	violet	violet	gray-						
II				blue	—	—	—	—	—	—
	Hpro	—	gray-brown	blue	red-blue	—	—	—	—	—
	Gly	violet	gray							
			br. ring	—	—	—	—	—	—	—
	Ser	red-viol.	red-viol.	—	—	brown	—	—	yellow	—
	Asp A	violet	blue	blue	pink	—	—	—	—	—
	Glu	red-viol.		—	—	—	—	—	—	—
	Tau	red-viol.		—	—	—	—	—	—	—
III	Asp	gray-green		—	—	—	—	—	—	—
	Cys	red-viol.	gray	—	—	—	—	—	—	—

Table 3. Quantitative determination of amino acids. Per cent organic matter in dry (105°C) soil 47.63.

Amino acids	No. of reliable measurements	Percentage of dry (105°C) soil × 10^3
Unknown	—	trace
Oct A	4	1.56
Leu	4	8.41
Ileu	4	2.04
Phe	4	2.46
Val	7	4.44
Met	9	6.58
But A	—	trace
EtAm	6	4.37
Try	—	trace
Tyr	—	trace
Pro	8	4.12
β-Ala	—	trace
α-Ala	12	6.35
Thr	12	4.96
Gly	4	5.25
Ser	6	3.79
Glu A	10	7.30
Asp A	8	6.66
Glu	—	trace
Tau	—	trace
Asp	—	trace
Cys	—	trace
Water-soluble amino acids, total		68.29

of soil micro-organisms polypeptides containing EtAm have been isolated.[22] The movement of a standard solution of this substance corresponded with the unknown substance in all solvents tested.

If there was any doubt about the identification, the unknown mixture was run with the addition of the amino acids which were believed to be identical with the unknown ones, and the bands in the mixed chromatogram were compared. The increase in intensity of the bands of the mixture containing the added known amino acids, located on the same circumference of the circle, indicates the presence of those particular amino acids in the unknown solution.

Quantitative estimation.

The procedure was essentially the same as that described by Giri *et al.*[23] The paper was divided into four equal sectors and known quantities of solutions were transferred to the sector arcs by help of micro pipettes, leaving some space (5 mm) between the adjoining points at the circumference, thus avoiding interference of amino acids from neighbouring sectors. In two of the sectors the unknown solution was spotted in the ratio of 1:2, and in the two others the standard solution in the same ratio. It is very important that for any one set of experiments the distance travelled by the solvent front is approximately the same. The paper was air-dried, placed on a clean glass plate, ninhydrin-solution (0.5 % in 95 % acetone) was applied with a piece of cotton wool, and the paper dried at 65°C for 30 min. Only those chromatograms where the bands were well separated and had little or no background colour were used for quantitative estimation.

The chromatograms were as far as possible protected from daylight. The boundary of the individual amino acid band was marked with a pencil, and the bands cut off carefully and placed in test tubes (one band in each tube). Bands of approximately identical areas for blanks were also cut off. From a burette 4 ml 75 % ethanol containing 0.05 mg $CuSO_4 \cdot 5H_2O$ per ml was added. The colour pigment formed with ninhydrin is quite soluble in 75 % ethanol and the cupric complex formed gives a bright red solution. The elution is complete in 10—15 min. The colour intensity of the solutions was determined in a Hilger Photoelectric Absorptiometer with the green filter No. 604 (wavelength 5200 Å), which gives maximum extinction. The readings were, after correction for blank, plotted in a diagram. The standard curves are linear within 2—12.5 μg. From the standard curve the quantity of the unknown can be read directly. Only those readings having the ratio 1:2 (not greater deviation than 5 %) were accepted. Standard curves were determined for each amino acid and each chromatogram.

In the case of proline a somewhat different procedure had to be used. This amino acid forms a yellow reaction product with ninhydrin. Some quantitative procedures for estimation of proline have been reported,[24,25] but they are rather difficultly applicable after paper chromatographic separation. A procedure described by Rao and Wadhwani [26] seemed promising. The chromatograms were colour developed with ninhydrin as described before, the yellow bands cut off, placed in test tubes, and 4 ml 50 % propanol was added. The intensity of the yellow colour elution was determined in a Beckman Spectrophotometer DU at 3500 Å.

RESULTS AND DISCUSSION

The qualitative and quantitative results are listed in Table 3. The values listed represent the average of four or more estimations.

As R_F values are rather irreproducable the qualitative estimation can not be based on these values, but tables of R_F values give information about the suitability of the solvents for separation in each special case. Various factors effecting the R_F values have been investigated and discussed by Rao and Giri.[27] The determination of the individual sequence of standard amino acids in each solvent system seems to leave no doubt about the correctness of the identification of the unknown acids.

Special caution must be exercised with regard to artifacts.[28] Such spots have been observed on paper chromatograms of amino acids, organic acids and bases, and inorganic ions. It is not impossible that the unidentified band in group I is an artifact.

In an investigation about factors effecting R_F values, Zimmermann [29] concluded that amino acids may influence the R_F values of each other. This problem was thoroughly investigated by Beck and Ebrey.[30] They assumed that amino acids react with each other and that the degree of reaction depends upon the ratio of their concentration and medium. The interfering effect may be the result of bonds between amino acids due to the amino and carboxyl groups. However, the correctness of this view may be discussed. In this case the chromatograms should not only show the spots corresponding to the "free" amino acids but also those of the products of the indicated reaction, and exact determination of amino acids chromatographically would be impossible without prior conversion into derivatives. Throughout this investigation no more bands were observed on chromatograms than there were amino acids in the standard mixture. A somewhat different colour at the boundary than in the middle of the band was often observed, and it is possible that this was due to a reaction product.

Although the humus compounds in the unknown solution were not positive to ninhydrin they may nevertheless give error. When relatively large amounts of the solution is spotted on the paper these substances form a barrier which retards the movement of the amino acids in the unknown solution in relation to that of the standards. It may also be assumed that the complex humus compounds contain free groups of different species which may form bands with the amino acids and exercise a further retardation. However, in such cases all doubt about the identification is eliminated when known amino acids are added to the spot of the unknown solution and the bands compared on the mixed chromatogram.

In quantitative estimations, loss by extraction may be an error that must be considered. However, a final extraction with one liter water, concentration *in vacuo* and negative ninhydrin reaction after prior chromatography indicated that the extraction was complete.

CONCLUSION

The analyses reported can hardly leave any doubt about the existence of free amino acids in the soil investigated. A great variety of different amino acids has been found, but the quantities must be characterized as very small.

We have not found any earlier reports about the existence of 2-amino-octoic acid and ethanol amine in soil.

The acid character of the soil (pH = 3.62) may lead to hydrolysis of proteins. Statistical proof for seasonal variations in soil pH has been reported,[31] and also seasonal variations in the total concentration of free amino acids.[32] The presence of free amino acids in soil is expected to have potential importance both to microbial nutrition and plant nutrition. Ghosh and Burris [33] have demonstrated that amino acids can be assimilated by certain higher plants.

Although the quantities of free amino acids in soil are relatively small, such compounds may be expected to be a vital linkage in the process of the decomposition of proteins to free ammonia, and in that way be of special significance for the metabolism of the soil micro-flora in a medium where roots, soil, and micro-organisms are intimately interrelated.

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REFERENCES

1. Scheiner, O. and Shorey, E. C. *J. Biol. Chem.* **8** (1910) 381.
2. Shorey, E. C. *U. S. Dep. Agr. Bur. of Soils Bull. 88.*
3 Payne, T. M. B., Rouatt, J. W. and Katz-Nelson, H. *Soil Sci.* **82** (1956) 521.
4. Putnam, H. D. and Schmidt, E. L. *Soil Sci.* **87** (1959) 22.
5. Grov, A. *Acta Chem. Scand.* **17** (1963) 2301.
6. Alvsaker, E. *Univ. Bergen Skrifter* **1948** No. 23, p. 12.
7. Anderson, H. H. *Anal. Chem.* **20** (1948) 1241.
8. Juvvik, P. and Michelsen, K. *To be published.*
9. Linskens, H. F. *Papierchromatographie in der Botanik*, Springer-Verlag, Berlin 1955, p. 109.
10. Consden, R., Gordon, A. H. and Martin, A. J. P. *Biochem. J.* **38** (1944) 224.

11. Krishnamurthy, K. and Swaminathan, M. J. *J. Sci. Ind. Res. (India)* **14 C** (3) (1955) 79.
12. Turba, F. *Chromatographisthe Metoden in der Protein-Chemie*, Springer-Verlag, Berlin 1954, p. 170.
13. Block, R. J. *Congr. intern. biochim. 2e Congr.*, Paris 1952, p. 171.
14. Giri, K. V. and Rao, N. A. N. *J. Indian Inst. Sci., Sect. A* **34** (1952) 95.
15. Moffat, E. D. and Lytle, R. J. *Anal. Chem.* **31** (1959) 926.
16. Ref.[9], p. 119.
17. Stegemann, H. and Griffin, H. F. *Naturwiss.* **45** (1958) 263; *Chem. Zentr.* **1959** 10023.
18. Block, R. J. *Food Industries* **22** (1950) 824.
19. Ref.[12], p. 180.
20. Sanger, F. and Tuppy, H. *Biochem. J.* **49** (1951) 463.
21. Schwartz, D. P. *Anal. Chem.* **30** (1958) 1855.
22. Fieser, L. F. and Fieser, M. *Organic Chemistry*, D. C. Heath and Company, Boston 1950, p. 456.
23. Giri, K. V., Radhakrishnan, A. N. and Vaidyanathan, C. S. *J. Indian Inst. Sci., Sect. A* **35** (1953) 145.
24. Pleshkov, B. P. and Fowden, L. *Nature* **183** (1959) 1445.
25. Chinard, F. P. *J. Biol. Chem.* **199** (1953) 91.
26. Rao, N. A. N. and Wadhwani, T. K. *J. Indian Inst. Sci., Sect. A* **37** (1955) 130.
27. Rao, T. and Giri, K. V. *J. Indian Inst. Sci., Sect. A* **35** (1953) 77.
28. Zweig, G. *Anal. Chem.* **31** (1959) 821.
29. Zimmermann, G. Z. *anal. Chem.* **138** (1953) 321.
30. Beck, M. T. and Ebrey, P. *Acta Chim. Hung.* **4** (1954) 231.
31. Gokhale, N. G. and Sen Gupta, A. K. *J. Indian Soc. Soil Sci.* **6** (1958) 103.
32. Dadd, C. C., Fowden, L. and Pearsall, W. H. *J. Soil Sci.* **4** (1953) 69.
33. Ghosh, B. P. and Burris, R. H. *Soil Sci.* **70** (1950) 187.

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