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Amino Acids in Soil

III. Acids in Hydrolyzates of Water-extracted Soil and Their Distribution in a Pine Forest Soil Profile

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Amino acids in hydrolyzates of water-extracted soil have been studied by a partition chromatography technique with circular filter papers as the stationary phase. The soil samples, taken from the F-, H-, and A-layers in a pine forest soil profile from Western Norway, were previously extracted with ether and cold water. The hydrolysis was carried out with 6 N hydrochloric acid at 120°C. The hydrolyzates were evaporated to dryness *in vacuo*, dissolved in water and desalted on an ion-exchange column. 21 ninhydrin-positive spots were observed and of these 16 were quantitatively determined. Inorganic cations in the hydrolyzates have been identified.

Earlier studies [1,2] have shown that a considerable part of the humic complex is composed of proteins. Soil extractions both with weak acids and bases for analysis of amino acids have been carried out,[3–8] but no reports deal with systematic studies of water-soluble and water-insoluble amino acids and proteins in soil.

In earlier qualitative and quantitative estimations of amino acids in water extracts of soil,[9,10] the technique used was circular paper chromatography, and the method was not only especially suitable, but simple and rapid too.

In the present study an attempt is made to obtain a qualitative and quantitative estimate of the amino acids in hydrolyzates of water-extracted soils from F-, H-, and A-layers in the same profile as mentioned above,[9,10] in order to get some information about the distribution and the individual variation of the acid-soluble amino acids in the whole profile. Like the previous investigations,[9,10] circular paper chromatographic procedures are applied, and the techniques, including mixed chromatograms and multiple development, were performed exactly as described before.[9]

EXPERIMENTAL

Soil samples. These were taken from the F-, H-, and A-layers in a pine forest soil profile from Western Norway. The profile has been described earlier.[9] The soil was first extracted with ether and then with cold water.

Paper, pipettes, cabinettes, standard solutions, solvents and colour reagents used were prepared as described before.[9]

Hydrolysis. To each gram of organic matter was added 20 ml 6 N hydrochloric acid, and the mixture was boiled on an oilbath (reflux) for 24 h at 120°C. The hydrolyzates were filtered through membrane filters, and the soil washed with water and filtered. The filtrates were evaporated to dryness *in vacuo* and the residues dissolved in water.

Procedure. The analyses were carried out exactly as earlier described.[9]

Desalting of the hydrolyzates. The hydrolyzates were found to contain interfering substances which gave trouble in the separation and identification of the amino acids. It was assumed that these substances were mostly inorganic, especially iron bound to the amino acids. No differences in movement of amino acids with or without iron could be observed, but the colour reaction of ninhydrin was markedly changed.

The desalting was carried out according to Buchanan,[11] using an ion-exchange column where the amino acids were displaced with piperidine. The ion-exchange resin, Dowex 50 Wx_4 20–50 mesh (wet), was washed with weak base, with acid and then with EDTA solution.[12]

A known quantity of hydrolyzate was passed through the column followed by distilled water until neutral effluent. 0.1 M piperidine was then added and the eluate collected. For control of the eluate and at the same time, if possible, to obtain a grouping of the amino acids, a fraction collector (LKB RadiRac Type 3400) with 2 ml portions in each tube was used. The eluation with piperidine was continued until negative ninhydrin reaction of concentrated eluate.

Investigation of inorganic cations. After displacement of the amino acids with piperidine the ion-exchange column was eluted with 3 N hydrochloric acid. The collected eluate was concentrated *in vacuo* and analyzed according to Feigl's spot tests [13] and Pollard and McOmie's chromatographic analysis.[14] The chromatograms were developed in the system butanol-benzoyl acetone. Both methods gave positive reaction to following ions: Fe, K, Na, Mg, Ca, Al, Zn, and Cu.

Qualitative estimation. The eluate in the different fraction tubes were tested with ninhydrin. The amino acids appeared after 550 ml eluate and were quantitatively out of the column after 1200 ml. The contents of tubes positive to ninhydrin were concentrated *in vacuo* and subjected to chromatographic analysis. However, neither separation nor groupings of the amino acids were obtained. The contents of all the fraction tubes belonging to the same hydrolyzate were then mixed and concentrated *in vacuo*. Chromatograms were made, and developed with the solvent systems: (*a*) BuOH:HAc:H_2O (4:1:1), (*b*) Water-saturated PhOH, (*c*) PhOH:BuOH:HAc:H_2O (20:20:8:40, upper layer), (*d*) *m*-Cresol saturated with 8.4 pH buffer, (*e*) MeCOEt:Py:H_2O (70:15:15), and (*f*) *t*-BuOH:MeCOEt:H_2O:HCOOH (160:160:39:1). 21 ninhydrin-positive spots were obtained which were identified as: Cys, Orn, Lys, Asp, His, Tau, Asp A, Ser, Glu A, Gly, Thr, Arg, α-Ala, Hpro, Pro, β-Ala, Met, Val, Phe, Leu, and Ileu.

The identification was based on:

1. The same movements (R_F values) as corresponding standards, in three different solvents.
2. Specific colour tests.
3. To two neighbouring sectors were applied equal quantities of the unknown mixture, and in addition, to one of the sectors was applied a standard amino acid with which a certain band in the unknown mixture was assumed to be identical. In the case of identity, the actual zone after chromatography and colour development increases in intensity in the sector with applied standard.

Quantitative estimation. A weighed quantity of the hydrolyzate was desalted on column as described above. The eluate containing amino acids was concentrated *in vacuo* and weighed. The qualitative tests showed that Asp, His, Tau, Met, and Phe appeared in too small concentrations for quantitative estimation. With the exception of Pro and Hpro, all the other amino acids were measured: The coloured bands on the chromatograms were cut off and extracted with 4 ml 75 % ethanol containing 0.05 mg $CuSO_4 \cdot 5H_2O$ per ml.

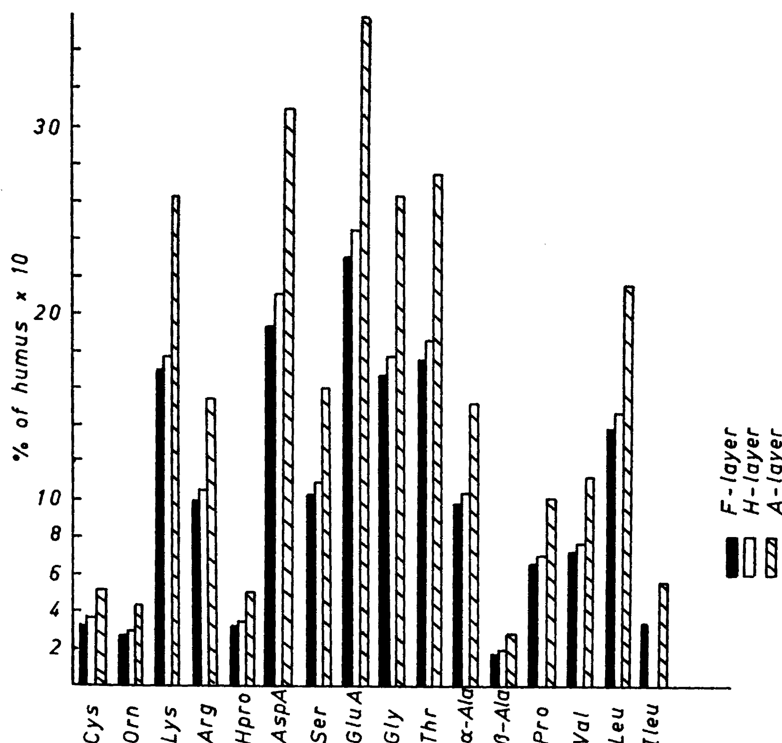

Fig. 1. Acid-soluble amino acids in the three profile layers. The amount of individual acids expressed as percentage of dry organic matter (humus).

The colour intensity was then measured with a Hilger Photoelectric Absorptiometer with the green filter No. 604 (wavelength 5200 Å).

Proline's and hydroxiproline's yellow bands, developed by treatment with ninhydrin, were cut off and extracted with 50 % propanol and the colour intensity measured with Beckman Spectrophotometer DU at 3500 Å.

RESULTS AND DISCUSSION

The qualitative and quantitative results are shown in Table 1. The values listed represent, in most cases, the average of four or more estimations. Fig. 1 shows the relative amounts of the individual amino acids in the respective layers, and Fig. 2 the total amounts in the three profile layers.

The ash contents of the dry (105°C) soils were, in F-layer: 30.07 %, H-layer: 52.45 %, and A-layer: 88.80 %.

Chromatographic analysis of the hydrolyzates without previous desalting was, as mentioned, difficult. The interfering substances were present in so high amounts that they would make the qualitative estimations uncertain, and also make the quantitative measurements quite unreliable. The desalting

Table 1. Quantitative determination of amino acids.

Amino acids	Amino acids expressed as percentage of organic matter (humus)			mg N bound to amino acids per g dry soil		
	F-layer	H-layer	A-layer	F-layer	H-layer	A-layer
Cys	0.30	0.36	0.48	0.25	0.20	0.06
Orn	0.25	0.29	0.40	0.38	0.30	0.09
Lys	1.62	1.79	2.36	2.17	1.63	0.51
Arg	0.95	1.05	1.52	2.12	1.61	0.55
Asp	trace	—	trace	—	—	—
His	trace	—	trace	—	—	—
Tau	trace	—	trace	—	—	—
Hpro	0.32	0.34	0.50	0.23	0.17	0.06
Asp A	1.86	2.10	2.98	1.37	1.05	0.35
Ser	1.02	1.09	1.56	0.95	0.69	0.23
Glu A	2.30	2.44	3.46	1.53	1.11	0.37
Gly	1.64	1.77	2.62	2.15	1.57	0.54
Thr	1.75	1.86	2.72	1.44	1.05	0.36
α-Ala	0.98	1.03	1.52	1.02	0.77	0.26
β-Ala	0.16	0.19	0.28	0.17	0.14	0.05
Pro	0.65	0.69	1.01	0.55	0.40	0.14
Met	trace	trace	trace	—	—	—
Val	0.66	0.76	1.08	0.55	0.43	0.14
Phe	trace	trace	—	—	—	—
Leu	1.38	1.47	2.06	1.03	0.75	0.25
Ileu	0.34	—	0.55	0.26	—	0.07
Total	16.18	17.23	25.10	16.17	11.87	4.03

on ion-exchange column was easy and effective; no interfering substances could be observed in the eluates. The chromatograms were "clean" and the identifications of the coloured bands were distinct and certain.

Tryptophane is destroyed on acid hydrolysis,[15] and could not be observed in the unknown mixture. There is some doubt about the listed values for cystine. This amino acid is found to be unstable during hydrolysis,[16,17] and the true amount must be assumed to be greater.

For the estimation of Hpro the chromatograms were developed with buffered *m*-cresol. The back-ground colour, after colour development, was relatively intense and affected the quantitative measurements.

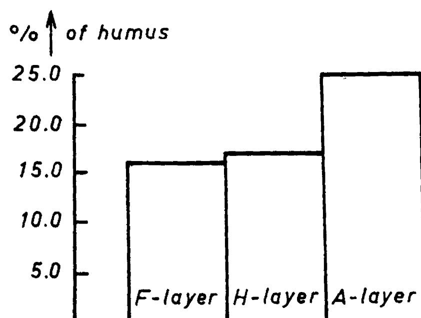

Fig. 2. The total quantities of acid-soluble amino acids in the three profile layers expressed as percentage of organic matter (humus).

Table 2.

Results from the earlier investigation of H-layer [1]				Results obtained in the present and earlier investigations [9,18]		
		% of dry soil	% of humus		% of dry soil	% of humus
Thickness of the layer	2 cm			3 cm		
pH	3.59			3.62		
Ash		10.82			52.37	
C		50.09	56.20		26.38	55.38
H		5.69	6.38		2.83	5.94
Humus/C (= f)	1.78			1.806		
C/H	8.80			9.30		
N total		1.29	1.45		1.195	2.516
N water-sol.		0.039	0.044		0.009	0.020
Proteins(acid soluble)			6.7		8.22	17.23
Proteins, total			8.8		8.288	17.43
C/N	38.9			22.0		

With the exception of Ileu, those amino acids which are present in measurable amounts are found in all the three layers. Both the individual and total amounts increase with depth, and the increase is appreciable from H- to A-layer in accordance with earlier observations made on podsol profiles from Western Norway.[1]

The relative increase of the individual amino acids from one layer to another downwards is surprisingly regular, and should indicate an approximately constant composition of the hydrolyzed complexes (proteins).

The quantities both of individual amino acids and the total amounts must be characterized as rather large and it is obvious that these compounds constitute a vital part of the humic complex, and that this part increases with the mineral content of the soil downwards in the profile.

In Table 2 some experimental results from an earlier study of H-layer from the same locality [1] are compared with the results obtained in the present and earlier investigations.[9,18]

Total humus may differ greatly within a relatively little area, but the elementary analyses of C and H show that the constitution of the humus does not vary much. This is in agreement with the factor f = humus/C and the ratio C/H.

The listed values, from the two separate investigations, were obtained with different methods. The nitrogen values from the present study refer only to protein-bound nitrogen. Water-soluble nitrogen amounts to only a small part of total nitrogen,[1] but increases with the mineral content of the soil. The wet climate of Western Norway should give rise to a relatively small amount of water-soluble nitrogen, especially in the uppermost layers.

Acid-insoluble proteins also constitute a relatively small part of the total,[1] and the nitrogen bound in these proteins therefore a small part of the total nitrogen.

The ratio C/N has been applied as a measure of the degree of decomposition of organic matter in soil,[1] a C/N value amounting to 20 or higher indicating a small degree of decomposition. The C/N ratio of 22 found in the present study indicates that the organic matter is rather little decomposed, but in the investigation [1] performed 18 years earlier, the C/N ratio was found to be 38.9 which indicates a still much smaller decomposition. (Calculations for the A-layer show: f = humus/C = 1.77, C/H = 9.78, and C/N = 15.8. Corresponding values from the earlier investigation [1] were: f = 1.79 and 1.77 (in A_1 and A_2 respectively), C/H = 9.6 and 7.6, and C/N = 26.3 and 26.4). This is in agreement with the total nitrogen and protein content estimated (approximately 1:2).

It must be assumed that the age of the profile, seasonal variations, and in what part of the year the soil samples are collected are of great importance with regard to the nitrogen content.

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