

STUDIES ON SOIL HUMIC ACIDS

I. THE CHEMICAL NATURE OF HUMIC NITROGEN

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(With One Text-figure)

The nitrogenous material in the humic (i.e. alkali-soluble, acid-insoluble) fraction of soil organic matter has received considerable attention, and various workers, notably Waksman & Iyer (1932) and Hobson & Page (1932), have concluded that humic nitrogen is largely protein and that humic acids are formed in soil by reaction between lignin and protein material. The ligno-protein theory of soil humic acids has received considerable support (see Bremner (1954) for a review of the literature on the subject), but it has not been generally accepted. For example, Mattson & Koutler-Andersson (1942, 1943, 1944-5) have rejected it and have presented some evidence to support their theory that humic acids are derived from lignin by autoxidation and ammonia fixation and that humic nitrogen is largely in the form of nitrogenous complexes formed by interaction between oxidized lignins and ammonia. Forsyth (1947) has also found reason to doubt the protein nature of humic nitrogen. He considered that humic acids as normally obtained, i.e. by acidification of alkali extracts of soil, are always contaminated by co-precipitated and absorbed material and purified his preparations by hydrolysing them with 2% hydrochloric acid for 4 hr. under reflux, a procedure which he claimed removed extraneous material but did not affect true humic acids. He found that the nitrogen contents of such purified preparations were not significantly affected when the preparations were methylated, esterified and acetylated by somewhat vigorous procedures and concluded that humic nitrogen could not be in the form of protein and must form part of the molecular structure.

The main object of the work reported in this series of papers was to obtain more precise information regarding the chemical nature of humic nitrogen. The present paper gives the results obtained in a study of the nitrogen distribution and amino-acid composition of humic acids isolated from 0.5M-sodium hydroxide and 0.1M-sodium pyrophosphate (pH 7.0) extracts of nine different soils and of the same preparations after pretreatment by Forsyth's technique. Preliminary results of this work have already appeared (Bremner, 1952).

MATERIALS AND METHODS

Soils. The materials used were as diverse as possible and consisted of three fen (nos. 1-3), three mineral (nos. 4-6) and three peat soils (nos. 7-9). Samples 1-3 were from the Cambridgeshire fen district. Sample 1 was from a peat containing shell fragments, samples 2 and 3 were from typical neutral fen soils. Sample 4 was from a continuous wheat plot (Broadbalk 2B) receiving farmyard manure annually, sample 5 from a permanent root plot (Barnfield 1C) receiving farmyard manure annually, and sample 6 from an old garden soil that had been cultivated with large additions of organic manure for almost 100 years. Sample 7 was from a peat overlying a lacustrine deposit of calcium carbonate, sedge (*Carex flacca*, *C. paniculata* and *C. spicata*) being the dominant vegetation. Sample 8 was from another sedge peat in the same region. Sample 9 was taken from the subsurface layer (9-15 in.) of a profile developed under vegetation consisting chiefly of *Molinia* and *Nardus* spp. The pH values of the samples were determined with the glass electrode, nitrogen contents by a micro-Kjeldahl procedure and calcium carbonate contents by the Schollenberger (1930) technique (Table 1).

Isolation of humic acids. The reagents used to extract the humic fractions from the soils were 0.5M-NaOH, the classical extractant, and neutral 0.1M-sodium pyrophosphate (Bremner & Lees, 1949). Before extraction soils 1-6 were leached with cold 0.1N-HCl to remove carbonates, exchangeable bases and inorganic nitrogen, and then washed thoroughly with distilled water; the acid-leached Burwell soil was leached with M-KCl to prevent dispersion of organic matter. The acid-leached soils were air-dried at room temperature and ground to pass a 2 mm. sieve. The humic fractions of the mineral soils (nos. 4-6) were isolated as follows. The soil was shaken with 0.1M-sodium pyrophosphate (pH 7.0) or 0.5M-NaOH (10 ml. extractant/g. of soil) for 20 hr., the suspension allowed to settle, and the supernatant liquid siphoned off, centrifuged to remove suspended material, and filtered with suction through a sinter-glass funnel.

The filtrate was brought to pH 2.0 by addition of dilute hydrochloric acid and the humic precipitate separated by centrifugation and washed on the centrifuge with 0.1N-HCl. The precipitate was re-dissolved in the reagent used for extraction, reprecipitated with dilute hydrochloric acid (pH 2.0) and washed on the centrifuge with 0.1N-HCl. This process was repeated 5 times. The precipitate was then frozen at -10°C . (Forsyth & Fraser, 1947), allowed to thaw on a filter with suction, and washed with 0.1N-HCl and then with water until it showed signs of dispersing. It was then dried *in vacuo* at room temperature and ground to a fine powder.

The above method of isolation proved unsuitable for the organic soils, and with these the following procedure was adopted. The soil was shaken with 0.1M-sodium pyrophosphate (pH 7.0) or 0.5M-sodium hydroxide (80 ml./g. of soil) for 20 hr. and the suspension filtered with suction through a Buchner funnel containing a layer of cottonwool over a sheet of fine muslin and then through a sinter-glass funnel

preparation was subsequently extracted with alcohol: benzene. Thus a preparation described as 6NE was obtained from soil 6 by extraction with 0.5M-NaOH and subsequently extracted in a Soxhlet with alcohol: benzene (1:1) for 36 hr.

Hydrolysis of preparations

Two methods of acid hydrolysis were employed. Method 1 was used for hydrolysis of protein material, the amino-acids liberated being subsequently estimated and identified. Method 2 was used for hydrolysis of amino-sugar material (Bremner & Shaw, 1954) and for estimation of amide nitrogen (Shore, Wilson & Stueck, 1935).

Method 1. The preparation was heated with 6N-HCl (5 ml./g. of preparation) in a sealed tube at 105°C . for 18 hr. The hydrolysis mixture was filtered through a weighed sinter-glass funnel with suction, the residue washed thoroughly with water, and the filtrate concentrated several times *in vacuo* to remove hydrochloric acid and made to volume.

Table 1. Description of soils used

No.	Soil	Site	pH	Percentage on moisture-free basis	
				N	CaCO ₃
1	Fen	Burwell	7.5	2.04	30.30
2	Fen	Swaffham	7.2	2.29	4.10
3	Fen	Littleport	7.1	1.50	3.04
4	Clay loam	Rothamsted	7.7	0.23	2.39
5	Clay loam	Rothamsted	7.6	0.27	0.70
6	Old garden	Rothamsted	6.9	0.43	1.29
7	Low-moor peat	Anglesey	6.3	2.82	0
8	Sedge peat	Anglesey	5.8	2.46	0
9	Mountain peat	Capel Curig	4.0	2.07	0

packed with cottonwool. The extract was centrifuged to remove clay colloids, refiltered, and acidified (pH 2.0) with dilute hydrochloric acid. The procedure was then as described above for mineral soils, the only difference being that the humic precipitate was washed with M-potassium chloride solution 0.1N with respect to hydrochloric acid instead of 0.1N-HCl to prevent dispersion of the organic matter.

Samples of some of the vacuum-dried preparations were extracted with alcohol: benzene (1:1) in a Soxhlet for 36 hr. to remove waxes and resins.

The nitrogen contents of the preparations were determined by a micro-Kjeldahl procedure using $\text{SeO}_2\text{:CuSO}_4\text{:5H}_2\text{O:K}_2\text{SO}_4$, 1:1:8, as catalyst and ash contents by incineration of weighed samples at 700°C . for 2 hr.

To facilitate reference to the preparations the following method of description has been adopted for all papers in this series. The description gives the number of the soil from which the preparation was obtained (Table 1), followed by N if the extractant was 0.5M-NaOH or P if the extractant was 0.1M-sodium pyrophosphate, followed by E if the pre-

The residue was dried to constant weight in an oven at 105°C . and samples of the dried material were taken for total-N and, in some cases, for ash determinations. Aliquots of the hydrolysate were taken for total-N, ammonia-N and α -amino-N determinations. The remainder of the hydrolysate was concentrated to small volume *in vacuo* and part of the concentrated solution desalted by the method of Consden, Gordon & Martin (1947). Samples of the undesalted and desalted solutions were then taken for paper chromatographic analysis for amino-acids.

Method 2. The preparation was heated with 6N-HCl (5 ml./g. of preparation) in a sealed tube at 100°C . for 6 hr. The procedure was then as in Method 1, aliquots of the hydrolysate being used for total-N, ammonia-N and amino-sugar-N determinations and for paper chromatographic analysis for amino-sugars.

Pretreatment of preparations and hydrolysis of pretreatment residues

Samples of some of the preparations were subjected to the pretreatment used by Forsyth (1947) for the purification of humic acid preparations and

the effect of the pretreatment studied by determining the amount of nitrogen dissolved and the nitrogen distribution of the pretreatment residue after hydrolysis with 6N-HCl. The pretreatment was carried out by hydrolysing with 2% (w/v) HCl (20 ml./g. of preparation) under reflux for 4 hr. The hydrolysis mixture was filtered through a weighed sinter-glass funnel with suction and the insoluble residue washed thoroughly with water, dried *in vacuo*, weighed, and analysed for total-N. The filtrate was made to volume and aliquots of the solution taken for total-N determinations. In a few cases an aliquot of the solution was evaporated to dryness *in vacuo*, the residue hydrolysed with 6N-HCl at 105° C. for 18 hr., and the hydrolysate concentrated several times *in vacuo* to remove hydrochloric acid, made to volume and the solution analysed for α -amino-N. The insoluble residue from the pretreatment was hydrolysed and analysed as described above in the section on hydrolysis of the preparations by Method 1, the only difference being that the hydrolysate was not analysed for ammonia-N.

Analysis of the hydrolysates

Total-N was determined by a micro-Kjeldahl method using $\text{SeO}_2\text{:CuSO}_4\cdot 5\text{H}_2\text{O:K}_2\text{SO}_4$, 1:1:8, as catalyst.

α -Amino-N (amino-acid-N) was determined by the manometric ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941).

Amino-sugar-N was estimated by the alkaline decomposition method of Tracey (1952) and by the colorimetric method of Elson & Morgan (1933) modified according to Blix (1948) and Immers & Vasseur (1950).

Ammonia-N was determined by the method of Pucher, Vickery & Leavenworth (1935) and by the microdiffusion method described by Bremner & Shaw (1954). The results obtained by these two methods were in close agreement.

Paper chromatographic analysis for amino-acids and amino-sugars

The technique used for paper chromatographic analysis for amino-acids was essentially that described by Dent (1948). Chromatograms were run in one direction with phenol in an atmosphere of ammonia and hydrocyanic acid, and in the other with 'collidine' in an atmosphere of diethylamine. The 'collidine' used was a mixture of equal parts of 2:4:6-collidine and the 2:4/2:5-lutidine supplied by Light and Co., the bases being redistilled before use. Whatman no. 4 filter-paper was used throughout. After development and drying the chromatograms were sprayed with ninhydrin (0.1%, w/v) in chloroform and the strengths of the spots detected were estimated visually from their size and colour in-

tensity using an arbitrary scale of 10 units. Proline and hydroxyproline, which give yellow and orange ninhydrin colours, respectively, were assessed as strong (S), medium (M) and weak (W). Identifications of the spots on the chromatograms were checked by marker experiments with pure amino-acids and by other techniques used in previous work (Bremner, 1950, 1955). Paper chromatographic analysis for amino-sugars was carried out by methods already described (Bremner, 1955; Bremner & Shaw, 1954).

RESULTS

The total nitrogen contents of the preparations and the amounts of acid-soluble-N, α -amino-N, ammonia-N, distillable-N, amide-N and amino-sugar-N liberated by their acid hydrolysis are given in Table 2. Amide-N has been estimated from the ammonia-N liberated by hydrolysis with 6N-HCl at 100° C. for 6 hr. The expression 'distillable-N' is used to refer to (amino-sugar + ammonia)-N liberated as ammonia by distillation with pH 11.1 Na_3PO_4 -borate buffer (Tracey, 1952).

Tests with several of the preparations showed that neither their total nitrogen contents nor their nitrogen distributions after acid hydrolysis were affected by further purification by reprecipitation or dialysis. Experiments with preparations 7N, 7P, 8N, 9N and 9P showed that less than 1% of their nitrogen was dialysable through Cellophane.

No volatile amines could be detected in the hydrolysates of the preparations by paper chromatographic analysis or by other methods previously described (Bremner, 1949a).

Ash was not determined when the amount of preparation isolated was small, as it was necessary to conserve material for other investigations. The ash contents of the preparations analysed, expressed as percentages on the moisture-free basis, were: 1N, 0.72; 1NE, 0.74; 1P, 0.26; 1PE, 0.20; 3N, 0.98; 3NE, 0.94; 7N, 0.83; 7P, 0.18; 8N, 0.94; 8NE, 0.90; 9N, 0.81; 9P, 0.28. The phosphorus contents of preparations 7N, 7P, 9N and 9P were determined by the method of Bolin & Stamberg (1944). The results, expressed as percentages on the moisture-free basis, were 7N, 0.06; 7P, 0.04; 9N, 0.06; 9P, 0.05.

From 10 to 40% of the material in the preparations was dissolved by the hydrolysis with 6N-HCl at 105° C. for 18 hr.; the nitrogen contents of the residues from the hydrolysis varied from 1.21 to 2.28%.

The amounts of nitrogen dissolved by pretreatment of the preparations with 2% HCl and the amounts of acid-soluble-N and α -amino-N liberated by hydrolysis of the pretreatment residues with 6N-HCl are given in Tables 3 and 4 respectively.

Table 2. *Analyses of humic acid preparations*

Acid-soluble-N, α -amino-N and ammonia-N estimated after hydrolysis with 6N-HCl at 105° C. for 18 hr. Distillable-N, amide-N and amino-sugar-N estimated after hydrolysis with 6N-HCl at 100° C. for 6 hr. Results expressed as percentages of total nitrogen in the preparations.

Preparation	Nitrogen content (%)*	Acid-soluble-N†	α -Amino-N	Ammonia-N	Distillable-N‡	Amide-N	Amino-sugar-N§	α -Amino-N as % of acid-soluble-N
1N	2.95 (2.97)	67.4 (66.5)	43.0	13.7	16.7	12.6	4.1	63.8
1NE	3.03 (3.05)	69.3	44.4	—	—	—	—	64.1
1P	1.92 (1.92)	56.3 (54.2)	31.8	12.8	16.1	11.5	4.6	56.5
1PE	1.91 (1.91)	58.0 (57.8)	33.5	13.1	—	—	—	57.8
2N	3.57	60.8 (59.8)	41.2	9.1	13.0	7.6	5.4 (4.9)	67.8
2P	2.63	58.7 (57.2)	34.5	8.8	12.8	8.0	4.8 (4.2)	58.8
3N	3.10	61.8 (59.4)	43.1	10.6	12.9	9.5	3.4	69.7
3NE	2.90 (2.93)	64.5	44.5	11.1	—	—	—	69.0
3P	1.94	51.2 (50.2)	29.0	10.3	13.1	9.1	4.0 (3.5)	56.6
3PE	2.05	50.0	28.5	10.2	—	—	—	57.0
4N	4.65	70.6	41.8	14.9	—	—	—	59.2
5N	4.10	77.4	47.2	15.3	—	—	—	61.0
6NE	3.14	80.7	47.5	15.9	—	—	—	58.9
6PE	2.65	62.5	34.9	12.4	—	—	—	55.8
7N	3.74 (3.77)	66.0 (65.3)	44.7	8.5	12.1	7.8	4.3 (4.0)	67.7
7P	2.15 (2.15)	46.0 (45.1)	26.3	11.9	13.4	10.8	2.6	57.2
8N	2.41 (2.43)	59.2 (57.3)	39.6	8.5	12.2	7.6	4.6	66.9
8NE	2.48 (2.50)	61.8	40.7	9.1	—	—	—	65.9
9N	2.31 (2.33)	56.3 (55.9)	31.2	8.4	15.9	7.8	8.1 (6.8)	55.4
9NE	2.95	62.8	32.0	9.3	15.2	7.9	7.3 (5.9)	50.9
9P	1.79 (1.80)	41.0	19.7	7.9	13.3	7.3	5.0 (4.5)	48.0

* Moisture-free basis; not corrected for ash. Figures in parentheses are nitrogen contents after correction for moisture and ash.

† Figures in parentheses represent amounts of acid-soluble-N after hydrolysis with 6N-HCl at 100° C. for 6 hr.

‡ Distillable-N represents (amino-sugar + ammonia)-N liberated as ammonia by distillation with pH 11.1 Na_2PO_4 -borate buffer (Tracey, 1952).

§ Distillable-N minus amide-N (Tracey, 1952). Figures in parentheses are results obtained by the colorimetric method of Elson & Morgan (1933) modified according to Blix (1948) and Immers & Vasseur (1950).

Table 3. *Amounts of nitrogen dissolved by pretreatment of humic acid preparations and total nitrogen contents of insoluble residues from pretreatment*

In the pretreatment the preparations were hydrolysed with 2% (w/v) HCl (25 ml./g. of preparation) for 4 hr. under reflux.

Preparation	N dissolved*	N content of insoluble residue (%)†
1NE	37.2	2.68
1PE	39.8	1.75
2N	34.3	3.07
3NE	38.0	2.35
3PE	33.0	1.89
7N	45.8	2.77
8N	22.3	2.28
8NE	23.1	2.36
9N	33.8	1.87
9NE	39.3	2.37

* As percentage of total nitrogen in preparation.

† Moisture-free basis; not corrected for ash.

Table 4. *Amounts of acid-soluble-N and α -amino-N liberated by acid hydrolysis of insoluble residues from pretreatment of humic acid preparations*

Residues were hydrolysed with 6N-HCl at 105° C. for 18 hr. Amounts of acid-soluble-N and α -amino-N liberated are expressed as percentages of total nitrogen in the residues.

Insoluble residue	Acid-soluble-N	α -Amino-N	α -Amino-N as % of acid-soluble-N
1NE	51.1	33.7	65.9
1PE	37.1	22.2	59.8
2N	44.3	26.4	59.6
3NE	41.0	27.1	66.1
3PE	30.3	18.5	61.1
7N	45.5	25.6	56.3
8N	46.6	31.1	66.7
8NE	48.9	32.0	65.4
9N	40.0	24.7	61.7
9NE	39.6	22.1	55.8

From 17 to 32% of the material in the preparations was dissolved by the pretreatment and from 9 to 17% of the material in the pretreatment residues was dissolved by the hydrolysis with 6N-HCl at 105° C. for 18 hr. The nitrogen contents of the insoluble residues from hydrolysis of the pretreated

preparations with 6N-HCl varied from 1.22 to 2.00%.

The identifications and strengths of the spots detected on two-dimensional phenol-'collidine' chromatograms of the desalted acid hydrolysates of the preparations and of the residues from pre-

treatment of the preparations are given in Tables 5 and 6, respectively. Chromatograms of the unsalted hydrolysates always showed some tailing of the spots, and for clarity the results obtained with these hydrolysates are included in Tables 5 and 6 only where they were markedly different from those obtained with the desalted hydrolysates. The positions of the ninhydrin-reacting substances detected on the two-dimensional chromatograms are shown diagrammatically in Fig. 1.

Histidine, which gives a weak reaction with ninhydrin, could not be detected on some of the two-dimensional chromatograms (Tables 5 and 6), but its presence in all of the hydrolysates examined was readily established by running one-dimensional chromatograms in butanol-acetic acid and spraying

amine and galactosamine, namely, *s*-collidine and butanol-NH₃ (Aminoff & Morgan, 1948). The results showed that both glucosamine and galactosamine were present in the hydrolysates, the concentration of glucosamine being considerably greater than that of galactosamine.

Identification of cysteic acid on chromatograms of the unsalted hydrolysates was confirmed by marker experiments with a specimen of cysteic acid synthesized by the method described by Clarke (1940). A weak spot due to cysteic acid was detected on chromatograms of the desalted hydrolysates after treatment with ammonium molybdate and hydrogen peroxide (Dent, 1948).

The substance occupying spot 21 (Fig. 1) appeared to be an α -amino-acid, as it could not be detected on

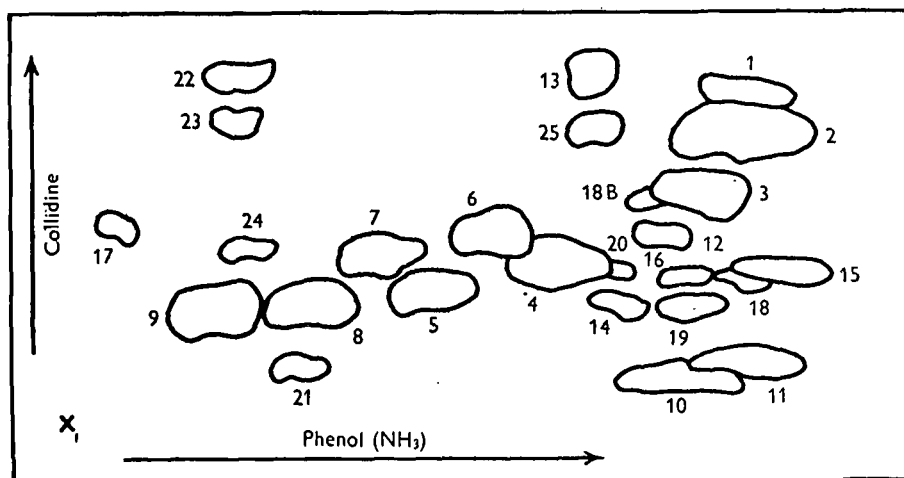


Fig. 1. Diagram showing positions of spots detected on two-dimensional phenol-'collidine' chromatograms of acid hydrolysates of humic acid preparations. A key to the numbers of the spots is given in Table 5.

with the Pauly reagents (Bremner, 1950). The presence of tyrosine in all of the hydrolysates was confirmed by the same method.

The presence of amino-sugars in all of the preparations examined was readily established by spraying one-dimensional butanol-acetic acid chromatograms of their hydrolysates with the Elson & Morgan (1933) hexosamine reagents (Partridge, 1948). Failure to detect an amino-sugar spot on two-dimensional phenol-'collidine' chromatograms of some of these hydrolysates (Table 5) is explained by the fact that both glucosamine and galactosamine are extensively decomposed on chromatograms run in these solvents (Aminoff & Morgan, 1951). The identity of the amino-sugar material in the hydrolysates of preparations 1N, 2N and 8N was investigated by running chromatograms in solvents which effect a separation of glucos-

chromatograms dusted with cupric carbonate (Crumpler & Dent, 1949). Its position was unaffected by hydrogen peroxide, indicating that it was not a sulphur-containing amino-acid (Dent, 1948). Its provisional identification as α, ϵ -diaminopimelic acid is based on the finding that its position on the two-dimensional phenol-'collidine' chromatograms was identical with that taken up by an authentic sample of α, ϵ -diaminopimelic acid added as a marker.

Identification of the substance occupying spot 19 (Fig. 1) as γ -aminobutyric acid was confirmed by marker experiments and by the cupric carbonate technique of Crumpler & Dent (1949). The identifications of β -alanine, α -amino-*n*-butyric acid, hydroxyproline and histidine were also confirmed by marker experiments.

Methionine and the leucine isomers cannot be distinguished on two-dimensional phenol-'collidine'

Table 5. Strengths of spots observed on two-dimensional chromatograms of desalted acid hydrolysates of humic acid preparations

Preparations were hydrolysed by heating with 6N-HCl at 105°C for 18 hr. Strengths of spots are on an arbitrary scale of 10 units. 1 = weak, 10 = strong. Proline and hydroxyproline strengths are indicated by S = strong, M = medium, W = weak; a dash (-) indicates that the substance was not detected. The numbers before each substance refer to the spot numbers in Fig. 1. Figures in parentheses are strengths of spots observed on two-dimensional chromatograms of undesalted hydrolysates.

	Preparation														
	1P	1PE	1N	1NE	2N	3PE	3NE	4N	7P	7N	8N	8NE	9P	9N	9NE
1 Phenylalanine	2	2	3	3	2	2	2	3	2	2	2	2	3	3	3
2 Leucine, etc.*	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
3 Valine	8	8	8	8	8	8	8	8	8	9	8	8	9	8	9
4 Alanine	9	9	10	9	9	9	9	8	9	10	9	9	10	9	10
5 Glycine	9	9	10	10	10	10	10	9	10	9	8	9	10	10	10
6 Threonine	4	4	3	3	3	4	3	3	5	5	6	5	5	5	5
7 Serine	6	6	5	6	6	6	5	5	7	7	7	6	7	8	8
9 Glutamic acid	9	8	9	9	9	8	9	9	9	8	8	8	9	8	9
10 Aspartic acid	3	3	5	4	4	5	4	4	4	4	3	3	4	3	4
11 Lysine	2	2	3	3	3	4	3	2	2	2	2	2	2	2	2
12 Arginine	<1	<1	<1	<1	<1	-	-	-	-	<1	<1	<1	<1	<1	<1
13 Histidine	1	1	1	1	1	1	1	1	<1	2	3	3	1	2	1
14 Tyrosine	2	2	2	2	2	2	2	2	2	2	1	1	2	2	2
15 β-Alanine	M	M	W	W	M	W	W	M	M	M	S	M	M	M	M
16 Proline	M	S	S	S	M	S	S	W	W	W	M	M	M	M	M
17 Hydroxyproline	(1)	(2)	(1)	(1)	(1)	(2)	(1)	(1)	(2)	(1)	(1)	(1)	(2)	(1)	(1)
18 Cysteic acid	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
18B Methionine sulphoxide	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
19 Methionine sulphonet†	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
20 γ-Aminobutyric acid	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
20 α-Amino-n-butyric acid	1	1	2	1	1	2	2	<1	2	1	2	1	2	1	1
21 α,ε-Diaminopimelic acid‡	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
22 Substance A	(2)	(2)	(2)	(2)	(1)	(2)	(1)	(1)	(2)	(3)	(2)	(2)	(3)	(3)	(3)
23 Substance B	(2)	(2)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(2)	(2)	(2)	(2)	(2)	(2)
24 Substance C	-	-	<1	<1	<1	-	-	-	-	-	-	-	-	-	-
25 Amino sugar§	<1	<1	-	-	-	-	-	-	-	-	1	1	<1	<1	<1

* Refers to spot in position occupied by leucine, isoleucine and methionine.

† Detected after ammonium molybdate-H₂O₄ treatment (Dent, 1948).

‡ Provisional identification.

§ Spot in position occupied by glucosamine and galactosamine.

chromatograms as their spots overlap. They can be readily distinguished, however, on one-dimensional chromatograms run in *tert.*-amyl alcohol (Work, 1949), and the presence of leucine and isoleucine in all of the hydrolysates examined was established by this method. Trace amounts of methionine were detected in some of the hydrolysates.

The ninhydrin-reacting substances occupying spots 22, 23 and 24 (Fig. 1) have not been identified. Spots 22 and 23 did not appear on chromatograms

DISCUSSION

The results given in Table 1 show that:

(1) Humic preparations isolated from alkali and pyrophosphate extracts of the same soil differ markedly in total nitrogen content and in nitrogen distribution after acid hydrolysis. The alkali-extracted preparation has a higher nitrogen content and a higher proportion of acid-soluble-N and α -amino-N.

Table 6. *Strengths of spots observed on two-dimensional chromatograms of desalted acid hydrolysates of insoluble residues from pretreatment of humic acid preparations*

In the pretreatment the humic acid preparations were heated with 2% (w/v) HCl for 4 hr. under reflux; the insoluble residues were then hydrolysed with 6N-HCl at 105° C. for 18 hr. Strengths of spots are on an arbitrary scale of 10 units. 1=weak, 10=strong. Proline and hydroxyproline strengths are indicated by S=strong, M=medium, W=weak; a dash (-) indicates that the substance was not detected. The numbers before each substance refer to the spot numbers in Fig. 1. Figures in parentheses are strengths of spots observed on two-dimensional chromatograms of undesalted hydrolysates.

		Pretreatment residue from									
		1PE	1NE	2N	3PE	3NE	7N	8N	8NE	9N	9NE
1	Phenylalanine	2	3	3	2	2	3	3	2	2	3
2	Leucine, etc.*	10	10	10	10	10	10	10	10	10	10
3	Valine	8	7	9	8	9	8	9	8	8	8
4	Alanine	9	7	9	9	9	9	9	9	10	10
5	Glycine	10	9	10	10	10	9	9	9	9	10
6	Threonine	4	3	4	4	3	4	5	4	5	5
7	Serine	6	5	5	6	5	5	6	5	7	8
8	Glutamic acid	9	8	8	8	8	8	7	7	8	8
9	Aspartic acid	9	9	7	8	8	8	8	8	8	8
10	Lysine	4	5	4	5	4	4	3	3	3	4
11	Arginine	3	4	3	4	2	2	2	2	2	2
12	Histidine	-	-	<1	-	-	-	-	-	1	<1
13	Tyrosine	1	1	2	1	1	2	2	2	2	1
14	β -Alanine	2	2	1	2	2	2	1	1	1	1
15	Proline	M	M	M	M	W	M	M	M	M	M
16	Hydroxyproline	S	S	M	S	S	M	M	M	W	W
17	Cysteic acid	-(2)	-(2)	-(2)	-(2)	-(2)	-(1)	-(1)	-(1)	-(2)	-(2)
18	Methionine sulfoxide	<1	<1	-	<1	-	<1	<1	<1	<1	<1
18B	Methionine sulfone†	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
19	γ -Aminobutyric acid	2	1	1	2	2	1	1	1	1	1
20	α -Amino- n -butyric acid	1 (<1)	1 (<1)	<1 (-)	1 (<1)	1 (<1)	2 (<1)	1 (<1)	1 (<1)	1 (-)	<1 (-)
21	α,ϵ -Diaminopimelic acid‡	<1	<1	<1	<1	<1	-	<1	<1	-	-
22	Substance A	-(1)	-(1)	-(<1)	-(1)	-(1)	-(2)	-(1)	-(1)	-(2)	-(2)
23	Substance B	-(<1)	-(<1)	-(<1)	-(<1)	-(<1)	-(1)	-(<1)	-(1)	-(1)	-(1)

* Refers to spot in position occupied by leucine, isoleucine and methionine.

† Detected after ammonium molybdate- H_2O_2 treatment (Dent, 1948).

‡ Provisional identification.

of the desalted hydrolysates; their positions on the chromatograms were unaffected by hydrogen peroxide or ammonium molybdate and hydrogen peroxide (Dent, 1948).

No purine or pyrimidine bases could be detected when the products liberated by hydrolysis of preparations 1N, 7P and 9P with 72% (w/w) $HClO_4$ at 100° C. for 1 hr. were analysed by the paper chromatographic techniques described by Wyatt (1951).

(2) The major fraction of the nitrogen liberated by acid hydrolysis of humic acid preparations is in the form of amino-acids; a smaller fraction is in the form of amino-sugars.

(3) The amounts of acid-soluble-N and ammonia-N liberated by acid hydrolysis of humic acid preparations with 6N-HCl at 105° C. for 18 hr. are not much greater than the amounts liberated by hydrolysis with 6N-HCl at 100° C. for 6 hr.

(4) From 31.2 to 47.5% of the nitrogen in the

alkali-extracted and 19.7 to 34.9 % of the nitrogen in the pyrophosphate-extracted preparations was liberated as α -amino-N by acid hydrolysis and presumably, therefore, was in the form of protein.

The finding that the nitrogen contents of alkali-extracted preparations are higher than those of pyrophosphate-extracted preparations is in harmony with the observations (Bremner, 1949*b*) that alkali extracts more nitrogen in relation to carbon from soil than does neutral pyrophosphate, and that there is little difference between the C/N ratio of a soil extract and the C/N ratio of its humic fraction.

Comparison of the nitrogen distributions of the alkali- and pyrophosphate-extracted preparations shows that although the alkali-extracted preparations have a considerably higher proportion of acid-soluble-N and α -amino-N, the proportions of ammonia-N, amide-N and amino-sugar-N are not greatly different. The method used for estimation of amide-N was essentially that recommended by Shore *et al.* (1935), but the results are of value only for purposes of comparison as some of the ammonia liberated by hydrolysis of the preparations with 6*N*-HCl at 100° C. for 6 hr. was undoubtedly formed by the decomposition of amino-sugars (see Bremner & Shaw, 1954) and other non-protein material. In this connexion it may be mentioned that tests have shown that some of the nitrogen fixed by lignin in its reaction with ammonia is liberated as ammonia by hydrolysis with 6*N*-HCl at 100° C. for 6 hr. It is of interest that whereas the amount of ammonia released by hydrolysis of soils with 6*N*-HCl increases steadily with time of hydrolysis (Bremner, 1949*a*), the amount of ammonia liberated by hydrolysis of humic acid preparations with 6*N*-HCl at 105° C. for 18 hr. is not much greater than the amount liberated by hydrolysis at 100° C. for 6 hr.

The fact that amino-sugars are unstable towards acid hydrolysis also complicates the estimation of amino-sugar-N in the preparations, but the results obtained by applying a correction factor of 1.25 to the amounts of amino-sugar-N found after acid hydrolysis are probably fairly reliable (see Bremner & Shaw, 1954), and these indicate that 3–10 % of the nitrogen in the preparations analysed was in the form of amino-sugars. The detection of amino-sugars in all the preparations examined confirms previous evidence (Bremner, 1949*a*) that soils contain amino-sugar complexes which, unlike chitin, are soluble in alkali and neutral pyrophosphate. It also suggests that some of the acetyl groups found in humic acid preparations (e.g. Gillam, 1940) may be in the form of *N*-acetyl amino-sugars.

Calculations from the results given in Table 1 show that 77.4–92.4 % of the nitrogen liberated by hydrolysis of the preparations was accounted for as (ammonia + amino-acid + amino-sugar)-N when these determinations were performed.

Attention may be drawn to the fact that a considerable fraction (20–60 %) of the nitrogen in the preparations was not dissolved by hydrolysis with strong acid. No method of establishing the chemical nature of this non-hydrolysable nitrogen is available, but tests have shown that most of the nitrogen fixed by lignin in its reaction with ammonia is resistant to hydrolysis with 6*N*-HCl and it seems likely that some of the non-hydrolysable nitrogen in humic preparations is in the form of such lignin-ammonia complexes.

Attempts to isolate the protein material from humic acid preparations, e.g. by removal of the non-protein material with mild oxidizing agents such as chlorine dioxide, have not so far been successful, but substances with the properties of peptides have been detected by paper chromatographic analysis of the products liberated by partial hydrolysis of humic preparations with concentrated hydrochloric acid at 35° C.

The results given in Tables 3 and 4 show that although a considerable fraction of the nitrogen in humic acid preparations is removed by the pretreatment used by Forsyth (1947) for the purification of humic acids, the residues from this pretreatment are not, as Forsyth believed, free from protein. Calculations from the results given in Tables 2, 3 and 4 show that 39–69 % of the α -amino-N in the preparations examined was removed by the pretreatment, and that on the average the residue from the pretreatment contained about 46 % of the α -amino-N present in the original preparation.

The results obtained by paper chromatography (Tables 5 and 6) show that humic preparations from different soils do not differ markedly in amino-acid composition and that the amino-acid composition of the residue from pretreatment of a humic preparation with 2 % hydrochloric acid is similar to that of the untreated material. The strongest spots on all chromatograms of the humic acid hydrolysates were those due to glutamic acid, aspartic acid, glycine, alanine, valine and the leucine isomers, and although the strengths of individual amino-acid spots are only roughly proportional to the amounts present on the chromatograms there can be little doubt that the major fraction of the α -amino-N in humic acid preparations is in the form of these seven amino-acids.

The detection of cysteic acid in hydrolysates of the humic preparations is of interest as neither cystine nor cysteic acid could be detected in desalted acid hydrolysates of soils (Bremner, 1950). The explanation of this would appear to be that cystine is oxidized to cysteic acid during the acid hydrolysis of soils or humic acid preparations, and that the cysteic acid so formed is lost if the hydrolysates are desalted before chromatography (see Table 5). Acid hydrolysates of humic acid preparations, unlike those of soils, need not be desalted before paper

chromatographic analysis, and cysteic acid appears on their chromatograms.

The detection of oxidation products of cystine and methionine on these chromatograms would appear to represent the only evidence so far obtained to support the view that part of the organic sulphur of soil is in the form of sulphur-containing amino-acids.

Apart from cysteic acid, the only ninhydrin-reacting substances found in the undesalted hydrolysates of the humic acid preparations and not detected in desalted acid hydrolysates of soils (Bremner, 1950) are the three unidentified substances A, B and C. Failure to detect substance C in soil hydrolysates is not very surprising, as it was detected in only a few of the humic acid hydrolysates and then only in trace amounts. Failure to detect substances A and B in desalted acid hydrolysates of soils is explained by the fact that, like cysteic acid, these substances are removed in the process of desalting (Tables 5 and 6).

It is of interest that most of the hydrolysates contained a substance whose behaviour on chromatograms was indistinguishable from that of α, ϵ -diaminopimelic acid, an amino-acid which appears to be peculiar to bacteria. A substance provisionally identified as α, ϵ -diaminopimelic acid has also been detected in acid hydrolysates of soils and composts (Bremner, 1950, 1955).

Attention may be drawn to the fact that the desalted hydrolysates invariably contained more α -amino-*n*-butyric acid than the undesalted hydrolysates (Tables 5 and 6), in some of which this amino-acid could not be detected. There are two possible explanations of this finding. One is that some α -amino-*n*-butyric acid was formed during the process of desalting; the other is that the undesalted hydrolysates, but not the desalted hydrolysates, contained salts or other substances that occupied the same position as α -amino-*n*-butyric acid on the two-dimensional phenol-'collidine' chromatograms and interfered with the reaction with ninhydrin.

The finding that γ -aminobutyric acid was released by hydrolysis of the preparations and of the residues from pretreatment of the preparations with 2% hydrochloric acid (Tables 5 and 6) is of considerable interest, as it provides evidence for the natural occurrence of chemically bound γ -aminobutyric acid. The same amino-acid was detected in acid hydrolysates of soils (Bremner, 1950), but this could not be taken as evidence for the natural occurrence of chemically bound γ -aminobutyric acid as it was not rigorously proved that the soils examined did not contain free γ -aminobutyric acid. This criticism does not apply to the results obtained with the preparations and their pretreatment residues, as there is no possibility that they contained free γ -aminobutyric acid. The possibility that the γ -aminobutyric acid

found in the hydrolysates of the humic preparations was an artefact formed by decomposition of glutamic acid or other amino-acids during acid hydrolysis was investigated, but no evidence of this could be obtained. For example, no trace of γ -aminobutyric acid could be detected after hydrolysis of glutamic acid, edestin or casein with 6N-HCl at 105° C. for 18 hr. when the substances were hydrolysed alone or in the presence of ten times their weight of lignin or ignited mineral soil. Moreover, tests with three soils showed that the amount of γ -aminobutyric acid liberated by their hydrolysis, as judged by the strength of the spot due to this amino-acid on two-dimensional chromatograms of the soil hydrolysates, was not increased by the addition of substantial amounts of glutamic acid, edestin or casein to the soils before hydrolysis. No evidence that any of the other ninhydrin-reacting substances detected in the humic acid hydrolysates were artefacts formed during acid hydrolysis was obtained in these experiments.

Failure to detect purine and pyrimidine bases in hydrolysates of the preparations indicates that only a small fraction of humic nitrogen is in the form of nucleic acids. This conclusion is supported by calculations from the phosphorus contents of preparations 7N, 7P, 9N and 9P, which show that not more than 3–5% of the total nitrogen in these preparations can be accounted for as nucleic acid-nitrogen even if it is assumed that all of the phosphorus is in the form of nucleic acids.

Further discussion of the results obtained in this investigation will be postponed until the results of other work on the preparations have been reported, but it may be stated here that all the results so far obtained are consistent with the view that humic acids as normally isolated from soil consist largely of high-molecular complexes formed by reaction of drastically altered plant lignin with ammonia and protein, together with polysaccharides containing amino-sugar and uronic acid units.

SUMMARY

1. The chemical nature of the nitrogen in humic acid preparations isolated from 0.5M-sodium hydroxide and 0.1M-sodium pyrophosphate (pH 7.0) extracts of nine different soils has been studied by determining the amounts of acid-soluble-N, ammonia-N, amino-sugar-N and α -amino-N liberated by acid hydrolysis of the preparations and by paper chromatographic analysis of their acid hydrolysates.

2. Humic acid preparations isolated from alkali and pyrophosphate extracts of the same soil differ markedly in total nitrogen content and in nitrogen distribution after acid hydrolysis. The alkali-extracted preparations have a higher nitrogen content

and a higher proportion of acid-soluble-N and α -amino-N.

3. A considerable fraction (20–60 %) of the nitrogen in the preparations examined was not dissolved by acid hydrolysis. The major fraction of the nitrogen dissolved was in the form of amino-acids.

4. At least 31–48 % of the nitrogen in the alkali-extracted preparations and 20–35 % of the nitrogen in the pyrophosphate-extracted preparations was in the form of protein. From 3 to 10 % of the nitrogen in the preparations was in the form of amino-sugars.

5. The results obtained by paper chromatographic analysis of acid hydrolysates of the preparations indicated that the protein materials in humic acids isolated from different soils by alkali

or pyrophosphate are similar in their amino-acid composition. The following nineteen amino-acids were detected in every hydrolysate examined: phenylalanine, leucine, isoleucine, valine, alanine, glycine, threonine, serine, aspartic acid, glutamic acid, lysine, arginine, histidine, proline, hydroxyproline, α -amino-*n*-butyric acid, β -alanine, γ -aminobutyric acid and tyrosine. Two unidentified ninhydrin-reacting substances, oxidation products of cystine and methionine, and amino-sugars were also detected in every hydrolysate examined. A third unidentified ninhydrin-reacting substance and a substance provisionally identified as α , ϵ -diaminopimelic acid were found in some of the hydrolysates.

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