Appendix

A. Derivation of equations (iv) and (v).

$$\bar{n} = \frac{[II] + 2[III]}{[M] + [II] + [III]},$$

where $[M]$ is the concentration of free metallic ions and II and III are the complexes illustrated in the introduction (p. 531). Then

$$\frac{\bar{n}}{1-\bar{n}} = \frac{[II] + 2[III]}{[M] - [III]} = \frac{[II]}{[M]},$$

when [III] is small, also

$$\frac{\bar{n}-1}{2-\bar{n}} = \frac{[III] - [M]}{2[M] + [II]} = \frac{[III]}{[II]},$$

when $[M]$ is small. Hence (from i)

$$K' = \bar{n}/1 - \bar{n}[Sc], \quad \text{(iv)}$$

provided [III] is small, and

$$K'' = (\bar{n} - 1)/(2 - \bar{n})\,[Sc] \quad \text{(v)}$$

provided $[M]$ is small.

B. Derivation of equation (xii).

When $\bar{n} = 1$, $[II] + 2[III] = [M] + [II] + [III]$; hence

$[M] = [III]$ and, as $K_s = [III]/[M]\,[Sc]^2$,

$$K_s = 1/[Sc]^2. \quad \text{(xii)}$$

The Amino-acid Composition of the Protein Material in Soil

By J. M. BREMNER

Chemistry Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

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Recent work (Kojima, 1947*a*; Bremner, 1949) has shown that a considerable fraction of the organic nitrogen of soil is in the form of protein, about one-third of it being liberated as α-amino nitrogen by acid or alkaline hydrolysis. No method of separating this protein material from other soil constituents has yet been found and information about its amino-acid composition is scattered and incomplete. The following amino-acids have been isolated as hydrolysis products of soil organic matter: leucine (Suzuki, 1906–8; Robinson, 1911; Lathrop, 1917; Kojima, 1947*b*), isoleucine (Robinson, 1911; Kojima, 1947*b*), valine (Kojima, 1947*b*), alanine and proline (Suzuki, 1906–8), arginine and histidine (Schreiner & Shorey, 1910*a*, *b*; Lathrop, 1917; Tokuoka & Dyo, 1937), lysine (Shorey, 1913; Lathrop, 1917), aspartic acid (Suzuki, 1906–8; Kojima, 1947*b*) and tyrosine (Lathrop, 1917). The presence of large amounts of extraneous material in soil hydrolysates makes preparative work so difficult that few workers have attempted more than qualitative isolation of some of the amino-acids present. In consequence the results obtained by isolation are too limited to permit deductions regarding the amino-acid distribution in soil.

The object of the present investigation was to identify the various amino-acids liberated by hydrolysis of soil protein and to determine whether or not the proteins in different soils are similar in their amino-acid composition. The method used for amino-acid analysis, that of partition chromatography on paper, permitted a comparison of the amino-acid distribution in a variety of soils.

Preliminary results of this work have already appeared (Bremner, 1950).

MATERIALS, METHODS AND RESULTS

Soils. The ten soil samples used differed greatly in pH value, organic-matter content and cultural history. The selection included four neutral fen soils (nos. 1–4), an acid fen soil (no. 8), an acid peat (no. 7) and a Russian chernozem (no. 9). The latter was from a bulk stock used by K. K. Gedroiz in his classical investigations on 'base exchange'. The three Rothamsted soils were selected to provide extreme contrasts in cultural history: sample 5 was from a continuous wheat plot (Broadbalk 2*B*) receiving farmyard manure annually, sample 6 was from unmanured land (Hoosfield) fallowed for 3 years after over a 100 years under alternating wheat and fallow, and sample 10 was taken in 1943 from an arable field (Sawyers II) down to grass from 1928 to 1940. The pH values of the samples were determined with the glass electrode, N contents by a micro-Kjeldahl procedure and $CaCO_3$ contents by the Schollenberger (1930) technique (Table 1).

Hydrolysis. The soils were hydrolysed by boiling under reflux for 24 hr. with 6 N-HCl (4 ml./g. of air-dried soil). In the case of sample 3, $CaCO_3$ was removed before hydrolysis by leaching with cold 0·1 N-HCl, which extracts insignificant amounts of organic N from soil. The hydrolysis mixtures were filtered, the residues washed thoroughly with hot water, and the filtrates concentrated several times *in vacuo* to remove HCl. The residues were dissolved in water and the solutions brought to pH 7·0 by addition of NaOH. The precipitates formed on neutralization were removed by filtration, washed with hot water, and the filtrates concentrated to small volume *in vacuo* and desalted by the electrical method of Consden, Gordon & Martin (1947). The brown precipitates formed during desalting were removed by filtration and the filtrates concentrated *in vacuo*. The N contents of the concentrated solutions were determined by a micro-Kjeldahl procedure and sufficient water was added to each to give a total N content of about 2 mg./ml.

Table 1. *Description of soils studied*

No.	Soil	Site	pH	Percentage on moisture-free basis N	$CaCO_3$
1	Fen	Swaffham	7·3	2·38	4·03
2	Fen	Methwold	7·2	2·62	1·37
3	Fen	Burwell	7·5	2·04	30·30
4	Fen	Littleport	7·2	1·52	3·04
5	Clay loam	Rothamsted	7·7	0·25	2·39
6	Clay loam	Rothamsted	7·5	0·10	0
7	Peat	Ponterwyd	4·1	2·50	0
8	Fen	Peterborough	5·0	1·78	0
9	Chernozem	Voronezh, U.S.S.R.	6·2	0·51	0
10	Clay loam	Rothamsted	5·5	0·18	0

Partition chromatography. The technique used for two-dimensional chromatography was essentially that of Dent (1948). The chromatogram was run in one direction with phenol in an atmosphere of NH_3 and HCN, and in the other with 'collidine' in an atmosphere of diethylamine. The 'collidine' used was the mixture described by Dent (1948), consisting of equal parts of 2:4:6-collidine and 2:4-lutidine. The solvents were used in the order given above; trials in which the order was reversed gave unsatisfactory results. Whatman no. 4 filter paper was used throughout. The chromatograms were sprayed with ninhydrin (0·1%, w/v) in $CHCl_3$ and the strengths of the spots detected were estimated visually from their size and colour intensity using an arbitrary scale of 10 units (Table 2). Proline and hydroxyproline, which give yellow and orange ninhydrin colours respectively, were assessed as strong (*S*), medium (*M*) and weak (*W*). The results of one of the two-dimensional chromatograms are shown diagrammatically in Fig. 1.

Identification of ninhydrin-reacting substances

γ-Aminobutyric acid. Most of the amino-acids were readily identifiable from the pattern of the spots, the chromatograms being compared with those of synthetic mixtures of pure amino-acids run under identical conditions and with Dent's (1948) 'map of the spots'. In many cases identifications were checked by adding pure amino-acids to the hydrolysates and observing that under identical conditions the pure substances ran exactly with the unknowns. The identity of γ-aminobutyric acid was confirmed in this way with a specimen of the pure amino-acid kindly supplied by Dr C. E. Dent. The presence of γ-aminobutyric acid in the hydrolysates was also demonstrated by the $CuCO_3$ technique of Crumpler & Dent (1949), which distinguishes α-amino-acids from other ninhydrin-reacting substances. Only one spot could be detected on papers dusted with $CuCO_3$; it occupied the position taken up by γ-aminobutyric acid on control chromatograms.

Methionine, cystine and leucine isomers. Methionine and the leucine isomers cannot be distinguished on two-dimensional phenol-'collidine' chromatograms as their spots overlap. Complete separation of these amino-acids could not be achieved on one-dimensional chromatograms using either benzyl alcohol or *n*-butanol as solvent (Consden, Gordon & Martin, 1944; Consden, Gordon, Martin, Rosenheim & Synge, 1945). They were readily distinguished, however, on one-dimensional chromatograms run in *tert.*-amyl alcohol (Work, 1949*a*), and the presence of leucine and isoleucine in all of the soil hydrolysates was established by this method. Methionine could not be detected even when the chromatograms were overloaded with the other amino-acids. Attempts to detect methionine by the H_2O_2 technique (Dent, 1947), which reveals sulphur-containing amino-acids, were also unsuccessful; neither the sulphone derived from methionine nor cysteic acid, which normally appear if methionine and cystine are present, could be detected on two-dimensional phenol-'collidine' chromatograms after oxidation with H_2O_2, even when the chromatograms were run with five times the usual quantity of hydrolysate. Before and after H_2O_2 treatment, however, most of the chromatograms showed a weak spot in the position taken up by the sulphoxide derived from methionine. The identity of this spot was confirmed by marker experiments with methionine, which is partly oxidized to this sulphoxide during the phenol run. The behaviour of methionine added to the hydrolysates towards H_2O_2 was found to be very erratic. In some cases it was oxidized to the sulphoxide, in others to the sulphone and occasionally to both. It is presumed that substances present in the hydrolysates interfered with oxidation. Whatever the reason, it was not possible to decide whether this sulphoxide was present as such in the original hydrolysates or was formed from methionine during the phenol run. If methionine was present in the hydrolysates the amount must have been very small. Had cystine been present it would have been revealed by the H_2O_2 technique, since it was readily detected as cysteic acid when added to the hydrolysates. The sensitive spot test for cystine described by Dent & Rose (1949) gave negative results when applied to the hydrolysates, and neither cystine nor methionine could be detected by spraying chromatograms with potassium iodoplatinate (Consden, Gordon & Martin, 1946; Winegard, Toennies & Block, 1948).

Histidine and tyrosine. Both amino-acids, which give weak reactions with ninhydrin, were only barely visible on some of the two-dimensional chromatograms. Their presence in the hydrolysates was readily confirmed, however, by means of one-dimensional chromatograms run in the butanol-acetic acid mixture used by Partridge (1948) for paper partition chromatography of sugars. Histidine and tyrosine are well separated on chromatograms run in this solvent and are readily detected by spraying first with diazotized sulphanilic acid and then with Na_2CO_3 solution (Pauly, 1904). Unsatisfactory results were obtained with one-dimensional chromatograms run in 'collidine' (Dent, 1947), since the paper strips could not be freed from traces of solvent by repeated drying at 100° and washing with benzene or acetone.

α, ε-Diaminopimelic acid. The substance occupying spot 10 (Fig. 1) could at first not be identified with any known ninhydrin-reacting substance. A similar substance was found in

Table 2. *Strengths of spots observed on two-dimensional chromatograms of the soil hydrolysates*

(Strengths of spots are on an arbitrary scale of 10 units. 1 = weak, 10 = strong. The numbers before each amino-acid refer to the spot numbers in Fig. 1. *S* = strong, *M* = medium, *W* = weak.)

Amino-acids Soil no. ...	1	2	3	4	5	6	7	8	9	10
1 Phenylalanine	4	3	4	3	3	3	4	3	3	4
2 Leucine, etc.*	10	10	10	10	9	9	10	10	10	10
3 Valine	9	9	9	9	9	9	9	9	9	9
4 Alanine	10	10	10	9	10	10	10	10	10	10
5 Glycine	9	9	9	8	8	9	9	9	10	9
6 Threonine	6	6	6	4	5	6	7	5	5	7
7 Serine	7	8	7	7	7	8	8	6	6	8
8 Glutamic acid	9	10	9	8	8	8	9	9	3	7
9 Aspartic acid	8	10	9	7	6	6	9	8	2	6
10 α, ε-Diaminopimelic acid	1	1	1	1	1	1	2	1	<1	2
11 Lysine	5	4	4	4	5	4	7	6	8	8
12 Arginine	4	4	5	4	4	4	5	5	5	6
13 β-Alanine	2	2	2	3	3	3	3	3	3	4
14 γ-Aminobutyric acid	2	1	2	2	2	3	2	3	2	3
15 α-Amino-*n*-butyric acid	2	2	<1	<1	1	<1	2	2	1	<1
16 Sulphoxide of methionine	1	1	1	<1	1	—	<1	<1	<1	—
17 Histidine	1	1	<1	1	<1	<1	3	<1	<1	<1
18 Tyrosine	2	1	1	2	2	<1	3	1	<1	<1
19 Proline	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*S*	*M*	*S*
20 Hydroxyproline	*M*	*M*	*W*	*W*	*W*	*W*	*W*	*S*	*W*	*W*
Amino sugar										
21 Glucosamine	1	<1	2	<1	<1	—	1	<1	<1	<1

* A composite spot which may contain methionine and the leucine isomers. An independent method showed that only isoleucine and leucine were present, the relative concentrations of these amino-acids being about 2:3.

alkaline hydrolysates of soil. Its position was unaffected by H_2O_2, indicating that it was not a sulphur-containing amino-acid. It appeared to be an α-amino-acid since it could not be seen on chromatograms dusted with $CuCO_3$ (Crumpler & Dent, 1949). Its position on the two-dimensional phenol-'collidine' chromatogram seemed to be identical with that of an unknown ninhydrin-reacting substance found by Work (1949*a*, *b*) in acid hydrolysates of the water- and ethanol-insoluble portion of *Corynebacterium diphtheriae*. The latter substance has recently been isolated in crystalline form and identified as α, ε-diaminopimelic acid (Work, 1950). When a sample of this new amino-acid, kindly supplied by Dr Elizabeth Work, was added to the soil hydrolysates it ran exactly with the unknown. The results of these marker experiments and of the H_2O_2 and $CuCO_3$ tests, together with the fact that the unknown was stable to prolonged hydrolysis with acid or alkali (5 N-NaOH), leave very little doubt as to the identity of the substance. Further identification by isolation and chemical characterization was not attempted.

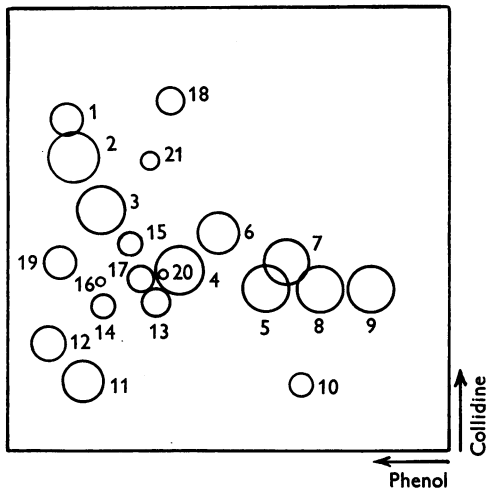

Fig. 1. Diagram of chromatogram obtained from acid hydrolysate of soil no. 7. The sample was placed at the bottom right-hand corner of the filter paper. Phenol was run from right to left followed by 'collidine' in an upward direction. The convention is used of representing the strength of the ninhydrin colour reaction by means of the size of the spot, using an arbitrary scale. A key to the numbers of the spots is given in Table 2.

Alkaline hydrolysis

Since tryptophan is destroyed during acid hydrolysis, alkaline hydrolysates of soil were examined for its presence. Soil samples 3 and 7 were used; before hydrolysis the former was leached with 0·1 N-HCl to remove $CaCO_3$. 5 N-NaOH (7 ml./g. of air-dried soil) was used for hydrolysis, which was carried out in sealed tubes at 100° for 30 hr. The hydrolysis mixtures were filtered, the residues washed thoroughly with hot water and the filtrates neutralized with HCl. From this stage the procedure was as described for acid hydrolysates. Two-dimensional chromatograms of the desalted hydrolysates differed from those of acid hydrolysates in that ornithine was present and serine, threonine, arginine and glucosamine were absent. Tryptophan could not be detected even when the chromatograms were overloaded with the other amino-acids. Judging from the strengths of the spots on the chromatograms, alkaline hydrolysates contained greater amounts of α-amino-*n*-butyric acid than did acid

hydrolysates. Preliminary results of an investigation of the effect of hot 5 N-NaOH on various amino-acids indicate that this is due to the formation of α-amino-*n*-butyric acid from threonine during alkaline hydrolysis.

D-*Amino-acid content of soil hydrolysates*

Acid hydrolysates of soils 3 and 9 were examined for the presence of D-amino-acids. α-Amino N was determined in the hydrolysates by the ninhydrin technique (Van Slyke, Dillon, MacFadyen & Hamilton, 1941) and D-amino-acid N by the method of Lipmann, Behrens, Kabit & Burk (1940), which utilizes the specific D-amino-acid oxidase of Krebs (1935). The enzyme preparation employed was obtained from sheep kidneys (Negelein & Brömel, 1939). The maximum amounts of D-amino-acid N present in acid hydrolysates of soils 3 and 9, calculated from the O_2 uptakes of samples in presence of the enzyme, were 1·4 and 1·7 % of the total α-amino N, respectively.

Free amino-acids in soil

Cold aqueous extracts of several of the soils were concentrated *in vacuo* at 40° and examined by paper partition chromatography. No free amino-acids could be detected. Tests with several amino-acids showed that they were rapidly destroyed when perfused through soil.

DISCUSSION

Since soil organic matter is formed by the action of micro-organisms on the plant and animal residues that find their way into the soil, it will contain, in addition to proteins derived from such residues, a considerable amount of protein material of microbial origin. It is not surprising, therefore, to find that soil hydrolysates contain most of the known naturally occurring amino-acids. Recent work has shown that α-aminobutyric acid, β-alanine and γ-aminobutyric acid are widely distributed in plant, animal and bacterial extracts. αε-Diaminopimelic acid, isolated by Work (1950) from cells of *Corynebacterium diphtheriae*, is probably a constituent of bacterial proteins. A substance with similar properties has been found by the writer in hydrolysates of rotted straw and by other workers in products of bacterial origin (see Work, 1950). The sulphoxide derived from methionine has been detected in various natural mixtures (Dent, 1948; Work, 1949*a*; Gordon, 1949). It is uncertain whether it is present as such in soil or is formed from methionine either during the phenol run or by catalytic oxidation during hydrolysis.

The detection of glucosamine, serine and threonine in the soil hydrolysates confirms previous evidence (Bremner, 1949) that 2-amino sugars and hydroxy-amino-acids occur in soil. Glucosamine probably exists in soil in the form of polysaccharide such as chitin.

Although tryptophan, cystine and methionine could not be detected in the soil hydrolysates, they are perhaps not entirely absent from soil. It may be that all three amino-acids are destroyed in the various secondary reactions which are possible during the hydrolysis of a complex heterogeneous material such as soil. Tryptophan undergoes some destruction whether acid or alkali is used for hydrolysis, and in the presence of carbohydrate its destruction by acid is complete. Small amounts of organic sulphur occur in soil and some of this is presumably in the form of amino-acids such as cystine and methionine.

Since several D-amino-acids have been found in products of microbial metabolism they may be expected to occur in soil. Although D-amino-acids were in fact detected in acid hydrolysates of soil, the amounts found were extremely low and could have arisen by the small degree of racemization of L-amino acids which takes place during acid hydrolysis of proteins.

Under the conditions of acid hydrolysis used in this work purines are decomposed giving rise to glycine (Markham & Smith, 1949). Some of the glycine found in soil hydrolysates is probably derived in this way since nucleic acids, which contain guanine and adenine, are believed to be present in soil (Shorey, 1913; Bottomley, 1919; Wrenshall & Dyer, 1941).

The failure of attempts to detect free amino-acids in the soils studied is in agreement with recent work by Quastel & Scholefield (1949) who found that most amino-acids are rapidly nitrified in soil.

Comparison of the results given in Table 2 shows that the protein materials in different soils do not differ greatly in amino-acid composition. The similarities in the amino-acid distribution of the various soils studied are much more striking than the differences. Indeed the only outstanding difference is that the Russian chernozem (9) has a lower content of aspartic and glutamic acids than the others. The three Rothamsted soils, in spite of their contrasting manurial and cultural histories and pH values, have similar amino-acid distributions, with a little less aspartic acid than the fen and peat soils. The four acid soils have a slightly higher lysine content than the others. Apart from these minor differences the general similarities suggest that, although soil organic matter is formed from a variety of materials under very different conditions, it tends to attain a more or less constant composition with respect to amino-acids, presumably through the activity of soil micro-organisms.

SUMMARY

1. The amino-acid composition of acid hydrolysates of ten different soils has been studied by paper partition chromatography.

2. The following 20 amino-acids were found in every hydrolysate examined; phenylalanine, leucine, isoleucine, valine, alanine, glycine, threonine, serine, aspartic acid, glutamic acid, lysine, arginine, histidine, proline, hydroxyproline, αε-diaminopimelic acid, α-amino-*n*-butyric acid, β-alanine, γ-aminobutyric acid and tyrosine. The sulphoxide derived from methionine and glucosamine were found in most of the hydrolysates.

3. Cystine, methionine and tryptophan could not be detected.

4. D-Amino-acids were detected in acid hydrolysates of soil, but the small amounts found could have arisen by racemization during hydrolysis.

5. No free amino-acids could be detected in any of the soils studied.

6. The results indicate that the protein materials in different soils are similar in their amino-acid composition.

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Studies in Congenital Porphyria

3. THE INCORPORATION OF ^{15}N INTO THE HAEM AND GLYCINE OF HAEMOGLOBIN

By C. H. GRAY, I. M. H. MUIR and A. NEUBERGER
The Department of Chemical Pathology, King's College Hospital, London, and The National Institute for Medical Research, Mill Hill, London

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In the two preceding papers of this series (Gray & Neuberger, 1950; Gray, Neuberger & Sneath, 1950) results were reported concerning the incorporation of ^{15}N into the coproporphyrin, uroporphyrin, stercobilin and hippuric acid of a patient suffering from congenital porphyria who had been fed isotopically labelled glycine. The present paper deals with the isotope content of the haem of the circulating haemoglobin and of the glycine from the globin as well as with haematological and other data concerning the patient. An attempt will be made on the basis of the new facts elicited during these investigations to define more clearly the nature of the metabolic lesion in congenital porphyria.