STUDIES ON SOIL ORGANIC MATTER

PART I. THE CHEMICAL NATURE OF SOIL ORGANIC NITROGEN

By J. M. BREMNER, *Chemistry Department, Rothamsted Experimental Station, Harpenden, Herts*

(With Four Text-figures)

The nitrogenous material of soil is predominantly organic in nature; the inorganic forms of soil nitrogen constitute at the most only 2–3 % of the total-N in soils of temperate regions. It has been assumed that much of the soil organic N is in the form of protein. Part of the soil N is rendered soluble by acid hydrolysis, and various workers have shown that this soluble-N can be separated into the classes recognized for protein hydrolysates, i.e. (1) ammonia and amide nitrogen, (2) basic (diamino)-N, (3) non-basic (monoamino)-N, and (4) humin-N. Shorey (1906), Jodidi (1911, 1912), Lathrop & Brown (1911), Schmuk (1914) and Kelley & Thompson (1914*a*, *b*) fractionated the N in soil hydrolysates by the Osborne-Harris (1903) modification of the Hausmann (1899) method of protein analysis, and Potter & Snyder (1915), Lathrop (1916) and Morrow & Gortner (1917) used the Van Slyke (1911–12, 1915) method. Although there is a superficial similarity between the N distributions in soil and protein hydrolysates, the only valid conclusion is that acid hydrolysates of soil contain ammonia and amino compounds which yield N_2 on treatment with nitrous acid at room temperature. Several amino-acids have been isolated from soil; leucine and iso-leucine (Robinson, 1911), arginine and histidine (Schreiner & Shorey, 1910*a*) and lysine (Shorey, 1913). Suzuki (1906–8) isolated alanine, leucine, aspartic acid and other amino-acids from the acid hydrolysate of a 'humic acid' preparation by Fischer's esterification method. Various amines (Shorey, 1912, 1913), purine bases (Schreiner & Shorey, 1910*b*), pyrimidine and pyridine derivatives (Shorey, 1906; Schreiner & Shorey, 1910*c*) and other nitrogenous organic compounds have also been isolated from soil, but the yields obtained have been too low to have much significance.

The main purpose of the present investigation was to determine what proportion of the organic-N of soil is in the form of protein. Direct extraction of the protein material did not appear practicable since there is evidence (Hobson & Page, 1932*a*, *b*, *c*) that the organic-N of soil is firmly attached to other fractions of soil organic matter. The alternative method, namely quantitative determination of the products formed on hydrolysis of the soil proteins, is complicated by the fact that soil organic matter contains nitrogenous complexes which are not protein in nature. It cannot be assumed, therefore, that the nitrogenous compounds found in soil hydrolysates are derived exclusively from protein material. Moreover, as Morrow & Gortner (1917) have pointed out, the presence of soil minerals and carbohydrates during the acid hydrolysis of a protein considerably alters the nitrogen distribution figures obtained. A minimal estimate of the protein content of soil can, however, be obtained by this method, since accurate and specific methods are at hand for the determination of the principal products of protein hydrolysis, i.e. amino-acids. The conditions of hydrolysis required for maximum liberation of amino-acids from soil were therefore studied, amino-acids being determined by the method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) from the CO_2 liberated on heating under specified conditions with ninhydrin. This method is very accurate and, unlike the nitrous acid method (Van Slyke, 1929), is specific for free amino-acids in that it requires the presence, in the free unconjugated state, of both the carboxyl and the neighbouring NH_2 or $NH\text{-}CH_2$ group.

Subsequent to the completion of the work described here, Kojima (1947*a*) reported the results of an investigation in which the ninhydrin method was used to establish the α-amino-acid character of products of hydrolysis of a highly organic muck soil. The conditions for maximum liberation of amino-acids were not determined. In the main her findings are in agreement with those of the present work, which gives the distribution of almost the whole of the N in six kinds of soil and also establishes the presence in soil hydrolysates of amino sugars and the absence of volatile bases other than NH_3. Kojima (1947*b*) later isolated several amino-acids in good yield from the soil used in her previous work.

EXPERIMENTAL

The soils listed below were sampled to 9 in., air-dried at room temperature and ground to pass a 2 mm. sieve.

The Hoosfield, Barnfield and Broadbalk samples were clay loams over clay-with-flints from the classical fields at Rothamsted. The plot from which

the Hoosfield sample was taken had lain fallow for 3 years; it had not been manured since 1851. The Barnfield and Broadbalk samples were from plots

Soil	pH	N content (as % of oven-dry soil)
Hoosfield	7·5	0·10
Barnfield	7·6	0·27
Broadbalk	7·7	0·25
Allotment	6·6	0·49
Littleport	5·6	1·50
Swaffham	7·3	2·38

that had received farmyard manure annually for a long period. The Allotment soil had been cultivated with large additions of organic manure for nearly 100 years; it adjoins Barnfield. The Littleport and Swaffham samples were from the Cambridgeshire fen district; the former was an acid sandy peat, the latter a typical neutral fen soil.

Hydrolysis of the soils

A preliminary investigation showed that 6 N-HCl or 8 N-H_2SO_4 used in the proportion 3 ml. acid/g. of soil gave maximal extraction of N. 6 N-HCl (3 ml./g. of air-dried soil) was used for hydrolysis, which was conducted under reflux at the boiling-point of the soil/acid mixture. A few drops of caprylic alcohol were added to prevent foaming and the mixture was shaken at intervals; it boiled smoothly and bumping was experienced only when hydrolysis was prolonged beyond about 48 hr. At the end of the hydrolysis period the mixture was cooled, filtered through a Buchner funnel with suction, and the residual soil washed thoroughly with distilled water. The filtrate and washings were combined and the excess acid removed in the usual way by repeated evaporation to a syrup *in vacuo*. The residue was taken up with water, made to volume, and aliquots were taken for total-N determinations.

Fractionation of the nitrogen in the soil hydrolysate

The N in the soil hydrolysate was fractionated by the method of Van Slyke (1911–12, 1915). The separation into basic and non-basic N was generally omitted, however, and the basic units were not determined. Thus the following fractions were distinguished by the method of separation employed:

(1) Insoluble-N.
(2) Soluble-N:
  (*a*) Ammonia-N;
  (*b*) Humin-N (N carried down with CaO precipitate);
  (*c*) Nitrogen in filtrate from humin precipitate (amino-N, α-amino-N, non-amino-N).

The NH_3 in the hydrolysate was removed by distillation with calcium hydroxide *in vacuo* at 40°; it was usually necessary to distil for 1 hr. to ensure complete removal. The NH_3 liberated was collected in N/70 or N/10 HCl and determined by back titration with standard alkali, methyl red masked by methylene blue being used as indicator. The residue from the distillation was filtered and the precipitate washed thoroughly with warm distilled water. The N present in the precipitate (humin-N) was then determined. The filtrate from the humin precipitate was neutralized with HCl and evaporated *in vacuo* to small volume. Total-N, amino-N and α-amino-N were determined on aliquots. In certain experiments the N in the filtrate from the humin precipitate was separated into basic and non-basic fractions by means of phosphotungstic acid, using the conditions recommended by Van Slyke, Hiller & MacFadyen (1941).

Alkaline hydrolysis

For comparison with acid hydrolysis, the effect of hydrolysing soils with alkali and with alkali under reducing conditions was also studied. 5N-NaOH was used for plain alkali hydrolysis and alkali-stannite (5 g. of $SnCl_2.2H_2O$/100 ml. of 5·5N-NaOH) for hydrolysis under reducing conditions (Lugg, 1938). The alkali digestions were carried out in soft glass test-tubes which were drawn out after addition of the soil samples. Interstitial air was then removed at the pump and the reagent admitted at low pressure (20 ml. of alkali or alkali-stannite/3 g. of soil). The tubes were sealed, leaving an air space of 1–2 ml. and immersed in boiling-water for 30 hr. The vessels were shaken vigorously during the early stages of hydrolysis to ensure that no solid was left out of contact with the reagent. At the end of the hydrolysis period the tubes were removed from the bath, cooled, and opened under 6N-HCl to avoid loss of NH_3. The acidified hydrolysis mixtures were filtered and the insoluble residues washed thoroughly with distilled water. The N contents of the residues and the total-N and α-amino-N contents of the filtrates were then determined.

To compare the N distributions in alkaline and acid hydrolysates of soil, 10 g. samples of the Swaffham soil were hydrolysed under reflux with 100 ml. of 5N-NaOH and 6N-HCl for 12 hr. The alkaline hydrolysis was carried out in an all-glass apparatus, and precautions were taken to ensure that NH_3 was not lost during the hydrolysis. At the end of the hydrolysis period the alkaline hydrolysis mixture was cooled and acidified with HCl. The N in both hydrolysates was fractionated by the method described above.

Analyses

Nitrogen was determined by a micro-Kjeldahl procedure using 1 : 1 : 8 SeO_2:$CuSO_4$:K_2SO_4 catalyst. Markham's (1942) microdistillation apparatus was used, NH_3 being collected in 2% (w/v) boric acid containing 25 ml./l. of Conway & O'Malley's (1942)

indicator. After about 15 ml. of distillate had been collected the contents of the receiver were titrated in a stream of CO_2-free air with N/140 H_2SO_4 from a 1·0 ml. burette. Frequent blank determinations on the reagents and control experiments with a standard (N/140) solution of $(NH_4)_2SO_4$ were carried out.

Amino-N was determined by the Van Slyke (1929) manometric nitrous acid method with 30 min. reaction time. Only 4 min. were allowed for deamination when amino-N was determined in the non-basic fraction of the humin-filtrate-N.

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

Periodate ammonia-N was determined by the method of Van Slyke, Hiller & MacFadyen (1941). Ammonia and humin were removed from the soil hydrolysates before analysis. The periodate-NH_3 was aerated (for 30 min.) into excess N/70 HCl and determined by back titration of the excess acid with standard alkali. The recoveries obtained with serine were 99 ± 1 %.

RESULTS

Individual fractions

(1) *Insoluble*-N

As Table 1 shows, a considerable fraction (13·2–31·8 %) of the total soil-N was not dissolved when the soils were boiled with 6N-HCl for 48 hr.; this fraction was not decreased significantly by hydrolysing with acid under reducing conditions. Although generally smaller, the quantity of insoluble-N was also considerable when hydrolysis was carried out with alkali alone or with alkali under reducing conditions (Table 2). Alkali hydrolysates were more strongly coloured than acid or alkali-stannite hydrolysates.

Table 1. *Nitrogen dissolved on hydrolysis of soils with* 6N-HCl *for* 48 *hr.*

Soil	N dissolved (% total soil-N)
Hoosfield	86·8
Barnfield	78·1
Broadbalk	76·3
Allotment	68·2 (69·7*)
Littleport	70·5 (72·2*)
Swaffham	68·8

* 10 g. of soil hydrolysed with 60 ml. of 6N-HCl plus 5 g. granulated tin.

Schmuk (1914) and Morrow & Gortner (1917) found that a dark-coloured material formed on the condenser walls during the hydrolysis of soils with acid under reflux, and Shorey (1930) has reported that this material contains N. The soils used in this work gave similar deposits on hydrolysis, and sufficient material for analysis (0·5 g.) was obtained by hydrolysing 100 g. of the highly organic Littleport soil with 300 ml. of 6N-HCl for 24 hr. The black film on the condenser was removed, washed thoroughly with water and dried *in vacuo*. The dried product was in the form of very thin leaflets and contained 56 % C, 0·3 % N and 14 % ash. The high ash content indicates that the condenser deposit was contaminated by creeping during the hydrolysis, since it is unlikely that a product of such high ash content would be volatile. No condenser deposits were formed when the soils were hydrolysed under reflux with alkali (5N-NaOH).

Table 2. *Nitrogen dissolved on hydrolysis of soils with alkali and with alkali under reducing conditions*

A, 5N-NaOH; B, alkali-stannite. Hydrolyses conducted in sealed tubes at 100° for 30 hr.

Soil	N dissolved (% total soil-N) A	B
Hoosfield	78·4	79·9
Allotment	68·4	73·7
Littleport	74·1	77·4
Swaffham	78·6	82·3

(2) *Soluble*-N

The major fraction of the organic-N was found to pass into solution very quickly when the soils were refluxed with 6N-HCl. The quantity dissolved was not increased significantly by prolonging the acid hydrolysis beyond about 12 hr. The rate of extraction of N from three of the soils is shown in Fig. 1.

Striking differences were observed between the colours of the solutions obtained by hydrolysing soil with acid for increasing periods of time, the colour becoming progressively lighter. This appeared to be due to precipitation of a finely divided dark brown material on prolonged boiling. For instance, the solution obtained by hydrolysing 20 g. of Littleport soil with 60 ml. of 6N-HCl for 1 hr. was dark red in colour. When this solution was refluxed alone a progressive precipitation of what may have been acid-insoluble humin of protein origin occurred with fading of the colour of the solution. Solutions obtained by hydrolysing the soils with 6N-HCl for 48 hr. were not dark brown, the usual colour of a protein hydrolysate, but pale yellow in colour.

(*a*) *Ammonia*-N. As Shorey (1930) has pointed out in his discussion of the results of acid hydrolysis of soil, 'there is little doubt that some ammonia is formed by the hydrolysis, but it is still a question whether or not some of the ammonia may not be formed from some as yet unrecognised constituent of the acid solution by the magnesium or calcium oxide used to drive off the ammonia'. It is also possible that soil hydrolysates contain volatile amines which are distilled with, and estimated as, ammonia in the procedures normally employed for the determination of ammonia. To decide these points, volatile base and NH_3 estimations were

made on various soil hydrolysates, and the results obtained by different methods of determining ammonia were compared. Pucher, Vickery & Leavenworth (1935) developed a method of estimating NH_3 which involves distillation *in vacuo* at 40° with a borax-NaOH-phosphate buffer solution. Under these conditions there is minimal interference in the NH_3 estimation by other substances such as urea, glutamine, allantoin and asparagine, which are easily decomposed with the production of NH_3. This method was used for the estimation of NH_3 in soil hydrolysates, distillations being conducted at pH 9·7. The results obtained by this method and by the customary procedures involving distillation with CaO or MgO are given in Table 3. Volatile base estimations were made by exhaustively distilling samples of hydrolysate, made alkaline with MgO, CaO or pH 9·7 buffer, into standard acid (N/70 HCl) and back titrating the excess acid with standard alkali (N/70 NaOH); methyl red masked by methylene blue was used as indicator. Distillations were conducted *in vacuo* at low temperature (40°) in an all-glass apparatus similar to that described by Pucher *et al.* (1935). NH_3 estimations were made by nesslerizing volatile base distillates and comparing with Nesslerized NH_3 solutions. The tints appeared to be identical with those given by NH_3. In the results presented in Table 3 volatile base is assumed to represent NH_3 and substituted NH_3.

Table 3. *Amounts of volatile base-*N *and ammonia-*N *(expressed as percentages of the total soil-*N*) present in hydrolysate of the Littleport soil after various periods of hydrolysis*

A, distilled with MgO; B, distilled with pH 9·7 buffer; C, distilled with CaO.

		Period of hydrolysis (hr.)				
		1	8	12	48	92
A	Volatile base-N (%)	9·03	13·33	14·84	20·65	22·19
A	Ammonia-N (%)	—	13·29	14·70	20·61	22·09
B	Volatile base-N (%)	—	13·20	14·71	—	21·88
B	Ammonia-N (%)	—	13·10	14·67	—	21·90
C	Volatile base-N (%)	9·0	13·28	—	—	22·2
C	Ammonia-N (%)	—	13·21	—	—	22·0

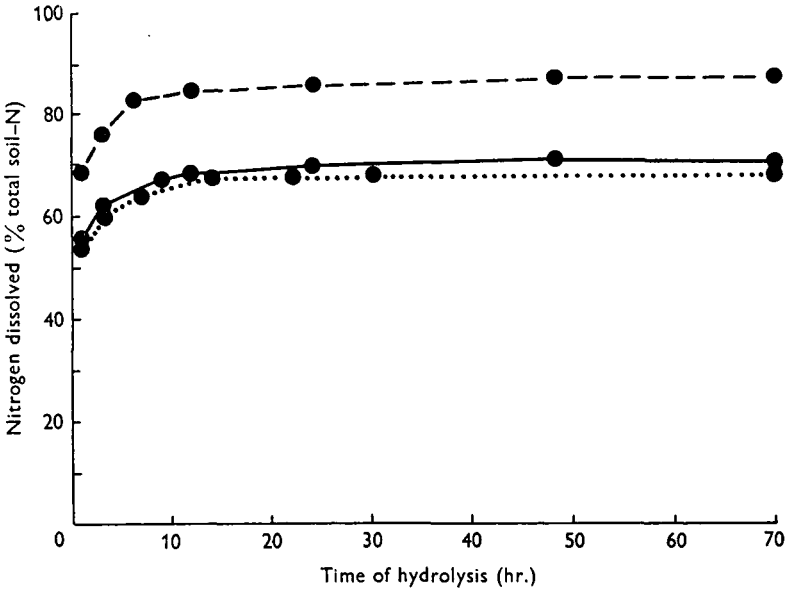

Fig. 1. Variation with time of the amount of N dissolved by acid hydrolysis of soil. ●– – –●, Hoosfield soil; ●—●, Littleport soil; ●· · · ·●, Allotment soil.

The effect of time on the quantity of NH_3 liberated by acid hydrolysis of the Hoosfield, Allotment and Littleport soils is shown in Fig. 2.

(*b*) *Humin*-N. Practically all the colour was removed from the soil hydrolysates by the humin precipitates. Since the colour of the hydrolysate was dependent upon the time of hydrolysis, the colour of the humin precipitate was also affected by this factor. Thus the solution obtained by hydrolysing 20 g. of the Swaffham soil with 60 ml. of 6N-HCl for 2 hr. gave a humin precipitate which was dark red in colour, whereas that obtained by a similar hydrolysis for 48 hr. yielded a pale yellow precipitate. The humin-N fraction decreased as hydrolysis was continued (Table 4). The results given in Fig. 3 indicate that this decrease is due, at least partly, to formation of NH_3.

(*c*) *Nitrogen in filtrate from humin precipitate.* The percentage of the total soil-N present in this fraction was found to vary with the time of hydrolysis; it

reached a maximum in about 12 hr. and then decreased slowly (Table 5). As Fig. 3 shows, this slow decrease is due to the steady production of NH_3 from the fraction. At least some of this NH_3 is produced by deamination of amino-acids. Fig. 4 presents graphically the results obtained by determining the amounts of α-amino-N present in hydrolysates of the Hoosfield and Littleport soils after various periods of hydrolysis. The quantity of α-amino-N liberated on acid hydrolysis reached a maximum in about 12 hr. and then decreased slowly, indicating that amino-acids were destroyed on prolonged hydrolysis. Table 6 gives the amounts of α-amino-N liberated on hydrolysis of the soils with 6N-HCl for 12, 24 and 48 hr. The α-amino-N contents of the filtrates from the humin precipitates were determined from the CO_2 evolved when 1 ml. samples of filtrate were heated with 50 mg. of ninhydrin in the presence of 50 mg. of pH 2·5 citrate buffer. The same results were obtained with pH 4·7 citrate buffer and at pH 1·0 (0·2 ml. of 6M-H_3PO_4). The quantity of CO_2 evolved on boiling 1 ml. of filtrate with citrate buffer in the absence of ninhydrin did not differ significantly from that obtained in the reagent blank determination.

Table 4. *Amounts of humin-*N *(expressed as percentages of the total soil-*N*) present in soil hydrolysates after various periods of hydrolysis*

Soil	Period of hydrolysis (hr.) 3	12	24	48
Hoosfield	20·2	17·8	16·0	13·6
Allotment	—	9·4	7·3	5·5
Littleport	8·6	7·5	7·4	7·3
Swaffham	—	6·0	4·7	—

Table 5. *Amounts of humin filtrate-*N *(expressed as percentages of the total soil-*N*) present in soil hydrolysates after various periods of hydrolysis*

Soil	Period of hydrolysis (hr.) 1	3	6	12	24	48	70
Hoosfield	32·1	39·2	44·6	45·3	44·7	43·9	—
Allotment	39·3	43·8	42·6	42·5	—	41·2	39·1
Littleport	—	43·2	—	45·5	43·9	42·6	—
Swaffham	—	—	—	50·1	—	45·1	—

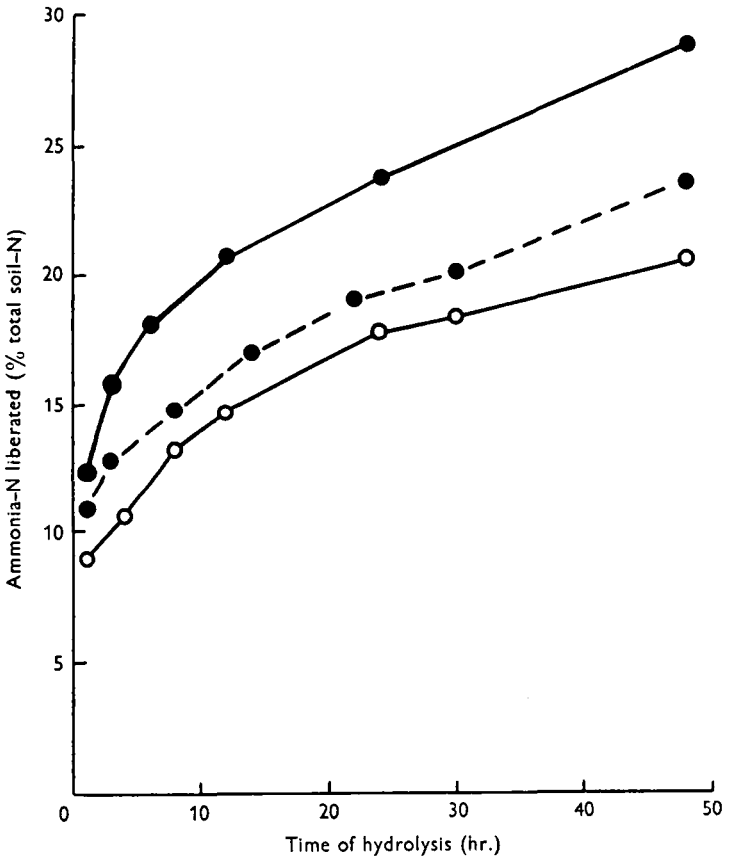

Fig. 2. Variation with time of the amount of NH_3 liberated by acid hydrolysis of soil. ●—●, Hoosfield soil; ○—○, Littleport soil; ●- - -●, Allotment soil.

Table 6. *Amounts of α-amino-N (expressed as percentages of the total soil-N) present in soil hydrolysates after various periods of hydrolysis*

Soil	Period of hydrolysis (hr.) 12	24	48
Hoosfield	24·2	23·9	23·8
Barnfield	30·1	—	29·6
Broadbalk	34·1	—	—
Allotment	31·3	30·9	30·1
Littleport	32·6	32·3	31·8
Swaffham	37·1	36·4	—

at pH 2·5, and a yellow colour which changed to red at pH 1·0. Similar amounts of α-amino-N were liberated by hydrolysing the soils with 5N-NaOH (Table 7).

After removal of NH_3 and humin from hydrolysates of the Swaffham and Littleport soils, the residual N was separated into basic and non-basic fractions by the phosphotungstic acid method; the amino-N and α-amino-N contents of the fractions were then determined. The results are given in Table 8, which also shows the amounts of NH_3 liberated when samples of the humin filtrates were

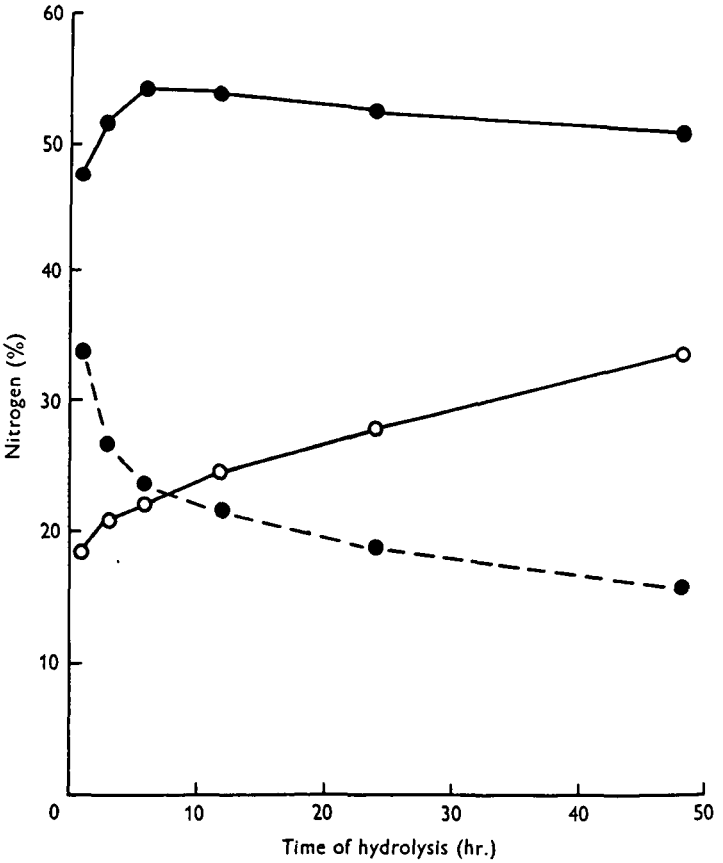

Fig. 3. Variation with time of the N distribution in the acid hydrolysate of Hoosfield soil. Results expressed as percentages of soil-N dissolved by acid. ●—●, humin filtrate-N; ○—○, ammonia-N; ●– – –●, humin-N.

Table 7. *Amounts of α-amino-N liberated by hydrolysis of soils with* 5N-NaOH

Hydrolyses conducted in sealed tubes at 100° for 30 hr.

Soil	α-Amino-N liberated (% total soil-N)
Hoosfield	23·1
Allotment	32·4
Littleport	32·9
Swaffham	34·0

Characteristic amino-acid colour reactions were observed during the ninhydrin-CO_2 determinations. Thus a blue colour developed at pH 4·7, a red colour treated with alkaline periodate. In the Swaffham soil hydrolysate the basic amino-N was 16·8 % of the total amino-N, and the basic α-amino-N constituted 13·6 % of the total α-amino-N; the corresponding figures for the hydrolysate of the Littleport soil were 17·6 and 9·9 %.

Comparison of the nitrogen distributions in alkaline and acid hydrolysates of soil

The results obtained by determining the N distributions in alkaline and acid hydrolysates of the Swaffham soil are given in Table 9. Tryptophane

Table 8. *Nitrogen distributions in acid hydrolysates of the Swaffham and Littleport soils*

A, Swaffham soil; B, Littleport soil. Soils hydrolysed with 6N-HCl for 24 hr.

	Percentage of			
	Total-N		Acid-soluble-N	
Fraction	A	B	A	B
Soluble-N	68·1	69·4	100	100
Ammonia-N	17·9	17·8	26·3	25·6
Humin-N	4·7	7·4	6·9	10·7
Humin filtrate-N	45·1	43·9	66·2	63·3
Basic-N	11·6	11·7	17·0	16·9
Non-basic-N	33·4	32·2	49·0	46·4
Total amino-N	36·4	34·0	53·5	49·0
Basic amino-N	6·1	6·0	9·0	8·6
Non-basic amino-N	30·3	28·0	44·5	40·3
Total α-amino-N	36·1	32·3	53·0	46·5
Basic α-amino-N	5·0	3·2	7·2	4·6
Non-basic α-amino-N	31·1	29·1	45·7	41·9
Periodate NH_3-N	7·2	7·0	10·6	10·1

amino-sugar colour tests which appear to be specific (Meyer, 1945). Thus red colorations developed when solutions obtained by acid hydrolysis of the soils,

Table 9. *Nitrogen distributions in alkaline and acid hydrolysates of the Swaffham soil*

A, 6N-HCl hydrolysis; B, 5N-NaOH hydrolysis. Hydrolyses conducted under reflux for 12 hr.

	Percentage total soil-N	
Fraction	A	B
Soluble-N	69·5	73·8
Ammonia-N	13·2	23·7
Humin-N	6·0	6·2
Humin filtrate-N	50·1	44·1
Amino-N	37·3	31·6
α-Amino-N	37·1	33·1

and of the humic and fulvic fractions of neutral 0·1M-sodium pyrophosphate extracts of the soils, were made alkaline, warmed with acetylacetone

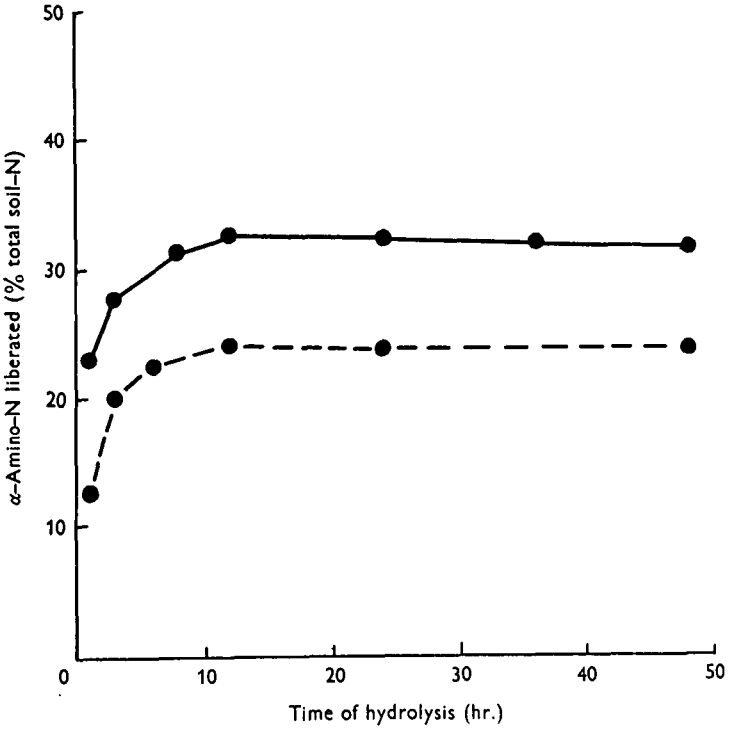

Fig. 4. Variation with time of the amount of α-amino-N liberated by acid hydrolysis of soil. ●—●, Littleport soil; ●- - -●, Hoosfield soil.

could not be detected in either alkaline or acid hydrolysates of this soil, or of any of the soils examined. Traces of tyrosine were, however, present in all hydrolysates.

The percentages of the Hoosfield, Littleport and Swaffham soils dissolved after various periods of hydrolysis with 6N-HCl are given in Table 10.

Indirect evidence for the presence of amino sugars was obtained by applying to soil products

Table 10. *Percentages of soils dissolved on hydrolysis with* 6N-HCl

	Period of hydrolysis (hr.)						
Soil	1	3	6	12	24	48	92
Hoosfield	13·2	14·8	16·1	16·2	16·4	17·0	—
Littleport	—	40·0	—	42·5	44·0	44·5	45·0
Swaffham	—	—	—	52·2	53·5	—	—

and treated with Ehrlich's reagent (Pauly & Ludwig, 1922). This colour test for 2-amino sugars cannot be used when certain pyrrole or indole derivatives are present in the test solution, but these substances are readily detected since they condense with Ehrlich's reagent in acid solution to yield coloured solutions without previous heating with the acetylacetone reagent. Such substances were not present in the soil hydrolysates but the presence of ferric salts caused the development of a faint orange colour in the test solutions on addition of the acetylacetone reagent; the ferric ion forms a coloured (yellow to orange-red) co-ordination complex with acetylacetone (Mellan, 1941). Of the total-N of the soils examined 2–6 % was in the form of 2-amino sugar as determined by the method of Palmer, Smyth & Meyer (1937). These figures cannot be regarded as accurate since the extent to which iron interfered in the estimations could not be assessed. They indicate, however, that only a small fraction of the total soil-N is in the form of 2-amino sugar material (e.g. chitin).

The Müller (1901) colour test for acetylated 2-amino sugars was given by the colourless solutions obtained by treating the fulvic (acid-soluble) fractions of pyrophosphate and alkali extracts of the soils with charcoal at pH 2 and filtering. This indicates that soils contain acetylated 2-amino sugars which are not in the form of chitin, since chitin is insoluble in pyrophosphate and alkali. The Müller colour test was also given by the acetylated products of acid hydrolysis of the soils and 2–4 % of the total-N of the soils examined was apparently in the form of 2-amino sugar as determined by the method of Zuckerkandl & Messiner-Klebermass (1931) which is based on the Müller reaction. This method is, however, inaccurate and it is possible that a greater fraction of the organic-N of soil is in the form of other amino sugars.

DISCUSSION

(1) *Insoluble nitrogen*

The amount of acid-insoluble (humin)-N formed when proteins are hydrolysed with acid rarely exceeds a few per cent, but is increased greatly if hydrolysis is carried out in the presence of carbohydrates. This is believed to be due to condensation of the furfural and amino-acids, particularly tryptophane, liberated during the hydrolysis. Although soil organic matter is known to contain considerable amounts of carbohydrates, such as pentosans and polyuronides, which yield furfural on acid hydrolysis, it does not appear likely that much of the insoluble soil-N found after acid hydrolysis is derived from protein material by secondary reactions, for the following reasons:

(i) The amount of acid-insoluble-N was not significantly decreased when acid hydrolysis was carried out under reducing conditions (Table 1). According to Hlaziwetz & Habermann (1873) humin formation during protein hydrolysis is decreased by the addition of tin (or $SnCl_2$) to the hydrolysis mixture.

(ii) The amount of insoluble-N was not greatly different when hydrolysis was carried out with alkali, or with alkali under reducing conditions (Table 2). Practically no insoluble-N is formed when proteins are hydrolysed with alkali or alkali-stannite (Lugg, 1938).

(iii) Tryptophane, which is not destroyed by alkali hydrolysis, could not be detected in alkaline hydrolysates of the soils. It is unlikely, therefore, that a significant fraction of the insoluble-N found after acid hydrolysis is in the form of tryptophane-carbohydrate condensation products.

(2) *Soluble nitrogen*

Morrow & Gortner (1917) found that 66·3–77·65 % of the total N of soils of different type was extracted when the soils were hydrolysed with 6N-HCl for 48 hr. The results obtained in the present investigation (Table 1) are not significantly different; the only notable exception is with the Hoosfield soil.

(*a*) *Ammonia nitrogen.* The amount of ammonia formed by acid hydrolysis of soil depends very largely on the time of hydrolysis; NH_3 is produced rapidly at first and then slowly over a long period (Fig. 2). It is difficult to decide what proportion of the NH_3 liberated is derived from the amide linkages of protein material. The methods that have been employed by Shore, Wilson & Stueck (1936) and others to determine the amide-N of proteins are not likely to yield reliable figures if applied to soil, since the NH_3 found in soil hydrolysates is probably not derived exclusively from protein material by deamidation and deamination reactions. Deamination reactions undoubtedly take place during hydrolysis (Fig. 4 and Table 6). The possibility that such reactions are accelerated by inorganic material dissolved from soil during acid hydrolysis seems to be excluded by the work of Morrow & Gortner (1917), who showed that the figure obtained for amide-N in a protein analysis is not appreciably changed if the protein is hydrolysed in the presence of twenty times its weight of ignited mineral soil.

The presence of hydroxyamino-acids in soil proteins is indicated by the fact that NH_3 is liberated when soil hydrolysates are treated with alkaline periodate (Table 8). Some of the NH_3 produced by acid hydrolysis of soil may, therefore, be derived from serine and threonine, since both of these hydroxyamino-acids undergo a progressive decomposition linear with time during acid hydrolysis (Rees, 1946).

A considerable fraction of the ammonia found in soil hydrolysates is probably produced by degrada-

tion of non-protein compounds during acid hydrolysis. Purine compounds are rapidly destroyed when boiled with strong acid (Graff & Maculla, 1935); for example, Gortner (1913) found that 15·27 % of the N of uric acid was converted to NH_3 by this treatment. It is not unlikely, therefore, that some of the NH_3 found in soil hydrolysates is derived from purine (and pyrimidine) bases liberated by acid hydrolysis of nucleic acids; it has been reported that such acids are present in soil (Shorey, 1913; Bottomley, 1919; Wrenshall & Dyer, 1941). Moreover, soil organic matter probably contains mucopolysaccharide or mucoprotein material of fungal or bacterial origin, and some of the NH_3 formed on acid hydrolysis of soil may be derived from the amino sugars in such material. The 2-amino sugars are extremely stable towards acid hydrolysis, but the amino sugars so far investigated which do not have the amino group in position 2 are relatively unstable (Fischer & Zach, 1911), and some of the bacterial amino sugars are known to yield NH_3 on hydrolysis.

It has been reported that certain amines (e.g. methylamine) give, with Nessler's reagent, coloured substances which are soluble in KI solutions and of different tints from that obtained with NH_3 (Charitschkov, 1906; Lugg, 1946). The presence in a soil hydrolysate of a significant amount of a volatile amine would probably be disclosed, therefore, by a departure from the normal tint on nesslerization of a volatile base distillate or by significant differences between the estimations of volatile base and ammonia. Since no such variations were observed (Table 3) it is evident that volatile amines could have been present only in traces in the soil hydrolysates. Moreover, the close agreement of the results obtained by the three different methods of estimating NH_3 indicates that it is most unlikely that NH_3 is formed from some as yet unrecognized constituent of the soil hydrolysate by the magnesium or calcium oxide used to drive off the ammonia.

(*b*) *Humin nitrogen.* Most protein preparations when boiled with acid yield a finely divided dark precipitate ('acid-insoluble humin') and a dark brown solution containing 'acid-soluble humin', which is largely precipitated as a dark-coloured material on partial neutralization of the solution. By analogy, the N in the precipitate formed on making a soil hydrolysate alkaline with CaO or MgO has been classified by most workers as 'humin-N'. The use of the term 'humin-N' in this respect is unfortunate since it implies that the N in this fraction of soil hydrolysates consists entirely of acid-soluble humin-N of protein origin, which is certainly not the case. Solutions obtained by acid hydrolysis of soil are not dark brown in colour and the precipitates formed on treating them with CaO or MgO do not contain any black material. It is unlikely, therefore, that the 'humin' fraction of soil hydrolysates contains significant amounts of true acid-soluble humin of protein origin.

(*c*) *Nitrogen in filtrate from humin precipitate.* As can be seen from Fig. 4 and Table 6 maximum liberation of α-amino-N from the soils was achieved by hydrolysing with 6 N-HCl for about 12 hr. In this period 24–37 % of the total-N of the soils was released as α-amino-N. Kojima (1947*a*) found that about 37 % of the N in the muck soil which she examined was liberated as α-amino-N by acid hydrolysis. The only compounds other than amino-acids which are known to yield CO_2 under the conditions employed for the determination of amino-acids with ninhydrin are urea, ascorbic acid and keto acids such as pyruvic or aceto-acetic. Such substances are not likely to occur in soil, but if present they would be destroyed during acid hydrolysis and would not interfere with the determination of α-amino-N in soil hydrolysates. Tests showed that the solutions used for ninhydrin-CO_2 determinations did not contain unstable organic compounds that decomposed on boiling with production of CO_2.

Since proteins are the only known class of natural compounds that yield amino-acids as the principal products of hydrolysis, the results given in Table 6 establish a *prima facie* case for the presence of protein-like material in soil, and indicate that approximately one-third of the total-N of the soils examined is in the form of such material. This figure must be regarded as minimal since destruction of amino-acids undoubtedly takes place during hydrolysis. Moreover, soil hydrolysates contain large quantities of NH_3 and some of this may be derived from the acid-amide (asparagine and glutamine) residues of protein material. If it were assumed that all of the NH_3 found in soil hydrolysates is formed from such residues it could be deduced from α-amino-N and ammonia-N determinations (Table 6 and Fig. 2) that as much as 45–50 % of the total-N of the soils examined is in the form of protein.

Two points of interest emerge from a comparison of the results obtained when the amino-N contents of the basic and non-basic fractions of soil hydrolysates were determined by the nitrous acid and ninhydrin methods (Table 8). In the basic fractions the figures obtained by the nitrous acid method were considerably higher than those obtained by the ninhydrin method, indicating that the basic fractions of soil hydrolysates contain amino compounds other than amino-acids. On the other hand, in the non-basic fractions the figures given by the ninhydrin method were slightly greater than those obtained by the nitrous acid method (see also Table 9). This discrepancy is probably due to the presence of aspartic acid, since it alone of the usual amino-acids yields 2 moles of CO_2 when heated with ninhydrin. The aspartic acid content of the non-basic fractions cannot be determined from the difference between

amino-N and α-amino-N figures since there is no evidence to suggest that the discrepancy between the figures is due entirely to the presence of this amino-acid.

The results of periodate oxidation (Table 8) show that approximately 7% of the total-N of the Swaffham and Littleport soils was in the form of hydroxyamino-acids (serine, threonine, hydroxylysine, etc.) or of other compounds containing the .CH(OH).CH(NH_2). group. The only compounds other than hydroxyamino-acids which have been tested and found to yield NH_3 on treatment with alkaline periodate are ethanolamine and glucosamine. If present in soil hydrolysates, ethanolamine would be removed during the distillation of ammonia and would not appear in the filtrate from the humin precipitate. Tests showed that 2-amino sugars were present in the soil hydrolysates. Some of the NH_3 liberated by periodate may, therefore, be derived from such material.

More NH_3 is produced by alkali than by acid hydrolysis, but the N distributions of alkaline and acid hydrolysates of soil are not greatly different (Table 9).

The quantitative determination of the individual amino-acids present in direct soil hydrolysates is likely to prove extremely difficult. As much as 50% of the soil is dissolved by acid hydrolysis (Table 10); the presence of so much extraneous inorganic and organic material in the soil hydrolysates makes it extremely difficult to apply any of the newer methods of amino-acid analysis.

The results reported here establish the protein nature of about one-third of the organic-N of soil, but the chemical condition of the rest of the organic-N remains obscure. The resistance of a significant fraction of the soil N to acid or alkali hydrolysis suggests that part of the organic-N is in the form of heterocyclic N compounds. The indications are that some amino sugar and nucleic acid materials are present in soil, but the exact amounts have yet to be determined.

Considering the incompleteness of present knowledge of the forms in which the organic-N of soil exists, it is difficult to find any justification for the practice of multiplying the total-N of a soil by 6·25 and returning the result as 'protein'.

SUMMARY

1. The acid hydrolysis of six soils with nitrogen contents ranging from 0·1 to 2·38% has been studied by determining the amounts of ammonia-, humin- and α-amino-N present in the soil hydrolysates after various periods of hydrolysis.

2. Under the conditions of hydrolysis employed (3 ml. of 6N-HCl/g. soil) the period required for maximum liberation of amino-acids from the soils was about 12 hr. 24·2–37·1% of the total-nitrogen of the soils examined was liberated as α-amino-N in this period. Further hydrolysis led to destruction of amino-acids. Similar amounts of α-amino-N were liberated by hydrolysis of the soils with alkali (5N-NaOH).

3. From 69 to 87% of the total-nitrogen of the soils was brought into solution by acid hydrolysis; the amount dissolved by hydrolysing with alkali or with alkali under reducing conditions (alkali-stannite) was not significantly different. It is concluded that most of the insoluble-nitrogen found after acid hydrolysis is not derived from protein material, and it is suggested that some of this nitrogen is in the form of heterocyclic nitrogen compounds.

4. Tests showed that volatile amines could have been present only in traces in the soil hydrolysates.

5. Tryptophane could not be detected in either acid or alkaline hydrolysates of the soils. Acid hydrolysates gave positive tests for amino sugars.

6. From the amounts of α-amino-N liberated by acid hydrolysis it is deduced that approximately one-third of the total-nitrogen of the soils examined was in protein-like combination. This figure is regarded as minimal.

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