STUDIES ON SOIL HUMIC ACIDS

II. OBSERVATIONS ON THE ESTIMATION OF FREE AMINO GROUPS. REACTIONS OF HUMIC ACID AND LIGNIN PREPARATIONS WITH NITROUS ACID

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The work reported in the first paper in this series (Bremner, 1955*a*) showed that a considerable fraction (20–48 %) of the nitrogen in humic acid preparations from different soils was in the form of amino-acid polymers and that a smaller fraction (3–10 %) was in the form of amino sugars. The object of the work reported in the present paper was to determine how much of the nitrogen in soil humic acids occurs as free amino groups, this information being required for the evaluation of theories regarding the chemical nature of soil humic acids and the mechanisms by which they may complex metals and react with clay colloids (see Bremner, 1954*a*, 1956). The methods used to detect and estimate free amino groups were the nitrous acid method of Van Slyke (1929) and the fluorodinitrobenzene technique developed by Sanger (1945) for the study of the terminal amino groups of proteins (see Table 1). As shown below, the results obtained by the Van Slyke method indicated that some constituent of humic acid preparations interfered with the estimation of free amino groups by this method and led to a study of the reaction of lignin with nitrous acid.

Preliminary results of this work have already appeared (Bremner, 1952).

EXPERIMENTAL

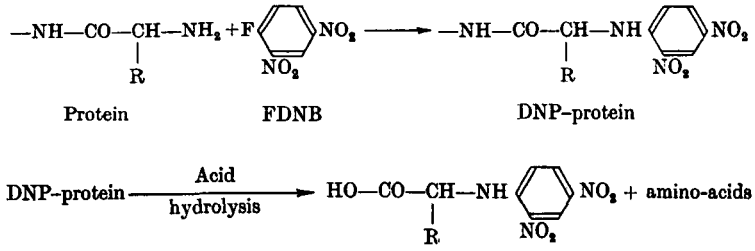
The humic acid preparations employed were isolated from 0·5 M-sodium hydroxide and 0·1 M-sodium pyrophosphate (pH 7·0) extracts of various mineral and organic soils. Details of the isolation procedures and analyses of the preparations are given in the previous paper (Bremner, 1955*a*), and to facilitate reference to the preparations the method of description adopted there will be followed here.

Table 1. *Methods used for detection and estimation of free amino groups*

1. Nitrous acid method (Van Slyke, 1929):

$$R.NH_2 + HNO_2 \rightarrow R.OH + H_2O + N_2^*$$

2. Fluorodinitrobenzene (FDNB) method (Sanger, 1945):



* Purified by treatment with alkaline permanganate, which removes NO formed by spontaneous decomposition of HNO_2, and determined manometrically.

† Separated by column or paper chromatography and estimated. ε-DNP-lysine is obtained if the protein has lysine residues with free ε-amino groups.

Reaction of humic acids with nitrous acid

The results obtained when the preparations were analysed for amino-nitrogen by the manometric nitrous acid method of Van Slyke (1929) are given in Table 2.

The analyses were performed on aliquots of solutions obtained by dissolving 1 g. samples of the air-dry preparations in 50 ml. of solvent, the alkali-extracted preparations being dissolved in 0·05 M-

sodium hydroxide and the pyrophosphate-extracted preparations in 0·1 M-sodium pyrophosphate (pH 7·0). Total nitrogen in the solutions was determined by a micro-Kjeldahl procedure and ammonia-nitrogen by a microdiffusion method previously described (Bremner & Shaw, 1955). The amounts of ammonia detected in the solutions were so small that it was not necessary to correct for interference from this source (see Van Slyke, 1912). Tests with several of the preparations showed that the same result was obtained whichever solvent was used, and tests with solutions of alanine in these solvents showed that the method gave quantitative recovery of amino-nitrogen. No hydrolysis of the preparations was observed in either solvent, the results obtained with solutions that had been allowed to stand for 24 hr. being the same as those obtained with freshly prepared solutions.

It can be seen from Table 2 that the results obtained by the Van Slyke method depended greatly on the reaction time adopted. The period required for complete reaction of the α-amino groups of amino-acids at temperatures of 20–25° C. is only 3–4 min., but the ε-amino group of lysine requires about 20 min. for complete reaction, and a period of 20–30 min. is generally used for the estimation of free amino groups in proteins. The values obtained with the humic preparations using a 15 min. period of reaction with nitrous acid should, therefore, be regarded as minimal if it is assumed that only the free amino groups of protein material are involved in the reaction, but even so they indicated that 12–30 % of the total nitrogen in the preparations examined was in the form of free amino groups. When attempts were made to confirm this estimate by determining the decrease in the nitrogen contents of the preparations on treatment with nitrous acid it was found that the preparations fixed nitrogen in their reaction with nitrous acid, the nitrogen content of the reaction product increasing with time of reaction. Some of the results obtained by studying the effect of nitrous acid treatment on the nitrogen contents of humic acid preparations are given in Table 3. They were obtained by analysing the products formed by

Table 2. *Apparent amino-nitrogen contents of humic acid preparations*

Preparation	N content (%)*	Reaction time (min.) Apparent amino-N (% of total-N) 5	15	30	60	Apparent amino-N content (%)*†
1N	2·95	—	14·8	—	—	0·44
1NE	3·03	—	14·5	—	—	0·44
1P	1·92	—	14·7	18·9	—	0·28
1PE	1·91	9·6	13·8	17·9	—	0·26
2N	3·57	12·3	14·8	17·4	22·2	0·53
2P	2·63	—	13·9	—	—	0·37
3N	3·10	9·7	12·5	16·2	22·2	0·39
3NE	2·90	—	12·8	—	—	0·37
3PE	2·05	—	14·0	—	—	0·29
4PE	4·53	12·1	15·1	—	—	0·68
6PE	2·65	13·4	16·9	—	—	0·45
7N	3·74	9·4	12·7	18·3	20·0	0·47
7NE	4·33	9·5	11·9	14·5	18·2	0·52
7P	2·15	10·0	14·7	18·3	25·0	0·32
7PE	2·31	9·3	12·6	18·0	23·6	0·29
8N	2·41	22·6	30·1	35·3	—	0·73
8NE	2·48	—	27·3	—	—	0·68
9N	2·31	16·0	19·5	24·0	30·0	0·45
9NE	2·95	15·0	18·2	20·6	26·1	0·54
9P	1·79	12·8	16·2	20·0	23·4	0·29
9PE	2·14	11·9	15·0	18·1	20·0	0·32

* Moisture-free basis; not corrected for ash.
† Calculated from results obtained using 15 min. period of reaction.

Table 3. *Nitrogen contents of products formed by reaction of humic acid preparations with nitrous acid*

Preparation	N content of preparation (%)*	Time of reaction (hr.)	N content of reaction product (%)*
1P	1·92	0·5	2·41
1P	1·92	2·0	3·10
1P	1·92	4·0	3·41
2P	2·63	1·0	3·24
2P	2·63	2·0	3·78
8N	2·41	2·0	3·44
8NE	2·48	0·5	2·98
8NE	2·48	2·0	3·50
9P	1·79	1·0	2·00
9P	1·79	2·0	2·47
9P	1·79	3·0	2·68
9N	2·31	1·0	2·70
9NE	2·95	2·0	3·43

* Moisture-free basis; not corrected for ash.

treating 1 g. samples of the preparations with 10 ml. of 3N-HCl and 4 ml. of 50 % (w/v) $NaNO_2$ at room temperature for various periods of time, the products being washed thoroughly with dilute HCl and water before analysis. Comparable results were obtained when the preparations were treated with sodium nitrite and acetic acid as in the Van Slyke method of analysis, but the products were difficult to filter and wash.

The identity of the gas evolved on treatment of the preparations with nitrous acid and estimated as nitrogen has not been fully established. The gas cannot contain carbon dioxide or nitric oxide as they would be absorbed by the alkaline permanganate used in the Van Slyke method, and tests using alkaline pyrogallol, alkaline sodium hyposulphite and Winkler's cuprous chloride solution as absorbents showed that it did not contain oxygen or carbon monoxide (see Peters & Van Slyke, 1932). It is probably nitrogen or nitrous oxide or a mixture of the two.

The following substances are known to interfere with the Van Slyke method of estimating amino groups by reacting with nitrous acid to yield gases that are insoluble in alkaline permanganate: ammonia and urea (Van Slyke, 1911); acetone and ethanol (Van Slyke, 1912); pyruvic acid (Clarke & Inouye, 1930); thioglycollic acid and α-thiolpropionic acid (Lough & Lewis, 1934); tannins (Rahn, 1932; Hulme, 1935; Stuart, 1935) and other phenolic compounds (Stuart, 1935; Carter & Dickman 1943; Fraenkel-Conrat, 1943); oximes (Schenck, 1950); and unsaturated fatty acids (Folch, Schneider & Van Slyke, 1940; Lea & Rhodes, 1954). As the humic preparations examined were isolated by a method involving repeated reprecipitation and washing with dilute acid and water, it did not appear very likely that they contained water-soluble interfering substances of low molecular weight, but to confirm this the effect of dialysing the preparations was studied, solutions of preparations 1P, 1N, 8N, 8NE, 9N and 9NE in 0·05N-NaOH being analysed before and after dialysis in cellophan sacs against distilled water at 5° C. for 36 hr. The results (Table 4) showed that material of low molecular weight capable of being removed by dialysis was not responsible for the high apparent amino-nitrogen values obtained with the preparations. Tests also showed that Soxhlet extraction of the preparations with ether, ethanol or benzene for 16 hr. did not significantly affect their amino-nitrogen contents as estimated by the Van Slyke method. The effect of light on analysis of the preparations by the Van Slyke method was investigated because Fraenkel-Conrat (1943) found that interference due to certain phenolic compounds was eliminated by carrying out the analysis in the dark. It was found that light had no influence on the analysis of the preparations, the results being the same whether the reaction chamber was exposed to strong light or was protected from light with black paper during the analysis. The effects of using freshly prepared alkaline permanganate for purification of the evolved gas and of increasing the time of contact of the gas with permanganate were also studied because Van Slyke (1912) found that perfectly fresh permanganate and a long period of contact with it were required for absorption of the gases evolved on treatment of ethanol and acetone with nitrous acid, and Stuart (1935) found that the amount of gas which was evolved and measured as nitrogen when phenols and tannins were analysed by the Van Slyke method decreased somewhat on long contact with permanganate. It was found that neither the use of freshly prepared permanganate nor an increase in the time of contact of the evolved gas with permanganate had any effect on the results obtained with the humic acid preparations.

Table 4. *Apparent amino-nitrogen contents of dialysed humic acid preparations*

Preparation	Apparent amino-N* (% of total N)	
	Undialysed	Dialysed
1P	14·6	14·0
1N	14·5	13·6
8N	30·2	24·2
8NE	27·5	25·2
9N	19·3	18·5
9NE	18·2	17·5

* As estimated by Van Slyke method using a reaction period of 15 min.

The above tests provided no definite clues to the identity of the material responsible for the high apparent amino-nitrogen values obtained with humic acid preparations by the Van Slyke method, but the dialysis experiment indicated that the material had a fairly high molecular weight. In view of the fact that lignin is a high-molecular substance and that lignin or lignin-derived material is believed to contribute substantially to the humic fraction of soil organic matter, it was therefore decided to investigate the effect of nitrous acid on lignin.

Reaction of lignin with nitrous acid

The reaction of lignin with nitrous acid was studied using the following preparations: native lignin, prepared from black spruce by the method of Brauns (1939); alkali lignin I, prepared from hardwood by the soda process (commercially available as Meadol MRM); alkali lignin II, prepared from pine wood by the sulphate process (commercially available as Indulin A); alkali lignin III, prepared from hardwood by the soda process (commercially available as Tomlinite); alkali lignin IV, prepared

from wheat straw by the method of Waksman & Iyer (1932); alkali lignin V, prepared from oat straw by the method of Bondi & Meyer (1948); acid lignin I, prepared from wheat straw by the method of Norman & Jenkins (1934*a*, *b*); acid lignin II, prepared from oat straw by the method of Crampton & Maynard (1938) as modified by Lancaster (1943).

The apparent amino-nitrogen contents of the alkali and native wood lignins as estimated by the Van Slyke method using various periods of reaction are given in Table 5. For analysis the alkali lignins were dissolved in 0·025 N-sodium hydroxide and the native lignin was dissolved in glacial acetic acid, aliquots of the solutions being analysed for total-nitrogen and ammonia-nitrogen as before. It can be seen that the Van Slyke method gave spurious results, the apparent amino-nitrogen contents of the preparations being considerably greater than their total nitrogen contents even when the reaction time

Table 5. *Apparent amino-nitrogen contents of lignin preparations*

Preparation	N content (%)*	Reaction time (min.) Apparent amino-N content (%)* 5	15	60
Native lignin	0·08	0·30	0·49	0·79
Alkali lignin I	0·12	0·24	0·40	0·67
Alkali lignin II	0·08	0·26	0·49	0·67
Alkali lignin III	0·07	0·27	0·44	0·73

* Moisture-free basis; not corrected for ash.

was only 5 min. It is also clear that lignin-like material may be largely responsible for the high apparent amino-nitrogen values obtained with humic acid preparations because the apparent amino-nitrogen contents of the lignin preparations estimated using a 15 min. period of reaction (mean value 0·45 %) are comparable with those of humic acid preparations similarly estimated (mean value 0·43 %). It was found that the analysis of the lignin preparations, like that of the humic acid preparations, was unaffected by light, and that colour changes noted during the reaction of humic acid preparations with nitrous acid were more pronounced during the reaction of lignin preparations. For example, the product from treatment of the originally buff-coloured native lignin with nitrous acid for 15 min. was light brown in colour.

The lignins isolated from straw were also found to react with nitrous acid with evolution of gas, but the results obtained with these preparations could not be readily interpreted as the untreated lignins contained substantial amounts of nitrogen.

It was found that lignins, like humic acids, fix nitrogen in their reaction with nitrous acid, the amount of nitrogen fixed increasing with time of reaction. The nitrogen contents of products formed by treating 2 g. samples of lignin preparations with 20 ml. of 3 N-HCl and 8 ml. of 50 % (w/v) $NaNO_2$ at room temperature for various periods of time are given in Table 6. Before analysis the products were washed thoroughly with dilute HCl and water. The results obtained with 'lignins' separated from humic preparations by the method of Norman & Jenkins (1934*a*, *b*) are also presented as they provide a further indication that it is probably the lignin-like material in humic acid preparations that is mainly responsible for their reaction with nitrous acid. Tests with samples of the commercial lignins pretreated by boiling under reflux with 3 % (w/w) H_2SO_4 for 2 hr. to remove carbohydrate and other hydrolysable impurities showed that under com-

Table 6. *Nitrogen contents of products formed by reaction of lignin preparations with nitrous acid*

Preparation	N content (%)*	Time of reaction (hr.)	N content of reaction product (%)*
Native lignin	0·08	1·0	1·95
Alkali lignin I	0·12	0·5	1·62
Alkali lignin I	0·12	2·0	1·80
Alkali lignin II	0·08	2·0	3·34
Alkali lignin II	0·08	5·0	3·79
Alkali lignin III	0·07	2·0	2·48
Alkali lignin III	0·07	5·0	3·47
Alkali lignin IV	0·71	1·0	2·10
Alkali lignin V	0·63	2·0	2·05
Acid lignin I	1·00	0·5	2·37
Acid lignin I	1·00	1·0	2·70
Acid lignin II	1·56	2·0	1·83
Humic acid lignin I†	2·15	2·0	2·68
Humic acid lignin II‡	2·22	2·0	2·74

* Moisture-free basis; not corrected for ash.
† Prepared from humic acid preparation 8NE by method of Norman & Jenkins (1934*a*, *b*).
‡ Prepared from humic acid preparation 9NE by method of Norman & Jenkins (1934*a*, *b*).

parable conditions they fixed slightly more nitrogen on treatment with nitrous acid than did samples not subjected to this pretreatment.

Some decomposition of lignin-nitrous acid reaction products was apparently caused by drying at 105° C., because products so dried were found to contain less nitrogen than when dried at room temperature. The total nitrogen analyses reported in Table 6 were obtained by determining nitrogen in the air-dry products by the Kjeldahl method and correcting the results for moisture. In view of the possibility that not all of the nitrogen in lignin-nitrous acid reaction products may be determinable by the Kjeldahl method, some analyses were performed by the Dumas technique, but the results with this method were very erratic and were generally lower than the corresponding Kjeldahl results. The Kjeldahl method gave very consistent results, and a modification

of it that included a pretreatment with reduced iron and sulphuric acid for the reduction of oxidized forms of nitrogen gave the same results as the normal procedure.

Methoxyl group estimations, performed by Dr Weiler and Dr Strauss, Oxford, showed that treatment of lignin with nitrous acid led to the destruction of methoxyl groups (Table 7). This destruction was reflected by the results obtained when

Table 7. *Methoxyl contents of products formed by reaction of lignin preparations with nitrous acid*

Preparation	Methoxyl content (%)*	Time of reaction (hr.)	Methoxyl content of reaction product (%)*
Alkali lignin I	22·4	2	15·5
Alkali lignin II	14·0	2	8·7
Alkali lignin II	14·0	5	7·6
Alkali lignin III	19·6	2	12·5
Alkali lignin III	19·6	5	10·4

* Moisture-free basis; not corrected for ash.

the amounts of aromatic aldehydes containing methoxyl groups produced by alkaline nitrobenzene oxidation of lignin-nitrous acid reaction products were estimated by the micro-method described by Stone & Blundell (1951). For example, the product from treatment of alkali lignin II with nitrous acid at room temperature for 5 hr. was found to yield only about half of the amount of vanillin obtained by nitrobenzene oxidation of the untreated lignin. It was also found that the carbon contents of lignin-nitrous acid reaction products were considerably lower than those of the untreated lignins. For example, the products from treatment of alkali lignin I (64·7 % carbon) and alkali lignin II (65·2 % carbon) with nitrous acid at room temperature for 5 hr. were found to contain 57·1 and 56·7 % carbon respectively.

Tests showed that treatment of humic acid preparations with nitrous acid also led to the destruction of methoxyl groups. For example, the products obtained by treating humic acid preparations 1N (3·1 % methoxyl), 8N (4·9 % methoxyl) and 9N (3·3 % methoxyl) with nitrous acid at room temperture for 2 hr. contained 1·9, 2·5 and 2·7 % methoxyl, respectively.

It was found that most of the nitrogen fixed by lignin in its reaction with nitrous acid was resistant to acid hydrolysis. The results obtained by determining the amounts of acid-soluble nitrogen and ammonia-nitrogen liberated by hydrolysing lignin-nitrous acid reaction products with 6N-HCl (10 ml./g. of product) in sealed tubes at 105° C. for 24 hr. are given in Table 8. Total-nitrogen and ammonia-nitrogen in the hydrolysates were determined as before.

It can be seen that only 27–36 % of the total nitrogen in the lignin-nitrous acid reaction products was liberated by hydrolysis with 6N-HCl, and that most of the nitrogen so released was in the form of ammonia. Only 8–11 % of the material in the products was dissolved by the hydrolysis and the nitrogen contents of the residues ranged from 1·0 to 2·7 %. Studies on the course of the hydrolysis with 6N-HCl at 105° C. showed that the amount of nitrogen dissolved was not significantly increased by prolonging the hydrolysis beyond about 24 hr. For example, when the product obtained by treatment of alkali lignin III with nitrous acid for 5 hr. was hydrolysed, 17·6 % of the total nitrogen was dissolved at 2 hr., 25·2 % at 9 hr., 34·0 % at 24 hr. and 37·5 % at 48 hr.

Products obtained by treating alkali lignins II and III with nitrous acid at room temperature for periods of 2, 3 and 5 hr. were analysed for oxime nitrogen by the method of Csaky (1948), which involves hydrolysis with 3N-H_2SO_4 at 100° C. for 6 hr. and estimation of the hydroxylamine liberated by hydrolysis by a colorimetric method based on oxidation of hydroxylamine to nitrite by iodine in acetic acid and estimation of the nitrite by means of the colour reaction with sulphanilic acid and 1-naphthylamine. The results indicated that about 1 % of the total nitrogen in the products examined was in the form of oximino groups. To confirm that hydroxylamine was liberated by acid hydrolysis of

Table 8. *Amounts of acid-soluble nitrogen and ammonia-nitrogen liberated by acid hydrolysis of lignin-nitrous acid reaction products*

Products were hydrolysed with 6N-HCl at 105° C. for 24 hr. Amounts of acid-soluble nitrogen and ammonia-nitrogen liberated are expressed as percentages of total nitrogen in the products.

Reaction product from*	N content (%)	Acid-soluble N	Ammonia-N	Ammonia-N as % of acid-soluble N
Alkali lignin I (2)	1·80	36·0	32·2	89·4
Alkali lignin II (2)	3·34	27·2	23·9	87·9
Alkali lignin II (5)	3·79	29·1	26·6	91·3
Alkali lignin III (2)	2·48	35·4	33·8	95·5
Alkali lignin III (5)	3·47	34·0	32·6	95·9

* Figures in parentheses are times (hours) of reaction with nitrous acid.

lignin-nitrous acid reaction products, 1 g. samples of products obtained by treating alkali lignins I, II and III with nitrous acid for various periods of time (1–5 hr.) were hydrolysed with 10 ml. of 3N-HCl at 100° C. for 6 hr., and the hydrolysates were concentrated to small volume *in vacuo* and examined for hydroxylamine by paper chromatographic techniques already described (Bremner, 1954*b*). Hydroxylamine was readily detected in every hydrolysate examined.

The amounts of ammonia-nitrogen liberated by distilling lignin-nitrous acid reaction products with 2N-NaOH (200 ml./g. of product) are given in Table 9. The distillations were carried out in a Kjeldahl apparatus, the level of the liquid in the flask being kept constant by adding water during the distillation. The ammonia evolved was collected in 2% (w/v) boric acid and determined by titration with 0·01N-HCl. It can be seen that the amounts of ammonia liberated by distillation with 2N-NaOH for 2 hr. were similar to the amounts liberated by hydrolysis with 6N-HCl at 105° C. for 24 hr.

Table 9. *Amounts of ammonia-nitrogen liberated by distillation of lignin-nitrous acid reaction products with alkali*

Products were distilled with 2N-NaOH (200 ml./g. of product) for 2 hr. Amounts of ammonia-nitrogen liberated are expressed as percentages of total nitrogen in the products.

Reaction product from*	Ammonia-N
Alkali lignin I (2)	30·8
Alkali lignin II (2)	24·1
Alkali lignin II (5)	24·9
Alkali lignin III (2)	31·5
Alkali lignin III (5)	34·0

* Figures in parentheses are times (hours) of reaction with nitrous acid.

Reaction of humic acids with 1-fluoro-2:4-dinitrobenzene (FDNB)

The reaction of humic acids with FDNB was studied using samples of preparations 1N, 1P, 2N, 3P and 8N. Before reaction with FDNB the preparations were extracted on a Soxhlet with ethanol for 16 hr. and then with ether for 6 hr. The method of reaction used, which was essentially that of Sanger (1945), was as follows. The alcohol-ether extracted preparation (5 g.) was shaken for 30 min. with $NaHCO_3$ (10 g.) and water (100 ml.). The suspension was then treated with FDNB (10 g., 7 ml.) dissolved in ethanol (200 ml.), and the mixture was shaken for 2 hr. at room temperature. The reaction product was separated by filtration, washed thoroughly with 90% (v/v) ethanol, absolute ethanol and ether, and extracted on a Soxhlet with ethanol (16 hr.) and ether (6 hr.). It was then hydrolysed with 6N-HCl (10 ml./g. of product) under reflux for 12 hr. and the hydrolysis mixture was filtered, the residue washed thoroughly with hot water, and the combined filtrate and washings were cooled and extracted three times with peroxide-free ether, each extract being washed in turn with water. The total ether fraction and an aliquot of the aqueous fraction were taken to dryness and examined for DNP-amino-acids by the paper chromatographic methods described by Blackburn & Lowther (1951) and Biserte & Osteux (1951), chromatography being carried out in boxes covered with black paper to avoid decomposition of DNP derivatives by light (Blackburn, 1949). The only substances detectable on chromatograms obtained from preparations 1N, 1P, 2N and 3P were 2:4-dinitrophenol and 2:4-dinitroaniline, but two other spots were detected on chromatograms obtained from preparation 8N. These spots were only visible when the chromatograms were overloaded, and attempts to identify the substances responsible for them were unsuccessful. Since it is known that DNP-proline is destroyed under the conditions of hydrolysis adopted, samples of the FDNB reaction products obtained with preparations 1N and 3P were hydrolysed in sealed tubes with 12N-HCl at 105° C. for 16 hr., a method of hydrolysis found by Porter & Sanger (1948) to cause less decomposition of DNP-proline. However, the latter could not be detected in the hydrolysates. To determine whether failure to detect free amino groups by the FDNB method was due to insufficient time of reaction, samples of preparations 1N and 8N were allowed to react with FDNB for 24 hr. The only noticeable effect of this increase in reaction time was that it led to an increase in the amounts of dinitrophenol and dinitroaniline found after hydrolysis.

DISCUSSION

The results obtained with the humic acid preparations by the Van Slyke nitrous acid method of estimating amino groups (Table 2) are suspect because from studies on the chemical nature of humic nitrogen and the decomposition of nitrogenous compounds in soil it appears very unlikely that such a large proportion of humic nitrogen is in the form of free amino groups. The deduction that some constituent of humic acid preparations interferes with the estimation of amino groups by the Van Slyke method is supported by the finding that lignin, like other phenolic substances, interferes in this method of analysis (Table 5), since lignin or lignin-derived material is believed to contribute substantially to the humic fraction of soil organic matter. The view that lignin-like interfering material occurs in humic acid preparations is strengthened by the finding that humic acids, like lignins, fix nitrogen and lose methoxyl groups in their reaction with nitrous acid. There seems little doubt that humic acid preparations contain some lignin-like material because it has been

demonstrated that, like lignins, they yield aromatic aldehydes (vanillin, syringaldehyde and *p*-hydroxybenzaldehyde) on oxidation with alkaline nitrobenzene (Bremner, 1955*b*).

Considering the inadequacy of present information regarding the chemical nature of humic acids and of lignins, it seems futile to indulge in much speculation regarding the nature of their reactions with nitrous acid. It appears likely that nitroso compounds are formed, but the possibility that nitro compounds are produced cannot be excluded, as several workers have found that phenolic compounds yield nitro derivatives when treated with nitrous acid (e.g. Rosenblatt, Epstein & Levitch, 1953). The reaction of lignin with nitrous acid does not appear to have been investigated in any detail, but the reaction with nitric acid has received considerable attention (see Brauns, 1952) and several workers have reported that nitration of lignin does not occur in the presence of urea, which destroys nitrous acid. This observation, together with the finding that nitrolignin cannot be reduced to an amino derivative and that it yields ammonia when treated with acid or alkali, has led Fuchs (1928, 1935) to advance the hypothesis that the nitration of lignin, like that of humic acids from coal, consists of a partial oxidation followed by the addition of nitrous acid to a tautomerized aromatic hydroxyl group with formation of a nitrosolignin. The results presented in this paper show that the reaction of lignin with nitrous acid is similar in many respects to its reaction with nitric acid. For example, it leads to the fixation of a considerable amount of nitrogen (Table 6), some of which is released as ammonia on treatment with acid or alkali (Tables 8 and 9), and is accompanied by the destruction of methoxyl groups (Table 7). Tests showed that it is also accompanied by the evolution of HCN, as is the nitration of lignin. It is also relevant in this connexion to note that Sarkar (1934) has observed that treatment of lignin with nitrous acid, like treatment with nitric acid, leads to the formation of oxalic acid in considerable quantity, and that work carried out by the author in conjunction with Mr K. Shaw has shown that the rates of mineralization in soil of the nitrogen in lignin-nitrous acid and lignin-nitric acid reaction products are practically identical.

Reference may be made here to a paper by Bondi & Meyer (1948). They report investigations on the nature of the nitrogen in alkali lignins from annual forage plants and conclude that it cannot be primary or secondary amine nitrogen 'as is shown by the resistance of lignin N to distillation of the lignins with concentrated NaOH and to treatment of the lignins with $NaNO_2$ (no liberation of N occurred and the N content of the lignin remained unchanged)'. No details of the experiments leading to these conclusions are given but if, as seems necessary, the tests with $NaNO_2$ were made with acid present, then the findings in this work are contrary to those reported here. This discrepancy cannot be attributed to differences in the methods used for isolating the lignins because the same results were obtained in the present investigation with a lignin preparation isolated by the method of Bondi & Meyer as with lignins isolated by other methods (see Table 6).

Further work is required to account for the liberation of what appears to be nitrogen or nitrous oxide on treatment of humic acid and lignin preparations with nitrous acid. It may be due to reduction of some of the nitrous acid to hydroxylamine or ammonia, as these compounds react with nitrous acid to yield nitrous oxide and nitrogen, respectively. It could also be due to the presence in lignin and humic acid preparations of reactive methylene groups (e.g. methylene groups adjacent to carbonyl groups), as these are known to react with nitrous acid to yield oximino groups which, in turn, react with nitrous acid to yield nitrous oxide and nitrogen (see Taylor & Baker, 1937; Schenck, 1950). Another possibility is that lignins and humic acids, like other phenolic substances, react with nitrous acid to form nitrosophenols which can tautomerize to quinone oximes and that the oximino groups in the latter are susceptible to decomposition by nitrous acid with the formation of nitrous oxide and nitrogen. Evidence that oximino groups are formed in the reaction of lignin with nitrous acid is provided by the detection of small amounts of hydroxylamine in acid hydrolysates of lignin-nitrous acid reaction products. To elucidate the mechanisms responsible for gas evolution with humic acid and lignin preparations it would probably be profitable first to investigate the mechanism with simple phenols. For further work with humic acids it would be useful to have sodium nitrite labelled with the heavy isotope of nitrogen, because determination of the isotope concentration in the gas evolved on treatment with labelled nitrite and acetic acid would reveal how much, if any, of the nitrogen or nitrous oxide liberated was derived from amino or other nitrogenous groupings in the preparations.

The finding that nitrogen is fixed and that a gas with the properties of nitrogen or nitrous oxide is liberated when the humic fraction of soil organic matter is treated with nitrous acid may have important implications. Several workers have suggested that nitrogen is lost from soil by production of nitrogen gas through interaction of nitrous acid with amino-acids or ammonia as in the Van Slyke method of analysis. The results reported here suggest that loss of nitrogen from soil may also occur through interaction of nitrous acid with the humic fraction of soil organic matter, and that some of the nitrogen found in this fraction may be in groups introduced by this reaction. These possibilities have

not been investigated, but recent work (Allison & Doetsch, 1951; Allison, Doetsch & Sterling, 1952) has shown that the nitrite-amino acid reaction is not likely to occur to any marked extent in soil as it requires a low pH and a high nitrite concentration, and this probably holds for the nitrite-humic acid reaction.

The finding that no free amino groups can be detected in humic acid preparations by the FDNB technique is in agreement with recent work by Sowden & Parker (1953) and Okuda & Hori (1954). The result obtained by this technique appears more reasonable than that given by the Van Slyke method and is to be expected if, as Waksman & Iyer (1932) have postulated, the humic fraction of soil organic matter is a ligno-protein complex in which the free amino groups in the protein material are combined with carbonyl groups in the lignin. However, failure to detect free amino groups in humic acid preparations by the FDNB method cannot be taken as conclusive evidence that such groups are absent because the method was designed for the detection of terminal amino groups in proteins, and humic acid preparations may contain other types of amino groups. Moreover, several workers have reported that not all of the ϵ-amino groups in some proteins are available to FDNB, although in some instances these groups are available to nitrous acid (cf. Salo, 1950; Bowes & Moss, 1953). Porter (1948) found that 30–50 % of the lysine ϵ-amino groups in certain globulins did not react with FDNB when these proteins were in the native state but that all the groups became reactive towards FDNB when the proteins were denatured. He suggested that the unreactivity of some of the amino groups in the native proteins was due to steric hindrance, the configurations of the polypeptide chains being such that they prevented a sufficiently close approach of the FDNB molecule. A similar phenomenon has been observed with collagen and gelatin (Bowes & Moss, 1953). It is possible, therefore, that failure to detect free amino groups in humic acid preparations by the FDNB technique is due to steric factors affecting the reactivity of the amino groups towards FDNB.

SUMMARY

1. Free amino groups in humic acid preparations isolated from 0·5M-sodium hydroxide and 0·1M-sodium pyrophosphate (pH 7·0) extracts of various soils have been estimated by the nitrous acid method of Van Slyke (1929) and the fluorodinitrobenzene technique of Sanger (1945).

2. The results obtained by the Van Slyke method using a reaction time of 15 min. indicated that from 12 to 30 % of the total nitrogen in the preparations examined was in the form of free amino groups. No free amino groups could be detected by the fluorodinitrobenzene technique.

3. It is shown that lignin interferes with the estimation of amino groups by the Van Slyke method, and it is suggested that lignin or lignin-derived material may be largely responsible for the high apparent amino-nitrogen values obtained with humic acid preparations by this method.

4. The reaction of humic acid with nitrous acid resembles the reaction of lignin with nitrous acid in that it is accompanied by the fixation of nitrogen and the destruction of methoxyl groups. The reaction of lignin with nitrous acid is similar in many respects to its reaction with nitric acid.

5. Only about one-third of the nitrogen fixed by lignin in its reaction with nitrous acid is removed by prolonged hydrolysis with 6N-HCl, and most of the nitrogen so released is in the form of ammonia. A small amount of the nitrogen liberated by acid hydrolysis is in the form of hydroxylamine.

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REFERENCES

ALLISON, F. E. & DOETSCH, J. (1951). *Proc. Soil Sci. Soc. Amer.* **15**, 163.
ALLISON, F. E., DOETSCH, J. H. & STERLING, L. D. (1952). *Soil Sci.* **74**, 311.
BISERTE, G. & OSTEUX, R. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 50.
BLACKBURN, S. (1949). *Biochem. J.* **45**, 579.
BLACKBURN, S. & LOWTHER, A. G. (1951). *Biochem. J.* **48**, 126.
BONDI, A. & MEYER, H. (1948). *Biochem. J.* **43**, 248.
BOWES, J. H. & MOSS, J. A. (1953). *Biochem. J.* **55**, 735.
BRAUNS, F. E. (1939). *J. Amer. Chem. Soc.* **61**, 2120.
BRAUNS, F. E. (1952). *The Chemistry of Lignin*. New York: Academic Press.
BREMNER, J. M. (1952). *J. Sci. Fd Agric.* **3**, 497.
BREMNER, J. M. (1954*a*). *J. Soil Sci.* **5**, 214.
BREMNER, J. M. (1954*b*). *Analyst*, **79**, 198.
BREMNER, J. M. (1955*a*). *J. Agric. Sci.* **46**, 247.
BREMNER, J. M. (1955*b*). *Z. PflErnähr. Düng.* **69**, 32.
BREMNER, J. M. (1956). *Soils & Fert.* **19**, 115.
BREMNER, J. M. & SHAW, K. (1955). *J. Agric. Sci.* **46**, 320.
CARTER, H. E. & DICKMAN, S. R. (1943). *J. Biol. Chem.* **148**, 453.
CLARKE, H. T. & INOUYE, J. M. (1930). *J. Biol. Chem.* **89**, 399.
CRAMPTON, E. W. & MAYNARD, W. A. (1938). *J. Nutr.* **15**, 383.

CSAKY, T. Z. (1948). *Acta chem. scand.* **2**, 450.
FOLCH, J., SCHNEIDER, H. A. & VAN SLYKE, D. D. (1940). *J. Biol. Chem.* **133**, xxxiii.
FRAENKEL-CONRAT, H. (1943). *J. Biol. Chem.* **148**, 453.
FUCHS, W. (1928). *BrennstChemie*, **9**, 178.
FUCHS, W. (1935). *ZellstFaser*, **32**, 86.
HULME, A. C. (1935). *Biochem. J.* **29**, 263.
LANCASTER, R. L. (1943). *N.Z. J. Sci. Tech.* **25A**, 137.
LEA, C. H. & RHODES, D. N. (1954). *Biochem. J.* **56**, 613.
LOUGH, S. A. & LEWIS, H. B. (1934). *J. Biol. Chem.* **104**, 601.
NORMAN, A. G. & JENKINS, S. H. (1934*a*). *Biochem. J.* **28**, 2147.
NORMAN, A. G. & JENKINS, S. H. (1934*b*). *Biochem. J.* **28**, 2160.
OKUDA, A. & HORI, S. (1954). *Trans. Fifth Int. Congr. Soil Sci.* **2**, 255.
PETERS, J. P. & VAN SLYKE, D. D. (1932). *Quantitative Clinical Chemistry*, **2**. London: Baillière, Tindall and Cox.
PORTER, R. R. (1948). *Biochim. biophys. Acta*, **2**, 105.
PORTER, R. R. & SANGER, F. (1948). *Biochem. J.* **42**, 287.
RAHN, H. (1932). *Planta*, **18**, 1.
ROSENBLATT, D. H., EPSTEIN, J. & LEVITCH, M. (1953). *J. Amer. Chem. Soc.* **75**, 3277.
SALO, T. P. (1950). *J. Amer. Leath. Chem. Ass.* **45**, 99.
SANGER, F. (1945). *Biochem. J.* **39**, 507.
SARKAR, P. B. (1934). *J. Indian Chem. Soc.* **11**, 407.
SCHENCK, M. (1950). *Hoppe-Seyl. Z.* **286**, 270.
SOWDEN, F. J. & PARKER, D. I. (1953). *Soil Sci.* **76**, 201.
STONE, J. E. & BLUNDELL, M. J. (1951). *Analyt. Chem.* **23**, 771.
STUART, N. W. (1935). *Plant Physiol.* **10**, 135.
TAYLOR, T. W. J. & BAKER, W. (1937). *Sidgwick's Organic Chemistry of Nitrogen*, 2nd ed. p. 172. Oxford: Clarendon Press.
VAN SLYKE, D. D. (1911). *J. Biol. Chem.* **9**, 185.
VAN SLYKE, D. D. (1912). *J. Biol. Chem.* **12**, 275.
VAN SLYKE, D. D. (1929). *J. Biol. Chem.* **83**, 425.
WAKSMAN, S. A. & IYER, K. R. N. (1932). *Soil Sci.* **34**, 43.

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