

## CHEMICAL STUDIES ON SOIL HUMIC ACIDS

### III. Nitrogen Distribution in Humic Acids

Kiyoshi TSUTSUKI and Shozo KUWATSUKA

*Faculty of Agriculture, Nagoya University, Nagoya, Japan*

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The levels of amino acid, ammonia, amino sugar, hydrolysable-N, non-hydrolysable-N, and total-N in humic acids obtained from various soils were determined to investigate their relationships to the degree of humification (*i.e.* *RF* values) or the types of humic acids. The levels of total-N, hydrolysable-N, and amino acid-N increased from Rp(2) type (obtained from Ao layer humus or rotted plants) to Rp(1) type humic acids (from A layer or plowed layer soils), and then decreased with increasing *RF* values in the order of Rp(1), P<sub>0</sub>, B, and A type humic acids. Most of the nitrogen constituents are presumed to be mineralized during humification. Non-hydrolysable-N increased slightly, but its ratio to total-N increased with *RF* values especially among A type humic acids. Yields of ammonia by hydrolysis decreased with increasing *RF* values. The amino acid compositions of 4 different humic acids were similar, and the ratio of amino acid-N to total-N was relatively constant among highly humified A type humic acids, so that proteinous structures in humic acids were presumed to be relatively stable. *Additional Index Words:* nitrogen constituents, amino acid composition, acid hydrolysis, humification.

Nitrogen in soil organic matter plays an important role as a source of nitrogen nutrition for plants in both natural and agricultural systems. Nitrogen transformation is also closely related to the process of humification. Consequently, the forms of nitrogen in soil organic matter including humic acids have been studied by many researchers for a long time (2, 8, 10). But relatively few researchers have investigated the role of nitrogen in the humification process of humic acids. Among them, KUMADA (5, 6) has studied the relationship between hydrolysable and non-hydrolysable nitrogen contents in humic acids and the degree of humification, though the number of samples was small.

The relationship between the total-N content and the degree of humification of humic acids has also been discussed in our previous paper (7), and it was shown that nitrogen content increases in the early stage of humification and then decreases with increasing degree of humification. In this paper, the amounts of hydrolysable- and non-hydrolysable-N, amino acids, and ammonia by 6 N HCl and 8 N H<sub>2</sub>SO<sub>4</sub> hydrolysis are reported, and the variation in the nitrogen distribution in relation to the process of humification is discussed.

## MATERIALS AND METHODS

1. *Humic acid samples.* The origins, methods of extraction and purification, elementary and oxygen-containing functional group compositions of humic acid samples used except Hichiso (No. 40) were reported previously (7, 12). This sample was obtained from the Bh layer of a podzolic soil in forest under natural vegetation of conifers at Hichiso in Gifu prefecture, Central Japan, extracted with 0.5 N NaOH at room temperature and purified by repeated washing by dil. HCl and treatment with cation exchange resin. Detailed analytical data of this sample shall be reported elsewhere.

2. *Determination of the amino acid composition and the nitrogen distribution of humic acids.* (1) Amino acid, ammonia, and amino sugar composition: Four humic acid samples, Temmondai (No. 2) from a humic volcanic ash soil, Kuragari (No. 18) from a brown forest soil, Kisokoma (No. 38) from F-layer humus in a forest soil, and Hichiso (No. 40) from podzol Bh-layer soil, were analyzed. Humic acid sample equivalent to about 800  $\mu\text{g}$  of N was weighed into a hard glass test tube, 2 ml of 6 N HCl containing 1  $\mu\text{mol}$  of norleucine as an internal standard was added and then the test tube was evacuated and sealed. The sealed tube was heated at 110°C for 20 hr. The hydrolysate was filtered through a filter paper into an evaporating flask, with about 10 ml of washing water, and dried up with a rotary evaporator at 60°C, followed by two times repetition of dissolution and drying. It was dissolved again in 5 ml of citrate buffer (pH 2.2), and a portion (0.25 ml) of the solution was chromatographed on Hitachi-034 liquid chromatograph for determining amino acid, ammonia, and amino sugar composition.

(2) Determination of the amounts of ammonia and total amino acids in 6 N HCl hydrolysate: Further 10 humic acid samples, different in *RF* values, types of humic acids, and the types of original soils (Table 3), were hydrolysed with 6 N HCl, and the hydrolysates were made up to 5 ml with citrate buffer (pH 2.2) by the same procedure mentioned above, except that an internal standard was not added. The amounts of ammonia and total amino acids were determined as follows. 0.5 ml of the hydrolysate solution was diluted with 2 ml of water, and 1 ml portions were transferred into two small vials. Into one portion was added 1 ml of water and stoppered tightly till analysis. Into the other was added 1 ml of 0.08 N NaOH to raise the pH value to 12 and it was put into a desiccator containing 1 N H<sub>2</sub>SO<sub>4</sub> and left overnight to remove ammonia. 0.25 ml portion of these hydrolysate solutions was injected into a Hitachi liquid chromatograph, of which the column part was substituted with Teflon tube, the sample solution was eluted by 4 N acetate buffer (pH 5.5) and the color was developed with ninhydrin reagent automatically. Calibration curves were made by standard ammonium sulfate and glycine solutions. Total amount of amino acids was determined by the coloration of the sample solution from which ammonia was removed. Using the calibration curve for glycine, the amount of ammonia was ob-

tained by subtracting total amino acid content from the apparent content of ammonia plus total amino acids, determined by the coloration of the sample solution without removing ammonia. The difference was divided by 0.770 which was the rate of coloration of ammonia to glycine.

(3) Determination of the nitrogen distribution by 8 N  $\text{H}_2\text{SO}_4$  hydrolysis: Thirty-four humic acid samples were hydrolysed by 8 N  $\text{H}_2\text{SO}_4$  to determine nitrogen distribution. The samples used were Nos. 2 to 6, 8 to 10, and 12 to 15 for A type (12 samples), 18 to 21 and 40 for B type (5 samples), 29, 31, 32, 34 to 36, and 39 for Rp(1) type (7 samples), 30, 33, 37, and 38 for Rp(2) type (4 samples), 24, 26, and 27 for  $\text{P}_0$  type (3 samples), and 11, 22, and 25 for  $\text{P}_{++++}$  type humic acids. Humic acid sample equivalent to about 400  $\mu\text{g}$  of N dissolved in 3 ml of 0.1 N NaOH was taken into a hard glass test tube, and 0.85 ml of conc.  $\text{H}_2\text{SO}_4$  was added. The test tube was evacuated and sealed, and heated at 110°C for 20 hr. The hydrolysate was made to 10 ml with water in a volumetric flask. The solution was transferred to a centrifuging tube with a glass stopper and centrifuged. The supernatant solution was analyzed for the total amino acid content by TNBS method. The resulting precipitate was washed three times with dil.  $\text{H}_2\text{SO}_4$  (1 : 100) by repeated centrifuge, and was analyzed for the non-hydrolysable-N by Kjeldahl method. Total-N content in humic acids was also determined by Kjeldahl method.

Total amino acid content was determined by TNBS method according to SATAKE and OKUYAMA (9) with some modifications. To 0.5 ml portion of the sample solution in 2 N  $\text{H}_2\text{SO}_4$  were added 2.5 ml of phosphate buffer (4 volume of 0.5 M phosphate buffer at pH 8.0 plus 1 volume of 2 N NaOH for neutralization) and 2 ml of 0.1% sodium trinitrobenzenesulfonate. After heating the solution at 40°C for 90 min, 5 ml of 6 N HCl was added. It was analyzed colorimetrically at 400 nm. Glycine was used to make the calibration curve.

## RESULTS AND DISCUSSION

### 1. Amino acid, ammonia, and amino sugar composition of humic acids

Amino acid compositions were compared among 4 humic acid samples (Table 1). Each molar amount of Asp, Glu, Gly, and Ala was about 10% or more of the total amount of amino acids. The composition of these 4 amino acids were very similar among the 4 humic acids though they were different in the degree of humification and origin. Each molar amount of Val, Ile, Leu, Phe, Pro, Ser, Thr, and Lys ranged from 4 to 8%, and that of Met, Tyr, His, and Arg ranged from trace to 4% of the total amino acids. These amino acid compositions were similar to those of humic acids obtained from a chernozem soil by KHAN and SOWDEN (3, 4), and from highly organic tropical volcanic soils by SOWDEN *et al.* (11). These results suggest that protein structure in humic acids is indifferent to the degree of humification and the origin of humic acids. The average molecular weight of amino acids in humic acids calculated from the average amino acid composition was 122, and each amino acid

Table 1. Relative molar distribution of amino acids in humic acids (%).

Name <i>RF</i> Type	Humic acid				Average	Standard deviation
	Temmondai 144 A	Kuragari 61 B	Hichiso 51 B	Kisokoma (F) 20 Rp(2)		
<b>Acidic</b>						
Aspartic acid	14.6	14.3	13.4	13.3	13.9	0.64
Glutamic acid	11.5	9.59	10.6	10.5	10.5	0.78
<b>Neutral (non-polar)</b>						
Glycine	12.6	12.8	11.6	12.3	12.4	0.51
Alanine	11.9	10.6	10.9	10.4	10.9	0.65
Valine	7.76	8.36	5.86	7.63	7.40	1.08
Isoleucine	4.13	4.16	4.66	4.86	4.45	0.37
Leucine	5.59	6.32	7.14	7.87	6.73	0.99
Phenylalanine	3.45	3.76	3.87	4.08	3.79	0.26
Proline	4.87	6.28	6.51	5.80	5.87	0.73
<b>Neutral (polar)</b>						
Serine	5.28	5.81	6.09	5.54	5.68	0.35
Threonine	6.60	5.36	7.18	5.57	6.18	0.86
Methionine	0.00	0.71	1.14	0.81	0.67	0.48
Tyrosine	0.89	1.40	1.77	2.17	1.56	0.55
Cystine	0.00	0.00	0.00	0.00	0.00	0.00
<b>Basic</b>						
Lysine	5.89	5.57	3.84	4.16	4.87	1.02
Histidine	1.67	1.55	2.66	2.68	2.14	0.61
Arginine	3.31	3.48	2.82	2.27	2.97	0.54

Table 2. Nitrogen and carbon distribution in humic acids.<sup>a</sup>

	Humic acid			
	Temmondai	Kuragari	Hichiso	Kisokoma (F)
<b>Nitrogen distribution</b>				
Amino acid-N	28.1	36.4	46.6	56.8
Amino sugar-N	1.99	1.24	0.92	2.19
Ammonia-N	15.3	13.7	8.51	15.3
<b>Carbon distribution</b>				
Amino acid-C	6.29	11.3	11.6	11.9
Amino sugar-C	0.73	0.60	0.36	0.71

<sup>a</sup> Values are expressed as percentage to total-N or total-C.

contained average values of 4.5 carbon atoms and 1.2 nitrogen atoms per molecule. The calculated C/N ratio of the proteinous fraction in humic acids was 3.3. These values can be used to calculate the amounts of C or N in amino acids from the yields of amino acids determined in molar amount.

For the same 4 humic acid samples, nitrogen and carbon distribution among total amino acids, amino sugars, and ammonia are presented in Table 2. The ratio of amino acid-N to total-N ranged from 28 to 57% decreasing with the increase in *RF* values. The ratio of amino acid-C to total-C were more or less similar for the 3 samples and ranged from 11.3 to 11.9%, except the highly humified Temmondai (No. 2) humic acid sample with a low value of 6.3%. The ratios of amino sugar-N to total-N, amino sugar-C to total-C, and ammonia-N to total-N ranged, respectively, from 0.92 to 2.2%, 0.36 to 0.76%, and 8.5 to 15.3%.

## 2. The relationship between the nitrogen distribution and the degree of humification or the types of humic acids

Nitrogen distribution was determined by 8 N H<sub>2</sub>SO<sub>4</sub> hydrolysis among 36 humic acids, and also by 6 N HCl hydrolysis among 13 humic acids (Table 3). Yields of amino acids in both hydrolysates showed very significant positive linear correlation ( $r=0.918^{***}$ ). Therefore, it is presumed that almost the same results may be obtained by using the two hydrolysis procedures.

(1) *Total-N content in humic acids.* Total-N contents were plotted against *RF* values (Fig. 1). The plots were grouped together according to their types, *i.e.* A, B, Rp(1), Rp(2), and P<sub>0</sub> type. Total-N contents increased from Rp(2) type to Rp(1) type, and then decreased among Rp(1), P<sub>0</sub>, B, and A type with increasing *RF* values. A very significant negative linear correlation ( $r=-0.715^{***}$ ) was found between total-N content and *RF* value among the latter 4 types of humic acids. As pointed out by KUMADA (5), the process of the humic acid formation could be divided into two stages, according to the change in total-N contents. In the first stage, corresponding to the change from Rp(2) type to Rp(1) type, humic acids with low nitrogen content are transformed to those rich in nitrogen, because easily utilizable materials such as polysaccharides may be exhausted by soil microbes, leaving behind the humic substances or the dead biomass richer in nitrogen. The color of humic acids, *i.e.* the degree of humification does not increase during this stage. In the next stage, nitrogen is released again with the remarkable increase in the deep color of humic acid solution.

(2) *Hydrolysable-N contents.* The nitrogen in the supernatant solution of 8 N H<sub>2</sub>SO<sub>4</sub> hydrolysate of humic acid sample was regarded as the hydrolysable-N. A very significant positive linear correlation ( $r=0.964^{***}$ ) was found between the hydrolysable-N and total-N contents. Similar to the total-N, hydrolysable-N contents increased from Rp(2) type to Rp(1) type, and then decreased among Rp(1), P<sub>0</sub>, B, and A type with increasing *RF* values (Fig. 2). A significant negative linear correlation ( $r=-0.769^{***}$ ) was found between hydrolysable-N content and *RF* value among the latter

Table 3. Nitrogen dis-

No.	Humic acid	Type	RF	Total-N	8N H <sub>2</sub> SO <sub>4</sub> hydrolysis	
					Hydrolysable-N	Amino acid-N
2	Temmondai	A	144	4.07%	2.14%	1.37%
7	Kinshozan-P-1	A	115	2.60		
8	Shitara black	A	114	2.67	1.54	0.91
13	Komagahara	A	89	2.77	1.71	1.85
16	Shitara brown	B	76	4.43	3.69	2.80
18	Kuragari	B	61	5.46	4.62	1.75
19	Kinshozan-F-1	B	60	3.70	2.79	2.28
21	Higashiyama (A)	B	47	2.75	1.70	1.92
29	Sanage-1	Rp(1)	32	5.75	4.94	2.96
32	Kinshozan-OH	Rp(1)	29	6.06	5.23	3.67
38	Kisokoma (F)	Rp(2)	20	3.72	3.53	2.19
26	Onobaru	P <sub>0</sub>	39	5.01	3.95	2.01
22	Tsubame-1	P <sub>++</sub>	69	4.86	4.26	2.18

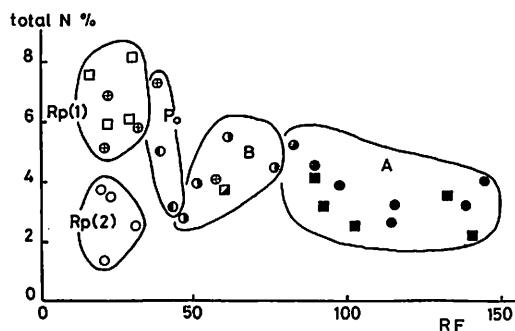


Fig. 1. Relationship between total-N % and RF value of humic acids.

4 types of humic acids. The ratio of hydrolysable-N to total-N ranged from 32 to 96%, and decreased with the increase in RF values, especially among A type humic acids (Fig. 3). There was no remarkable difference between hydrolysable-N contents of Rp(1) and Rp(2) type humic acids. A very significant negative linear correlation ( $r = -0.749^{***}$ ) was found between the ratio of hydrolysable-N to total-N and RF value.

(3) *Non-hydrolysable-N contents.* The nitrogen in the precipitates of the 8N H<sub>2</sub>SO<sub>4</sub> hydrolysates of humic acids was regarded as non-hydrolysable-N. Non-hydrolysable-N contents in humic acids increased with increasing RF values as shown by the significant positive linear correlation between them ( $r = 0.492^{***}$ ) (Fig. 2). The

tribution in humic acids.

6N HCl hydrolysis			$\frac{\text{Ammonia-N}}{\text{Total-N}} \times 100$	$\frac{\text{Amino acid-N} + \text{ammonia-N}}{\text{Hydrolysable-N}} \times 100$
Amino acid-N	Ammonia-N	Amino acid-N + ammonia-N		
1.17%	0.641%	1.81%	15.7%	84.6%
0.79	0.492	1.28	18.9	
1.36	0.650	2.01	24.4	131
1.81	0.763	2.57	27.6	150
2.16	0.858	3.02	19.4	81.8
2.01	0.748	2.76	13.4	59.7
2.09	0.756	2.85	20.4	102
1.93	0.550	2.48	20.0	146
3.40	0.732	4.13	12.7	83.6
3.52	0.860	4.38	14.2	83.8
2.14	0.565	2.71	15.2	76.8
2.46	1.142	3.60	22.8	91.1
2.04	0.972	3.01	20.4	70.7

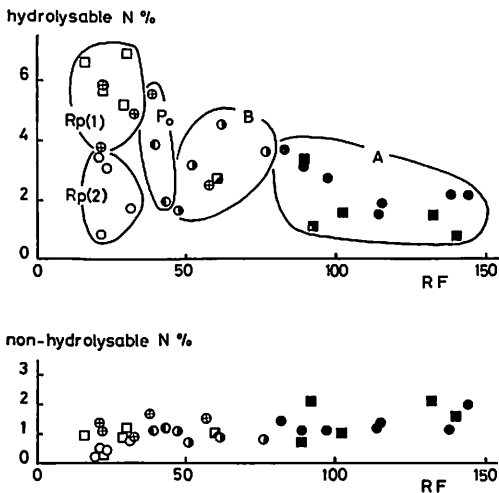


Fig. 2.

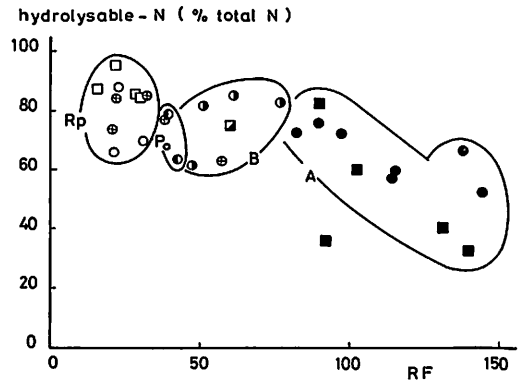


Fig. 3.

Fig. 2. Relationships between hydrolysable- and non-hydrolysable-N % and RF value of humic acids.

Fig. 3. Relationship between the ratio of hydrolysable-N to total-N and RF value of humic acids.

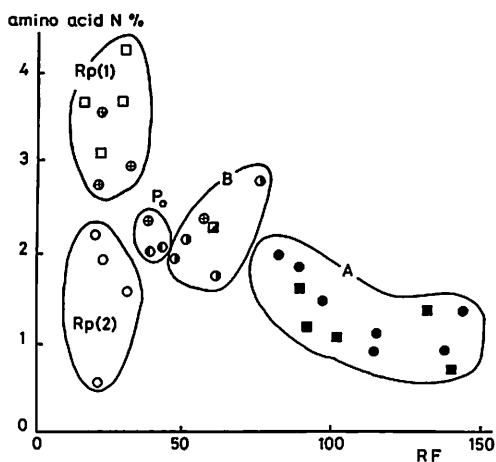


Fig. 4.

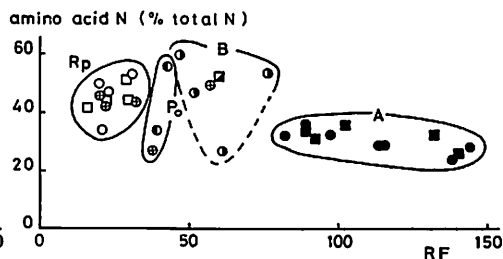


Fig. 5.

Fig. 4. Relationship between amino acid-N % and *RF* value of humic acids.

Fig. 5. Relationship between the ratio of amino acid-N to total-N and *RF* value of humic acids.

increase in the non-hydrolysable-N content, however, was equivalent to only about 15% of the decrease in the hydrolysable-N as indicated by the slopes of their regression lines against *RF* value ( $b=0.0049$  for non-hydrolysable-N and  $b=-0.033$  for hydrolysable-N, respectively). Therefore, it is considered that most part of the hydrolysable-N in humic acids were released from the humic acid fraction during humification. The ratio of non-hydrolysable-N to total-N ranged from 4 to 68% and increased with increasing *RF* value, especially among A type humic acids (Fig. 3). The contribution of non-hydrolysable-N to the color of humic acids is thought to be small, taking into account the small rate of increase of the former.

(4) *Amino acid-N contents by 8 N H<sub>2</sub>SO<sub>4</sub> hydrolysis.* The amino acid-N content showed very highly linear positive correlations with both the hydrolysable-N and total-N content ( $r=0.883^{***}$  and  $r=0.862^{***}$ , respectively). Similar to the total-N and hydrolysable-N, the amino acid-N content increased from Rp(2) type to Rp(1) type, and then decreased among Rp(1), P<sub>0</sub>, B, and A type with increasing *RF* value (Fig. 4), and a very significant negative linear correlation ( $r=-0.861^{***}$ ) was found between the amino acid-N content and *RF* value. The ratio of amino acid-N to total-N ranged from 25% to 60% (Fig. 5). A significant negative linear correlation ( $r=-0.597^{**}$ ) was found between the ratio and *RF* value among Rp(1), P<sub>0</sub>, B, and A type humic acids. The ratio for A type humic acids was lower than that for other type humic acids and decreased gradually with increasing *RF* value, but the trend of decrease was not so distinct among Rp(1), P<sub>0</sub>, and B type humic acids. Comparing this ratio with the ratio of hydrolysable-N to total-N, which decreased with increasing *RF* value, especially sharply among A type humic acids, it is presumed that amino



acids are rather stable constituents among the acid-hydrolysable nitrogen constituents.

(5) *Ammonia by 6 N HCl hydrolysis.* Ammonia was determined only for the 6 N HCl hydrolysates of 14 humic acid samples but not for the 8 N H<sub>2</sub>SO<sub>4</sub> hydrolysates (Table 3). Among the Rp(1), P<sub>0</sub>, B, and A type humic acids, the yields of ammonia were similar and no significant linear correlation was found between the yields of ammonia and the RF values ( $r = -0.563$  N.S.). In addition, no significant linear correlation existed between the ratio of ammonia-N to total-N and RF value among Rp(1), P<sub>0</sub>, B, and A type humic acids. But this ratio tended to increase from Rp type to B type, and decreased with increasing RF value among A type humic acids. This decrease among A type humic acids corresponds to the sharp decrease in the ratio of hydrolysable-N to total-N among A type humic acids as shown in Fig. 3. Some ratios of the sum of amino acid-N and ammonia-N to hydrolysable-N (Table 3) exceeded 100% among A and B type humic acids. This may be because each of the amino acid, ammonia, and hydrolysable-N content was determined by different method. These ratios gave rather lower values among Rp type humic acids. This result, however, may not necessarily mean that Rp type humic acids contain more hydrolysable nitrogenous constituents other than amino acid and ammonia, because it is known that amino compounds are likely to react with sugars and phenolics (1, 2), which are abundant in Rp type humic acids (13) and difficult to remove from humic acids before hydrolysis.

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