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Permanganate Oxidation of Methylated and Unmethylated Fulvic Acid, Humic Acid, and Humin Isolated from Raw Humus

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Methylated and non-methylated raw humus and its fractions humin, humic acid, and fulvic acid have been oxidized by potassium permanganate at pH 9–10. After methylation with diazomethane and separation by preparative thin-layer and gas chromatography, the oxidation products found were 8 benzenecarboxylic acid methyl esters, 13 methoxy-benzenecarboxylic acid methyl esters, 7 1,2-dimethoxy-benzenecarboxylic acid methyl esters, 11 dicarboxylic acid dimethyl esters, and 2 dimethoxy-carbomethoxy-diazines. Qualitatively no major differences were found between the various humic fractions even though these fractions represent materials with large differences in solubility and molecular weights. The differences found between methylated and non-methylated samples were greater than the difference between the various humic fractions. In general, a higher yield of benzenecarboxylic acids was found from non-methylated material, and the higher homologs of the dicarboxylic acids ($C_{11}-C_{14}$) were found in this material only. 1,2-Dimethoxy-benzenecarboxylic acid methyl esters were detected among the oxidation products of methylated fractions only. A small amount of 4 different methoxy-benzenecarboxylic acids was found from unmethylated fractions, whereas methylation prior to oxidation sharply increased the total yield of this group of compounds. The dimethoxy-carbomethoxy-diazines were found from methylated material only and believed to be an artefact from the methylation with diazomethane.

For methylated material, the most pronounced difference between the various humic fractions was the decrease of the total amount of dimethoxy-benzenecarboxylic acids and the increase in the amount of dicarboxylic acids in the order humin, humic acid, and fulvic acid. The increase in the amount of dicarboxylic acids was mainly due to an increase in the amount of the lower homologs. For all humic samples, condensed aromatic structures are evidently of importance. One such structure is exemplified, based on the finding that 1,2,3,5-benzenetetracarboxylic acid is an oxidation product of non-methylated materials only. The methoxyl content (2 %) of humic materials is due to a limited number of structures, mainly those giving 2-methoxy-1,4-benzenedicarboxylic acid and 6-methoxy-1,2,4-benzenetricarboxylic acid by oxidation. Structures with 1,2-dimethoxy

substitution on the benzene ring are not present. The possibility of small amounts of 1-methoxy-2-hydroxy substituted structures cannot be excluded. 1,2-Dihydroxy substituted aromatic entities contribute significantly to the humic structure (30 – 50 % of the oxidation products) and are twice as abundant as the hydroxy substituted structures. The phenolic entities are linked together by aliphatic chains or attached to cycloaliphatic rings, the most prominent chain length is 6 – 8 CH_2 units. The aliphatic part of the humic material amounts to 17 – 44 % of the oxidation products from methylated materials.

The classical method of fractionating soil organic matter involves extraction with bases to give fractions like fulvic acids, humic acids, and humin. This method and the further separation into subfrations are reviewed by Kononowa.[1] Very little is known about the detailed chemical structure of these fractions.

From a naturally occurring fulvic acid, small amounts of 21 different phenolic and benzenecarboxylic acids were detected by chromatographic separations.[2] Recently, permanganate oxidation of methylated fulvic and humic acids and humin was shown to give a mixture of 63 – 76 % benzenecarboxylic, 32 – 20 % phenolic and 5 – 4 % aliphatic carboxylic acids.[3] A comparison has been performed between the yields of the different oxidation products resulting from permanganate oxidation of methylated and non-methylated acid resistant humic residue.[4] The results demonstrated that more structural information was obtained by this method than by oxidation of methylated samples alone.

The purpose of this work has been to investigate the composition of different humic fractions by oxidation of both methylated and non-methylated samples.

A comparison has been made between original raw humus (sample A) and its fractions: the alkali insoluble humic residue, humin, (sample B); the alkali extracted, acid insoluble humic acid (sample C), and the alkali extracted, acid soluble fulvic acid. The last mentioned material was separated into two fractions, the high molecular weight matter (fraction D) and the material able to pass through the dialysis bag used for purification (fraction E). Part of each fraction was methylated with diazomethane to give fractions AM, BM. a.s.o.

The yield of the different fractions was humin (B) 61 %, humic acid (C) 12 %, fulvic acid (D) 11 %, and low molecular weight fulvic acid (E) 9 %. The analytical data, Table 1, show that humic acid contains more C, N, and methoxyl than the two fulvic acids. After methylation, both fraction C and E have higher methoxyl contents than fraction D. Since the O content (calculated by difference) in fractions D and E is higher than in fraction C, the data indicate that fraction D contains more oxygen-containing functional groups which fail to react with diazomethane in methanol than do fractions C and E. This is verified by the carbohydrate analysis which demonstrates that twice as much carbohydrate (43 %) is present in fraction D as in fractions C and E.

Table 1. Elementary composition, methoxyl, carbohydrate, and ash contents of raw humus (A), humin (B), humic acid (C), fulvic acid (D), and low molecular weight fulvic acid (E).

Element or group	A	B	C	D	E
C	36.6	34.3	49.6	40.5	40.7
H	4.76	4.37	5.97	5.78	6.06
N	1.25	0.83	2.98	1.72	1.80
OCH_3	2.1	2.0	2.3	1.8	
Carbohydrate	22.8	20.0	17.3	42.7	21.7
Ash	31.0	31.0	5.8	6.3	7.7
OCH_3 after methylation	11.0	10.6	20.2	14.1	24.2

Prior to oxidation the water soluble fulvic acids, samples D and E, were investigated by gel separation. Sephadex G25 was used with water as eluent. According to the chromatogram. Fig. 1, sample D consisted of high molecular weight compounds. The difference in the two samples seemed obvious, al-

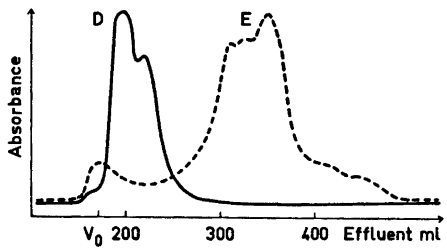

Fig. 1. Separation of high (D) and low (E) molecular weight fulvic acids on Sephadex G25.

though, because of the possibility of adsorption of humic compounds to the dextran gel, as shown by Lindquist,[5] the E sample might not necessarily contain low molecular weight compounds only. By gel separation of the methylated sample E, (sample EM) only 6 % of the fraction was eluted as low molecular weight material, *i.e.* at the same elution volume as methylbenzoate. Sephadex LH20 was used with methanol as eluent. Only trace amounts of the lower molecular weight fraction, and nothing of the other fractions isolated by separation on Sephadex LH20, was eluted from a gas chromatographic column programmed from 150° to 300°C. The results demonstrate that the major part of sample E also consists of relatively high molecular weight or nonvolatile compounds, even after methylation with diazomethane.

The original raw humus and its fractions, both methylated and non-methylated, were oxidized with potassium permanganate at 90°C at pH 9–10. The oxidation products were extracted, methylated, and separated by preparative thin-layer chromatography, followed by preparative gas chromatography.

Table 2. Yields of compounds isolated from methylated and non-methylated humic fractions after oxidation. A, raw humus; B, humin; C, humic acid; D, fulvic acid; and E, low molecular weight fulvic acid. Methylated fractions are marked with M. Yields refer to 100 g samples.

Component No	Compounds	AM mg	BM mg	CM mg	DM mg	EM mg	A mg	B mg	C mg	D mg
1	1,2-Benzenedicarboxylic acid dimethyl ester			13	18			2		
2	1,2,3-Benzenetricarboxylic acid trimethyl ester			17	17	27	28	25	6	14
3	1,2,4-Benzenetricarboxylic acid trimethyl ester	46	40	79	68	35	70	60	110	110
4	1,2,3,4-Benzenetetracarboxylic acid tetramethyl ester	59	40	69	66	43	150	62	100	68
5	1,2,3,5-Benzenetetracarboxylic acid tetramethyl ester					12	75	47	93	68
6	1,2,4,5-Benzenetetracarbozylic acid tetramethyl ester	29		23	42	17	37	31	56	40
7	Benzenepentacarboxylic acid pentamethyl ester	24		26	33	45	61	33	97	36
8	Benzenehexacarboxylic acid hexamethyl ester					17	12		8	
9	4-Methoxy-benzoic acid methyl ester	150	250	450	270	210				
10	3-Methoxy-1,2-benzenedicarboxylic acid dimethyl ester	130	120	100	49	50				
11	4-Methoxy-1,3-benzenedicarboxylic acid dimethyl ester	130	110	250	140	120				
12	2-Methoxy-1,4-benzenedicarboxylic acid dimethyl ester	29			38	5	7	7	15	23
13	4-Methoxy-1,2,3-benzenetricarboxylic acid trimethyl ester	250	170	160	79	15				
14	3-Methoxy-1,2,4-benzenetricarboxylic acid trimethyl ester	110	95	72	52	43				
15	5-Methoxy-1,2,4-benzenetricarboxylic acid trimethyl ester			81						
16	6-Methoxy-1,2,4-benzenetricarboxylic acid trimethyl ester	87	10	81	55	46	15	25	51	27
17	2-Methoxy-1,3,5-benzenetricarboxylic acid trimethyl ester			22	17	32				1
18	5-Methoxy-1,2,3,4-benzenetetracarboxylic acid tetramethyl ester	52	23	32	30	40				
19	4-Methoxy-1,2,3,5-benzenetetracarboxylic acid tetramethyl ester	120	84	110	65	75		3		4
20	3-Methoxy-1,2,4,5-benzenetetracarboxylic acid tetramethyl ester		6	20	24	18				
21	Methoxy-benzenepentacarboxylic acid pentamethyl ester	8			7	20				
22	3,4-Dimethoxy-benzoic acid methyl ester	330	600	960	510	460				
23	3,4-Dimethoxy-1,2-benzenedicarboxylic acid dimethyl ester	96	90	69	36	26				
24	4,5-Dimethoxy-1,2-benzenedicarboxylic acid dimethyl ester	390	260	650	210	330				
25	4,5-Dimethoxy-1,3-benzenedicarboxylic acid dimethyl ester	210	390	710	540	220				
26	4,5-Dimethoxy-1,2,3-benzenetricarboxylic acid trimethyl ester	310	280	240	150	81				
27	Dimethoxy-benzenetricarboxylic acid trimethyl ester	140	110							
28	Dimethoxy-benzenetetracarboxylic acid tetramethyl ester	17	17		13	13				
29	Chloro-3,4-dimethoxy-benzoic acid methyl ester	140	240	60	120	32				
30	Dichloro-3,4-dimethoxy-benzoic acid methyl ester				2	3				
31	Succinic acid dimethyl ester	19	5	42	170	260			18	
32	Glutaric acid dimethyl ester	60	15	73	110	310	6	4	61	

Table 2. Continued.

33	Adipic acid dimethyl ester	180	87	180	230	390	65	44	200	
34	Pimelic acid dimethyl ester	190	160	390	160	370	150	130	240	
35	Suberic acid dimethyl ester	160	170	410	95	230	200	170	370	33
36	Azelaic acid dimethyl ester	160	170	420	110	170	190	120	310	54
37	Sebacic acid dimethyl ester						50	30	110	22
38	Hendecaneidioic acid dimethyl ester						26	21	61	17
39	Dodecanedioic acid dimethyl ester						23	22	34	12
40	Tridecaneidioic acid dimethyl ester						8	14	11	7
41	Tetradecanedioic acid dimethyl ester						3	8	5	6
42	Dimethoxy-carbomethoxy-diazine I	200	140	37	36	120				
43	Dimethoxy-carbomethoxy-diazine II	130			23	130				

Table 3. Groups of compounds isolated from methylated and non-methylated humic fractions after oxidation. Yields refer to 100 g samples. The contribution of each group relative to the total amount of identified compounds is given in per cent.

Compounds		Fraction								
		AM	BM	CM	DM	EM	A	B	C	D
Benzenepolycarboxylic acid polymethvl esters	g	0.16	0.08	0.23	0.24	0.20	0.43	0.26	0.47	0.34
	%	4	2	4	7	5	37	31	24	63
Methoxy-benzenepolycarboxylic acid poly-methyl esters	g	1.07	0.87	1.38	0.83	0.67	0.02	0.03	0.07	0.05
	%	27	23	23	23	17	2	3	4	9
Dimethoxy-benzenepolycarboxylic acid poly-methyl esters	g	1.63	1.99	2.69	1.58	1.17				
	%	41	54	46	44	30				
Dicarboxylic acid dimethyl esters	g	0.77	0.62	1.52	0.88	1.73	0.72	0.56	1.42	0.15
	%	20	17	26	24	44	61	66	72	28
Dimethoxy-carbomethoxy-diazines	g	0.33	0.14	0.04	0.06	0.15				
	%	8	4	1	2	4				
Total	g	3.96	3.70	5.85	3.59	3.92	1.17	0.85	1.96	0.54
	%	100	100	100	100	100	100	100	100	100

The chemical structure and yield of all components identified are shown in Table 2. The results refer to 100 g samples where fractions A and B contain 31 % ash, and fractions C, D, and E show ash contents of 5.8 – 7.7 %. The identity of each compound was established by comparing thin-layer and gas chromatographic retention data, IR and mass spectra with those of known standards. No standards were available for compounds 18, 27 – 30, and 38 – 43. In these cases identification was tentatively based on the spectra obtained.

The infrared spectra of compounds 18 and 27 – 30 indicate that they all are aromatic esters. The molecular ion is easily recognized in all the mass spectra and the base peak is at M – 31. The fragmentation pattern of compound 18 is similar to that of compounds 19 and 20. The same relation is found between the mass spectra of compounds 26 and 27. Compounds 29 and 30 also show the characteristic "doublet" of the molecular ion and the base peak (M – 31), and with intensities indicating a mono- and dichloro substituted compound, respectively.

Compounds 28 – 41 give the infrared spectra typical of dicarboxylic acid dimethyl esters. The base peak in the mass spectra is as M – 31, and the molecular ion is of low intensity. In all spectra, a strong peak at *m/e* 74 is found; this is due to γ-hydrogen rearrangement of the methyl ester. Gas chromatographic retention data support the identity of compounds 38 – 41.

By mass spectroscopy the molecular formula of compounds 42 and 43 are determined to $C_8H_{10}N_2O_4$(M.W. 198). According to NMR spectra both compounds contain two methoxyl and one carbomethoxy group. The carboxyl absorption is verified by their IR spectra (1720 cm^{-1}). The last proton resonates at $\tau = 2.17$ ppm and $\tau = 2.57$ ppm for compounds 42 and 43, respectively. The results therefore indicate a substituted diazine structure, although the position of the two nitrogen atoms in the ring is uncertain.

The components isolated can be classified as benzenecarboxylic acid methyl esters (compounds 1 – 8), methoxy-benzenecarboxylic acid methyl esters (compound 9 – 21), dimethoxy-benzenecarboxylic acid methyl esters (compounds 22 – 30), aliphatic dicarboxylic acid dimethyl esters (compound 31 – 41), and dimethoxy-carbomethoxy-diazines (compounds 42 – 43). The results have been summarized in Table 3.

DISCUSSION

For all the isolated fractions the most prominent benzenecarboxylic acid methyl esters are compounds 4 and 3, followed by the methyl ester of benzenepentacarbozylic acid. These structures are schematically represented by I, II, and III in Fig. 2. The only exception is the methylated humin fraction (BM) where compounds 6 and 7 are not present in detectable amounts. The same is the case with compound 2. Disregarding these exceptions, the differences found between the humic fractions isolated were smaller than the differences introduced between methylated and non-methylated fractions. Especially noteworthy is the absence of compound 5 in methylated fractions (except for the small amount found in fulvic acid (EM)). This indicates the presence of condensed structures such as IV of Fig. 2. Depending on whether this structure has been methylated or not prior to oxidation, the products

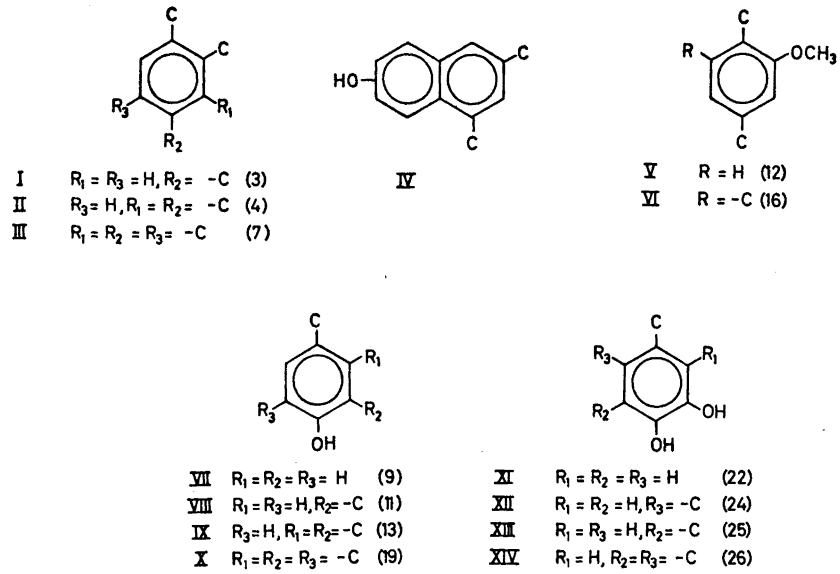

Fig. 2. Some of the major structural units in humus and humic fractions. The symbol —C denotes carboxyl or a group giving carboxyl by oxidation. The number in brackets gives the compound formed by oxidation and refers to Table 2.

will be a methoxy-benzenecarboxylic acid or 1,2,3,5-benzenetetracarboxylic acid. Different substitution and/or different ring systems might yield the oxidation products identified depending on whether the phenolic structure is methylated or not. The importance of these condensed systems, also possibly similar to those believed to be present in lignin,[6,7] is shown by the fact that the yields of benzenecarboxylic acids in general are higher for the unmethylated samples relative to the methylated samples (Tables 2 and 3).

Methoxy-benzenecarboxylic acid methyl esters are found in significant amounts among the products from oxidation of methylated samples only. Since the only methoxy derivatives found for the non-methylated samples are compounds 12,16, 17, and 19, at least part of the methoxyl content of the humic fractions (Table 1) is present in structures giving these four methoxy-benzenecarboxylic acids by oxidation. Whether the humic fractions are methylated or not prior to oxidation, the yields of compounds 12 and 16 remain fairly constant, this in contrast to the yields of the other methoxy-benzenecarboxylic acid methyl esters isolated (Table 2). This indicates that nearly all the methoxy substituted structures identified are originating from humic phenols (VII—XIV, Fig. 2). It follows, further, that the methoxyl content in humic fractions is due to a limited number of structures only, mainly those (V, VI of Fig. 2) giving compounds 12 and 16, respectively, by oxidation. Of course, also structures containing 1-methoxy-2-hydroxy substitution on the benzene ring may contribute to the methoxyl content of humus, but the data given here fail to clarify this particular question. Di-

methoxy substitution on benzene rings in humus is definitely ruled out by the absence of dimethoxy-benzenecarboxylic acids among the oxidation products from non-methylated fractions.

For all the isolated fractions, compound 9 is the most prominent among the methoxy-benzenepolycarboxylic acid polymethyl esters, followed by compounds 11, 13, 10, and 19. According to Table 3 the total yield of this group of compounds is remarkably constant for the humic fractions BM, CM, and DM (fraction EM is somewhat lower) with regard to their different chemical properties.

From Table 3 it is evident that structures with 1,2 dihydroxy (or possibly 1-methoxy-2-hydroxy) substitution on the benzene ring are contributing significantly to the structure of the raw humus used in this study, yielding 30 – 54 % of the oxidation products. The different aromatic acids isolated have different stability to alkaline permanganate oxidation;[7] their contribution to the humic structure is, therefore, not necessarily reflected by their yields.

Two main features are evident from Table 3, namely the increasing contribution of dimethoxy derivatives for fractions BM, CM, and DM relative to AM, and the decreasing relative amount from fraction BM to fraction EM (from 54 – 30 %). The oxidation of the different fractions is performed under the same condition, and thus believed to give comparable results, even though the possibility exists that the oxidation is influenced by the different solubilities of the humic fractions. The observed yields of samples AM and BM indicate that the alkali treatment of raw humus introduces free phenolic groups available for later methylation. The relative contributions of compounds 22 and 25 both increase from sample AM to BM (from 8.3 to 15.8 % and from 5.3 to 10.5 % of the total yield, respectively). The compounds 23, 24, and 26 show no significant increase. The results, therefore, indicate that structures producing compounds 22 and 25 by oxidation partly exist as 1,2 dihydroxy (1-methoxy-2-hydroxy) substituted entities and partly as structures where the one phenolic group is bound for example in an ester linkage. The other oxidation products in this group probably exist as *o*-diphenols in humus. Therefore, compound 27 most probably is 5,6-dimethoxy-1,2,4-benzenetricarboxylic acid trimethyl ester, and compound 28 5,6-dimethoxy-1,2,3,4-benzenetetracarboxylic acid tetramethyl ester.

Compounds 29 and 30 have previously been found among the oxidation products from a raw humus, hydrolyzed with hydrochloric acid,[4] and were therefore believed to be an artefact from that treatment. However, if 3,4-dimethoxy-benzoic acid and manganese dioxide in water are treated according to the procedure for oxidation mixtures in this work, both compounds 29 and 30 are synthetized if the reaction mixture gets too acidic.[8] Both components are therefore artefacts from the isolation procedure, and should correctly be calculated as component 22, which is the most prominent of all the oxidation products found.

As seen from Table 3 the percentage of dimethoxy-benzenecarboxylic acid methyl esters decrease from fraction BM to EM. With few exceptions this decrease is reflected among all compounds 22 – 26. Thus, as for the methoxy substituted components, neither does the dimethoxy derivatives show any

definite difference between the various humic fractions based on the relative amount of the different dimethoxy-benzenecarboxylic acid methyl esters.

The percentage of dicarboxylic acid dimethyl esters increases from fraction BM to EM with a possible exception for fraction DM. This indicates that the more soluble humic fractions, *i.e.* the fulvic acids, contain more aliphatic units than the less soluble humic acids and humin. By urea adduct formation, only straight chain dicarboxylic acids were shown to be present among the oxidation products from the same humic material after acid hydrolysis.[4] The stability of sebacic acid when oxidized at the same conditions as in this study resulted in a recovery of 43 % material, which, after methylation, consisted of 90 % sebacic acid dimethyl ester and 3.6 % suberic acid dimethyl ester together with decreasing amounts of the lower homologs down to succinic acid dimethyl ester.[4] The actual contribution of the aliphatic part found here is, therefore, definitely larger than indicated by the yields and must also include the different chain lengths.

The accuracy of the yields for compound 31, and also to some degree compound 32, should not be overemphasised as, unavoidably, some losses by evaporation occurred during the isolation procedure. Except for these two components, there is a general shift in yields towards shorter chain lengths from fraction BM to EM (compounds 36 – 33 in Table 2). The increase in the aliphatic part of the more soluble humic fractions is thus mainly due to an increase of shorter chain lengths, this at the expense of the content of 1,2-dihydroxy substituted entities.

The long chain dicarboxylic acid dimethyl esters compounds 37 – 41, are products of unmethylated material only. This is in accordance with earlier findings, and supports the assumption of phenolic components in humus being linked together by aliphatic chains or, to a smaller extent, cycloaliphatic rings attached to one phenolic structure.[4] Oxidation of the phenolic structure will first break down the benzene ring thus giving an aliphatic carboxylic acid only. Methylation of the phenolic structure, followed by oxidation, will, due to the greater stability of the methyl ether, more easily break the aliphatic C – C bonds. If the bond between the α and β carbon atom is oxidized, the products will be one methoxybenzene carboxylic acid plus one aliphatic carboxylic acid, at least two CH_2 units shorter than the oxidation product from the non-methylated structure. If the aliphatic chain links two penolic structures together, the effect of methylation is doubled. According to the data given in Table 2 the most prominent chain lengths consist of 6 – 8 CH_2 units with predominance of shorter chains in the more soluble humic fraction. Still, the explanation fails to comply well with the complete absence of compounds 31 – 34 for fraction D and its low yield of dicarboxylic acid (Table 3).

Compounds 42 and 43 are found among the oxidation products of methylated materials only. The question arises whether these compounds are artefacts due to isolation procedure. In general, 70 – 80 % of the nitrogen in soil organic matter can be accounted for, mainly as amino acids, amino sugars and ammonia after acid hydrolysis. The chemistry of the residual nitrogen is not known, although several teories have been forwarded.

If the diazine ring is part of the humic structure, one would expect it to appear also with other methylation agents, for instance dimethyl sulphate.

This is not the case, as repeated methylations of humin by dimethyl sulphate failed to give compounds 42 and 43 among the methylated oxidation products. Diazomethane is known to increase the nitrogen content of humic materials,[4,9] even if the increase for the fractions used in this study was too small to be significant (except for fraction DM). Diazomethane adds to double bonds to give five-membered rings with nitrogen in neighbouring position.[10] These components are usually labile at higher temperatures. Diazomethane might add to some specific structures in humus with, for example, subsequent rearrangements to six membered rings during oxidation or in the gas chromatograph. Whether this is the case or not is hard to decide from the present information. This point will probably be better understood when more structural information is available about the position of the nitrogen atoms in the diazine ring.

Low in methoxyl content (Table 1), fraction D was repeatedly treated with diazomethane in order to increase the methoxyl content. This fraction was also the only one responding with significant increase in the nitrogen content (8 %) to methylation. In spite of this fact, the yield of compound 42 and 43 is relatively low for fractions DM. The origin of the dimethoxy-carbomethoxy-diazines is, therefore, still a matter of conjecture, although, most probably, they are artefacts from the diazomethane treatment.

The results given here clearly demonstrate the complexity of humic materials, and also the relatively slight differences between the various humic fractions.

EXPERIMENTAL

Material. The ether and ethanol extracted raw humus, investigated earlier,[11] was also used in this study, sample A.

A sample of the humus (150 g) was percolated at room temperature with 0.5 N sodium hydroxide until an almost colorless eluate was obtained (2.5 l for 4 days). In order to reduce oxygen absorption and condensation reactions,[1] the whole extraction unit was kept under nitrogen, and 9 N sulphuric acid was continuously added to the eluate to maintain a pH of 3 – 4. The residue was repeatedly suspended in dilute sulphuric acid at pH 2 – 3 and filtrated to exchange sodium ions, and then thoroughly washed with distilled water and dried. Sample B, yield 91 g.

To isolate the humic acids, the extract was adjusted to pH 1.5 with sulphuric acid and centrifuged. The precipitate was stirred with water and then again centrifuged. Finally, the precipitated humic acid, suspended in water, was dialyzed against distilled water until free of sulphate ions. Yield of dry matter, sample C, 18 g.

The filtrate from humic acid precipitation was concentrated to 500 ml and dialyzed against distilled water (Kalle dialysis bag) until the dialyzate was negative on sulphate ions. The desalted material gave the higher molecular weight part of fulvic acid after drying, sample D. Yield 16 g.

The fulvic acid dialysate was evaporated to 50 ml at 30°C, 12 mmHg, and adjusted to pH 2 – 3 by adding sodium bicarbonate. The mixture was treated with 200 ml of methanol, and the precipitate, mainly sodium sulphate, was filtered off. The filtrate was concentrated and again treated with methanol (200 ml) and filtered. The process was repeated several times, and gave a methanol solution of fulvic acid sample E. This sample could not be completely dried without destructions taking place. It was therefore methylated with diazomethane before drying. Yield sample EM 14 g.

Methylation. The dried and ground samples (5 – 10 g) were suspended in 250 ml of methanol and exhaustively treated with diazomethane in ether at 5°C until maximum methoxyl content (1 – 3 days). The methylation gave no significant rise in nitrogen content (less than 5 %) except for fraction D, where the increase was 7.5 %.

To sample B (10 g), suspended in 250 ml dioxane + 150 ml water at 65°C, was added droppwise 33 ml dimethyl sulphate and a solution of 20 g sodium hydroxide in 80 ml water under stirring. After 2 h the humic sample was filtered off, rinsed with water, and again methylated as above.

Carbohydrate analysis. The samples (50 mg) were subjected to hydrolysis in 10 ml 0.5 N hydrochloric acid for 5 h. After filtration the solution was diluted to 250 ml. The hydrolysis dissolves a major part of the soil carbohydrates and also almost eliminates interfering background from humic substances. Aliquots (1 ml) of the diluted filtrate were analysed for carbohydrate according to the phenol-sulphuric acid method (Table 1).

Each hydrolysate was concentrated to 1 ml at 12 mmHg and 40°C. 1 and 10 μl aliquots were spotted on Whatman No. 1 paper and developed by the descending technique in ethyl acetate : acetic acid : water (3:3:1) for 24 h. After drying, the spots were developed by spraying with aniline phthalate reagent and drying at 105°C. For fractions A, B, C, and D the chromatograms appeared identical. Arabinose, galactose, glucose, mannose, rhamnose, and xylose were identified and estimated to be present in approximately equal amounts. Smaller amounts of fucose and ribose were also present together with higher molecular weight carbohydrate material, as demonstrated earlier.[11] Fraction E contained the same carbohydrates as mentioned above but obviously in smaller amounts.

Oxidation. The sample (5 – 10) g was suspended in 250 – 500 ml of water containing 5 – 10 g of sodium carbonate, to which was added dropwise a solution of 5 % potassium permanganate under stirring at 90°C, until no more reagent was consumed (6 – 8 h). The reaction mixture was acidified with hydrochloric acid and sodium bisulfite added to reduce manganese dioxide. The warm solution was filtered and the oxidation residue dried and weighed. The filtrate (250 – 500 ml) was extracted 4 times with 200 ml of chloroform : acetone (1:1) and 4 times with 200 ml of ethyl acetate. Each extract was dried with sodium sulphate, evaporated to dryness, and the yield recorded. The extracted acids were dissolved in methanol and methylated with diazomethane. Avoiding high temperatures, the ester mixture was taken to dryness and the yield recorded.

Yields: 10.0 g of fraction AM: 0.93 g acids, 0.85 g esters, and 4.5 g oxidation residue; 10.0 g BM: 0.86 g acids, 0.85 g esters, 4.7 g residue; 10.0 g CM: 1.14 g acids, 1.05 g esters, 0.4 g residue; 10.0 g DM: 0.66 acids, 0.67 g esters, 0.01 g residue; 8.9 g EM: 1.02 g acids, 0.83 g esters, 0.01 g residue; 10.0 g A: 0.61 g acids, 0.36 g esters 3.6 g residue; 10.0 g B: 0.31 g acids, 0.24 g esters, 4.3 g residue; 6.1 g C: 0.48 g acids, 0.41 g esters, 0.02 g residue; 5.0 g D: 0.18 g acids, 0.13 g esters, and 0.01 residue.

Chromatographic techniques. Gel fractionation of samples D and E was carried out on a 2.5 × 85 cm column with Sephadex G25. Water was used as eluent at a flow rate of 12 ml/h (Fig. 1). Sample EM was investigated on a column of Sephadex LH 20 (2.5 × 90 cm) in methanol, flow rate 16 ml/h. The eluates were continuously monitored at 254 mμ.

The ester mixtures obtained by methylation of the extracted oxidation products were separated by preparative TLC on aluminum oxide (activated at 150°C for 20 h, thickness of coating absorbent 0.5 mm). The solvent system used was toluene : ethylacetate (3:1). After separation, each plate was inspected under a UV lamp and a number of fractions were marked out (3 – 5), scraped off the plate and extracted with ethyl acetate.

Each subfraction was further separated by preparative gas chromatography (Aerograph model 1520 B_1 TC detector, 300 × 0.4 mm s.s. column packed with 4 % OV-17 on Chromosorb W HMDS, 60/80 mesh, programmed from 150 to 300°C at a rate of 10°C per min, helium being used as carrier gas). The material eluted from the column, according to recorder response, was collected in glass tubes, and analyzed by IR as micro KBr pellets on a Unicam SP 200 spectrophotometer equipped with a beam condenser, and by mass spectrometry on a Perkin Elmer Hitachi RMU-6N mass spectrometer equipped with a heated direct inlet probe.

Quantitative estimates of each major compound were made by measuring peak areas on the gas chromatogram by triangulation, and by comparing these with the peak area of reference compounds injected in known amounts. The origins of the reference compounds were identical to those described previously.[2,4]

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