The Chemical Degradation of a Humic Acid[1]

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A humic acid (HA) extracted from the A_1 horizon of a Brunizem soil was degraded in the unmethylated and methylated form by sequential reaction with oxidants of increasing strength. The HA was first oxidized with alkaline cupric oxide; the products were then further degraded by oxidation with first alkaline $KMnO_4$ and then with H_2O_2 in alkaline solution. Unmethylated HA was also degraded by Na-amalgam reduction. The degradation products were extracted into organic solvents, methylated, and separated by preparative gas chromatography into relatively pure components which were analyzed by mass spectrometry and micro-i.r. spectrophotometry. A matching of the mass and i.r. spectra and gas chromatographic retention times of the isolated components with those of authentic specimens led to their identification.

The experimental data show that the HA contains a relatively easily degradable portion, which comprises guaiacyl and syringyl units and which may be lignin-derived (about 10% of the total weight). This material is degraded by CuO–NaOH oxidation and Na-amalgam reduction. The bulk of the HA structure, however, consists of a more condensed, chemically complex "core", which degrades on more drastic oxidation into complex phenolic and benzenepolycarboxylic acids. It is likely that the "core" originates in part from condensed lignin and in part from products of microbial synthesis. Of the methods investigated, the CuO–NaOH and the $KMnO_4$ oxidation of methylated HA appear most promising for providing information on the chemical structure of the HA.

Un acide humique (HA) extrait du sol de l'horizon A_1 de Brunizem, a été dégradé sous forme non méthylée et méthylée selon une séquence réactionnelle utilisant des oxydants de force croissante. Le HA a d'abord été oxydé par CuO en milieu alcalin; les produits obtenus ont été ensuite degradés par oxydation permanganique et par H_2O_2 en solution alcaline. Le HA non méthylé a été également dégradé par réduction à l'amalgame de Na. Les produits dégradés ont été extraits par des solvants organiques, méthylés puis séparés par chromatographie en phase gazeuse préparative en composés relativement purs qui ont été analysés par spectrométrie de masse et microspectrophotométrie i.r. La comparaison des spectres de masse et i.r. et des temps de rétention de la chromatographie en phase gazeuse des composés isolés avec ceux de specimens authentiques ont suffi à leur identification

Les données expérimentales montrent que le HA comprend une partie relativement facile à degrader qui contient des unités guaiacyle et syringyle, qui peuvent être dérivées de la lignine (environ 10% du poids total). Ce matériel est dégradé par oxydation avec CuO–NaOH et réduction par l'amalgame de Na. Le gros de la structure HA consiste cependant en un noyau plus condensé, chimiquement complexe, qui se dégrade par oxydation plus brutale en phénols complexes et acides benzène polycarboxyliques. Il est probable que ce noyau tire son origine en partie de la lignine condensée et en partie des produits de synthèse microbienne. Parmi les méthodes étudiées, l'oxydation par CuO–NaOH et $KMnO_4$ de HA méthylé est une source d'information pleine de promesse sur la structure chimique de HA.

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Introduction

Humic substances are widely distributed in nature and arise from the chemical and biological degradation of plant and animal residues and from synthetic activities of microorganisms. The bulk of the organic matter in most soils and waters consists of humic substances which are amorphous, dark brown or black, hydrophilic, acidic, polydisperse substances of molecular weights ranging from a few hundred to tens of thousands. Based on their solubility in dilute alkali and acid, humic substances are usually subdivided into three main fractions: (*a*) humic acid (HA), which is soluble in dilute alkaline solution but is precipitated by acidification of the alkaline extract; (*b*) fulvic acid (FA), which is that humic fraction which remains in the aqueous acidified solution, *i.e.*, it is soluble in both acid and base; and (*c*) the humic fraction that cannot be extracted by dilute base and acid, which is referred to as humin (1). Important characteristics exhibited by all humic fractions are resistance to microbial degradation, and the ability to form stable water-soluble and water-

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insoluble salts and complexes with metal ions and hydrous oxides, and to interact with clay minerals and organic chemicals often added by man, which may be toxic pollutants.

Since quantitatively HA is the most important humic fraction, it is not too surprising that a considerable amount of research has been done on this subject. Yet, in spite of the existence of a voluminous literature, going back more than 200 years (1), very little is known about the synthesis and chemical structure of HA's. The relatively recent availability of powerful and sophisticated analytical tools such as the gas chromatographic–mass spectrometric (g.c.–m.s.) system and the g.c.–m.s.-computer system prompted us to take a new look at this problem in the expectation that significant advances in our knowledge of the chemical structure of HA's, a relatively "old" but neglected problem in chemistry, should now be possible.

Thus, we set out to degrade a HA extracted from a Brunizem soil by oxidative methods of increasing strength. The first method that we used was the relatively mild alkaline cupric oxide (CuO–NaOH) oxidation which had been pioneered by Pearl (2) on lignosulfonates and various lignin preparations. In a second series of experiments we methylated the CuO–NaOH oxidation products and then oxidized these again with alkaline $KMnO_4$ solution at p*H* 10. In a third set of experiments we went one step further and oxidized the CuO–NaOH + $KMnO_4$ oxidation products with H_2O_2 at p*H* 10. This approach was similar to that taken recently by Larsson and Miksche (3), who used a sequence of oxidants of increasing strength on birch lignin. A fourth method that we employed was alkaline $KMnO_4$ oxidation which has been used on coal (4), lignin (5), and humic substances (6). Another approach that we investigated was reduction of the HA with Na-amalgam, a relatively mild method that has been used previously on HA with varying degrees of success (7). Finally, we applied each of the oxidative degradation procedures to HA that was methylated prior to oxidation in order to evaluate the effects of methylation on the quality and quantity of products formed. Following each degradation, the products were methylated, extracted into organic solvents, separated by preparative gas chromatography, and identified by matching their mass and micro-i.r. spectra with those of authentic specimens. Our principal objective was to uncover the method or methods that would provide the most meaningful information on the chemical structure of the HA.

Experimental

HA

The soil sample from which the HA was extracted was taken from the A_1 horizon (0–23 cm) of a Brunizem soil (3.84% C; p*H* = 6.0) from a grassland area in the province of Buenos Aires, Argentina. Methods of extraction, fractionation, and purification were analogous to those described previously (8). Elemental analysis (%) of the purified HA (0.73% ash) showed 54.99 C; 4.38 H; 3.29 N; 0.84 S; 36.50 O; and 1.18 OCH_3. One gram of HA contained 4.75 mequiv CO_2H, 2.36 mequiv phenolic OH, 1.99 mequiv alcoholic OH, and 1.37 mequiv C=O. The ratio of optical densities at 465 and 665 nm (E_4/E_6) was 3.92 and the free radical concentration was 11.73×10^{17} spins/g (8).

(I) CuO–NaOH Oxidation

To 500 mg of HA dissolved in 50 ml of 2 *N* NaOH solution in a 300 ml stainless steel autoclave equipped with a magnetic stirrer, 2.5 g of CuO was added. The system was heated to 170 °C with constant stirring over a period of 1 h and then maintained at that temperature for 2 h. Following cooling to room temperature, suspended CuO was removed by centrifugation and washed with distilled H_2O. The supernatant solution plus washings were saturated with NaCl and extracted successively with 3 × 100 ml aliquots of 2:1 (v/v) chloroform–acetone after acidification to p*H* 6 and 2. When the p*H* was lowered to 2, a fraction of the water-soluble oxidation products became insoluble. The insoluble material, (Ii) was removed by filtration and the clear supernatant solution extracted with chloroform–acetone. The two organic extracts were first dried over anhydrous Na_2SO_4 and then taken to dryness in a rotary evaporator, dissolved in a small volume of CH_3OH, and methylated with an ether solution of diazomethane generated from Diazald. Yields of benzene-soluble, methylated extracts were: 32.3 mg for the extract at p*H* 6, and 27.2 mg for the extract at p*H* 2. The two extracts were combined (fraction I, see Fig. 1). The insoluble fraction (Ii) weighed 150 mg, contained 39.3% ash and, on a dry, ash-free basis, analysis (%) showed 61.62 C; 4.56 H; 2.18 N; 0.68 S; 30.96 O; and 1.19 OCH_3. The major components in fraction I were separated by preparative gas chromatography and identified by mass spectrometry and micro-i.r. spectrophotometry as described below.

(II) CuO–NaOH + $KMnO_4$ Oxidation

The CuO–NaOH oxidation of 500 mg of HA was carried out as described under method I. Fractions I and Ii were methylated with diazomethane and then oxidized with aqueous 4% $KMnO_4$ solution (6). The oxidation products were extracted from acidified aqueous solution with ethyl acetate, remethylated with diazomethane, dissolved in benzene, separated by preparative gas chromatography, and identified by mass spectrometry and micro-i.r. spectrophotometry as described below. Yields of methylated, benzene-soluble fractions II*a* (from I) and II*b* (from Ii) were 20.5 and 65.6 mg, respectively.

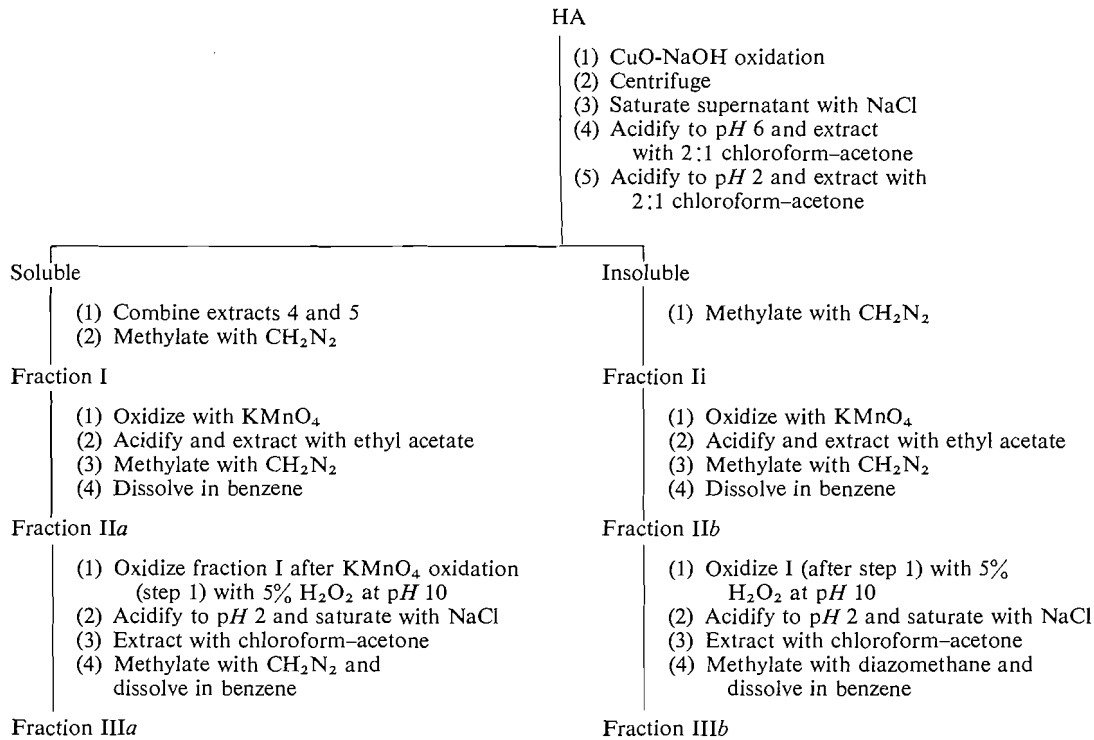


FIG. 1. Oxidation and separation of fractions I, Ii, IIa, IIb, IIIa, and IIIb.

(III) CuO–NaOH + $KMnO_4$ + H_2O_2 Oxidation

Fractions I and Ii were prepared as described above and then oxidized with 4% $KMnO_4$ solution. Water-soluble oxidation products were further oxidized by contact with 5% H_2O_2 solution at pH 10 for ½ h at room temperature with intermittent shaking and then warmed briefly to 50 °C. The excess H_2O_2 was destroyed by the addition of a few drops of dilute $KMnO_4$ solution. The resulting solutions were treated briefly with SO_2, acidified to pH 2 with 6 *N* H_2SO_4 solution, saturated with NaCl, and extracted three times with 100 ml of 2:1 (v/v) chloroform–acetone. The organic extracts were dried, methylated with diazomethane, dissolved in benzene, and analyzed as described under method I. Yields were as follows: fraction IIIa (from IIa), 2.1 mg and fraction IIIb (from IIb), 23.1 mg.

(IV) $KMnO_4$ Oxidation

Five hundred milligrams of HA was refluxed for 8 h with 125 ml of 4% (w/v) aqueous $KMnO_4$ solution (pH 10). Following oxidation, the excess $KMnO_4$ was destroyed by careful addition of small volumes of CH_3OH. The insoluble MnO_2 was removed by filtration and washed with small aliquots of hot water. The filtrate plus washings were acidified to pH 2, saturated with NaCl, extracted with 3 × 100 ml portions of 2:1 (v/v) chloroform–acetone, dried, methylated, dissolved in benzene, and analyzed as described below. The benzene-soluble, methylated oxidation product (fraction IV) weighed 34.9 mg.

(VI) Na-amalgam reduction

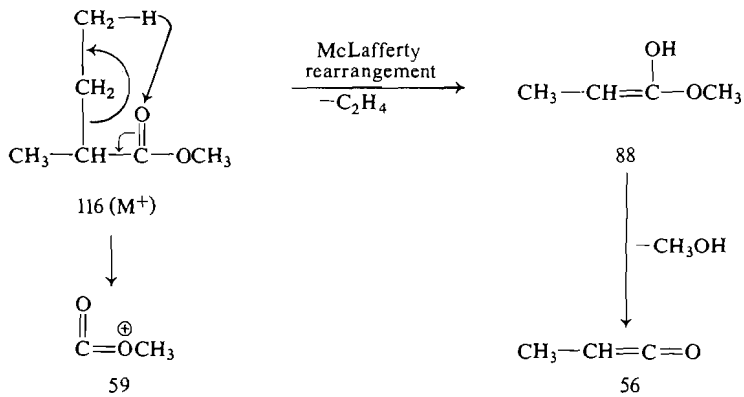
One gram of HA, dissolved in 100 ml of 1% NaOH solution, was refluxed with vigorous stirring under N_2 over 100 g of 5% Na-amalgam (BDH reagent grade) for 3 h. After cooling, the supernatant solution was separated from the insoluble residue by filtration, acidified to pH 2, and extracted successively with three 75-ml portions of diethyl ether. The ether extract was dried over anhydrous Na_2SO_4, and then methylated with diazomethane. For additional purification, the methylated reduction products were extracted with three 75-ml portions of benzene to yield fraction VI (39.50 mg). The latter was then separated by preparative gas chromatography and identified as described below.

Oxidation of Methylated HA

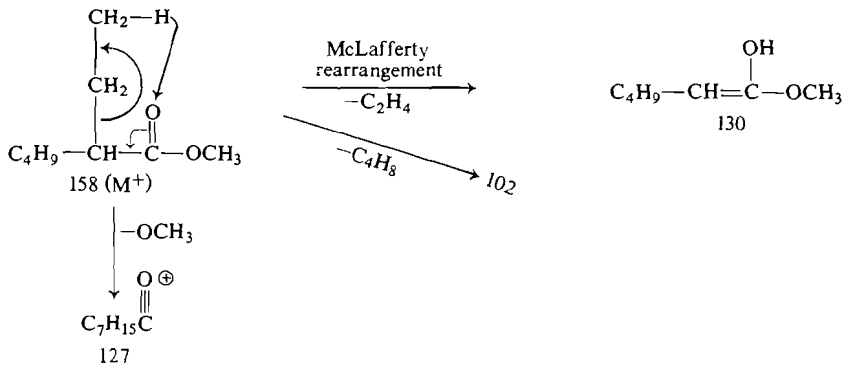
Methods I, II, III, and IV were also applied to HA that was methylated with diazomethane (increase in OCH_3 = 14.37%) prior to oxidation. The fractions so obtained are referred to as Im, IIm, IIIm, and IVm (see Table 2 and Fig. 1). Fraction Vm was prepared by oxidizing 500 mg of methylated HA with aqueous 4% $KMnO_4$ solution as described under IV. Water-soluble oxidation products were then further oxidized with 5% H_2O_2 at pH 10 as in method III.

Separation and Identification of Degradation Products

Each methylated fraction was first analyzed as a 2.5% solution in benzene by analytical gas chromatography (Hewlett-Packard, Model 402, flame ionization detector,



SCHEME 1



SCHEME 2

1800 × 4 mm glass column packed with 3% OV 17 on Chromosorb W HMDS, 60–80 mesh, programmed from 90–310 °C at a rate of 7.5 °C/min, N_2-flow = 40 ml/min), and then separated as a 10% solution in benzene by preparative gas chromatography under the same conditions. Materials representing the major gas chromatographic peaks were eluted from the gas chromatographic column, collected in capillary tubes, and analyzed by mass spectrometry and by micro-i.r. spectrophotometry as described earlier (9). Mass and i.r. spectra of unknowns were matched with those of standards of known structures. As an additional check, each unknown was co-chromatographed (on the gas chromatograph) with the known compound to which it corresponded. In some instances fractions were analyzed directly on a g.c.–m.s.-computer system. Quantitative estimates of each compound were made by triangulation of peak areas on the gas chromatograms. All solvents were purified by distillation through high-efficiency columns.

The known compounds used as standards for the mass spectrometric and i.r. spectrophotometric identification of the unknowns were either prepared as described earlier (9) or purchased commercially. Each compound was purified by suitable methods. We were unable to obtain appropriate standards for components **27** and **29**, so that the identification of these compounds is tentative only.

Compounds **1** and **5** were identified by g.c.–m.s.-computer (Shrader Analytical and Consulting Laboratories, Detroit, Michigan). The mass spectrum of **1** suggested the fragmentation pattern shown in Scheme 1. The mass spectrum for **5** suggested similarly the fragmentation pattern depicted by Scheme 2. The gas chromatographic separation of fraction Vm is shown in Fig. 2.

Analytical Methods

C and H were determined by dry-combustion, N by the automated Dumas method, S by oxygen-flask combustion, and O was calculated by difference. The OCH_3-content was measured by the Zeisel method.

Results

(A) Degradation of Unmethylated HA

(I) CuO–NaOH Oxidation

Major degradation products resulting from the CuO–NaOH oxidation of the HA are listed in Table 1 under method 1. Since the oxidation products were methylated prior to the gas chromatographic separations, and since the OCH_3-content of the untreated HA was low, it is likely that in the initial HA's the functional group in

TABLE 1. Compounds (mg) produced by the degradation of 1 g of unmethylated HA

No.	Compound	Method* I	II *a*	II *b*	II *a* + *b*	III *a*	III *b*	III *a* + *b*	IV	VI
8	1,2-Benzenedicarboxylic acid dimethyl ester	0	1.24	2.55	3.79	0.02	0	0.02	4.12	0
9	1,3-Benzenedicarboxylic acid dimethyl ester	0	0.37	0	0.37	0	0	0	0.68	0
10	3,4-Dimethoxybenzaldehyde	3.82	0	0	0	0	0	0	0	2.50
11	3,4-Dimethoxyacetophenone	4.16	0	0	0	0	0	0	0	0
12	3,4-Dimethoxybenzoic acid methyl ester	1.94	1.77	0	1.77	0	0	0	0	1.72
13	3,5-Dimethoxybenzoic acid methyl ester	0	0	0	0	0	0	0	0	1.50
15	2,3,4-Trimethoxyacetophenone	5.39	0	0	0	0	0	0	0	0
16	3,4,5-Trimethoxybenzaldehyde	4.53	0	0	0	0.13	0	0.13	0	2.00
17	3,4,5-Trimethoxyacetophenone	8.15	0	0	0	0	0	0	0	0.32
18	3,4,5-Trimethoxyphenylacetone	0	0	0	0	0	0.72	0.72	0	2.60
19	3,4,5-Trimethoxybenzoic acid methyl ester	8.65	0.57	0	0.57	0.27	2.16	2.43	0	7.25
20	*N*-Methyl-benzylsulfonamide	7.66	3.49	0	3.49	0.23	0.46	0.69	0	3.25
21	1,2,3-Benzenetricarboxylic acid trimethyl ester	0	5.36	13.35	18.71	0	4.76	4.76	32.52	0
22	1,2,4-Benzenetricarboxylic acid trimethyl ester	0	2.63	12.19	14.81	0.04	3.05	3.09	6.50	0
23	1,3,5-Benzenetricarboxylic acid trimethyl ester	0	0.59	0	0.59	0.67	4.12	4.79	0	0
24	Dibutyl phthalate	32.97	0	0	0	0	0	0	0	0
26	1,2,3,4-Benzenetetracarboxylic acid tetramethyl ester	0	3.59	28.48	32.07	0.33	11.68	12.01	6.45	0
27	1,2,4,5-Benzenetetracarboxylic acid tetramethyl ester	0	0	10.50	10.50	0	0	0	4.99	0
28	1,2,3,5-Benzenetetracarboxylic acid tetramethyl ester	0	0	5.73	5.73	0.25	2.17	2.42	0.11	0
30	Dimethoxy-benzenetetracarboxylic acid tetracarboxylic acid tetramethyl ester	0	1.30	4.16	5.46	0	1.27	1.27	0	0
31	Benzenepentacarboxylic acid pentamethyl ester	0	3.77	26.95	30.72	0.17	6.54	6.71	0.68	0
32	Aceto-benzenepentacarboxylic acid pentamethyl ester	0	0.37	8.79	9.16	0	0	0	0	0
34	Benzenehexacarboxylic acid hexamethyl ester	0	1.75	4.26	6.01	0.15	2.90	3.05	0.27	0
Identified		77.27	26.80	116.96	143.76	2.26	39.83	42.09	56.32	21.14
Total weight of products		119.73	41.00	131.19	172.19	4.07	46.09	50.16	69.85	39.50
% of products identified		65	65	89	83	56	86	84	81	54

*I = CuO–NaOH oxidation.
II = CuO–NaOH + $KMnO_4$ oxidation.
III = CuO–NaOH + $KMnO_4$ + H_2O_2 oxidation.
IV = $KMnO_4$ oxidation.
VI = Na-amalgam reduction.
a = degradation products soluble in organic solvents after CuO–NaOH oxidation.
b = degradation products insoluble at p*H* 2 after CuO–NaOH oxidation.

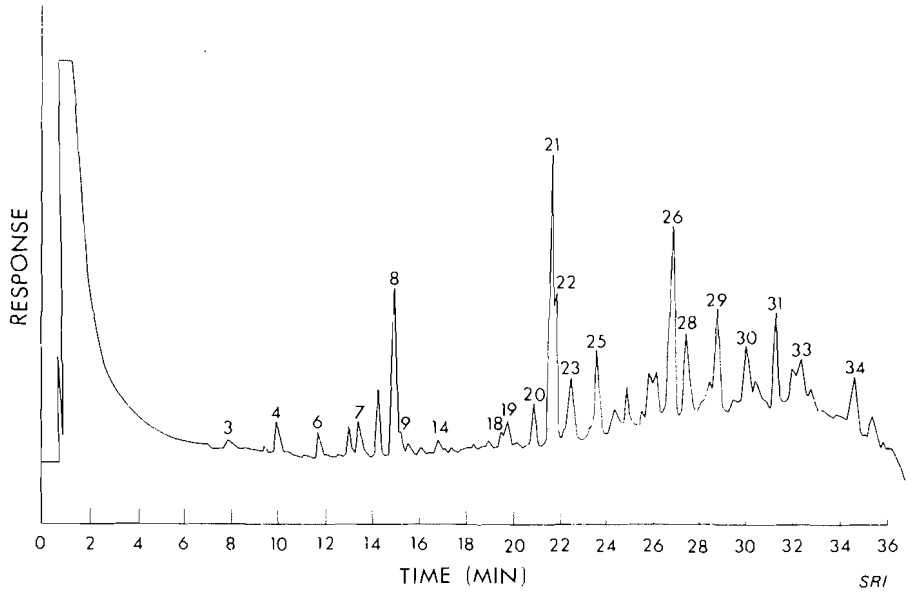


FIG. 2. Gas chromatographic separation of fraction IVm. Numerals above peaks refer to compounds identified in Fig. 3.

the 4-position on the aromatic rings of compounds **10–12** and **15–19** (see Fig. 3) was OH rather than OCH_3. Thus, the major CuO–NaOH oxidation products have guaiacyl and syringyl structures with CHO, $COCH_3$, and CO_2H groups in the 1-position. The weight ratio of guaiacyl (**10–12**) to syringyl (**16–19**) structures was 0.47, indicating a preponderance of syringyl units in the oxidation mixture. Other compounds identified were **20**, which contains both S and N, and about which little is known, and dibutyl phthalate (**24**) which was isolated in relatively large amounts. In view of recent work in this laboratory (10) which showed that HA's can adsorb large amounts of dialkyl phthalates, it is likely that **24** is not a structural HA component but that it is either firmly adsorbed on the HA surface or, less likely, a contaminant.

(II) CuO–NaOH + $KMnO_4$ Oxidation

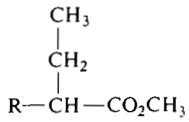
Further oxidation of methylated fraction I with $KMnO_4$ led to the disappearance of phenolic compounds **10**, **11**, **13**, **15**, **16**, and **17** and to losses of **12** and especially of **19** (Table 1, II*a*). At the same time varying amounts of benzenepolycarboxylic acid methyl esters **8**, **9**, **21–23**, **26**, **30–32**, and **34** were formed, apparently from more complex chemical structures, by the more vigorous oxidation with $KMnO_4$. This was especially so for the CuO–NaOH pretreated material that coagulated at p*H* 2 and that was insoluble in organic solvents. The oxidation ($KMnO_4$) mixture from the latter material (Table 1, II*b*) was free of phenolic compounds but contained relatively large amounts of benzenecarboxylic acid methyl esters, principally the tri-(**21**, **22**), tetra-(**26**) and penta-(**31**) forms. These data indicate the occurrence of more condensed chemical structures in II*b* than in II*a*.

(III) CuO–NaOH + $KMnO_4$ + H_2O_2

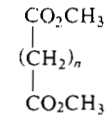
Additional oxidation of II*a* with H_2O_2 led to substantial losses of phenolic as well as benzenecarboxylic components (III*a*). In the case of fraction II*b*, H_2O_2 oxidation produced small amounts of C_6C_3-compound **18** and somewhat greater amounts of **19**, but lowered the yields of most benzene – carboxylic acid methyl esters (III*b*, compare with II*b*). It appears that the additional treatment with H_2O_2 in alkaline solution was too drastic.

(IV) $KMnO_4$ Oxidation

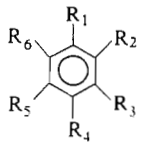
The $KMnO_4$ oxidation of the unmethylated HA produced small amounts of benzenecarboxylic acid methyl esters ranging from the di- to the hexa-forms (IV). Especially noteworthy was the formation of relatively large amounts of 1,2,3-benzenetricarboxylic acid trimethyl ester (**21**), accounting for 3.3% of the initial HA. Failure to isolate phenolic compounds under these conditions is not surprising since the pres-



1 R = CH_3
5 R = C_4H_9



3 $n = 3$
4 $n = 4$
6 $n = 5$
7 $n = 6$
14 $n = 8$



2 $R_1 = CO_2CH_3$; $R_4 = OCH_3$; $R_2 = R_3 = R_5 = R_6 = H$
8 $R_1 = R_2 = CO_2CH_3$; $R_3 = R_4 = R_5 = R_6 = H$
9 $R_1 = R_3 = CO_2CH_3$; $R_2 = R_4 = R_5 = R_6 = H$
10 $R_1 = CHO$; $R_3 = R_4 = OCH_3$; $R_2 = R_5 = R_6 = H$
11 $R_1 = COCH_3$; $R_3 = R_4 = OCH_3$; $R_2 = R_5 = R_6 = H$
12 $R_1 = CO_2CH_3$; $R_3 = R_4 = OCH_3$; $R_2 = R_5 = R_6 = H$
13 $R_1 = CO_2CH_3$; $R_3 = R_5 = OCH_3$; $R_2 = R_4 = R_6 = H$
15 $R_1 = COCH_3$; $R_2 = R_3 = R_4 = OCH_3$; $R_5 = R_6 = H$
16 $R_1 = CHO$; $R_3 = R_4 = R_5 = OCH_3$; $R_2 = R_6 = H$
17 $R_1 = COCH_3$; $R_3 = R_4 = R_5 = OCH_3$; $R_2 = R_6 = H$
18 $R_1 = CH_2COCH_3$; $R_3 = R_4 = R_5 = OCH_3$; $R_2 = R_6 = H$
19 $R_1 = CO_2CH_3$; $R_3 = R_4 = R_5 = OCH_3$; $R_2 = R_6 = H$
20 $R_1 = CH_2SO_2NHCH_3$; $R_2 = R_3 = R_4 = R_5 = R_6 = H$
21 $R_1 = R_2 = R_3 = CO_2CH_3$; $R_4 = R_5 = R_6 = H$
22 $R_1 = R_2 = R_4 = CO_2CH_3$; $R_3 = R_5 = R_6 = H$
23 $R_1 = R_3 = R_5 = CO_2CH_3$; $R_2 = R_4 = R_6 = H$
24 $R_1 = R_2 = CO_2C_4H_9$; $R_3 = R_4 = R_5 = R_6 = H$
25 $R_1 = R_2 = R_4 = CO_2CH_3$; $R_3 = OCH_3$, $R_5 = R_6 = H$
26 $R_1 = R_2 = R_3 = R_4 = CO_2CH_3$; $R_5 = R_6 = H$
27 $R_1 = R_2 = R_4 = R_5 = CO_2CH_3$; $R_3 = R_6 = H$
28 $R_1 = R_2 = R_3 = R_5 = CO_2CH_3$; $R_4 = R_6 = H$
29 $R_1 = R_2 = R_3 = R_4 = CO_2CH_3$; $R_5 = OCH_3$; $R_6 = H$
31 $R_1 = R_2 = R_3 = R_4 = R_5 = CO_2CH_3$; $R_6 = H$
33 $R_1 = OCH_3$; $R_2 = R_3 = R_4 = R_5 = R_6 = OCH_3$
34 $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = CO_2CH_3$

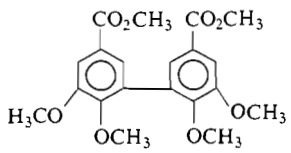


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FIG. 3. Chemical structures of compounds identified.

ence of electron-donating OH groups on aromatic rings makes these rings susceptible to attack by electrophilic $KMnO_4$ (4).

(VI) Na-Amalgam Reduction

The major reduction products were phenolic compounds **19**, **18**, and **10** (Table 1, VI). Compound **20** was another major product identified. It was also one of the major components isolated from the mixture resulting from the CuO–NaOH oxidation (I). Thus, the reduction of HA yields both guaiacyl (**10**, **12**) and syringyl (**16–19**) structures, with the latter clearly predominant. Of some interest is the isolation of small amounts of **13**, which is not a "typical" lignin-degradation product.

(B) Degradation of Methylated HA

(Im) CuO–NaOH Oxidation

The CuO–NaOH oxidation of methylated HA (Table 2, Im) yielded the same type of compounds as that of the unmethylated HA (Table 1) but in greater amounts. Again there was a predominance of syringyl (**16–19**) over guaiacyl (**10–12**) moieties. In addition, small amounts of phenolic compound **2** and larger quantities of branched fatty acid methyl esters **1** and **5** were identified in the oxidation mixture. The most abundant products were **20** and **24**. The total weight of oxidation products soluble in organic solvents was about twice that obtained from the CuO–NaOH oxidation of the unmethylated HA. Only about 65% of the oxidation products was identified (Table 2, Im). Most of the compounds that we have so far been unable to identify eluted from the gas chromatographic column at < 200 °C. It is apparent that methylation of the HA prior to CuO–NaOH oxidation increased the yields of oxidation products significantly.

(IIm) CuO–NaOH + $KMnO_4$ Oxidation

Further oxidation with $KMnO_4$ of the products from the CuO–NaOH oxidation that were soluble in chloroform–acetone (IIm*a*) resulted in the virtual disappearance of most of the compounds that eluted at <200 °C and also led to either the disappearance or a reduction in concentration of the phenolic compounds and of **20**. Simultaneously small amounts of benzenecarboxylic acids were formed. The CuO–NaOH pretreated fraction that was insoluble in organic solvents yielded on further oxidation with $KMnO_4$ (IIm*b*), small amounts of aliphatic dicarboxylic acid methyl ester **7**, methylated phenolic acids **12** and **19** and benzenecarboxylic acids, mainly the tetra-**26** and penta-**31** forms.

(IIIm) CuO–NaOH + $KMnO_4$ + H_2O_2

More drastic oxidation of fraction IIm*a* with H_2O_2 produced small amounts of methylated aliphatic dicarboxylic acids (**3**, **4**, **6**, **7**), phenolic compounds (**12**, **18**, and **19**) and benzenecarboxylic acids, some of which contained $OH(OCH_3)$ groups (**25**) (Table 2, IIIm*a*), indicating the occurrence in the HA of complex phenolic structures. Fraction IIIm*b* was similar in composition to IIIm*a*.

(IVm) $KMnO_4$ Oxidation

The permanganate oxidation of methylated HA (Table 2, IVm) yielded small amounts of methylated suberic acid (**7**), complex phenolic acids (**29**, **33**, and **35**) and relatively large amounts of benzenecarboxylic acids, ranging from di- to hexa-types. The most abundant products were the 1,2,3,4-benzenetetra-**26**, the -penta-**31**, and 1,2,4- and 1,2,3-tri-**22** and -**21** benzenecarboxylic acid methyl esters. Of special interest was the isolation of **35**, the only biphenyl compound that we were able to detect.

(Vm) $KMnO_4$ + H_2O_2 Oxidation

Further oxidation of IVm with H_2O_2 yielded fraction Vm which was similar in composition to IVm except that it contained additional small amounts of phenolic compounds **18**, **19**, **25**, **29**, and **30**, aliphatic dicarboxylic acid esters **3**, **4**, **6**, **7**, and **14** but lower concentrations of benzenecarboxylic acid methyl esters. The additional treatment with H_2O_2 appeared to favor, at least to a small extent, the formation of phenolic compounds.

Discussion

The CuO–NaOH oxidation of the unmethylated and methylated HA yields small amounts of guaiacyl and syringyl monomers with up to three C atoms in the aliphatic side chain (Table 1, I; Table 2, Im); in addition, small yields of guaiacyl compounds with a biphenyl structure are obtained by the permanganate oxidation of methylated HA (Table 2, IVm). The CuO–NaOH oxidation of the unmethylated and methylated HA affords phenolic compounds which account for about 3.1 and 6.0% of the weight of the initial HA, respectively. Qualitatively, the compounds identified resemble those isolated by Pearl (2) from products resulting from the CuO–NaOH oxidation of coniferous

TABLE 2. Compounds (mg) produced by the oxidation of 1 g of methylated HA

No.	Compound	Method*								
		Im	IIm			IIIm			IVm	Vm
			a	*b*	*a* + *b*	*a*	*b*	*a* + *b*		
1	2-Methylbutyric acid methyl ester	13.32	0	0	0	0	0	0	0	0
2	*p*-Methoxybenzoic acid methyl ester	4.24	0	0	0	0	0	0	0	0
3	Dimethyl glutarate	0	0	0	0	0.34	0.07	0.41	0	0.20
4	Dimethyl adipate	0	0	0	0	0.75	0.14	0.89	0	1.00
5	2-Butylbutyric acid methyl ester	9.70	0	0	0	0	0	0	0	0
6	Dimethyl pimelate	0	0	0	0	0.48	0.14	0.62	0	0.70
7	Dimethyl suberate	0	0	1.02	1.02	0.24	2.30	2.54	0.57	1.33
8	1,2-Benzenedicarboxylic acid dimethyl ester	0	0.82	0	0.82	1.10	0.73	1.83	3.47	5.48
9	1,3-Benzenedicarboxylic acid dimethyl ester	0	0	0	0	0.30	0.22	0.52	0.85	0.68
10	3,4-Dimethoxybenzaldehyde	3.42	0	0	0	0	1.67	1.67	0	0
14	Dimethyl sebacate	0	0	0	0	0	0.41	0.41	0	0.53
11	3,4-Dimethoxyacetophenone	8.68	0	0	0	0	0	0	0	0
12	3,4-Dimethoxybenzoic acid methyl ester	12.41	3.65	1.33	4.98	1.96	1.27	3.23	0	0
16	3,4,5-Trimethoxybenzaldehyde	14.08	0	0	0	0	0	0	0	0
18	3,4,5-Trimethoxyphenylacetone	5.12	0.71	0	0.71	0.68		0.68	0	1.38
19	3,4,5-Trimethoxybenzoic acid trimethyl ester	15.56	1.14	0.16	1.30	1.98	3.85	5.83	0	2.53
20	*N*-Methyl-benzylsulfonamide	32.08	13.98	1.73	15.71	1.50	0	1.50	1.42	3.73
21	1,2,3-Benzenetricarboxylic acid trimethyl ester	0	1.45	0	1.45	0.98	3.11	4.09	14.28	11.75
22	1,2,4-Benzenetricarboxylic acid trimethyl ester	0	0.94	0	0.94	0.76	0.92	1.68	15.75	5.38
23	1,3,5-Benzenetricarboxylic acid trimethyl ester	0	1.45	0	1.45	2.50	0.59	3.09	1.55	4.23
24	Dibutyl phthalate	20.20	0	0	0	0	0	0	0	0
25	3-Methoxy-1,2,4-benzenetricarboxylic acid trimethyl ester	0	1.92	0	1.92	8.54	1.40	9.94	0	3.78

TABLE 2. (*Concluded*)

No.	Compound	Method*								
			IIm			IIIm				
		Im	*a*	*b*	*a* + *b*	*a*	*b*	*a* + *b*	IVm	Vm
26	1,2,3,4-Benzenetetracarboxylic acid tetramethyl ester	0	1.59	8.82	10.41	0	3.86	3.86	36.24	12.93
27	1,2,4,5-Benzenetetracarboxylic acid tetramethyl ester	0	0	0	0	0	0	0	9.03	0
28	1,2,3,5-Benzenetetracarboxylic acid tetramethyl ester	0	0.61	0	0.61	0.28	1.90	2.18	3.39	3.90
29	5-Methoxy-1,2,3,4-benzenetetracarboxylic acid tetramethyl ester	0	0	0	0	0.30	0.85	0.30	4.79	3.10
30	Dimethoxy-benzenetetracarboxylic acid tetramethyl ester	0	0	0	0	0	0.92	0.92	0	3.18
31	Benzenepentacarboxylic acid pentamethyl ester	0	0.61	6.39	7.00	0.74	0.98	1.72	16.25	5.10
32	Aceto-benzenetetracarboxylic acid tetramethyl ester	0	0.45	2.53	2.98	0.06	0.62	0.68	0	0
33	Methoxy-benzenepentacarboxylic acid pentamethyl ester	0	0	0	0	0	0	0	5.02	3.23
34	Benzenehexacarboxylic acid hexamethyl ester	0	0	0.76	0.76	0.18	0	0.18	4.67	4.00
35	Dehydrodiveratric acid dimethyl ester	0	0	0	0	0	0	0	2.12	0
Identified		138.81	29.32	22.74	51.96	23.67	25.95	48.77	119.40	78.14
Total weight of oxidized products		213.22	38.95	26.52	65.47	28.28	28.74	57.02	148.20	90.0
% of products identified		65	75	86	79	84	90	86	81	86

*Im = CuO–NaOH oxidation.
IIm = CuO–NaOH + $KMnO_4$ oxidation.
IIIm = CuO–NaOH + $KMnO_4$ + H_2O_2 oxidation.
IVm = $KMnO_4$ oxidation.
Vm = $KMnO_4$ + H_2O_2 oxidation.
a = oxidation products soluble in organic solvents after CuO–NaOH oxidation.
b = oxidation products insoluble at p*H* 2 after CuO–NaOH oxidation.

and hardwood lignosulfonates. Quantitatively the amounts of phenolics isolated from the HA are low. For example, Pepper and Karapally (11) found average yields (as % of Klason lignin) of 15.1 for **10**, 3.7 for **11**, and 2.0% for **12** (compared to 0.4, 0.4, and 0.2, and 0.3, 0.9, and 1.2%, respectively, from unmethylated and methylated HA, see Table 1, I and Table 2, Im), when they oxidized spruce potolignin with CuO–NaOH. Chang and Allan (12) report that the CuO–NaOH oxidation of spruce lignin yields 15.90 of **10**, and 3.91% of **11**, while that of aspen lignin produces 7.82 of **10**, 1.96 of **11**, 20.0 of **16**, and 5.34% of **17**. On the other hand Greene and Steelink (13) oxidized two HA's with CuO–NaOH and identified 0.57 and 0.01% of **10**, 0.41 and 0.21% of **12** and 0.05% and a trace of **16**, but failed to detect any of the other compounds listed in Table 1, I. Greene and Steelink (13) identified in addition small amounts of *p*-hydroxybenzaldehyde, *m*- and *p*-hydroxybenzoic acid (**2**), and 3,5-dihydroxybenzoic acid (**13**) for a total of 1.43% phenolics for one HA sample and 0.58% for another HA sample. These amounts are lower than those identified in this investigation although they are of the same general magnitude. Reduction of the HA with Na-amalgam (Table 1, VI) affords 1.8% of phenolic compounds, with syringyl units predominating over guaiacyl moieties. Thus, all experimental data point to the occurrence of lignin or lignin-derived material in the HA. Probably the best indication for the presence of lignin-derived units in the HA is the isolation of 0.2% of dehydrodiveratric acid dimethyl ester (**35**) from products resulting from the $KMnO_4$ oxidation of methylated HA (IVm). According to reports in the literature (12), yields of **35** on permanganate oxidation of a variety of methylated lignins, with and without prior alkaline hydrolysis, range from 0.2 to 4.0%. If we accept an average value of 2.0%, the contribution of lignin-derived units to the total weight of the HA as indicated by the isolation of **35** is about 10%.

An objection to the interpretation advanced above arises from recent work in our laboratory (14) which showed that the Na-amalgam reduction of "HA's" synthesized by fungi grown on a glucose–asparagine medium in the laboratory (and which had never been in contact with lignin) also produced small amounts (0.7%) of guaiacyl structures **10** and **12** and syringyl monomers **16–19**. Thus, the isolation of phenolic compound **10–12** and **16–19** is not an unequivocal proof for the presence of lignin-derived units in the HA. It may be relevant at this point to refer to other work that we have done recently (14) in which we investigated the permanganate oxidation of "HA's" synthesized in the laboratory by *Aspergillus niger*, *Epicoccum nigrum* and *Stachybotrys chartarum* grown on a glucose–asparagine medium. The oxidation products of methylated fungal "HA's" produced practically the same types but, per unit weight, relatively greater amounts of aliphatic acids but smaller amounts of phenolic and benzenecarboxylic compounds than those listed in Table 2 under IVm. The only exception was our failure to detect even traces of dehydrodiveratric acid dimethyl ester (**35**), which the fungi were apparently unable to produce. We believe, therefore, that the isolation of **35** is the most reliable indication of the presence of lignin-derived material in HA.

The permanganate oxidation of unmethylated HA (IV) produces 5.6% benzenecarboxylic acids (as esters) which is higher than the yields of 0.1 to 3.8% obtained by Read and Purves (5) from various lignins under the same experimental conditions. It has been postulated that aromatic rings containing oxygen are destroyed by $KMnO_4$ and that benzenecarboxylic acids originate from rings in which ring-C atoms are bonded to other C atoms but not to O atoms. The suggestion has also been made that in the case of lignin, benzenecarboxylic acids are formed from side chains of condensed units having conidendrin-like structures as a result of dehydrogenation (12) or from cyclolignans (3). Whether and to what extent this applies to the HA remains to be investigated.

Larsson and Miksche (3) have recently shown that the permanganate oxidation of methylated birch lignin that had first been pre-oxidized with CuO–NaOH yielded more than three times as much **12** and nine times as much **19** as that of methylated lignin that had not been so pretreated. They ascribe the increased yields to the degradation of structures bonded via phenolic oxygens to other lignin components in addition to structures with free phenolic OH groups (the latter are apparently degraded by $KMnO_4$ oxidation of methylated lignin). When we used this approach on the HA (Table 1, I and III) we were unable to detect even traces of **12** and the

yield of **19** was reduced to 25% of that obtained with CuO–NaOH alone. Thus, the HA does not appear to contain many "bonded" phenolic oxygens.

Other oxidation products from the methylated HA are small amounts of *p*-methoxy(OH)-benzoic acid methyl ester (**2**), a typical lignin degradation product (2) and somewhat larger amounts of branched butyric acid methyl esters (**1**, **5**) with CH_3- and C_4H_9-groups in the side chains. In addition, small amounts of aliphatic dicarboxylic acid esters, ranging from glutaric ($n = 3$) to sebacic acids ($n = 8$) are formed. Some of the aliphatic compounds may arise from chains that link aromatic or hydroaromatic rings or from the degradation of saturated ring structures. Furthermore, the oxidation of methylated HA produces a number of methoxy-(OH)-benzenecarboxylic acid esters (**25**, **29**, **33**) which are indicative of the occurrence of complex phenols in the HA. The major oxidation products from methylated HA's, however, are benzenecarboxylic acid methyl esters, ranging from the di- to the hexa-forms. For example, the $KMnO_4$ oxidation of the methylated HA (Table 2, IVm) produces 0.1% aliphatic dicarboxylic acid methyl ester, 1.2% phenolic ethers and esters, but 10.6% benzenecarboxylic acid methyl esters. Of the latter 4% are dicarboxylic, 30% tricarboxylic, 47% tetracarboxylic, 15% pentacarboxylic, and 4% hexacarboxylic. Carbon skeletons of possible precursors of the major oxidation products are shown in Fig. 4.

Relatively large amounts of **20** (up to 3.2% of the initial weight) were isolated from the methylated HA. How **20** fits into the chemical structure of HA is not known at this time. Judging from the N content (3.29%), protein could constitute ±20% of the weight of the HA, although a more realistic figure would be 10% (1). In addition, the HA could also contain a few per cent of carbohydrates. Since recent studies (14) have shown that most of the N-compounds and carbohydrates could be removed by hydrolysis with 6 *N* HCl without causing significant changes in analytical and structural characteristics of HA's, it is likely that proteins and carbohydrates are not structural HA components but are adsorbed on the large HA surfaces.

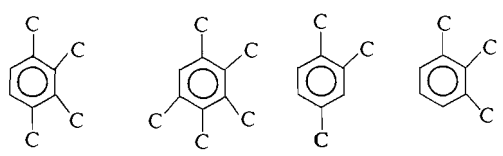

FIG. 4. Carbon skeletons of major products resulting from the $KMnO_4$ oxidation of methylated HA.

The experimental results reported herein show that the HA contains an easily degradable portion which consists of guaiacyl and syringyl units and which may be lignin-derived (about 10% of the total weight). This material is degraded by CuO–NaOH and Na-amalgam. The bulk of the HA-structure, however, consists of a more condensed chemically complex "core" which degrades on more drastic oxidation into complex phenolic and benzenecarboxylic acids. Whether the "core" consists of condensed lignin or whether it is of microbial origin awaits further investigation. It is likely that it is a mixture of the two.

Of the methods that we have examined in this investigation the most promising appear to be: (*a*) the CuO–NaOH oxidation of methylated HA, which produces high yields of products, about half of which elute from the gas chromatographic column at relatively low temperatures (<200 °C), and a substantial portion of which still remains to be identified and (*b*), the permanganate oxidation of methylated HA which provides meaningful information on the chemical structure of the complex "core" of the HA.

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