TABLE II

XENYL ESTERS

Isomer			*p*-Butyl	*o*-Butyl	*p*-Ethanol	*o*-Ethanol
Crystal form			Large white opaque flakes	Heavy yellow liquid	White powder	Small white plates
M. p., °C.			73	B. p., 137 (18 mm.)	107	68
Yield, %			88	30	88	60
Formula			$C_{16}H_{18}O$		$C_{14}H_{14}O_2$	
Analyses, %	Carbon	Calcd.	84.90	84.90	78.47	78.47
		Found	84.80	84.03	78.37	77.97
	Hydrogen	Calcd.	8.02	8.02	6.59	6.59
		Found	8.00	7.87	6.60	6.54

Trixenyl Esters of Phosphoric Acid.—Approximately 0.3 mole sodium xenate was allowed to react with 0.1 mole phosphorus oxytrichloride in anhydrous toluene solution. About half the solvent was removed by distillation, after completion of the reaction, and anhydrous ethyl alcohol added to aid precipitation of the ester, on cooling. Recrystallization was from anhydrous ethyl alcohol. For analysis, the esters were digested with concentrated sulfuric acid, and the resulting phosphoric acid determined by the common gravimetric methods.

Butyl and Hydroxyethyl Xenyl Ethers.—These were prepared by the method of Sowa, Hinton and Nieuwland,[3] because previous runs with sodium xenate and alkyl halides under anhydrous conditions gave small yields.

Summary

The butyl and hydroxyethyl ethers and the complete phosphate esters of *o*- and *p*-xenols have been prepared and some of their physical characteristics described.

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(3) Sowa, Hinton and Nieuwland, THIS JOURNAL, **54**, 2019 (1932).

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[CONTRIBUTION FROM THE DIVISION OF INDUSTRIAL AND CELLULOSE CHEMISTRY, MCGILL UNIVERSITY]

Studies on Lignin and Related Compounds. XI. The Nature of Lignite Humic Acid and of the So-called "Humic Acid" from Sucrose[1,2]

BY MARK PLUNGUIAN AND HAROLD HIBBERT

Introduction

Natural humic acids have been defined[3,4] as those dark brown amorphous substances, resulting from the natural decomposition of plant residues, which are soluble in dilute alkali and which may be precipitated from these solutions by mineral acids.

Synthetic humic acids may be defined as the dark-brown, alkali-soluble amorphous substances obtained in the laboratory by the vigorous action of mineral acids and oxidizing agents on carbohydrates,[5] proteins,[6] phenols,[7] furans[8] and solid hydrocarbons.[9]

For one hundred years after Braconnot[10] first described the preparation of synthetic humic acids from carbohydrates, the identity of these acids with, or their close relationship to, the naturally occurring humic acids was quite generally assumed to be true in spite of the complete lack of chemical evidence for such belief. The ease of formation of these synthetic humic acids from carbohydrates in the laboratory led to the other tacit assumption that the origin of the natural humic acids should be looked for in the carbohydrate part of the decaying plant material and that wood cellulose chiefly was the precursor of natural humic acid. This assumption remained unchallenged until Fischer and Schrader[11] in 1921 put forward their "lignin theory" of the origin of humic acid and of coal. According to this theory the cellulose is largely destroyed by bacterial decomposition, the lignin fraction of the plant substance being more resistant to decay. The nu-

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(1) This work was presented at the Cleveland, Ohio, meeting of the American Chemical Society, September, 1934.

(2) Abstracted from a thesis presented by Mark Plunguian to McGill University in partial fulfilment of the requirements for the Ph.D. degree, October, 1934.

(3) S. Odén, *Kolloidchem. Beihefte*, II, 75–260 (1919).

(4) W. Fuchs, *Kolloid-Z.*, **52**, 248, 350; **53**, 124 (1930).

(5) (a) M. Conrad and M. Guthzeit, *Ber.*, **19**, 2844 (1886); (b) W. B. Bottomley, *Biochem. J.*, **9**, 260 (1915); (c) R. S. Hilpert and E. Littman, *Ber.*, **67**, 1551 (1934).

(6) R. A. Gortner and E. R. Norris, THIS JOURNAL, **45**, 550 (1923).

(7) Wm. Eller and K. Koch, *Ber.*, **53B**, 1469 (1920).

(8) J. Marcusson, *ibid.*, **54B**, 542 (1921).

(9) N. A. Orlov and V. V. Tischenko, *J. Applied Chem.* (*U. S. S. R.*), **6**, 112 (1933).

(10) H. Braconnot, *Ann. chim. phys.*, **12**, 172 (1819).

(11) F. Fischer and H. Schrader, *Brennstoff-Chem.*, **2**, 27 (1921).

cleus of the lignin molecule is supposed to remain intact, but during "humification" it is modified, and also possibly polymerized, to form humic acid. Further humification then leads to the formation of lignite, or brown coal, and finally to hard coal.

In view of the possible close relationship existing between lignin and humic acid on the one hand, and between the naturally occurring and the synthetic humic acids on the other, it seemed of interest to undertake a rigorous chemical characterization of the two types of humic acid.

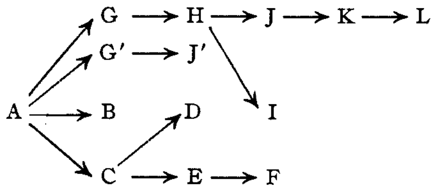
Lignite humic acid was isolated from Ontario lignite by extraction with alkali and precipitation of the alkaline solution with dilute sulfuric acid. Sugar humic acid was prepared by the action of 72% sulfuric acid on sucrose. Both types of humic acid were purified carefully by the use of electrodialysis and repeated precipitation of the dioxane and acetone solutions of the humic acid products with ether. Well-characterized, variously methylated and acetylated derivatives of lignite and sugar humic acid were then prepared. The criterion employed for the homogeneity of the various derivatives of the two types of humic acid was that of a constant methoxyl value (within the allowable experimental error) after successive solution in dioxane and precipitation with ether. The homogeneity of the original sugar humic acid, which does not contain any methoxyl groups, was tested by the standard of constant carbon and hydrogen content after successive solution in dioxane and precipitation with ether. All methylation experiments were repeated several times until a constant methoxyl value was obtained.

Discussion of Experimental Results

In Tables I and II are given the methoxyl and acetyl values of the various derivatives prepared from lignite and sugar humic acid, respectively. On the basis of the given methoxyl values of the methylated derivatives the lowest molecular weight of the two types of humic acid which will satisfy the analytical data can be calculated. From the combustion analyses of the original unmethylated products it is then possible to obtain the empirical formula, or smallest "building unit." In this way, lignite humic acid was found to have the empirical formula $(C_{82}H_{69}O_{28})_x$ with a calculated molecular weight of $(1502)_x$, and sugar humic acid the empirical formula $(C_{59}H_{49}O_{23})_x$, with a calculated molecular weight of $(1125)_x$.

TABLE I

AVERAGE METHOXYL AND ACETYL CONTENT OF LIGNITE HUMIC ACID AND ITS DERIVATIVES



	Methoxyl Groups	Methoxyl %	Acetyl Groups	Acetyl %
A, original lignite humic acid	1	1.74		
B, acetylated derivative of (A)	1	1.64	6	14.70
C, (A) methylated with $(CH_3)_2SO_4$	7	13.78		
D, acetylated derivative of (C)	7	13.46	2	4.96
E, (C) methylated with diazomethane	11	21.35		
F, (E) hydrolyzed with NaOH	7	14.05		
G, (A) methylated with diazomethane	11	20.20		
G′, (A) partially CH_2N_2 methylated	..	18.55		
H, (G) hydrolyzed with NaOH	7	13.61		
I, Acetylated derivative of (H)	7	12.39	2	5.64
J, (H) methylated with $(CH_3)_2SO_4$	8	15.72		
J′, (G′) methylated with $(CH_3)_2SO_4$	8	15.30		
K, (J) methylated with diazomethane	11	20.32		
L, (K) hydrolyzed with NaOH	8	15.60		

Structure of Lignite Humic Acid.—An approximate molecular weight for lignite humic acid can be obtained from a consideration of the methoxyl values found for the various methylated, hydrolyzed and re-methylated products.

Diazomethane, as is well known, can be used to distinguish phenolic from aliphatic hydroxyl groups, since it exerts little or no action on the latter except in the case of certain acidic tertiary aliphatic groups[12] and enolic substances.[12a]

Lignite humic acid shows a somewhat abnormal behavior toward diazomethane as compared with dimethyl sulfate. Thus methylation with the latter reagent increases the methoxyl content to 13.78% (C), while on further treatment with diazomethane the value rises to 21.35% (E), due to methylation of carboxyl groups. Hydrolysis of (E) with sodium hydroxide gives a product (F)

(12) H. Meerwein and G. Hinz, *Ann.*, **484**, 1–25 (1931).

(12a) Certain hydroxylic cyclic derivatives, other than phenols, react readily with diazomethane, for example, kojic acid [Armit and Nolan, *J. Chem. Soc.*, **134**, 3023–3031 (1931)]. Since humic acids probably contain pyrone rings, great care is necessary in drawing conclusions regarding the nature of their hydroxyl groups based on the use of diazomethane. (H. H.)

having the same methoxyl (14.05%) as product (C) (see Table I).

On the other hand, if the original lignite humic acid is treated directly with diazomethane until completely methylated with this reagent, the resulting product (G) has a methoxyl value of 20.20%, due to simultaneous methylation of both hydroxyl and carboxyl groups. Hydrolysis of (G), involving saponification of the ester groups, yields a derivative (H) containing only 13.61% methoxyl, or a value corresponding to that found for (C), thus pointing to the complete absence of free aliphatic hydroxyl groups. However, on remethylation of this hydrolyzed product (H) with dimethyl sulfate, the methoxyl value increases to 15.72% (J), indicating that under the influence of the diazomethane, followed by hydrolysis, certain structural changes have occurred resulting in the formation of at least one free hydroxyl group. Advantage can be taken of this fact in order to determine the approximate molecular weight of the original lignite humic acid.

Methylation of the latter substance (J) with diazomethane yields a product (K) having a methoxyl value of 20.32%, corresponding to that of the derivative (G).

However, the ratio of methoxyl ester to ether groupings present in (K) is different from that in (G) since, on hydrolysis of the former, a substance (L) is obtained having a methoxyl value of 15.60%, this higher value indicating that a smaller number of ester methoxyl groups has been removed.

On the basis of these facts it is possible to separate the methylated derivatives of lignite humic acid into three groups according to their methoxyl content. One group includes compounds C, F and H (Table I) with an average methoxyl content of 13.81%. Another group comprises compounds J, J′ and L with an average methoxyl content of 15.51%, while a third group includes the compounds E, G and K with an average methoxyl content of 20.62%.

In order to obtain the minimum "building-unit" molecular weight of lignite humic acid, the difference in methoxyl between the two lower percentages, namely, 1.70% may be assumed to represent the difference associated with *one* (OCH_3) group. On this basis the results obtained agree most closely when it is assumed that 13.81% methoxyl represents seven (OCH_3) groups, and 15.51% methoxyl eight (OCH_3) groups. The average molecular weight of methoxyl-free lignite humic acid may then be calculated as follows

Let P = percentage of methoxyl in methylated lignite humic acid
N = number of (OCH_3) groups in one molecule
and M = molecular weight of methoxyl-free lignite humic acid
$\therefore P = (N \times 31 \times 100)/(M + (N \times 14))$
or $M = (3100N/P) - 14\,N$

This may now be applied to all of the methylated derivatives.

For $P = 13.81$ and $N = 7$
$M = (3100 \times 7/13.81) - 7 \times 14 = 1572 - 98 = 1474$
For $P = 15.51$ and $N = 8$
$M = (3100 \times 8/15.51) - 8 \times 14 = 1599 - 112 = 1487$
For $P = 20.62$ and $N = 11$
$M = (3100 \times 11/20.62) - 11 \times 14 = 1653 - 154 = 1499$

Thus, on the basis of all the methoxyl values found, there is obtained an average molecular weight for methoxyl-free lignite humic acid of 1487. However, lignite humic acid actually contains methoxyl to the extent of 1.74%. If it is assumed that this is due to one (OCH_3) group, then the molecular weight of the lignite humic acid is 1487 + 14 = 1501.

On the basis of this experimentally derived value for the molecular weight, combined with the empirical formula derived from the combustion data, it can be seen from Table I that acetylation of original lignite humic acid introduces 14.70% (CH_3CO), which corresponds to six acetyl groups. The methoxyl content of lignite humic acid, after treatment with dimethyl sulfate, is seen to increase from 1.74 to 13.78%. This percentage corresponds to seven methoxyl groups or an increase of six methoxyl groups over the one originally present in lignite humic acid. Thus both acetylation and dimethyl sulfate methylation of original lignite humic acid point to the presence in this compound of six free hydroxyl groups. However, acetylation of lignite humic acid, premethylated with dimethyl sulfate (product C) yields a compound (D) containing two acetyl groups. There are present, therefore, in the original lignite humic acid, besides the six free hydroxyl groups, two other potentially available hydroxyl groups which are not methylatable with dimethyl sulfate but are acetylatable. The methoxyl content of product (C), on exhaustive methylation with diazomethane, increases from

13.78 to 21.35%, and, on alkaline hydrolysis, decreases again to 14.05%. This difference corresponds to four ester methoxyl groups; in other words, there are present in lignite humic acid four carboxyl groups.

Furthermore, exhaustive diazomethane methylation of the original lignite humic acid increases the methoxyl content from 1.74 to 20.20% (product G), and, on alkaline hydrolysis, the methylated derivative is converted to a product (H) with a methoxyl content of 13.61%. This again indicates that of the ten new (OCH_3) groups introduced by diazomethane four are ester methoxyls. The six free active hydroxyl groups present in the original lignite humic acid are thus seen to be acidic in character, probably phenolic or enolic, since they are methylatable with diazomethane.

Acetylation of product (H), premethylated with diazomethane, serves to introduce two acetyl groups, thus showing the same behavior as the analogous product (C) premethylated with dimethyl sulfate.

When the attempt is now made to methylate product (H) with dimethyl sulfate, the methoxyl content increases from 13.61 to 15.72% (J) and, on exhaustive methylation with diazomethane, to a product (K) with 20.32% methoxyl. Alkaline hydrolysis of product (K) again decreases the methoxyl content to 15.60%. These results may be explained if it is assumed that during the course of operations involving the synthesis of the hydrolyzed, diazomethane methylated derivative (H) a change takes place whereby one of the two potentially available hydroxyl groups undergoes methylation and one carboxyl group disappears. Methylation with dimethyl sulfate thus takes place to give one additional (OCH_3) group or a total of eight such groups. Subsequent methylation with diazomethane then serves to esterify the three carboxyl groups yielding product (K) with a total of eleven methoxyl groups. Alkaline hydrolysis of this product (K) saponifies the three ester methoxyl groups yielding product (L) which is presumably identical in structure with product (J).

It follows from these analytical data that the smallest lignite humic acid "building unit" must contain one methoxyl, four carboxyl and eight hydroxyl groups, of which two are apparently associated with keto-enol isomerism, while the remaining six are characterized by high acidity such as is associated with the phenol hydroxyl group. The empirical formula of lignite humic acid may therefore be expanded to $C_{77}H_{54}O_{11}(OCH_3)(OH)_8(COOH)_4$.

Structure of Sugar Humic Acid.—The calculation of the molecular weight of sugar humic acid is somewhat simpler than with lignite humic acid.

By reference to Table II, it is seen that complete methylation of the original sugar humic acid (M) with dimethyl sulfate gives a product (O) containing 10.51% (OCH_3). On the other hand, complete methylation of (M) with diazomethane involving methylation of both carboxyl and phenolic groups, if present, yields a derivative (P) having a methoxyl value of 13.05. If the assumption is made that the difference in methoxyl content ($13.05 - 10.51 = 2.54\%$) represents one methoxyl group, it is possible to calculate, as before, a minimum molecular weight for the original sugar humic acid.

For 10.51% and four (OCH_3) groups

$$M = (12{,}400/10.51) - 56 = 1124$$

For 13.05% and five (OCH_3) groups

$$M = (15{,}500/13.05) - 70 = 1118$$

or an average calculated molecular weight of 1121.

TABLE II

METHOXYL AND ACETYL CONTENT OF SUGAR HUMIC ACID DERIVATIVES

$$\begin{tikzcd}
& P \arrow[r] \arrow[d] \arrow[dr] & T \arrow[r] & U \\
M \arrow[ur] \arrow[dr] & S & Q \arrow[r] & R \\
& N \arrow[rr] & & O
\end{tikzcd}$$

	Methoxyl		Acetyl	
	Groups	%	Groups	%
M, original sugar humic acid	..	0.27	..	...
N, acetylated derivative of (M)	..	...	4	12.30
O, (N) methylated with $(CH_3)_2SO_4$	4	10.51		
P, (M) methylated with CH_2N_2	5	13.05		
Q, acetylated derivative of (P)	5	11.61	3	9.06
R, (Q) hydrolyzed with NaOH	1	5.38		
S, (P) hydrolyzed with NaOH	1	5.37		
T, (P) methylated with $(CH_3)_2SO_4$	4	10.53		
U, (T) methylated with CH_2N_2	8	21.90		

If this molecular weight is now combined with the combustion data (p. 534) for the original sugar humic acid, it is found that the formula $(C_{59}H_{49}O_{23})_x$ best satisfies the experimental data.

It can be seen from Table II that acetylation of the original sugar humic acid introduces 12.30%

acetyl, corresponding to four (CH_3CO) groups and that deacetylation of this product (N) and methylation with dimethyl sulfate in alkaline solution introduces 10.51% methoxyl, corresponding to four methoxyl groups. Complete methylation of original sugar humic acid with diazomethane yields product (P) with five (OCH_3) groups and 13.05% methoxyl and acetylation of this compound (P) yields product (Q) with 9.06% acetyl and 11.61% methoxyl, or a ratio of (CH_3CO) to (OCH_3) of 3:5, showing that the diazomethane methylated derivative of sugar humic acid still contained three free hydroxyl groups. If, instead of acetylating, product (P) is methylated with dimethyl sulfate in alkaline solution, product (T) is obtained with 10.53% methoxyl corresponding to four (OCH_3) groups. Since product (P) still contained three free hydroxyl groups which would be methylated with dimethyl sulfate, it is evident that the mild hydrolysis during methylation served to saponify four ester methoxyl groups and that sugar humic acid, therefore, contains four carboxyl groups. This is also indicated by the fact that further methylation of product (T) with diazomethane yields the fully methylated derivative (U) with a methoxyl content of 21.90% which corresponds to eight methoxyl groups.

It follows from these analytical data that the smallest sugar humic acid "building unit" must contain four carboxyl and four hydroxyl groups, of which three are aliphatic and one of a much more acidic type. The empirical formula of sugar humic acid therefore may be expanded to $C_{55}H_{41}O_{11}(OH)_4(COOH)_4$.

Mild alkaline hydrolysis of the esterified sugar humic acid serves to saponify the ester groups. However, when the hydrolysis is carried out at a somewhat elevated temperature, a deep-seated change takes place whereby the carbon content increases by about 2% and the methoxyl content is much higher than expected (products R and S). Assuming that the higher methoxyl content (5.38%) corresponds to *one* (OCH_3) group, the empirical formula of product (S), based on the carbon and hydrogen analysis, was found to be $(C_{33}H_{31}O_{11})_x$.

The authors wish to thank Dr. Margaret E. Greig for her assistance in connection with the acetyl determinations.

Experimental

All analyses reported are calculated on an ash-free basis.

(a) Lignite Humic Acid, Isolation and Purification.—The "woody" lignite, still showing the wood structure, was obtained from the lignite deposit on the Onakawana River in Northern Ontario.[13] It was broken up in a pebble mill to a fine powder and screened through a 200-mesh screen. The powder was dried to constant weight in a vacuum oven at 60°; 300 g. of it was then extracted with (1:1) alcohol-benzene for twenty-four hours in a Soxhlet apparatus. The extracted lignite was dried in the air and then washed with methyl alcohol on the Büchner funnel. It was then extracted five times with water warmed to 50–60° or until the extract was only faintly yellow.

The humic acid was extracted by shaking the lignite powder overnight with one liter of 4% sodium hydroxide solution. The alkaline extract was diluted with ten times the amount of water. It was filtered with suction, first through a pad formed of its own insoluble residue and then a second time using a pad of fuller's earth. The clear dark filtrate was acidified with 10% hydrochloric acid, which caused a voluminous precipitate to settle out. The residue from the first alkaline extraction was again treated in a similar manner for seven consecutive extractions. The combined humic acid precipitates were washed free of chloride ion first by centrifuging and finally by suspending in water several times and filtering; yield of crude lignite humic acid, dry basis, 63.0 g.

A separate quantitative extraction was carried out on a smaller sample of lignite. The same procedure as described above was followed, except that after nearly complete extraction with alkali, the lignite residue was shaken overnight with 2% hydrochloric acid and then again extracted with alkali. Much larger yields of humic acid were thereby obtained. Yield of humic acid before hydrochloric acid treatment, 12.7%; yield of humic acid after hydrochloric acid treatment, 53.6%.

The 63 g. of crude lignite humic acid was dialyzed in four portions as follows. The humic acid was dissolved in 1% sodium hydroxide solution and dialyzed in an ordinary parchment bag using flowing distilled water, warmed to 40–50°, in order to eliminate the soluble silicates. This was continued for nine days or until the colloidal solution of humic acid in the parchment dialyzer had become acid to litmus paper. It was then transferred to an electrodialysis apparatus and the dialysis continued for eight days, using flowing distilled water in both electrode compartments.

Anal. Before dialysis: ash, 3.7. After dialysis: ash, 0.72, 0.79.

The dialyzed humic acid was so finely dispersed in the water that it could not be centrifuged or filtered. The water was therefore removed by distillation at 50–60° under reduced pressure in a special anti-foaming distilling apparatus. The following *general* purification procedure was then carried out. The moist residue was dissolved in acetone, the solution centrifuged, filtered to remove a small insoluble residue and the humic acid precipitated by pouring into ten times its volume of anhydrous ether. The brown, flocculent precipitate was centrifuged, washed twice with low-boiling petroleum ether (30–50°) and the product dried, *incompletely*, in the vacuum desiccator over sulfuric

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(13) C. Tasker, "Technical Investigation of Northern Ontario Lignite," Ontario Research Foundation, Toronto, Ontario, 1933.

acid. This incomplete drying was necessary in order to prevent the material from becoming insoluble in all organic solvents. The small sample taken for analysis was always further dried to constant weight in the Abderhalden over fresh phosphorus pentoxide at 12–15 mm. pressure. The temperature employed was usually 100°. The acetylated compounds were dried at 56°.

Anal. Ash, 0.79; OCH_3, 1.71.

The general purification procedure was repeated on the same material, using as the solvent dioxane which had been distilled twice over metallic sodium and to which 2% water had been added.

Anal. Calcd. for $C_{31}H_{66}O_{27}(OCH_3)$: C, 65.53; H, 4.63; OCH_3, 2.06. Found: C, 65.56, 65.60; H, 4.67, 4.59; OCH_3,[14] 1.77, 1.72; ash, 0.75.

A qualitative test for nitrogen in three separate preparations of purified lignite humic acid showed the absence of this element. The woody lignite as mined in Northern Ontario contains 0.40–0.57% nitrogen.[13]

(b) **Acetylation of Original Lignite Humic Acid.**—Two grams of the dry, original, purified lignite humic acid was dissolved partially in 20 cc. of anhydrous pyridine (freshly distilled over barium oxide) and acetylated by adding 10 cc. of acetic anhydride (freshly distilled over phosphorus pentoxide). After shaking for twenty-four hours at room temperature, the mixture was centrifuged, the solution filtered and the acetylated product precipitated by dropping into a large volume of well-stirred ice water. This crude product was washed thoroughly on the filter and then purified according to the general procedure by dissolving in 30 cc. of dioxane containing 5 cc. of pyridine, filtering and precipitating with ether. The last traces of pyridine were removed from the precipitate by extracting thoroughly with ether. The ether in turn was removed by washing with petroleum ether; yield, 1.0 g. The samples taken for analysis were dried to constant weight in the Abderhalden at 56°. The acetylated product is insoluble in cold dilute alkali but readily soluble in pyridine.

Anal. Calcd. for $C_{77}H_{56}O_{13}(OCH_3)(OCOCH_3)_6(CO–O–CO)_2$: C, 65.70; H, 4.52; OCH_3, 1.80; CH_3CO, 15.0. Found: C, 66.13, 66.13; H, 4.35, 4.25; OCH_3, 1.59, 1.68; CH_3CO,[15] 15.3, 14.4, 14.0, 15.1; ash, 1.0.

(c) **Methylation of Original Lignite Humic Acid with Dimethyl Sulfate.**—1.6 g. of the purified lignite humic acid was dissolved in 75 cc. of dilute aqueous alkali in a three-necked flask and methylated with 20 cc. of dimethyl sulfate and 30 cc. of 30% sodium hydroxide solution. The two reagents were added dropwise over a period of five hours with continuous mechanical stirring, the reaction being carried out at 20–25°. The solution was then made acid to Congo red with dilute sulfuric acid. The resulting precipitate was centrifuged and washed on the filter with hot water until free from sulfates.

A small sample was removed for analysis and further purified by solution in dioxane and precipitation with ether. The remainder of the methylated product was dissolved in dilute alkali and again methylated with dimethyl sulfate. This was repeated until a constant methoxyl value was obtained which was the case with the fourth methylation: *first*, 12.3; *second*, 13.43; *third*, 13.77; *fourth*, 13.78.

Anal. Calcd. for $C_{31}H_{60}O_{21}(OCH_3)_7$: C, 66.60; H, 5.15; OCH_3, 13.68. Found: C, 66.59, 66.67; H, 5.07, 5.10; OCH_3, 13.78, 13.79; ash, 1.55.

(d) **Acetylation of Lignite Humic Acid Premethylated with Dimethyl Sulfate.**—This was carried out by dissolving the material in 15 cc. of pyridine, adding to it 8 cc. of pure acetic anhydride and allowing to stand for forty-eight hours. The crude product obtained by precipitation of the solution with ice water was washed thoroughly with water, dissolved in dioxane containing 10% pyridine and precipitated with anhydrous ether. It was insoluble in pure dioxane and in cold dilute alkali.

Anal. Calcd. for $C_{77}H_{54}O_{11}(OCH_3)_7(OCOCH_3)_2(CO–O–CO)_2$: C, 67.55; H, 4.97; OCH_3, 13.28; CH_3CO, 5.26. Found: C, 67.25, 67.18; H, 5.10, 4.84; OCH_3, 13.52, 13.40; CH_3CO, 5.35, 4.57; ash, 1.4.

(e) **Diazomethane Methylation of Lignite Humic Acid Premethylated with Dimethyl Sulfate.**—1.3 g. of methylated lignite humic acid (OCH_3, 13.78%) was dissolved in dioxane and methylated at 10° by passing through it diazomethane generated from 10 cc. of nitrosomethyl urethan and 20 cc. of 6% sodium glycolate over a period of two hours. The methylation was conducted in an atmosphere of nitrogen. The dioxane solution was allowed to stand for twenty-four hours at room temperature and the methylation then repeated, using only 5 cc. of nitrosomethyl urethan. A third and fourth methylation were carried out before a constant methoxyl value was obtained; yield, 0.9 g.

Anal. Calcd. for $C_{77}H_{56}O_{13}(OCH_3)_7(COOCH_3)_4$: C, 67.24; H, 5.47; OCH_3, 20.77. Found: C, 66.02, 66.47; H, 6.00, 5.94; OCH_3, 21.33, 21.38; ash, 2.0.

(f) **Action of Alkali on Fully Methylated and Esterified Lignite Humic Acid.**—0.7 g. of the fully methylated and esterified derivative (OCH_3, 21.35%) was suspended in 50 cc. of dilute sodium hydroxide solution (about 0.5%) containing some acetone and warmed to 60° under reflux until solution took place; 30% sodium hydroxide solution was then added in small portions to the humic acid solution maintained at 60° over a period of two hours until the alkali concentration was approximately 4%. It was isolated and purified by the general procedure; yield of hydrolyzed product, 0.4 g.

Anal. Calcd. for $C_{77}H_{56}O_{13}(OCH_3)_7(COOH)_4$: C, 66.60; H, 5.15; OCH_3, 13.68. Found: C, 67.05, 66.84; H, 5.46, 5.43; OCH_3, 13.90, 14.20; ash, 0.29.

(g) **Methylation of Original Lignite Humic Acid with Diazomethane.**—Fifteen grams of the original purified humic acid was dissolved in 200 cc. of dioxane containing 5% water and methylated at 10° with diazomethane generated from 10 cc. of nitrosomethyl urethan. This methylation was repeated eight times before a constant methoxyl value was obtained; *second*, 16.55, 16.41; *third*, 18.56, 18.33; *fourth*, 19.38, 19.48; *fifth*, 19.39; *sixth*, 19.93, 19.93; *seventh*, 20.10; *ninth*, 20.20, 20.17.

During the methylation a part of the humic acid separated out of solution. This was filtered off after completion of the methylation and was not investigated further.

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(14) Vieböck and Schwappach method: E. P. Clark, *J. Assoc. Off. Agr. Chem.*, **15**, 136 (1932).

(15) R. Kuhn and H. Roth, *Ber.*, **66**, 1274 (1933).

The methylated product, isolated by the general procedure, was redissolved in dioxane and precipitated with ether; yield of reprecipitated methylated lignite humic acid, 7.7 g.

Anal. Calcd. for $C_{77}H_{56}O_{13}(OCH_3)_7(COOCH_3)_4$: C, 67.24; H, 5.47; OCH_3, 20.77. Found: C, 66.30, 66.22; H, 5.61, 5.62; OCH_3, 20.21, 20.19; ash, 0.43.

A preliminary diazomethane methylation of the original purified lignite humic acid was carried out on a smaller sample (1.6 g.) prior to undertaking the exhaustive methylation described above. An apparently constant value was reached after the third methylation; *second,* 18.5, 18.57; *third,* 18.53. Further methylation was therefore discontinued. This product was purified (yield, 1.3 g.) and subjected directly to methylation with dimethyl sulfate, without isolating the intermediate hydrolysis product. Methoxyl content after three successive methylations: *first,* 15.47; *second,* 14.94; *third,* 16.72. Due to the methoxyl content fluctuating so unaccountably, the product was saponified with 4% sodium hydroxide solution at room temperature; OCH_3, 15.67, 15.45. The saponification was then repeated at 60°; yield, 0.5 g.

Anal. Calcd. for $C_{31}H_{59}O_{20}(OCH_3)_8$: C, 66.75; H, 5.23; OCH_3, 15.50. Found: C, 66.64, 66.78; H, 5.36, 5.33; OCH_3, 15.22, 15.36; ash, 0.20.

(h) Saponification of Diazomethane Methylated Lignite Humic Acid.—Five grams was saponified with 4% sodium hydroxide solution in 50% aqueous acetone at 60°; yield, 4.6 g.

Anal. Calcd. for $C_{77}H_{56}O_{13}(OCH_3)_7(COOH)_4$: C, 66.60; H, 5.15; OCH_3, 13.68. Found: C, 66.88, 66.77; H, 5.50, 5.47; OCH_3, 13.56, 13.66; ash, 0.32.

(i) Acetylation of the Diazomethane Methylated and Later Saponified Lignite Humic Acid.—One gram of the material (OCH_3, 13.61%) was acetylated and purified as before; yield, 0.9 g.

Anal. Calcd. for $C_{77}H_{54}O_{11}(OCH_3)_7(OCOCH_3)_2(COOH)_4$: C, 66.10; H, 5.15; OCH_3, 13.0; CH_3CO, 5.15. Found: C, 66.40, 66.56; H, 5.40, 5.54; OCH_3, 12.37, 12.41; CH_3CO, 5.98, 4.94, 5.99; ash, 0.35.

The somewhat lower methoxyl and higher acetyl content actually found, as compared with the calculated values, would seem to indicate that, during acetylation, demethoxylation with accompanying partial acetylation had taken place to a limited extent.

(j) Methylation with Dimethyl Sulfate of Lignite Humic Acid Premethylated with Diazomethane and then Saponified.—2.3 grams of the product (OCH_3, 13.61%) was dissolved in 100 cc. of dilute alkali and methylated at a controlled temperature of 20–25° with 20 cc. of dimethyl sulfate and 30 cc. of 30% sodium hydroxide solution: OCH_3, 15.32. The methylation was then repeated and the product purified; yield, 1.8 g.

Anal. Calcd. for $C_{73}H_{56}O_{14}(OCH_3)_8(COOH)_3$: C, 66.75; H, 5.23; OCH_3, 15.50. Found: C, 67.04, 66.94; H, 5.22, 5.42; OCH_3, 15.61, 15.82; ash, 0.45.

(k) Diazomethane Methylation of Lignite Humic Acid Premethylated with Diazomethane, then Saponified and Subsequently Methylated with Dimethyl Sulfate.—1.3 grams of this product (OCH_3, 15.72%) was methylated four times in succession at 10° with diazomethane. OCH_3: *third methylation,* 20.07; *fourth,* 20.35, 20.27; yield, 1.1 g.

Anal. Calcd. for $C_{73}H_{56}O_{14}(OCH_3)_8(COOCH_3)_3$: C, 67.24; H, 5.47; OCH_3, 20.77. Found: C, 66.13, 65.99; H, 6.02, 5.95; OCH_3, 20.35, 20.28; ash, 0.17.

(l) Saponification of the Product Obtained in Experiment (k).—0.5 gram of the methylated lignite humic acid (OCH_3, 20.32%) was hydrolyzed by heating with 50 cc. of 4% sodium hydroxide solution for two hours at 60°; yield, 0.35 g.

Anal. Calcd. for $C_{73}H_{56}O_{14}(OCH_3)_8(COOH)_3$: C, 66.75; H, 5.23; OCH_3, 15.50. Found: C, 66.98, 66.98; H, 5.84, 5.72; OCH_3, 15.53, 15.67; ash, 0.27.

(m) Preparation of Sucrose Humic Acid.—This was prepared according to the following method: 100 g. of sucrose was dissolved in 200 cc. of 72% sulfuric acid (sp. gr. 1.64) at room temperature and the solution allowed to stand at 20–25° for three hours. It was diluted with 400 cc. of water and heated to the boiling point. As the dilute solution approached this stage, the synthetic humic acid separated out as a chocolate-brown precipitate, considerable foaming taking place. After boiling for ten minutes the foaming subsided. The solution was then cooled to room temperature, centrifuged and the precipitate washed free from sulfuric acid; yield, dry basis, about 10 g.[16]

The crude sugar humic acid was dissolved in 1% sodium hydroxide and the solution filtered. The filtrate was acidified with sulfuric acid whereby a voluminous dark-brown flocculent precipitate separated out. This was centrifuged and washed free of sulfate. The moist humic acid was dissolved in dioxane, centrifuged, filtered and precipitated with ether.

Thus prepared, sugar humic acid is a light-brown powder which readily acquires an electrostatic charge. It is insoluble in dry dioxane or acetone, but becomes readily soluble when 2–5% water is added to these solvents. However, when the humic acid is dried at 100° or even in the desiccator over concentrated sulfuric acid, it becomes insoluble in all solvents.

Anal. Found: C, 63.07, 63.36; H, 4.27, 4.26; OCH_3, 0.54, 0.48; ash, 2.8.

A suspension of the sugar humic acid in water was then electrodialyzed for five days, when the ash had decreased to 0.6%. The suspension was filtered and the moist precipitate (30 g., dry basis) was dissolved in 500 cc. of acetone. This material was kept in solution throughout the course of the investigation in order to protect it as much as possible from changing over into the insoluble humic acid. Whenever any dry humic acid was required, part of this stock solution was precipitated with ether, washed, first with fresh ether and then with petroleum ether, and the dry humic acid used immediately for the purpose in hand.

Anal. Found: C, 63.17, 63.14; H, 4.32, 4.39; ash, 0.6.

Part of the sugar humic acid used for previous elementary analyses was redissolved in acetone and the centrifuged, filtered solution precipitated with dry ether.

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[16] The yield of humic acid from fructose by the same method of preparation was 18.0%. This would therefore seem to support the contention of Hilpert and Littman[50] that the sugar humic acid was derived mainly from the fructose part of the sucrose molecule.

Anal. Calcd. for $C_{59}H_{49}O_{23}$: C, 62.91; H, 4.39. Found: C, 63.07; H, 4.31; OCH_3, 0.29, 0.25; ash, 0.6.

(n) **Acetylation of Sugar Humic Acid.**—Two grams of purified sugar humic acid was partly dissolved in 40 cc. of pyridine and acetylated with 20 cc. of pure acetic anhydride. The acetylated product was triturated with acetone, and ether was then added to the mixture. The product was centrifuged, washed with ether, then with petroleum ether, dried and analyzed: yield, 1.1 g.

Anal. Calcd. for $C_{55}H_{41}O_{11}(OCOCH_3)_4(CO\text{–}O\text{–}CO)_2$: C, 63.95; H, 4.25; CH_3CO, 13.68. Found: C, 64.20, 64.30; H, 4.33, 4.26; CH_3CO, 12.63, 11.96; ash, 1.2.

(o) **Methylation of Sugar Humic Acid with Dimethyl Sulfate.**—Two grams of purified acetylated sugar humic acid was subjected to a simultaneous deacetylation and methylation with dimethyl sulfate in alkaline solution. The product was methylated nine times in succession with the following results: OCH_3: *first*, 3.56; *second*, 4.68; *third*, 8.34; *fourth*, 8.73; *fifth*, 11.58; *sixth*, 12.68; *seventh*, 12.67; *eighth*, 13.80; *ninth*, 12.36.

It was evident that the carboxyl groups were being partially methylated in the dilute alkaline solution. The product from the ninth methylation was therefore hydrolyzed with warm 4% sodium hydroxide solution and the material purified; yield, 0.3 g.

Anal. Calcd. for $C_{55}H_{41}O_{11}(OCH_3)_4(COOH)_4$: C, 63.99; H, 4.87; OCH_3, 10.50. Found: C, 63.75, 63.53; H, 4.84, 4.94; OCH_3, 10.50, 10.51; ash, 0.15.

(p) **Diazomethane Methylated Sugar Humic Acid.**—About 3 g. of original purified sugar humic acid was partly dissolved by shaking with 100 cc. of dioxane containing about 3% water. The solution was centrifuged, filtered and methylated by passing through it at 10° diazomethane generated from 10 cc. of nitrosomethyl urethan. The methylation was repeated three times before a constant methoxyl content was obtained: *second*, 12.56; *third*, 12.93, 13.08; *fourth*, 13.15, 12.95; yield, 2.5 g.

Anal. Calcd. for $C_{55}H_{41}O_{11}(OH)_3(OCH_3)(COOCH_3)_4$: C, 64.24; H, 4.98; OCH_3, 12.97. Found: C, 63.48, 63.59; H, 5.57, 5.19; OCH_3, 13.15, 12.95; ash, 0.20.

(q) **Acetylation of Sugar Humic Acid Premethylated with Diazomethane.**—This was carried out by dissolving 0.9 g. in 15 cc. of dry pyridine and adding to it 8 cc. of pure acetic anhydride. After standing for forty-eight hours, the acetylated product was isolated by precipitation with ice water and centrifuging. It was purified by washing thoroughly with water, dissolving in dioxane containing 5% pyridine and precipitating with dry ether; yield, 0.8 g.

Anal. Calcd. for $C_{55}H_{41}O_{11}(OCOCH_3)_3(OCH_3)(COOCH_3)_4$: C, 63.51; H, 4.95; OCH_3, 11.72; CH_3CO, 9.75. Found: C, 63.56, 63.33; H, 5.36, 5.14; OCH_3, 11.63, 11.59; CH_3CO, 9.47, 8.65; ash, 0.20.

(r) **Action of Alkali on Acetylated Diazomethane-Methylated Sugar Humic Acid.**—0.1 gram of the product described in the last paragraph was dissolved in 10 cc. of 4% sodium hydroxide solution and warmed at 60° for two hours. The reaction product was precipitated by addition of sulfuric acid, isolated and purified by precipitation with ether from its solution in dioxane–pyridine; yield, 0.07 g.

Anal. Calcd. for $C_{32}H_{28}O_{10}(OCH_3)$: OCH_3, 5.14. Found: OCH_3, 5.36, 5.23.

(s) **Action of Alkali on Diazomethane Methylated Sugar Humic Acid.**—0.6 gram of this product (OCH_3, 13.05%) was heated with 4% sodium hydroxide solution at 60° for two hours and the product isolated as described under experiment (r); yield, pure, 0.25 g.

Anal. Calcd. for $C_{32}H_{28}O_{10}(OCH_3)$: C, 65.65; H, 5.17; OCH_3, 5.14. Found: C, 65.65, 65.78, 65.64, 65.91; H, 5.25, 5.18, 5.11, 5.09; OCH_3, 5.31, 5.41; ash, 0.45.

(t) **Action of Dimethyl Sulfate and Alkali on Sugar Humic Acid Premethylated with Diazomethane.**—Simultaneous saponification and dimethyl sulfate methylation were carried out on 1 g. of the sugar humic acid premethylated with diazomethane to a methoxyl content of 13.05%. The methoxyl value was constant after the second methylation; yield, 0.8 g.

Anal. Calcd. for $C_{55}H_{41}O_{11}(OCH_3)_4(COOH)_4$: OCH_3, 10.50. Found: OCH_3, 10.58, 10.48.

(u) **Fully Methylated Sugar Humic Acid.**—This was obtained by subjecting the dimethyl sulfate methylated product (OCH_3, 10.53%) to exhaustive methylation with diazomethane. A constant methoxyl value was reached after the fourth successive methylation. This product contained an abnormally high ash content due to the attempt to remove excess water from the dioxane solution by means of anhydrous magnesium sulfate.

Anal. Calcd. for $C_{55}H_{41}O_{11}(OCH_3)_4(COOCH_3)_4$: C, 64.97; H, 5.29; OCH_3, 20.04. Found: C, 64.31, 63.82; H, 5.39, 4.92; OCH_3, 21.87, 21.93; ash, 13.1.

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Summary

1. Lignite humic acid was isolated from Ontario lignite by extraction with alkali. Sugar humic acid was prepared from sucrose by the action of 72% sulfuric acid. Both types of humic acid were subjected to careful purification.

2. Well-defined derivatives of the two types of humic acid were prepared by methylation, acetylation and alkaline hydrolysis.

3. Lignite humic acid has the empirical formula $(C_{82}H_{69}O_{28})_x$ or $[C_{77}H_{54}O_{11}(OCH_3)(OH)_8(COOH)_4]_x$ and contains one methoxyl, four carboxyl and eight hydroxyl groups. Of the hydroxyl groups, two are apparently associated with keto-enol isomerism, while the remaining six are characterized by high acidity.

4. Sugar humic acid has the empirical formula $(C_{59}H_{49}O_{23})_x$ or $[C_{55}H_{41}O_{11}(OH)_4(COOH)_4]_x$ and contains four carboxyl and four hydroxyl groups. Of the latter, three are aliphatic and one is of a much more acidic type.

5. Alkaline hydrolysis of diazomethane methylated sugar humic acid at 60° leads to a decomposition product having the empirical formula $(C_{33}H_{31}O_{11})_x$ or $[C_{32}H_{28}O_{10}(OCH_3)]_x$.

6. The evidence obtained indicates clearly that the two types of humic acid have markedly different structures.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, TEACHERS COLLEGE, COLUMBIA UNIVERSITY]

Studies of Crystalline Vitamin B₁. III. Cleavage of Vitamin with Sulfite

BY ROBERT R. WILLIAMS, ROBERT E. WATERMAN, JOHN C. KERESZTESY AND EDWIN R. BUCHMAN

When in the fall of 1933 supplies of crystalline vitamin B_1 became available[1] to us for study, careful consideration was given to the choice of cleavage reactions adapted to reveal facts about its constitution. Such care was considered necessary not only for the sake of economy of vitamin but also because the lability of the substance suggested that drastic treatments would probably cause splitting at several points in the molecule. This indication appeared to be confirmed by the experience of others, notably Van Veen,[2] that a miscellany of substances is produced by the more usual oxidative and hydrolytic reagents.

A review of our experience extending over many years in connection with isolation of the vitamin offered a promising suggestion. Several years ago an attempt to utilize sulfurous acid as a preservative against bacterial decay of rice polish extracts had led to a very prompt and complete loss of the antineuritic activity at room temperature. In a preliminary experiment, a few milligrams of the crystalline vitamin dissolved in two portions of sodium sulfite solution were allowed to stand at room temperature under toluene for thirty-six hours after adjustment to *p*H 1 and *p*H 4, respectively. The solutions were then made alkaline with baryta to remove sulfite ion. The neutralized filtrate of the solution which had been adjusted to *p*H 1 had lost about half its activity as shown by injection of polyneuritic rats; that at *p*H 4 was quite devoid of activity.

We have since had considerable experience with this reaction of the vitamin and find that it proceeds readily at about *p*H 5, under which conditions it is complete at room temperature in twenty-four to forty-eight hours; at steam-bath temperature it is complete in an hour or less according to a single experiment. The use of saturated aqueous sulfurous acid gives only very slow destruction of activity. After three months at room temperature, 50% yields of cleavage products were obtained. Under favorable conditions, it proceeds quantitatively to form crystalline products according to the equation

$$C_{12}H_{16}N_4OS + H_2SO_3 \longrightarrow \underset{\text{I}}{C_6H_9N_3SO_3} + \underset{\text{II}}{C_6H_9NOS}$$

With sulfite at *p*H 5.0, the yield of each cleavage product has reached 97% of the calculated and the products are well defined. The constancy of these results from a quantitative standpoint and the corresponding degree of destruction of physiological activity which occurs simultaneously afford strong assurance of the essential purity of the crystalline vitamin. The reaction has been carried out thirteen times. A detailed description of the isolation of the cleavage products is given in the experimental part.

I is an acidic substance, sparingly soluble in water and insoluble in other solvents; II is a basic substance soluble in water but extractable by means of chloroform from an alkaline aqueous solution. It has been dealt with principally as the hydrochloride which can readily be obtained in a crystalline condition. Results of investigation of the constitution of both the acidic and basic cleavage products will be reported in papers now in preparation.

Experimental

1.000 gram of vitamin was dissolved in 15 cc. of sodium sulfite solution containing sufficient excess sulfurous acid to bring the *p*H to 4.8–5.0. The total sulfite content was 2.6 *N*. After standing overnight at room temperature the liquid had deposited copious amounts of the sparingly soluble acidic cleavage product in crystalline form. After standing for several days, the crystalline product was collected, washed and dried; weight 535.8 mg.

The mother liquor and washings were brought to *p*H 10 with strong sodium hydroxide and the alkaline solution extracted seven times with 50 cc. of chloroform each time.

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[1] R. R. Williams, R. E. Waterman and J. C. Keresztesy, THIS JOURNAL, **56**, 1187 (1934).

[2] A. S. Van Veen, *Rec. trav. chim.*, **51**, 279 (1932).