Effect of Storing the Fungal Culture in the Refrigerator upon Yield of Crystalline Product and Rate of Synthesis.—The fungal culture was stored in the refrigerator and samples were withdrawn at weekly intervals and used for synthesis experiments. The yield of crystalline product obtained by boiling the aqueous methanol solution (I) for eight hours was determined. Figure 3 shows that the yield of crystalline product decreased markedly as the length of storage time increased. Practically no crystals were obtained when fungal cultures which had been stored for five weeks were used for the synthesis.

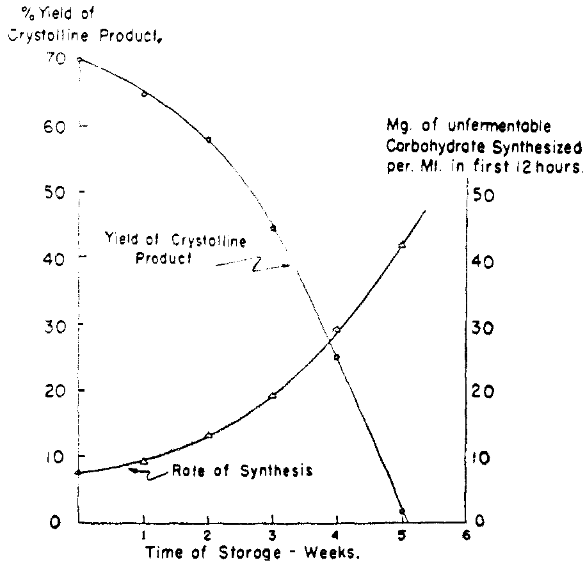

Fig. 3.—Effect of storing the fungal culture upon rate of synthesis and yield of crystalline product.

On the other hand, the rate of synthesis, as determined by the mg. of unfermentable carbohydrate synthesized in the first 12 hours of incubation (*cf.* Fig. 2), increased markedly as the length of storage time increased (Fig. 3).

Because the unfermentable carbohydrate synthesized tended to disappear after all the maltose had been consumed,[3] the maximum amount that could be synthesized could hardly exceed 60 mg. per ml. When the fungal culture which had been stored in the refrigerator for three weeks or more was used for the synthesis, this limit (60 mg. per ml.) was reached in less than 72 hours and the reaction had to be stopped correspondingly earlier. The possibility that the synthesis had already proceeded beyond the limit before it was stopped, however, could not be excluded. Whether the yield of crystalline product was affected by over incubation was not determined.

Paper Chromatography.—A modification of the apparatus described by Ma and Fontaine[14] was used for preparing ascending-type paper chromatograms. The elution solvent, a mixture of pyridine, butanol and water (2:3:1.5, respectively) and the developing agent, a 0.5% solution of 3,5-dinitrosalicylate in 4% sodium hydroxide, were developed by Dr. R. J. Dimler and associates of the Northern Regional Research Laboratory.

A 10% solution of the sugar was spotted five times on a strip of Whatman No. 1 filter paper and eluted for sixteen hours at room temperature (25 to 30°). The strip was air-dried, sprayed with the developing agent, air-dried again, and heated at 105° for 10 minutes.

Acknowledgments.—The authors wish to thank Dr. M. L. Wolfrom of Ohio State University for his helpful advice and Dr. R. J. Dimler of Northern Regional Research Laboratory for the paper chromatographic method.

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[14] R. M. Ma and T. D. Fontaine, *Science*, **110**, 232 (1949).

Louisville, Ky. Received September 18, 1950

[Contribution from the Pulp Mills Research Project and the Department of Chemistry and Chemical Engineering of the University of Washington]

Non-degradative Oxidation of Lignin Sulfonates

By D. M. Ritter

In order to obtain information concerning the non-benzenoid portion of lignin derivatives, sodium peracetoxy lignin sulfonate was oxidized with mercuric acetate, quantitatively and without molecular degradation. The product was a carboxy dihydroxylated lignin sulfonate from which methylated and acetylated derivatives and a potassium salt were prepared. The product and its methylated derivatives underwent spontaneous γ-lactone formation. It was concluded that each C_{18} unit of the lignin sulfonate ion contains at least four contiguous aliphatic carbon atoms, the terminal one of which bears the hydroxyl group of a primary alcohol. The fourth carbon in the group may bear one of the aromatic rings.

Oxidation of basic lignin sulfonate solutions with metallic oxides has contributed substantially to an understanding of the aromatic character of lignin derivatives and of the substituents in the ring.[1] However, those obviously complex and multiple reactions give products which until now have offered little information concerning the aliphatic portions of lignin. The present study of the action of mercuric acetate on lignin sulfonate derivatives furnishes more experimental information concerning the non-benzenoid parts of the lignin structure.

When a lignin sulfonate salt in 1 *N* acetic acid solution was treated with mercuric acetate at room temperature, two reactions were observed. One of these was a mercuration,[2] and the other was an oxidation characterized by the slow separation of mercurous acetate. The rate of oxidation was increased to the useful point and the mercuration was suppressed by performing the reaction with sodium peracetoxy lignin sulfonate dissolved in a boiling solution of sodium acetate and mercuric acetate in glacial acetic acid. In the 24 hours required for complete reaction no methoxyl loss occurred[3] nor was degradation in molecular size observed as shown by the diffusion constants, $D = 7.1$ mm.^{2}/day, before the reaction, and $D = 7.2$ mm.^{2}/day, after the oxidation.

The oxidation product (I) and its derivatives had compositions given in Table I. The potassium sulfonate-carboxylate salt (II) was water soluble, but the barium salt was practically insoluble. After repeated treatment of (I) with diazomethane[4,5] the

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[1] W. Lautsch, E. Plankenhorn and F. Klink, *Angew. Chem.*, **53**, 450 (1940); I. A. Pearl, This Journal, **64**, 1429 (1942); **71**, 2196 (1949); **72**, 1427, 2309 (1950).

[2] The mercuration reaction, which has been studied by Dr. K. A. Wright, will be the subject of a separate communication.

[3] *Cf.* Table III.

[4] E. G. King, F. Brauns and H. Hibbert, *Can. J. Research*, **13B**, 88 (1935).

[5] D. M. Ritter, E. D. Olleman, D. E. Pennington and K. A. Wright, This Journal, **72**, 1347 (1950).

TABLE I

COMPOSITIONS OF CARBOXY-DIHYDROXYLATED LIGNIN SULFONATE AND DERIVATIVES

No.	Proposed name	Empirical formula	Analyses, % C	H	S	MeO	Cation
I	Ammonium dihydroxylated lignin sulfonate carboxy-lactone	$C_{17}H_{15}O_2(OH)_2(OCH_3)_{1.8}\left(\mathrm{C}\begin{matrix}{}^{/\!\!/O}\\{}_{\diagdown O\diagdown}\end{matrix}\right)(SO_3NH_4)_{0.72}$	52.51	5.10	5.12	12.13	1.97[a]
II	Potassium dihydroxylated lignin sulfonate carboxylate	$C_{17}H_{15}O_2(OH)_3(OCH_3)_{1.8}(COOK)(SO_3K)_{0.72}$				10.50	13.70[b,c]
III	Methylated (I)	$C_{17}H_{14.4}O_{1.2}(OH)(OCH_3)_{2.86}\left(\mathrm{C}\begin{matrix}{}^{/\!\!/O}\\{}_{\diagdown O\diagdown}\end{matrix}\right)(SO_3H)_{06.}(NH_3)_{0.7}$	55.20	5.90	4.44	19.44	2.23[a]
IV	Methylated (III)	$C_{17}H_{13.4}O_{1.5}(OCH_3)_4\left(\mathrm{C}\begin{matrix}{}^{/\!\!/O}\\{}_{\diagdown O\diagdown}\end{matrix}\right)(SO_3H)_{0.7}(NH_3)_{0.5}$	55.65	5.85	4.70	26.0	1.66[a]
V	Acetylated dimethyloctadecylammonium salt corresponding to II					7.76	10.9[d,e]

[a] Nitrogen. [b] Potassium. [c] Calculated for II on the basis of hydrolyzed lactone from I; $K = 12.75$. [d] Acetyl. [e] Ratio Ac/MeO = 1.01.

TABLE II

LACTONE FORMATION AND HYDROLYSIS

Formula No.[a]	Form analyzed	Conditions of preparation Process	Temp., °C.	Time, hr.	Quantities present milliequiv. $\frac{\text{MeO}^b}{n}$	S	T. A.[c]	Ratios $\frac{\text{SO}_3\text{Hxn}^b}{\text{MeO}}$	$\frac{\text{T. A.xh}^b}{\text{MeO}}$	Lactone,[d] %
I	Free acid	Dil. NaOH hydrolysis[e]	25	10	1.51	..	1.24	0.40[f]	0.82	9
I	Free acid	Dil. NaOH hydrolysis[e]	25	72	1.79	..	1.67	.40[f]	.93	11.5
I	Free acid	Dil. NaOH hydrolysis[e]	90	1	1.70	..	1.66	.40[f]	.98	3
I	Free acid	Acid-catalyzed esterfn.	25	720	1.70	..	0.87	.40[f]	.51	82
III	Free acid	Acid-catalyzed esterfn.	25	72	1.25	0.46	.68	.37	.54	73
III	Free acid	Acid-catalyzed esterfn.	35	24	0.75	.28	.33	.37	.44	89
III	Salt[g]	Dil. NaOH hydrolysis	90	1	0.75	.28	.51	.37	.68	50
III	Salt[g]	Dil. NaOH hydrolysis	90	1	1.25	.46	.91	.37	.73	43
III	Salt[g]	Dil. NaOH hydrolysis	90	2	1.25	.46	.95	.37	.76	38
III	Salt[g]	Dil. NaOH hydrolysis	90	8	1.25	.46	1.18	.37	.945	9
IV	Free acid	Acid-catalyzed esterfn.	25	24	0.795	.28	.32	.35	.40	92
IV	Salt[g]	Dil. NaOH hydrolysis[e]	90	1	0.795	.28	.575	.35	.72	43

[a] See Table I. [b] n = number of MeO groups per C_{18}. [c] Total acid. [d] Estimated on basis of equation: % lactone = $100\left[1.00 - \frac{R_{\mathrm{T.a.}} - R_{\mathrm{SO_3H}}}{1.00 - R_{\mathrm{SO_3H}}}\right]$ where the R's are the respective ratios in Columns 9 and 10. [e] Solution de-ashed following hydrolysis. [f] Estimated from Formula I, Table I. [g] Total acid determined by back titration of excess basc.

TABLE III

MATERIAL BALANCES

Derivative, sodium sulfonate	Electron change[a]	Methoxyl recovery, %
Lignin[b]	1.95–2.08[d]	104[f]
Peracetoxy lignin[c]	8.08[e]-8.12[e]	103[f]
Hemimethoxy acetoxy lignin[c]	7.00[e]	..
Permethoxy lignin[b]	1.84[e]	98[g]

[a] The number of electrons exchanged for each C_{18} unit, based on C_{18} = $^1/_2$MeO for lignin sulfonates and for the peracetoxy derivative; C_{18} = $^1/_3$MeO for the hemiethoxy derivative and C_{18} = $^1/_4$MeO for the permethoxy derivative. Determined by weighing the mercurous acetate formed in the reaction. [b] In 50% acetic acid solution. [c] In glacial acetic acid. [d] After reaction of one hour. [e] After reaction for 25 hours. [f] Determined after dialysis. [g] Determined before dialysis.

product III was obtained in which the phenolic hydroxyl groups were methylated. This was shown by the identity between the ultraviolet absorption spectra of (III) in neutral and in 1 N sodium hydroxide solution.[5] Repeated methylation of (I) or (III) with dimethyl sulfate in alkali[4,6] gave the product (IV). A salt with dimethyloctadecylamine,[7] derived from the salt (II) was acetylated in pyridine with acetic anhydride to give (V).

The behavior of product (I) and its methylated derivatives (III) and (IV) in acidic and basic solutions is shown in Table II. In addition to the sulfonate group evidence can be seen for the presence of one new acidic function for each C_{18} unit. This acidic group had the properties of a lactone-forming carboxyl group, since it diminished in amount upon standing in acid solution, and it was fully restored by sufficiently long heating in dilute alkaline solution. At the end-points of conductimetric titrations, where the salt (II) was present at about 0.05 N concentration, the pH was 8.0–8.5. Methylation of the phenolic hydroxyl groups interfered not at all with the spontaneous lactone formation. With all possibility of aromatic δ-lactone formation thus eliminated[8] the substance (I) appeared to be an aliphatic γ-lactone.[9,10] This conclusion was confirmed by comparison of the infrared absorption

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(6) G. H. Tomlinson and H. Hibbert, THIS JOURNAL, **58**, 340 (1936).

(7) Use of organic solvent-soluble amine salts of this type is based upon work by Dr. A. Markham and Dr. J. L. McCarthy of this Laboratory.

(8) R. Fittig and H. Hochstetter, *Ann.*, **226**, 357 (1880).

(9) Erwin Ott in J. Houben, "Die Methoden der organische Chemie," Vol. 3, 2nd edition, p. 644 ff.; H. Johansson and H. Sebelius, *Ber.*, **51**, 480 (1918); A. Kailan, *Z. physik. Chem.*, **94**, 111 (1919).

(10) The spontaneity of the process disposed of the possibility that esterification between units was involved. Such esterifications as cross-linking and condensation reactions have equilibrium constants and velocities of the same magnitude as ordinary esterifications. *Cf.* P. J. Flory, THIS JOURNAL, **61**, 3334 (1939). According to Kenyon behavior in acid and alkali similar to this was found to be characteristic of the poly-γ-lactones from vinyl acetate–acrylic acid and vinyl acetate–maleic acid interpolymers. The δ-lactone from the head-to-head polymerized α-chloroacrylic acid, once hydrolyzed, would not re-form. W. O. Kenyon, G. P. Waugh and L. M. Minsk, 118th Meeting A. C. S. *Abstracts of Papers* 7J.

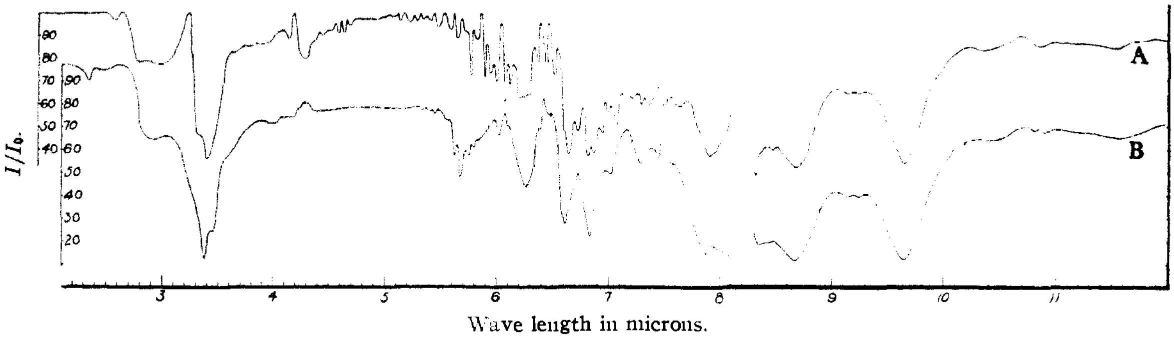

Fig. 1.—Infrared spectra of octadecyldimethylammonium salts in chloroform: A, lignin sulfonate; B, dihydroxylated lignin sulfonate carboxy lactone.

spectra shown in Fig. 1. Oxidation of lignin sulfonate ion introduced a new band at 5.65 μ characteristic of a γ-lactone carbonyl group,[11] and a re-enforcement at 6.2–6.3 μ was interpreted as arising from a carboxy salt.[12]

From the data in Table III it was apparent that extensive reaction with mercuric acetate occurred only when the aliphatic hydroxyl groups were free.[5,13] Retention of methoxyl groups by the oxidized permethoxy lignin sulfonate ions showed that the small amount of oxidation observed in that case did not involve the methoxyl-bearing aliphatic carbon atoms. It is proposed, therefore, *that in each C_{18} unit of the lignin sulfonate ion the approximately one aliphatic hydroxyl group is a primary alcohol and that it was oxidized to a carboxyl group in the reaction with mercuric acetate.*

Methylated derivative (IV) and acetylated derivative (V), the former of which completely retained its lactone-forming property, showed that for every primary alcohol group oxidized, two new hydroxyl groups appeared.[5,14]

Therefore, the two half reactions involved appear to be[15]

$$-CH_2OH + H_2O \longrightarrow -COOH + 2H^+ + 4e^-$$
$$2{>}CH_2 + H_2O \longrightarrow 2{>}CHOH + 2H^+ + 4e^-$$

thus accounting for the almost exactly eight electrons exchanged for every two methoxyl groups or each C_{18} unit in the lignin sulfonate ion. Since one of the hydroxyl groups was introduced *gamma* to the carboxyl group, the non-benzenoid portion of the lignin sulfonate ion must contain at least four contiguous carbon atoms.

The ultraviolet absorption spectra shown in Fig. 2 can be interpreted to support the viewpoint that the lactone-forming hydroxyl group is on a carbon atom linked to one of the aromatic rings. After the oxidation, Curve C shows both hyperchromic and slight hypochromic effects on the 280 mμ aromatic band. After standing in 1 *N* HCl until lactone-formation was completed a hypochromic effect was observed as shown in Curve B. Reactions at carbon atoms other than those linked to the ring, directly or through conjugation, are little likely to produce shifts such as that from Curve C to Curve B.

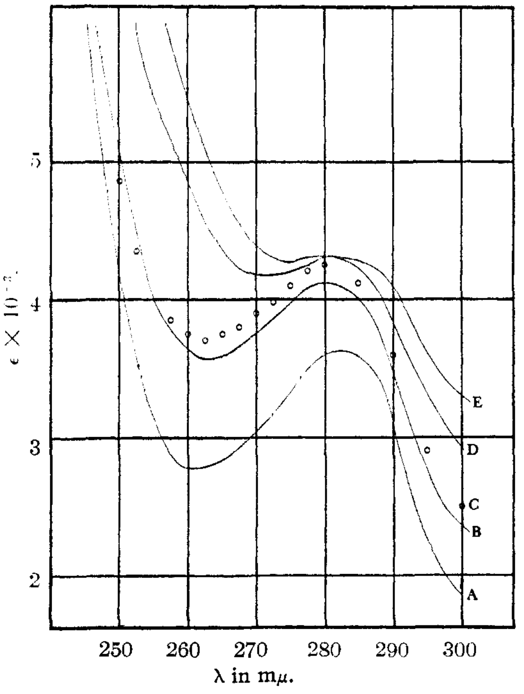

Fig. 2.—Ultraviolet spectra of lignin sulfonate ion in water A, and of dihydroxylate lignin sulfonate-carboxylate derivatives, the lactone in 1 *N* HCl B, the carboxylic acid in 1 *N* HCl C, the sulfonate-carboxylate ion in 1 *N* K_2CO_3 D, and in 1 *N* KOH E.

Experimental Part

Lignin Sulfonate Derivatives.—Hemimethoxy, permethoxy and peracetoxy lignin sulfonates were prepared as described previously.[4,5,6] The starting material in each case was an ammonium lignin sulfonate obtained by dialysis[16]

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[11] R. B. Woodward, This Journal, **72**, 3327 (1950).

[12] H. M. Randall, R. G. Fowler, Nelson Fuson and J. R. Dangl, "Infra Red Determination of Organic Structures," D. Van Nostrand Co., New York, N. Y., 1949.

[13] In glacial acetic acid solution in the presence of a Lewis acid, such as mercuric acetate, acetoxy groups can be considered equivalent to free hydroxyl groups for purposes of reaction mechanism and related kinetics. *Cf.* W. F. Luder and S. Zuffanti, "Electronic Theory of Acids and Bases," John Wiley and Sons, New York, N. Y., 1946, p. 123 ff.

[14] The generalized formula for lignin sulfonic acid can be expressed as $C_{18}H_{16}O_2(OH)_2(OCH_3)_2SO_3H$.

[15] Under appropriate circumstances mercuric ion will accomplish the transformation: (a) CH → COH; (b) COH → CO; (c) COH → COOH; (d) CH–CH → C=C. *Cf.* R. Stoermer in J. Houben, "Die Methoden der organische Chemie," Band II, Auflage III, G. Thieme, Leipzig, 1925, p. 219. For examples in the alkaloid series *cf.* J. Gadamer, *Ber. pharm. Ges.*, **29**, 156 (1919); *C. A.*, **13**, 2414 (1919); J. Gadamer and G. Von der Bruck, *Arch. Pharm.*, **261**, 117 (1923); H. Dieterle and P. Dickens, *Arch. Pharm.*, **264**, 257 (1926). Use in the sterol field is exemplified by A. Windaus, U. Riemann and G. Zuhlsdorff, *Ann.*, **552**, 135 (1942).

[16] Q. P. Peniston and J. L. McCarthy, This Journal, **70**, 1327 (1948); A. Markham, Q. P. Peniston and J. L. McCarthy, *ibid.*, **71**, 3599 (1949).

from the calcium-base sulfite waste liquor discharged from a soft cook of Western hemlock *tsuga heterophylla*. The starting material and derivatives conformed in all respects with prior descriptions.[5] Action of acetic anhydride in pyridine was used as an alternative procedure for preparing the peracetoxy derivative. The product obtained was indistinguishable from that prepared by the action of acetic anhydride in acetic acid–sodium acetate solution.

Oxidation Procedure.—By use of moderate agitation over a period of several days sodium peracetoxy lignin sulfonate (58 g. of air-dried material, 175.2 milliequiv. of methoxyl) was dissolved in 3.5 l. of glacial acetic acid containing 60 g. of anhydrous sodium acetate. Solid mercuric acetate (319 g., one mole) was added, and the solution was heated to its boiling point for 24 hours.

The solution was concentrated at 2.5 cm. pressure to 750–1000 ml. volume. It was left standing for 24 hours to permit the bulk of the mercurous acetate to crystallize. This was removed by centrifuging, and the moist cake was washed with a 2% sodium acetate solution in glacial acetic acid until the washings were colorless. To remove a small remaining amount of mercurous acetate the concentration and crystallization steps were repeated with the combined solution and washings. Thereafter, the solution was dialyzed through "Cellophane" until free of all but traces of mercury. During the dialysis a very small amount of mercurous acetate crystallized from the solution. The dialyzed solution was filtered and de-ashed,[17] and the oxidized lignin sulfonate ions were extracted into chloroform (500 ml.) containing octadecyldimethylamine[7] (30 g.). The aqueous phase was washed twice with a 5% solution of the amine in chloroform (100 ml.). The combined solution and washings were extracted with 500 ml. of 0.2 *N* sodium hydroxide solution, and the chloroform phase was washed twice with 100-ml. portions of 0.1 *N* sodium hydroxide solution. The solution after de-ashing contained 151 milliequivalents of MeO, representing a product yield of 86.3%.

The degree of oxidation was determined by weighing the well washed and dried mercurous acetate. The material balances were based upon methoxyl analyses made on aliquots taken at appropriate points. Diffusion constants used to judge whether degradation had occurred were determined by the gel diffusion method.[18]

Functional Derivatives of Carboxy Dihydroxylated Lignin Sulfonate Ion.—The methylated derivatives III, IV (Table I) were prepared by repeated treatment with diazomethane[4] or dimethyl sulfate and alkali[4,6] until the methoxyl content reached a constant value.

To acetylate, the free acid was transferred to chloroform using octadecyldimethylamine.[7] The chloroform was evaporated; the solid residue was dissolved in pyridine and treated with acetic anhydride at room temperature for a week. Then to completely acetylate the carboxy dihydroxylated lignin sulfonate, the reaction was carried out on the steam-bath for 18 hours. The solid was recovered by precipitation in ether followed by six thorough washes with ether. Acetyl determinations were made in an improved apparatus described by Weisenberger.[19] Acid hydrolysis was used.

Lactone Formation and Hydrolysis.—Conductimetric titration was used to observe the acidimetric properties of carboxy dihydroxylated lignin sulfonic acid. Total acidity was easily determined in that way, but, as was expected,[20] no end-point was found between the carboxylic acid and the sulfonic acid.

The lactones were formed by allowing de-ashed[21] solutions to stand at room temperature. The lactones were hydrolyzed by heating their alkaline solutions on the steam-bath for a period established by trial. The initial hydroxide ion concentration was adjusted so that after hydrolysis its concentration was 0.1 *N*.

Preparation of Salts.—If a basic solution of hydrolyzed lactone was de-ashed by means of H^+ ion-charged sulfonate type ion-exchange resin,[17] the resulting acid solution could be used to prepare the carboxylate sulfonate salts, provided this was done at once before lactone-formation became appreciable. The potassium salt was made by passing the acid solution over a K^+ ion-charged carboxylic acid resin.[21] Compound (II) in Table I was a salt prepared in that way.

Ultraviolet and Infrared Spectra.—The ultraviolet absorption spectra shown in Fig. 2 were determined on a Beckman DU quartz spectrophotometer. Curve A was obtained on refined ammonium lignin sulfonate[16] Curves D and E were obtained on solutions of Salt II (Table I) in 1 *N* potassium carbonate and 1 *N* potassium hydroxide, respectively. Curve C was obtained by measuring Salt II freshly dissolved in 1 *N* hydrochloric acid, and Curve B was determined on the same solution after a month. In all cases the reference solvent was the appropriate electrolyte solution.

The infrared spectra in Fig. 1 were determined on a Beckman IR-2 spectrometer with sodium chloride optics. Data from the instrument were fed into a Brown recording potentiometer by means of which the chloroform solvent and the octadecyldimethylammonium salt solutions were separately but synchronously recorded on the same chart. I/I_0 values were calculated directly from the chart readings. Figure 1 is a plot of the data obtained. The substances measured were the octadecyldimethylammonium salts of lignin sulfonic acid and the dihydroxylated lignin sulfonate carboxy lactone corresponding to line 1, Table II, having 30% lactone.

Acknowledgment.—The author wishes to thank the staff of the Chemistry Division, Pulp Mills Research Project, for their assistance, particularly Mrs. E. D. Olleman, who first observed lignin sulfonate oxidation with silver acetate, and Dr. K. A. Wright, who first observed the oxidative side reaction incident to mercuration. Some of the laboratory work was done by Mr. T. D. Parsons, and Mr. M. E. Taylor carried out many of the microanalyses.

SEATTLE, WASHINGTON RECEIVED OCTOBER 28, 1950

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[17] This was done with "Ionex" No. 2 cation exchange resin supplied by Dow Chemical Co., Midland, Mich.

[18] V. F. Felicetta, A. Markham, Q. P. Peniston and J. L. McCarthy, THIS JOURNAL, **71**, 2879 (1949).

[19] E. Weisenberger, *Mikrochemie ver Mik. Acta*, **30**, 241 (1942); **33**, 51 (1947).

[20] A. Katchalsky and J. Gillis, *Rec. trav. chim.*, **68**, 879 (1949); F. T. Wall and E. H. de Butts, *J. Chem. Phys.*, **17**, 1330 (1949).

[21] The resin used was IRC-50 supplied by Resinous Products Division, Rohm and Haas Company, Philadelphia 5, Pennsylvania.