column was eluted with 1500 ml. of 30% ethanol. The effluent was evaporated to dryness, repeating after addition of absolute ethanol; yield 0.42 g., $[\alpha]^{25}D$ +88°, non-reducing. Three-hundredths gram of the obtained material was hydrolyzed in 1 ml. of 0.05 *N* hydrochloric acid and spot-checked. As the hydrolysis proceeded, a gentiobiose spot appeared on the paper chromatogram but no cellobiose spot was found.

Acetolysis of Cellulose Triacetate.—Ten grams of the cellulose triacetate used for the production of the water-soluble polysaccharide was dissolved in a mixture of 34 ml. of glacial acetic acid, 10 ml. of acetic anhydride and 6 ml. of concentrated sulfuric acid. The solution was allowed to stand at room temperature for 140 hours, and filtered through a sintered glass funnel. The filtrate was poured with vigorous stirring into 500 ml. of ice-water. The precipitate was filtered, washed, and dissolved in 400 ml. of chloroform. The solution was washed thrice with 500 ml. of water, 400 ml. of 1% sodium bicarbonate solution and again with 500 ml. of water. The chloroform was removed by distillation under vacuum. The acetolyzate was separated and dried as usual; yield 7 g.

One gram of the acetolyzate was deacetylated in 14 ml. of 0.2 *M* sodium methoxide. After complete deacetylation the mixture was neutralized with dilute acetic acid, diluted with water, de-ionized and concentrated under vacuum to a thin sirup. To the sirup was added 70 ml. of absolute ethanol. The precipitate was separated and the filtrate evaporated to dryness, repeatedly adding absolute ethanol. Both the precipitate and the residue were spot-checked. On the chromatogram the precipitate from ethanol was found to be a mixture of cellobiose, cellotriose and their higher homologs, and the residue, a mixture of glucose, cellobiose and cellotriose. No gentiobiose was indicated on the chromatogram.

Hydrolysis of Cellulose Regenerated from Cellulose Triacetate.—Two grams of cellulose triacetate was deacetylated completely in 30 ml. of 0.2 *M* sodium methoxide in methanol. The regenerated cellulose was dissolved in 72% sulfuric acid at room temperature. The solution was diluted with water to 1 l., and refluxed for 4 hours. Twenty-five milliliters of the solution was withdrawn and neutralized with barium carbonate. The solution was filtered and the filtrate concentrated to 5 ml. The solution was spot-checked. On the chromatogram the solution was found to be a mixture of glucose, cellobiose and cellotriose.

APPLETON, WISCONSIN

[CONTRIBUTION FROM THE INSTITUTE OF PAPER CHEMISTRY]

Studies on Lignin and Related Products. X.[1] Further Studies on the Isolation of Compounds from Lignin Oxidation Mixtures by Chromatographic Techniques[2,3]

BY IRWIN A. PEARL AND DONALD L. BEYER

RECEIVED JUNE 17, 1954

Further studies have been made of chromatographic fractionation of lignin oxidation mixtures, with separation of a variety of products.

In an attempt to obtain better preliminary separation of the acid fraction alkaline cupric oxide oxidations of lignosulfonate materials a large scale experiment was performed on the use of an anion-exchange resin in ethanolic solution as described earlier.[4] An ethanol solution comprising the combined total ether extracts from the many individual oxidations for fermented spent sulfite liquor reported earlier,[5] was filtered to remove dehydrodivanillin and then passed through a column of Duolite A-2.[4] The column was washed with ethanol and then with water, collecting each washing separately. Finally the column was washed with 4% sodium hydroxide solution followed by water. A flow sheet giving the preliminary separations employed is pictured in Fig. 1.

Fractions IV, V and VII were fractionated by means of techniques reported earlier,[6,7] but it soon became obvious that such techniques employing only adsorption chromatography on Magnesol were not sufficient for the complete fractionation of the phenolic and acidic mixtures. The original mixture contained a great many phenolic and carboxylic compounds in small concentration in the presence of an overwhelming amount of vanillic acid. Under these conditions the vanillic acid was dispersed in all of the above fractions and was obtained along with vanillin, 5-formylvanillin, 5-carboxyvanillin, vanillil, a compound melting at 110–120° which yielded only vanillic acid upon further chromatography, and several unidentified crystalline compounds. It was at this point that recourse was made to cellulose column and paper chromatographic methods.

Fractions I, II and III were separated by chromatography on Magnesol into fractions which were analyzed by means of cellulose powder and paper chromatography employing butanol saturated with 2% aqueous ammonia. Vanillin and acetovanillone were found to be the chief components of these fractions along with an unidentified compound having an R_f value of 0.87.

It soon became apparent from these paper chromatographic studies and from analogous studies on the oxidation of lignin model substances[1] that, for a particular developer, R_f values of a pure compound and the compound in admixture are usually different. This difference might be of considerable magnitude, especially with compounds of similar R_f values. The adulterants appear to act as components of the developing system until they are completely removed as independent spots by development. When the R_f values are very close, the adulterant may merely push the desired compound to a higher R_f without any separation whatsoever. Thus, simultaneous paper chromatography of a lignosulfonate oxidation fraction and known pure compounds will yield spots for identical

(1) For paper IX of this series, see THIS JOURNAL, **76**, 2224 (1954).

(2) Presented before the Division of Cellulose Chemistry at the 126th Meeting of the American Chemical Society, New York, N. Y., September 12–17, 1954.

(3) The results reported here are from a research program at this Institute sponsored by the Sulphite Pulp Manufacturers' Research League. Acknowledgment is made for their permission to publish these results.

(4) I. A. Pearl and D. L. Beyer, THIS JOURNAL, **75**, 2630 (1953).

(5) I. A. Pearl and D. L. Beyer, *Tappi*, **33**, 544 (1950).

(6) I. A. Pearl, THIS JOURNAL, **71**, 2196 (1949).

(7) I. A. Pearl and E. E. Dickey, *ibid.*, **74**, 614 (1952).

compounds at different R_f values. Therefore, *extreme caution must be practiced in the interpretation of such chromatograms.*

Two-dimensional chromatograms help obviate discrepancies in R_f values. Under ideal conditions in which a developing system in one dimension will completely separate all components, albeit the R_f values might be fallacious, the second dimension with the same developing system will develop the already separated compounds to their true R_f values. However, ideal conditions very seldom prevail, and better results are usually obtained if the second dimension is developed with a different system. We have found 10:3:3 butanol–pyridine–water and butanol saturated with 2% aqueous ammonia very satisfactory systems for the development of two-dimensional chromatograms of lignosulfonate oxidation fractions. We have determined the R_f values at 20° for these two systems of pure compounds isolated from lignosulfonate mixtures. These values were determined by means of bis-diazotized benzidine (BDB), 2,4-dinitrophenylhydrazine (DPH), ferric chloride, and ferric chloride–potassium ferricyanide spray reagents described earlier[1] and ultraviolet light. These R_f values together with pertinent comments are given in Table I.

TABLE I

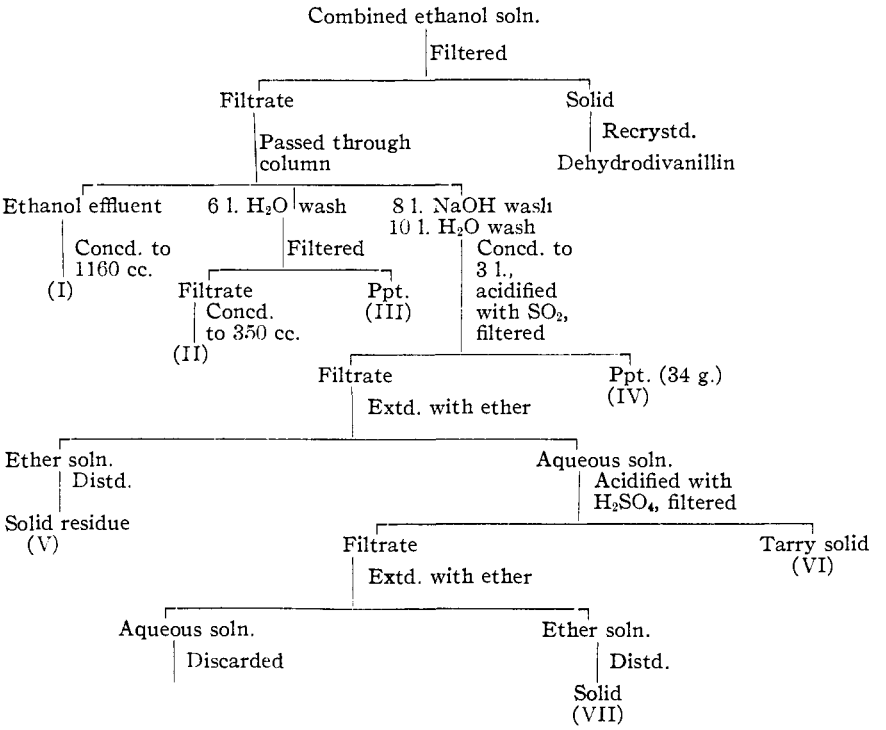
R_f VALUES OF LIGNOSULFONATE OXIDATION PRODUCTS FOR TWO-SOLVENT SYSTEMS

Compound	BuOH–2% aq. ammonia	10:3:3 BuOH–pyridine–H_2O	Comments
Dehydrodivanillic acid	0.00	0.07	
5-Carboxyvanillic acid	.00	.14	Blue fluorescence (UV)
Vanillic acid	.10	.41	Brown to BDB
Compound VI[7]	.10	.83	
5-Carboxyvanillin	.12	.37	Yellow fluorescence (UV)
4,4'-Dihydroxy-3,3'-dimethoxychalcone	.18	.80	Yellow fluorescence (UV)
Vanillil[a]	.19	.87	Negative fluorescence (UV)
5-Formylvanillin	.20	.53	White fluorescence (UV)
Dehydrodivanillin	.31	Streak	Negative fluorescence (UV)
Syringaldehyde	.37	0.79	
Vanillin	.44	.87	Reddish violet to BDB
Vanillovanillone	.47	.86	
Acetovanillone	.60	.85	Bright red to BDB

[a] Vanillil does not give a spot with DPH.

Two-dimensional chromatograms also tend to offset the effect of inequalities in concentration of components of a mixture. Heavy concentrations will form long spots in the first dimension, thus making it very difficult to determine an R_f value. However, in the second dimension these long spots become wide spots with average concentration more nearly that of the materials originally present in smaller concentration.

Fig. 1.—Flow sheet of initial separation of combined ethanol solution.



Experimental

All melting points are uncorrected.

Starting Material.—The total ether solubles, obtained from each experiment in a large study on the cupric oxide oxidation of fermented (*Torulopsis utilis*) spent sulfite liquor reported earlier,[5] had been dissolved in ethanol and stored. These ethanol solutions were combined to give 2000 cc. of solution containing 480 g. of solids representing the oxidation products of a great many experimental conditions.

Initial Separation.—A glass tube, 70 mm. in diameter and 4 ft. in length, was packed with 2 lb. of Duolite A-2 resin and prepared exactly as described earlier.[4] The separation is indicated in Fig. 1.

Representative Fractionation by Adsorption Chromatography. Analysis of Fraction V.—Ten grams of Fraction V was boiled with 300 cc. of water, and the clear solution was decanted from the little tar which adhered to the flask. A mixture of oil and crystals separated from the solution upon cooling. The insoluble material was separated by decantation and washed several times with water. The dried product (2.5 g.) was dissolved in 10:1 benzene–ethanol, chromatographed on a column of acid-washed Magnesol (44 mm. in diameter by 224 mm. in length), and developed with 320 cc. of 10:1 benzene–ethanol as described earlier.[7] Although 4 bands were indicated by ferric chloride streak and by ultraviolet light, elution of the individual extruded bands yielded only pure vanillic acid, melting at 209–210° and not depressing a mixed melting point with pure vanillic acid.

The original cooled aqueous solution was extracted twice with 250 cc. of benzene, and the benzene extract was concentrated to a small volume and cooled. The crystals were filtered (1.20 g.) and recrystallized from ethanol to yield fine needles of vanillil melting at 228–229° and not depressing a mixed melting point with authentic vanillil.[8] The benzene filtrate was chromatographed on acid-washed Magnesol and developed with 325 cc. of 20:1 benzene–ethanol. Streaking with ferric chloride and with 2,4-dinitrophenylhydrazine and examination under ultraviolet light indicated four bands. These were sectioned from the extruded column and individually eluted with acetone. Acetone eluates were

(8) I. A. Pearl, THIS JOURNAL, 74, 4260 (1952).

evaporated. The first section (186 mg.) was covered with benzene and filtered to remove crystals of vanillic acid. The benzene solution upon rechromatographing yielded a yellow product melting at 110–120° which, upon further chromatography, yielded only vanillic acid melting at 209–210°. The second section (1.15 g.) was recrystallized from water to yield vanillic acid melting at 209–210°. The third section (390 mg.) was recrystallized from petroleum ether (b.p. 65–110°) to give acetovanillone melting at 110–111° and not depressing a mixed melting point with authentic acetovanillone. Section four, weighing 106 mg. and having a crude melting point of 109–128°, was boiled with petroleum ether and filtered. Recrystallization of the precipitate from water gave fine white crystals melting at 193–194°. The compound has not been identified. Concentration and cooling of the petroleum ether yielded needles of pure acetovanillone melting at 111–112°. The effluent upon evaporation and recrystallization gave more of the 193–194° melting compound.

The aqueous solution, after the above-described benzene extraction, was then extracted with ether, and the ether was dried and distilled to yield 7.12 g. of residue. The residue was boiled with an excess of 10:1 benzene–ethanol and filtered to leave 2 g. of crystals which were recrystallized from water to yield pure 5-carboxyvanillin melting at 256–257° and not depressing the melting point of a mixture with authentic 5-carboxyvanillin.[9] The benzene–ethanol filtrate was chromatographed on a large column of acid-washed Magnesol and developed with a large excess of 20:1 benzene–ethanol. Elution of the Magnesol yielded only a product which upon repeated recrystallization from water gave light brown crystals melting at 222–226° and giving a purple color with ferric chloride. The product has not been identified. The effluent, upon evaporation and boiling with benzene, gave vanillic acid as a precipitate (3.7 g.) and a filtrate which upon chromatography yielded crystals, melting at 113–118°, which have not been identified.

Other fractions shown in Fig. 1, except fractions I, II and III, were analyzed in an analogous manner.

(9) I. A. Pearl and D. L. Beyer, THIS JOURNAL, **74**, 4263 (1952).

Representative Fractionation by Paper Partition and Cellulose Column Chromatography. Analysis of Fraction I.—Fraction I (10 g.) was dissolved in benzene and chromatographed on acid-washed Magnesol (70 mm. in diameter by 330 mm. in length), and developed with 1300 cc. of 20:1 petroleum ether (b.p. 65–110°)–ethanol. Ferric chloride, 2,4-dinitrophenylhydrazine and alkaline permanganate streak reagents indicated four bands. The column was cut into four sections, and each section was eluted with acetone. The acetone eluates were evaporated to dryness. The four residues were dissolved in acetone and spotted on Whatman No. 1 filter paper in quadruplicate, developed in a descending system with butanol saturated with 2% aqueous ammonia, and the chromatograms sprayed separately with three reagents as described earlier.[1] Results were: section 1, trace of vanillic acid at R_f 0.10; section 2, strong vanillin at R_f 0.48, and a strong spot at R_f 0.78; section 3, strong vanillin at R_f 0.48, spot at R_f 0.65 and at R_f 0.87; and section 4, light vanillin spot at R_f 0.48, acetovanillone at R_f 0.60, and spot at R_f 0.87.

All spots were eluted from the unsprayed chromatogram as described earlier.[1] Rechromatographing of the eluted R_f 0.78 spot of section 2 and of the R_f 0.65 spot of section 3 under the same conditions gave only spots of pure acetovanillone at R_f 0.60. The R_f 0.87 spots of sections 3 and 4 on rechromatographing gave spots at only R_f 0.87. All eluates were identified by comparison with authentic samples (mixed melting points and ultraviolet absorption spectra).

Larger amounts (3.0 g.) of sections 3 and 4 were also chromatographed on a large column of cellulose powder and developed with butanol saturated with 2% aqueous ammonia, as described earlier.[1] Samples of the effluent were collected in an automatic fraction collector and individually spotted on paper. In this manner the same materials were isolated in a pure state in large amounts. It is interesting to note that the materials came off the column in the following order: R_f 0.87, vanillin at R_f 0.48, acetovanillone at R_f 0.60, and vanillin at R_f 0.48.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Cholesteryl Naphthylcarbonates[1]

BY KWAN-CHUNG TSOU

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Cholesteryl and cholestanyl 2-naphthylcarbonate and cholesteryl 1-napthylcarbonate were prepared as chromogenic substrates for the study of cholesterol esterase. Preliminary data on the enzymatic hydrolysis of these substrates are given. Pyrolysis of cholesteryl 2-naphthylcarbonate yields mainly 2-naphthol, carbon dioxide and $\Delta^{3,5}$-cholestadiene, together with small amounts of a hitherto undescribed $\Delta^{2,5}$-cholestadiene, cholesteryl 2-naphthyl ether, 2,2′-dinaphthylcarbonate, and impure dicholesteryl ether. Characterization and properties of the new cholestadiene are described.

Since cholesterol and esters of cholesterol[2] are important constituents of living cells and tissues and human blood, enzymes involved in formation and hydrolysis of esters of cholesterol are of interest, and methods for demonstrating such enzymatic activity would be important in studying the role of cholesterol in health and disease. Esters of cholesterol with naphthylcarbonic acids are particularly suitable as substrates, since on enzymatic hydrolysis they should yield first naphthoxyformic acids and then naphthols which can be measured colorimetrically after coupling with a suitable diazonium salt.

Cholesteryl 1- and 2-naphthylcarbonate were prepared by the reaction of cholesterol with the corresponding naphthylchlorocarbonate in pyridine with protection against moisture. Cholestanyl 2-naphthylcarbonate was prepared similarly. Enzymatic studies on these substrates[3] were carried out in tissue homogenates at both *p*H 7.4 and 5.4.[4] Hydrolysis is very slow and is not complete even after 20 hours incubation, owing, at least in part, to the poor solubility of the esters. Klein[4] also noted slow hydrolysis of synthetic cholesteryl esters when in colloidal suspension.

Since Reichstein and co-workers[5] have found that

(1) This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Department of Health, Education and Welfare, (C312-C8).

(2) L. F. Fieser, *Science*, **119**, 710 (1953).

(3) Performed by Drs. H. A. Ravin and A. M. Seligman, Beth Israel Hospital, Boston, Mass.

(4) These two *p*H's were chosen because Klein (*Z. physiol. Chem.*, **254**, 1 (1938)) found that mammals have at least two cholesterol esterases. One acts in acid solution (*p*H 5.3) and is present in liver, spleen, kidney and other tissues; the other is active at a neutral *p*H and is apparently limited to the pancreas.

(5) J. von Euw, A. Lardon and T. Reichstein, *Helv. Chim. Acta*, **27**, 821 (1944); A. Lardon and T. Reichstein, *ibid.*, **28**, 1420 (1945).