recrystallized from ethyl acetate. A sample prepared from authentic 4-*O*-methyl-D-glucose had m.p. 156–158° and a mixed melting point of 156–159°.

In an attempt to make other derivatives of 3- and 4-*O*-methyl-D-glucose for comparison, the dibenzyl mercaptals were made by the usual procedure from authentic specimens. That from 3-*O*-methyl-D-glucose was recrystallized with difficulty from ethanol containing a few drops of water and had m.p. 66–69°. The mercaptal from 4-*O*-methyl-D-glucose readily crystallized from absolute ethanol, and had m.p. 158–159°.

Methyl 2-*O*-(2,3,4,6-Tetra-*O*-methyl-α-D-glucopyranosyl)-3,4-di-*O*-methyl-D-xylopyranoside.—To a portion (864 mg.) of the neutral disaccharide methyl glycoside was added sodium hydroxide (6 ml., 40%) and dimethyl sulfate (0.5 ml.). The mixture was stirred at high speed at a temperature of 45–55° and 0.5-ml. portions of dimethyl sulfate added every 15 minutes for 1.5 hours. This treatment was repeated twice and after 4–5 hours the bath temperature was raised to 80° for 0.5 hours. This cycle was repeated twice and finally after neutralization to *p*H 8.9 with sulfuric acid the product was isolated by continuous extraction with chloroform overnight. The partly methylated sirup (675 mg.), which showed only weak infrared absorption in the hydroxyl region, was dissolved in methyl iodide (20 ml.) and Drierite (*ca.* 1 g.) added. Silver oxide (5 g.) was added in small portions over 9 hours to the refluxing and stirred solution. After three such methylations the infrared spectrum showed no hydroxyl absorption. The distilled methylated sugar (517 mg.) had b.p. 170–180° (bath temp.) at 0.01 mm. and OMe, 55.7%; calcd. $C_{18}H_{34}O_{10}$; OMe, 52.9%. We are indebted to Mr. Y. Tanaka for the infrared spectra.

Hydrolysis of Methylated Disaccharide.—The methylated disaccharide (437 mg.) was dissolved in sulfuric acid (25 ml., 1 *N*) and hydrolyzed on the steam-bath for 11 hours, [α] 84.8°, constant. The sirup (396 mg.) obtained after neutralization ($BaCO_3$) showed two components of R_f 0.68 and 0.83 in solvent A. The mixture was separated on prewashed Whatman 3MM paper using the same solvent. The faster component was extracted with ether and the slower one with ethanol.

Identification of 2,3,4,6-Tetra-*O*-methyl-D-glucose.—Careful chromatographic examination of the fraction having R_f 0.83 showed the presence of a small amount of a faster component (tri-*O*-methyl-L-rhamnose ?) and because of this it was not possible to obtain the tetra-*O*-methyl-D-glucose crystalline. A small amount of *N*-phenyl-2,3,4,6-tetra-*O*-methyl-D-glucosylamine was obtained with m.p. 122–132° and when mixed with an authentic sample 130–136°.

Identification of 3,4-Di-*O*-methyl-D-xylose.—The component with R_f 0.68 was chromatographically pure and was oxidized with bromine in the ordinary way for 48 hours. The derived 3,4-di-*O*-methyl-D-xylono-δ-lactone distilled at 140–160° (bath temp., 0.01 mm.), crystallized spontaneously and had m.p. and mixed m.p. 64–66°.

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[CONTRIBUTION FROM THE INSTITUTE OF MICROBIOLOGY, RUTGERS, THE STATE UNIVERSITY]

The Specific Polysaccharide of Type VI Pneumococcus. I. Preparation, Properties and Reactions[1]

BY PAUL A. REBERS AND MICHAEL HEIDELBERGER

RECEIVED NOVEMBER 24, 1958

Purified specific polysaccharide of type VI pneumococcus, S VI, $[\alpha]_D$ +129°, contains D-galactose, D-glucose, L-rhamnose, a polyol definitely identified as ribitol and phosphorus in approximately equimolar amounts. The P occurs as a labile diester, and alkaline hydrolysis yields a stable, serologically inactive monoester, $[\alpha]_D$ +106°. Periodate oxidation of S VI produces formaldehyde but no formic acid; the galactose is attacked, but the glucose and rhamnose are resistant. After alkaline hydrolysis of S VI, glucose and rhamnose are still attached to the phosphorus-containing portion in periodate-resistant linkages in the original ratio, but now, besides formaldehyde, formic acid is liberated, apparently from the galactose.

S VI seems to be a linear polymer of the unit —[—4 or 2-O-D-galactopyranosyl-(1 → 3)-O-D-glucopyranosyl-(1 → 3)-O-L-rhamnopyranosyl(1 → 1 or 3)-ribitol-3 or 1-O-P(OH)(=O)-O—]$_x$—.

Pneumococci of the serological Types II, V and VI form a triad, of which Types V and VI were originally considered sub-types of II.[2] Because the serological relationships reflect differences and similarities in the capsular specific polysaccharides which, among pneumococci, are the principal determinants of immunological type specificity,[3] a study of the fine structures of these three carbohydrates[4] is considered likely to clarify the as yet obscure relation of chemical constitution to the immunological specificities and cross reactivities involved and to serve as a model for similar investigations. The chemistry of S II and S V is being unravelled by Prof. Maurice Stacey and Dr. S. A. Barker, respectively, of the University of Birmingham, England, and some progress with S II has already been recorded.[5] The present paper is concerned with initial stages of the chemical study of S VI.

Experimental

Materials and Methods.—S VI, isolated by the phenol process,[6] was generously supplied by E. R. Squibb and Sons, through the kindness of Mr. T. D. Gerlough. Type VI anti-pneumococcal horse serum was furnished by the Division of Laboratories, New York State Department of Health. Thanks are also due to Dr. N. K. Richtmyer for many of the samples of polyols and to Dr. F. Smith for the sample of L-threitol.

(1) This study was carried out under a grant from the National Science Foundation.

(2) O. T. Avery, *J. Exper. Med.*, **22**, 804 (1915); G. Cooper, M. Edwards and C. Rosenstein, *ibid.*, **49**, 461 (1929).

(3) M. Heidelberger and O. T. Avery, *ibid.*, **38**, 73 (1923); **40**, 301 (1924); O. T. Avery and M. Heidelberger, *ibid.*, **38**, 81 (1923); **42**, 367 (1925); M. Heidelberger, W. F. Goebel and O. T. Avery, *ibid.*, **42**, 727 (1925).

(4) Hereinafter referred to as S II, S V and S VI.

(5) K. Butler and M. Stacey, *J. Chem. Soc.*, 1537 (1955).

(6) T. D. Gerlough, U. S. Pat. 2,340,318 (Feb. 9, 1944); J. W. Palmer and T. D. Gerlough, *Science*, **92**, 155 (1940).

Nitrogen in all polysaccharide samples was determined by a micro-Kjeldahl method.[7] Inorganic phosphorus was determined by the method of Fiske and Subbarow[8] or by a modification[9] in which *p*-semidine replaces sodium 1-naphthol-2-amino-4-sulfonate. Organic phosphorus was estimated after digestion with sulfuric acid and 30% hydrogen peroxide[9,10] which had been absorbed with kaolin in order to reduce its P content.[11]

All centrifugations were carried out at 0° unless otherwise noted. Measurements of optical activity were made at room temperature, 25–30°, in a Rudolph Model 80 polarimeter with a sodium lamp. Unless otherwise designated, water was used as solvent and the concentration was 1–2%.

Oxidations by periodate were carried out as suggested by Dyer.[12] Periodate consumption was determined according to Fleury and Lange.[13] Any formic acid liberated was estimated by addition of ethylene glycol and titration under N_2 with 0.01 *N* $Ba(OH)_2$ to the initial *p*H. Formaldehyde was identified and determined by precipitation with dimedone[12,14] or by the use of chromotropic acid.[15]

Dialyses were carried out in Visking cellophane tubing, ³/₄'' diameter, that had been pretested for leaks.

*p*H was determined with a Model G Beckman *p*H meter.

Paper chromatograms were run with Whatman No. 1 filter paper by a descending technique at room temperatures (25–30°). The solvent mixtures used were: (A) BuOH:pyridine:H_2O, 6:4:3[16]; (B) BuOH:EtOH:H_2O:NH_3, 40:10:49:1[17]; (C) s-collidine–H_2O.[17] Sugars were located on chromatograms with reagents: (D) aniline–trichloroacetic acid[18]; (E) aniline hydrogen phthalate.[19] (F) Polyols[20] as well as sugars[17] were detected with ammoniacal silver nitrate; (G) phosphate esters were located by a modification of the Hanes–Isherwood procedure.[21]

Paper electrophoresis was carried out in borate buffer (0.05 *M*, *p*H 10) on Whatman No. 3[22] or glass paper.[23]

Purification of S VI.—Of the methods tried, precipitation by alcohol in the presence of calcium acetate yielded the best results. A 7% aqueous solution was centrifuged at 105,000 times gravity for 1 hr. in a Spinco ultracentrifuge. After addition of calcium acetate at *p*H 5 to the clear supernatant up to 5% (w./v.), 95% ethanol was added to 0.75 vol. The small precipitate of high N content was removed by centrifugation and the main fraction was precipitated by an additional 0.15 of the new volume of alcohol. After four such precipitations, it showed $[\alpha]_D$ +121 to 127°, 0.06 to 0.2% N and 4.0 to 4.2% P, as compared with +113°; 0.3 to 0.4, and 4.0 to 4.2, respectively, reported by Brown.[24] Fusion with sodium failed to show sulfur or halogens. Three g. of purified material was obtained from 8 g. of starting product. The N content could be reduced to 0.06% without change in the other properties by additional fractional precipitations with alcohol or glacial acetic acid in the presence of calcium acetate.

The ultraviolet absorption of a 1% aqueous solution of purified S VI showed no peaks between 2300–3600 Å. The extinction coefficient at 2600 Å., calculated per mole of P was 6.5. The value for yeast RNA[25] is 8710. Since DNA shows about the same value,[25] the amount of nucleic acid in the S VI would be roughly 6.5/8710, or <0.1%.

Electrophoretic Homogeneity of S VI.—In a Perkin-Elmer Model 38 apparatus at *p*H 5.2 in *M*/10 acetate buffer, S VI showed one stationary and one rapidly moving component. The former was serologically inactive and gave a negative Molisch reaction, suggesting that it was due to a salt effect, while the mobile component was strongly positive with antiserum and in the Molisch test. While this does not prove the homogeneity of purified S VI, it indicates that the serologically active carbohydrate contains charged groups.

Ash Determination.—Sulfated ash of the Ca salt of S VI was 7.6 to 9.4%, equivalent to 2.2 to 2.8% Ca; *i.e.*, one mole of Ca per 2 moles of P.

Reducing Function.—Twenty mg. of S VI was analyzed for reducing groups with alkaline ferricyanide.[26] The color corresponded to 12 μg. of glucose, or 0.06% as reducing end groups.

Uronic Acids.—One ml. of a solution of S VI containing 85.5 μg. was treated with sulfuric acid and carbazole.[27] Only ¹/₆₀ as much color was produced as by an equivalent weight of glucurone.

A summary of data on S VI is given in Table I.

TABLE I

SUMMARY OF RESULTS

	Original S VI	Alkali-hydrolyzed S VI
$[\alpha]^{25}_D$ [a]	+129°	+106°
Exptl. equiv. wt.[b]	782, 791	338, 340
Calcd. equiv. wt.[c]	740	370
pK' [d]	2.0	pK_1', 1.5; pK_2', 5.7
Uronic acids, %	<2	
Acetyl,[e] %	0.4	
Reducing end groups, %	0.06	0.08
$PO_4^{\equiv}$ after phosphatase, %	0	50
Rhamnose, %	22.5, 22.3	22.5, 22.6
Glucose, %	24, 25.4	
Galactose, %	29	
Total hexose, by cysteine[f]	41.6, 40.1	41.3, 41.9
$NaIO_4$ oxidation[g]		
	Moles per mole P	
Consumption, 2 wk.	2.08, 2.12	
3 wk.	2.10, 2.15	3.17, 3.14
4 wk.	2.08	3.11, 3.04
HCOOH production	0	0.9, 1.0
CH_2O liberation[h]	1.1, 1.2	1.0
Resistant sugars	Gluc. and rham.	Gluc. and rham.
Susceptible sugars	Galactose	Galactose

[a] Free acids, c = 0.52 and 0.86, resp., in H_2O. [b] The salts were converted to the free acids with Dowex 50 and titrated potentiometrically with $Ba(OH)_2$ under N_2. See Fig. 1. [c] Calcd. for the phosphate diester and monoester, resp., 4.2% P. [d] From Van Slyke's formulas

$$K_1' = \frac{[H^+](B + [H^+])}{C - (B + [H^+])},\ pK_2' = pH - \log \frac{B}{C - B'}$$

D. D. Van Slyke, *J. Biol. Chem.*, **52**, 525 (1922); A. B. Hastings and D. D. Van Slyke, *ibid.*, **53**, 269 (1922). [e] Acc. to M. Heidelberger, F. E. Kendall and H. W. Scherp, *J. Expt. Med.*, **64**, 559 (1936). [f] Calculated as glucose. [g] 0.5–1.2 mg./ml. S VI or derivative in 0.02 *M* $NaIO_4$ at 0°, initial *p*H 5.4. [h] M.p. of dimedone deriv., 190–191°, m.p. of mixture with authentic dimedone deriv., 190–191°.

Acid Hydrolysis of S VI.—Two hundred and forty mg. of S VI was dissolved in 2 *N* H_2SO_4. The solution, $[\alpha]_D$ +117°,

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(23) D. R. Briggs, E. F. Garner and F. Smith, *Nature*, **178**, 155 (1956).

(24) R. Brown, *J. Immunol.*, **37**, 445 (1939).

(25) J. N. Davidson, "The Biochemistry of the Nucleic Acids," 2nd Ed., Methuen, London, 1950, p. 58.

(26) O. Folin, *J. Biol. Chem.*, **81**, 231 (1929); S. Nussenbaum and W. Z. Hassid, *Anal. Chem.*, **24**, 501 (1952); F. Smith, "Methods of Biochemical Analysis," ed. by D. Glick, Interscience Publishers, Inc., New York, N. Y., 1956, Vol. III, p. 186.

(27) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).

was heated at 100°. After 1 hr., $[\alpha]_D$ +44° and was unchanged after an additional 0.5 hr. The solution was neutralized to *p*H 7 with $Ba(OH)_2$, Super-Cel added, the precipitate filtered off and washed; paper chromatography of the supernatant with solvent C for 48 hr. gave 3 spots with reagent D, corresponding to galactose, glucose and rhamnose. As a result of a letter from Prof. J. K. N. Jones reporting a polyol in another pneumococcal carbohydrate, a second chromatogram, run in solvent B and sprayed with reagent F, was found to give a new spot suggesting that it was a polyol or polyol anhydride.

The main solution was evaporated *in vacuo* to a sirup which was dissolved in methanol and treated with *n*-butanol until faintly turbid. Paper chromatograms of the crystals which separated were made with solvents A, B and C, and indicated a component corresponding to galactose. The crystals, $[\alpha]^{24}_D$ +80° (equil., *c* 2 in H_2O), melted at 162° and showed a mixed melting point with authentic D-galactose of 162°.

Further additions of butanol to the supernatant resulted in deposition of glucose and galactose. The supernatant was evaporated to a sirup *in vacuo*, dissolved in *n*-butanol and ether added until slightly turbid. Small crystals formed in 1 wk., m.p. 148°, and showing a mixed melting point of 147° with authentic D-glucose. The supernatant was poured off and additional ether was added. Large crystals formed in another week, m.p. 82–85°; mixed melting point with authentic L-rhamnose hydrate, 82–84°.

The precipitate of $BaSO_4$ was extracted with 50 ml. of 0.1 *N* HCl, 2.2 ml. of 25% $Ba(OAc)_2$ was added to the filtered extract, and the precipitate adjusted to *p*H 7.2 with KOH. Salts of the phosphate ester remained in solution. After evaporation *in vacuo* to 5 ml., 95% EtOH was added until turbid (12 ml.). After 3 days at −10° the precipitate was centrifuged off, washed with 75% EtOH and dissolved in 0.1 *N* H_2SO_4. P determinations indicated 0.6 mg. in the precipitate and 3.6 mg. in the ethanolic supernatant and washings. Barium salts were removed from an aliquot of the supernatant with Na_2SO_4, after which wheat-germ acid phosphatase[28] was found to liberate 33% of the organically linked P in 20 hr. at 25°. After 1 month at −10° additional precipitate separated from the ethanolic supernatant; yield 11.3 mg., 5.4% P. After treatment with 0.1 *N* H_2SO_4 and removal of $BaSO_4$, an aliquot was mixed with 0.1 *M* $NaIO_4$. Periodate consumption after 24 hr. at room temperature was 2.8 moles per mole P. A pentitol-1-PO_4 should consume three moles of $NaIO_4$. Formaldehyde liberation was estimated with chromotropic acid.[15] After 10 minutes at room temperature, 0.9 moles of CH_2O were released per mole P, but after 2 hr. at 100° the figure was twice as great. Ribitol 1-phosphate should produce 1 mole of CH_2O at room temperature and 2 moles at 100°.[29]

The amount of sugar in the phosphate ester was determined as roughly 1% with phenol and sulfuric acid,[30] with which polyols would not be expected to react. Reducing groups were also tested for by alkaline ferricyanide, with the same result. A 0.2-ml. aliquot was also hydrolyzed 17 hr. with 2 *N* H_2SO_4 at 100°, neutralized and chromatographed at 25° with the aid of solvent B and spray F (Table II). It will be seen that the chromatographic behavior of the hydrolysate was different from that of any of the polyols tested. Although closest to glycerol, the substance migrated more rapidly in borate buffer during electrophoresis on glass paper.[23] Dr. Richtmyer suggested the possibility of anhydride formation during hydrolysis, as reported for ribitol 1-phosphate,[31] which yields chiefly anhydroribitol when heated with *N* HCl and ribitol at *p*H 4. To test the result at an intermediate *p*H, 1 g. of S VI was hydrolyzed by heating in 0.1 *N* H_2SO_4 at 100° for 12.5 hr., when specific rotation and reducing sugars were essentially constant and 43% of inorganic phosphate was liberated. The solution was neutralized to *p*H 7.5 with *ca.* 0.4 *N* $Ba(OH)_2$ and the precipitate was centrifuged off and washed. The supernatant and washings were concentrated *in vacuo* to 15 ml. and 3 vol. of 95% ethanol were added, yielding a white precipitate. After 16 hr. at 6° this was centrifuged off and redissolved in H_2O. Reducing sugar phosphates were removed by precipitation with 0.5 ml. of phenylhydrazine reagent[32] at 100° for 15 minutes. The supernatant was mixed with 8 ml. of 95% EtOH, and the white flocculent precipitate centrifuged off, dissolved in H_2O and reprecipitated with EtOH, again dissolved in water and passed through a column of Dowex 50 (H^+). Analysis showed 7.4 mg. of P. An attempt was made to form a crystalline dicyclohexylammonium derivative, but a granular amorphous product was obtained. This was treated with 0.02 *M* $NaIO_4$ for 7 days at room temperature. Two and nine tenths moles of $NaIO_4$ were consumed and 2.2 moles of formic acid liberated per mole of P, close to the values expected for ribitol 1-phosphate.

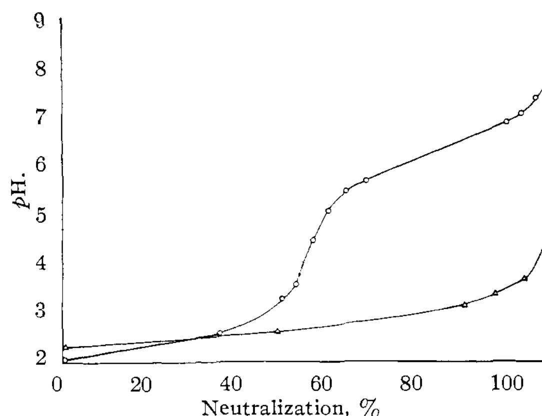


Fig. 1.—Titration curves of S VI before and after alkaline hydrolysis: O, alkali S VI, *c* = 8.65 mg./ml.; Δ, original S VI, *c* = 5.2 mg./ml.

TABLE II

CHROMATOGRAPHIC BEHAVIOR OF POLYOLS IN BuOH: EtOH:H_2O:NH_3 AT 25°

	R_f after 24 hr.	R_r[a] after 44 hr.
1-Desoxy-L-mannitol	0.32	1.0
1-Desoxy-D-galactitol	.32	
1-Desoxy-D-glucitol	.27	
1-Desoxy-D-altritol	.31	
1-Desoxy-D-talitol	.29	
L-Arabinitol		0.66
Xylitol		.60
Ribitol		.64
L-Threitol		.86
Erythritol		.90
Glycerol		1.16
"Hydrolysate"	.34	1.12
Acid-treated ribitol		1.10, 0.65

[a] Distance moved relative to 1-desoxy-L-mannitol.

Enzymatic hydrolysis of the derivative was effected by "Polidase S"[33] in 0.1*N* ammonium acetate buffer.[34] Paper chromatography, solvent B, spray F, indicated ribitol.

Hydrolysis of the S VI polyol phosphate and an authentic sample of ribitol 1-phosphate was next effected by heating 0.1 *N* H_2SO_4 solutions of each at 100° for 27 hr. Paper chromatographic analysis of the hydrolysates, solvent B, spray F, gave in each case a spot with the R_f of ribitol and another close to the origin, suggesting incomplete hydrolysis; there were no faster moving components. However, hydrolysis in 2 *N* H_2SO_4 for 1.5 hr. at 100° indicated a faster component as major product. Ribitol, heated in 2 *N* H_2SO_4 solution for 5 hr. at 100°, also produced a small amount of fast-moving component corresponding to anhydroribitol (Table II).

(28) Worthington Biochemical Corp., Freehold, N. J.

(29) P. Fleury and J. Courtois, *Bull. soc. chim. France*, **8**, 397 (1941).

(30) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

(31) J. Baddiley, J. G. Buchanan and B. Carss, *J. Chem. Soc.*, 4058 (1957).

(32) One ml. of phenylhydrazine, 0.9 ml. of acetic acid, 1 g. of NaOAc·$3H_2O$ and 5.0 ml. of H_2O; *p*H 5.2.

(33) A mixture of enzymes from Schwartz Laboratories, dialyzed against buffer to remove impurities.

(34) J. Baddiley, J. G. Buchanan, B. Carss and A. P. Mathias, *J. Chem. Soc.*, 4583 (1956).

Additional hydrolyses of alkali-treated S VI (see below) were run at pH 4 and these resulted in the isolation of a crystalline, four-component, phosphate-free substance. Crystalline ribitol, m.p. and mixed m.p. with an authentic sample, 101°, has been isolated from this material.

Percentage Composition of S VI.—A sample of hydrolysate was analyzed in duplicate for D-glucose by means of a glucose oxidase–peroxidase-*o*-dianisidine reagent,[35] showing 24 and 25.4% of the weight of sample before hydrolysis. No color was produced in controls of rhamnose and galactose. Rhamnose, determined without preliminary hydrolysis[36] was found to be 22.5–23%. Total hexose ("basic cysteine" reaction)[37] was 41%, calculated as glucose. Since glucose was 25% and since a standard solution of galactose gave 55% as much color as glucose, the galactose content equals (41–25)/0.55 or 29%. Calculation of the polyol by difference indicates 0.7 mole per mole P. Since the polyol had no detectable optical activity, the optical rotation of the hydrolysate could be calculated from the rotations of its sugar components: $0.29 \times 80.2 + 0.25 \times 52.7 + 0.23 \times 8.9 = +39$. The observed $[\alpha]_D$ was +42 to +44.

Action of Alkali on S VI. a. NaOH.—Ten ml. of S VI, 5.6 mg./ml., was mixed with 10 ml. of 2 *N* NaOH and kept under N_2 at room temperature. Serological activity was abolished in 3 hr., but no inorganic phosphate could be detected. The solution remained clear on addition of 4 vol. of 95% EtOH and 0.2 vol. of 20% $Ca(OAc)_2$, whereas S VI turned turbid and flocked with only 2 vol. of EtOH. Dialysis of 13.8 ml. of alkali-S VI, 2.6 mg./ml., in 0.5 *M* NaCl, against 150 ml. of 0.5 *M* NaCl at 5° for 24 hr. showed that 78% of the carbohydrate, tested for by the phenol–sulfuric acid reaction,[30] had passed through.

The relative viscosity of S VI in 0.02 *N* NaOH at 29.4°, measured in an Ostwald viscosimeter, decreased rapidly, becoming constant in 3 to 4 hr. Five min. after addition of alkali and mixing, the time of flow was 188 sec.; after 4 hr., 117 sec., after 20 hr., 116 sec.

b. 0.02 *N* $Ba(OH)_2$.—10.0 ml. of S VI, 10.1 mg./ml., was mixed with 0.5 ml. of *ca.* 0.4 *N* $Ba(OH)_2$ and allowed to stand under N_2 at 25–30°. Precipitation of antiserum no longer occurred after 3 days. Paper electrophoresis of the material in 0.05 *M* potassium borate buffer, pH 10, for 2 hr. at 500 v., followed by drying and spraying with reagent G for phosphates, showed a single spot. Acid groups liberated were estimated with later samples by titration of the excess alkali with oxalic acid. After 3 days, 1.01 equivalent of alkali was neutralized per mole of P, a value unchanged within 5% by reaction for 7 or 45 days at room temperature, or by heating for 10 hr. at 100° in a sealed tube under N_2, in accord with the known stability of phosphate monoesters toward further alkaline hydrolysis.[38] The rhamnose content, total hexoses and reducing groups of the alkali-hydrolyzed S VI agreed with the values for S VI, indicating that no loss of sugars had occurred.

Forty mg. of the Ba salt was converted to the acid by addition of H_2SO_4 to pH 1.7. The solution was passed through a column of Duolite A4. The effluent plus washings yielded only 3 mg. or 8%, of solid. Since this contained 8% of the total P, it was evident that neutral fragments were absent. Elution of the column by 0.2 *N* H_2SO_4 removed 85% of the adsorbed material.

Effect of Acid Phosphatase on Alkali-treated S VI.—Wheat-germ phosphomonoesterase,[28] which had no effect on S VI at pH 5.2 in 0.1 *M* NaOAc buffer, acted slowly upon alkali-hydrolyzed S VI. After 1 day, 24%; 6 days, 45%; 12 days, 50% of $PO_4^{\equiv}$ had been formed.

Oxidation of S VI with Periodate.—The results are shown in Table I. Fifteen % of the P was rendered dialyzable, and this material is under study.* Glucose and rhamnose were resistant, but galactose was found to have been destroyed after reduction of the product, hydrolysis and paper chromatography, solvent C, spray E,[39] as well as by paper electrophoresis in borate buffer at pH 10.

Ratio of Glucose to Rhamnose in Alkali-hydrolyzed S VI after Periodate Oxidation.—Periodate and iodate were removed with lead acetate, excess lead with H_2S and reduction effected with $NaBH_4$. Cations were removed by passage through Dowex 50 (H^+) and the effluent was adsorbed on Duolite A4(OH^-). A negative Molisch test indicated complete adsorption. After elution with 0.2 *N* H_2SO_4 the eluate and washings were concentrated ten fold and heated at 100° for 18 hr. Inorganic P amounted to only 43% of the total. Analysis of the hydrolysate for rhamnose,[36] glucose[35] and for total P gave ratios of 0.9:0.9:1.0, showing that hydrolysis of S VI by alkali does not increase the susceptibility of the glucose or rhamnose to attack by periodate.

Results and Discussion

The purified S VI was shown to be free of nucleic acids and proteins by its low ultraviolet absorption and nitrogen content and free of neutral polysaccharides by its electrophoretic behavior and almost complete absorption on Duolite A4 after alkaline hydrolysis. Repeated fractionation by alcohol caused optical rotation and phosphorus to reach constant values which were unchanged by further fractionation with glacial acetic acid.

The S VI titrated as a monobasic acid, $pK' = 2.0$, as would be expected for a phosphate diester.[40] Methods of hydrolysis were chosen with regard to earlier studies on sugar phosphates and sugar alcohols. Thus, alkaline hydrolysis of methyl glucoside 3- and 6-phosphates[41] proceeded without anhydride formation or Walden inversion. However, hydrolysis of ribitol 1(5)-phosphate by *N* HCl has been reported to give 1,4-anhydroribitol, whereas hydrolysis at pH 4 produced ribitol.[31] Since alkaline hydrolysis at high temperatures might result in degradation and isomerization, acid hydrolysis of S VI was effected first. The sugars liberated were D-galactose, D-glucose and L-rhamnose, as shown by paper chromatography and isolation of the crystalline sugars. A polyol phosphate was also obtained. Enzymatic hydrolysis of this, as well as hydrolysis with 0.1 *N* H_2SO_4, liberated a polyol with the same R_f as ribitol, whereas hydrolysis in 2 *N* acid resulted in a compound, probably an anhydride, with a much higher R_f.[31] Authentic samples of ribitol 1(5)-phosphate showed the same divergent behavior on hydrolysis with 0.1 *N* and 2 *N* H_2SO_4, a rather surprising result in view of the mechanism proposed for anhydride formation.[31] Oxidation of the polyol phosphate indicated a pentitol-1-PO_4 from the amount of $NaIO_4$ consumed and the formic acid and formaldehyde liberated. Finally, ribitol was isolated in crystalline form. Because of the well-known ease of isomerization of polyhydroxy phosphate esters by treatment with hot acid, it remains uncertain whether or not the ribitol, as it exists in S VI, has the phosphorus linked to a primary alcohol group or to the adjacent one.

Alkali, even at room temperature and at low concentration, caused extensive changes in S VI. In this respect it is similar to S XVIII[42] and to the polyribophosphate of *Hemophilus influenzae* Type

(35) A. S. Keston, Abstracts A. C. S., Division of Biol. Chem., April 1956, p. 31C; J. D. Teller, *ibid.*, September 1956, p. 69C. Sold as "Glucostat" by the Worthington Biochemical Corp.

(36) Z. Dische and L. B. Shettles, *J. Biol. Chem.*, **192**, 579 (1951); **175**, 595 (1948).

(37) Z. Dische, *ibid.*, **181**, 379 (1949).

(38) G. M. Kosolapoff, "The Chemistry of Organophosphorus Compounds," John Wiley and Sons, Inc., New York, N. Y., 1950.

* NOTE ADDED IN PROOF: In another run only 5% of P and carbohydrate became dialyzable.

(39) J. K. Hamilton and F. Smith, THIS JOURNAL, **78**, 5910 (1956).

(40) O. Meyerhof and J. Suranyi, *Biochem. Z.*, **178**, 427 (1926); W. D. Kumler and J. J. Eiler, THIS JOURNAL, **65**, 2355 (1943).

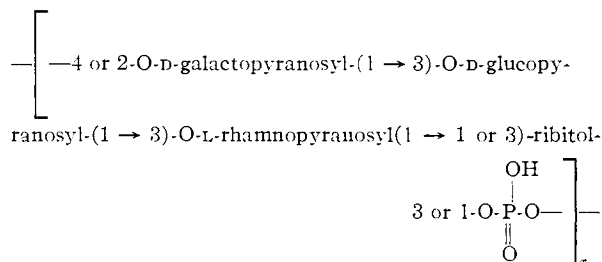
(41) E. E. Percival and E. G. V. Percival, *J. Chem. Soc.*, 875 (1945).

(42) H. Markowitz and M. Heidelberger, THIS JOURNAL, **76**, 1317 (1954).

b.[43] Mild alkaline treatment renders all three substances serologically inactive and susceptible to attack by phosphatase. The action of alkali on polyribophosphate was shown to result in dialyzable, non-reducing, chromatographically homogeneous fragments. Alkali-treated S VI likewise consisted of similar fragments which no longer separated upon the addition of alcohol, failed to precipitate antiserum and showed a decreased viscosity. The titration curve and pK' of the fragments resembled those of a phosphate monoester[40] and existence of this linkage was confirmed by the action of phosphatase.

Periodate oxidation of S VI suggested that the glucose and rhamnose were joined in 1,3-like linkages. The liberation of formaldehyde indicated that a -CHOH-CHOH group was present. Since formic acid was not produced, S VI has very few end groups, while the simultaneous disappearance of the galactose indicated that this sugar is bound in 1,4- or 1,2- linkage. After alkaline hydrolysis of S VI, an additional mole of $NaIO_4$ was consumed and one mole of formic acid was released, with no change in the amount of formaldehyde. This would be expected if the 2- or 4-OH of galactose were set free by alkaline hydrolysis, as, for example, in cleavage of the phosphate diester, S VI. The production of formaldehyde might come from oxidation of a 1,3-linked ribitol.

The following formula is tentatively proposed for the structure of S VI



The ribitol phosphate residue might have either the D- or L-configuration.

Structural studies are continuing and these indicate the correctness of the order given to the glucose and rhamnose residues.

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(43) S. Zamenhof, G. Leidy, P. L. Fitzgerald, H. E. Alexander and E. Chargaff, *J. Biol. Chem.*, **203**, 695 (1953).

[CONTRIBUTION FROM THE PRAIRIE REGIONAL LABORATORY, NATIONAL RESEARCH COUNCIL OF CANADA]

Studies of Lignin Biosynthesis Using Isotopic Carbon. VIII. Isolation of Radioactive Hydrogenolysis Products of Lignin[1]

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RECEIVED DECEMBER 8, 1958

After the feeding of several C^{14}-labeled compounds to wheat and maple, degradation of lignin by hydrogenolysis led to the isolation of dihydroconiferyl and dihydrosinapyl alcohols. The radioactivity of these products was taken to indicate the degree of incorporation of the administered compounds into lignin. The pattern revealed was very similar to that previously found when the activity of the oxidative degradation products, vanillin and syringaldehyde, was used, reinforcing the validity of conclusions from the oxidative degradation. However, labeled sinapic acid, which is converted to lignin yielding syringaldehyde, did not form lignin degradable to dihydrosinapyl alcohol. The active syringaldehyde was shown not to arise from sinapic acid bound to lignin with alkali-labile linkages. Further evidence was obtained that vanillin can be incorporated into lignin, probably by condensation with a two-carbon metabolite. Acetic acid was converted only slightly to the lignin giving rise to hydrogenolysis products; hence the Birch–Donovan acetate pathway does not contribute significantly to lignification.

Introduction

Previous papers of this series[2] have reported comparisons of the efficiencies with which several plant species can convert numerous C^{14}-labeled compounds to lignin. Although the criterion of the conversion efficiency should, in theory, be the specific activity of an intact lignin, it is very doubtful whether such a compound has ever been isolated, and the ill-defined nature and variable composition of lignins isolated by standard methods have made this approach, in our opinion, impractical. As an alternative we have turned to lignin degradation products of low molecular weight which have known structures and either are crystalline or form crystalline derivatives. The use of these degradation products enables a direct comparison of specific radioactivities on a molar basis between the administered compound and the lignin (as represented by its derivative).

This alternative approach, too, suffers from certain disadvantages. There is no known reaction by which lignin can be degraded in anything approaching quantitative yield to products of known structure. The best reaction in this respect is nitrobenzene oxidation in alkaline medium, which yields phenolic aldehydes: vanillin, syringaldehyde and *p*-hydroxybenzaldehyde. It is this reaction which we have used in our studies to date. If one accepts the theory that the unit of the lignin polymer contains nine skeletal carbon atoms, the isolation of substituted benzaldehydes as lignin degradation products has the additional drawback that only seven-ninths of the unit is available for study, the other two carbon atoms being lost in the

(1) Presented in part at the Eighth Annual Research Conference on Plant Physiology, Hamilton, Ontario; November, 1957. Issued as N.R.C. No. 5147. For Part VII of this series see reference 2e.

(2) (a) S. A. Brown and A. C. Neish, *Nature*, **175**, 688 (1955); (b) *Can. J. Biochem. Physiol.*, **33**, 948 (1955); (c) **34**, 769 (1956); (d) D. Wright, S. A. Brown and A. C. Neish, *ibid.*, **36**, 1037 (1958); (e) S. A. Brown, D. Wright and A. C. Neish, *ibid.*, **37**, 25 (1959).