sec-butyl radical albeit to a minor extent.[18] The air oxidation of aromatic Grignard reagents also leads to by-products typical of free radicals[19] as does the anodic oxidation of alkyl Grignard reagents.[20] The other products of homolytic scission of the peroxyalkylboron compounds are $-\overset{|}{B}O\cdot$ and $-\overset{|}{B}O_2\cdot$ (reaction 3). These radicals may react to form –BOH compounds, also products of the oxidations.

(18) Some examples of alkyl free radical isomerizations are: J. A. Berson, C. J. Olsen and J. S. Walia, *J. Am. Chem. Soc.*, **82**, 5000 (1960); C. G. Overberger and H. Gainer, *ibid.*, **80**, 4561 (1958); D. Y. Curtin and M. J. Hurwitz, *ibid.*, **74**, 538 (1952).

(19) C. Walling and S. A. Buckler, *ibid.*, **77**, 6032 (1955).

(20) W. V. Evans, R. Pearson and D. Braithwaite, *ibid.*, **63**, 2574 (1941); W. V. Evans and R. Pearson, *ibid.*, **64**, 2865 (1942).

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The Specific Polysaccharide of Type VI Pneumococcus. II.[1] The Repeating Unit[2]

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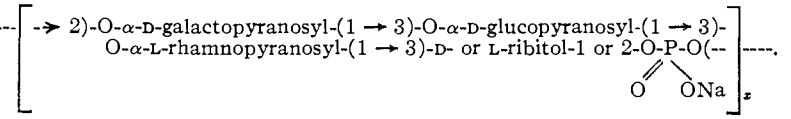
Evidence is given that the structural formula of the specific polysaccharide of type VI pneumococcus is:



Alkali converts the polymer to a monomer with a phosphate monoester linkage and this in turn is split by intestinal alkaline phosphatase into inorganic phosphate and a crystalline, non-reducing, chromatographically homogeneous galactoglucorhamnoribitol in 94% of the theoretical yield. The structure of the repeating unit was established by methylation, oxidation with periodate and degradation.

Because of the importance of the specific capsular polysaccharide of Type VI pneumococcus, S VI, for the understanding of the relation between chemical constitution and immunological specificity of the three related pneumococcal types II, V and VI, the study of its fine structure has been continued. In the first paper of this series[1] the following most probable formula was postulated: -E4 or 2-O-D-gal-*p*-(1 → 3)-O-D-glu-*p*-(1 → 3)-O-L-rham-*p*-(1 → 1 or 3)-ribitol-3 or 1-OPO(OH)-O∃-x. This conclusion was based on oxidation of S VI with periodate and hydrolysis with dilute alkali. The latter yielded non-reducing, dialyzable fragments which were homogeneous by paper electrophoresis and which contained galactose, glucose, rhamnose, ribitol and monoesterified phosphate. Additional data, resulting in a more explicit structure, are now given.

Experimental

Materials and Methods.—S VI was supplied by E. R. Squibb and Sons through the kindness of T. D. Gerlough. Lot 172 was purified as before,[1] but additional precipitations with methanol at low salt concentrations were necessary to remove N-containing impurities. Especial thanks are due Prof. Fred Smith for reference standards of methylated sugars.

Methoxyl was determined according to Steyermark.[3] Infrared analyses were made with a Perkin–Elmer Model 21 recording infrared spectrophotometer at the Squibb Institute for Medical Research by Dr. N. H. Coy and Mr. C. Sabo. Thanks are also due Dr. R. H. Marchessault and Mr. N. W. Walter of the American Viscose Corporation for X-ray diagrams. Paper chromatographic analyses were carried out as before,[1] and the following additional solvents were used: (A) 2-Butanone:H_2O azeotrope[4] and (B) Benzene:EtOH:H_2O 200:47:15[5]

Reducing sugars were located with aniline hydrogen phthalate and non-reducing sugars or polyols with periodate spray reagents.[6]

Chemical Hydrolysis of the Phosphate Ester Group.—This was carried out before it was known that the phosphate ester could be completely hydrolyzed enzymatically. A solution of 1.0 g. of S VI, Ca^{++} salt, in 80 ml. of H_2O was treated under N_2 with 10 ml. of *ca.* 0.4 *N* $Ba(OH)_2$ for 4 days at room temperature. This converted the phosphate diester linkages to monoesters. The *p*H was adjusted to 4.0[7] with 2 *N* H_2SO_4 and the mixture heated in a glass-stoppered flask at 100°. Inorganic phosphate and reducing sugars as glucose were determined at 24 hr. intervals. After 24 hr., 59% of the P was liberated as inorganic phosphate and after 48 hr., 83% inorganic P and 7% of reducing sugar were formed. The reducing sugar rose to 13% after 72 hr., whereupon the hydrolysate was deionized with Dowex 50 and Duolite A4 and evaporated to dryness *in vacuo*. Neutral fragments, 0.811 g.; theoretical, 0.887 g. The residue was dissolved in water and separated on a 25 × 200 mm. charcoal–Celite column.[8] Elution with 200 ml. of water and 100 ml. of 5% ethanol gave 64 mg. of material, $[\alpha]^{26}D$ +41° in H_2O, which indicated galactose and ribitol by paper chromatography. Elution with 300 ml. of 15–25% ethanol gave 598 mg. of a crystalline non-reducing substance, after drying *in vacuo* at 65°, $[\alpha]^{26}D$ +110°, (*c* 1, in H_2O). Continued elution with 25% ethanol gave 85 mg. of reducing sirup, $[\alpha]^{26}D$ +129° (in H_2O). After recrystallization of the former fraction from isopropyl alcohol–water, the air-dry crystals melted with foaming at 140–145°. Further recrystallization from the same solvent, ethanol–water, or methanol gave a fusion point of 138–140°, unchanged by further crystallizations.

(1) Paper I: P. A. Rebers and M. Heidelberger, *J. Am. Chem. Soc.*, **81**, 2415 (1959).

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

(3) A. Steyermark, *J. Assoc. Offic. Agr. Chemists*, **39**, 401 (1956).

(4) L. Boggs, L. S. Cuendet, I. Ehrenthal, R. Koch and F. Smith, *Nature*, **166**, 520 (1950).

(5) G. A. Adams, *Can. J. Chem.*, **33**, 56 (1955).

(6) J. A. Cifonelli and F. Smith, *Anal. Chem.*, **26**, 1132 (1954); J. Baddiley, J. G. Buchanan, R. E. Handschumacher and J. F. Prescott, *J. Chem. Soc.*, 2818 (1956); J. Baddiley, J. G. Buchanan and B. Carss, *ibid.*, 4138 (1957).

(7) J. Baddiley, J. G. Buchanan and B. Carss, *ibid.*, 4058 (1957).

(8) R. L. Whistler and D. F. Durso, *J. Am. Chem. Soc.*, **72**, 677 (1950).

After drying *in vacuo* for 2 hr. at 65° a definite fusion point was no longer obtained. The specific rotation of the vacuum-dried samples varied from +117 to +124°. Paper chromatography with butanol–ethanol–water (BEW) or butanol–pyridine–water (BPW) showed only one component, as also did electrophoresis in 0.05 *M* borate buffer, at *p*H 9.2 and 400 v.

On hydrolysis of 10 mg. of the recrystallized substance at 100° for 3 hr. in *N* H_2SO_4, $[\alpha]^{26}D$ fell to +34.° Paper chromatography with BPW showed galactose, glucose, ribitol and rhamnose. Hydrolysis of 104 mg. at 100° for 6 hr. in *N* H_2SO_4 and separation on 10 sheets of 7 × 22″ Whatman No. 3 filter paper was carried out. The sugars were located on guide strips and the sections extracted with methanol. The solvent was evaporated *in vacuo*, and the fractions were dried to constant weight over P_2O_5 *in vacuo;* 31 mg. was obtained corresponding to galactose, 29 mg. to glucose, 34 mg. to rhamnose and 16 mg. to ribitol. Calculation of the mole ratios gave: galactose, 1.07; glucose, 1.0; rhamnose, 1.29; and ribitol, 0.7. Deviation of the last two values from 1 possibly was due to incomplete separation. The ribitol content of another sample was determined after hydrolysis by analysis of the formaldehyde produced by oxidation with periodate in acid solution for 10 min. at room temperature.[9] Since sugars also give small amounts of CH_2O by this procedure, a ribitol standard was prepared with equimolar amounts of glucose, galactose and rhamnose. The hydrolysate gave analysis for 25% ribitol, theoretical 23%.

Determination of rhamnose with cysteine and sulfuric acid[10] gave 27%, theoretical 25%.

The ribitol fraction, recrystallized from methanol–isopropyl alcohol, melted at 100°, alone or mixed with an authentic specimen of ribitol. The D-galactose fraction was recrystallized from methanol; m.p. 163–164° and mixed m.p. with galactose m.p. 162–163°. L-Rhamnose was characterized as the *p*-nitroanilide[11a] melting at 220°, not depressed by admixture with an authentic specimen and showing $[\alpha]^{26}D$ +294° (*c* 0.2, in pyridine, lit. value $[\alpha]^{26}D$ +308° in pyridine). Glucose was also identified as its *p*-nitroanilide,[11b] m.p. 182–184°, $[\alpha]^{26}D$ −207°, (*c* 0.1, in pyridine); mixed m.p. 182° with authentic glucose *p*-nitroanilide, m.p. 182°, $[\alpha]^{26}D$ −202° in pyridine.

Intact S VI failed to liberate inorganic phosphate after heating 24 hr. at 100° at *p*H 4. This was expected, since phosphate diesters are usually stable at this *p*H.

C, H Analysis and Molecular Weight of Galactoglucorhamnoribitol.—Found: After drying 2 hr. at 65° *in vacuo*, C, 41.8; H, 6.8; calcd. for galactoglucorhamnoribitol·$2H_2O$: C, 41.94; H, 7.0. Mol. wt., found, ebullioscopic in 2-butanone, 638; calcd. 658.6.

Loss in weight after drying at 65°, 3 hr., 5.9%, 9 hr., 6.6%. The residue is hygroscopic. An acetate, prepared with pyridine and acetic anhydride, failed to crystallize.

Enzymatic Hydrolysis of the Phosphate Ester.—Calf-intestinal phosphatase[12] was purified by dialysis against distilled water at 4° and precipitation with an equal volume of acetone in the presence of sodium acetate.[13] After 3 precipitations it was dried at room temp. under N_2 and stored at −15°.

S VI Ca salt, 352 mg., dried *in vacuo* over P_2O_5 to constant weight, was dissolved in 25 ml. of water and depolymerized with 1 ml. of 2 *N* NaOH under N_2 for 4 days at room temp. Glycine, 450 mg., and $MgCl_2 \cdot 2H_2O$, 60 mg., were added; the *p*H was adjusted to 9.0 with HCl. Purified alkaline phosphatase, 9 mg., 406 "millimol units"[14] per mg., dissolved in 3.0 ml. buffer (1.5% glycine, 0.1% $MgCl_2$, *p*H 8.8) was added and the mixture sterilized by filtration through a Swinny hypodermic adapter[15] into a sterile test-tube. After 2.5 weeks at 23–27°, the enzyme was precipitated by addition of an equal volume of acetone. The precipitate was centrifuged off, washed 3 times with 50% aqueous acetone and discarded. Acetone was removed by evaporation *in vacuo* to near dryness. The residue was dissolved in water, deionized with Dowex 50 and Duolite A4 and evaporated *in vacuo*. The product crystallized rapidly. After drying to const. wt. *in vacuo* the yield was 296 mg.; $[\alpha]^{22}D$ +115° (*c* 1, in H_2O). After crystallization from MeOH, fusion point, 134–135°, air dry; dried to const. wt. at 65°, $[\alpha]^{22}D$ +118° (*c* 1 in H_2O). Paper chromatography with BEW or BPW showed only one component with the same mobility as produced by heating alkali-degraded S VI at *p*H 4. X-Ray powder diagrams of both samples were the same. The *d* spacings in Å. and intensities (n = normal, s = strong, weak and very weak lines omitted) derived from the enzymatically produced material in a powder diagram with a G. E. Universal camera and Cu Kα radiation were: 9.0, s; 7.5, n; 6.1, n; 4.55, s; 4.35, s; 4.05, n; 3.70, n; 3.35, n; 3.10, n; 2.76, n. The darkest reflections correspond roughly to the interplanar spacing at 4.44 which separates the planes of glucose rings in mercerized cellulose. This suggests that the galactoglucorhamnoribitol may form a linear planar chain, as do the glucose rings in cellulose.[16]

Only traces of inorganic phosphate were liberated on treatment of intact S VI with the same enzyme preparation at *p*H 9.

The theoretical phosphorus content of S VI Ca^{++} salt is 4.4%, by analysis, 4.2%,

Calcd. equiv. wt. of S VI, Ca^{++} salt, 4.2% P: 738.

Mol. wt. of galactoglucorhamnoribitol·$2H_2O$: 658. Theoretical yield 352 × 658 ÷ 738 = 314 mg. Actual yield 296 mg., or 94%,

Methylation.—Galactoglucorhamnoribitol, 225.9 mg., was methylated twice with Ag_2O and CH_3I in N,N-dimethylformamide.[17] The methoxyl content after the first run was 48% and after the second, 49.5%; theoretical, 50.1%. The yield was 212 mg. of sirup, $[\alpha]^{25}D$ +80° (*c* 2, in $CHCl_3$). An infrared spectrum of the methylated derivative in CCl_4 (25 mg./ml.) showed a broad peak at 850–860 $cm.^{-1}$, suggesting α-linkages,[18] and a small hump at 910 $cm.^{-1}$.

The methylated derivative was refluxed with 2% HCl in methanol for 5 hr., after which $[\alpha]^{27}D$ +38°. No further change occurred after another 2 hr. HCl was removed with Ag_2CO_3 and the precipitate centrifuged off and washed with MeOH. The combined solutions were evaporated *in vacuo*. The glycosides were converted to the free sugars with *N* H_2SO_4 at 100°, $[\alpha]^{28}D$ +55, 5.5 hr., unchanged after 8 hr. An oil (tetramethylribitol?) collected on the surface and was removed by extraction with petroleum ether, yield 12.7 mg. Paper chromatographic analysis of the water-soluble fraction for 6 hr. at 30° in solvent B[5] gave three spots with aniline hydrogen phthalate: red, 0.79[19]; brown, 0.30[19]; and red, 0.21.[19] The separation was not as good with BEW or solvent A.

Separation of the Components in the Water-soluble Fraction.—Inasmuch as more of the tetramethylribitol was expected, a partial separation was performed in a 30-tube hand-operated Craig countercurrent distribution apparatus[19a] with solvent B, modified 980:620:454 so as to give equal volumes of upper and lower phases.[20] After 84 extractions, the fractions were examined by paper chromatography with the results

Fraction no.	Wt. dry residue, mg.	Probable components
0–4	81	2,4,6-tri-O-Me-D-glucose
		2,4-di-O-Me-L-rhamnose
5–7	24	Mixture of same 3 components
8–11	27	2,3,4,6-tetra-O-Me-D-galactose
12–14	22	Tetra-OMe gal and other subst.
15–84	46	Tetra-O-Me-ribitol

(9) M. Lambert and A. C. Neish, *Can. J. Research*, **28B**, 83 (1950).

(10) Z. Dische and L. B. Shettles, *J. Biol. Chem.*, **175**, 595 (1948).

(11) (a) M. Frèrejacque, *Compt. rend.*, **207**, 638 (1938); (b) F. Weygand, W. Perkow and P. Kuhner, *Chem. Ber.*, **84**, 594 (1951).

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(13) R. K. Morton, *Biochem. J.*, **57**, 595 (1954).

(14) O. A. Bessey, O. H. Lowry and M. J. Brock, *J. Biol. Chem.*, **164**, 321 (1946); Sigma Chemical Co. Tech. Bull. 104.

(15) Millipore Filter Corp., Bedford, Mass.

(16) R. H. Marchessault, personal communication.

(17) R. Kuhn, H. Trischmann and I. Löw, *Angew. Chem.*, **67**, 32 (1955).

(18) S. A. Barker, E. J. Bourne, D. H. Whiffen, "Methods of Biochemical Analysis," Vol. III, ed. by D. Glick, Interscience Publishers, Inc., New York, N. Y., 1956.

(19) Ratio of the distance moved as compared to 2,3,4,6-tetra-O-methyl-D-glucose.

(19a) H. O. Post Scientific Supply Co., Maspeth, Long Island, N. Y.

(20) K. A. Varteressian and M. R. Fenske, *Ind. Eng. Chem.*, **28**, 928 (1936).

Fractions 0–4 were separated on 10 sheets of Whatman No. 3, 7″ × 22″ paper. After 24 hr. at *ca.* 25° with solvent B, complete separation was obtained. The sugars were extracted with methanol, the solvents removed *in vacuo* and the fractions dried to constant weight. The slower component, 30 mg., crystallized, $[\alpha]^{25}D$ +71°. After recrystallization from ethyl acetate and petroleum ether, m.p. 124°, m.p. of 2,4,6-tri-O-methyl-D-glucose, 123–126°.[21] Although 2,3,6- and 2,4,6-tri-O-methyl-D-glucose show almost identical melting points and rotations, they may easily be separated by paper chromatography in 24 hr. with solvent B. A standard 2,3,6 derivative produced a brown spot with aniline phthalate 8″ from the starting line, whereas the known 2,4,6-trimethylglucose and the product from S VI each gave red spots at 7″. The resistance of the glucose in S VI to attack by periodate is also indicative of its 1,3-linkage.

The faster component, 26.7 mg., $[\alpha]^{25}D$ 0° (in H_2O) was converted to the anilide.[22] It melted sharply at 141°, lit. value for anilide of 2,4-di-O-methyl-L-rhamnose 141–142.5°; mixed m.p., 138°; at c = 0.6 in 95% EtOH, $[\alpha]^{25}D$ +141° (2.5 min.); +120° (20 min.), +52° (4.5 hr.); +8° (48 hr.), lit. value $[\alpha]^{20}D$ +136° → 4° (39 hr.).

Since the high specific rotation, $[\alpha]D$ +108°, c = 0.7 in H_2O, and chromatographic behavior suggested that fraction 8–11 was 2,3,4,6-tetra-O-methyl-D-galactose, its anilide was prepared and recrystallized from ethyl acetate; m.p. 193°, mixed m.p. 190°; compared with 191° for the standard substance.[23] The petroleum ether extract and fractions 15–84 presumably contain 1,2,4,5 tetra-O-methylribitol, an as yet undescribed substance.

Linkage of Ribitol.—Oxidation of galactoglucorhamnoribitol in acid solution with periodate for 40 min. at 27°[9] gave 2.3 moles of CH_2O. CH_2O is produced by oxidation of $-CHOH-CH_2OH$ groupings, of which there are two in ribitol linked in the 3-position. Only one mole of CH_2O would have been formed if the ribitol had been linked in the 1- or 2-position.

Determination of the Galactose Linkages in S VI.—Previous studies on the oxidation of S VI with periodate[1] did not differentiate between 1,2- and 1,4-linked galactose. Degradation of 454 mg. of S VI was carried out by oxidation with 100 ml. of 0.05 M $NaIO_4$ at 0° for 3 weeks.[24] Iodate and periodate were precipitated with calcium acetate buffer, pH 5, and after centrifugation the supernatant, $[\alpha]^{22}D$ +40°, was treated with two 500-mg. portions of $NaBH_4$ during 16 hr. Calcium borate was removed by centrifugation and washed with water. The supernatant, pH 9.7, was neutralized with HOAc to pH 7.2; $[\alpha]^{26}D$ +47°. After dialysis against distilled water at 26°, the inside solution showed $[\alpha]^{26}D$ +38°, indicating some loss. Treatment with N H_2SO_4 at room temp. for 4 days released an aldehyde giving a positive Schiff test and abolished activity with Type VI antipneumococcal horse serum. The aldehyde was completely removed by extraction with butanol. After neutralization of the aqueous layer with $Ba(OH)_2$, the $BaSO_4$ was centrifuged off, washed with water, and the supernatant and washings were evaporated *in vacuo* to a sirup. This was extracted with ethanol; soluble, 166 mg., alcohol-insoluble, probably Ba salt of a phosphate ester, 66 mg. Paper chromatographic analysis of the alcohol-soluble portion with BEW indicated glycerol and a slow-moving component. The fraction resembling glycerol was separated from the oligosaccharide on a charcoal–Celite column, 23 mg. coming off with water. The oligosaccharide was eluted with 5% EtOH. Glycerol was confirmed by preparation of its p-nitrobenzoate, according to Lewis,[25] m.p. 191°, reported 192–194°; mixed m.p. 191.5–192°. Since free glycerol would result from 1,2-galactose and free threitol if the linkages are 1,4-, the galactose in S VI is bound 1,2-. The mobility of the single spot due to the oligosaccharide in BEW in relation to glucose was 0.6; the yield, 54 mg.; $[\alpha]^{26}D$ +60°. Hydrolysis of an aliquot and paper chromatography indicated glucose, rhamnose and erythritol, the last owing to degradation of the 1,3- or 2,3-linked ribitol in S VI.

Determination of Anomeric Linkages in Galactoglucorhamnoribitol.—Enzymatic Hydrolysis.—Almond emulsin,[26] 5.5 mg., was dissolved in 5 ml. of 0.2 M acetate buffer, pH 5, 31 mg. of the S VI derivative was added and optical rotation was followed with time: enzyme alone, α = −0.58°; 5 min. after addition of derivative, α + 0.18°; 1 hr., α + 0.13°; 3.5 hr., α + 0.05°. Paper chromatography with BPW and paper electrophoresis of the mixture in 0.1 M borate buffer indicated that only galactose was split off. The accompanying decrease in rotation shows that it was alpha-linked.

Degradation with Periodate.—The action of periodic acid or its salts on galactoglucorhamnoribitol in aqueous solution, either at room temperature or at 5°, occurred with extensive over-oxidation but was slower and more easily regulated in methanol. Consumption of periodate with 0.02 M HIO_4 in methanol at 5° was as follows: 1 day, 3.3 moles; 2 days, 3.3; 7 days, 3.7; 14 days, 4.0; 21 days, 4.4. Oxidation in 50% aqueous methanol: 1 day, 3.8; 2 days, 4.1. Accordingly, 89 mg. of the substance was treated with 0.02 M HIO_4 in 84 ml. of 50% aqueous methanol for 24 hr. at 5°. The solution was neutralized to about pH 7 (brom thymol blue) in the cold with $Ba(OH)_2$. The precipitate of $Ba(IO_3)_2$ and $Ba(IO_4)_2$ was centrifuged off and washed with 50% aqueous methanol. Three portions of 100 mg. each of $NaBH_4$ were added at room temp. to the soluble portion during 48 hr., neutralizing with acetic acid after each addition and finally making up to 0.1 N HCl and letting stand for 3 days. The solution was neutralized with N NaOH and passed through a charcoal (Darco G-60)-Celite column. Elution with 10% ethanol gave 49 mg., $[\alpha]^{22}D$ +57° (c 1%, in H_2O). Hydrolysis of 3 mg. with N H_2SO_4 for 3 hr. at 100° and paper chromatography with BPW and BEW showed glucose, rhamnose and glycerol, the last arising from the oxidation and subsequent reduction of 3-linked ribitol.

Repetition of the degradation procedure with 35 mg. of glucorhamnoglycerol and purification on a charcoal column gave 17.9 mg. of a rhamnoglycerol, $[\alpha]^{22}D$ −42°, with 0.72 of the mobility of glycerol in BEW.

Discussion

Treatment of S VI with alkali converts a carbohydrate made polymeric by phosphate diester bridges to a monomeric derivative with phosphate monoester linkages. When this is heated at pH 4 most of the phosphate is made inorganic while most of the glycosidic linkages are unattacked. A non-reducing, chromatographically homogeneous galactoglucorhamnoribitol remains and this may be crystallized easily. It contains the same components, with the exception of phosphate, in the same ratio as does S VI. Purified intestinal alkaline phosphatase gave a much cleaner hydrolysis of the phosphate monoester, inorganic phosphate and the galactoglucorhamnoribitol being produced exclusively. This is the first example, we believe, in which the repeating unit of a heteropolysaccharide has been isolated in almost quantitative yield. However, the repeating unit of the specific polysaccharide of Type III pneumococcus, later shown to be cellobiuronic acid,[27] was isolated in 76% yield[28] and that of S VIII, O-β-D-glucopyranosyluronic acid-(1 → 4)-O-β-D-glucopyranosyl-(1 → 4)-O-α-D-glucopyranosyl-(1 → 4)-D-galactopyranose, in 0.5% yield.[29] The almost quantitative yield of repeating unit from S VI strongly supports the widely held view that heteropolysaccharides

(21) W. N. Haworth and W. G. Sedgwick, *J. Chem. Soc.*, 2573 (1926).

(22) K. Butler, P. F. Lloyd and M. Stacey, *Chemistry and Industry*, 107 (1954); *J. Chem. Soc.*, 1531 (1955).

(23) J. C. Irvine and D. McNicoll, *ibid.*, **97**, 1449 (1910).

(24) F. Smith and R. Montgomery, "Chemistry of Plant Gums and Mucilages," Reinhold Publishing Corp., New York, N. Y., 1959, pp. 194–223.

(25) B. A. Lewis, "Studies on Periodate Oxidation Products of Carbohydrates," Ph.D. Thesis, Univ. of Minnesota, 1957.

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(27) R. D. Hotchkiss and W. F. Goebel, *J. Biol. Chem.*, **121**, 195 (1937).

(28) M. Heidelberger and W. F. Goebel, *ibid.*, **70**, 613 (1926).

(29) J. K. N. Jones and M. B. Perry, *J. Am. Chem. Soc.*, **79**, 2787 (1957).

may be composed of multiples of simple repeating units.

Methylation of the galactoglucorhamnoribitol with methyl iodide and silver oxide in N,N-dimethylformamide[17] gave a derivative with the expected methoxyl content. After hydrolysis, 2,3,-4,6-tetra-O-methyl-D-galactose, 2,4-di-O-methyl-L-rhamnose and 2,4,6-tri-O-methyl-D-glucose were identified. These data exclude furanose rings since each of the methylated sugars contains a methyl group in position 4. Thus, a non-reducing galactopyranose end-group is joined to the 3-position of a glucopyranose or rhamnopyranose, and this in turn to the 3-position of the other, while the last is joined to ribitol by a glycosidic linkage. Although suitable standard derivatives of tetra-O-methylribitol are not available, the position of attachment of the sugar to the ribitol can be deduced from the reaction of non-methylated galactoglucorhamnoribitol with periodate. The rapid production of two moles of formaldehyde shows that two $-CHOH-CH_2OH$ groupings are present. This could occur only if the ribitol, also, is linked in the 3-position. A sample of S VI was tested by Dr. Baddiley with the periodate-Schiff reagents, and it gave the color expected for a 3-linked ribitol.[30]

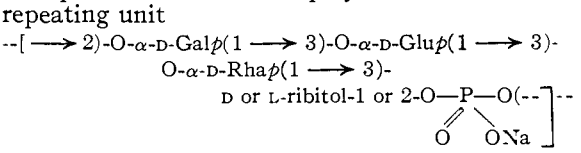
Periodate consumption and production of formaldehyde and formic acid before and after cleavage of S VI with alkali indicate, indirectly, a phosphate diester bridge between galactose and phosphate.[1] Isolation of relatively small amounts of reducing sugar phosphates as insoluble phenylosazones[1] in addition to ribitol phosphate supports this view.

Smith has shown recently that it is possible to remove selectively the fragments which are attacked by periodate from sugar residues which are resistant.[24] Treatment of S VI by this procedure gave free glycerol, formaldehyde, another aldehyde, probably glyceraldehyde and a glucorhamnoerythritol. Since only the galactose and ribitol are attacked by periodate, the degradation products must arise from these. Ribitol linked to phosphate in its 1- or 2-position and to a sugar in position 3 would be degraded to formaldehyde and erythritol, the latter glycosidically bound to glucose or rhamnose; whereas a 1,2-linked galactose should produce free glycerol and glyceraldehyde. Since a 1,4-linked galactose would have given free erythritol, the isolation of free glycerol from the S VI and its characterization as the crystalline *p*-nitrobenzoate shows that the galactose is linked 1,2.

(30) J. Baddiley, personal communication.

The arrangement and anomeric configuration of the sugars in the galactoglucorhamnoribitol were also established by stepwise degradation with periodate.[24] Periodate oxidation at either 4° or room temperature with HIO_4, $NaIO_4$ or NH_4IO_4 buffered to *p*H 7, proceeded with rapid consumption of the first 3–4 moles of periodate and continued until the sugars had been completely oxidized. Better control was obtained in 50% aqueous methanol at 4° with HIO_4. After reduction and hydrolysis[24] a glucorhamnoglycerol, $[\alpha]^{22}D$ +57°, was obtained. Repetition of the reaction with this produced a rhamnoglycerol, $[\alpha]^{22}D$ −42°, [M] = −10,000°. Isolation of the latter shows that rhamnose is glycosidically linked to ribitol in the original substance, and since α-methyl-L-rhamnopyranoside has a negative rotation, [M] = −11,140°, whereas β-methyl-L-rhamnopyranoside has [M] = +17,000°, the S VI derivative is undoubtedly the α-anomer. Previous work has shown that the glycerol derivative of another sugar, namely, α-D-galactopyranosyl-2-glycerol,[31] has a molecular rotation similar to that of α-methyl-D-galactopyranoside. Inasmuch as the optical rotation of the glucosylrhamnoglycerol is positive, $[\alpha]D$ +57°, the increase from $[\alpha]D$ −42° is doubtless due to the presence of an α-glucopyranose ring. Since galactoglucorhamnoribitol has a still higher rotation, $[\alpha]D$ +115°, an α-galactopyranose ring most likely is present. This is consistent with the finding that enzymatic hydrolysis of galactose from galactoglucorhamnoribitol decreased the optical rotation.

Although it is not yet possible to assign a configuration to the ribitol or to fix the position of its phosphate linkage in S VI, the polysaccharide may be represented as a linear polymer of the following repeating unit



The above arrangement of "head to tail" is favored over "head-to-head and tail-to-tail" since alkali-degradation of S VI produces a phosphate monoester in which almost all of the phosphate is attached to the ribitol. The immunological consequences of this formula already have been discussed.[32]

(31) E. W. Putman and W. Z. Hassid, *J. Am. Chem. Soc.*, **76**, 2221 (1954).

(32) M. Heidelberger and P. A. Rebers, *J. Bacteriol.*, **80**, 145 (1960).