Cross Reactive Human Blood-Group H(O) Specific Polysaccharide from *Sassafras albidum* and Characterization of Its Hapten*

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ABSTRACT: A novel, nitrogen-free, homogeneous polysaccharide isolated from *Sassafras albidum* possesses blood-group H(O) activity equal to that of human H(O) substances when measured with eel anti-H(O) serum in quantitative precipitin, immune diffusion, and hemagglutination inhibition tests and shows some reaction with human anti-H(O) serum. It does not inhibit other anti-H(O) reagents and is free of additional blood-group specificities. In a minority of injected chickens and goats it elicits specific anti-H(O) antibodies. It is a potent lipemia clearing agent and prevents the growth of certain viruses. Some of its physical properties are similar to those of the human blood-group substances. Chemically, however, there is little relation to human blood-group H(O) antigen, which contains the H(O) specific L-fucose.

Sassafras polysaccharide, on the contrary, has no fucose but contains the haptenic sugar 3-*O*-methyl-D-galactose, which was found only once before in Nature and whose crystallization and full characterization are herein described. Other major components are rhamnose, xylose, galactose, galacturonic acid, L-arabinose, and acetyl. The antigen is considerably more acid resistant than is the human H(O) substance. Short term hydrolysis increases its blood-group activity by removal of terminal arabinose and exposure of 3-*O*-methyl-D-galactose. Rhamnose seems to form the core. The identical serological activities of Sassafras and human H(O) active antigens are reflected in the similar serological activities of their haptenic sugars. Surprisingly, at concentrations exceeding 0.5 μmole, 3-*O*-methyl-D-galactose, as previously 3-*O*-methyl-D-fucose, precipitated the eel anti-H(O) antibody of most eel sera, giving a typical quantitative precipitin curve. Nearly 100% of the active anti-H(O) antibody could be recovered from the precipitate. *O*-Methylation at the appropriate C atom and/or *O*-methyl glycosidation abolished the precipitating ability but yielded potent neutralizing haptens. The present study gives some insight as to possibilities and limits of immunochemical methods.

Terminal α-L-fucopyranosides are predominantly involved in the human blood-group H(O) specificity of mammalian secretions. Rege *et al.* (1964) and Lloyd and Kabat (1964) have shown that L-fucosyl-α-(1→2)-β-D-galactosyl structures are involved in the determinant groups of water-soluble mammalian H(O) substances.

It has been shown that substances with human H(O) specificity, as determined with heterologous eel anti-H(O) serum, are not confined to animals but are frequently found in gram-negative bacteria and, in two instances, even in higher plants (Springer, 1958; Springer *et al.*, 1961). Some bacteria possess antigens whose determinant groups are closely similar to human blood-group substances both serologically and chemically (Springer, 1958; Springer *et al.*, 1964b). The higher plant substance previously isolated by us from *Taxus cuspidata*, the Japanese yew, is quite different from mammalian substances with blood-group H(O) activity; its serologically determinant group is 2-*O*-methyl-L-fucose, not previously found in Nature (Springer *et al.*, 1956; Springer, 1958). Highly active material was obtained from autumn twigs only.

The properties of the second blood-group H(O) cross-reactive substance isolated from a higher plant, the lauraceous tree *Sassafras albidum*, and its relation to other blood-group H(O) specific substances form the subject of this report. The isolated macromolecule proved to be a polysaccharide which could be obtained from twigs throughout the year without demonstrable variations in activity and amount. It behaved as a single component in the systems tested, and its composition differed from human blood-group substances. It lacks both nitrogen and fucose, and the structure and properties of its blood-group specific hapten are unique.

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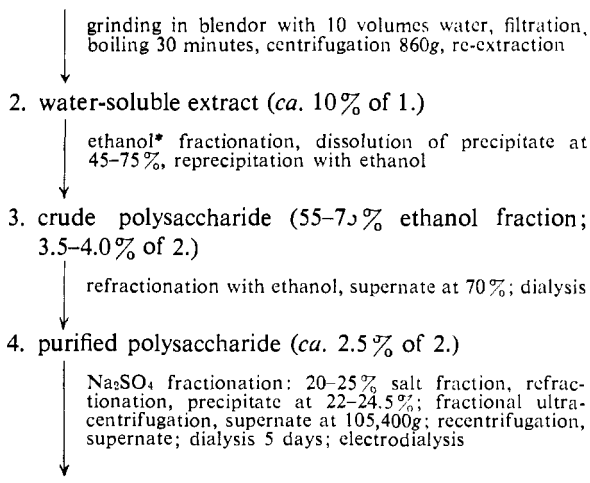

FIGURE 1: Preparation of blood-group H(O) specific polysaccharide from *Sassafras albidum*. Asterisk indicates 0.05–0.1% sodium acetate throughout.

Materials and Methods

Isolation of Blood-Group Active Polysaccharide. Sassafras twigs were collected in the Philadelphia, Pa. area. They were either processed within 4 hours after collection or kept at −20° until used. The leafless twigs were found to have little or no microbial contamination when tested by standard procedures at 22–25° and at 37°.

The isolation procedure depicted in Figure 1 was adopted. Only water-soluble materials were investigated. The solutions were concentrated under reduced pressure to a dry weight of 2.0–2.5% and fractionated with absolute ethanol. Highly active material amounting to about 10% of the water-soluble extract precipitated in the 45–75% ethanol range. Fractions other than those obtained at this ethanol concentration amounted to 80–90% of the starting material and possessed between 5 and 20% of its activity; they were re-extracted and refractionated. The most highly active material precipitated at a final ethanol concentration of between 55 and 75%. Ultracentrifugal analysis showed it was not yet homogeneous. It was made 2.5% in water. The fraction not precipitable by 70% ethanol possessed the highest activity (fraction 4, Figure 1). After exhaustive dialysis it was precipitated in ten volumes of 100% ethanol which contained 0.05% sodium acetate. The precipitate was made 2.5% in water and fractionated with Na_2SO_4 at 37°. The largest (80%) and most active fraction precipitated between 20 and 25% concentration of Na_2SO_4. After dialysis and refractionation with Na_2SO_4, the material precipitating at a salt concentration between 22.0 and 24.5% was collected and dialyzed. Fractional ultracentrifugation of a 1.13% solution yielded material of highest activity in the supernate at 105,400*g* (Beckman-Spinco SL preparative ultracentrifuge). This highly purified polysaccharide (step 5 in Figure 1, laboratory number Ca 606) was used either as such for chemical and physical analyses or after desalting by electrodialysis (Wood, 1956) at 200 v through parchment membranes (A. H. Thomas catalog No. 7961) and freeze dried.

Analytical Procedures. All macromolecular samples used for physical and chemical analyses were dried to constant weight at 110° and 10^{-1}–10^{-2} mm over P_2O_5. Optical rotations, uncorrected, were measured with a Perkin-Elmer Model 141 digital readout polarimeter. Melting points, uncorrected, were obtained on a Fisher-Johns melting point apparatus. Ultraviolet absorption of 0.1% aqueous solutions was measured in a Beckman DU spectrophotometer.

The following physical measurements were performed by Dr. G. Bernardi: sedimentation, diffusion, and infrared spectra as described previously (Bernardi and Springer, 1962). Viscosity was measured at 20° using both a four-bulb viscometer built according to Eigner (1960; see also Freund and Bernardi, 1963) and a capillary viscometer operated at a constant pressure of 26 cm of water. In the first case, the average velocity gradients associated with each bulb were determined according to Kroepelin (1929) and found to range from 20 to 100 sec^{-1}; in the second case the gradient was equal to 1200 sec^{-1}. Light scattering and the specific refractive index increment were determined as described by Bernardi *et al.* (1961; Bernardi, 1964). The solvent used throughout by Dr. Bernardi was 0.13 M NaCl + 0.01 M phosphate buffer, pH 6.8.

The following quantitative tests were used: Reducing values were determined by the method of Folin and Malmros (1929), modified by use of Duponol (ME Dry) instead of gum ghatti, and that of Park and Johnson (1949). Methylpentose was determined by spectrophotometry (Dische and Shettles, 1951). Uronic acid was determined by Dische's carbazole method B (1950). Deacetylation was carried out for 3 hours at 23–25° in 0.1 N NaOH according to Whiteside and Baker (1960). De-*O*-methylation with 48% HBr was performed as by Hough *et al.* (1950) except that neutralization was done with MB-3 monobed resin. Elemental and other analyses were performed by Huffman Microanalytical Laboratories, Wheatridge, Colo. Hydrolyses were carried out with 0.5 and 1.0 N sulfuric acid and 1.0 N acetic acid at 95–100° in sealed vessels (*vide infra*). The samples were neutralized either immediately or following thorough dialysis at 4°. Neutralization of the inorganic acid to pH 5.5 was done with resins, Amberlite IR-45 (OH^-) or Duolite A-2 (CO_3^{2-}), or with $Ba(OH)_2$. Acetic acid hydrolysates were adjusted to pH 5–6 by repeated evaporation in a desiccator. Periodate consumption was determined spectrophotometrically and formaldehyde production was measured with chromotropic acid (*cf.* Kabat, 1961). Appropriate controls and standards were included. Lipid extraction was performed in a Soxhlet apparatus for 24 hours with 1000 volumes of ether–ethanol (1:1 v/v) followed by extraction for 24 hours with the same volume of chloroform–methanol (1:3 v/v). A

thimble extraction using solvents alone was also carried out.

Enzymatic Analyses. Inactivation attempts were made with coffee bean α-galactosidase under the conditions described by Springer *et al.* (1964b) and an enzyme preparation from *Lactobacillus bifidus* var. Pennsylvanicus (Gyorgy *et al.*, 1954); both are known to inactivate human blood-group substances. The effects of β-galactosidase from *Diplococcus pneumoniae* (Hughes and Jeanloz, 1964), maltase (Nutritional Biochemicals), and bacterial α-amylase (B-grade, Calbiochem) were investigated under the conditions specified by the supplier.

Paper Chromatography, Paper Electrophoresis, and Qualitative Color Reactions. Comparative paper chromatography was performed by the descending technique on Whatman No. 1 paper. Preparative chromatographic separation was achieved on acid- and solvent-washed Whatman 3 MM or Schleicher and Schuell 589 Green R paper (57 × 46 cm, 22.5 × 18.25 in.); 150 mg of material was applied per sheet. After development, guide strips were cut and stained, the areas of the desired sugar(s) were marked and eluted, and the eluates were cleaned as described earlier (Springer *et al.*, 1964a).

Descending chromatography was performed in: (a) 1-butanol–ethanol–water (5:1:4) (Partridge, 1948), (b) 1-butanol–pyridine–water (6:4:3) (French *et al.*, 1950), and (c) these reagents in the proportion 10:3:3 (Hough *et al.*, 1950); (d) 90% aqueous phenol (v/w) (Partridge, 1948) containing 0.08% 8-hydroxyquinoline in an atmosphere saturated with NH_3 and NaCN (Block, 1950); (e) ethyl acetate–pyridine–water (8:2:1) (White and Secor, 1953); (f) the same components in the proportion 10:4:3 (Whistler and Hickson, 1955); (g) 1-butanol–acetic acid–water (2:1:1) (Hough *et al.*, 1950); (h) benzene–1-butanol–pyridine–water (1:5:3:3) (Hough, 1950). Methyl ethers of hexoses were separated in (i) 2-butanone–water (88.7:11.3) saturated with Borax (Dutton and Tanaka, 1962); (k) pyridine–ethyl acetate–glacial acetic acid–water (5:5:1:3) was used in chromatography of the uronic acids after the tank had been saturated with pyridine–ethyl acetate–water (11:40:6) (Fischer and Dörfel, 1955). The developed chromatograms were stained for carbohydrates with aniline oxalate (Horrocks, 1949) or silver nitrate according to Trevelyan *et al.* (1950) and Benson *et al.* (1952) or with triphenyltetrazolium chloride (Trevelyan *et al.*, 1950; Wallenfels, 1950), which was employed as by Wallenfels, except that the spraying reagent was a freshly prepared mixture of 1 part of 0.5% triphenyltetrazolium chloride in absolute ethanol and 1 part of 1 N NaOH in 75% ethanol; *p*-anisidine hydrochloride was used to detect uronic acids (Hough *et al.*, 1949) and ninhydrin to check for amino acids (Toennies and Kolb, 1951).

Paper electrophoresis at 6–7 v and 0.25–1 ma/cm was carried out for 6–12 hours horizontally; at high tensions, 44–50 v/cm, the paper strips were run in vertical position for 50 minutes in water-cooled Varsol-containing chambers. Whatman No. 1 paper was used and the following electrolytes: (a) pyridine–acetic acid–water (10:4:8.6) pH 5.3 (Katz *et al.*, 1959), (b) 0.1 M sodium borate pH 9.2 (Mabry *et al.*, 1963), (c) Na-Veronal, 0.06 M, pH 8.6 (Durrum, 1950), (d) sodium citrate hydrochloride, 0.1 M, pH 2.8 (Schmid *et al.*, 1959), and (e) glycine–NaOH, 0.1 M, pH 11.0 (Sorensen, 1909). The polysaccharide was located with sodium metaperiodate–potassium permanganate (Wolfrom and Miller, 1956) or other appropriate reagents. Glucose, 2,3-di-*O*-methylfucose, tetra-*O*-methylglucose, and starch were included as controls.

Immunochemical Procedures. Blood-group activity of the substances described below was assessed by established hemagglutination inhibition, precipitin, and precipitation inhibition techniques (Heidelberger *et al.*, 1933; Springer, 1956; *cf.* Kabat, 1961; Springer and Williamson, 1962; Springer *et al.*, 1964a).

ANTI-H(O) REAGENTS AND OTHER ANTISERA. Various lots of eel serum and seed extracts from *Lotus tetragonolobus*, *Cytisus sessilifolius*, and a human anti-H(O) serum were obtained, prepared, and preserved by procedures previously described (Springer and Williamson, 1962; Springer *et al.*, 1964a). The source and properties of the other agglutinins used in this study have been reported before (Springer, 1956; Springer *et al.*, 1961; Springer *et al.*, 1964a). Type XIV antipneumococcus horse serum No. 635, bled 5/25/1939, from the New York State Department of Health was clarified by centrifugation before use.

ERYTHROCYTES AND SOLUTIONS. Human erythrocytes were obtained, stored, and used as described previously (Springer *et al.*, 1964a). In all precipitin and precipitation-inhibition tests 0.10 M aqueous sodium chloride containing 0.05 M phosphate buffer, pH 7.2 (buffered saline), was used as diluent and solvent of antigen and haptens. Also, this buffer contained a final concentration of 0.25% phenol and 0.005% merthiolate.

HEMAGGLUTINATION INHIBITION TESTS. The procedures have been described (Springer *et al.*, 1964a). L-Fucose was included as standard in all assays. All active materials were tested at least three times. Preparations that did not inhibit 4 minimum hemagglutinating doses of serum at concentrations of 5 mg/ml were considered to be inactive. Activities are expressed on a weight basis and in terms of dilution of the inhibiting material before addition of serum and erythrocyte suspension.

PRECIPITIN TESTS. The ability of the Sassafras polysaccharide and human blood-group H(O) ovarian cyst glycoprotein to precipitate the eel anti-H(O) blood-group antibody was determined by the classical quantitative precipitin technique. Three of the blood-group H(O) glycoproteins were given by Professor Morgan; an H(O) ovarian cyst glycoprotein prepared in this laboratory, Ca IIb 2 + 3 PF (for some of its physicochemical properties see Springer *et al.*, 1964a), as well as some less active fractions from cyst glycoprotein were also employed. A highly blood-group H(O) active Taxus polysaccharide preparation, *Escherichia coli* O_{128} lipopolysaccharide (Springer and Horton, 1964), and 3-*O*-methyl-D-fucose and 3-*O*-methyl-D-galactose (Kolecki and Springer, 1965) were also included in the precipitin studies.

Known amounts of antigen were added in buffered

saline to individual conical tubes calibrated at 2.5 ml and containing 0.5 ml of high-titer eel serum which had been decomplemented and absorbed with blood-group A_1B erythrocytes as described previously (Springer *et al.*, 1964a) and centrifuged at 860*g* at 4° for 12–20 hours. Where indicated, Lotus reagent [anti-H(O) titer 1:16–1:32] centrifuged for 2 hours at 13,000*g* was employed. The samples were twirled and incubated at 37° for 1 hour and then for 7–12 days at 4° with daily agitation.

Precipitates were recovered by centrifugation and washed, and their protein was determined with the Folin-Ciocalteu reagent (Heidelberger and MacPherson, 1943). Precipitates obtained with monosaccharides were washed only once. Eel serum or Lotus reagent alone and antigen alone served as controls. Duplicate samples were run and measured in each test. All tests were carried out under sterile conditions. Some samples were cultured on blood agar plates; no growth was observed.

The human anti-H(O) serum used had almost as high a titer (1:64–1:128) as the eel anti-H(O) serum with human blood-group O erythrocytes; because of the small quantity of this serum, microprecipitin tests were done after its prior clarification. Sassafras polysaccharide C-70 and human blood-group H(O) substance Ca IIb were dissolved and filled to 30 mm in capillaries of 0.5–0.9 mm diameter; subsequently, an equal amount of antibody was introduced at the same end. The capillaries were embedded vertically in plasticine. Negative controls consisted of appropriately diluted serum alone and 0.1% antigen solution alone. Similar microprecipitation with eel anti-H(O) serum was also carried out. The samples were incubated for 30 minutes at 37° and then at 1–2° for up to 3 weeks. Such experiments were also performed with the pre- and postimmunization sera of goats W and B, 4 germfree and 4 ordinary chickens (*vide infra*), and with Type XIV antipneumococcus horse serum which included as an additional antigen Type S XIV pneumococcus polysaccharide.

PRECIPITATION INHIBITION TESTS. The ability of haptenic substances to inhibit precipitation of both human blood-group H(O) ovarian cyst glycoprotein and Sassafras polysaccharide by eel serum was determined quantitatively (*cf.* Kabat, 1961) as described previously (Springer *et al.*, 1964a). Precipitates were washed and protein was determined as in the precipitin test. Duplicate samples were measured in each test. The percentage of inhibition was computed from the difference in amount of protein precipitated in the presence and in the absence of inhibitors.

AGAR GEL DIFFUSION. The two-dimensional double-diffusion procedure (*cf.* Ouchterlony, 1958, 1962) was used. Flat-bottom Petri dishes were layered with 0.8% IONAGAR (Oxoid) containing 3.75% glycine and merthiolate 1:10,000. Serum or plant extract, 0.2–0.4 ml, was added to the center basin. Antigen solutions in 0.1–0.4-ml volumes and ranging from 0.05 to 2.0 mg were added to six peripheral wells. The plates were then incubated in a water-saturated atmosphere either at room temperature or at 4–6° and observed for 3 weeks.

IMMUNIZATION. Crude Sassafras polysaccharide and H(O) specific human ovarian cyst glycoprotein (Ca II) preparations were used with or without 0.6% alum [$KAl(SO_4)_2 \cdot 12H_2O$] after neutralization with NaOH. Control animals received injections of alum alone. Ordinary and germfree White Leghorn chickens and goats were injected after blood had been obtained immediately prior to an immunization attempt. The birds were given 1–2 mg each, divided evenly into 2 doses, within 48 hours; the first was given intraperitoneally and the second subcutaneously; they were bled 10 days later. In some instances booster injections of 1 mg were given subcutaneously; bleeding was 10–11 days later. Two female (W and B) goats weighing about 40 pounds each were given 2.0 mg of crude Sassafras polysaccharide plus alum subcutaneously; 24 hours later, 2.0 mg intravenously; and again 24 hours later, 2.0 mg subcutaneously. Two additional goats of the same weight were immunized subcutaneously: over a period of 11 days goat (C) was given a total of 38 mg and goat (D) 96 mg of polysaccharide without adjuvant. Blood was withdrawn 10–13 days later and 1 month after this bleeding. Sera were tested either unabsorbed or after absorption with A_1B erythrocytes as described (Springer and Horton, 1964). Pre- and postimmunization specimens were tested simultaneously.

Experimental and Results

General and Physical Properties of the Homogeneous Sassafras Polysaccharide. A maximal reducing value of 54% (Park-Johnson) after hydrolysis at 100° for 4 hours in 1 N HCl and analytical data of C, 47.0, and H, 5.82, indicated that the blood-group specific Sassafras macromolecule is a polysaccharide which, besides sugar, contained 11.33% acetate (Kuhn and Roth determination), 2.07% additional methyl (from CCH_3), and 4.00% of *O*-methyl, indicative of large amounts of *C*- and *O*-methylated sugars. No P or S and less than 0.25% N were found. The polysaccharide was a light brown fluff which lost 13–14% of its weight at 110° *in vacuo*. Weight constancy was reached in less than 20 hours. Acid hydrolysis (1 N HCl, 4 hours) formed 6–11% humin. Between pH 5.0 and 9.0 the material dissolved slowly but completely at concentrations of 1.25% in water and with all electrolytes tested. A 0.5% aqueous solution of the dialyzed polysaccharide had the same pH as water; it was clear, brown, and lost 7.5% of its weight on electrodialysis and again had the same pH as electrodialyzed water. Its activity was not measurably affected by autoclaving at 120° (15 lb) for 20 minutes at pH 7.0. The polysaccharide showed no absorption maximum between 220 and 625 mμ. The exhaustively dialyzed polysaccharide was homogeneous by immunochemical criteria, in the ultracentrifuge, and on electrophoresis; it migrated to the anode in borate buffer. After 101 minutes at 59,780 rpm the polysaccharide showed only one sharp sedimenting boundary (Figure 2). The sedimentation coefficient was $s_{20,w} = 2.84$ S (*c* 0.62). The sedimentation data were extrapolated to zero concentration since a marked concentration de-

TABLE I: Physical Data of Highly Purified Blood-Group H(O) Active Sassafras Polysaccharide.

$[\alpha]_D^{23}$ (degrees)	+63.2 (*c* 0.25, H_2O, 1 dm)
$s_{20,w}$ (S)	8.5 (*c* 0)
$D_{20,w}$ (Fick units)	2.0 (*c* 0.5)
Mol wt	2.5×10^5 (from *s* and *D*)[a]
η_{sp}/c (dl/g)	2.5 (*c* 0.018; $\bar{G} = 0$)[b]
d*n*/d*c* (ml/g)	1.766×10^{-5} (λ 5461 A; 20°)
Mol wt	4.6×10^5 (light scattering)
f/f_0	2.6

[a] Assuming a partial specific volume $\bar{v} = 0.60$ ml/g.
[b] No shear dependence was found in the velocity gradient range 20–100 sec^{-1}; η_{sp}/c was found to be equal to 1.4 dl/g.

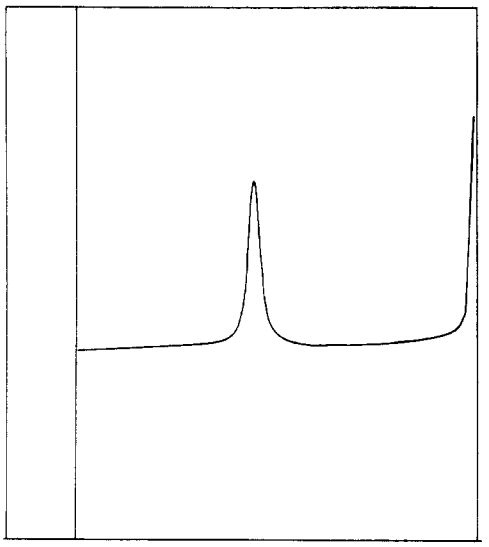

FIGURE 2: Sedimentation pattern of Sassafras polysaccharide Ca 606; *c* 0.50 in 0.13 M NaCl + 0.01 M $K^+PO_4^{3-}$ buffer, pH 6.8; 101 minutes after start; 59,780 rpm.

pendence was found. From sedimentation and diffusion values a frictional ratio f/f_0 of 2.6 was calculated (Table I).

The infrared spectrum of Sassafras polysaccharide showed the following main features: (1) a strong absorption at about 1000–1200 cm^{-1} due to C–O stretching and C–O–H bending, characteristic of all sugar derivatives; (2) a strong band at 1240 cm^{-1} due to *O*-acetyl groups; (3) a band at 1370 cm^{-1} to be attributed to methyl groups; (4) a band at 1400 cm^{-1} due to ionized carboxyl groups; this band is centered at 1410 cm^{-1} and is much stronger when the polysaccharide is cast from alkaline solutions; (5) a band at 1620 cm^{-1} due to carboxyl groups and/or moisture; (6) a band at 1740 cm^{-1} which is much stronger when casting the polysaccharide from acid solutions; this should be due to nonionized carboxyl groups and also due to the C=O of the *O*-acetyl groups.

The most relevant bands of human cyst glycoprotein Ca IIb 2 + 3 PF are the 1370 cm^{-1} methyl band and the monosubstituted amide bands at 1650 (amide I) and 1560 cm^{-1} (amide II), which are due to the peptide moiety of the product.

The native substance had a reduction value of 6.8% as glucose. Lipid extraction yielded only negligible amounts of material. Each component of the macromolecule was determined quantitatively. Only those monosaccharides which could possibly cause or contribute to the blood-group specificity of the polysaccharide were fully characterized chemically.

Isolation, Crystallization, and Characterization of the Blood-Group Active Monosaccharide of Sassafras. Acid hydrolysis of Sassafras polysaccharide readily released dialyzable blood-group H(O) active material from the macromolecule. Paper chromatographic properties of the Sassafras active component (SAC) were similar to those of xylose except in solvent d where the active material migrated faster than xylose; upon staining with aniline oxalate it gave a brown hexose color rather than the pink of pentose. After preliminary experiments this highly active component was isolated as follows: 44 g of crude polysaccharide was divided into several lots and hydrolyzed at a concentration of 1% in 1 N H_2SO_4 at about 96° for 2–6 hours. The supernate was neutralized to pH 5.5 with $Ba(OH)_2$, the $BaSO_4$ was centrifuged off, and the resulting supernate was cleaned with Darco G-60 and MB-3 monobed resin. Additional impurities were sedimented at 33,000*g*. The over-all yield of soluble material was 16.6 g (38%) which was streaked on Whatman 3 MM paper, separated in solvent (d), and dried, and residual phenol was removed by dipping the paper in ether. The appropriate area was eluted and the eluate was cleaned, restreaked, separated in solvent d, washed with ether, and again eluted. After treatment with Darco G-60 and MB-3 resin, 747 mg (4.5% of the hydrolysate) which was chromatographically about 90% pure was obtained. Pure sugar resulted after additional separation cycles using the same procedure, except that Schleicher and Schuell 589 Green R paper was used and development time was 7–8 hours. This was followed by a final paper chromatographic purification using solvent c. The colorless syrup was dextrorotatory and reduced ferricyanide; the triphenyltetrazolium chloride reaction was positive, indicating lack of substitution at C-2 (Feingold *et al.*, 1956). Spot tests for nonterminal and ω-deoxy sugars (Edward and Waldron, 1952) and for ketoses (Dedonder, 1952) were negative. The sugar was oxidizable by periodate as shown by the Wolfrom–Miller test (1956). An analysis performed on the syrup showed it contained one OCH_3 group per monosaccharide but no CCH_3.

The blood-group H(O) active syrup had an R_{fucose} of 0.86 in solvent a, 0.94 in c, 1.06 in d, and 0.9 in e. Its paper chromatographic and serological properties differed from 2-*O*-, 3-*O*-, and 6-*O*-methyl-D-glucose. The parent sugar was shown to be galactose by paper chro-

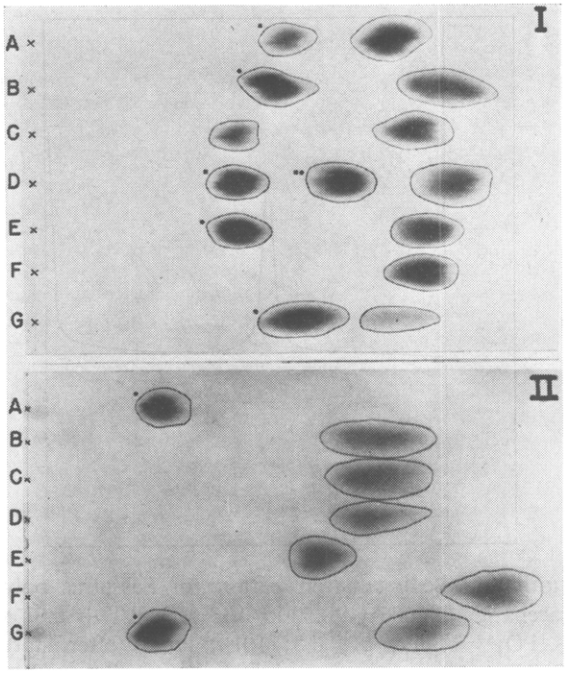

FIGURE 3: Chromatographic identification of the blood-group specific Sassafras active component (SAC). Paper, Whatman No. 1; stain, aniline oxalate. (I) Demethylated SAC and reference hexoses, developed 36 hours in 1-butanol–pyridine–water (10:3:3): A, allose•, altrose; B, glucose•, idose; C, demethylated SAC; D, galactose•, mannose••, fucose; E, galactose•, demethylated SAC; F, SAC; G, gulose•, talose. (II) SAC and reference mono-*O*-methylgalactoses; developed 65 hours in 2-butanone–water (88.7:11.3) saturated with Borax: A, galactose•; B, SAC; C, SAC, 3-*O*-methyl-D-galactose; D, 3-*O*-methyl-D-galactose; E, 4-*O*-methyl-D-galactose; F, 2-*O*-methyl-D-galactose; G, galactose•, 6-*O*-methyl-D-galactose.

matography of the partially de-*O*-methylated product in solvents c, d, and e. Solvent c was especially valuable since galactose could be clearly separated from the 7 isomeric aldohexoses (Figure 3I). Therefore, the intact monosaccharide was compared chromatographically and serologically with all known mono-*O*-methyl ethers of D-galactose. As can be seen in Figure 3II the blood group active monosaccharide from Sassafras had the same chromatographic properties as 3-*O*-methylgalactose.

Crystals of active sugar were obtained from an acetone–methanol solution of highly purified syrup at 4°. They were dried on porcelain dishes in an acetone–methanol atmosphere. The final optical rotation was $[\alpha]_D^{23}$ +92.1° (*c* 1.0, water, 1 dm) (authentic 3-*O*-methyl-D-galactose $[\alpha]_D$ +86° (Hirst *et al.*, 1951); +108.6° (Reber and Reichstein, 1945)); SAC mp 140–142°, authentic 3-*O*-methyl-D-galactose mp 140.5–142.5°; mmp 140–143°.

Anal. Calcd for $C_7H_{14}O_6$: C, 43.3; H, 7.2; OCH_3, 16.0. Found for 3-*O*-methyl-D-galactose from Sassafras: C, 43.1; H, 7.2; OCH_3, 14.8.

Periodate oxidation of SAC in parallel with authentic 3-*O*-methyl-D-galactose revealed, within the limits of experimental error, an equal periodate consumption/μmole of each sugar. Similarly, 0.63 μmole of formaldehyde was liberated by both SAC and by 3-*O*-methyl-D-galactose, further proof of a primary alcohol at C-6; however, these values amounted to only about two-thirds of theory. Finally, the infrared spectrum (determined in KBr disks with a Perkin-Elmer 237 recording spectrophotometer) of SAC was identical with that of 3-*O*-methyl-D-galactose but not with those of 2-*O*-methyl-, 4-*O*-methyl-, and 6-*O*-methyl-D-galactose.[1]

Isolation and Crystallization of Arabinose. D-Arabinose but not L-arabinose has been found to possess slight H(O) activity in tests with the eel and Lotus agglutinins (Watkins and Morgan, 1952; Springer and Williamson, 1962). The arabinose was, therefore, isolated from the polysaccharide.

A 1% solution of crude polysaccharide in 1 N H_2SO_4 was hydrolyzed for 1 hour at 94–96°. The hydrolysate was then dialyzed twice against 100 volumes of water and the outside fluids were concentrated *in vacuo*. After neutralization with $Ba(OH)_2$, 8.85 g of small molecular size material was recovered. Following chromatographic separation of the arabinose-rich area, isolation of arabinose was achieved by repeated chromatographic separation in solvent d and elution. After treatment with charcoal and MB-3 ion-exchange resin, the sugar was dried, dissolved in a minimum amount of hot methanol–water, and crystallized at 4°. Recrystallization from methanol followed: mp 158–158.5°, authentic L-arabinose (Mann) mp 155.5–157.5°, mmp 156–158°; the final optical rotation was $[\alpha]_D^{20}$ +100.5 ± 3° (*c* 1.02, water, 1 dm). The isolated L-arabinose possessed no blood-group H(O) activity.

Anal. Calcd for $C_5H_{10}O_5$: C, 40.00; H, 6.71. Found: C, 40.27, H, 6.61.

None of the other monosaccharides composing the polysaccharide possessed H(O) specificity (Watkins and Morgan, 1952; Springer and Williamson, 1962; Springer *et al.*, 1964a). In the present study D-galacturonic acid buffered to pH 7.2 was also inactive. The identity of all sugars was ascertained by paper chromatography in at least three different solvents with inclusion of the proper, reference compounds. In addition, feasible, colorimetric tests were performed. Rhamnose was identified in solvents a, c, and g and measured quantitatively. 3-*O*-Methyl-D-galactose was determined in solvents d, i, and a; its quantity was assessed by OCH_3 determination. Uronic acid was detected in solvent d

[1] We informed Professor E. A. Kabat that the blood-group specific monosaccharide from the Sassafras polysaccharide was a D-galactose methyl ether with the methyl substituent at one of two possible positions. He correctly suggested several months after this, on the basis of his studies of molecular models, that the sugar ". . . would be 3-*O*-methyl-D-galactose . . .," without knowing that we had crystallized and fully characterized this sugar in the interim.

TABLE II: Constituents of Blood Group H(O) Active Sassafras Polysaccharide.

Structural Units	Quantitative Determination by[a]	%	Moles[b]/Mole of Sassafras Polysaccharide
3-*O*-Methyl-D-galactose	Methoxyl (Zeisel)	25.0	355
Rhamnose	D.S.[c] colorimetry (Kuhn–Roth)	26.0 (22.6)	445
Xylose	F.M.[d] after paper chromatography	9	171
Galactose	F.M. after paper chromatography	8	124
Galacturonic acid	Dische[e] colorimetry	5.6	78
L-Arabinose	F.M. after paper chromatography	5	95
Glucose	P.J.[f] after paper chromatography	2	31
Acetyl	Kuhn–Roth	11.33	660

[a] For description of procedures see text. [b] Minus water. [c] Dische–Shettles method. [d] Folin–Malmros procedure. [e] Dische B reaction. [f] Park–Johnson procedure.

TABLE III: Hemagglutination Inhibiting Activity of Blood-Group H(O) Active Polysaccharides.

Polysaccharide	Minimum Amount (mg/ml) Giving Complete Inhibition of 4 Doses of Anti-							
	H(O) Eel Serum	H(O) Lotus Extract	H(O) Cytisus Extract	H(O)	A_1	A_2	B	$Rh_0(D)$
					Human Sera			
Sassafras albidum								
Water-soluble extract	0.05–0.1	N.a.[a]						
Highly purified polysaccharide (C-70)	0.002–0.005	N.a.	N.a.	N.a.	N.a.	N.a.	N.a.	2.5
Taxus cuspidata								
Highly purified polysaccharide (XXI)	0.002–0.005	N.a.	N.a.	N.a.	N.a.	2.5	N.a.	N.a.
Human ovarian cyst								
Morgan 277/P1/WS	0.001–0.002	0.02–0.05						
Ca IIb 2 + 3 PF	0.002–0.005	0.05–0.1	0.005–0.01	0.002–0.005	5	0.3	N.a.	N.a.
Human meconium	0.01–0.03				N.a.		N.a.	
Lipopolysaccharide								
Escherichia coli O_{128} *Salmonella poona*	0.005–0.01	±5	2.5	N.a.	2.5	N.a.	0.6	N.a.
Escherichia coli O_{86}:B7	N.a.	N.a.	N.a.	N.a.	5		0.001	N.a.

[a] No activity as defined in text.

and identified as galacturonic acid in solvent k and c as well as spectrophotometrically. Galactose and glucose were separated and identified in solvent c and less clearly in solvents d, e, and f. Xylose and arabinose were identified in solvents c, d, e, and h. Quantitative colorimetric determination was carried out on the total hydrolysate for the assessment of the component sugars. The sugars were separated on ammonia-washed (*cf.* Dubois *et al.*, 1956) Whatman No. 1 filter paper in suitable solvents and eluted with water. Xylose and 3-*O*-methylgalactose did not separate in the solvent used for quantitative determination, therefore, they were measured as one and the xylose content was derived by using the OCH_3 value as a measure of the 3-*O*-methyl-D-galactose present. Test procedures and results are listed in Table II. It is evident that 3-*O*-methyl-D-galactose and rhamnose account for about one-half of the molecule. There was almost twice as many acetate as 3-*O*-methyl-D-galactose molecules. Xylose was the third most common sugar. This was followed by galactose, which amounted to about one-third of the 3-*O*-methyl-D-galactose, then arabinose, galacturonic acid, and finally glucose.

Attempts to Degrade the Sassafras Polysaccharide.

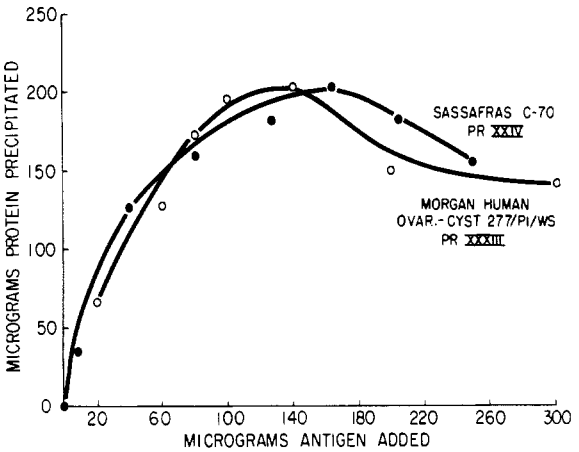

FIGURE 4: Precipitin curves obtained with eel anti-H(O) serum (a) and human and Sassafras H(O) active substances.

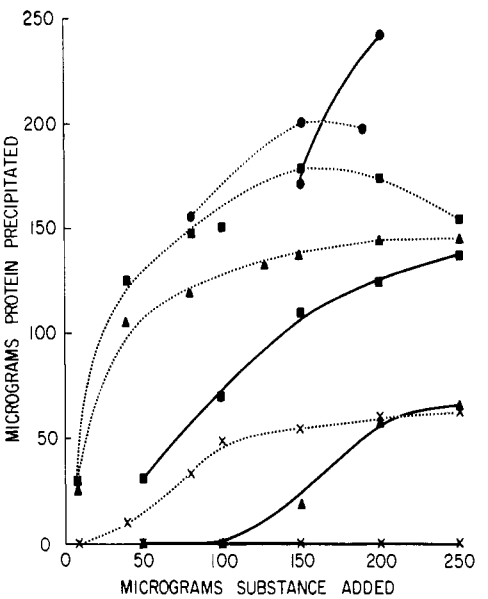

FIGURE 5: Decreased capacity of 1 N acetic acid hydrolyzed Sassafras and Taxus polysaccharides to precipitate eel anti-H(O) antibodies. Sassafras (P VII 60–75% IV): ···●···, unhydrolyzed; ···■···, hydrolyzed 2 hours; ··▲···, hydrolyzed 7.5 hours; ···×···, hydrolyzed 22 hours. Taxus (PT Pool B 65–70% D)—●—, unhydrolyzed; —■—, hydrolyzed 2 hours; —▲—, hydrolyzed 7.5 hours; —×—, hydrolyzed 22 hours (antibody, 0.5 ml of eel serum; antigen, highly purified Sassafras or Taxus).

Polysaccharide Ca 606 lost no more than 50% of its activity upon hydrolysis for 3 hours at 96° between pH 2.0 and 11.0, whereas there was >95% inactivation at pH 1.0 and at pH 14.0. The first sugars liberated on hydrolysis at pH 1.0 and pH 2.0 were large amounts of L-arabinose, some 3-*O*-methyl-D-galactose, xylose, and traces of rhamnose. Further hydrolysis released almost all arabinose, xylose, and substantial amounts of 3-*O*-methyl-D-galactose during the first 2 hours. Oligosaccharide mixtures which were released during the first 6 hours of hydrolysis possessed 2–10% of the H(O) activity of 3-*O*-methyl-D-galactose and had $R_{lactose}$ of 0.20–0.84 in solvent c. They were not investigated chemically. Between the third and sixth hour most of the galactose and 3-*O*-methyl-D-galactose and uronic acid were liberated as well as some rhamnose. The largest amounts of rhamnose and glucose were released only after this time. Hydrolysis at pH 9 and pH 11 liberated only trace amounts of hexose and material migrating slower than glucose on paper chromatograms. Deacetylation did not alter the activity of the Sassafras polysaccharide. None of the enzymes investigated decreased the activity of the polysaccharide.

Immunochemistry. HEMAGGLUTINATION INHIBITION. Results for the macromolecular substances are depicted in Table III. Highly purified Sassafras polysaccharides (C-70 and comparable preparations) and the most highly active blood-group H(O) glycoproteins from pseudomucinous human ovarian cysts are of similar activity when measured with eel serum but not with any of the other sera. The biological properties of the polysaccharide isolated from Taxus and Sassafras were analogous in that they inhibited no other anti-H(O) agglutinin than that from the eel (Table III and earlier observations by Springer, 1956, 1958). In contrast, the human cyst substances strongly inhibited all anti-H(O) reagents investigated. Highly purified Sassafras polysaccharide weakly inhibited anti-Rh_0(D) antibodies (Springer and Tegtmeyer, 1964) but, like the Taxus polysaccharide, it had no effect on any of the agglutinins of other erythrocyte antigens listed in the table and also none on human anti-M, -N, -P, and -S antibodies. By comparison, cyst preparation Ca IIb 2 + 3 PF inhibited faintly the agglutination of A_1 cells and that of A_2 erythrocytes by homologous antisera to the extent of 1% of most H-anti-H(O) agglutinations.

Inhibition given by the blood-group specific Sassafras hapten 3-*O*-methyl-D-galactose (the authentic sugar and SAC) is shown in Table IV, which for comparison also lists the activity of the blood-group H(O) specific sugars of human glycoprotein and Taxus as well as other active methyl ethers of D-galactose and of L- and D-fucose. As can be seen, 3-*O*-methyl-D-galactose is as active as L-fucose when determined with eel serum but inactive in tests with *Lotus tetragonolobus* extract. None of the other *O*-methyl ethers of D-galactose was more active than 3-*O*-methyl-D-galactose. 2-*O*-Methyl-D-galactose, 2,3-di-*O*-methyl-D-galactose, and *O*-α-D-galactopyranosyl-(1→3)-D-galactose were active in the Lotus system, although the disaccharide did not inhibit eel anti-H(O) serum. D-Galactose was inactive in both systems while L-galactose showed significant activity with the Lotus extract.

QUANTITATIVE PRECIPITIN REACTION. Highly purified Sassafras polysaccharide gave a precipitin curve with eel anti-H(O) serum virtually identical with that ob-

TABLE IV: Hemagglutination Inhibiting Activity of Blood-Group H(O) Specific Monosaccharides.

Test Substance	Minimum Amount (mg/ml) Completely Inhibiting 4 Hemagglutinating Doses	
	Eel Serum Agglutinin	Lotus Extract Agglutinin
L-Fucose	0.1–0.2	0.1
3-*O*-Methyl-D-galactose[a]	0.1	>5
2-*O*-Methyl-D-galactose	2.5–5	2.5–5
2,3-Di-*O*-methyl-D-galactose	0.1	5
3,4-Di-*O*-methyl-D-galactose	>5	Not tested
2,3,4-Tri-*O*-methyl-D-galactose	0.3	>5
O-α-D-Galactopyranosyl-(1→3)-D-galactose	>5	1.2–2.5
L-Galactose	>5	0.3
2-*O*-Methyl-L-fucose	0.05	0.05–0.1
3-*O*-Methyl-L-fucose	0.05–0.1	0.05
3-*O*-Methyl-D-fucose	0.05	>5
2,3-Di-*O*-methyl-L-fucose	0.05	0.05
2,3-Di-*O*-methyl-D-fucose	0.05	1.2–2.5
2,3,4-Tri-*O*-methyl-D-fucose	1.2–2.5	±5

[a] Methyl substituents at C-4; at C-6; at C-2 and C-4; and at C-2, -4, and -6 did not activate the inactive parent compound D-galactose.

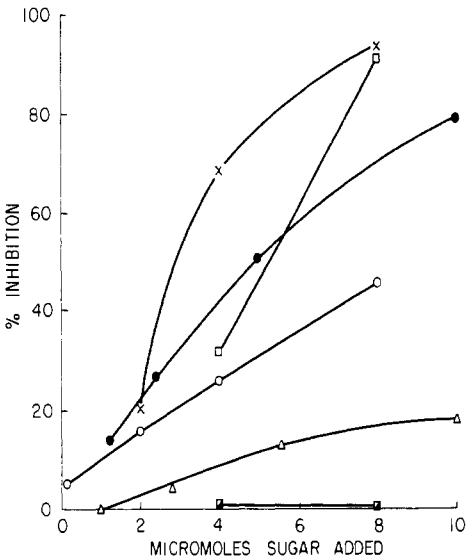

FIGURE 6: Blood-group H(O) specific hapten inhibition of anti-H(O) antibody precipitation with Sassafras polysaccharide. ●—●, L-fucose; □—□, 2-*O*-methyl-L-fucose; ◪—◪, 2-*O*-methyl-D-fucose; ○—○, 3-*O*-methyl-D-fucose; ×—×, methyl α-L-fucopyranoside; △—△, methyl β-L-fucopyranoside (antibody, 0.5 ml of eel serum D + E; antigen, 145 μg of highly purified Sassafras pool C).

tained with the ovarian cyst material 277/P1/WS from Professor Morgan (Figure 4). The H(O) cyst glycoprotein Ca IIb precipitated about one-half as much antibody protein in the equivalence zone.

The Lotus reagent gave a typical precipitin curve with human cyst substances Ca IIb 2 + 3 PF and Morgan 370. The maximal amount of plant protein, 56 μg, was precipitated with an addition of 100 μg of blood-group mucoid Ca IIb. In comparison, 100 μg of eel anti-H(O) antibody protein were precipitated in the area of equivalence, between 80 and 100 μg of antigen added for this particular eel antiserum. Sassafras or Taxus polysaccharide did not precipitate with the Lotus reagent.

E. coli O_{128} antigen, in keeping with its lower hemagglutination-inhibiting activity, precipitated about one-half as much eel antibody protein as Sassafras polysaccharide C-70 or cyst 277/P1/WS in the area of maximal precipitation. The curve showed a consistent and slow increase in antibody protein precipitation. The Taxus polysaccharide precipitated as much eel antibody protein as did Sassafras C-70 at the point of maximal precipitation of the latter. However, Taxus did precipitate some additional antibody with a maximum of about 240 μg of antibody protein upon addition of 200 μg of polysaccharide; this was the area of equivalence, since thereafter the amount of precipitated antibody protein decreased. The precipitin curve given with the Taxus polysaccharide was not symmetrical.

Precipitin reactions were also carried out with Sassafras and Taxus polysaccharides partially degraded with 1 N acetic acid. The results agreed with those obtained in the hemagglutination inhibition and agar gel diffusion assays described below; Figure 5 shows that Taxus polysaccharide was inactivated more rapidly than Sassafras antigen.

MICROPRECIPITIN REACTION. Precipitation with eel serum was visible within 48 hours and increased up to the seventh day. Both Sassafras and ovarian cyst substances precipitated at concentrations of 10 mg–10 μg/ml. The amount of precipitate was maximal at 1 mg/ml. When homogeneous Sassafras polysaccharide hydrolyzed with 0.1 N HCl for 30 minutes was used as precipitinogen, the amount of precipitate was nearly doubled in the range of 0.5–5 mg/ml and precipitation was still observed at an antigen concentration of 0.1 μg/ml, a hundredfold increase of activity over the unhydrolyzed material. Prolonged hydrolysis led to a decrease and ultimate end of the precipitating ability of the residual polysaccharide.

All antigens investigated precipitated Type XIV antipneumococcus horse serum in the range of 1 μg–10 mg/ml. The homologous pneumococcus S XIV antigen precipitated at 0.1 μg/ml. Partially degraded Sassafras polysaccharide showed a slight increase in precipitability after hydrolysis for 30 minutes with 0.1 N HCl; thereafter, precipitability disappeared.

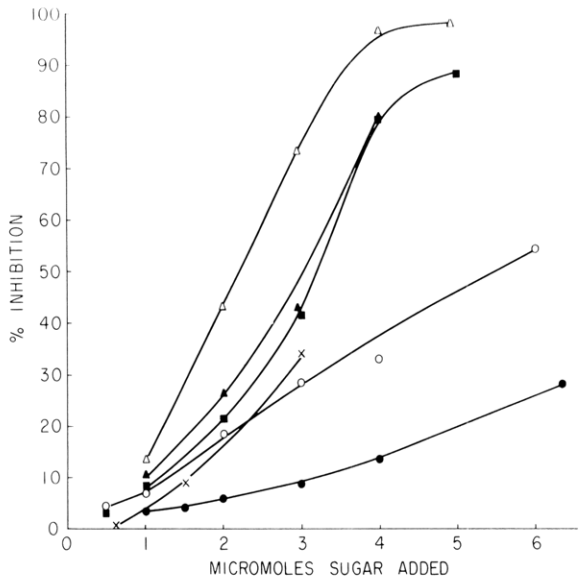

FIGURE 7: Blood-group H(O) specific hapten inhibition of anti-H(O) antibody precipitation with Sassafras polysaccharide. ●—●, L-fucose; ×—×, 3-*O*-methyl-L-fucose; ○—○ 3-*O*-methyl-D-galactose (SAC); ▲—▲, 2,3-di-*O*-methyl-D-fucose; △—△, 2,3-di-*O*-methyl-L-fucose; ■—■, 2,3-di-*O*-methyl-D-galactose (antibody, 0.5 ml of eel serum m′ 12/64; antigen, 65 μg of Sassafras Ca 606).

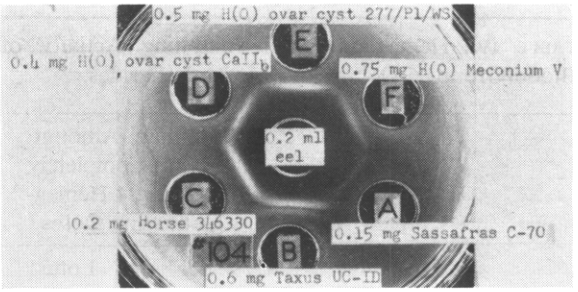

FIGURE 8: Reactions of blood-group H(O) active macromolecules with eel anti-H(O) serum compared by immune diffusion.

Precipitation was observed for both human and Sassafras antigens with the human anti-H(O) serum at levels of 0.1–10 mg/ml for the human antigen and 0.5–10 mg/ml for the Sassafras substance. Precipitation experiments with sera obtained after immunization with Sassafras polysaccharide are listed under "Immunization."

PRECIPITATION INHIBITION. The inhibition of precipitation of eel anti-H(O) serum by blood-group H(O) specific human ovarian cyst substance has been studied and reported earlier (Springer and Williamson, 1962; Springer *et al.*, 1964a). However, the effect of the blood-group specific hapten from Sassafras had not been investigated previously. It was now found that this sugar, 3-*O*-methyl-D-galactose, behaved like 3-*O*-methyl-D-fucose in the eel serum–ovarian cyst system. Because of lack of hapten, one duplicate test at seven concentration levels was carried out. Inhibition of precipitation was observed between 0.1 and 0.5 μmolar hapten concentration; the latter concentration gave 23% inhibition. At higher concentrations no inhibition but precipitation by the hapten was discernible with this serum pool (*cf.* Kolecki and Springer, 1965).

Figures 6 and 7 list hapten inhibition of precipitation of two different eel sera with highly purified Sassafras polysaccharide. The sugars predominantly responsible for the H(O) specificity of ovarian cyst and Taxus substances (Watkins and Morgan, 1952; Springer and Williamson, 1962; Springer *et al.*, 1964a) were also included. The inhibiting activity of L-fucose when measured with eel serum D + E was over twice that shown with eel serum m′ 12/64 which had an unusually low hemagglutinin titer (1:16). None of the inhibitors was quite as potent with any of the sera tested when Sassafras was used as precipitating antigen instead of the ovarian cyst antigen which was used previously (Springer *et al.*, 1964a). The most powerful inhibitor was 2,3-di-*O*-methyl-L-fucose, closely followed by its D enantiomorph and by 2,3-di-*O*-methyl-D-galactose. Digitalose at 5 μmoles gave >30% inhibition in antiserum D + E (Figure 6). Also, 3-*O*-methyl-D-galactose gave an almost straight line curve (with eel serum m′ 12/64) and was a better inhibitor than L-fucose (Figure 7); however, it was evident from visual inspection that the composition of the precipitate at levels above 2.0 μmoles of hapten was a mixture of the smooth antigen–antibody complex and of the granular monosaccharide–antibody precipitate (*cf.* Kolecki and Springer, 1965).

With ovarian cyst substance as well as Sassafras polysaccharide as precipitinogen methyl α-L-fucopyranoside was highly active as an inhibitor, while the β anomer was less active than L-fucose (Figure 6). The precipitation-inhibiting capacity of monosaccharide constituents of the human blood-groups other than L-fucose, namely *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and D-galactose, was also assessed. They possessed about 10% of the L-fucose activity. A similar activity was exhibited by D-arabinose. D-Fucose and 2-*O*-methyl-D-fucose in concentrations up to 10 μmoles gave no significant inhibition.

AGAR GEL DIFFUSION. Some of the results of the precipitation reaction of blood-group H(O) specific antigens in a solid medium are depicted in Figure 8. All blood-group H(O) active antigens formed a distinct line with the exception of the Taxus preparation; it gave two bands. The Sassafras band fused with the preparation of human meconium and ovarian H(O) macromolecules. It formed a spur with the faster of the two Taxus bands; in other tests at different concentrations there was fusion. The faster Taxus band fused with the blood-group preparation from horse stomach. The Taxus polysaccharide frequently only partially fused when compared with human ovarian cyst H(O)

TABLE V: Hydrolysis by 1 N Acetic Acid (96°) of Blood-Group H(O) Specific Sassafras and Taxus Polysaccharides.

Duration of Hydrolysis (Hours)	Amount of Ethanol Precipitable Material Recovered (%)		H(O) Activity[a] of Ethanol Precipitable Material (mg/ml)[b]		Reactivity in Ouchterlony Plates[a]	
	Sassafras	Taxus	Sassafras	Taxus	Sassafras	Taxus
0	100	100	0.002	0.005	++	++
2	95	85	0.002–0.005	0.01	++	++
4	90	77	0.002–0.005	0.01–0.02	++	++
7.5	83	60	0.005	0.1–0.2	++	N.r.[c]
11	75	55	0.05	0.6	N.r.	N.r.
22	69	42	0.2	5	N.r.	N.r.
50	60		0.3		N.r.	N.r.

[a] Using eel serum. [b] See Table III. [c] No reaction.

substance. The horse stomach glycoprotein gave a band which fused with the ovarian cyst substance line. The bands of both ovarian cyst glycoprotein preparations fused with one another and with the human H(O) meconium preparation. Lipopolysaccharide of *E. coli* O_{128}, not shown in the figure, also gave only one band which fused with all other H(O) active antigens tested. Fusion was generally obtained when their concentrations were closely similar. Antigens of other blood group specificities did not react (Springer *et al.*, 1964b). Table V shows that Sassafras polysaccharide retained almost unaltered activity after mild acid hydrolysis for up to 7.5 hours in agar gel and hemagglutination inhibition tests, whereas the Taxus polysaccharide was largely inactivated.

Agar gel diffusion studies were also carried out with the *Lotus tetragonolobus* extract. Blood-group H(O) active substances from mammals but not those from higher plants reacted with it. Type XIV antipneumococcus horse serum detected its homologous antigen but none of the other antigens investigated.

The precipitin reaction between eel serum and H(O) active Sassafras, Taxus, *E. coli*, and ovarian cyst antigens was inhibited by the H(O) active haptens methyl α-L-fucopyranoside and L-fucose but not by D-glucose when they were present in the agar at a concentration of 40 μmoles/ml.

IMMUNIZATION. Of nine germ-free birds with no preexisting anti-H(O) agglutinins injected with Sassafras polysaccharide, two formed specific agglutinins against erythrocytes of blood group O. The highest titer after the serum had been absorbed with A_1B erythrocytes was 1:8. Among four ordinary chickens, three formed significant anti-H(O) agglutinins of a titer not exceeding 1:4. The ovarian cyst substance proved to be of higher antigenicity in that a significant titer resulted in all seven germ-free birds; the highest titer was 1:64 and was obtained in two chickens. One of two ordinary chickens showed a significant titer increase, from <1:1 to 1:16. Alum injection alone did not increase the anti-H(O) titer. In most instances the highest titer was obtained after a booster injection. Only one of the four goats immunized (C) showed a fourfold increase in titer from 1:2 to 1:8 after absorption.

Even though the absorbed sera of the two goats which were investigated in the precipitin test did not show a rise in agglutination titer against human erythrocytes of blood group O, their absorbed postimmunization sera precipitated Sassafras polysaccharide at concentrations from 5 μg to 5 mg/ml. No precipitate was given by the preimmunization sera. The sera of eight chickens immunized with Sassafras polysaccharide, including those which showed a significant increase in anti-H(O) agglutinins, did not precipitate Sassafras antigen.

Discussion

The novel Sassafras antigen described in this paper is the second blood-group cross-reactive poly-saccharide from a higher plant. The activity of both plant polysaccharides was as high as that of the best human preparations but was demonstrable with eel anti-H(O) serum only. It was not established that the precipitation of Sassafras antigen with human anti-H(O) serum was H(O) specific. It is noteworthy that the molecular weight of the Sassafras polysaccharide is similar to that of human H(O) substance which was found to be 3.2×10^5 (Annison *et al.*, 1952). The discrepancy between the molecular weights by light scattering and by hydrodynamic measurements is in part due to the polydispersity of the Sassafras polysaccharide. Furthermore, the molecular weight arrived at by Svedberg's formula is likely to be low because the diffusion coefficient used was obtained at a single, rather high concentration. Also, from the value of $d\eta/dc$ it appears that the assumed value of $\bar{\nu}$ is too low. The Sassafras polysaccharide in solution is probably a polydisperse system of random coils. These have a nearly spherical over-all shape at low-shear gradient, but may be deformed into asymmetrical particles at high shear. The relatively high value calculated for the

frictional ratio should be mainly due to hydration. The results obtained by infrared analysis agree well with the analytical chemical data.

There was a great difference between the chemical composition of the human and the Sassafras blood-group H(O) antigens. Galactose is the only sugar which the Sassafras polysaccharide shares with the human blood-group substances; however, this sugar is not primarily involved in blood-group H(O) activity and by itself is inactive (*cf.* Watkins and Morgan, 1952; *cf.* Kabat, 1956; Springer and Williamson, 1962, 1963). While L-fucose is the monosaccharide of paramount importance for the specificity of human blood-group H(O) cyst substances isolated from secretions, this study has shown that 3-*O*-methyl-D-galactose is responsible for the H(O) specificity of the Sassafras polysaccharide. In addition, we found the disaccharide *O*-α-D-galactopyranosyl-(1→3)-D-galactose, which is responsible for B specificity in human blood-group substances (Painter *et al.*, 1962), to possess some activity in the anti-H(O) specific Lotus system (Table IV). There is one isolated earlier study awaiting confirmation (Masamune *et al.*, 1957) which suggests that another structure, "sphingosine-*N*-melibioside," with a nonreducing terminal α-galactopyranosyl group, possessed H(O) specificity when measured with chicken and goat anti-H(O) sera.

3-*O*-Methyl-D-galactose has been found in Nature only once before, specifically in the mucilage of the slippery elm (*Ulmus fulva*) (Hirst *et al.*, 1951), a tree not related to the Sassafras tree. The 3-*O*-methyl-D-galactose content of the polysaccharide from *Sassafras albidum* is about twice that found in the *Ulmus fulva* polysaccharide. Of the Sassafras antigen's other monosaccharides only arabinose could have been blood-group H(O) active (Watkins and Morgan, 1952; Springer and Williamson, 1962), but it proved to be the L enantiomorph which does not possess H(O) specificity. Another fundamental difference between the Sassafras polysaccharide and the human H(O) glycoprotein is the former's lack of hexosamines and amino acids. Nevertheless, there was no difference between these two substances by the immunochemical criteria of activity in hemagglutination inhibition, agar gel diffusion, and even the strictly quantitative precipitin tests when eel sera were employed. The finding of close immunochemical similarity with three different methods of chemically unrelated substances seems to point to limitations of immunological methods, at least when heterologous reagents are used. This observation is of special importance for studies on blood-group active substances, since it appears that a significant portion of all blood-group antibodies cross react and are therefore heterologous (for references see Springer *et al.*, 1964a). However, the unreliability of immunochemical reagents may be apparent rather than real, since we have adduced evidence (Springer *et al.*, 1964a) that the minimum complementary structure for the eel antibody is smaller than a monosaccharide and may therefore be found on a variety of otherwise unrelated monosaccharides. This minimum structure appears to consist of a methyl substituent attached equatorially to a pyranose ring; adjoining is an ether oxygen. On a contiguous C atom is an axial oxygen-carrying substituent *cis* to the methyl group. Such a structure is present in L-fucose, 3-*O*-methyl-D-galactose, 3-*O*-methyl-D-fucose, and 2-*O*-methyl-α-L-fucose. 2-*O*-Methyl-L-fucose is the blood-group H(O) specific component in the polysaccharide of *Taxus cuspidata* (Springer *et al.*, 1956) and 3-*O*-methyl-D-fucose when present in terminal position is responsible for the blood-group H(O) activity of various cardiac glycosides and the antibiotic chartreusin (Springer and Williamson, 1963). We have been unable to determine the linkage of 3-*O*-methyl-D-galactose in the Sassafras macromolecule as α, β, or both, since none of the enzymes tested released this sugar and we have found previously as also in this study that α- and β-glycosides of *O*-methyl ethers of monosaccharides in the D series of fucose can both possess high and essentially equal activities (Springer *et al.*, 1964a). No attempt has been made to determine whether or not the 3-*O*-methyl-D-galactose is attached *via* the same linkage and to the same sugar, galactose, as is L-fucose in the human substances where this linkage is α-(1→2); α-(1→2) linked fucosyl radicals were found to be the most active (Rege *et al.*, 1964; Lloyd and Kabat, 1964). It is unlikely that more than a minority of the 3-*O*-methyl-D-galactose molecules are attached to galactose in the Sassafras polysaccharide, since there are only one-third as many galactose molecules as there are of its 3-*O*-methyl ether. The galactose is not an artifact. HCl (1N) for 4 hours at 100° did not de-*O*-methylate the *O*-methylhexose.

Approximately 92% of the Sassafras macromolecule can be accounted for as carbohydrate and acetyl; based on a molecular weight of 2.5×10^5 as determined by hydrodynamic measurements and a 3-*O*-methyl-D-galactose content of 25%, there are 355 molecules of this blood-group active monosaccharide in the macromolecule (Table II). Similarly, approximately 350 L-fucose units are found in each molecule of H(O) substance (Morgan and Watkins, 1953). However, in contrast to the human blood-group H(O) glycoprotein, the blood-group specific sugar of the Sassafras polysaccharide is not only in terminal position; part of it appears to be masked by inactive arabinose and other units. This is indicated by the increase in activity during the first 30 minutes of hydrolysis at pH 1.0 which removed *ca.* 90% of the arabinose. The ease with which arabinose is split off by mild acid suggests that it is present in the macromolecule in furanose form.

The results obtained with the antipneumococcus Type XIV antiserum indicate that part of the galactose is β-linked in terminal position or that 3-*O*-methyl-D-galactose cross reacts with this serum. Cross reactivity of mammalian blood group substances with Type XIV antipneumococcus horse serum has been ascribed to the presence of β-D-galactopyranosyl in terminal position (Watkins and Morgan, 1956; Allen and Kabat, 1959). The high cross-reacting activity indicates that the specific sugar is linked either 1:4 or 1:3 to the subterminal unit. Our finding that the Taxus and Sassafras polysaccharides cross react with Type XIV antipneumo-

coccus horse serum in the microprecipitin test confirms earlier observations (Heidelberger, 1959; and personal communication). Both polysaccharides failed to react with this serum in agar gel diffusion tests, indicating the lower sensitivity of this method.

Since there are more moles of acetyl than of any one sugar, it is necessary either to assign more than one acetyl to some units of one of the two most abundant sugars, or to assume acetylation of more than one kind of sugar. That the acetyl is not involved in the blood-group H(O) activity of the antigen has been shown by the deacetylation study.

The activity of the Sassafras polysaccharide persisted much longer on acid hydrolysis than did that of the Taxus polysaccharide (Table V, Figure 5) or the human blood-group H(O) substance (Annison *et al.*, 1952; *cf.* Kabat, 1956). This persistence of activity during hydrolysis is probably due to not terminally bound 3-*O*-methyl-D-galactose, although it may also be more acid resistant than L-fucose on the human substance or 2-*O*-methyl-L-fucose on the Taxus polysaccharide. Stepwise acid hydrolysis suggests that surprisingly most of the rhamnose constitutes the "core" of this polysaccharide.

The strikingly similar activities of the macromolecules from humans and higher plants studied here have a parallel in the approximately equal activity, in the hemagglutination inhibition tests, of the constituent blood-group specific haptens of these materials: L-fucose, 3-*O*-methyl-D-galactose, 2-*O*-methyl-L-fucose, and 3-*O*-methyl-D-fucose (Table IV). It will also be seen from this table that, similar to our earlier findings on fucose ethers (Springer and Williamson, 1962, 1963; Springer *et al.*, 1964a), mono-*O*-methylation of D-galactose on positions other than C-2 or C-3 did not lead to active compounds. Additional methylation at C-2 of the 3-*O*-methylated monosaccharides of the D series did not change the activity in the eel serum system but led to *de novo* appearance of some activity when determined with the Lotus reagent; *O*-methylation at C-4 inactivated 3-*O*-methyl- and 2-*O*-methyl-D-galactose, while 2,3,4-tri-*O*-methyl-D-galactose (Table IV) was active. The similarity of 3-*O*-methyl-D-galactose to 3-*O*-methyl-D-fucose in the eel serum system was extended to quantitative precipitation inhibition results. It was found that 3-*O*-methyl-D-galactose as well as 3-*O*-methyl-D-fucose gave significant inhibition which increased in proportion to the amount of monosaccharide added at levels up to 0.5 μmole with all sera tested. Above this level these two sugars precipitated the majority of eel anti-H(O) sera in a manner similar to human H(O) substance and gave a typical quantitative precipitin curve. All anti-H(O) agglutinins and precipitins could be removed by these haptens. Virtually 100% of the antibody was recovered from the dialyzed precipitate. The recovered antibody agglutinated erythrocytes of blood-group O and precipitated blood group H(O) active Sassafras and human ovarian cyst antigens as well as digitalose (G. F. Springer and B. Kolecki, 1965, in preparation). The mechanism underlying this precipitation is not understood, since it is difficult to see how a lattice can be formed between the nonaggregated low molecular weight hapten molecules, which are free of characteristic repeating units, and the eel antibody. Both precipitating haptens carry at least one apolar substituent which may be involved in hydrophobic bonding. It has been assumed by Karush (1962) that the interaction of any apolar group in an antigenic determinant with an antibody would make a large contribution to the stability of the resulting complex. The complexity of the situation is evident since certain modifications such as 2-*O*-methylation or methyl glycosidation of the two 3-*O*-methyl sugars abolished their precipitating ability, giving potent neutralizing haptens (Kolecki and Springer, 1965).

Immunization experiments showed that the Sassafras polysaccharide's blood-group H(O) antigenicity was much lower than that of human ovarian cyst H(O) glycoprotein injected under the same circumstances. Since goat immune sera precipitated the homologous Sassafras polysaccharide without showing a rise in agglutinin titer against O erythrocytes, structures other than those possessing H(O) specificity must have induced these precipitins.

Highly purified Sassafras polysaccharide was also investigated for other biological properties. It was a potent lipemia clearing agent *in vivo*, exceeding heparin by a factor of 2500 (for methods see Schuler and Springer, 1957). It was free of any anticoagulant effect *in vitro* as well as *in vivo*. It possessed antiviral properties *in vivo* but was inactive in virus hemagglutination inhibition tests. Its protective activity was especially pronounced in mice infected with Columbia SK polio virus where it was effective in doses as low as 0.01 mg/kg of body weight. In the chick embryo this material prevented growth of influenza viruses—without exhibiting toxicity toward the embryo. Highly purified Sassafras polysaccharide was not pyrogenic in rabbits at concentrations of 1 mg/kg of body weight and was free of anticomplementary activity against all nine complement components (Nelson, 1965).

The results reported here show that inferences from a given serological specificity as to its chemical basis have to be drawn with caution. The relation of one active macromolecule to another which exhibits similar properties in a given biological system also must be verified by several methods.

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The Mechanism of the Bacterial C-1,2 Dehydrogenation of Steroids. III. Kinetics and Isotope Effects*

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ABSTRACT: The kinetics of the C-1,2 dehydrogenation of 3-keto steroids with cell-free preparations of *Bacillus sphaericus* in the presence of menadione and 2,6-dichlorophenolindophenol as electron acceptors indicate that the enzyme undergoes intermediate reduction–oxidation. Lineweaver–Burk plots of $1/V$ *vs.* 1/acceptor gave parallel slopes for several fixed concentrations of steroid and the replot of intercepts *vs.* 1/steroid gave $1/V_{max} > 0$. Deuterium isotope effects were studied by enrichment procedures, by steroidal product isolation, and by spectrophotometric kinetic assays. In the case of 5α-3-keto steroids, a significant kinetic isotope effect was found for deuterium at the 1α but not the 2β position while for the Δ^4-3-ketone, deuterium at either position affected the rate at V_{max}. The kinetic evidence, which is in accord with the previously proposed enolization–hydride abstraction mechanism, indicates that the rate-determining step in the over-all sequence appears in the steroid dehydrogenation step rather than in the enzyme reoxidation step.

Previous studies from this laboratory (Hayano *et al.*, 1961; Ringold *et al.*, 1962, 1963) demonstrated that the enzymatic introduction of a double bond into the C-1,2 position of 5α- or Δ^4-3-keto steroids by sonically disrupted cell-free preparations of *Bacillus sphaericus* (ATCC 7055) in the presence of added external electron acceptors, preferably menadione, proceeds *via* a *trans*-diaxial elimination of the 2β,1α-hydrogen atoms. Tritium-incorporation studies established that in the absence of an electron acceptor a preferential introduction of heavy isomer into the 2β position can be effected. This, coupled with a number of mechanistic considerations, led to the proposal of a dehydrogenation mechanism consisting of a two-step process of enolization followed by hydride abstraction, a mechanism essentially identical with the nonenzymatic C-1,2 dehydrogenation of steroids by dichlorodicyanoquinone (Ringold and Turner, 1962).

In this paper we report a spectrophotometric assay for the reaction which couples the reduction of 2,6-dichlorophenolindophenol with reduced menadione. Utilizing this assay the kinetics of reaction with a 5α-3-keto steroid, a Δ^4-3-keto steroid, and with their corresponding 1α- and 2β-mono- and -polydeuterio derivatives have been studied. In addition, the isotope effects have been determined by enrichment and by steroidal product isolation procedures, which have in general given good agreement with the spectrophotometric method. The kinetic evidence has been found to be consistent with the general mechanism for two-substrate systems in which the enzyme exists in oxidized and reduced states, the steroid reacting with the oxidized form and the external electron acceptor with the reduced form. The primary deuterium isotope effects,

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