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Synthesis and Immunochemistry of Fucose Methyl Ethers and Their Methylglycosides*

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L-Fucopyranose in α-glycosidic linkage has been thought to be responsible for blood-group-H(O) specificity of human blood-group mucoids as determined with heterologous anti-H(O) reagents from the eel and *Lotus tetragonolobus*. This conclusion was based on hapten studies. However, some L- and D-fucose-*O*-methyl ethers were as active as L-fucose. Therefore the stereospecific requirements for the activities of fucose methyl ethers and their methylglycosides were systematically investigated. Eight fucose-*O*-methyl ethers and fourteen methylglycopyranosides of fucose were synthesized and characterized. The majority of these sugars were crystallized and ten are novel. Quantitative precipitin-inhibition tests in the eel serum anti-H(O)—H(O) substance system were only in qualitative accord with hemagglutination-inhibition tests. A number of methylated L- and D-fucoses and their methylglycopyranosides were potent inhibitors. The enantiomorphs of 3-*O*- and 2,3-di-*O*-methylfucoses had nearly identical activities. 3-*O*-Methylfucose precipitated the anti-H(O) antibody of some eel sera and may thus be the smallest uncharged antibody-precipitating hapten yet found. The complementary structure for the eel serum antibody is probably smaller than a monosaccharide. It seems to consist of a methyl substituent attached equatorially to a pyranose; there is an ether oxygen adjoining the methyl and an axial, oxygen-carrying substituent *cis* to the methyl group is on a contiguous C atom. An *O*-methyl at C-4 is compatible with activity. Hapten requirements for activity in the *Lotus* system differ; a substituent at C-2 is a precondition in the D series. A furanoid structure substituted with three *O*-methyl groups is equally active in both series. While appropriate methyl substitution increases the activity of highly active fucose compounds, it can inactivate slightly active fucose ethers.

Simple substances which combine specifically with antibodies or antibodylike reagents are thought to be identical with or very closely related to the immunologically determinant group of an antigen. This concept is based on Landsteiner's classical studies (1920). More recently it has been successfully applied to the elucidation of the nature of the antigenically determinant groupings of blood-group-specific substances (cf. Kabat, 1956; cf. Morgan, 1960).

It was first observed by Morgan and Watkins (1953) that L-fucose inhibited the agglutination of human blood-group-O erythrocytes by anti-H(O) agglutinins in eel serum and in seed extracts of *Lotus tetragonolobus*. This inhibition was thought to possess considerable specificity in that L-fucose was the only active sugar of the four constituent monosaccharides of blood-group mucoids. Kuhn and Osman (1956) confirmed and extended the earlier findings of Morgan and Watkins and postulated the following necessary conditions for the serological activity of fucosides: (a) The fucose residue must belong to the L series; (b) it must be in the pyranose form; (c) if linked, it must be present in α-glycosidic linkage. Other sugars were thought to be inactive.

Springer *et al.* (1956) reported that the polysaccharide with high blood-group-H(O) activity isolated from *Taxus cuspidata* contained no L-fucose, but the serologically active sugar was 2-*O*-methyl-L-fucose, not then known to occur in nature.

Earlier studies (Springer and Williamson, 1962) had shown that previously held ideas on the stereospecificity of serological reactions do not apply to the eel serum antibody and apply only to a limited extent to the *Lotus tetragonolobus* agglutinin, at least if the smallest structure which is complementary to the combining site of an antibody is considered to be a monosaccharide. While L-fucose and some of its derivatives are highly active substances, high and sometimes equal activities are also found among substituted members of the D series in both hemagglutination and precipitation-inhibition tests. Kabat (1962) has offered an explanation for these observations of equal biological activity of enantiomorphs.

These studies suggested limitations of heterologous serological reagents for the prediction of the nature of immunochemically specific groups. The ultimate rigid chemical characterization of serologically specific structures, therefore, is indispensable, especially in the assessment of blood-group substances, since experimental evidence has been adduced (Springer *et al.*, 1959, 1962; for theoretical aspects of this problem see

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Wiener, 1951; Kabat, 1956; Springer, 1960) that a significant proportion of anti-blood-group antibodies of all individuals are cross-reacting and therefore of heterologous nature.

Because of the unexpected biological properties of enantiomorphous methylfucoses and the dependence of serological activity on the position of methoxyl groups on the fucose molecule, it was considered important to investigate systematically the stereospecific requirements for the activities of fucose methyl ethers, their methylglycosides, and closely related compounds. The methylglycosides were of particular interest since, in biological systems, one anomer is usually of much higher activity than the parent compound while the other anomer is of considerably lower activity or even inactive.

Therefore most of the methyl ethers of L- and D-fucose and their methylglycosides were synthesized, the majority of the latter for the first time. Four of the nine fucose ethers and over one-half the novel glycosides were obtained in crystalline form. The synthetic procedures and the immunochemical properties of the resulting end products are the subject of this paper.

Experimental Procedures and Results

Organic Syntheses

Both members of each pair of enantiomorphs were synthesized by the same procedure. All experiments, including the syntheses, were carried out repeatedly. All samples used for physical and chemical analyses were dried to constant weight at 23–25° *in vacuo* at about 10^{-2} mm Hg over phosphorus pentoxide and paraffin. Melting points were determined on a Fisher-Johns melting-point apparatus and were not corrected. All optical rotations were measured with a Perkin-Elmer Model 141 digital readout polarimeter except those for methyl 2-*O*-methyl-α-L- and D-fucopyranosides, which were determined with a standard Hilger polarimeter. The values were not corrected. Elemental analyses were performed by Huffman Microanalytical Laboratories, Wheatridge, Colo., Micro-Tech Laboratories, Skokie, Ill., and M. Manser, Herrliberg, Zurich. Comparative paper chromatography was performed 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 of paper.

Most of the methyl ethers and their glycosides were eluted from the paper chromatograms with water; the more volatile compounds, however, were eluted with acetone. The acetone eluates contained only negligible amounts of impurities from paper. The water eluates were cleaned by heating 0.5–1.0% aqueous sugar solutions with acid-washed Darco G-60 charcoal (*ca.* 10 mg/100 mg sugar), filtration by gravity, and subsequent treatment of vacuum-concentrated filtrate with mixed H^+–OH^- exchange resin (Amberlite MB-3). Most syrups were ultimately dissolved in acetone and the last residues were removed by centrifugation. Further purification by distillation at 10^{-3} mm Hg was carried out if indicated.

Reducing Sugars

These were stained with aniline oxalate (Horrocks, 1949) after descending paper chromatography in the following solvents: (a) 1-butanol-ethanol-water (5:1:4) (Partridge and Westall, 1946); (b) toluene-1-butanol (1:1) saturated with water (Krauss *et al.*, 1960);[1] and (c) benzene-1-butanol-pyridine-water (1:5:3:3) (Gardiner and Percival, 1958). At least one enantiomorph of each ether was included as an authentic reference sample. Where indicated, the desired sugar was separated from contaminating monosaccharides by preparative paper chromatography on Whatman 3 MM paper in solvent (b) and subsequent elution with water.

Synthesis of Methyl Ethers of Fucose[2]

*2-O-Methyl-*L- *and* D-*fucose.*—The syntheses of these methyl ethers were performed as described by Springer and Williamson (1962). The yields of crystalline 2-*O*-methylfucoses were 40% of theory based on starting material.

*3-O-Methyl-*D-*fucose.*—The isolation of digitalose from various natural sources was carried out as reported previously (Springer and Williamson, 1962).

*2,3-Di-O-methyl-*L- *and* D-*fucose.*—These ethers were synthesized by the following modifications of the methods of Schmidt and Wernicke (1944) and Springer and Williamson (1962): Acetonization was carried out in the presence of 1% water. Methylations were performed by repeated use of Purdie's reagents (Purdie and Irvine, 1903); freshly prepared silver oxide was used (Helferich and Klein, 1926). The syrup which resulted after methylation contained approximately 50% methyl ethers other than 2,3-di-*O*-methylfucose, as determined by paper chromatography. Pure 2,3-di-*O*-methylfucose was separated from this mixture by preparative paper chromatography and obtained as a pure colorless syrup upon distillation at 10^{-3} mm Hg. The yield amounted to about 18% of theory based on the starting material; 2,3-di-*O*-methyl-D-fucose crystallized from acetone–petroleum ether (about 8:1 at −20°, mp 75–76°). The enantiomorph crystallized (mp 75–76°) under the same conditions after addition of traces of crystalline 2,3-di-*O*-methyl-D-fucose to more than 500 mg of syrupy sugar. Traces of mother liquor were removed as described for the methylglycosides. The respective $R_{\text{tetramethylglucose}}$ (R_G) values for 2,3-di-*O*-methyl-L- and D-fucose were 0.76 and 0.75 in solvent (a), 0.53 and 0.54 in solvent (b), and 0.84 and 0.84 in solvent (c). The optical rotations were $[\alpha]_D^{25}$ −100.8° (*c*, 1.05, water) and $[\alpha]_D^{25}$ +105.6° (*c*, 1.06, water) for the L and D enantiomorphs, respectively.

Anal. Calcd. for $C_8H_{16}O_5$: C, 50.00; H, 8.33; OCH_3, 32.29. Found for 2,3-di-*O*-methyl-L-fucose: C, 49.97; H, 8.36; OCH_3, 33.25. Found for 2,3-di-*O*-methyl-D-fucose: C, 49.70; H, 8.19; OCH_3, 31.64.

*2,3,4-Tri-O-methyl-*L- *and* D-*fucose.*—These known methyl ethers (Schmidt *et al.*, 1943) have been synthesized from the methylglycosides of mono- and di-*O*-methyl ethers of fucose to prove their ring structures (see under Methylglycosides). The parent glycosides were completely methylated with Purdie's reagents and the resulting crystalline products were hydrolyzed with 0.1 N HCl for 90 minutes at 100°. Amberlite IR-45 (OH^-) was used to neutralize the reaction mixture. The average yield of 2,3,4-tri-*O*-methylfucoses exceeded 90%; these ethers were identified by chromatography in solvent (a). The R_G values were 0.89 for both enantiomorphs.

[1] Saturation of the papers with water-acetone before applying the spots (see Krauss *et al.*, 1960) was omitted since resolution of sugar mixtures was satisfactory without this procedure.

[2] Crystalline L- and D-fucose were the starting materials unless otherwise indicated. These were purchased from Fluka, A. G., Buchs, Switzerland; Mann Laboratories, Inc., New York, U.S.A.; and Th. Schuchardt Co., Munich, Germany; before use the identity and purity of the initial purchases were ascertained by chromatography and measurement of optical rotation.

TABLE I
PROPERTIES OF METHYLGLYCOSIDES OF FUCOSE METHYL ETHERS

Glycoside	Melting Point (°C)	$[\alpha]_D^0$	Concn (%)	Paper Chromatography[a] R_G[b]	R_{Fu}[c]	Elemental Analyses (%) C Calcd	(%) C Found	(%) H Calcd	(%) H Found	(%) OCH_3 Calcd	(%) OCH_3 Found
Methyl 2-*O*-methyl-α-D-fucopyranoside[e]		+173.6 (26°)	1.17[d]		1.80	50.00	49.72	8.33	8.41	32.29	30.50
Methyl 2-*O*-methyl-β-D-fucopyranoside[e]	98.5–99.5	+3.46 (23°)	1.27[f]		2.17	50.00	49.76	8.33	8.38	32.29	30.95
Methyl 2-*O*-methyl-α-L-fucopyranoside[e]		−179.0 (26°)	1.17[d]		1.80	50.00	50.06	8.33	8.28	32.29	30.55
Methyl 2-*O*-methyl-β-L-fucopyranoside[e]	98–99	+17.15 (26°)	0.49[f]		2.17	50.00	49.32	8.33	8.14	32.29	30.00
Methyl 3-*O*-methyl-α-D-fucopyranoside	98.5–100.5	+198.5 (23°)	0.65[f]		1.63	50.00	50.10	8.33	8.14	32.29	30.30
Methyl 3-*O*-methyl-β-D-fucopyranoside[e]	108–110	+9.94 (23°)	0.31[f]		1.42	50.00	48.51	8.33	8.26	32.29	31.55
Methyl 2,3-di-*O*-methyl-α-D-fucopyranoside[e]		+190.0 (24°)	1.27[g]	0.85		52.43	52.55	8.74	8.88	45.15	45.02
Methyl 2,3-di-*O*-methyl-β-D-fucopyranoside[e]		+0.74 (24°)	1.21[g]	0.97		52.43	52.21	8.74	8.79	45.15	44.93
Methyl 2,3-di-*O*-methyl-α-L-fucopyranoside		−189.5 (29°)	1.38[g]	0.85		52.43	51.65	8.74	8.97	45.15	43.27
Methyl 2,3-di-*O*-methyl-β-L-fucopyranoside[e]	78	−1.23 (30°)	0.98[g]	0.96		52.43	51.69	8.74	8.76	45.15	44.18
Methyl 2,3,4-tri-*O*-methyl-α-D-fucopyranoside[e]	97–99	+206.8 (27°)	2.03[g]			54.55	55.40	9.09	9.07	56.36	56.37

[a] Solvent: 2-butanone-water (4:1). [b] $R_{tetramethylglucose}$. [c] $R_{methyl\text{-}\alpha\text{-}fucopyranoside}$. [d] In water. [e] Novel compound. [f] In methanol. [g] In acetone.

*2,3,5-Tri-O-methyl-*L- *and* D-*fucose.*—Only the preparation of the L enantiomorph has been reported previously (Gardiner and Percival, 1958). Both enantiomorphs have now been obtained from those 2,3-di-*O*-methylfucose glycosides which moved fastest on chromatograms thus establishing their 1,4 ring structure. Syntheses and identification of 2,3,5-tri-*O*-methylfucoses were carried out by the same procedure as those of the 2,3,4-tri-*O*-methylfucoses described. The over-all yield of 2,3,5-tri-*O*-methylfucoses was 60–65% of theory. In solvent (a) the R_G value was 1.01 for both enantiomorphs of 2,3,5-tri-*O*-methylfucose. The novel methyl ether 2,3,5-tri-*O*-methyl-D-fucose gave $[\alpha]_D^{25}$ −36.8° (*c*, 0.61, ethanol).

Anal. Calcd. for $C_9H_{18}O_5$: C, 52.43; H, 8.74; OCH_3, 45.15. Found: C, 51.71; H, 8.83; OCH_3, 42.34.

Methylglycosides

All glycosides were prepared by refluxing with continuous stirring 1 part of the parent sugar and 40 parts of anhydrous methanol in the presence of 4 parts of cation-exchange resin in the H^+ form (IR-120 or Duolite C-20) (cf. Osman *et al.*, 1951). The refluxing periods for the syntheses of the glycosides of the various fucose ethers varied and are listed with the description of their syntheses. The chosen refluxing times were arrived at in quest of an optimal yield of the pyranosidic glycosides and an equal proportion of both pyranosidic anomers.

All methylglycoside preparations were nonreducing, i.e., free of parent sugar, but they contained mixtures of furanosides and pyranosides. The pyranosides were separated from the furanosides and divided into their anomers on Whatman 3 MM paper by descending preparative paper chromatography using 2-butanone-water (4:1) as solvent (Boggs *et al.*, 1950). The solvent was not allowed to run off the chromatograms. In order to obtain distinct separation of some of the glycopyranoside mixtures the chromatogram had to be dried and rerun in the same solvent. It is indicated in the preparation of the various glycosides when this became necessary. Those glycosides which possessed 1,2 glycol structures were stained with sodium metaperiodate–potassium permanganate–benzidine reagent as described by Wolfrom and Miller (1956). Glycosides which were not oxidizable by the periodate ion were stained with iodine vapor (Brante, 1949). All methylglycosides of methyl ethers of fucose were distilled at 10^{-3} mm Hg. Some of the glycosides crystallized spontaneously during distillation; these were recrystallized from ether-acetone (5:1), spread on porcelain dishes, and kept in an atmosphere saturated with ether-acetone (10:1) in order to remove adhering traces of mother liquor. The boiling points of the methyl ether methylglycosides at 10^{-3} mm Hg were as follows: 60–75° for the mono-*O*-methylated glycosides, 45–55° for the di-*O*-methylated glycosides, and 40–45° for the tri-*O*-methylated glycopyranosides. The physicochemical properties and elemental analyses of all novel glycopyranosides and the known methyl 2,3-di-*O*-methyl-α-L-fucopyranoside (Conchie and Percival, 1950) are listed in Table I. Lack of serological activity and higher mobility than that of the pyranosides on paper chromatograms were characteristic of all furanosides.

Methyl-α- and β-fucopyranosides.—These were available from previous experiments (Springer and Williamson, 1962). Crystalline methyl-β-L-fucopyranoside was prepared from its crystalline potassium acetate complex. The salt was removed with Amberlite MB-3 (H^+-OH^-), the resin was separated by filtration, and the K^+-free filtrate (as determined by flame photometry) was concentrated *in vacuo*. The glycoside crystallized spontaneously and was recrystallized from ether-methanol (5:1 at 4°; mp 121°).

Methyl 2-O-Methyl-α- and β-fucopyranosides.—The mixture of methyl 2-*O*-methylfucosides was obtained by the glycosidation procedure described above; refluxing time was 24 hours. These glycosides were streaked on Whatman 3 MM paper and the pyranosidic anomers were separated from each other by running the paper chromatograms twice. Differentiation between pyranosides and furanosides was made

on the basis of periodate oxidation. The methyl 2-*O*-methyl-β-L- and D-fucopyranosides were eluted and crystallized after vacuum distillation. The α anomers were obtained as colorless syrups.

Methyl 3-O-Methyl-α- and β-D-fucopyranosides.—Glycosides of 3-*O*-methyl-D-fucose (digitalose) were prepared, separated, and purified by the same procedure as those of 2-*O*-methylfucose. The refluxing time was 10 hours and the chromatograms were run three times. Since neither the pyranosides nor the furanosides are oxidizable by periodate ions, the pyranosides were distinguished from the furanosides by methylation, optical rotations of the fully methylated glycopyranosides, and subsequent hydrolysis of all the completely methylated glycosides. The furanosides yielded 2,3,5-tri-*O*-methylfucose while from the pyranosides 2,3,4-tri-*O*-methylfucose was obtained. These tri-*O*-methyl ethers were identified by paper chromatography. Both pyranosidic anomers of digitalose were obtained as crystals.

Methyl 2,3-Di-O-methyl-α- and β-fucopyranosides.—Here the refluxing time was 20 hours, and it sufficed to run each chromatogram once for distinct separation of the anomeric pyranosides from one another and from the furanosides. The pure pyranosides were recognized and distinguished from the furanosides by preparation and characterization of their fully methylated derivatives as described for the digitalosides; methyl 2,3-di-*O*-methyl-β-L-fucopyranoside crystallized from diethyl ether at −20°. The isolation of only one of these four sugars in pure form, the methyl 2,3-di-*O*-methyl-α-L-fucopyranoside, has been reported previously (Conchie and Percival, 1950).

Methyl 2,3,4-Tri-O-methyl-α- and β-fucopyranosides.—With the exception of methyl 2,3,4-tri-*O*-methyl-α-D-fucopyranoside, which is a novel compound and whose properties are listed in Table I, these glycosides are known and were synthesized by Schmidt *et al.* (1943) and James and Smith (1945). In the present study all four glycosides were obtained as intermediates while establishing the ring structure of compounds described above. The methylglycopyranosides of the 2,3,4-tri-*O*-methylfucoses not listed in the table had the following properties: for methyl 2,3,4-tri-*O*-methyl-β-D-fucopyranoside, mp 101.0–101.5°, $[\alpha]_D^{27}$ +11.16° (*c*, 0.97, acetone); methyl 2,3,4-tri-*O*-methyl-α-L-fucopyranoside, mp 96.5–98°, and methyl 2,3,4-tri-*O*-methyl-β-L-fucopyranoside, mp 100–101°.

Methyl 2,3,5-Tri-O-methyl-L- and D-fucofuranosides.—The anomers of these hitherto undescribed glycosides were obtained by complete methylation of the methyl-furanosides of various fucose methyl ethers using Purdie's reagents. The corresponding pairs of anomers did not separate distinctly on paper chromatograms with the solvents employed in the present study. Because of the lack of serological activity of the anomeric mixtures no further attempts were made to separate them.

Immunochemistry

Only chromatographically pure haptens were used. Their activity was assessed by established hemagglutination-inhibition and precipitation-inhibition techniques (Springer, 1956; cf. Kabat, 1961; Springer and Williamson, 1962). The compounds whose preparation was described under Organic Syntheses and samples obtained from others were employed in the immunochemical studies.

Anti-H(O) Reagents.—Eel serum and seed extracts from *Lotus tetragonolobus*, *Ulex europeus*, *Laburnum alpinum*, and *Cytisus sessilifolius* were obtained, prepared, and preserved by the procedures previously described (Springer and Williamson, 1962), except that the eel sera were also procured from two additional lots of eels which were bought in the fall of 1962 along the Eastern Seaboard. The final serum pools were decomplemented at 56° for 35 minutes and absorbed with A_1B erythrocytes. These different pools varied somewhat in their affinity for different haptens. An adjustment for these variations was made by correction to standards of L-fucose and methyl-α-L-fucopyranoside wherever possible. A human anti-H(O) serum was also investigated.

Solutions.—The diluent or the erythrocyte-suspending solution in all hemagglutination-inhibition tests was 0.15 M aqueous sodium chloride containing 0.025 M sodium phosphate buffer, *p*H 7.2. In all precipitation-inhibition tests 0.10 M aqueous sodium chloride containing 0.05 M phosphate buffer, *p*H 7.2, was used as solvent of antigen and haptens.

Erythrocytes.—Human group-O erythrocytes from citrated blood obtained from three donors and stored for less than 2 weeks were washed three times by standard procedures and then used as a 0.5% suspension.

Hemagglutination-Inhibition Procedure.—In brief, the procedure employing 0.1 ml throughout was as follows: Twofold serial dilutions of test substances were made, and a different 0.1-ml serological pipet was used for each tube in a titration series. To the titrated solutions 4–8 minimum hemagglutinating doses of anti-H(O) reagent were added, and the samples were shaken and incubated for 2 hours at room temperature (22–26°). Human group-O erythrocytes were then added, and the test mixtures again were shaken and read microscopically after a further 1–2 hours' incubation at room temperature. Each titration series included controls consisting of a serum standard, diluted to 4–8 minimum hemagglutinating doses, and then titrated in 2-fold geometrical dilutions, as well as an erythrocyte suspension in saline. L-Fucose and methyl-α-L-fucopyranoside were included as standards in all assays. All materials were tested at least three times with eel serum and *Lotus* reagents and twice with human anti-H(O) serum and with anti-H(O) agglutinins extracted from *Ulex europeus*, *Laburnum alpinum*, and *Cytisus sessilifolius* seeds.

Under the conditions of the experiments, specificity and reproducibility were found to be wanting at concentrations of more than 5 mg of inhibitor per ml or less than 4 minimum hemagglutinating doses of serum. Hapten preparations that did not inhibit 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. Final concentrations of inhibitor are obtained by dividing the given values by 3.

Table II lists the effectiveness in the hemagglutination-inhibition assays of the active L- and D-fucose methyl ethers and their methylglycosides as measured with eel serum and *Lotus* agglutinins. For comparison L-fucose, its methylglycopyranosides, and previously described blood-group active macromolecules (Springer and Williamson, 1963) are also included. It can be readily seen that a number of methyl ethers were as active or even more so than L-fucose when determined with eel serum. These were found in the L as well as the D series. It is noteworthy that a tri-*O*-methylated D-fucose with a methoxyl group on C-4 showed significant activity. The enantiomorphs of 3-*O*-methylfucose and 2,3-di-*O*-methylfucose were of equal activity. In contrast, with the *Lotus* reagent only the L isomers showed high activity and only those fucose ethers of the D series with a methoxyl group at C-2

TABLE II
BLOOD-GROUP-H(O) ACTIVITY OF FUCOSE METHYL ETHERS AND THEIR METHYLGLYCOSIDES

Test Substance	Minimum Amount (mg/ml) Completely Inhibiting 4 Hemagglutinating Doses	
	Eel Serum Agglutinin	*Lotus* Extract Agglutinin
L series:		
L-Fucose	0.1	0.05–0.1
Methyl-α-L-fucopyranoside	0.02	0.01
p-Aminophenyl-α-L-fucopyranoside	0.02	0.05
Methyl-β-L-fucopyranoside	0.3	0.1
2-*O*-Methyl-L-fucose	0.05	0.05–0.1
Methyl 2-*O*-methyl-α-L-fucopyranoside	0.02	0.05
Methyl 2-*O*-methyl-β-L-fucopyranoside	0.15	0.1
3-*O*-Methyl-L-fucose	0.05–0.1	0.05
2,3-Di-*O*-methyl-L-fucose	0.05	0.05–0.1
Methyl 2,3-di-*O*-methyl-α-L-fucopyranoside	0.02	0.05
Methyl 2,3-di-*O*-methyl-β-L-fucopyranoside	0.3	0.15
2,3,4-Tri-*O*-methyl-L-fucose	±5	>2.5
2,3,5-Tri-*O*-methyl-L-fucose	>5	0.6–1
D series:		
2-*O*-Methyl-D-fucose	2.5	0.75
3-*O*-Methyl-D-fucose	0.05	>5
Methyl 3-*O*-methyl-α-D-fucopyranoside	0.02–0.05	>5
Methyl 3-*O*-methyl-β-D-fucopyranoside	0.02	>5
2,3-Di-*O*-methyl-D-fucose	0.05	1.2–2.5
Methyl 2,3-di-*O*-methyl-α-D-fucopyranoside	0.05	>5
Methyl 2,3-di-*O*-methyl-β-D-fucopyranoside	0.15	>5
2,3,4-Tri-*O*-methyl-D-fucose	1.2–2.5	±5
2,3,5-Tri-*O*-methyl-D-fucose	>5	0.6–1
Blood-group H(O)-active polysaccharides:		
Human ovarian-cyst mucoid	0.002–0.005	0.02–0.05
Taxus cuspidata twig polysaccharide	0.002–0.005	>5
Sassafras albidum twig polysaccharide	0.002–0.005	>5
E. coli O_{128} lipopolysaccharide	0.005–0.01	±5

had any activity; this included one tri-*O*-methyl ether, 2,3,5-tri-*O*-methyl-D-fucose, which was of the same activity as its enantiomorph. Fucoses substituted at C-4 showed no significant activity when tested with the *Lotus* reagent. The following fucose ethers were inactive as determined with either reagent: 2,5-di-*O*-methyl-L-fucose, 3,4-di-*O*-methyl-L-fucose, and 3,5-di-*O*-methyl-L-fucose.

With eel anti-H(O) serum, methyl 2-*O*-methyl-α-L-fucopyranoside, methyl 2,3-di-*O*-methyl-α-L-fucopyranoside, methyl 3-*O*-methyl-α-D-fucopyranoside, methyl 3-*O*-methyl-β-D-fucopyranoside, and methyl 2,3-di-*O*-methyl-α-D-fucopyranoside all were of high and similar activity, which was of the same order as that of methyl-α-L-fucopyranoside. Within the limits of experimental error, none of the glycosides of highly active methyl ethers of fucose was more active than the parent sugar, though at least one of the anomers was always as effective an inhibitor as the parent compound. In the L series the methyl-α-pyranosides were about ten times as active as the β anomers, while such a regularity was not observed in the D series. Here methylglycosidation led to inactivation of the 2-*O*-methyl derivative, while both pyranosidic anomers of 3-*O*-methyl-D-fucose retained full activity. Glycosidation of 2,3-di-*O*-methyl-D-fucose yielded an α-pyranoside of unchanged and a β-pyranoside of decreased activity. In the *Lotus* system high activity was found for L-fucose derivatives only and no methyl ether glycoside was as active as methyl-α-L-fucopyranoside. The *Lotus* reagent did not differentiate clearly between the parent methyl ether and its anomeric glycopyrano sides in the L series; they all showed similar activity. Methylglycosidation led to inactivation of those methyl ethers of the D series which showed some activity.

In no instance did methylglycosidation activate an inactive fucose ether. Methylglycosidation of the active tri-*O*-methyl ethers resulted in inactive compounds. All furanosides tested were inactive in both systems. None of the anti-H(O) reagents, other than those from the eel and *Lotus*, were inhibited by any of the haptens tested. Among the macromolecules, besides the human blood-group mucoid which inhibited all agglutinins, only the *E. coli* O_{128} lipopolysaccharide had some activity against *Cytisus sessilifolius* agglutinin as well as against eel anti-H(O).

Precipitation-Inhibition Tests.—The ability of the fucose methyl ethers and their methylglycosides to inhibit precipitation of human blood-group H(O) ovarian-cyst mucoid by eel serum (Springer *et al.*, 1961) was determined by means of the quantitative hapten-inhibition technique (cf. Kabat, 1961). All inhibitions were carried out in the equivalence zone towards the region of antibody excess. Known amounts of sugar were added in 0.5-ml volumes to 0.5 ml of either of two serum pools of high titer (1:128–1:256) with closely similar immunochemical properties and incubated in an ice bath for 30 minutes. After the addition of 150 μg of human ovarian-cyst H(O) substance (Springer *et al.*, 1954)[3] in 0.5 ml of buffered saline, the samples were

[3] The H(O) ovarian-cyst substance described previously (Springer *et al.*, 1954) had been further purified by ethanol and sodium sulfate fractionations. The most highly active fraction was used in the precipitation-inhibition tests. Its hemagglutination-inhibiting activity is listed in Table II. A physicochemical analysis of this material by Dr. G. Bernardi in a Spinco Model E analytical centrifuge showed only one peak of $s_{20,w}$ = 7.6 S (*c*, 0.62%, 0.13 M NaCl + 0.01 M PO_4^{-3}, *p*H 6.8), the boundary was asymmetric. Analytical data were: C, 47.7%; H, 6.7%; N, 5.12%; CCH_3, 6.6%; methylpentose, 20.4%; hexosamine, 28.8%; sialic acid, <1%.

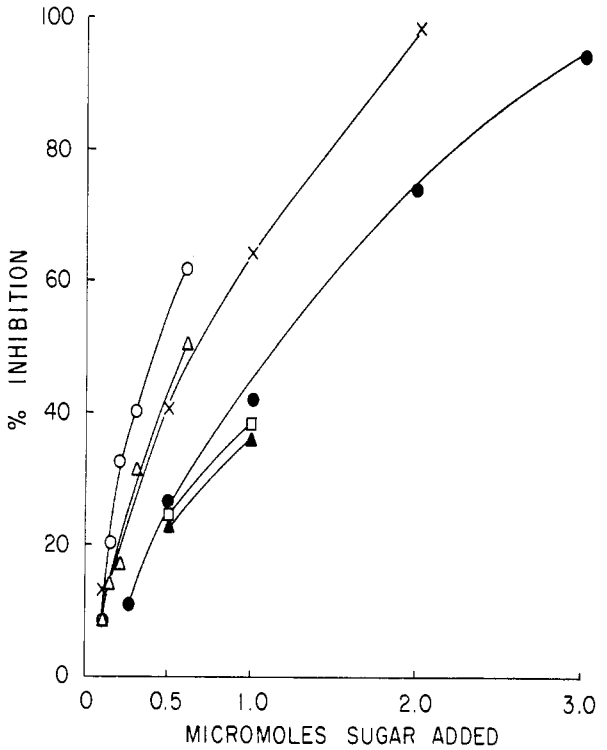

FIG. 1.—Inhibition of precipitation by mono-*O*-methyl ethers of fucose and deoxyfucoses. ●, L-fucose; ×, 2-*O*-methyl-L-fucose; △, 3-*O*-methyl-L-fucose; ○, 3-*O*-methyl-D-fucose; □, 2-deoxy-L-fucose; ▲, 2-deoxy-3-*O*-methyl-D-fucose. (Antigen, 150 μg Ca IIb 2 + 3; antiserum, 0.5 ml eel H′.)

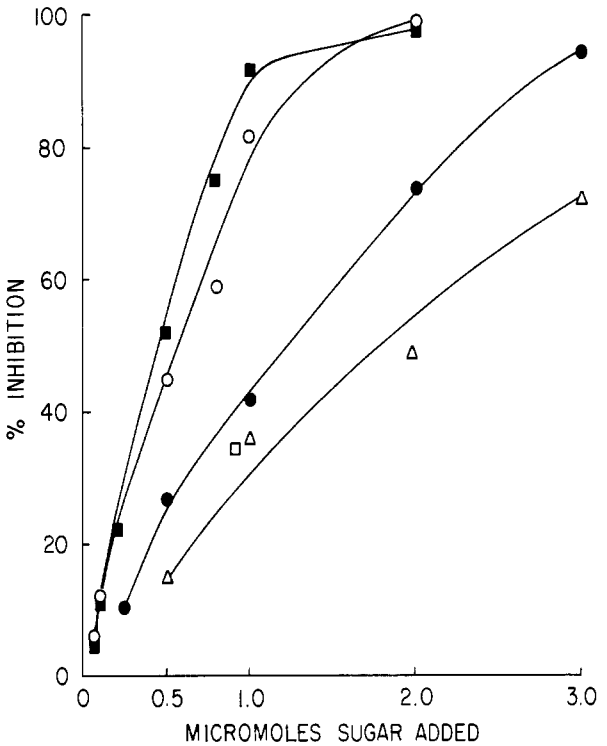

FIG. 2.—Inhibition of precipitation by di-*O*-methyl ethers of fucose and fucosamines. ●, L-fucose; ■, 2,3-di-*O*-methyl-L-fucose; ○, 2,3-di-*O*-methyl-D-fucose; □, 2-amino-2-deoxy-L-fucose; △, 2-acetamido-2-deoxy-L-fucose. (Antigen, 150 μg Ca IIb 2+3; antiserum, 0.5 ml eel H′.)

incubated for another hour in the ice bath and then for 7–9 days at 4° with daily agitation. Precipitates were washed and protein was determined with the Folin-Ciocalteu reagent as described by Heidelberger and MacPherson (1943a,b) since it was found that eel-antibody protein determined by this method paralleled that measured with the Kjeldahl procedure (G. F. Springer and B. L. Readler, unpublished data). Eel serum alone and antigen alone, as well as those reagents incubated together, served as negative and positive controls, respectively. The percentage of inhibition was computed from the difference of N precipitated in the presence and absence of sugars. Duplicate samples were measured in each test and the experiment was repeated at least once.

The pertinent results of the quantitative precipitin-inhibition tests in the human blood-group H(O) substance–eel serum anti-H(O) system are shown in Figures 1 through 4. Fucose, the highly active mono-*O*-methyl ethers of both enantiomorphs of fucose, and two deoxyfucoses are listed in Figure 1. The depicted fucose mono-*O*-methyl ethers all gave over 40% inhibition at levels of 0.6 μM of added sugar and exceeded 60% inhibition at the 1 μM level. They were approximately one and one-half to two times as active as L-fucose, the sugar considered to be responsible for H(O) activity of human blood-group mucoids. The precipitin-inhibition curves for the two 3-*O*-methyl fucoses are carried only to the 0.6 μM level since, unexpectedly, with some of the eel sera but not with others, at higher concentrations the haptenic sugar itself began to precipitate anti-H(O) antibodies. This effect, if it occurred, was much more pronounced for 3-*O*-methyl-D-fucose than for its enantiomorph (G. F. Springer and B. Kolecki, to be published). 2-*O*-Methyl-L-fucose was of closely similar activity and much more potent than 2-*O*-methyl-D-fucose, which inhibited only slightly (*ca.* 10% at 4 μM). While 2-*O*-methyl-L-fucose was considerably more active than L-fucose, 2-deoxy-L-fucose was less active than L-fucose as was 2-deoxy-3-*O*-methyl-D-fucose (diginose) and 2-acetamido-2-deoxy-L-fucose (Figs. 1 and 2). In contrast to 3-*O*-methyl-D-fucose, the 2-deoxy-3-*O*-methyl-D-fucose did not precipitate with any of the eel sera investigated. The enantiomorphous di-*O*-methyl-fucoses listed in Figure 2 were of high and closely similar activity. They were over twice as active as L-fucose at concentrations of 0.25 μM to 1.25 μM and exceeded 90% at 1.5 μM. 2-Amino-2-deoxy-L-fucose was tested in duplicate determinations at only one level (owing to lack of sugar) 0.91 μM, where it inhibited slightly less than L-fucose. Slight, consistent activity amounting to less than 20% in the quantitative assay even at a concentration of 4 μM was exhibited by a number of reducing sugars tested, in addition to 2-*O*-methyl-D-fucose, namely, 2,3,4-tri-*O*-methyl-D-fucose, 2-acetamido-2-deoxy-D-arabinose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-ribose, 2-acetamido-2-deoxy-D-talose, D-fructose, 6-deoxy-L-talose and sedoheptulose. D-Arabinose inhibited somewhat more at the 4 μM level. 3-Deoxy-L-fucose (colitose) and 3-deoxy-D-fucose (abequose) were both of faint activity which did not exceed the limit of error of the assay. Inactive up to 4 μM were D-fucose, 2-keto-L-fucose (angustose), L-galactose, D-galactose, L-arabinose, 2-acetamido-2-deoxy-D-glucose, and 2-deoxy-D-ribose. A number of fucose ethers which had no activity in the hemagglutination-inhibition assay were not tested.

Of the seven glycopyranosides of fucose and fucose mono-*O*-methyl ethers shown in Figure 3, five were over two to three times as active as L-fucose, and two of these were members of the D series. The two most highly and almost equally active glycopyranosides on a molecular-weight basis were methyl 3-*O*-methyl-β-D-

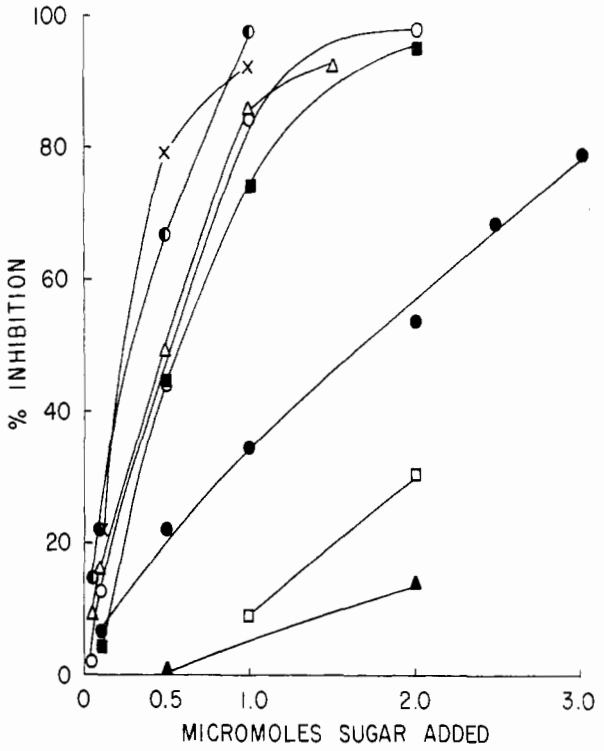

FIG. 3.—Inhibition of precipitation by glycopyranosides of fucose and fucose mono-*O*-methyl ethers. ●, L-fucose; ○, methyl-α-L-fucopyranoside; ×, *p*-aminophenyl-α-L-fucopyranoside; ▲, methyl-β-L-fucopyranoside; ■, methyl 2-*O*-methyl-α-L-fucopyranoside; □, methyl 2-*O*-methyl-β-L-fucopyranoside; △, methyl 3-*O*-methyl-α-D-fucopyranoside; ◐, methyl 3-*O*-methyl-β-D-fucopyranoside. (Antigen, 150 μg Ca IIb 2+3; antiserum, 0.5 ml eel [*HLJ*].)

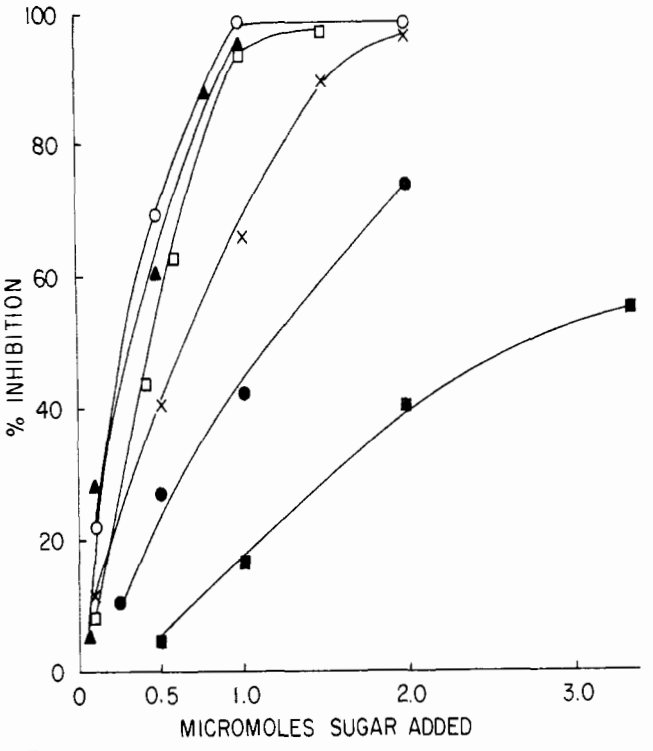

FIG. 4.—Inhibition of precipitation by methylglycopyranosides of di-*O*-methylfucoses. ●, L-fucose; ▲, methyl-α-L-fucopyranoside; ○, methyl 2,3-di-*O*-methyl-α-L-fucopyranoside; ■, methyl 2,3-di-*O*-methyl-β-L-fucopyranoside; □, methyl 2,3-di-*O*-methyl-α-D-fucopyranoside; ×, methyl 2,3-di-*O*-methyl-β-D-fucopyranoside. (Antigen, 150 μg Ca IIb 2+3; antiserum, 0.5 ml eel H′.)

fucopyranoside and *p*-aminophenyl-α-L-fucopyranoside, while on a weight basis the former glycoside was clearly more active. Of nearly identical activity were methyl 3-*O*-methyl-α-D-fucopyranoside and methyl-α-L-fucopyranoside. The two glycopyranosides which had significant but lower activity than L-fucose were both β anomers and belonged to the L series of fucose and fucose ethers. The α and β anomers of methyl-D-fucopyranoside and methyl 2-*O*-methyl-D-fucopyranoside were inactive at levels up to 4 μM. Figure 4 reproduces the inhibition curves obtained with methylglycopyranosides of di-*O*-methylated fucoses. The most highly active glycopyranoside was methyl 2,3-di-*O*-methyl-α-L-fucopyranoside which was closely similar in activity to methyl 3-*O*-methyl-β-D-fucopyranoside, the most active methylglycoside of the fucose mono-*O*-methyl ethers. Both gave over 95% inhibition at 1.0 μM concentration and exceeded the activity of methyl-α-L-fucopyranoside at all levels. Also highly active was methyl 2,3-di-*O*-methyl-α-D-fucopyranoside, while both β anomers of 2,3-di-*O*-methylfucopyranosides were of lower activity. The β-L-enantiomorph was only about one-third as active as the β-D enantiomorph.

Methylglycosides of the tri-*O*-methylfucoses had no activity in the hemagglutination-inhibition tests and were not investigated.

DISCUSSION

The chemical syntheses described in this paper have produced, among others, nine new, adequately characterized methylglycopyranosides of fucose methyl ethers and one new fucose methyl ether. In addition to these new compounds, three previously synthesized carbohydrates, methyl 3-*O*-methyl-α-D-fucopyranoside (Tamm, 1949), and the enantiomorphous 2,3-di-*O*-methylfucoses (Schmidt and Wernicke, 1944; Gardiner and Percival, 1958) have been obtained in crystalline form for the first time.

The serological activities observed for some fucose methyl ethers and their glycosides were high when compared to simple haptens in other blood-group systems. On a weight basis, the activities relative to H(O) substance of a given fucose derivative showed apparent differences, depending on whether they were measured in the precipitin or hemagglutination test. Thus, in the hemagglutination-inhibition tests, the activity of the most potent fucose methyl ethers did not amount to more than 10% of that of blood-group-specific macromolecules of human or higher plant origin, while in the precipitin test approximately equal activity was found for monosaccharides and macromolecules (*vide infra*). On a molar basis this difference appeared to be more pronounced in the hemagglutination assay: approximately 10^{-1} μM of the most active monosaccharides and their glycosides gave complete hemagglutination inhibition while about 10^{-5} μM of human blood-group H(O) substance (based on a molecular weight of 300,000) were needed for complete hemagglutination inhibition. Therefore the number of monosaccharide molecules required to neutralize completely the agglutinins is about 10,000 times as great as the number of molecules of H(O) substance. This calculation, however, does not take into account that one molecule of the human H(O) substance used in this study contains about 20% fucose, i.e., approximately 350 fucose units per H(O)-substance molecule. If all the fucose radicals on the macromolecules were free to react, then the concentration of highly active fucose and fucose ether glycosides needed to neutralize H(O)

eel agglutinins would amount to only about thirty times that of the fucose residues of the macromolecule. In the hemagglutination-inhibition test the proportion arrived at by comparison on a molecular basis is of the same order of magnitude, therefore, as is that obtained by comparing free sugars and macromolecules on a weight basis. The precipitin test as used in this study is less sensitive, by one decadic logarithm, than the hemagglutination-inhibition assay. However, if a comparison is made between the weight of a sugar such as methyl 3-*O*-methyl-β-D-fucopyranoside which gives >95% inhibition at 1 μM concentration and the weight of blood-group H(O) substance giving maximal precipitation, it is found that both compounds are of approximately equal activity; whereas a comparison between free sugar and H(O) substance on a molar basis gives a ratio similar to that found in the hemagglutination-inhibition test. Corresponding calculations for the blood-group active polysaccharide from *Sassafras albidum* and its haptenic monosaccharide gave similar results. One of the factors limiting the value of these estimates is that undoubtedly, not all fucose molecules on the macromolecule are free to react (cf. Morgan and Watkins, 1953). The determining groups of the intact H(O) active substances are therefore more closely complementary to the eel serum antibody than even the most active monosaccharide methyl ethers and their glycosides.

The present studies shed no light on the important problem as to whether or not the glycosides investigated here are equivalent to the entire structure which is complementary to the eel serum antibody. The lower activity of the haptenic structures as compared to intact H(O) active macromolecules is not necessarily due to their incompleteness; it is conceivable that in order to obtain full activity the appropriate arrangement of the active structures in space is attained only if they are fixed to the macromolecule, as has been suggested for the human blood-group A substance isolated from erythrocytes (Kościelak, 1963).

Since one of the two anomeric methylglycopyranosides of L-fucose is 5–10 times as active in the hemagglutination-inhibition test as is the parent sugar, it was reasonable to expect that methylglycosidation of the active fucose methyl ethers may result in similar activation. As is evident from Table II and Figures 1 through 4, this was not the case. None of the methylglycosides of any fucose ether was more than twice as active as the parent compound in the hemagglutination-inhibition test (an insignificant difference with this method). In the quantitative precipitin-inhibition test the increase in activity of the most active glycosidic anomer did not exceed 50% of the activity of the parent compound. This figure was reached with only some methylglycopyranosides of fucose mono-*O*-methyl ethers. The reducing 2,3-di-*O*-methylfucoses were of higher activity and the corresponding increase in inhibiting activity of the most potent methylglycoside was less than that observed for the fucose mono-*O*-methyl ethers. It appears that two *O*-methyl groups in appropriate position may convey close to maximal activity to the fucose molecule. Accordingly, no tested fucose derivative was more active than methyl 3-*O*-methyl-β-D-fucopyranoside. The most highly active fucose-derived compounds were of similar activities and had closely resembling inhibition curves. It is noteworthy that methyl-α-L-fucopyranoside, a sugar with no *O*-methyl ether group, was among the most active compounds. There were members of the L as well as the D series among the most active compounds, which included α and β anomers of the D series but only α anomers of the L series. All active methyl-β-glycopyranosides of the L series were less active than L fucose, although their activity increased in parallel with that of the parent compound, i.e., it was dependent on the extent of its *O*-methylation.

The presence of a methyl group is a necessary but not an adequate prerequisite to confer activity on a sugar in the eel anti-H(O)–O erythrocyte system. Only compounds with at least one methyl group have been found to be active. While originally the activity was thought to be confined to α-L-fucopyranosyl structures, it became apparent during our studies (cf. Springer and Williamson, 1962) that D-fucose derivatives also could show high activity. It was recently recognized in this laboratory that the C-methyl substituent on C-5 as in the fucose molecule is not a necessary radical for the activity of a sugar molecule as long as the latter contains a methyl group in another appropriate position. Thus 3-*O*-methyl-D-galactose was recognized as a highly H(O)-active hapten (Springer, 1964; Springer *et al.*, 1964) and the sugar responsible for at least the major part of the H(O) specificity of the polysaccharide from *Sassafras albidum* (Springer, 1958). C-methyl and *O*-methyl groups appear therefore to be interchangeable to a degree. To increase activity of the L-fucose molecule *O*-methylation is an effective step provided the methyl groups are in a suitable position. Even the inactive D-fucose molecule may become more active than L-fucose by proper *O*-methyl substitution; *O*-methylation at C-2 and C-3 in general led to a molecule more active than L-fucose, the sole exception was 2-*O*-methyl-D-fucose, a monosaccharide with little activity. Methylation, however, also can lead to inactivation of fucose molecules, thus OCH_3 groups at C-4 and C-5 were not in general compatible with H(O) activity. None of the di-*O*-methyl ethers of fucose tested which had one *O*-methyl either on C-4 or C-5 showed any activity. It is remarkable, therefore, that contrary to these findings and earlier suppositions (Watkins and Morgan, 1952; Springer and Williamson, 1962) a fucose methylated at C-4, namely, 2,3,4-tri-*O*-methyl-D-fucose, was of significant activity and that the enantiomorph also was slightly active. Furthermore, 2,3,4-tri-*O*-methyl-D-galactose and 2,3,4-tri-*O*-methyl-L-arabinose also showed considerable potency in neutralizing the action of eel anti-H(O) serum on group O erythrocytes. The tests so far indicate that if one substituent is at C-4, three *O*-methyl groups are needed to convey activity to either galactose or fucose; the other two must be at C-2 and C-3.

While the anomeric glycopyranosides of L-fucose and L-fucose ethers showed relations of activity customary for such compounds in biological systems, in that one glycoside was much more and the other considerably less active than the parent compound, such a relation could not be observed in the D series. Rather, the glycopyranosides of highly active fucose ethers were both highly active while both anomeric pyranosides of the weakly active 2-*O*-methyl-D-fucose were inactive. Activation of L-fucose by α-glycosidation was achieved not only with the comparatively small methyl group but to an even larger extent by the *p*-aminophenyl radical. From this observation it may not be concluded that the nature of the substituent at C-1 is unimportant; its great influence was shown by Kuhn and Osman (1956) who found oligosaccharides with terminal α-fucopyranosyl structures to be inactive. In this laboratory (Springer, 1964), 3-*O*-methyl-D-galactose was found to be highly active but 3-*O*-α-D-galactopyranosyl-D-galactose was inactive; in the *Lotus* system, in contrast, only the latter compound gave some inhibition.

It is difficult to define the responsible structure com-

mon to all the sugars possessing the capacity to combine with the anti-H(O) antibody in eel serum. The present discussion is restricted to fucoses and fucose derivatives. As has been pointed out by Kabat (1962) substitution of a methoxyl group on C-2 and C-3 results in D and L compounds which with conventional scale models possess a closely similar profile and a second area (in addition to that at C-6) of low hydrogen bonding at about 180° from C-6. The observed activities were therefore explained by the similarities of profile of closely packed models of some L- and D-fucose methyl ethers. Findings reported here on fucose derivatives generally can be reconciled with this interpretation. It is to be noted that both anomeric methylglycopyranosides of the highly active methyl ethers of D-fucose were potent inhibitors of eel anti-H(O) antibodies. The higher activity of the β anomer of methyl 3-*O*-methyl-D-fucopyranoside as compared to that of the parent compound and the α anomer corresponds to the observations with the L-fucose glycopyranosides, even though the difference between parent compound and α anomer was minimal. The higher activity of methyl 2,3-di-*O*-methyl-α-D-fucopyranoside as compared to the β anomer, however, was unexpected and does not agree with presently accepted theories since the absolute configuration of α-L and β-D at C-1 is the same (cf. Pigman, 1957). The activities of the parent compound and the two anomeric methylglycopyranosides of 2,3-di-*O*-methyl-D-fucose are almost alike. The inhibition curve of the parent sugar lies precisely between that of the α- and β-glycoside. The differences between the three curves are in the order of 10% and therefore may not be significant.

Clearly, methylglycosidation of this di-*O*-methyl compound has little influence on its activity, indicating that over 90% of the binding energy is accounted for by those parts of the sugar which do not carry the glycoside function. The anomeric form of the sugar is thus of only minor importance in the determination of specificity in these examples. These results differ from those for anomers of the L series reported in this study and for glycosides of different sugars (Goebel *et al.*, 1934). The findings point to possible limitations of the methods of serological inhibition in the determination of the nature of serologically determinant carbohydrate structures in that a number of chemically different groups may exhibit the same specific serological activity. On the other hand, great differences in serological activity of H(O)-specific haptens were found for L-fucopyranosyl-α-(1 → 2)-D-galactose and L-fucopyranosyl-α-(1 → 2)-D-galactopyranosyl-β-(1 → 4)-D-glucose. The latter compound had only one-thirtieth the activity of the former (Watkins and Morgan, 1962); it does not appear feasible to explain this difference by steric hindrance, owing to the glucose moiety.

The present studies indicate that the whole sugar molecule probably does not combine firmly with antibody and it is thus not necessary for activity (see also Springer and Williamson, 1962). It was noted by these authors that the ring oxygen next to the equatorial C-methyl group of L-fucose has a prominent position and may thus be involved in antibody-hapten interactions. The ether oxygen of 3-*O*-methyl-D-fucose fitted into the imprint made, into plaster casts, by the ring oxygen of L-fucose, thus making complementary the O-methyl group of digitalose to the C-methyl group of L-fucose. All substances active in the eel serum—O-erythrocyte system have in common a pyranose structure with an equatorial CH_3 substituent and a neighboring ether oxygen group. An axial substituent, in *cis* position capable of hydrogen bonding, is on a contiguous carbon atom. The presence of an additional structure such as this or of substantial parts of it may lead to an increase in activity as in the case of 2-*O*-methyl-L-fucose, which is considerably more active than L-fucose (Fig. 2). It may also be assumed that the α form of 2-*O*-methyl-L-fucose reacts with the eel serum antibody since the configuration at C-1 and C-2 would then fulfill the requirements delineated above. Such an interpretation would explain the higher activity of the α-glycopyranosides of L-fucose and its methyl ethers as well as that of methyl 2,3-di-*O*-methyl-α-D-fucopyranoside as compared to their anomers. These attempts at interpreting the requirements for activity of sugar molecules in the eel serum system are far from perfect and complicated not only by the heterologous nature of the serum but also because the six-membered rings of sugars are not rigid structures in any conformation (cf. Eliel, 1956); thus the haptenic sugar may mold itself to the shape of the antibody-combining site and vice versa.

It should be remembered that the observed similarity in contour of certain enantiomorphs of fucose methyl ethers, as determined with closely packed atomic scale models (e.g., Fisher-Hirschfelder-Taylor), disregards the following: the internuclear C–C distance as compared to the C–O–C distance, the free rotation, and the angle of methoxyl and C-methyl groups to one another and in relation to the pyranose ring are entirely different. This becomes quite evident when inspection and measurements on Dreiding-stereo models (Swissco Instruments; Glenville, Ill.) are carried out.

An unexplained and surprising phenomenon is the precipitation of eel anti-H(O) antibody of some eel serum pools by 3-*O*-methyl fucoses. Preliminary experiments showed that the precipitate consisted of the anti-H(O)-specific protein in eel serum and that its amount was proportional to the concentration of hapten (G. F. Springer and B. Kolecki, to be published). The precipitation was much more effectively induced by the D enantiomorph but it was not demonstrable under the experimental conditions used at sugar concentrations below 0.6 μM, where it functioned as an inhibitor. Digitalose appears to be the smallest hapten yet reported that is able to precipitate antibody. To the authors knowledge these are the only uncharged units yet described. The smallest haptens previously found to precipitate antisera all had a high negative charge and were somewhat larger than the haptens of the present study (Landsteiner and Van der Scheer, 1932; Heidelberger and Kendall, 1933). It is tempting to speculate that precipitation may be owing to the ability of the hapten to participate in lattice formation via its two methyl groups; but it is difficult to reconcile such an interpretation not only with the failure of the highly active methylglycopyranosides of digitalose to precipitate any eel anti-H(O) antibody, but in addition, with the failure of the very closely related 2-deoxy-3-*O*-methyl-D-fucose to precipitate at any of the concentrations tested.

One of the more remarkable findings of this study was the activity of some C-4 substituted fucoses which was demonstrable in the eel serum system but not with *Lotus* agglutinins. For moderate activity in the *Lotus* system, on the other hand, not even the pyranose ring was necessary, as is shown by the significant and equal activity of the enantiomorphous 2,3,5-tri-*O*-methyl-fucoses. Presumably these highly methylated compounds are present in the furanose ring form, which may exist in coplanar rings resembling the Haworth formula. It is to be noted that the activities of these compounds are lower by one decadic logarithm than the highest observed for L-fucopyranose derivatives, but no compound in the D series was more active than 2,3,5-

tri-*O*-methylfucose. None of the glycosides of D-fucose was active when tested with *Lotus* agglutinin.

The lack of parallelism between hemagglutination inhibition and precipitation inhibition is evident when one compares the activities of methyl-α-L-fucopyranoside in both tests relative to L-fucose. Another example is methyl-β-L-fucopyranoside, which was proportionally more active in the hemagglutination-inhibition test. The activity differences between the anomeric methylglycopyranosides of 2-*O*-methyl-L-fucose and 2,3-di-*O*-methyl-L-fucose were considerably larger in the hemagglutination assay than in the precipitin test. Also, up to 2-fold difference in activity could be pinpointed with only the precipitin test, as the hemagglutination-inhibition test is too inaccurate. The different results obtained with hemagglutination and precipitin tests were uniformly qualitative rather than quantitative.

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