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Biochem. J. (1962) **85**, 282

Immunochemical Significance of L- and D-Fucose Derivatives

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The elucidation of the chemical structures responsible for the serological independence of the various blood-group characters and the specific combining sites of their complementary antibodies constitutes a fundamental challenge of immunochemistry. Knowledge of the structures determining specificity may yield information on the forces decisive in antigen-antibody interactions, as well as on genetical mechanisms controlling blood-group characters.

It was first observed by Watkins & Morgan (1952) and by Morgan & 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 shown to possess considerable specificity in that L-fucose was the only active sugar of the four constituent monosaccharides of blood-group mucoids. Of numerous other sugars only 2-deoxy-L-fucose, 6-deoxy-L-talose, D-arabinose and D-fructose showed some, though less, activity; D-fucose was inactive. Watkins & Morgan noted that methyl α -L-fucopyranoside was 4-5 times as active as L-fucose; the β -glycoside was less active than the parent compound. Kuhn & Osman (1956) confirmed and extended the earlier findings of Watkins & Morgan and postulated the following necessary conditions

for the serological activity of fucosides: (1) The fucose residue must belong to the L-series; (2) it must be in the pyranose form; (3) if linked, it must be present in α -glycosidic linkage.

Springer, Ansell & Ruelius (1956) reported that the polysaccharide with high blood-group H(O) activity isolated from *Taxus cuspidata* contained no L-fucose, but instead serologically active 2-O-methyl-L-fucose, not then known to occur in Nature. It is now recognized that this sugar is widely distributed in plants (Andrews & Hough, 1958): it has also been found in mycobacteria (MacLennan, Randall & Smith, 1961). It was also found that di-O-methylation of L-fucose at C-2 and C-3 was compatible with high serological activity; introduction of a third methyl group at C-4 led to complete inactivation of the compound (Springer, Williamson & Ruelius, 1959).

In view of the apparent influence of methyl groups on the serological activity of the fucose molecule, we decided to investigate the serological properties of various fucose ethers, glycosides and other derivatives. The effect of substituents in the various positions of the fucose molecule might yield information on the nature of the forces acting between haptens and antibodies or antibody-like substances, not only for the blood-group H(O) system but for other antigen-antibody systems as well.

During the extensive study on L-fucose derivatives reported here, the surprising observation was

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made that, although D-fucose and its glycosides, in agreement with earlier results mentioned above, are quite inactive in the eel-serum system, certain methyl ethers of D-fucose are as active as their L-enantiomorphs. The eel and *Lotus* reagents have been successfully used in the prediction of the structure of D-fucose methyl ethers in the antibiotic chartreusin and in the cardiac glycoside streblosid (Springer, Williamson & Ruelius, 1960; Springer, 1959).

The present paper describes serological properties of derivatives of both L- and D-fucose. The results obtained with some D-fucose ethers disagree with classical concepts of the stereospecificity of antigen-antibody reactions (see also Addendum).

MATERIALS AND METHODS

Sugars obtained from others. Many sugars and derivatives were gifts and the sources are acknowledged in the Tables. D- and L-Fucose were purchased from Th. Schuchardt Co., Munich, Germany, and from Mann Laboratories Inc., New York, U.S.A., respectively. Numerous sugars not closely related to fucose were also of commercial origin.

Sugars synthesized in this Laboratory. The same steps were employed for the preparation of comparable derivatives of the enantiomorphous isomers of fucose. All syntheses were carried out more than once. The identity of known reducing sugars synthesized was determined by comparative paper chromatography with authentic samples in the following three solvent systems: butan-1-ol-ethanol-water (5:1:4, by vol.) (Partridge & Westall, 1946), butan-1-ol-pyridine-water (6:4:3, by vol.) (R. L. Whistler & E. Feinler, personal communication) and ethyl acetate-pyridine-water (8:2:1, by vol.) (Jermyn & Isherwood, 1949), and subsequent staining with aniline oxalate (Horrocks, 1949). Glycosides were separated in butan-2-one-water (Boggs, Cuendet, Ehrenthal, Koch & Smith, 1950) and stained with periodate-potassium permanganate-benzidine reagent (Wolfrom & Miller, 1956). Digitalose glycosides, which are not oxidizable by periodate ion, were stained with iodine vapour (Brante, 1949). Preparative separation was achieved on acid-washed sheets of Whatman 3MM or Schleicher and Schuell 589 Green R paper (22½ in. × 18½ in.).

Melting points were determined on a Fisher-Johns melting-point apparatus and are not corrected. Elementary analyses were performed by M. Manser, Herrliberg-Zurich, Switzerland, and by the Micro-analytical Division of Ciba Pharmaceutical Inc., Summit, N.J., U.S.A. No material was used unless we had shown it to be chromatographically pure, except D-galactose, methyl β-D-fucopyranoside and D-psicose. L-Fuculose, regenerated (Reichstein, 1934) from its dinitrophenylhydrazine given by Prof. S. S. Cohen, contained 5–10% of L-fucose, as did a L-fuculose sample given by Prof. Reichstein. Pure L-fuculose was obtained from these materials by elution from developed paper chromatograms. D-Psicose, 2,3-di-O-methyl-D-glucose and 6-deoxy-D-glucose were prepared from derivatives according to the advice of Prof. Reichstein.

Methyl α- and β-L- and -D-fucopyranosides. These were prepared by refluxing 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, Hobbs & Walston, 1951) with continuous stirring for 24 hr. After filtration, the solution was concentrated until spontaneous crystallization of methyl α-fucopyranoside occurred (cf. MacPhillamy & Elderfield, 1939). Recrystallization from ethyl acetate resulted in pure methyl α-fucopyranoside, m.p. 154–157°, $[\alpha]_D^{25} + 189^\circ$ and –188.6° for the D- and L-isomers respectively (c 1 in water, 1 dm.) (Found for methyl α-L-fucoside: C, 47.0; H, 7.8; OMe, 17.2. Found for methyl α-D-fucoside: C, 47.2; H, 7.7; OMe, 17.4. C₇H₁₄O₅ requires C, 47.2; H, 7.9; OMe, 17.4%). Methyl β-D-fucopyranoside was obtained in a syrupy mixture with the furanosides after removal of most of the α-anomer. No efforts were made to purify this serologically inactive syrup. Methyl β-L-fucopyranoside was not obtained in this way, since the crystalline potassium acetate complex was available (Table 1); this had m.p. 209–211° and contained 30% of potassium acetate, calc. from K content (flame photometer).

2-O-Methyl-L- and -D-fucose. These were prepared from the corresponding methyl α-fucopyranosides by the following steps: methyl 3,4-O-isopropylidene-α-fucopyranoside was obtained by the procedure of Schmidt & Wernicke (1946). The product distilled at 72–79°/0.02–0.04 mm. Hg. Methyl 3,4-O-isopropylidene-2-O-methyl-α-fucopyranoside was prepared from this material by repeated methylation with Purdie's reagents (Purdie & Irvine, 1903); freshly prepared silver oxide (Helfferich & Klein, 1926) was used. The completely methylated product was hydrolysed at a concentration of 3.5–4% with 0.5N-sulphuric acid at 100° for 6–7 hr. After neutralization with barium hydroxide, the aqueous solution was dried *in vacuo* and the resulting crystalline mass was recrystallized from absolute ethanol. 2-O-Methyl-L-fucose melted at 153.5–155°; $[\alpha]_D^{25} - 91^\circ$ (c 1 in water, 1 dm.); 2-O-methyl-D-fucose melted at 155–157°; $[\alpha]_D^{25} + 88^\circ$ (c 1 in water, 1 dm.) (Found for 2-O-methyl-L-fucose: C, 47.2; H, 8.0; OMe, 17.7. Found for 2-O-methyl-D-fucose: C, 47.6; H, 8.2; OMe, 17.3. C₇H₁₄O₅ requires C, 47.2; H, 7.9; OMe, 17.4%).

Methyl 2-O-methyl-α- and -β-fucopyranosides. These were prepared in the same way as were the glycosides of fucose described above. However, only a syrupy mixture resulted; separation and isolation of the two pyranosides could be effected by chromatography in butan-2-one-water. Differentiation between furanosides and pyranosides was made on the basis of periodate oxidation, *R_F* values and serological activity. Optical rotations of the α-pyranosides were $[\alpha]_D^{25} - 179.5 \pm 5^\circ$ (c 1.17 in water, 1 dm.) and $[\alpha]_D^{25} + 173.1 \pm 5^\circ$ (c 1.17 in water, 1 dm.) for the L- and D-enantiomorph respectively. The β-pyranosides had specific rotations of $[\alpha]_D^{25} + 10 \pm 5^\circ$ (c 0.24 in methanol, 1 dm.) and $[\alpha]_D^{25} + 5 \pm 3^\circ$ (c 0.38 in methanol, 1 dm.) for the L- and D-enantiomorph respectively.

2,3-Di-O-methyl-L- and -D-fucose. The L- and D-fucose dibenzyl mercaptals were synthesized by the method described for the L-isomer by Schmidt, Mayer & Distelmeier (1943). The recrystallized products melted at 186–190° and yielded 4,5-O isopropylidene-D- and L-fucose dibenzyl mercaptals (m.p. 103–105°) by the procedure of Schmidt & Wernicke (1944); methylation of these compounds gave their 2,3-di-O-methyl derivatives as yellow oils in low yields. Conversion of these substances into free 2,3-di-O-methylfucoses was accomplished according to the

above authors except that anions were removed by Amberlite IR-4B (OH⁻) resin. The solutions were concentrated *in vacuo* and partially decolorized with Darco G-60. The dried syrups were distilled at 120°/0.01 mm. Hg to yield pale-yellow oils; these were shown by chromatographic analysis to contain about one-third of methylated sugars other than 2,3-di-*O*-methylfucose, which was identified by reference compounds. Pure 2,3-di-*O*-methyl-L- and -D-fucoses, therefore, were obtained by preparative chromatography.

Isolation of 3-*O*-methyl-D-fucose. This sugar was obtained from the naturally occurring pure cardiac glycosides, strospesid (Rittel, Hunger & Reichstein, 1952) and panstrosid (Rosselet, Hunger & Reichstein, 1951), and an antibiotic, chartreusin (Sternbach, Kaiser & Goldberg, 1958). Purified digitalinum verum and commercial digitalin (Nutritional Biochemicals Corp.) were also used. All glycosides were hydrolysed and rehydrolysed separately by refluxing in 10% concentration with Kiliani's (1930) mixture. After extraction with water, all solutes were combined and concentrated by distillation *in vacuo*, which also removed most of the volatile acids. Neutralization was achieved by treatment of the solution with Amberlite IR-45 (OH⁻) ion-exchange resin, followed by a small amount of IR-120 (H⁺). Panstrosid and strospesid yielded essentially pure digitalose. Chartreusin and digitalin hydrolysates (the latter after 15–20 hr. incubation at 37° with Fleischmann's yeast to remove most of the glucose) were chromatographed on a cellulose column (Hough, Jones & Wadman, 1949), 4 cm. × 140 cm., with butan-1-ol-ethanol-water as the mobile phase. Fractions containing predominantly digitalose were combined, and chromatographically pure digitalose was isolated from paper sheets developed with butan-1-ol-ethanol-water. The resulting yellow syrup was further purified by boiling an aqueous solution with acid-washed Darco G-60 charcoal and treatment with Amberlite MB-3 Monobed resin; $[\alpha]_D^{25} + 105^\circ$ (*c* 2.58 in water, 1 dm.) (Found: C, 45.85; H, 8.0; OMe, 17.5. C₇H₁₄O₆ requires C, 47.2; H, 7.9; OMe, 17.4%).

Methyl 3-*O*-methyl- α - and - β -D-fucopyranosides. Glycosides of digitalose were prepared by the procedure described above for the glycosidation of fucose and under the same conditions. Separation of the resulting glycosides was achieved as described for the 2-*O*-methylfucose glycosides. The serologically inactive furanosides were not further investigated. Crystalline glycopyranosides were obtained with the following optical rotations: $[\alpha]_D^{20} + 180 \pm 5^\circ$ and $+6 \pm 5^\circ$ (*c* 0.64 and 0.169 respectively in methanol, 1 dm.). The compound with the higher positive rotation was assumed to be the α -anomer.

Haemagglutination inhibition. The method for studying inhibition of blood-group-specific haemagglutination is based on standard procedures and was described by Springer, Rose & György (1954) and Springer, Williamson & Brandes (1961). In brief, the testing procedure, employing 0.1 ml. throughout, was as follows: Two-fold serial dilutions of test substance were made, a different 0.1 ml. serological pipette being used for each tube in a titration series. The titrated solutions (or suspensions) were shaken and incubated for 2 hr. at room temperature (22–26°) with 4–8 minimum doses of haemagglutinins. Human group O erythrocytes were then added, the test mixtures shaken, and read microscopically after a further 1–2 hr. incubation at room temperature. Each titration series included controls

consisting of a serum standard, diluted to 4–8 minimum haemagglutinating doses and then titrated in twofold geometrical dilutions, as well as a suspension of the erythrocytes in aq. 0.85% sodium chloride. L-Fucose was included as standard in all assays. All materials, except 5-*O*-methyl-L-fucose, were tested at least three times and most were tested more than five times.

Eel serum and extracts of *Lotus tetragonolobus* were used as anti-H(O) reagents. Live eels weighing over 1 lb. were purchased during summer time along the Eastern Seaboard of the U.S.A., and sera were obtained by methods previously described (Miyazaki, 1930; Jonsson, 1944). Before use, the complement activity of the sera was destroyed by heating at 56° for 45 min. (because of their potent haemolysin) and the sera were subsequently absorbed with an equal volume of fresh, washed A₁B erythrocytes. One-half to two-thirds of the eel sera were of high enough titre (1:128 and greater) to be employed in the present experiments. The sera were stored at 4° after sterilization with phenol and thiomersal (0.25% phenol and 1:20 000 thiomersal, final concentrations). *Lotus tetragonolobus* seeds were ground finely in a mortar and extracted for 2 hr. at 37° with 10 vol. of the buffered saline described below. The supernatant solution was used for the tests.

The diluent and the erythrocyte-suspending solution in all tests was aq. 0.85% sodium chloride, containing 0.025 M-phosphate buffer, pH 7.2. Human O erythrocytes from citrated blood obtained from three donors and stored for less than 2 weeks were washed three times and then used as 0.5% suspension throughout.

Hapten preparations that did not inhibit at concentrations of 5 mg./ml. were considered to be inactive. Under the conditions of the present experiments, specificity and reproducibility were found to be wanting at concentrations of more than 5 mg. of inhibitor/ml., or less than four minimum haemagglutinating doses of serum. 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.

Precipitation inhibition. The ability of various monosaccharides and their derivatives to inhibit precipitation of human cyst mucoid with high blood-group H(O) activity by eel serum (Springer, Readler & Williamson, 1961) was determined by means of the quantitative hapten-inhibition technique (cf. Kabat, 1961). Inhibitions were carried out in the equivalence zone towards the region of antibody excess. Known amounts of sugar in aq. 0.85% sodium chloride were added in 0.5 ml. volumes to 0.5 ml. of serum of high titre and incubated in an ice bath for 30 min. After the addition of 110 μ g. of human ovarian-cyst H(O) substance in 0.5 ml. of aq. 0.85% NaCl, the samples were incubated for another hour in the ice bath and then 7–9 days at 4° with daily agitation. Precipitates were washed as described and protein was determined with the Folin-Ciocalteu reagent (cf. Heidelberger & MacPherson, 1943*a, b*; Kabat, 1961), since we found that measurements by this method agreed with those calculated from Kjeldahl nitrogen determinations. Eel serum alone and antigen alone, as well as these 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 ex-

periment was repeated at least once with the exception of the test done with 3-*O*-methyl-L-fucose, where sufficient material was available for one duplicate determination only.

RESULTS

The results obtained with derivatives of L- and D-fucose in the O(H)-anti-H(O) system (eel and *Lotus* reagents) are listed in Tables 1-3 for the haemagglutination-inhibition tests; results of the quantitative studies on the precipitation-inhibiting capacity of haptenic sugars in the eel serum-human H(O)-mucoid system are depicted in Fig. 1.

Haemagglutination-inhibition studies with eel serum

L-Fucose and derivatives. In agreement with earlier observations (cf. Springer, 1958), L-fucose possessed 1-5% of the activity of blood-group H(O)-active macromolecules (Table 1). Similarly, methyl α -L-fucopyranoside was 5-10 times as active as L-fucose (Watkins & Morgan, 1952). A second α -glycoside, *p*-aminophenyl α -L-fucopyranoside, showed the same level of activity. Methyl β -L-fucopyranoside, however, possessed only trace

activity. Two non-cyclic derivatives, namely L-fucose dibenzyl mercaptal and L-fucitol, and the furanosidic compound L-fuculose (6-deoxy-L-tagatose) were found to be inactive.

Fig. 1. Inhibition of precipitation by sugars [110 μ g. of human H(O) ovarian-cyst mucoid (Morgan 370) plus 0.5 ml. of eel serum] in a total volume of 1.5 ml. ●, 2-*O*-Methyl-L-fucose; □, methyl α -L-fucopyranoside; ○, 3-*O*-methyl-L- or -D-fucose; △, L-fucose; ▲, D-fucose; ×, D-galactose; ■, *N*-acetyl-D-galactosamine.

Table 1. *Fucose derivatives with high blood-group H(O) activity in the haemagglutination-inhibition tests*

Test substance	Minimum amount (mg./ml.) giving complete haemagglutination inhibition*	
	Eel-serum agglutinin	<i>Lotus</i> -extract agglutinin
L-Series		
L-Fucose†	0.1	0.05-0.1
Methyl α -L-fucopyranoside‡	0.01-0.02	0.01
Methyl β -L-fucopyranoside (potassium acetate complex)¶¶	1	0.1-0.2
<i>p</i> -Aminophenyl α -L-fucopyranoside‡‡	0.02	0.05
2- <i>O</i> -Methyl-L-fucose‡	0.05	0.05-0.1
Methyl 2- <i>O</i> -methyl- α -L-fucopyranoside‡	0.05	0.05-0.1
Methyl 2- <i>O</i> -methyl- β -L-fucopyranoside‡	0.2	0.1
3- <i>O</i> -Methyl-L-fucose‡‡	0.05-0.1	0.05
2,3-Di- <i>O</i> -methyl-L-fucose‡	0.1	0.05-0.1
2-Deoxy-L-fucose	0.1	0.2
2-Keto-L-fucose (angustose)§§	1	0.75
2-Amino-2-deoxy-L-fucose (L-fucosamine)**	0.2	0.3
D-Series		
2- <i>O</i> -Methyl-D-fucose‡	2.5	0.75
3- <i>O</i> -Methyl-D-fucose§	0.05-0.1	> 5
Methyl 3- <i>O</i> -methyl- α -D-fucopyranoside‡	0.05-0.1	> 5
Methyl 3- <i>O</i> -methyl- β -D-fucopyranoside‡	0.05	> 5
2,3-Di- <i>O</i> -methyl-D-fucose‡	0.03	1
2-Deoxy-3- <i>O</i> -methyl-D-fucose (D-diginose)	0.15	> 5
Blood-group H(O)-active polysaccharides		
Human ovarian-cyst mucoid	0.001	0.01-0.02
<i>Taxus cuspidata</i> twig polysaccharide	0.001-0.002	> 5
<i>Sassafras albidum</i> twig polysaccharide	0.001-0.002	> 5

* Inhibitor concentration before dilution with agglutinins and erythrocyte suspension. For final dilution divide values by 3.

† Purchased.

§ Isolated in this Laboratory.

¶ From Dr N. K. Richtmyer.

‡‡ From Dr E. E. Percival.

§§ From Dr H. Yüntsen.

¶¶ From Dr H. W. Ruelius.

‡ Synthesized in this Laboratory.

|| From Professor T. Reichstein.

** From Professor R. Kuhn.

‡‡ From Professor O. Westphal.

||| From Professor O. Th. Schmidt.

Table 2. *Fucose derivatives without significant H(O) activity in the haemagglutination-inhibition tests*

Test substance	Minimum amount (mg./ml.) giving complete haemagglutination inhibition*	
	Eel-serum agglutinin	<i>Lotus</i> -extract agglutinin
L-Series		
L-Fucose dibenzyl mercaptal†	> 5	> 5
3-Deoxy-L-fucose (colitose)††	5- > 5	2.5- > 5
5-O-Methyl-L-fucose††	< 2.5	Not tested
	(one test only)	
3,4-Di-O-methyl-L-fucose††	> 1	> 1
2,3,4-Tri-O-methyl-L-fucose††	> 2.5	> 2.5
2,3,5-Tri-O-methyl-L-fucose††	> 5	Not tested
2,5 } -Di-O-methyl-L-fucose††	> 5	> 2.5
3,5 }		
L-Fucitol¶	> 5	> 5
L-Fuculose*†	> 5	> 5
D-Series		
D-Fucose†	> 5	> 5
Methyl α -D-fucopyranoside†	> 5	> 5
Methyl β -D-fucopyranoside†	> 5	> 5
Methyl 2-O-methyl- α -D-fucopyranoside†	> 5	> 5
Methyl 2-O-methyl- β -D-fucopyranoside†	> 5	> 5
3-Deoxy-D-fucose (abequose)††	> 5	2.5-5
2-Amino-2-deoxy-D-fucose (D-fucosamine) hydrochloride*†	> 5	> 5

*† From Dr D. A. L. Davies.

*† See text.

Other footnotes are as in Table 1.

Table 3. *Sugars other than fucose derivatives possessing some blood-group H(O) activity in the haemagglutination-inhibition tests*

Test substance	Minimum amount (mg./ml.) giving complete haemagglutination inhibition*	
	Eel-serum agglutinin	<i>Lotus</i> -extract agglutinin
Related to L-fucose		
L-Galactose¶	> 5	0.3
6-Deoxy-L-talose	2	1
6-Deoxy-D-altrose	> 5	5
D-Ribose†	> 5	< 5
D-Arabinose†	2.5	2.5-5
2-Deoxy-D-ribose†	2.5-5	5
D-Fructose†	2	> 5
3-Deoxy-D-fructose**	2.5-5	> 5
2,6-Dideoxy-D-ribo-hexose (digitoxose)	> 5	2.5-5
2,6-Dideoxy-3-O-methyl-D-ribo-hexose (cymarose)	> 5	2.5-5
D-glycero-D-fructo-Heptose (sedoheptulose)¶	2.5	> 5
O- α -D-Glucopyranosyl-(1 $\rightarrow$ 3)-D-fructose (turanose)†	5	> 5
Related to D-fucose		
D-Galacturonic acid†	< 5	> 5
Not obviously related to fucose		
D-Allose	> 5	2.5
6-Deoxy-D-allose	> 5	1
D- Psicose (D-allulose)	> 5	2.5
6-Deoxy-D-glucose	> 5	2.5-5
2-O-Methyl-D-glucose	> 5	0.75
3-O-Methyl-D-glucose	> 5	5
2,3-Di-O-methyl-D-glucose	> 5	1
6-Deoxy-3-O-methyl-D-glucose (D-thevetose)	> 5	1-2.5
2,3,4,6-Tetra-O-methyl-D-glucose	> 5	0.75
D-glycero-D-allo-Heptose¶	> 5	2.5
N-Acetyl-D-glucosamine†	> 5	2.5
O- β -D-Galactopyranosyl-(1 $\rightarrow$ 4)-N-acetyl-D-glucosamine*§	> 5	5

*§ From Dr R. Tomarelli.

Other footnotes are as in Table 1.

As can be seen in Tables 1 and 2, *O*-methylation at C-2 of L-fucose somewhat increases activity. *O*-Methylation at C-3 and di-*O*-methylation at C-2 and C-3 yielded compounds whose activity was closely similar to that of L-fucose. *O*-Methylation on C-4 and C-5 in combination with *O*-methylation on C-2 or C-3 or both resulted in derivatives showing no activity. A single determination on 5-*O*-methyl-L-fucose showed a trace of activity; the sample had been separated by Gardiner & Percival (1958) by column chromatography from the slightly faster 2,3-di-*O*-methyl and slightly slower 2-*O*-methyl ethers, both of which are active.

Reduction at C-2 of L-fucose or amination of 2-deoxy-L-fucose at C-2 did not decrease the H(O)-inhibiting capacity significantly. Oxidation of C-2, however, lowered the activity tenfold. L-Fucose reduced at C-3 (colitose) was almost inactive as an inhibitor of haemagglutination.

The methyl α -pyranoside of 2-*O*-methyl-L-fucose was about ten times as active as the β -anomer. Angustmycin A, the naturally occurring *N*-glycoside of '2-keto-L-fucose' (angustose), showed no activity.

D-Fucose and derivatives. In agreement with earlier observations (Watkins & Morgan, 1952; Kuhn & Osman, 1956), we found D-fucose and its methyl α -glycoside to be inactive. We also noted that methyl β -D-fucopyranoside was inactive. However, 3-*O*-methyl-D-fucose was somewhat more active than L-fucose. Reduction of 3-*O*-methyl-D-fucose at C-2 led to a slight decrease in activity, whereas additional *O*-methylation at C-2 increased the activity fractionally. Mono-*O*-methylation at C-2 of D-fucose led to a compound of low activity. The unmethylated D-fucose derivatives 3-deoxy-D-fucose (abequose) and 2-amino-2-deoxy-D-fucose were inactive.

The methyl glycosides of 3-*O*-methyl-D-fucose were almost equally active. Naturally occurring cardiac glycosides with β -linked D-digitalose in the terminal position, e.g. panstrosid, strosposid and chartreusin, were also tested and found to be as active as 3-*O*-methyl-D-fucose (Springer *et al.* 1960). Streblosid, a β -glycoside of 2,3-di-*O*-methyl-D-fucose, also exhibited high activity. The methyl glycosides of 2-*O*-methyl-D-fucose were inactive.

Haemagglutination-inhibition studies with Lotus tetragonolobus extract

L-Fucose and derivatives. Results in the L-fucose series are comparable with earlier observations on the compounds described by Morgan & Watkins (1953), except that methyl β -L-fucopyranoside showed only about 10 % of the activity reported by these investigators.

In addition, the following L-fucose derivatives showed activities in the *Lotus* system that were

significantly different from those exhibited in the eel-serum system: methyl β -L-fucopyranoside was 5–10 times as active and *p*-aminophenyl α -L-fucopyranoside was only about one-half as active. Angustmycin A was as active as the free sugar angustose. The plant polysaccharides, which were of high activity against eel agglutinins, were inactive when tested with *Lotus*.

D-Fucose and derivatives. The activities, in the *Lotus* system, of D-fucose derivatives *O*-methylated at C-2 or C-3, or both, differed greatly from those observed with eel serum. This is in contrast with the similar activities exhibited by L-fucose derivatives in these two systems.

2-*O*-Methyl-D-fucose, although of trace activity only in the eel test, showed appreciable activity when tested with *Lotus* extract. This activity of 2-*O*-methyl-D-fucose is not affected by additional *O*-methylation at C-3. D-Fucose mono-*O*-methylated at C-3, however, was inactive, as was 2-deoxy-3-*O*-methyl-D-fucose (diginose), although both were highly active when tested with eel anti-H(O).

Among the glycosides of D-fucose and of D-fucose ethers tested, only streblosid (containing β -linked terminal 2,3-di-*O*-methyl-D-fucose) showed some activity in the *Lotus* test (cf. Springer, 1959).

Haemagglutination-inhibition studies with eel serum and Lotus extract on sugars other than fucose

Active sugars. Most sugars showing any activity when tested with either the eel or the *Lotus* reagent are structurally closely related to fucose, although their trivial names do not indicate this relationship. They are listed in Table 3, which also contains 11 monosaccharides and 1 disaccharide, active against *Lotus* reagent alone, whose close relation to fucose is not obvious. With the exception of 6-deoxy-D-allose, 2-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-D-glucose, this activity was very low. As is discussed below, all these carbohydrates conform to one of two basic structures.

Inactive sugars. The following sugars and derivatives, in addition to those already listed, were inactive in both tests: cladinoses (2,6-dideoxy-3-*C*-methyl-ribo-hexose), mycarose (2,6-dideoxy-3-*C*-methyl-lyxo-hexose), L-arabinose, D- and L-xylose, D- and L-lyxose, D-altrose, D-glucose, D-mannose, D-gulose, D-galactose, L-talose, methyl α - and β -D-galactopyranosides, α -D-galactose 1-phosphate, D-galactose 6-phosphate, D-glucuronolactone, D-glucuronic acid, D-galactosamine hydrochloride, *N*-acetyl-D-galactosamine, D-glucosamine hydrochloride, methyl *N*-acetyl- α - and - β -D-glucosaminide, 6-amino-6-deoxy-D-glucose hydrochloride, 3-acetamido-3,6-dideoxy-D-glucose, D-glucosaminuronic acid, mycosamine hydrochloride, D- and L-tagatose, L-sorbose, L-rhamnose, 6-deoxy-D-talitol, galactitol, D-mannitol, D-glycero-L-glucose, heptose,

D-glycero-D-gulo-heptose, D-glycero-D-and-L-galacto-heptose, D-glycero-L-manno-heptose, D-glycero-D-tagato-heptulose, D-glycero-D-sorbo-heptulose and α -glucoheptonolactone.

The following sugars, which were tested only as β -glycosides linked to a steroid aglycone, were inactive in both tests: 6-deoxy-3-O-methyl-L-glucose, 6-deoxy-3-O-methyl-L-talose, and 3,4-di-O-methyl-D-galactose.

Among the oligosaccharides investigated, turanose was active against eel serum only; the other fructose-containing oligosaccharides, sucrose [α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside], raffinose [α -D-galactopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside] and melezitose [α -D-glucopyranosyl-(1 $\rightarrow$ 3)- β -D-fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside], showed no activity either against eel-serum agglutinins or the *Lotus* reagent. β -D-Galactosyl-(1 $\rightarrow$ 4)-N-acetyl-D-glucosamine showed slight activity when tested with *Lotus* agglutinins. Trehalose (α -D-glucopyranosyl- α -D-glucopyranoside), melibiose [α -D-galactopyranosyl-(1 $\rightarrow$ 6)-D-glucose], cellobiose [β -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose], lactose [β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose] and β -D-galactosyl-(1 $\rightarrow$ 4)-N-acetyl-D-mannosamine were inactive in both systems.

Precipitation-inhibition studies

The striking results of the quantitative precipitin-inhibition studies in the H(O) substance-anti-H(O) system are shown in Fig. 1. The active monosaccharides, except L-fucose, give more than 75% inhibition at levels of 2 μ moles of added sugar. 2-O-Methyl-L-fucose is the most active sugar, possessing slightly higher activity than methyl α -L-fucopyranoside, although this glycoside is 2-5 times as active as 2-O-methyl-L-fucose in the haemagglutination-inhibition test. Also, L-fucose is approximately one-half as active as methyl α -L-fucopyranoside, even though the former compound possesses only 10-20% of the activity of the latter in the haemagglutination-inhibition test.

The 3-O-methyl ether of L-fucose was considerably more active than L-fucose and almost as active as methyl α -L-fucopyranoside; even more surprisingly 3-O-methyl-D-fucose gave a curve identical with that of the L-enantiomorph. N-Acetyl-D-galactosamine, D-galactose and D-fucose showed no significant inhibition up to the 6-8 μ moles level (see Fig. 1).

DISCUSSION

These results point to the profound influence that relatively small changes of the structure of serologically active haptens may have on their specificity. They recall earlier work begun at the

Rockefeller Institute (Goebel, Avery & Babers, 1934; Goebel & Hotchkiss, 1937), in which it was shown that a change in configuration of one carbon atom of a sugar hapten could have a decisive influence on its serological specificity. Of the large number of sugars tested for their ability to inhibit eel serum or *Lotus* anti-H(O) extracts, fucose derivatives were the only ones giving high inhibition (< 0.3 mg./ml.). Low levels of inhibition were observed for a number of compounds in both systems. Although those active against eel serum were all structurally related to fucose, this was not always true for the *Lotus* system.

As can be seen from Tables 1 and 2 and Fig. 1, the L-fucose molecule can undergo a limited number of modifications that maintain or even enhance its activity in both systems. Thus a methoxyl or amino group or a hydrogen atom, instead of the hydroxyl group, on C-2 of L-fucopyranose is compatible with high activity, whereas an oxo group or reversal of hydroxyl and hydrogen on C-2, without other alterations of the molecule, impairs activity in both systems. On C-3 the possible groups compatible with activity appear to be limited to hydroxyl, methoxyl and perhaps other similarly protruding groups.

Introduction of a methoxyl group into either C-4 or C-5 of L-fucose results in an inactive molecule (Table 2). 5-O-Methyl-L-fucose (a syrup of which showed a trace of activity in the single test performed, explainable by contamination with as little as 1% of an active methyl ether) is prevented from forming a pyranose ring by its C-5 substituent, and thus should be totally inactive according to the tenets of earlier workers (Kuhn & Osman, 1956). This contention is supported by the inactivity of other 5-O-methyl-L-fucose derivatives, by the acyclic compounds L-fucitol and L-fucose dibenzyl mercaptal and by the furanosidic L-fuculose.

The inhibition of antigen-antibody reactions by haptens has been considered to be stereospecific (cf. Landsteiner, 1947). Hence the postulates by Kuhn & Osman (1956), that only L-fucose and its derivatives would be active against eel serum, conformed to accepted ideas. However, as indicated by Springer *et al.* (1959, 1960), certain D-fucose derivatives possess an inhibitory activity in the eel-serum system higher than that of L-fucose; as is shown above the enantiomorphs of 3-O-methyl-fucose are of equal activity. This unexpected observation appears to be irreconcilable with the classical theory of serological specificity. The planar sugar formulae do not explain this effect. However, some clarification may be obtained if puckered structures of the pyranose ring are considered (see Addendum).

The methyl α - and β -glycosides of 3-O-methyl-D-fucose were of similar activities but less active than

the free digitalose (Table 1). The methyl glycosides of 2-*O*-methyl-D-fucose were inactive. This is in contrast with the findings on unsubstituted L-fucose, where α -methyl glycosidation led to a compound much more active than the starting sugar and β -glycosidation much decreased this activity. The methyl α -glycoside of 2-*O*-methyl-L-fucose, however, is not significantly more active than 2-*O*-methyl-L-fucose. These findings, therefore, may indicate that a dimethylated structure (*C*- or *O*-methyl) has achieved nearly maximum activity.

A related effect of fucose methyl ethers was also noted in the *Lotus* system. Again, D-fucose and its glycosides are quite inactive, whereas D-fucose mono-*O*-methylated on C-2 (but not C-3) shows significant activity, as does 2,3-di-*O*-methyl-D-fucose. The activation of D-fucose by 2-*O*-methyl-ation in the *Lotus* system is low (Table 1).

Among the sugars not directly derived from fucose, the moderate activity of D-fructose, which in the pyranose form has the same basic ring configuration as L-fucopyranose (*cis*-hydroxyl groups on C-3 and C-4 below the plane of the ring and a hydrogen atom above the plane at C-5) (Watkins & Morgan, 1952), and of a number of fructose derivatives in eel serum is noteworthy. We found that the fructose structure in reducing pyranosidic form as it occurs in sedoheptulose and turanose (Table 3) is active. Although in the latter instance D-fructose is linked at C-3 to glucose, this does not interfere with its activity. Other sugars containing the skeleton structure recognized by Watkins & Morgan (1952), e.g. 2-deoxy-D-ribose, D-arabinose and 6-deoxy-L-talose, are also usually active in the eel serum. Exceptions to this generalization indicate the importance of other positions on the pyranose ring, as demonstrated by the inactivity of D-ribose and L-galactose.

The substitution of ring C-1 of the basic L-fucopyranose ring structure with the CH₂•OH grouping as in D-fructose is incompatible with activity in the *Lotus* system. Otherwise, the *Lotus* system appears to be less selective towards changes on C-2 and C-5 of the ring proper, as is shown by the compounds listed in the upper part of Table 3. If, however, the substituent on C-5 contains more than one carbon atom and is below the plane of the ring, the molecule becomes inactive, as for D-*glycero*-L-*galacto*-heptose.

Although the explanations advanced above cover most of the observed activities or their absence, there remain two groups of compounds active in the *Lotus* system that do not fit in any of the above categories. The first group comprises four sugars of the D-allose configuration (Table 3). Models of the most likely conformation and plaster moulds made of them show that the hydroxyl

group at C-4 and the larger substituent at C-5 of both D-allose and 6-deoxy-D-allose (C-1 conformation) match the C-4 hydroxyl group and the C-5 methyl group of L-fucose (1-C conformation) respectively (Reeves, 1951). However, models of inactive sugars can also show a partial fit in the L-fucopyranose imprint.

The second group consists of seven glucose derivatives (including an active disaccharide where the D-glucose derivative is linked through C-4) each carrying methyl group(s) (Table 3), which probably has a bearing on the activity of the molecule. These have to hold a specific position in the molecule, as numerous methylated sugars and other simple methyl-containing compounds were inactive.

The configurational requirements for activity in the *Lotus* system are not evident, since 2-*O*-methyl-D-glucose does not possess the *cis*-hydroxyl groups on C-3 and C-4 in equatorial and axial positions respectively that are present in L-fucose. Instead, the hydroxyl groups at C-3 and C-4 of 2-*O*-methyl-D-glucose are both equatorial and *trans*. In both α -L-fucose and α -2-*O*-methyl-D-glucose the methyl groups are equatorial and adjacent to paired hydroxyl groups at C-3 and C-4 and one hydroxyl group is axial and *cis* to the vicinal *O*-methyl group. Models show conformations of the structures of α -L-fucose and α -2-*O*-methyl-D-glucose under consideration to be similar, which may account for the activities observed.

Models also show that in general the ring oxygen atom 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 atoms of 3-*O*-methyl-D-fucose and of 2-*O*-methyl-D-glucose fit into the imprint made by the ring oxygen atom of L-fucose, and the *O*-methyl group is complementary to the *C*-methyl group of L-fucose.

The precipitation-inhibition studies reported here put inhibition by sugars in the H(O)-anti-H(O) system on a strictly quantitative basis. Surprisingly, not only is L-fucose about half as active as its methyl α -L-glycopyranoside, but also, in contrast with observations in the haemagglutination-inhibition test, the 2-*O*- and 3-*O*-methyl ethers of L-fucose are as potent inhibitors as is methyl α -L-fucopyranoside. It is therefore impossible to predict whether or not the α -glycosidic structure is the one responsible for H(O) activity of all macromolecular blood-group substances, including those of infra-human species. Conceivably, from the results presented here, a C-3- or perhaps C-2-linked reducing fucose or fucose derivative is an active component.

Taxus polysaccharide, in which 2-*O*-methyl-L-fucose is believed to be the component mainly re-

sponsible for H(O) activity in the eel-serum system (Springer *et al.* 1956), does not neutralize the *Lotus* agglutinins. This points to a structural arrangement of 2-*O*-methyl-L-fucose on the macromolecule that makes it accessible only to the eel reagent. Free 2-*O*-methyl-L-fucose is as good an inhibitor of *Lotus* agglutinin as of eel serum; the *Lotus* reagent is less able to differentiate between α - and β -glycosidic linkages than is the eel agglutinin.

It was hoped that the present study would contribute to the understanding of the significant forces in hapten-antibody interactions. Although the reagents used are heterologous, results obtained with them may be useful because the agglutinins are directed against comparatively small structures and the reagents are not produced by artificial immunization. It may be possible to deduce some features of general significance from these experiments, especially since monosaccharides and their simple glycosides inhibit eel-serum and *Lotus* extract at lower concentrations than does any homologous system reported. Obviously homologous systems should also be investigated and to this end antigens containing fucose derivatives are being prepared in this Laboratory.

If hydrogen-bonding of the hydroxyl groups with the appropriate groups of the antibody molecule is considered to be of importance in hapten-antibody interactions, then the only hydroxyl group of L-fucose necessary for hydrogen-bonding is that at C-4. It may be assumed from the model studies mentioned above that the ring oxygen atom or the *O*-methyl oxygen atom may also partake in the hydrogen-bonding. If, however, the hydroxyl group at C-3 is substituted, it appears necessary for the preservation of activity to have a substituent capable of hydrogen-bonding, because reduction of OH to H leads to almost complete loss of activity (Tables 1 and 2). A larger substituent than a hydrogen atom may bring the sugar molecule into close-fitting juxtaposition with the complementary area of the antibody molecule, thus allowing for extensive van der Waals interaction (Pauling, 1947) in addition to hydrogen-bonding. As pointed out by Pauling, however, steric restrictions give hydrogen bonds greater stereochemical significance than the less selective van der Waals forces. This lack of selectivity may account in part for the rather extensive changes of the basic structure which are compatible with some activity. It is unlikely that the whole sugar molecule combines closely with antibody and that it is thus necessary for activity.

The detailed shape of the antibody-combining site for either reagent is not obvious. The possibility that the combining site of the antibody may not be a rigid structure but a flexible one capable of moulding itself to the shape of the hapten, and

vice versa, as suggested by Wallenfels, Lehmann & Malhotra (1960) for the action of *Escherichia coli* β -galactosidase on galactosides, must be considered.

SUMMARY

1. The hapten activity of methyl ethers of L- and D-fucose, their glycosides, and of other sugars not directly related to fucose, has been investigated in the heterologous eel-serum- and *Lotus tetragonolobus* anti-H(O)-human O erythrocyte-haemagglutination systems. In addition, inhibition by monosaccharides in the eel serum-human H(O) ovarian-cyst mucoid precipitin system was measured quantitatively for the first time. The relative inhibitory activities of fucose derivatives in the precipitin-inhibition assay did not strictly parallel those found in the haemagglutination test.

2. Haemagglutination-inhibition studies showed, in agreement with earlier observations, L-fucopyranoside in α -glycosidic linkage to be the most active haptenic structure of the L-fucose series in both the eel-serum and *Lotus* systems.

3. Some changes on the L-fucose molecule at C-2 and C-3 are compatible with high activity and others are not; substitution at C-4 or C-5 led to inactive compounds. Methyl α -glycosidation did not activate *O*-methylfucoses.

4. The D- and L-enantiomorphs of 3-*O*-methylfucose were of equal activity in the eel anti-H(O) haemagglutination-inhibition system; equal activities were also found in the precipitation-inhibition system. These findings are contrary to classical concepts of stereospecificity. Similar observations were made with the 2,3-di-*O*-methylated derivatives. In the *Lotus* system, significant activation of D-fucose occurred only by *O*-methylation of C-2.

5. Two groups of sugars unrelated to fucose showed low to moderate activity in the *Lotus* system only. These were sugars possessing the D-allose configuration and D-glucose derivatives possessing *O*- or *C*-methyl groups. A large number of other sugars were inactive in both systems.

6. Explanations for the observed activities and their deviations from accepted theory were attempted by molecular-model studies (see also Addendum) and by applying conformational theory.

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ADDENDUM

Structural Similarities of Methylated D- and L-Fucose Derivatives as seen in Three-Dimensional Models

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The concept of stereospecificity in biological phenomena, notably in enzymic and immunological reactions, is so deeply rooted that it is assumed to hold in all circumstances. The findings

of Springer & Williamson (1962) of exceptions to this rule, in their studies of the role of D- and L-fucose and their derivatives in reactivity with eel anti-H(O) or with the *Lotus* haemagglutinin to

inhibit haemagglutination of O erythrocytes or precipitation by H(O) substances, require an explanation. This seems to be provided by a study of three-dimensional close-packed models of D- and L-fucose, their 2-O-methyl, 3-O-methyl and 2,3-di-O-methyl derivatives shown in Fig. 1.

In each case, for orientation, the left-hand set of photographs A, C, E and G show the models arranged as mirror images, whereas in the right-hand set (B, D, F and H) the L-isomer has been rotated through an angle of about 180° to indicate the way in which each of the two isomers is thought to react with the eel or *Lotus* combining site. The oxygen of the hydroxyl group on C-1 is marked with a small piece of white tape and positions of C-2, -3 and -6 are numbered for convenience in orientation. The region of interaction is assumed to be with the non-reducing end of the compounds, e.g. an antibody or *Lotus* combining site interacting at the left.

In Fig. 1B, the differences between D- and L-fucose are quite evident, the methyl group of the D- and L-compound being oppositely located, so that the two hydroxyl groups on C-2 and C-3 of the L-form occupy the relative position of the methyl

group of the D-form and vice versa. Thus the two molecules present substantially different contours. Moreover, the methyl group would have a much lower tendency to form hydrogen bonds than the hydroxyl groups, which would make for greater difference in complementarity than the molecules show.

In Fig. 1D the substitution of a methoxyl group on C-2 has given the molecules a similarity in contour not present in the unsubstituted fucoses. This results in the D- and L-compounds' having a very similar profile and a second area of low hydrogen-bonding at about 180° from C-6.

In Fig. 1F substitution of a methoxyl group on C-3 has also created a similarity in profile not previously present. The 3-O-methyl-L- and -D-fucoses appear somewhat triangular, with C-6 and C-3 being similar in outline and non-hydrogen-bonded. This similarity between the D- and L-derivatives with respect to C-3 and C-6 is also seen with the 2,3-di-O-methyl compounds (Fig. 1H).

It is thus not surprising that the observed activities may be explained by the similarities of the models of the methylated D- and L-derivatives. These similarities with the eel anti-H(O) are especially striking with 3-O-methyl-D- and -L-fucose and the 2,3-di-O-methyl derivatives, all of which have activities even higher than that of L-fucose by haemagglutination inhibition and quantitative precipitin assays. 2-O-Methyl-D-fucose, however, shows only a slight increase in potency over D-fucose. Quantitative precipitin tests were not done on 2,3-di-O-methylfucose. It would appear, therefore, that the similarity in contour between C-3 and C-6 of the methylated derivatives is more important to the complementarity than that between C-2 and C-6.

In the *Lotus* system there is obviously an increased complementarity of the 2-O-methyl-D- and 2,3-di-O-methyl-D-derivatives compared with that of D-fucose and 3-O-methyl-D-fucose, but these do not approach the activity of the L-series. Thus the similarity in contour of the D- and the L-series between C-2 and C-6 of the methylated derivatives seems more important than that between C-3 and C-6, although the overall effect is less than that with the eel anti-H(O).

In the immunochemical studies, the reaction of both the eel anti-H(O) and the *Lotus* haemagglutinin with O erythrocytes and with H(O) substance was only a fortuitous discovery. The basis for the complementarity of the *Lotus* haemagglutinin is unknown, and even in the eel anti-H(O) the chemical nature of the antigenic determinant toward which the antibody specificity is directed is not known. Accordingly, in neither case is it certain that the potency of any of the compounds

Fig. 1. Mirror-image forms. A, D- and L-Fucose; C, 2-O-methyl-D-fucose and 2-O-methyl-L-fucose; E, 3-O-methyl-D-fucose and 3-O-methyl-L-fucose; G, 2,3-di-O-methyl-D-fucose and 2,3-di-O-methyl-L-fucose. In B, D, F and H, the L-derivatives in A, C, E and G respectively have been rotated about 180° to show the D- and L-derivatives in the position in which they would react with an antibody or *Lotus* combining site on the left-hand side of the molecules. The oxygen atoms on C-1 are marked with a piece of white tape.

studied even approaches that ultimately obtainable. Although the D- and L-methylated derivatives resemble each other in contour more than do D- and L-fucose, it cannot be assumed that they are identical. It would be of interest to obtain by immunization homologous antibody with specificities directed against the D- and L-methylated fucoses and to determine whether the structural similarities in Fig. 1 used in the explanation of these cross-

reaction studies would hold for an homologous antigen-antibody system.

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Studies on Isocitrate Oxidation in Mitochondria of Normal Rat Liver and Azo-Dye-Induced Hepatomas

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Previous studies on isocitrate oxidation in normal rat-liver mitochondria have resulted in a controversy about the cofactor requirement and pathway of such oxidation. Kaplan, Swartz, Frech & Ciotti (1956) as well as Purvis (1958*a, b*) have provided considerable evidence to indicate that the oxidation of isocitrate is NADP-dependent, and that the primary dehydrogenase reaction is followed by a transfer of electrons via a transhydrogenase enzyme to NAD, which is in turn linked through a cytochrome system to oxygen.

On the other hand, Ernster (1959) maintains that a NAD-dependent isocitrate dehydrogenase is also present, and has recently shown (Ernster & Glasky, 1960) that, in pyridine nucleotide-depleted rat-liver mitochondria, isocitrate can be oxidized aerobically via a NAD-linked and NADP-independent pathway at a rate similar to that observed for isocitrate oxidation in intact mitochondria.

In view of these controversial findings, it was considered of importance to re-investigate the problem of isocitrate oxidation in normal rat-liver mitochondria, and the results of such research are reported in this paper.

Certain experiments on the oxidation of isocitrate in rat-hepatoma mitochondria induced by 4-dimethylamino-3'-methylazobenzene and 4-dimethylaminoazobenzene are also reported, and these results, as well as those on the normal rat-liver mitochondria, are contrasted with the findings of Hawtrey & Silk (1960, 1961) on the oxidation of isocitrate in Ehrlich ascites-tumour-cell mitochondria.

Vignais & Vignais (1961) have reported that the oxidation of isocitrate by pyridine nucleotide-depleted rat-liver mitochondria could be restored by NAD or NADP. Results in this paper support these findings, but differ in certain aspects, which are more fully dealt with in the Discussion.

MATERIALS AND METHODS

Male and female albino rats (wt. 200–250 g.) of the Wistar strain were provided by the National Nutrition Research Institute, Pretoria. The animals were housed in wire cages at 20° and fed *ad lib.* on the Institute's stock diet (protein, 20%; ash, 7.4%; main component: maize meal, 56%) and tap water. The 4-dimethylamino-3'-methylazobenzene-induced hepatoma was obtained from male rats of the above strain which had been fed on the stock diet containing 0.054% of the dye for a period of 8–9 months. The 4-dimethylaminoazobenzene-induced hepatoma was obtained from rats kindly supplied by Professor J. Gillman of the University of Witwatersrand, Johannesburg. Fully developed tumours induced by the feeding of azo dyes were grossly necrotic and gave poor results. All experiments reported were therefore carried out with hepatoma material contaminated to a slight extent with normal liver tissue.

Both the source and method of assay of the following materials have been described by Hawtrey & Silk (1961): hexokinase, cytochrome *c*, AMP, ADP, Amytal [sodium 5-ethyl-5-(3-methylbutyl)barbiturate], antimycin A, DL-isocitrate, nicotinamide, 2,6-dichlorophenol-indophenol and digitonin. NAD and NADP were supplied by C. F. Boehringer und Soehne, Germany, and assayed by the cyanide-addition method of Ciotti & Kaplan (1957). Reduced NADP was also obtained from C. F. Boehringer und Soehne and its purity determined by measurement of *E* at 340 mμ, with a millimolar extinction coefficient of 6.3.