THE CONVERSION OF D-GLUCOSE TO L-FUCOSE BY AEROBACTER CLOACAE*

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(Received for publication, August 19, 1957)

L-Fucose (6-deoxy-L-galactose) is widely distributed in nature as a component of serum glycoproteins (2), the blood group substances (3), and many plant (4) and bacterial polysaccharides (5). The pathway of biosynthesis of this compound is unknown.

Dudman and Wilkinson (5) reported that Aerobacter cloacae NCTC 5920 produces a polysaccharide which contains approximately equal quantities of L-fucose, D-glucose, D-galactose, and uronic acid. In the present studies, this organism was grown on specifically labeled glucose as the sole source of carbon. The distribution of isotope in the L-fucose was determined and compared with the distribution in D-glucose and D-galactose of the polysaccharide.

The results of these experiments indicate that the specifically labeled D-glucose carbon chain is converted to that of L-fucose, D-glucose, or D-galactose without significant inversion or randomization of the isotope.

EXPERIMENTAL

Culture Conditions—A culture of A. cloacae NCTC 5920 was kindly provided by Dr. J. F. Wilkinson of the University of Edinburgh. The organism was grown in media as previously described (5). D-Glucose autoclaved separately as a 50 per cent solution was added to the sterile salt solution. The medium (1 liter) was inoculated with 10 ml. of a 24 hour liquid culture, and was incubated for 24 hours at 37° on a New Brunswick rotary shaker at 210 r.p.m.

The organism was grown in the presence of glucose-1-C\textsuperscript{14} (0.0485 µc. per mg.) or glucose-6-C\textsuperscript{14} (0.089 µc. per mg.). The radioactive sugars were kindly supplied by Dr. H. S. Isbell of the National Bureau of Standards.

Isolation of Polysaccharide—The viscous culture (1 liter) was centrifuged in the cold at 20,000 X g for 15 minutes to remove the cells. The clear

* A preliminary report (1) has been presented. The Rackham Arthritis Research Unit is supported by a grant from the Horace H. Rackham School of Graduate Studies of the University of Michigan. This investigation was supported in part by a grant (No. A-512) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, and a grant from the Michigan Chapter, Arthritis and Rheumatism Foundation.
supernatant fluid was stirred at room temperature with a glass rod during the addition of 2 volumes of acetone and the polysaccharide collected on the rod as a fibrous "clot." After being washed several times with acetone, the material was dissolved in approximately 200 ml. of water in a Waring blender (5 minutes at high speed) and dialyzed overnight against cold running tap water.

Further purification of the polysaccharide was afforded by subjecting it to an electrodeposition technique. This procedure has previously been used for the isolation of hyaluronic acid (6) and will be described in detail elsewhere. The polysaccharide was deposited on the channel membrane of the electroconvection apparatus as a gel, was removed, and lyophilized. At this stage of purification the polysaccharide contains approximately 1 per cent nitrogen.

Isolation of Monosaccharides—The polysaccharide was dissolved in 25 ml. of 96 per cent formic acid and sealed in a Pyrex test tube. After being heated for 24 hours at 100°, the formic acid was removed in vacuo, and the syrup was treated for an additional 6 hours with 25 ml. of 1 N sulfuric acid at 100° (5). The sulfate ion was removed with barium hydroxide, and the filtrate was concentrated in vacuo to a syrup.

The hydrolysate was dissolved in approximately 3 ml. of water and applied to a cellulose column (3.5 X 40 cm.) which was prepared as previously described (7). The monosaccharides were eluted with an isopropanol-water mixture (9:1) at a rate of approximately 1 ml. per minute; 10 ml. fractions were collected with an automatic fraction collector. Aliquots of each fraction were examined for reducing sugar by the method of Nelson (8). L-Fucose emerged from the column in a symmetrical peak between 400 and 575 ml. of effluent; a mixture of glucose and galactose emerged between 600 and 850 ml. The sugars were identified by paper chromatography with use of the isopropanol-water solvent described above. The materials were obtained as syrups by combination of appropriate fractions and removal of the solvent in vacuo. The yields of monosaccharides obtained at this stage from 1 liter of growth medium were as follows: L-fucose 25 mg., a mixture of D-glucose 15.5 mg., and D-galactose 20 mg. (by analysis). The methyl pentose isolated from the hydrolysate of the polysaccharide was further characterized as L-fucose by preparation of the o-nitrophenylhydrazone derivative (9). o-Nitrophenylhydrazone of authentic L-fucose: m.p., 182-183°, [a]D measured at 20.8°; o-nitrophenylhydrazone of isolated methyl pentose: m.p., 182-183°, [a]D measured at 19.0°.

The mixture of glucose and galactose was dissolved in 3.5 ml. of 0.2 M phosphate buffer, pH 5.6, which contained 11 mg. of glucose oxidase.

1 Performed by Mr. Donald Watson, to whom the authors are grateful.
2 Dee-O, Takamine Laboratory, Clifton, New Jersey. The samples were kindly provided by Dr. Sheldon Rennert. The solid (32 mg.) was stirred with 10 ml. of
The mixture was incubated for 2 hours at 37° with vigorous shaking, after which 3.5 ml. each of 0.2 M zinc sulfate and 0.2 M barium hydroxide were added, and the precipitate was removed by centrifugation. The supernatant fluid, containing a mixture of galactose and gluconic acid, was passed through a column of 20 ml. of Dowex 1, HCO₃⁻ resin (200 to 400 mesh). The column was washed with 50 ml. of water, and the washings containing the galactose were concentrated in vacuo to a syrup. Gluconic acid was eluted by shaking the resin in a flask with 75 ml. of 2 N hydrochloric acid solution for 1 hour. The resin was removed by filtration, and the filtrate and washings were concentrated in vacuo to a syrup.

The efficiency of the above procedure was determined by separation of a mixture of equal quantities of galactose and uniformly labeled glucose-C¹⁴. The respective benzimidazole derivatives were isolated as described below and their specific activities determined. The galactobenzimidazole exhibited no detectable radioactivity, and the activity per millimole of the glucobenzimidazole was identical with that of the glucose (6750 c.p.m.).

Preparation and Degradation of L-Fuco-, D-Galacto-, and D-Glucobenzimidazole—The benzimidazole derivatives of fucose and galactose were prepared by a modification of the "direct method" with cupric acetate being used as the oxidant, while gluconic acid was converted to its benzimidazole derivative by the method for aldonic acids (10).

The crude reaction mixtures (which initially contained from 15 to 50 mg. of carbohydrate) were placed on columns containing 20 ml. of Dowex 50-H⁺ resin (200 to 400 mesh) and were eluted with gradients produced by the addition of 2.87 N hydrochloric acid to 1 liter of 0.10 N hydrochloric acid in a mixing vessel. The fractions were examined for the benzimidazole derivatives by determining the absorbancies at 277 nm (11). The benzimidazoles emerged from the columns in the range of 300 to 500 ml. of effluent.

The fractions containing the derivatives were pooled, concentrated 0.2 M phosphate buffer, pH 5.6, in an ice bath for approximately 10 minutes. The mixture was filtered, and 3.5 ml. of the filtrate were used.

A typical reaction mixture for the "direct method" consisted of the following: 0.28 mmole of aldose; 0.83 mmole of o-phenylenediamine; 0.5 mmole of cupric acetate; 4.0 mmoles of glacial acetic acid; water to a total volume of 2.0 ml. The mixture was maintained at 53° for 14 hours. Copper ion was removed from the mixture by treatment with hydrogen sulfide before chromatography. A typical reaction mixture for the gluconic acid consisted of the following: 0.28 mmole of gluconic acid; 0.60 mmole of o-phenylenediamine dihydrochloride; 0.01 ml. of ethanol; water to a total volume of 0.2 ml. The mixture was heated for 2 hours at 135°.

Preliminary experiments indicated that mixtures containing fuco-, gluco-, and galactobenzimidazoles were not significantly separated from each other by this procedure. Consequently, the individual sugars must be isolated before preparation of the respective derivatives.
centrated in vacuo, crystallized, and further purified by recrystallization (11). The respective melting points of the benzimidazole derivatives of fuco-, gluco-, and galactobenzimidazoles prepared from the sugars isolated from the polysaccharide were 254°, 215°, and 245° and were not depressed when these compounds were mixed with derivatives of authentic sugars.

The chromatographic procedure employed for the purification of the derivatives permits the use of excess o-phenylenediamine rather than equivalent amounts as have previously been used (12).

Degradation of the benzimidazoles was conducted by previously described procedures (11-14) after the addition of the respective non-radioactive derivative as carrier. Gluco- and galactobenzimidazoles, when oxidized with periodate, yield 2-benzimidazolealdehyde from hexose carbon 1 and carbon 2, formic acid from carbons 3, 4, and 5, and formaldehyde from carbon 6. Fucobenzimidazole is oxidized to comparable fragments, except that only carbons 3 and 4 yield formic acid while carbons 5 and 6 yield acetaldehyde. The benzimidazole (0.41 mmole) was suspended in 10 ml. of water to which an equivalent amount of sulfuric acid was added. To this solution, 4 mmoles of sodium periodate were added and the mixture was incubated in the dark at 4° for 60 minutes. The final pH of the reaction mixture was about 3.5. The consumption of periodate in the presence of fuco-, gluco-, and galactobenzimidazoles was followed by the titrimetric technique (15); theoretical periodate consumption was attained after 30 minutes. No further consumption of periodate could be detected within 2 hours. The excess periodate was decomposed by the addition of excess sodium arsenite and the mixture was adjusted to pH 7.5 by the addition of a 5 per cent sodium bicarbonate solution. The mixture was allowed to stand in ice for 1 hour, after which 2-benzimidazolealdehyde was recovered by centrifugation; m.p. 241-242°.

The supernatant solution contained formic acid and either acetaldehyde or formaldehyde. These compounds were isolated and degraded as described below. 2-Benzimidazolealdehyde was oxidized to 2-benzimidazolecarboxylic acid with permanganate in pyridine solution. Under these conditions, the product was obtained in higher yield than when the oxidation was conducted by suspending the aldehyde in water (14). The aldehyde (0.30 mmole) was refluxed with 7.5 ml. of purified, anhydrous pyridine until the solid completely dissolved. Potassium permanganate solution (2.2 ml. of 0.51 N) was added, and the mixture was heated on a steam bath for 30 minutes. Pyridine was removed by steam distillation, and the residue was treated with a small amount of Norit A and filtered. The clear, colorless solution was adjusted to pH 4 to 5 with acetic acid and was allowed to stand at 0° for several hours. The white needles of 2-benzimidazolecarboxylic acid were obtained in 75 to 80 per cent yield; m.p. 174°.
2-Benzimidazolecarboxylic acid was quantitatively decarboxylated by being heated in a glass tube in ethylene glycol vapors. The carbon dioxide, which represents carbon 2 of the hexoses, was trapped in barium hydroxide solution. The remaining crystalline benzimidazole, which contains carbon 1 of the aldoses, was recrystallized from water; m.p. 169–170°.

Formaldehyde (carbon 6 of glucose or galactose) or acetaldehyde (carbon 5 and carbon 6 of fucose) was steam-distilled from the supernatant solution which remained after removal of the 2-benzimidazolealdehyde. Formaldehyde was converted to its dimedon derivative (16); m.p. 189–190°. The acetaldehyde was divided into two portions, one of which was converted to the dimedon derivative (16); m.p. 142°; the other portion (17) was treated with sodium hypoiodite to yield iodoform (carbon 6 of fucose); m.p. 113°.

Formic acid (carbon atoms 3, 4, and 5 of glucose or galactose and carbons 3 and 4 of fucose) was selectively oxidized to carbon dioxide by treatment of the steam distillation residue with mercuric acetate (18).

**Determinations of Radioactivity**—Measurements were made with a model 210 Flo-Window counter, Packard Instrument Company, and the results are expressed as counts per minute at infinite thickness as barium carbonate. All samples were counted for periods of time sufficient to give a standard error of ±2 per cent. All samples, not otherwise converted to carbon dioxide, were subjected to combustion by the method of Gabourel et al. (19), and counted as barium carbonate.

**RESULTS AND DISCUSSION**

After growth of the organism in the presence of glucose-1-C\textsuperscript{14} or glucose-6-C\textsuperscript{14}, the polysaccharide was harvested and hydrolyzed, and the monosaccharides were isolated as the respective benzimidazole derivatives. The distribution of C\textsuperscript{14} in the carbon skeletons of the aldoses are presented in Tables I and II. It is evident from the data that C\textsuperscript{14} was found only in carbon 1 of the L-fucose, D-glucose, and D-galactose when the organism was grown on glucose-1-C\textsuperscript{14}, and that the isotope appeared only in carbon 6 of the monosaccharides when glucose-6-C\textsuperscript{14} was the carbon source. Further, within the errors of the methods employed, there was no significant dilution of the C\textsuperscript{14} content of the isolated monosaccharides when compared with the glucose used in the growth media.

Wilkinson,\textsuperscript{5} and Segal and Topper,\textsuperscript{5} working independently, have reported similar results with a strain of *Aerobacter aerogenes* which produces a fucose-containing polysaccharide. In their experiments glucose-1-C\textsuperscript{14} or glucose-6-C\textsuperscript{14} was provided as the sole source of carbon for growth, and the L-fucos was found to be predominantly labeled in the carbon 1 or carbon 6.

\textsuperscript{5} Private communications.
position. A small but significant degree of randomization of the label was observed in the L-fucose and D-glucose isolated from the polysaccharide. In the experiments conducted by these investigators, the organism was grown for 48 hours rather than for the 24 hour periods used in our experiments. The longer growth period may explain the randomization.

**TABLE I**

*Conversion of D-Glucose-1-\(^{14}\)C to L-Fucose, D-Glucose, and D-Galactose of Polysaccharide*

<table>
<thead>
<tr>
<th></th>
<th>L-Fucose</th>
<th>D-Glucose</th>
<th>D-Galactose</th>
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<tbody>
<tr>
<td>CHO</td>
<td>94</td>
<td>CHO 100</td>
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<td>0</td>
<td>HCOH 0</td>
<td>HCOH 0</td>
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<tr>
<td>C(_{1-6})</td>
<td>97</td>
<td>C(_{1-6}) 99</td>
<td>C(_{1-6}) 95</td>
</tr>
</tbody>
</table>

*The growth medium contained 48.4 \(\mu\)c. of glucose-1-\(^{14}\)C per liter. The glucose carbon 1 exhibited a specific activity of 5550 c.p.m. when determined as barium carbonate at infinite thickness. For purposes of clarity, this value was assigned an arbitrary value of 100 and the specific activities in Table I are presented on a relative basis.

† The specific activity of L-fucose carbon 5 was determined by difference; carbons 5 and 6 were determined as the acetaldehyde-dimedon derivative and carbon 6 as iodoform. See the text for the details of the degradations.

Hauser and Karnovsky (20) have studied the conversion of D-fructose-6-\(^{14}\)C to another 6-deoxyaldose, L-rhamnose, which is produced as a constituent of a rhamnolipide by *Pseudomonas aeruginosa*. Essentially all of the label was found in the carbon 6 of the rhamnose. It was also demonstrated that specifically labeled glycerol was converted to L-rhamnose, the results suggesting a condensation of two 3-carbon intermediates to form the 6-carbon chain of L-rhamnose (21).

The results of the experiments reported here suggest a direct conversion of D-glucose to L-fucose, involving neither inversion nor fragmentation of the D-glucose carbon chain. A direct pathway requires a change in con-
figuration of the hydroxyl groups attached to carbon atoms 2, 3, and 5 of d-glucose, and reduction to a methyl group of carbon 6. To our knowledge, there has been no report of an enzyme-catalyzed reduction at carbon 6 of the hexoses. On the other hand, analogous reactions have been reported for enzymatic epimerizations: d-glucose → d-fructose → d-mannose via the 6-phosphate esters (22); d-glucose → d-galactose via the uridine deriv-

Table II
Conversion of d-Glucose-6-C\textsuperscript{14} to L-Fucose, D-Glucose, and D-Galactose of Polysaccharide

<table>
<thead>
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<th>Relative specific activity*</th>
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<tr>
<td><strong>L-Fucose</strong></td>
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<td>CHO</td>
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<td>HCOH</td>
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<td>HCOH</td>
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<tr>
<td>HOCH</td>
</tr>
<tr>
<td>CH\textsubscript{3}</td>
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<tr>
<td>C\textsubscript{1-6}</td>
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* The growth medium contained 88.8 μc. of glucose-6-C\textsuperscript{14} per liter. The glucose carbon 6 exhibited a specific activity of 10,500 c.p.m. when determined as barium carbonate at infinite thickness. For purposes of clarity, this value was assigned an arbitrary value of 100 and the specific activities in Table II are presented on a relative basis.

† The specific activity of L-fucose carbon 5 was determined by difference; carbons 5 and 6 were determined as the acetaldehyde-dimedon derivative and carbon 6 as iodoform. See the text for details of the degradations.

atives (23); L-ribulose → d-xylulose via the 5-phosphate esters (24, 25); L-arabinose → d-xylose via the uridine derivatives (26).

The isotope data also agree with the formation of L-fucose by cleavage of the d-glucose carbon chain to yield fragments which are then recombined. This pathway requires that there be no equilibration of the smaller units. The cleavage of hexose diphosphate by aldolase, for example, yields trioses which are in equilibrium and which would ultimately give rise to doubly labeled L-fucose if these compounds were involved in the synthesis. In this respect, it is of interest to note that Hough and Jones (27) demon-
strated the formation of 6-deoxy-D-fructose and 6-deoxy-L-sorbose by the condensation of dihydroxyacetone phosphate with DL-lactaldehyde in the presence of an aldolase preparation from peas. The conversion of 6-deoxy-L-sorbose to L-fuculose would require only one epimerization, at carbon 3.

Since L-fucose has the configuration of L-galactose, another possibility for the pathway of biosynthesis of L-fucose was the following sequence of reactions: D-glucose $\xrightarrow{1} D$-galactose $\xrightarrow{2} L$-galactose $\xrightarrow{3} L$-fucose. Reaction 1 is known (23); Reaction 2 has been proposed (28); Reaction 3 would involve the reduction of the carbinol group (carbon 6) of L-galactose to a methyl group. This type of inversion is known; D-glucose is converted to L-ascorbic acid (29); D-xylulose is converted to L-xylulose (30). In the organisms that have been studied, this over-all pathway has been eliminated as significant in the biosynthesis of L-fucose, since the process would result in inversion of the glucose carbon chain yielding L-fucose with a labeling pattern opposite to that of the glucose in the medium.

Studies are now in progress to determine the enzymatic mechanism of biosynthesis of L-fucose.

SUMMARY

1. Aerobacter cloacae NCTC 5920 was grown on glucose-1-C$^{14}$ or glucose-6-C$^{14}$. The extracellular polysaccharide was isolated and hydrolyzed, and the benzimidazole derivatives of the component sugars, L-fucose, D-glucose, and D-galactose, were isolated. The C$^{14}$ distribution in the aldoses was determined by degradation of the benzimidazoles. The procedures for the preparation and degradation of the benzimidazoles were modified so that microquantities of aldoses could be used.

2. D-Glucose-1-C$^{14}$ was converted to L-fucose-1-C$^{14}$ and D-glucose-6-C$^{14}$ was converted to L-fucose-6-C$^{14}$ without significant dilution of the radioactivity. Similar results were observed with the other hexose components of the polysaccharide, D-glucose and D-galactose. It has been suggested that D-glucose is converted to L-fucose either directly without cleavage of the carbon chain or via a pathway involving the cleavage of the glucose carbon chain to fragments, not in equilibrium with each other, which are subsequently recombined to form L-fucose.

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