Conversion of Glucose to Sorbitol and Fructose by Liver-derived Cells in Culture*

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Conversion of glucose to fructose and sorbitol is documented in rat hepatoma-derived cultured cells (HTC cells). After addition of 5.5 mM [U-14C]glucose to incubation medium, labeled sorbitol and fructose accumulated intracellularly at a linear rate over a period of 60 min. The sugars were isolated, identified, and quantitated by paper chromatography, gas-liquid chromatography, and enzymatic phosphorylation of fructose. Primary culture of adult rat hepatocytes was analyzed both in terms of enzyme activities that mediate the formation of sorbitol and fructose and in terms of the catabolism of these sugars. Both types of culture (as well as extracts of intact rat liver) exhibited enzymatic activities catalyzing the conversion of glucose to sorbitol (aldose reductase) and sorbitol to fructose (sorbitol dehydrogenase). However, the cultures differed strikingly with regard to the catabolism of sorbitol and fructose. The conversion of labeled sorbitol to metabolites in HTC cells was negligible; by contrast, hepatocytes in primary culture utilized the sugars at rates comparable to that of glucose, which may account for the lack of their accumulation in primary culture. The findings suggest that the conversion of glucose to sorbitol and fructose by HTC cells may represent a retained normal liver function, one which is amplified by the inability of HTC cells to dispose of these sugars.

Fructose and sorbitol are widespread in human diet, and their metabolism has been studied in detail in hepatic tissue, where the sugars are antiketogenic (1) and alter lipid oxidation (2, 3). With regard to the metabolic inter-relation of these sugars, sorbitol is oxidized to fructose in a reversible reaction mediated by sorbitol dehydrogenase (EC 1.1.1.14) (4). These findings are part of an extensive body of information on the uptake and utilization of exogenous sorbitol or fructose by various tissues.

By contrast, relatively little is known of the formation of these sugars in either hepatic tissue or other animal cells. Endogenous production of free fructose and sorbitol is exhibited by accessory reproductive organs (seminal vesicle, placenta) in several species (1, 5), and conversion of glucose to sorbitol has been documented in erythrocytes (6), lens (7), and aortic wall (8). The pathway of carbon flow from glucose to sorbitol and fructose is unclear but may involve direct reduction of glucose to sorbitol, a reaction presumably catalyzed by an enzyme termed aldose reductase (EC 1.1.1.21) (9). Alternatively, it has been suggested that the glucose metabolite, fructose 6-phosphate, may be converted to free fructose by a tissue phosphatase (10).

In recent studies (11), we have examined the fate of [U-14C]glucose in adult rat hepatocytes in primary monolayer culture (12) and in HTC cells—a cell line derived from rat hepatoma 7288c (13). After incubation of the cultures for varying periods with labeled glucose (5.5 mM), formation of labeled metabolites was analyzed quantitatively by two-dimensional paper chromatography-autoradiography. These findings demonstrated strikingly different patterns of glucose metabolism between primary cultures and HTC cells, including large differences in the amount of glucose utilized and the distribution of 14C into specific metabolic products (11). In addition, the HTC cells exhibited a unique highly labeled area, in which radioactivity increased linearly with time and which constituted a major fraction of glucose metabolites other than lactate and CO2. Hepatocytes in primary culture failed to exhibit similar labeling in the corresponding area of the chromatogram (11).

We now have identified this labeled material from HTC cells as a mixture of fructose and sorbitol. Because these co-migrate in the previously employed chromatographic system (11, 14, 15), we have employed additional procedures to separate the individual sugars. The kinetics of carbon flow into these sugars indicate that formation of sorbitol precedes that of fructose. In contrast to primary hepatocyte cultures, sorbitol and fructose are metabolized very poorly or not at all by HTC cells, which may account for accumulation of the labeled sugars in this cell type.

**Experimental Procedures**

Materials—[U-14C]Glucose (306 Ci/mol) was obtained from American and [U-14C]sorbitol (213 Ci/mol) and [U-14C]fructose (199 Ci/mol) were from New England Nuclear. The labeled sugars were supplied in ethanol/water (9:1), which was reduced to dryness under nitrogen prior to use. Glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, and ATP were from Sigma Chemical Co., St. Louis, MO. Hexokinase (yeast, type VI) was from Sigma.

Cell Cultures—Adult rat hepatocytes in primary monolayer culture were prepared as described previously (12). Hepatoma tissue culture cells were obtained as needed from stocks maintained by the Cell...
Culture facility, University of California, San Francisco. The latter cell line was free of mycoplasma (16, 17). All cultures were established in 35-mm plastic Petri dishes (Lux Scientific, Thousand Oaks, CA) coated with rat-tail collagen, in a modified Medium 199, as described elsewhere (11).

Analysis of Metabolism of 1-C-labeled Substrates by Cultured Cells—The techniques for these analyses have been described in detail previously (11, 14, 15). Briefly, labeled substrate was introduced into the culture medium at high specific activity (approximately 40 Ci/mol) and at a concentration of 5.5 mM. After a period of incubation ranging from 5 to 60 min, the labeled medium was removed and frozen for later chromatographic analysis. The cell monolayer was washed several times with a solution of sodium chloride, and the washed material was killed with 80% acetic acid, and 1-C-labeled metabolites were chromatographically analyzed. The latter was carried out by descending two-dimensional paper chromatography on Whatman No. 1 paper. The solvent system used for the first dimension consisted of H-O-saturated phenol adjusted to pH 4.4 with glacial acetic acid to which EDTA was added to a final concentration of 1 mM. Solvents for the second dimension were a 1:1 mixture of n-butyl alcohol-water (70:30) and propionic acid-water (180:20). The papers were developed for 24 h in each direction. After drying, the papers were placed on x-ray film for 1 to 2 weeks. Labeled spots, visualized by autoradiography, were cut from the paper and their radioactivity was quantitated with an automated strip-scanning apparatus (18).

This system resolves most of the labeled metabolites of glucose obtained from cultured liver cells (11). However, fructose and sorbitol, while separately cleanly from glucose itself, fail to separate from each other, necessitating the use of secondary procedures. These are described below and were employed to analyze the composition of the labeled spot revealing the position of fructose and sorbitol in the above chromatographic system.

Gas-Liquid Chromatography—This procedure was carried out on an 11-foot, 0.1-inch diameter metal column, containing 5% SE-30 on Chromosorb G, 70/80 mesh, at a temperature of 195°C and a helium flow rate of 30 ml/min (Varian 1800 Aerograph). Standard sugars and unknown C-labeled compounds eluted from paper chromatograms were converted to trimethylsilyl derivatives with Tri-$1 %$ (Pierce Chemical Co.). Five to ten microliters of solution containing approximately 5 μg of sugar were injected into the instrument. The effluent was monitored by thermoconductivity and collected in toluene for determination of radioactivity by liquid scintillation spectrometry (Packard Tri-Carb). The lag time between thermoconductivity measurements and emergence of the sample from the column was negligible. The R<sub>i</sub> values (minutes) for the sugar standards were: sorbitol, 25; glucose, 20; galactose, 18; sorbose, 18; fructose, 15; mannose, 14. By this approach, glucose, fructose, and sorbitol were well separated and could be identified unambiguously. However, the procedure was only semiquantitative because of tailing of fructose (average recovery of labeled material was 54%). Therefore, the following two procedures were introduced for separation and accurate quantification of fructose and sorbitol.

One-Dimensional Paper Chromatography—Fructose and sorbitol standards were mixed with the material eluted from the two-dimensional chromatogram, which represented the combined fructose/sorbitol radioactivity, and spotted on paper. Chromatograms were developed for 96 h in a descending solvent system consisting of n-butanol:55% ethanol:water:diethylamine (80:10:20:1). Labeled compounds were visualized by autoradiography and quantitated as in the two-dimensional chromatographic system described above. Standard sugars were visualized by dipping the chromatograms in a silver nitrate solution (100 ml of acetone, 0.5 ml of saturated AgNO<sub>3</sub>, and enough water to dissolve the precipitate) and then spraying the paper with 9% NaOH in ethanol (v/v). When the autoradiographically visualized unknown and the standard spot were superimposable, the two were considered identical. In this solvent system, fructose and sorbitol were well separated, running 26 cm and 21 cm from the origin, respectively.

Enzymatic Phosphorylation of Fructose—In a mixture of fructose and sorbitol, fructose undergoes selective phosphorylation in the presence of hexokinase and ATP. The phosphorylated compound is readily separated from free sugars by the two-dimensional paper chromatographic system. Phosphorylation of the hexose was carried out in a mixture containing 12.0 mM triethanolamine, 4.0 mM MgCl<sub>2</sub>, 1.4 mM ATP, 1.3 mM standard glucose or fructose, and 0.1 mg of hexokinase, in a volume of 0.4 ml (pH 7.6). The eluted labeled material from the fructose/sorbitol area of the chromatogram (0.1 ml) was added and incubated at 37°C for 1 h. Control experiments demonstrated that standard labeled glucose or fructose, initially subjected to the chromatographic procedure and eluted from the paper, was converted quantitatively to the corresponding monophosphate derivatives under these conditions. The incubated solution then was analyzed by two-dimensional paper chromatography. Material remaining in the free sugar area was identified as sorbitol by re-elution and co-chromatography with sorbitol standard in the one-dimensional system described above.

Other Procedures—Conversion of 1-C-labeled fructose or sorbitol to 14CO<sub>2</sub> by cultured cells was quantitated as described previously (11, 19). Sorbitol dehydrogenase (20), aldose reductase (21), and protein (22) were measured by the methods referenced.

RESULTS

Conversion of Glucose to Fructose and Sorbitol by Cultured Liver Cells—Chromatographic analysis of the glucose-derived 1-C-labeled metabolites from HTC cells revealed a unique and highly labeled spot (11). The spot was localized to the region of fructose and sorbitol, which migrate together in the standard two-dimensional system employed, as noted under "Experimental Procedures." The amount of label increased linearly over a period of 60 min (Fig. 1). By contrast, only traces of label in the same area were exhibited by chromatograms of primary hepatocyte cultures. These failed to increase with time and were present at a comparable level in nonincubated labeled culture medium, consistent with slight contamination of the [U-14C]glucose stock by labeled fructose and sorbitol.

The data of Table I indicate the magnitude of carbon flux into the fructose/sorbitol spot, relative to some other glucose metabolites in HTC cells. While lactate and CO<sub>2</sub> are the major products of glucose metabolism in these cells (11), the fructose/sorbitol spot accounts for a substantial proportion of the remaining labeled substances. Comparable data for primary cultures are included in Table I. In contrast to the analysis of cell-associated metabolites, neither of the media from the two types of culture exhibited progressive, time-dependent accumulation of 14C in the fructose/sorbitol area of the chromatogram. However, the sensitivity of this evaluation was limited by the presence of measurable "background" radioactivity in the media (due to slight impurities in the commercial preparations, as noted above).

Confirmation of the identity of the labeled spot from HTC cells as well as proof that it contained both fructose and sorbitol—was obtained by gas-liquid chromatography and a one-dimensional paper chromatographic analysis of the ma-

Fig. 1. Conversion of [U-14C]glucose to [14C]sorbitol and [14C]fructose. Labeled glucose was added to the medium of HTC monolayer cultures, as described under "Experimental Procedures." At the indicated time points, individual plates were analyzed for labeled sorbitol plus fructose.
Formation of labeled metabolites of [U-14C]glucose by liver-derived cells in culture

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Fructose</th>
<th>Hexose</th>
<th>Aspartic acid</th>
<th>Lactic acid</th>
<th>Total metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary hepatocyte</td>
<td>n.d.7</td>
<td>1.8</td>
<td>2.1</td>
<td>48.5</td>
<td>76.6</td>
</tr>
<tr>
<td>HTC cells</td>
<td>90.1</td>
<td>13.4</td>
<td>1.9</td>
<td>4526.0</td>
<td>5183.0</td>
</tr>
</tbody>
</table>

- Data for hexose monophosphate includes both glucose 6-phosphate and fructose 6-phosphate, which are present in approximately equal amounts.
- Lactic acid values represent the sum of labeled lactate in the cells and in the incubation medium.
- Total metabolites (cell + culture medium) includes 14CO2, measured as described previously (11, 19).
- n.d., not detected. Cultures were incubated for 60 min with [U-14C]glucose and then analyzed as described under "Experimental Procedures."

Separation and quantitation of fructose and sorbitol in a mixture of the compounds

HTC cells were incubated for 60 min with [U-14C]glucose. After separation of labeled metabolites by two-dimensional chromatography, the material in the fructose sorbitol area of the chromatogram was eluted and further analyzed by one of the indicated procedures, as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Method of analysis</th>
<th>Fructose</th>
<th>Sorbitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-dimensional paper chromatography (n = 4)</td>
<td>65 ± 3</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>Hexokinase phosphorylation (n = 3)</td>
<td>67 ± 2</td>
<td>39 ± 2</td>
</tr>
</tbody>
</table>

The Route of Formation of Fructose and Sorbitol in Liver Cell Culture—Postulated pathways for the flow of glucose carbon into fructose and sorbitol include direct reduction of glucose to sorbitol (9) and conversion of fructose phosphate to free fructose, a reaction presumably catalyzed by a phosphatase (10). These possibilities represent distinct routes of carbon flow, which may be distinguished experimentally. By the former route, formation of sorbitol would precede that of fructose, whereas the reverse would hold true for a pathway involving a fructose phosphatase. As an approach to this question, we examined the time-course of transfer of 14C from glucose to sorbitol or fructose. As shown in Fig. 2, 14C appeared predominantly in sorbitol at early time points (2 to 10 min) after addition of labeled glucose to the culture medium. With longer periods of incubation, 14C appeared increasingly in fructose until approximatly 65% of the total label was in fructose. This represents an apparent equilibrium, in that the ratio of [14C]fructose/[14C]sorbitol was essentially unchanged at 2 h of incubation. These data indicate that labeling of sorbitol precedes that of fructose. Since this pathway presumably involves aldose reductase, this enzyme activity was assayed, with xylose as substrate (Table III). Comparable activities were exhibited with glucose as substrate, although much higher concentrations of the substrate were required (400 to 800 mM). The data indicate that primary hepatocyte cultures and liver homogenate, as well as HTC cells, contain aldose reductase activity. Sorbitol dehydrogenase, which mediates the conversion of sorbitol to fructose, was readily detectable in HTC cells, primary culture, and liver homogenate. Fructose phosphatase activity was assayed in whole cell sonicate with [U-14C]fructose 6-phosphate as substrate, and the products of the reaction were isolated by the standard two-dimensional paper chromatographic technique (see "Experimental Procedures"). The results (not shown) indicated that all cultures

Table III

Aldose reductase in extracts of whole liver or liver-derived cultured cells

<table>
<thead>
<tr>
<th>Source of extract</th>
<th>Km (mM)</th>
<th>Vmax (nmol NADPH oxidized × min × mg−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole liver</td>
<td>250</td>
<td>2.6</td>
</tr>
<tr>
<td>Primary culture</td>
<td>125</td>
<td>3.2</td>
</tr>
<tr>
<td>HTC cells</td>
<td>75</td>
<td>8.3</td>
</tr>
</tbody>
</table>

For the reason that, in crude extracts, free phosphate could result in part from conversion of fructose 6-phosphate to glucose 6-phosphate and dephosphorylation of the latter compound (25). Both primary cultures and HTC cells exhibited measurable glucose-6-phosphate activity (unpublished observations).
Fructose Production by Liver Cells

FIG. 3. Metabolism of [U-14C]sorbitol by liver-derived cells in culture. Cultures were prepared, as described elsewhere (11), and studied at confluence. Labeled sorbitol (30 μCi/ml) was introduced into culture medium containing 5.5 mM glucose. After 60 min of incubation, cultures were processed for paper chromatography, as described under "Experimental Procedures." Top panel, adult rat hepatocytes in primary monolayer culture; bottom panel, HTC cells. Abbreviations: O, origin; UDPGA, uridine-5'-diphosphoglucuronic acid; FDP, fructose 1,6-diphosphate; UDPG, uridine-5'-diphosphoglucone; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 3-PGA, d-phosphoglyceric acid; GA, glucuronic acid; PEP, phospho(enol)pyruvate; GP, glycerol 3-phosphate; ASP, aspartic acid; CIT, citric acid; MAL, malic acid; PUM, fumaric acid; GLUT, glutamic acid; GLUC, glucose; GLN, glutamine; ALA, alanine; LAC, lactic acid.

were capable of converting fructose 6-phosphate to free fructose. Thus, this process—although seemingly unlikely from the results of the kinetic studies shown in Fig. 2—cannot be excluded on the basis of enzymatic analysis.

Utilization of Fructose and Sorbitol by Liver Cell Cultures—In cells incubated with [U-14C]glucose, accumulation of labeled fructose or sorbitol could reflect overproduction of the sugars, a block in their utilization, or a combination of these factors. Utilization of uniformly labeled [14C]fructose or [14C]sorbitol was examined, both by paper chromatographic analysis and by production of 14CO2. With either approach, HTC cells exhibited minimal metabolism of the labeled sugars. By contrast, hepatocytes in primary culture converted sorbitol to numerous labeled intermediates (Fig. 3); similar autoradiograms (not shown) were obtained from cultures incubated with [U-14C]fructose. These data were confirmed by measurement of 14CO2 production from labeled fructose or sorbitol (Fig. 4).

DISCUSSION

The findings of this study document production of fructose and sorbitol by HTC cells, an established cell line derived from a rat hepatoma. The sugars were identified by paper chromatography in two different solvent systems, by gas-liquid chromatography, and by the selective modification of fructose in a hexose-phosphorylating system. With regard to the route of formation of fructose and sorbitol, kinetic analysis of the flow of 14C from glucose into these compounds indicated clearly that sorbitol is formed prior to fructose. The formation of fructose, therefore, presumably occurs by oxidation of sorbitol in the reaction mediated by sorbitol dehydrogenase (4).

The fact that sorbitol and fructose are formed in HTC cells does not necessarily establish a basis for the progressive accumulation of these sugars, which was linear for periods of incubation up to 2 h (Fig. 1) (2). This finding is compatible with the existence of large pools of fructose and sorbitol to which glucose carbon makes only a minor contribution over an extended time period (14) or with poor metabolism of these sugars by HTC cells. As a test of the latter possibility, the conversion of labeled fructose and sorbitol to their respective metabolites was examined and found to be negligible in HTC cells. It is reasonable to infer from these data that sorbitol and fructose accumulate in HTC cells because their further metabolism is blocked.

The failure of HTC cells to metabolize sorbitol and fructose suggests that these cells lack the ability to carry out the normal liver functions of fructose phosphorylation and/or conversion of fructose 1-phosphate to triose phosphates (2). The consequences of this loss of function are unknown at the present time. However, it has been shown that administration of exogenous fructose in vivo alters lipid metabolism in liver, shifting the metabolism of fatty acids in the direction of triglyceride formation while decreasing their oxidation (2, 3). It is reasonable to assume that endogenous fructose would exert similar effects if exogenous and endogenous fructose are compared.

1 G. A. Levine, M. J. Bissell, and D. M. Bissell, unpublished data.
metabolized by the same route. Thus, the block in fructose metabolism in HTC cells may be associated with altered lipid metabolism.

A major question presented by these studies concerns the extent to which findings in HTC cells can be extrapolated to the liver in primary culture or *in vivo*. Production of fructose and sorbitol in tissue culture may be peculiar to established cell lines. However, this process is absent from BRL cells, a nonneoplastic cell line from rat liver (24) in which glucose metabolism closely resembles that in HTC cells, apart from formation of sorbitol and fructose (11). Since HTC cells were derived from tumor tissue, the process may be specific for neoplastic cells. However, a second tumor cell line—7800 cells—derived also from rat hepatoma (25) failed to exhibit conversion of labeled glucose to sorbitol or fructose. These data suggest that a capacity for the formation of these sugars is linked neither to extended propagation of cells in culture nor to cells of neoplastic origin.

Alternatively, the capacity for conversion of glucose to sorbitol and fructose may be present in normal liver and fortuitously retained by HTC cells. Despite being broadly "de-differentiated," HTC cells demonstrate individual characteristics of the intact liver, including steroid-inducible tyrosine aminotransferase and glucose-6-phosphatase activities (13, 26). If the capacity for the formation of sorbitol and fructose falls into this category of retained hepatic functions, the fact that it is absent from other liver-derived cell lines is not surprising. Liver-derived cells are remarkably heterogeneous with regard to their pattern of tissue-specific functions (27), and the preservation of a particular characteristic in an individual cell line may occur as a random process. If indeed the formation of free sorbitol and fructose is a normal hepatic function, the lack of detectable pools in the primary cultures may be due to small steady state pool sizes brought about by the ability of hepatocytes in primary culture to rapidly dispose of these sugars (Figs. 3 and 4).

To evaluate further whether the formation of fructose and sorbitol by HTC cells might be unique to this cell type or rather a characteristic of normal liver, we assayed enzyme activities that appear to be related to this process. Although dephosphorylation of fructose occurred in HTC cells, in hepatocyte primary cultures, and in liver homogenate, the kinetic studies of fructose and sorbitol formation with HTC cells indicated that formation of sorbitol preceded formation of fructose. Also, preliminary experiments with inhibitors of sorbitol dehydrogenase (4) have confirmed that the correct sequence is glucose, sorbitol, and then fructose. Therefore, our attention was directed at an examination of aldose reductase. This enzyme is active towards an extraordinarily broad range of substrates (1, 7) with the available data suggesting the existence of a single enzyme only (7). Both HTC cells and normal liver contain this activity, the findings for liver confirming earlier studies (28). Regardless of the source of the enzyme, its $K_s$ with glucose as substrate far exceeded the physiological concentrations of glucose (5.5 mm) introduced in these studies. The quantitative differences between aldose reductase from HTC cells and from liver, while present, may not provide a basis for assuming that production of sorbitol and fructose is unique to HTC cells. Rather, the major difference between the cell line and normal liver may be the inability of HTC cells to metabolize fructose or sorbitol. On this basis, the conversion of glucose to sorbitol and fructose by liver remains a distinct possibility, one with potentially important implications for hepatic lipid metabolism (2).

**REFERENCES**

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