2-DESOXY-D-GLUCOSE METABOLISM IN LEUCONOSTOC MESENTEROIDES

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Received for publication January 7, 1955

2-Desoxy-d-glucose has been reported to inhibit glucose fermentation by yeast cells, as determined by CO₂ formation, although exhibiting little effect upon glucose respiration or upon glucose fermentation by cell-free yeast extracts (Cramer and Woodward, 1952; Woodward, 1952). The inhibitor was not degraded under the conditions employed. These authors also demonstrated the phosphorylation of 2-desoxy-d-glucose by yeast hexokinase preparations in the presence of adenosine triphosphate; thus, the sugar analogue is not completely inert at the enzymatic level.

During investigations into the nature of the hexose monophosphate pathway of Leuconostoc (DeMoss et al., 1951, 1953), it seemed likely that experiments with phosphorylated analogues of the actual metabolic intermediates might be useful in elucidating the precise nature of the reactions involved. The equivalent pathway in yeast has been postulated (Horecker and Smyrniotis, 1950) to involve 3-keto-6-phosphogluconate as the intermediate between 6-phosphogluconate and ribulose-5-phosphate. It was anticipated that the results of analogue experiments might aid in more positive identification of the intermediate compound although this aim was not attained in fact.

The present report describes some experiments with 2-desoxy-d-glucose and its derivatives, using Leuconostoc mesenteroides as the biological agent.

MATERIALS AND METHOD

Leuconostoc mesenteroides strain 39 was grown, harvested, and extracted as previously described (DeMoss et al., 1951). Crude gluconokinase preparations were extracted by sonic disintegration of cells grown in the same medium, with the exception that potassium gluconate replaced glucose as the energy source. The glucose-6-phosphate dehydrogenase preparation was described previously (DeMoss et al., 1953). Yeast hexokinase, prepared according to the method of Berger et al. (1946), was a gift of Dr. M. Pullman.

Ca₃(PO₄)₂ gel was prepared according to the method of Keilen and Hartree (1938). Protein was estimated by the spectrophotometric method of Warburg and Christian (1941).

2-Desoxy-d-glucose, generously furnished by Dr. F. B. Cramer, was determined by a modification of the diphenylamine reaction of Dische (1930). For a discussion of this method, see Racker (1952). To a 2.0 ml sample containing 20-170 μg 2-desoxy-d-glucose were added 4.0 ml diphenylamine reagent (1 g dissolved in 100 ml glacial acetic acid plus 2.75 ml concentrated H₂SO₄). After mixing, the solution was held 50 min in a boiling water bath, cooled to room temperature, and read at 515 mμ in an Evelyn colorimeter. Figure 1 shows the spectrum of the color obtained. The effect of heating time is depicted in figure 2, while figure 3 illustrates the standard curve obtained with increasing amounts of 2-desoxy-d-glucose. After the experiments were concluded, a method for determination of 2-desoxyglucose involving 3,5-diaminobenzoic acid dihydrochloride was described by Cramer and Neville (1953). 2-Desoxy-d-glucose may be separated from glucose chromatographically, using Whatman no. 1 paper in a solvent consisting of ethanol: methanol: formic acid: water (8:6:1:6 v/v), and detected by the p-anisidine hydrochloride or diphenylamine spray reagents of Hough et al. (1950). 2-Desoxy-d-glucose was prepared by bromine oxidation of 2-desoxy-d-glucose.

Color reactions are also given by 2-desoxy-glucose with resorcinol (Roe, 1943) and cysteine-carbazole (Dische and Borenfreund, 1951) as shown in figure 1.

RESULTS AND DISCUSSION

Preparation of 2-desoxy-d-glucose-6-phosphate.

The results of Cramer and Woodward (1952)
2-DESOXY-D-GLUCOSE METABOLISM

Figure 1. Spectra of 2-desoxy-D-glucose reactions with diphenylamine (157 µg sugar, 10 min heating period), resorcinol (328 µg sugar), and cysteine-carbazole (164 µg sugar).

Figure 2. 2-Desoxy-D-glucose-diphenylamine reaction as a function of heating time (43.8 µg sugar).

were confirmed by manometric demonstration of 2-desoxy-D-glucose phosphorylation catalyzed by highly purified yeast hexokinase in the presence of adenosine triphosphate and Mg++. The phosphorylation is also demonstrable with cell-free extracts of L. mesenteroides. On the basis of analogy with glucose phosphorylation, it is assumed that the product of the kinase reaction is 2-desoxy-D-glucose-6-phosphate.

A mixture of 600 units yeast hexokinase, 1,000 µmoles adenosine triphosphate, 1,500 µmoles MgCl₂, 500 µmoles NaHCO₃, and 500 µmoles 2-desoxy-D-glucose in a total volume of 21.5 ml was incubated at 37°C until CO₂ evolution ceased. Without further treatment, the reaction mixture was passed repeatedly through a 100 by 34 mm diameter Dowex-50-X2 (200-400 mesh) column until the eluate was free of adenosine derivatives, as indicated by a lack of absorption at 260 nm. The Dowex-50 column had first been washed thoroughly with 2 N HCl until the eluate was free of 260 nm absorbing material, and subsequently with water until free of Cl⁻. The resulting solution, containing 2-desoxy-D-glucose-6-phosphate but no adenine or ribose derivatives, was adjusted to pH 8.0, diluted to 200 ml with distilled water, and passed through a 150 by 34 mm diam Dowex-1-X2 (200-400 mesh) column. The column was washed with 100 ml water and the 2-desoxy-D-glucose-6-phosphate eluted with a solution containing 0.01 N formic acid and 0.03 M sodium formate. Figure 4 illustrates the behavior of the phosphate ester according to analyses of the eluate fractions with the diphenylamine reagent. To the combined eluate fractions containing 350 µmoles 2-desoxy-D-glucose-6-phosphate was added 0.55 ml of 25 per cent barium acetate. The pH was adjusted to 8.2, and 4
volumes of 95 per cent ethanol were added. After standing at 2 C for several hours, the precipitate was collected by centrifugation, washed successively with 95 per cent ethanol, absolute ethanol, and ether, and dried in vacuo over CaCl₂. From phosphorus and 2-desoxy-D-glucose determinations, the dried salt (125 mg) was calculated to be 95 per cent barium-2-desoxy-D-glucose-6-phosphate, representing a 65 per cent yield, based on the initial amount of sugar added.

Triphosphopyridine nucleotide reduction. Crude enzyme preparations from L. mesenteroides were tested for ability to catalyze pyridine nucleotide reduction with 2-desoxy-D-glucose-6-phosphate or glucose-6-phosphate as substrate. The data of table 1 show that only triphosphopyridine nucleotide is reduced by the desoxy analogue. It should be pointed out that L. mesenteroides cells contain a glucose-6-phosphate dehydrogenase which is nonspecific in its pyridine nucleotide requirement.

Partial separation of dehydrogenases. An arbitrary fractionation procedure was designed to demonstrate separation, rather than purification, of the enzymes responsible for the triphosphopyridine nucleotide reductions observed (table 1).

All manipulations were performed at 2 C or in an ice bath.

A suspension of 5 g (wet weight) freshly harvested L. mesenteroides cells in 50 ml 0.1 M K₂HPO₄ was treated for 20 min in a 200 watt Raytheon sonic oscillator, centrifuged 30 min at 9,000 × G, and the supernatant dialyzed overnight against 4 L 0.05 M K₂HPO₄ (fraction A). Fraction A was adjusted to pH 6.0, with n-acetic acid, and the nucleic acid precipitated by addition of 0.05 volume M MnCl₂. The supernatant obtained after centrifugation was fractionally precipitated by addition of solid (NH₄)₂SO₄. Fractions J and L, representing the precipitates obtained at 0.50-0.65 and 0.65-0.80 saturation, respectively, were combined and treated with Ca₃(PO₄)₂ gel (0.5 mg/mg protein). After centrifugation, the supernatant (fraction M) containing the total activity was again treated with Ca₃(PO₄)₂ gel (5 mg/mg protein) and centrifuged, yielding fraction O, the supernatant. The sedimented gel was successively eluted with 0.1 M phosphate buffers of pH 6.5 (fraction P), and pH 7.0 (fraction Q). Analysis of the various fractions obtained (table 2) demonstrated partial separation of the two enzymes.

Specificity of the dehydrogenase. The fraction O analysis shows that 2-desoxy-D-glucose-6-phosphate dehydrogenase has been almost completely removed from the glucose-6-phosphate dehydrogenase. Other purified glucose-6-phosphate de-
hydrogenase preparations from *L. mesenteroides* were similarly low in activity with 2-desoxy-D-glucose-6-phosphate as substrate. Fraction Q represents an effective enrichment of 2-desoxy-D-glucose-6-phosphate dehydrogenase activity. From these results, it may be concluded that 2-desoxy-D-glucose-6-phosphate is not a substrate for the glucose-6-phosphate dehydrogenase of fraction O. However, since in fraction Q, the glucose-6-phosphate dehydrogenase activity is greater than that of 2-desoxy-D-glucose-6-phosphate dehydrogenase, at least two possibilities exist: (1) a triphosphopyridine nucleotide specific dehydrogenase is present and capable of catalyzing oxidation of either glucose-6-phosphate or 2-desoxy-D-glucose-6-phosphate, (2) the triphosphopyridine nucleotide specific, 2-desoxy-D-glucose-6-phosphate specific dehydrogenase is contaminated with the pyridine nucleotide nonspecific, glucose-6-phosphate specific dehydrogenase.

### Table 2

**Partial separation of dehydrogenases from Leuconostoc mesenteroides**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity*</th>
<th>G6P</th>
<th>2DG6P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Crude dialyzed extract</td>
<td>0.06</td>
<td>3.9</td>
<td>65.0</td>
</tr>
<tr>
<td>J (NH₄)SO₄ ppt (0.5-0.65)</td>
<td>0.29</td>
<td>18.7</td>
<td>64.5</td>
</tr>
<tr>
<td>L (NH₄)SO₄ ppt (0.65-0.8)</td>
<td>0.50</td>
<td>33.3</td>
<td>66.6</td>
</tr>
<tr>
<td>N 1st Ca₃(PO₄)₂ gel supernatant</td>
<td>0.47</td>
<td>25.8</td>
<td>54.9</td>
</tr>
<tr>
<td>O 2nd Ca₃(PO₄)₂ gel supernatant</td>
<td>0.03</td>
<td>37.9</td>
<td>263.0</td>
</tr>
<tr>
<td>P Ca₃(PO₄)₂ gel eluate, pH 6.5</td>
<td>0.08</td>
<td>2.1</td>
<td>26.2</td>
</tr>
<tr>
<td>Q Ca₃(PO₄)₂ gel eluate, pH 7.0</td>
<td>1.06</td>
<td>5.0</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Assay protocol: 150 μmoles Tris(hydroxymethyl)aminomethane, pH 7.5; 10 μmoles MgCl₂; 0.5 μmoles triphosphopyridine nucleotide; 20 μmoles 2-desoxy-D-glucose-6-phosphate or 10 μmoles glucose-6-phosphate; 0.1-0.5 units of enzyme; total volume, 3.0 ml. The reaction was followed as the increase in extinction (E) at 340 mμ after addition of substrate.*

*Specific activity (μmol ΔA₄₁₂/TPN per min per mg protein) was calculated from the values obtained during the 15-30 sec interval after initiation of the reaction.*

As shown in Table 2, the specific activity of the 2-desoxy-D-glucose-6-phosphate dehydrogenase increases in fractions O, P, and Q, while that of glucose-6-phosphate dehydrogenase decreases. These data are insufficient for the conclusion that the 2-desoxy-D-glucose-6-phosphate dehydrogenase is not active with glucose-6-phosphate as substrate.

Whether or not the enzyme in question is specific for 2-desoxy-D-glucose-6-phosphate has not been determined. 2-Desoxyhexoses apparently have not been reported to occur natu-

### Table 3

**Effect of 2-desoxy-D-glucose and 2-desoxy-D-glucose-6-phosphate on triphosphopyridine nucleotide reduction by glucose-6-phosphate**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ΔEm per Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>2DG, 10 μM</td>
<td>0.008</td>
</tr>
<tr>
<td>2DG6P, 10 μM</td>
<td>0.007</td>
</tr>
<tr>
<td>G6P, 10 μM</td>
<td>0.074</td>
</tr>
<tr>
<td>G6P + 2DG</td>
<td>0.074</td>
</tr>
<tr>
<td>G6P + 2DG6P</td>
<td>0.082</td>
</tr>
</tbody>
</table>

*Protocol: As for Table 1, except crude dialyzed extract was replaced by 2 μg glucose-6-phosphate dehydrogenase (specific activity 51.2).*

### Table 4

**Gluconokinase and 6-phosphogluconate dehydrogenase activities in Leuconostoc mesenteroides cell-free extracts**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>μM CO₂ per Hour*</th>
<th>ΔEm per Min†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2-Desoxy-D-glucinate</td>
<td>2.2</td>
<td>0.0</td>
</tr>
<tr>
<td>D-Glucinate</td>
<td>5.2</td>
<td>0.220</td>
</tr>
</tbody>
</table>

*Protocol: * Warburg, 30 C, 5 per cent CO₂-95 per cent N₂ atmosphere. Main compartment: 99 μmoles NaHCO₃; 20 μmoles adenosine triphosphate; 10 μmoles MgCl₂; 0.1 ml dialyzed cell-free extract of gluconate grown *L. mesenteroides*; total volume 1.8 ml. Side arm: 10 μmoles substrate.

† Beckman spectrophotometer, 18 C, 100 μmoles tris(hydroxymethyl)aminomethane, pH 7.5; 0.45 μmoles diphosphopyridine nucleotide; 10 μmoles adenosine triphosphate; 30 μmoles MgCl₂; 0.1 ml dialyzed cell-free extract of gluconate grown *L. mesenteroides*; 10 μmoles substrate; total volume, 3.0 ml.
Cell-free extracts of *Leuconostoc mesenteroides* contain an enzyme which catalyzes reduction of triphosphopyridine nucleotide by 2-desoxy-D-glucose-6-phosphate. The enzyme can be partially separated from a glucose-6-phosphate dehydrogenase which is not active on the 2-desoxy analogue.

2-Desoxy-D-gluconate phosphorylation is catalyzed by gluconokinase in crude extracts from gluconate adapted *L. mesenteroides*. The product of phosphorylation does not effect diphosphopyridine nucleotide reduction in an extract which contains active 6-phosphogluconate dehydrogenase.

REFERENCES


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**SUMMARY**

2-Desoxy-D-glucose-6-phosphate has been prepared and isolated by enzymatic and ion exchange procedures.


