2-DESOXY-D-GLUCOSE METABOLISM IN LEUCONOSTOC MESENTEROIDES

R. D. DeMOSS AND M. E. HAPPEL

McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland

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 Keilin 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 H₃SO₄). After mixing, the solution was held 50 min in a boiling water bath, cooled to room temperature, and read at 515 μm 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-gluconate 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)

1 Contribution no. 107 from the McCollum-Pratt Institute. This investigation was supported in part by a research grant (G-3852) from the National Institutes of Health, Public Health Service.
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Figure 1. Spectra of 2-desoxy-D-glucose reactions with diphenylamine (157 \( \mu \)g sugar, 10 min heating period), resorcinol (328 \( \mu \)g sugar), and cysteine-carbazole (164 \( \mu \)g sugar).

Figure 2. 2-Desoxy-D-glucose-diphenylamine reaction as a function of heating time (43.8 \( \mu \)g 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 \( \mu \)moles adenosine triphosphate, 1,500 \( \mu \)moles MgCl\(_2\), 500 \( \mu \)moles NaHCO\(_3\), and 500 \( \mu \)moles 2-desoxy-D-glucose in a total volume of 21.5 ml was incubated at 37 C until CO\(_2\) 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 \( \mu \)moles 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

\[ \mu g \ 2-\text{DESOXY-D-GLUCOSE} \]

Figure 3. The range of 2-desoxy-D-glucose concentration determinable in the diphenylamine reaction.
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-o-glucose determinations, the dried salt (125 mg) was calculated to be 95 per cent barium-2-desoxy-o-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-o-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).

![Diagram](http://jb.asm.org/)

**Figure 4.** Elution of 2-desoxy-o-glucose-6-phosphate from a Dowex-1-X2 column. Determined by diphenylamine method.

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>Substrate</th>
<th>ΔE₄₅₀ per Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN</td>
<td>G6P, 10 μM</td>
<td>0.035</td>
</tr>
<tr>
<td>TPN</td>
<td>G6P, 10 μM</td>
<td>0.037</td>
</tr>
<tr>
<td>DPN</td>
<td>2DG6P, 30 μM</td>
<td>0.0</td>
</tr>
<tr>
<td>TPN</td>
<td>2DG6P, 6 μM</td>
<td>0.010</td>
</tr>
<tr>
<td>TPN</td>
<td>2DG6P, 30 μM</td>
<td>0.017</td>
</tr>
</tbody>
</table>

**Protocol:** 150 μmoles Tri(hydroxymethyl)-aminomethane, pH 7.8; 10 μmoles MgCl₂; 0.5 μmoles di- or triphosphopyridine nucleotide; glucose-6-phosphate (G6P) or 2-desoxy-o-glucose-6-phosphate (2DG6P) as indicated; 0.1 ml crude dialyzed extract; total volume 3.0 ml. The reaction was followed as the increase in optical density at 340 mμ after addition of substrate.

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-o-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 still lower 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>2DG6P</th>
<th>G6P</th>
<th>2DG</th>
<th>GAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Crude</td>
<td>0.06</td>
<td>3.9</td>
<td>65.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J (NH4)SO4</td>
<td>0.29</td>
<td>18.7</td>
<td>64.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L (NH4)SO4</td>
<td>0.50</td>
<td>33.3</td>
<td>66.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N 1st Ca3(PO4)2 gel supernatant</td>
<td>0.47</td>
<td>25.8</td>
<td>54.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O 2nd Ca3(PO4)2 gel supernatant</td>
<td>0.03</td>
<td>37.0</td>
<td>283.0</td>
<td></td>
<td></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>
<td></td>
<td></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>
<td></td>
<td></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 nm after addition of substrate.*

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 nat-

**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>ΔE660 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>μmol CO2 per Hour*</th>
<th>ΔE660 per Min†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2-Desoxy-d-glucurate</td>
<td>2.2</td>
<td>0.0</td>
</tr>
<tr>
<td>n-Glucurate</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; 0.48 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.
ural in biological systems; thus 2-desoxy-D-glucose may represent an artificial substrate in the present studies. However, the possibility exists that 2-desoxypentose phosphate may be active as a substrate for the dehydrogenase. A crude preparation of adenine desoxyribose-5'-phosphate was tested as substrate, both before and after hydrolysis in n HCl. No triphosphopyridine nucleotide reduction was observed in either test. The limited amount of desoxyribotide precluded purification, and the negative result may have been due to some inhibitory substances present in the preparation. The question remains open concerning a possible role of the enzyme in desoxyribose metabolism.

In view of the inhibitory effect of 2-desoxy-D-glucose on glucose fermentation by yeast cells observed by Cramer and Woodward (1952), the effect of the analogue on glucose-6-phosphate dehydrogenase was tested. As shown in table 3, neither 2-desoxy-D-glucose nor the phosphorylated derivative inhibits significantly the glucose-6-phosphate dehydrogenase purified from L. mesenteroides, as measured by triphosphopyridine nucleotide reduction.

Studies on 2-desoxy-D-gluconate-6-phosphate. The product of the 2-desoxy-D-glucose-6-phosphate dehydrogenase reaction has not been isolated but is presumed to be 2-desoxy-D-gluconate-6-phosphate.

Using crude cell-free extracts of gluconate grown L. mesenteroides, the phosphorylation of 2-desoxy-D-gluconate by adenosine triphosphate could be demonstrated manometrically (table 4). However, when the phosphorylated product was incubated with diphosphopyridine nucleotide, no reduction was observed spectrophotometrically. Under the same conditions, D-gluconate-6-phosphate did effect diphosphopyridine nucleotide reduction (table 4). These results indicate that the diphosphopyridine nucleotide specific 6-phosphogluconate dehydrogenase from L. mesenteroides does not catalyze dehydrogenation of 2-desoxy-D-gluconate-6-phosphate.

SUMMARY

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

DIsCHe, Z. 1930 Über einige neue charakteristische Farbrreaktionen der Thymonukleinäsure und eine Mikromethode zur Bestimmung derselben in tierischen Organen mit Hilfe dieser Reaktionen. Mikrochemie, 8, 4-32.
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