ANAEROBIC GLYCOLYSIS BY ENZYME PREPARATIONS OF BRUCELLA SUIS

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The applicability to bacteria of the Embden-Meyerhof scheme of carbohydrate dissimilation has been investigated by many workers, but evidence for the occurrence of phosphorylated hexose intermediates in anaerobic glycolysis by bacteria has been incomplete and indirect. Virtanen and Tikka (1) isolated what they believed to be a hexose monophosphate from a system containing phosphate, glucose, and dried Escherichia coli cells. Phosphoglyceric acid has been isolated as an intermediate in the dissimilation of glucose by Aerobacter, Citrobacter, Escherichia, Propionibacterium, Serratia, and other genera (2-8).

Utter and Werkman (9) investigated the aldolase and isomerase equilibria with enzyme preparations of E. coli. Bard and Gunsalus (10) reported that ferrous ions are required for the function of aldolase prepared from Clostridium perfringens. The occurrence of aldolase suggests that the Embden-Meyerhof system functions in these organisms.

The rôle of phosphate in carbohydrate metabolism has been studied with resting cells of Streptococcus faecalis by O’Kane and Umbreit (11) and with Propionibacterium pentosaceum by Barker and Lipmann (8).

Doudoroff et al. (12) obtained evidence for the formation of glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate when dried cell preparations of a non-glucose-fermenting E. coli mutant metabolized maltose under anaerobic conditions. Colorimetric techniques were used, however, to identify and estimate the amounts of phosphorylated intermediates formed.

Leloir and coworkers (13-16) investigated the activity of glucose-1,6-diphosphate in the reaction between glucose-1-phosphate and glucose-6-phosphate when catalyzed by phosphoglucomutase. Glucose diphosphate was considered the coenzyme in this reaction and was formed by partially purified enzyme preparations from E. coli, Aerobacter aerogenes, and Klebsiella pneumoniae acting on glucose-1-phosphate. These workers postulated that glucose diphosphate was formed by transphosphorylation between 2 molecules of glucose-1-phosphate.

The coenzyme involved in the conversion of galactose-1-phosphate to
glucose-1-phosphate has been shown to contain uridine, two phosphate groups, and glucose (17). The coenzyme has been found in animal tissues and yeast not adapted to galactose and these workers suggest that it may have other functions besides being the coenzyme of "galactomald Enase."

The work reported here concerns the hexose phosphate esters which occur in the anaerobic dissimilation of glucose and galactose by cell-free enzyme preparations of Brucella suis. Techniques involving paper partition chromatography and ion exchange resins reported by us (18) were utilized to show that hexose phosphate esters were formed.

**Methods and Materials**

*Cultural and Enzymatic Methods*—Cultures of *B. suis* were grown for approximately 40 hours on a shaker at 37° in Fernbach flasks which were fitted with rubber stoppers, each holding three cotton-plugged thistle tubes and a siphon arrangement. The medium was composed of 2 per cent of casein acid partial hydrolysate, 1 per cent of glucose, and 0.2 per cent of autolysed yeast. Each flask contained 500 ml. of medium which provided 3 to 4 gm. of wet cell paste.

Because of the hazard involved in pipetting or pouring viable cells of *B. suis* from Fernbach flasks, the culture was siphoned into a receiving flask and dispensed into centrifuge tubes by means of a second siphon system. The cells were washed twice in m/15 phosphate buffer, pH 7.0, and centrifuged in duraluminum cups at approximately 3400 X g for ½ hour. The cells were finally resuspended in m/15 phosphate buffer, pH 8.0, so that 5 gm. of cells (wet weight) were contained in 20 ml. of liquid. The cells were stored at 5° until used.

The enzyme preparation was made in the Raytheon oscillator by treating 20 ml. of cell suspension at maximum voltage output for ½ hour at 9000 cycles. The pH of the cell suspension changed to 7.4 during sonic treatment. Ice water was circulated through the instrument during operation and the enzyme preparation was kept cold until it was used. Cell debris and residual intact cells were removed by centrifugation at 23,000 X g for 1 hour at 5°. In several experiments a lower centrifugation speed, 5000 X g, was employed in an attempt to increase enzyme activity. Very few intact cells remained in suspension after 1 hour's centrifugation at 5000 X g, although some of the larger particulates remained in the preparation. Nitrogen analysis showed that the sonic treatment converted approximately 75 per cent of the cell nitrogen to a "soluble" form which remained in the supernatant solution on centrifugation.

1 Yeast-75, Vito Products Company, Chicago, Illinois.
2 Raytheon Manufacturing Company, Waltham, Massachusetts.
Fractionations and Chemical Analyses—The products of fermentation were fractionated with barium acetate and alcohol as recommended by Umbreit et al. (19). Preliminary chemical analyses of the isolated fractions were made prior to the identification of the phosphorylated intermediates by paper chromatography.

The phosphorylated intermediates were assayed colorimetrically after separation into a barium-insoluble fraction (containing fructose diphosphate) and a barium-soluble, alcohol-insoluble fraction (containing the glucose monophosphates and fructose monophosphate).

Reducing values were determined by the method of Folin and Malmros (20).

Fructose esters were determined by the method of Roe (21). The amount of each of the fructose esters was calculated by the use of factors. Fructose esters were hydrolyzed at 42° in 1 N hydrobromic or hydrochloric acid for 3 weeks. This technique permitted the hydrolysis of fructose esters without appreciable destruction of the liberated fructose (18). Identification of the ketose portion of the ester as fructose was subsequently accomplished by paper chromatography.

The Fiske and Subbarow method (22) or its modification by King (23) was used to assay the inorganic, total, and 7 minute-hydrolyzable phosphate.

EXPERIMENTAL

The experiments were carried out in 125 ml. Erlenmeyer flasks which contained the following components of the enzyme-substrate system: 4 or 8 mg. per ml. of glucose or galactose, 0.008 M adenosinetriphosphate, 0.004 M MgSO4, 0.00016 M MnSO4, 0.00008 M FeSO4, 0.0064 M NaHCO3, 0.053 M NaF, and 12.5 ml. of enzyme preparation. The final volume was 24.9 ml.

1 ml. of the contents of each of the flasks was removed immediately after the addition of the enzyme and mixed with an equal volume of 10 per cent trichloroacetic acid in a volumetric flask. This sample was used for zero time determinations. The experimental flasks were flushed for 15 minutes with a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide and then closed during incubation. Incubation was at 37° for 4 hours on the shaking apparatus.

The enzyme action was stopped by adding an equal volume of cold 10 per cent trichloroacetic acid, and after centrifugation the material was fractionated with barium acetate and alcohol. The phosphorylated hexose compounds in the fractions were identified by chromatographic techniques reported by us (18).
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Results

The results of the analytical determinations by colorimetric and chromatographic methods (Tables I and II) show that enzyme preparations of *B. suis* metabolize glucose and galactose under anaerobic conditions to the initial hexose phosphate intermediates of the Embden-Meyerhof scheme. Approximately 15 per cent of the added substrate was metabolized. As shown in Table I, the hexose phosphates accounted for most of the metabolized substrate. Adenosinetriphosphate was essential in the system; in its absence only a small amount of substrate disappeared and only small amounts of phosphorylated intermediates were formed (Table I). In early experiments it was found that greater amounts of the phosphorylated esters were produced when the incubation time was increased from 1 to 4 hours and the substrate concentration increased from 100 to 200 mg.

The results obtained by paper chromatographic methods of analysis are shown in Table II. Fructose diphosphate was identified in the barium-insoluble fraction with either glucose or galactose as the substrate. Fructose-6-phosphate was found in the barium-insoluble, alcohol-insoluble fraction and identified by the specific ketose reagent and by paper chromatography. The fructose was identified by the ketose reagent on the paper chromatogram at *R*<sub>f</sub> 0.51 following the mild hydrolysis of the barium fractions in 1 N acid at 42° for 3 weeks. The pure fructose controls had *R*<sub>f</sub> values of 0.52.

Glucose-1-phosphate was characterized by the lability of the phosphate and the appearance of a glucose spot on the paper chromatogram. This ester was found when either glucose or galactose was the substrate. As shown in Table II, well defined spots at *R*<sub>f</sub> 0.38 were found on a chromatogram of the concentrate of the sugar liberated by hydrolysis in 1 N acid at 100° for 9 minutes; control spots of glucose gave the same *R*<sub>f</sub> value. Analytical determinations of easily hydrolyzable phosphate and reducing sugar were made on the barium-insoluble, alcohol-insoluble fraction. Although increases were observed, the ratios of phosphorus to reducing sugar were not consistent from one experiment to another; in some instances the inorganic phosphate in the fraction was high. Glucose-6-phosphate, found in the same fraction with glucose-1-phosphate, fructose-6-phosphate, and pentose phosphate, was partially hydrolyzed by 1 N acid in 3 hours at 100°. Chromatographic analysis of the hydrolysate when the aldose reagent was used showed four well defined spots (Table II). The brown spot at *R*<sub>f</sub> 0.38 was the same in color and *R*<sub>f</sub> as that of the control of pure glucose, indicating that glucose-6-phosphate was partially hydrolyzed by the 3 hour treatment. The brown spot of the hydrolysate at *R*<sub>f</sub> 0.08
### Table I

**Anaerobic Dissimilation of Carbohydrate by Enzyme Preparations of Brucella suis**

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Substrate</th>
<th>Adenosine-triphosphate (mM)</th>
<th>Reducing values (glucose equivalents)</th>
<th>Fructose diphosphate</th>
<th>Fructose mono-phosphate</th>
<th>Barium-soluble, alcohol-insoluble fraction (mg.)</th>
<th>Barium-insoluble, alcohol-insoluble fraction (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose</td>
<td>0.2</td>
<td>Initial: 98 mg.</td>
<td>2.6</td>
<td>0.8</td>
<td>17 mg.</td>
<td>6.2 mg.</td>
</tr>
<tr>
<td>1</td>
<td>Galactose</td>
<td>0.2</td>
<td>Metabolized: 98 mg.</td>
<td>6.9</td>
<td>0.2</td>
<td>13 mg.</td>
<td>2.6 mg.</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>0.2</td>
<td>Control: 1 mg.</td>
<td>2.7</td>
<td>0.2</td>
<td>1 mg.</td>
<td>0.8 mg.</td>
</tr>
<tr>
<td>4</td>
<td>Glucose</td>
<td>0.2</td>
<td>Before 7 min. hydrolysis: 199 mg.</td>
<td>24.5</td>
<td>6.8</td>
<td>31 mg.</td>
<td>9.6 mg.</td>
</tr>
<tr>
<td>4</td>
<td>Galactose</td>
<td>0.2</td>
<td>After 7 min. hydrolysis: 31 mg.</td>
<td>20.1</td>
<td>6.8</td>
<td>31 mg.</td>
<td>10.5 mg.</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>0</td>
<td>Control: 1 mg.</td>
<td>5.9</td>
<td>1.1</td>
<td>1 mg.</td>
<td>0.2 mg.</td>
</tr>
<tr>
<td>0</td>
<td>None</td>
<td>0</td>
<td>Control: 1 mg.</td>
<td>2.0</td>
<td>0.2</td>
<td>0 mg.</td>
<td>0.2 mg.</td>
</tr>
</tbody>
</table>

The experiments were carried out in flasks which contained, in addition to the adenosinetriphosphate and substrate, the following substances: 0.004 M MgSO₄, 0.00016 M MnSO₄, 0.00008 M FeSO₄, 0.0004 M NaHCO₃, 0.053 M NaF, and 12.5 ml. of enzyme preparation. The final volume was 24.9 ml. The flasks were incubated with shaking at 37° under an atmosphere of 5 per cent carbon dioxide and 95 per cent nitrogen.

### Table II

**Chromatographic Identification of Phosphorylated Sugar Esters**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Hydrolysis in N HCl</th>
<th>Reagent</th>
<th>RF of unknown</th>
<th>Control</th>
<th>Compound</th>
<th>Compound indicated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barium-insoluble</td>
<td>42°, 3 wks.</td>
<td>Ketose</td>
<td>0.51</td>
<td>0.52</td>
<td>Fructose</td>
<td>Fructose diphosphate</td>
</tr>
<tr>
<td>Barium-insoluble</td>
<td>42°, 3 “”</td>
<td>“”</td>
<td>0.51</td>
<td>“”</td>
<td>“”</td>
<td>Fructose-6-phosphate</td>
</tr>
<tr>
<td>Barium-insoluble</td>
<td>100°, 9 min.</td>
<td>Aldose</td>
<td>0.38</td>
<td>0.38</td>
<td>Glucose</td>
<td>Glucose-1-phosphate</td>
</tr>
<tr>
<td>Barium-insoluble</td>
<td>100°, 3 hrs.*</td>
<td>“”</td>
<td>0.38</td>
<td>“”</td>
<td>“”</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>Barium-insoluble</td>
<td>100°, 3 “”</td>
<td>“”</td>
<td>0.08</td>
<td>0.08</td>
<td>“”</td>
<td>“”</td>
</tr>
<tr>
<td>Barium-insoluble</td>
<td>100°, 3 “”</td>
<td>“”</td>
<td>0.59</td>
<td>0.59</td>
<td>Ribose</td>
<td>Ribose phosphate</td>
</tr>
<tr>
<td>Barium-insoluble</td>
<td>100°, 3 “”</td>
<td>“”</td>
<td>0.20</td>
<td>“”</td>
<td>“”</td>
<td>“”</td>
</tr>
</tbody>
</table>

The chromatograms were developed for 20 hours at room temperature, 24-27°, with 75 per cent aqueous phenol and Whatman No. 1 paper.

* The 3 hour hydrolysis was carried out on the fraction reprecipitated by barium and alcohol after the initial 9 minute hydrolysis.
was the unhydrolyzed glucose-6-phosphate which had leaked through the anion exchanger, as previous experience had demonstrated. The other spots, which had the typical reddish brown color of aldopentoses, appeared at \( R_f \) 0.59 and 0.20 and were found to be due to pentose and pentose phosphate respectively. Analyses of materials eluted from parallel chromatograms in the area of \( R_f \) 0.20 gave a pentose to phosphorus ratio of 6.4 (theoretical value, 5). These pentose spots probably arose from the adenylic acid which was originally present in the barium-soluble, alcohol-insoluble fraction. The pentose and purine values of the fraction were equivalent.

The brown spot at \( R_f \) 0.08 was glucose-6-phosphate (18). Ultraviolet spectrophotometric assays at \( \lambda \) 2600 made on the area of \( R_f \) 0.08 disclosed the absence of purines and, therefore, the absence of nucleotide phosphorus. Pentose was not found in significant amounts and fructose esters would have been destroyed by the 3 hour hydrolysis.

**DISCUSSION**

Although the phosphorylated hexose esters of the Embden-Meyerhof scheme were identified following the anaerobic dissimilation of glucose and galactose by enzyme preparations of \( B. \) suis, there is no assurance that other metabolic routes are not also in operation in the intact cell. It is generally recognized that enzyme preparations such as those employed in this study merely show enzymic capability and may not necessarily portray the exact sequence of events that takes place in the living cell.

The technique of preparing enzymes by sonic means has been considered by some investigators to be quite harsh and capable of destroying certain enzyme systems. The sonic disintegration of cells was the only permissible means of preparing enzymes in this work because of safety considerations.

**SUMMARY**

Phosphorylated hexose esters of the Embden-Meyerhof scheme are formed during the initial steps of anaerobic dissimilation of glucose and galactose by enzyme preparations of \( B. \) suis. The phosphorylated intermediates were identified by techniques employing paper partition chromatography and ion exchange resins. Adenosinetriphosphate was essential in the anaerobic system which also contained substrate, \( \text{Mg}^{++}, \text{Mn}^{++}, \text{Fe}^{++}, \) phosphate buffer, and sodium fluoride. Evidence was obtained for the occurrence of glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate.
BIBLIOGRAPHY

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