THE SYNTHESIS OF GLUCOSE-6-PHOSPHATE AND 6-PHOSPHOGluCONATE

BY J. E. SEEGMILLER AND B. L. HORECKER

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Maryland)

(Received for publication, March 26, 1951)

In studies of the enzymatic oxidation of 6-phosphogluconate, relatively large quantities of this substance in high purity were required. This led to a search for a convenient preparation of glucose-6-phosphate. Available methods for the synthesis of glucose-6-phosphate, reviewed by Lardy et al. (1, 2), are laborious and not well suited to the preparation of large quantities of this substance.

A procedure has now been developed, based on the phosphorylation of glucose with polyphosphoric acid. Pyrophosphoric acid has been reported to act as a phosphorylating agent for high molecular weight alcohols (3). More recently polyphosphoric or pyrophosphoric acid has been used by Cherbuliez and Weniger (4) in the preparation of phosphoenolpyruvic acid, phosphocholine, phosphoethanolamine, and glycerophosphoric acid. In the direct phosphorylation of glucose with polyphosphoric acid, esterification occurs not only at the 6 position but at other positions as well. Since glucose-6-phosphate is resistant to acid hydrolysis (5), other esters formed could be removed by this means with little loss of glucose-6-phosphate. Selective hydrolysis with acid has been used by LePage and Umbreit (6) for the preparation of glucose-6-phosphate from Embden ester.

The oxidation of glucose-6-phosphate to 6-phosphogluconate with excess bromine for 48 hours at room temperature according to Robison and King (7) resulted in a product which, by enzymatic assay, was only 50 to 70 per cent pure and contained much inactive esterified phosphate. The yield of 6-phosphogluconate was correspondingly low. A study of the rate of oxidation of glucose-6-phosphate by bromine confirmed the finding of Warburg and Christian (8) that glucose-6-phosphate was completely oxidized in 1 to 2 hours at room temperature. With the enzymatic assay described above, the quantitative formation of 6 phosphogluconate could be demonstrated and a pure product obtained in high yield.

**Methods**

**Materials**—Tetraphosphoric (polyphosphoric) acid was supplied by the Monsanto Chemical Company.

Triphosphopyridine nucleotide (TPN) (purity 86 per cent) was pre-
pared by a method involving purification by ion exchange chromatography of a crude liver fraction. Glucose-6-phosphate dehydrogenase (Zwischenferment) free of phosphogluconic dehydrogenase was prepared by the method of Kornberg (9). Phosphogluconic dehydrogenase was purified from yeast (10).

Determinations—The assay for glucose-6-phosphate was carried out with 20 μM (micromoles) of MgCl₂, 0.13 μM of TPN, and Zwischenferment (0.13 mg. of protein) in 0.04 M glycylglycine buffer, pH 7.4, in a total volume of 1.55 cc. The change in density at 340 μM on addition of glucose-6-phosphate was measured with the Beckman spectrophotometer, and the concentration was calculated from the extinction coefficient of 6.22 × 10⁶ cm.² mole⁻¹ (11). 6-Phosphogluconate was determined in the same manner, except that Zwischenferment was replaced by phosphogluconic dehydrogenase (0.3 mg. of protein). Inorganic phosphate was determined by the method of Fiske and Subbarow (12). Total phosphate was measured by ashing with H₂SO₄-HNO₃ mixture.

Preparation of Glucose-6-Phosphate

Phosphorylation—40 cc. of water were added to 400 gm. of anhydrous polyphosphoric acid and the mixture was stirred mechanically in an ice bath until the temperature had dropped to 5-10°. 174 gm. of anhydrous glucose (0.97 mole) were added without delay; the mixture was stirred slowly for 16 hours at room temperature (25°) and the reaction stopped by the addition of 1 liter of water. This solution contained 132 mM of glucose esterified at the 6 position, determined on an aliquot after hydrolysis at 100° for 45 minutes in 2.4 N hydrobromic acid.

Removal of Inorganic Phosphate—520 gm. of anhydrous sodium carbonate were added to the reaction mixture with mechanical stirring. To aid in the removal of CO₂ the warm mixture (about 60°) was evacuated on the water pump for several minutes. The neutralized solution was diluted with 2.6 liters of water, the pH now 7.3 to 7.6, and cooled overnight in the cold room at 3° with stirring. To reduce the rate of cooling, the reaction mixture was placed in a 10 liter water bath which was initially about 40°. The dense slurry of sodium polyphosphate crystals which resulted was cooled in an ice bath with stirring for 4 to 6 hours and filtered with suction; the crystals were permitted to drain thoroughly. By this procedure 85 to 90 per cent of the inorganic phosphate was removed. The filtrate contained 112 mM of ester hydrolyzable to glucose-6-phosphate.

Hydrolysis—The filtrate (2.5 liters) was treated with 554 cc. of concentrated HBr (8.7 N), refluxed for 16 hours, and cooled. 93 mM of glucose-6-phosphate remained.

1 A. Kornberg and B. L. Horecker, unpublished.
Precipitation of Barium Salts—The brown hydrolysis mixture was treated at room temperature with 500 gm. of barium carbonate, with capryl alcohol to reduce foaming. This mixture was stirred for 4 hours, during which time the pH rose to 6.2 to 6.4, and filtered. The residue was washed twice by suspending it in 100 cc. portions of water. The filtrate and washings containing the barium salt of glucose-6-phosphate were combined and treated with 4 volumes of 95 per cent ethanol, and the flocculant precipitate was allowed to settle. The precipitate was centrifuged, washed with 1 liter of 95 per cent ethanol, and packed hard on the centrifuge. The precipitate containing barium glucose-6-phosphate was extracted four times with 150 cc. portions of water and the insoluble brown residue discarded. A precipitate which formed when the extracts were combined was dissolved by the further addition of water and the solution was decolorized at room temperature for 2 hours with 1 to 2 gm. of activated charcoal (Merck). The filtrate was treated with 4 volumes of 95 per cent ethanol and the precipitate was washed with absolute ethanol and ether and dried in air. This product contained no inorganic phosphorus and 83 to 93 per cent of the organic phosphate was glucose-6-phosphate. The purity of the dry barium salt, based on enzymatic assay, was 77 to 89 per cent and the yield was 36 to 39 gm. (73 mM). Some further purification was effected by conversion to the potassium salt.

Preparation of Potassium Salt—5 gm. of the barium salt dissolved in 30 cc. of water were converted to the free acid by passage through an Amberlite cation exchange resin (IR-100, hydrogen ion form) in a column 37 cm. × 8.3 sq. cm. The glucose 6 phosphoric acid was washed through with water at a rate of 5 cc. per minute. Recovery was essentially quantitative in the first 500 cc. following the appearance of acid in the effluent. The effluent was evaporated to a thick syrup at 15 mm. pressure in a 35-40° bath. This syrup was dissolved in 400 cc. of 95 per cent ethanol and chilled, and 0.1 x KOH in absolute methanol was added with stirring until the supernatant became green to added brom thymol blue. The precipitate was centrifuged, washed with absolute ethanol and ether, and dried in vacuo. On the assumption that the product was principally the monopotassium salt, the purity of the dry salt by enzymatic assay was 82 to 90 per cent. If some dipotassium salt were present, the purity would be higher. The potassium salt was somewhat hygroscopic and less convenient to store than the barium salt.

Yield and Purity—The original phosphorylated mixture contained very little glucose-6-phosphate as determined by enzymatic assay, presumably because the glucose was esterified at several positions or because of the presence of pyrophosphoric ester linkages. As is shown in Fig. 1, glucose-6-phosphate was formed during the early stages of acid hydrolysis, while
the total organic phosphate decreased. In this experiment, glucose-6-phosphate accounted for 98 per cent of the total esterified phosphate at the conclusion of hydrolysis.

![Graph showing hydrolysis of phosphorylated glucose.](image)

**Fig. 1.** Hydrolysis of phosphorylated glucose. The hydrolysis was carried out in 1.6 N HBr essentially as described in the preparation procedure. Glucose-6-phosphate was determined on neutralized aliquots by enzymatic assay as described in the text. Organic phosphate was determined on the aliquot after removal of inorganic phosphate by neutralizing with barium carbonate and barium hydroxide. A small correction for loss due to coprecipitation was calculated from the loss of glucose-6-phosphate.

**Table I**

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Glucose added</th>
<th>Ba glucose-6-phosphate</th>
<th>Purity*</th>
<th>Glucose-6-phosphate Total phosphate (\times 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>10.6</td>
<td>81</td>
<td>88</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>10.0</td>
<td>85</td>
<td>93</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>14.8</td>
<td>89</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>15.9</td>
<td>80</td>
<td>86</td>
</tr>
<tr>
<td>5</td>
<td>174</td>
<td>38.9</td>
<td>77</td>
<td>83</td>
</tr>
<tr>
<td>6</td>
<td>174</td>
<td>36.1</td>
<td>79</td>
<td>85</td>
</tr>
</tbody>
</table>

* Corrected for moisture loss in a high vacuum at 57°. These determinations were made in the microanalytical laboratory of the National Institutes of Health under the supervision of Dr. W. C. Alford.

The phosphorus content of the barium salt was 7.15 to 7.28 per cent compared with the theoretical value of 7.85 per cent. On this basis it was 91 to 93 per cent pure and compared favorably with other preparations described. However, during the operations following acid hydrolysis, a small part of the glucose-6-phosphate was apparently converted to esters...
inactive in the highly specific enzymatic test. One-third to one-half of this inactive phosphate has been identified as fructose-6-phosphate. The yield and purity of the final product in a number of preparations are shown in Table I.

Preparation of 6-Phosphogluconate

20 gm. of barium glucose-6-phosphate (purity 79 per cent) were dissolved in 142 cc. of water and the small insoluble residue centrifuged. To the supernatant were added 23.6 gm. of barium carbonate and 2.8 cc. of bromine, and the mixture was kept for 2 hours at room temperature with frequent shaking. Excess bromine was removed by aeration and barium carbonate filtered and washed. The filtrate and washings were adjusted to pH 3.5 by the addition of 3.1 cc. of glacial acetic acid and precipitated by the addition of 940 cc. of ethanol. The mixture was cooled to 0° and centrifuged, and the precipitate was washed with 200 cc. of 80 per cent ethanol and dried in vacuo over potassium hydroxide and calcium chloride. A small additional amount of 6-phosphogluconate was recovered from the supernatant by treating it with saturated barium hydroxide solution (124 cc.) until it was pink to phenolphthalein, and the precipitate was centrifuged, washed with 80 per cent ethanol, and dried.

The dried precipitates were combined and dissolved in 1 liter of water with the aid of 3.5 cc. of glacial acetic acid. Helium was passed through the acidified solution to remove remaining carbon dioxide. With continued aeration with helium, 179 cc. of saturated barium hydroxide were slowly added until a permanent pink phenolphthalein end-point was reached. 354 cc. of absolute ethanol were added, and, after 1 hour at room temperature, the precipitate was filtered with suction, washed with ethanol, and dried. 20.3 gm. of barium 6-phosphogluconate were obtained. By enzymatic assay with phosphogluconic dehydrogenase, the purity was 92 to 96 per cent, corrected for moisture loss in a high vacuum at 77°. The yield was 85 to 90 per cent. Enzymatic assay with Zwischenferment placed the glucose-6-phosphate content at less than 0.5 per cent. 6-Phosphogluconate accounted for 99 per cent of the total phosphate.

The authors are indebted to Dr. C. W. Sondern of the White Laboratories for the initial suggestion that pyrophosphoric acid be used as a phosphorylating agent.

SUMMARY

Glucose-6-phosphate has been synthesized by direct phosphorylation with polyphosphoric acid. By this method 36 to 39 gm. of the barium salt can readily be prepared in a single laboratory scale run.
The purity of the barium salt based on its phosphorus content was 91 to 93 per cent, but in the specific enzymatic test a part of this phosphate was found to be inactive. The purity based on enzymatic assay was 77 to 89 per cent.

Barium 6-phosphogluconate prepared in 85 to 90 per cent yield from barium glucose-6-phosphate by a modification of the method of Robison and King was 92 to 96 per cent pure and free of other phosphate esters.

BIBLIOGRAPHY

THE SYNTHESIS OF
GLUCOSE-6-PHOSPHATE AND
6-PHOSPHOGLUCONATE
J. E. Seegmiller and B. L. Horecker


Access the most updated version of this article at http://www.jbc.org/content/192/1/175.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/192/1/175.citation.full.html#ref-list-1