HOG KIDNEY GLUCONOKINASE

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Previous studies (1, 2) have indicated that gluconate is extensively metabolized in the mammal without prior conversion to glucose. The interpretation of experiments performed with labeled gluconate has rested upon the assumption (3) that gluconate is converted to 6-phosphogluconate in the course of its metabolism. The enzyme, gluconokinase, which catalyzes this reaction has been partially purified from Escherichia coli (4) and yeast (5), but has not previously been demonstrated in mammalian systems. In the present study, the phosphorylation of gluconate according to Equation 1 has been shown to occur in extracts of mammalian tissues and the enzyme, gluconokinase, has been partially purified from hog kidney.

\[
\text{Gluconate} + \text{ATP} + \text{Mg}^{++} \rightarrow 6\text{-phosphogluconate} + \text{ADP}
\]

Equation 1

A brief summary of some of these results has appeared (6).

Materials and Methods

The following nucleotides, except where otherwise noted in the text, were obtained from either the Sigma Chemical Company or the Pabst Laboratories: ITP, UTP, GTP, ADP, DPNH, TPN, and crystalline ATP. Phosphoenol pyruvate was prepared by an unpublished procedure of Mr. William E. Pricer of this Institute. It contained no detectable free pyruvate. 6-Phosphogluconate was prepared by bromine oxidation of glucose-6-phosphate (7). We are indebted to Dr. Nelson K. Richtmyer and Dr. James W. Pratt of this Institute for crystalline salts or lactones of the following sugar acids: L-gluconate, D-arabonate, L-arabonate, D-xylonate, D-alronate, D-talonate, D-allonate, D-gulonate, D-mannone, and D-glycero-D-glucoheptonate. Glucurononlactone was obtained from the Corn Products Refining Company and potassium gluconate from the Mallinckrodt Chemical Works. Phosphogluconic dehydrogenase was prepared from brewers' yeast through the ammonium sulfate step as described by Horricker and

1 The following abbreviations are used: ATP, adenosine triphosphate; UTP, uridine triphosphate; ITP, inosine triphosphate; GTP, guanosine triphosphate; ADP, adenosine diphosphate; IDP, inosine diphosphate; TPN, triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine.
Smyrniotis (8). It was most stable when stored in the cold as a lyophilized powder. Crystalline lactic dehydrogenase was obtained from the Worthington Biochemical Corporation. This preparation contained a very active phosphoenolpyruvic kinase and traces of ATPase and adenylate kinase. Hexokinase was obtained from the Pabst Laboratories and glucose-6-phosphate dehydrogenase from the Sigma Chemical Company. ATP was determined by means of the combined action of hexokinase and glucose-6-phosphate dehydrogenase (9) and ITP by the modification of this method described by Berg and Joklik (10). ADP and IDP were determined by the phosphoenolpyruvic kinase-lactic dehydrogenase method described by Kornberg and Pricer (11).

RESULTS AND DISCUSSION
Purification of Enzyme

Assay—In the routine assay, a unit of enzyme is defined as that quantity which forms 1 μmole of 6-phosphogluconate in 10 minutes at 34°. 6-Phosphogluconate was determined spectrophotometrically by means of 6-phosphogluconic dehydrogenase essentially as described by Horricker and Smyrniotis (8). The assay mixture contained 40 μmoles of maleate buffer, pH 6.2, 6 μmoles of MgCl₂, 10 μmoles of ATP, 2.5 μmoles of gluconate, and the enzyme preparation to be assayed in a total volume of 1 ml. The reaction was carried out at 34° for 10 minutes and was stopped by heating the mixture in a boiling water bath for 2 minutes. A control incubated without gluconate was included in each assay. To measure the 6-phosphogluconate formed, an aliquot (usually 0.2 ml.) was added to a 1.0 cm. spectrophotometer cell containing 50 μmoles of glycylglycine buffer, pH 7.5, 1.5 μmoles of TPN, 6 μmoles of MgCl₂ and water to make 0.97 ml. An initial reading was made at 340 μm, and 0.03 ml. (0.1 mg. of protein) of 6-phosphogluconic dehydrogenase was added. The increase in optical density at 340 μm was used to calculate the amount of 6-phosphogluconate synthesized on the basis of an extinction coefficient of 6.22 × 10⁸ sq. cm. per mole (12) for reduced pyridine nucleotides.

An alternative spectrophotometric assay combining the action of gluconokinase and 6-phosphogluconic dehydrogenase was used in several experiments. Relatively large amounts of dehydrogenase were required in this procedure and therefore it was necessary that this preparation be essentially free from yeast gluconokinase. In this assay a unit of enzyme may be defined as that amount which causes an optical density change of 1.0 per minute at 340 μm in a 1.0 cm. cell. The reaction mixture contained 1.5 μmoles of TPN, 6 μmoles of MgCl₂, 40 μmoles of maleate buffer, pH 6.2, 2 mg. of 6-phosphogluconic dehydrogenase, 10 μmoles of ATP, 2.5
μmoles of gluconate, and the enzyme to be assayed in a total volume of 1.0 ml. The reaction was started by the addition of the latter enzyme and readings were made at 1 minute intervals for several minutes.

Protein was determined by the method of Bücher (13). Proportionality between enzyme concentration and activity was obtained with both assays. The proportionality of the routine assay procedure is indicated in Fig. 1, A. Fig. 1, B demonstrates that the reaction rate is linear with time when measured by this procedure. Throughout this paper, enzymatic activity is expressed in terms of the routine assay unit of Fig. 1, A.

**Purification of Enzyme**

Gluconokinase activity was present in dialyzed extracts of rat kidney and liver. Hog kidney had approximately the same activity as that of the rat and provided a more convenient starting material. The results of a typical fractionation are presented in Table I.

Four to five kidneys which had been stored at −15° were thawed and
the cortices were separated. 450 gm. of cortex were treated in the Waring blender with 700 ml. of 0.1 M phosphate buffer, pH 7.4, for 2 minutes. The suspension was centrifuged at 2° for 40 minutes at 4700 r.p.m. The turbid supernatant fluid was carefully decanted and the residue reextracted in the Waring blender for 1 minute with 750 ml. of cold water. The mixture was centrifuged as above, the supernatant fluid combined with the first extract, and the total volume brought to 1700 ml. with cold water (Crude extract, Table I).

First Acetone Fractionation—The crude extract was adjusted to pH 5.0 with approximately 21 ml. of 2 N acetic acid and, after 5 minutes at 3-5°, the pH was brought to 5.5 with 4 ml. of 5 N KOH. The precipitate which formed in this process was not centrifuged. The mixture was chilled to 0°, and 800 ml. of acetone, previously cooled to −10° to −15°, were added over a period of 30 minutes. The mixture was cooled to −8° during the addition and, after 5 minutes at this temperature, the precipitate was centrifuged and discarded. Two successive fractionations were made at −10° with 172 ml. of acetone each. These additions took 5 minutes and the mixtures were stirred for 5 minutes before centrifugation. The precipitates were discarded and the supernatant fluid (approximately 2500 ml.) was treated with 1340 ml. of acetone added over a period of 10 minutes at −11° to −12°. After being stirred for 5 minutes, the precipitate was collected by centrifugation, dissolved in approximately 150 ml. of 0.02 M phosphate buffer, pH 7.4, and twice dialyzed for 2 hours against 2 liters of this buffer. This fraction (First acetone Fraction IV) may be frozen overnight with less than 3 per cent loss in activity.

Ammonium Sulfate Fractionation—Acetone Fraction IV was diluted with water to contain 10 mg. of protein per ml. and the pH adjusted to 7.5 with 2.5 M ammonium hydroxide. A precipitate which sometimes formed at this point was removed by centrifugation. The solution (304 ml.) was

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**Table 1**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units*</th>
<th>Specific activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>755</td>
<td>0.009</td>
</tr>
<tr>
<td>1st acetone Fraction IV</td>
<td>340</td>
<td>0.125</td>
</tr>
<tr>
<td>Ammonium sulfate Fraction II</td>
<td>180</td>
<td>0.32</td>
</tr>
<tr>
<td>2nd acetone Fraction II</td>
<td>100</td>
<td>0.64</td>
</tr>
</tbody>
</table>

* A unit is defined as that amount of enzyme which forms 1 μmole of 6-phosphogluconate in 10 minutes at 34° under the conditions described in the text.

† Expressed as units per mg. of protein.

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2 Glass-distilled H2O was used throughout.
brought to 48 per cent saturation by the addition of 84 gm. of solid ammonium sulfate over a period of 40 minutes, the pH being kept at 7.4. The precipitate was centrifuged and discarded. The supernatant solution (325 ml.) was brought to 56 per cent saturation with 15.3 gm. of ammonium sulfate as above and, after centrifugation, the precipitate was dissolved in 23 ml. of 0.02 M phosphate buffer, pH 7.4 (Ammonium sulfate Fraction II).

Second Acetone Fractionation—The ammonium sulfate fraction was dialyzed overnight against 10 liters of slowly flowing distilled water. Usually less than 10 per cent of the activity was lost during this operation. Water was added to the dialyzed enzyme to give a solution (116 ml.) containing 3 to 5 mg. of protein per ml. The pH was adjusted to 5.4 with a few drops of 0.1 N acetic acid and 62.5 ml. of acetone, chilled to $-10^\circ$, were added over a period of 10 minutes. During the addition, the mixture was cooled to $-8^\circ$ and, after 5 minutes, the mixture was centrifuged and the precipitate discarded. 39.4 ml. of acetone were added to the supernatant solution (165 ml.) at $-10^\circ$ over a 15 minute period and, after being stirred for 5 minutes, the precipitate was collected by centrifugation and dissolved in 0.025 M glycylglycine buffer, pH 7.4 (15.5 ml., Second acetone Fraction II).

The final preparation contained a highly active adenylate kinase and nucleoside diphosphokinase (14). Various preparations contained little or no ATPase and no hexokinase activity. When stored for 3 weeks, the enzyme lost less than 10 per cent of its activity at $-15^\circ$ and approximately 50 per cent at 3$^\circ$. Preparations which had been frozen and thawed sometimes became slightly turbid at pH 6.2. The component causing this turbidity could be removed by centrifugation at pH 5.5 with little loss in gluconokinase activity.

Identity of Product

The identification of the product as 6-phosphogluconate through its activity as substrate for 6-phosphogluconic dehydrogenase was confirmed by paper chromatography. The barium salt of the presumed 6-phosphogluconate was isolated by repeated alcohol precipitation and chromatographed, in parallel with synthetic 6-phosphogluconate, in the methanol-formic acid and methanol-ammonia solvents described by Bandurski and Axelrod (15). Both substances gave identical $R_F$ values of 0.32 and 0.57 in the respective solvents. When mixed with synthetic 6-phosphogluconate (7), a single spot of the same $R_F$ was obtained.

Properties of Enzyme

Effect of pH—The enzyme had maximal activity at pH 6.2 (Fig. 2). The same pH optimum was obtained in either maleate or phosphate buffer.
Fig. 2. pH optimum of gluconokinase. The routine assay of Fig. 1, A was used, except that each tube contained 80 μmoles of phosphate buffer.

Fig. 3. ATP-Mg++ relationship. Each tube contained 0.75 unit of enzyme, specific activity 0.64. ATP was included at three levels: 0.005 M, ▲; 0.001 M, ●; and 0.0001 M, ○. The Mg++ concentration was varied as indicated. The other components were the same as in the routine assay of Fig. 1, A. Incubations were performed at room temperature for 10 minutes.

Metal Activation—In the absence of added divalent cations, different preparations of the enzyme exhibited from less than 1 to approximately 10 per cent of the maximal activity observed in the presence of Mg++. Mn++,

Zn$^{++}$, Co$^{++}$, and Ca$^{++}$ could substitute for Mg$^{++}$ with decreasing effectiveness, the last being one-third as active as Mg$^{++}$.

**Mg$^{++}$-ATP Relationship**—At given ATP levels, maximal activity was obtained when the ratio of Mg$^{++}$ to ATP was 1:2. At higher relative concentrations, Mg$^{++}$ was inhibitory (Fig. 3). This relationship of the concentrations of Mg$^{++}$ to ATP corresponds to that found by Hers (16) for fructokinase at low concentrations of alkaline cations. High levels of ATP, tested in the presence of optimal Mg$^{++}$ concentrations, were found to accelerate the reaction beyond that predicted for Michaelis-Menten (17) kinetics (Fig. 4). This activating effect may be due to the removal of an inhibitory metal ion by the formation of a complex with ATP (18), as suggested by the data on Cu$^{++}$ ion inhibition discussed below.

**Inhibition of Gluconokinase**—The data presented in Table II indicate that the enzyme is inhibited by Cu$^{++}$ ions and by p-chloromercuribenzoate. Experiment 2 demonstrates that 0.01 M ATP partially protects the enzyme from Cu$^{++}$ ion inhibition. That the lack of protection in the presence of 0.0001 M ATP is not owing to the concomitant decrease in the Mg$^{++}$ ion concentration is demonstrated in Experiment 3, which shows that the protective effect of 0.01 M ATP is enhanced when the concentration of Mg$^{++}$ ions is reduced. These results are consistent with the suggestion mentioned above that the activating effect of high concentrations of ATP might be owing to the binding of a heavy metal contaminant in the test system.
The inhibition of gluconokinase by p-chloromercuribenzoate was not prevented by the presence of Mg$^{++}$ plus gluconate or Mg$^{++}$ plus ATP. Fluoride (0.01 M) and iodoacetate (0.001 M) did not inhibit the enzyme.

**Substrate Specificity**—The purified enzyme is devoid of activity toward all of a number of substances tested except d-gluconate. The specificity was studied by testing for the formation of ADP in the presence of the test substrate by means of the lactic dehydrogenase-pyruvic kinase test system. Because the purified enzyme contained a very active adenylate kinase and AMP was present as an impurity, an initial formation of ADP was always observed in the absence of substrate. This reaction was allowed to go to completion over a period of 5 to 10 minutes before the test substrate was added. A typical test is illustrated in Fig. 5. The following compounds have been tested and found to be without activity as substrates: D-glycerate, D-glucose, D-glucuronate, L-gluconate, D-arabonate, L-arabonate, D-altronate, D-talonate, D-allonate, D-gulonate, D-galactonate,

### Table II

**Inhibition of Gluconokinase**

Each tube contained approximately 0.12 unit of gluconokinase, specific activity 0.58. In the first three experiments, the conditions were the same as in the routine assay, except for the variation in the concentrations of ATP and Mg$^{++}$ as indicated. In Experiment 4, the enzyme was preincubated with p-chloromercuribenzoate for 10 minutes in the presence of 50 μmoles of glycylglycine buffer, pH 7.0, at 34°. The components of the routine assay were then added to start the incubation. All the tubes were incubated for 15 minutes.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Inhibitor</th>
<th>Inhibitor concentration</th>
<th>ATP μmole per l.</th>
<th>MgSO4 μmole per l.</th>
<th>6-Phosphogluconate formed μmole per l.</th>
<th>Inhibition per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cupric acetate</td>
<td>0.1</td>
<td>1.0</td>
<td>5</td>
<td>0.18</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>0.5</td>
<td>1.0</td>
<td>5</td>
<td>0.089</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>0.1</td>
<td>1.0</td>
<td>0.05</td>
<td>0.063</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>p-Chloromercuri-</td>
<td>0.0005</td>
<td>0.001</td>
<td>0.05</td>
<td>0.074</td>
<td>0</td>
</tr>
</tbody>
</table>

...
Fig. 5. Substrate specificity of gluconokinase. Each cuvette contained 40 μmoles of maleate buffer, pH 6.2, 0.5 μmole of ATP, 0.25 μmole of MgSO₄, 1 mg. of lactic dehydrogenase-pyruvic kinase, 0.4 μmole of phosphoenol pyruvate, 0.15 μmole of DPNH, and 0.15 unit of gluconokinase, specific activity 0.64. At the first arrow, 2.5 μmoles of test substrate (n-xylonate, ○; d-glycero-d-glucoheptonate, ●) were added to two cuvettes and H₂O to a third. The final volume was 1 ml. At the second arrow, H₂O and 2.5 μmoles of d-gluconate were added as indicated. Corrections have been made for density changes owing to dilution at the points of addition.

Fig. 6. Effect of gluconate concentration. The conditions were as described for the spectrophotometric assay. Each cuvette contained 0.11 unit of enzyme, specific activity 0.6.
d-mannionate, d-xylionate, and d-glycero-d-glucoheptonate. The compounds tested include all the d-hexonic acids except idonic acid. The adaptive gluconokinase of E. coli has also been found (4) to exhibit strict specificity for d-gluconate.

Maximal activity was obtained with gluconate concentrations of approximately $1.7 \times 10^{-3}$ M, and the $K_i$ estimated from the Lineweaver-Burk plot was $1.4 \times 10^{-4}$ M (Fig. 6). By combining the action of gluconokinase with 6-phosphogluconic dehydrogenase, gluconate could be quantitatively determined as illustrated in Fig. 7. The reaction was run at pH 7.0, which is intermediate between the pH optima of the two enzymes. Because of the presence of hexokinase and glucose-6-phosphate dehydrogenase in the 6-phosphogluconic dehydrogenase preparation, this procedure cannot be directly applied to the analysis of gluconate in the presence of glucose.

![Figure 7](http://www.jbc.org/)
Nucleotide Specificity—UTP, ITP, and GTP were active in the gluconokinase reaction; the activities relative to ATP were 1.3, 0.6, and 0.25, respectively. The interpretation of these results was complicated by the presence of nucleoside diphosphokinase in the purified enzyme and the likelihood that ADP was a contaminant of the nucleotides used. A sample of enzyme, treated with Dowex 1 chloride to remove any ADP which might be associated with the enzyme, was tested with UTP which had been chromatographed on Dowex 1 (20) and was free from adenine nucleotides. Although the treatment with the resin resulted in a 37 per cent loss in activity, the ratio of the activity of UTP to ATP was unchanged.

TABLE III
Stoichiometry of Gluconokinase Reaction

All the tubes contained 40 μmoles of maleate buffer, pH 6.2, and 6 μmoles of MgCl₂. In addition, the complete system contained, in Experiment 1, 1.8 μmoles of gluconate, 1.95 μmoles of ITP, and 0.5 unit of gluconokinase and, in Experiment 2, 3.1 μmoles of gluconate, 3.7 μmoles of ITP, and 1.0 unit of gluconokinase. The final volume was 1.0 ml. After incubation at 34° for approximately 30 minutes, the reaction was stopped by heating at 100° for 2 minutes. Aliquots were removed and assayed for ITP, IDP, and 6-phosphogluconate as described under “Materials and methods,” and for free gluconate as described in Fig. 7. All the values are expressed as micromoles.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Conditions</th>
<th>Δ 6-phosphogluconate</th>
<th>Δ gluconate</th>
<th>Δ ITP</th>
<th>Δ IDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Complete</td>
<td>+0.90</td>
<td>-0.93</td>
<td>-0.80</td>
<td>+0.90</td>
</tr>
<tr>
<td></td>
<td>Gluconate omitted</td>
<td>0</td>
<td>0</td>
<td>-0.07</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Complete</td>
<td>+2.46</td>
<td>-2.30</td>
<td>-2.20</td>
<td>+2.20</td>
</tr>
<tr>
<td></td>
<td>Gluconate omitted</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Reaction Stoichiometry

Because of the presence of adenylic kinase in the purified enzyme, balance experiments were performed with ITP. The results, presented in Table III, are in accord with Equation 1.

SUMMARY

1. The phosphorylation of gluconate by adenosine triphosphate (ATP) in the presence of Mg²⁺ and an enzyme prepared from hog kidney has been studied. The enzyme, gluconokinase, has been partially purified, and the product of this reaction has been identified as 6-phosphogluconate.

2. Only d-gluconate, of a number of sugar acids tested, serves as sub-

³ We are indebted to Dr. Wayne Kielley of the National Heart Institute for this material.
strate for this enzyme, but several divalent metals can substitute for Mg$$^{++}$$ and uridine triphosphate can replace ATP. Inosine triphosphate and guanosine triphosphate can substitute for ATP with decreasing effectiveness, but indirect participation in the reaction via nucleoside diphosphokinase has not been excluded.

3. At each level of ATP the optimal molar concentration of Mg$$^{++}$$ is one-half that of ATP. Higher concentrations of Mg$$^{++}$$ are inhibitory.

4. The enzyme is inhibited by Cu$$^{++}$$ ions and by $p$-chloromercuribenzoate, but not by fluoride or iodoacetate. ATP partially protects the enzyme from Cu$$^{++}$$ inhibition. The relationship of this protection to the activating effect of high concentrations of ATP is discussed.

5. The enzyme has been used for the quantitative determination of gluconate spectrophotometrically.

BIBLIOGRAPHY

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