Studies on Possible Phosphoryl Enzyme Formation in Catalysis by Hexokinase, Pyruvate Kinase, and Glucose 6-Phosphatase

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One possible mode of enzymic phosphoryl transfer involves formation of phosphoenzymes as intermediates. Thus far, however, convincing evidence for the formation of such intermediates has been obtained only for the phosphoglucomutase reaction (1, 2) and, to a lesser extent, for the phosphoglyceromutase reaction (3). Suggestive evidence has been reported for the participation of phosphoenzyme intermediates in certain phosphatase reactions. Participation of such intermediates was postulated by Hass and Byrne (4) and by Neuhaus and Byrne (5) for the glucose 6-phosphatase and phosphoserine phosphatase reactions, respectively. The findings of Agren et al. (6-8) that radioactively labeled inorganic orthophosphate becomes attached to serine residues of alkaline phosphatase supports their contention that the catalysis involves phosphorylated enzyme intermediates. The catalysis of phosphoryl transfer by phosphatases (4, 5) is in accord with participation of phosphoenzyme intermediates, but may also be explained by alternate mechanisms (9).

Isotope exchange studies have given evidence against formation of phosphoenzymes in kinase reactions, as shown by the lack of catalysis of exchange of pyruvate-C14 with phosphoenolpyruvate by pyruvate kinase (10), of acetate-C14 with acetyl phosphate by acetate kinase (11), of glucose-C14 with glucose 6-phosphate by hexokinase (12), and of radioactively labeled adenosine diphosphate with adenosine triphosphate by creatine kinase (13). Further, Callaghan and Weber (14) recently demonstrated that P32 of the terminal phosphoryl group of ATP is not incorporated into rabbit muscle myokinase during catalysis. In contrast, Agren and Engström (15, 16) demonstrated that P32-labeled phosphoserine could be isolated from relatively crude yeast hexokinase and muscle phosphorylase preparations after incubation with ATP32 or P32, respectively, and postulated formation of catalytically active phosphophosphate intermediates. Krebs et al. (17) and Rall et al. (18), however, found no incorporation of P32 into purified muscle and liver phosphorylase during glycogen phosphorylase. One purpose of the studies presented herein was to find if formation of phosphoenzymes could be demonstrated with purified hexokinase and pyruvate kinase under conditions in which such formation would be expected if the phosphoenzymes were catalytic intermediates.

A second purpose of our studies was to find if O18 from H2O is incorporated into the phosphoryl group of glucose 6-phosphate during the glucose-inhibited hydrolysis by glucose 6-phosphatase. The kinetic and exchange data (4) could be explained by participation of a firmly but noncovalently bound inorganic orthophosphate instead of the postulated phosphoryl enzyme. If such were the case, however, catalysis of the incorporation of glucose into unhydrolyzed glucose 6-phosphate would be accompanied by incorporation of water oxygen into the phosphate group if the enzyme did not distinguish among the 4 inorganic orthophosphate oxygens.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**

**Enzyme Preparations and Assays.** Hexokinase was prepared from Fleischmann's baker's yeast according to the method of Darrow and Colowick (19) and crystallized by the procedure of Robbins and Boyer (20). The enzyme was stored at 4°C as a suspension in the ammonium sulfate-crystallizing medium. Before use, an aliquot of the suspension was centrifuged and the precipitated enzyme dissolved in an appropriate volume of buffer.

Darrow and Colowick report that their best preparation, a four times crystallized hexokinase, catalyzed phosphorylation of approximately 600 umoles of glucose per minute per mg of protein at 30°, as assayed by colorimetric measurement of H+ release and with use of the A320 of enolase for calculation of protein concentration (19). In a similar assay, but at 25°, the preparations used herein had an activity of approximately 250; with approximation of a temperature correction this corresponds to roughly 7 of the activity reported by Darrow and Colowick. In the titrimetric assay of Kunitz and McDonald (21, 22), our...
preparations had an activity of 22 to 26 μmoles per minute per mg of protein at 5°, and with use of the A_260 reported by McDonald (21), as based on original data of Berger et al. with purified hexokinase (23), for estimation of protein. Kunitz and McDonald reported an activity of 14.4 for their best preparation under the same conditions.

Neither the assays of Darrow and Colowick (19) nor of Kunitz and McDonald (21, 22) were satisfactorily reproducible in our hands, and with the ready availability of appropriate enzymes, a coupled assay with pyruvate kinase and lactate dehydrogenase appears preferable. For such an assay, the enzyme centrifuged from the crystallizing solution is dissolved in a small volume of cold water (e.g., 25 μl for 0.25 mg of enzyme), and an aliquot removed with a micropipet and diluted with 50 mM Tris buffer, pH 7.5 for measurement of A_260 for estimation of protein concentration. An aliquot measured with the same micropipet is diluted with an appropriate volume of cold 50 mM Tris buffer, pH 7.5, containing 5 mM glucose and 2 mg of bovine serum albumin per ml. A 10- to 50-μl aliquot of the cold, freshly diluted hexokinase is added with an adder-mixer (24) to a 3.0-ml volume at 25° in a spectrophotometer cell containing in 50 mM Tris, pH 7.5, 75 mM KCl, 2 mM ATP, 2 mM MgCl₂, 0.3 mM P-enolpyruvate, 10 mM glucose, approximately 0.15 mM DPNH, and an excess of pyruvate kinase and lactate dehydrogenase. For the latter, 10 μl each of pyruvate kinase and of lactate dehydrogenase of A_260 = to about 6.0 suffices. The lactate dehydrogenase and pyruvate kinase are added 1 to 2 minutes before the hexokinase, and after the hexokinase addition, the change in wave length at 340 μm soon becomes linear and the maximal slope is recorded. As measured with a recording spectrophotometer, linearity of hexokinase concentration was observed up to a ΔA_260 of 0.7 per minute. In this assay the hexokinase used for the experiments reported in Fig. 2 had an activity of 210 μmoles of ADP formed per minute per mg of protein.

Pyruvate kinase was prepared from rabbit muscle by the method of Tietz and Ochoa (25) through Step 4 with slight modifications in the last heating procedure (26). After Step 4, the resulting supernatant solution was treated with sufficient solid ammonium sulfate to precipitate the enzyme completely. Pyruvate kinase was stored at 4° as an ammonium sulfate slurry adjusted to pH 7.0 and when used, was centrifuged and dissolved in an appropriate volume of buffer solution. This preparation had a specific activity equivalent to 240 when assayed at pH 7.0 (26). For some of the work, pyruvate kinase from C. F. Boehringer, and Soehne, Mannheim, Germany was used.

Rat liver microsomes were used as the source of glucose 6-phosphatase activity (27); preparations and assays were made as described previously (4).

Materials—ATP, equally labeled in the β- and γ-phosphate groups, was prepared by the method of Pressman (28). Glucose-1-C₁⁴, obtained from the National Bureau of Standards, was found by chromatographic (29) and strip-scanning techniques to be free of radioactive contaminants. 6-Deoxy-6-fluoro-D-glucose had been synthesized previously by a modification (30) of the method of Helferich et al. (31, 32). P-enolpyruvate was purchased as the crystalline silver-barium salt from C. F. Boehringer and Soehne and converted to the potassium salt by treatment with Dowex 50-H⁺ resin followed by neutralization with KOH (26). Crystalline disodium ATP was obtained from the Pabst Laboratories, and the disodium salt of glucose-6-P was purchased from the Sigma Chemical Company.

Procedures for Attempted Isolation of Phosphoryl Enzymes—Experimental procedures representative of the various approaches used in attempting to isolate a phosphorylated hexokinase and pyruvate kinase intermediate are as follows:

A. Ammonium Sulfate Precipitation—Hexokinase reactions were initiated by adding labeled ATP to otherwise complete incubation mixtures which had been equilibrated at room temperature. After 20 to 30 seconds, the protein was completely precipitated by the addition of a saturated ammonium sulfate solution in sufficient volume to give 0.8 saturation. Unless otherwise stated, the ammonium sulfate solution was buffered at pH 3.9 with 0.1 M citrate to minimize further enzymatic activity (33). The incubation tubes were chilled in an ice bath and all subsequent enzyme manipulations were performed at 0°. The insoluble enzyme was centrifuged at 10,000 x g for 20 minutes and an aliquot of the supernatant solution assayed for radioactivity. The protein pellet was dissolved in a volume of 0.4 saturated ammonium sulfate solution (pH 3.9) equivalent to the original incubation mixture and reprecipitated with the same volume of saturated ammonium sulfate solution used previously. This process of washing the enzyme and assaying for liberated radioactivity was repeated three to four more times. After the last precipitation, the protein was dissolved in 1 to 2 ml of buffer (usually 0.1 M phosphate, pH 7.0) and an appropriate aliquot was assayed for radioactivity. Typical results obtained by this procedure are illustrated in Table I. As a control, a similar procedure was followed but with the major portion (99.5 per cent) of hexokinase added after the addition of ammonium sulfate to a reaction mixture containing a catalytic amount of enzyme. The short incubation with the catalytic amount of enzyme assured a distribution of radioactivity among substrates similar to the experiments with all the hexokinase added initially.

Pyruvate kinase samples were treated as above with the following modifications: Five minutes after the addition of enzyme, the incubation mixture (2.2 ml, total volume) was chilled in an ice bath and divided into two 1.0-ml aliquots. The protein in each aliquot was precipitated with ammonium sulfate (pH 8.0) in the manner described above. One protein sample was dissolved in a 0.25 saturated ammonium sulfate solution, the other in 0.01 M KOH (to insure complete stoppage of the reaction), and the precipitation procedure continued as outlined above. After the fourth wash, each of the precipitates was dissolved in 1.0 ml of its respective solubilizing reagent and 0.5-ml aliquots were plated for radioactivity analyses. The remaining 0.5 ml of each

### Table I

**Representative removal of ATP by successive protein precipitations**

<table>
<thead>
<tr>
<th>Fraction assayed</th>
<th>Hexokinase precipitation</th>
<th>Trichloroacetic acid procedure</th>
<th>Total radioactivity, c.μ.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>First supernatant</td>
<td>1.51 \times 10⁷</td>
<td>3.94 \times 10⁷</td>
<td></td>
</tr>
<tr>
<td>Second supernatant</td>
<td>2.31 \times 10⁵</td>
<td>1.65 \times 10⁵</td>
<td></td>
</tr>
<tr>
<td>Third supernatant</td>
<td>4.00 \times 10⁴</td>
<td>1.75 \times 10⁴</td>
<td></td>
</tr>
<tr>
<td>Fourth supernatant</td>
<td>200</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>Fifth supernatant</td>
<td>100</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>Sixth supernatant</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>Final precipitate</td>
<td>320</td>
<td>810</td>
<td></td>
</tr>
</tbody>
</table>
solution was used for an additional wash plus a final wash in the presence of $10^{-3}$ M ATP. After this, the remaining protein was analyzed for radioactivity.

**B. Trichloroacetic Acid Precipitation—**Hexokinase reaction ingredients, minus ATP, were pipetted into the barrel of a 5.0-ml syringe equipped with a No. 26 needle. After the addition of ATP$^{32}$, the syringe contents were briefly mixed by inversion and quickly expelled as a fine stream into 20 ml of a rapidly and turbidly stirred 0.6 M trichloroacetic acid solution. This process insured the formation of an extremely fine protein precipitate with minimal ATP$^{32}$ occlusion. The insoluble enzyme was centrifuged and an aliquot of the supernatant solution was assayed for radioactivity. The resultant protein pellet was washed into a glass Potter-Elvehjem type homogenizer with 3.0 ml of 0.6 M trichloroacetic acid and dispersed for 3 minutes with a rapidly rotating glass pestle. The suspended protein particles, along with several 0.6 M trichloroacetic acid rinses, were pipetted into an appropriate tube and centrifuged. This process of redispersing and centrifuging was repeated four more times and, as illustrated in Table I, was sufficient to allow for removal of most of the occluded radioactive nucleotide from the protein. After the last wash, the protein was dissolved in 9.0 ml of 1 N NaOH and an aliquot was assayed for radioactivity. A control experiment was run in which labeled ATP and a mixture of the other reaction ingredients were simultaneously expelled from two separate syringes into trichloroacetic acid as described above.

The extent of hexokinase action during incubation was checked by determining the radioactivity associated with labile (ATP and ADP) and stable (glucose-6-P) phosphate compounds. A 0.1-ml aliquot of the first supernatant solution was added to 0.9 ml of 2 N HCl and heated in a boiling water bath for 10 minutes. Inorganic phosphate was then isolated from organic phosphate by the phase separation technique of Martin and Doty (34) and an aliquot of each phase was plated for determining radioactivity. The radioactivity associated with the organic phosphate (lower phase) was normally sufficient to account for the complete phosphorylation of glucose in the allotted reaction time.

**C. Dialysis—**The hexokinase reaction was initiated by adding ATP$^{32}$ to an otherwise complete reaction mixture. After the addition of radioactive nucleotide, the reaction was stopped within a few seconds by adding 3.0 ml of a saturated urea solution (25°C) to the incubation medium. This solution was then dialyzed at 2°C on a rocking dialyzer against 130 ml of 6 M urea. Periodically (every 6 to 18 hours), the dialysate was changed and assayed for radioactivity. After the tenth dialysate change, 0.1 M KCl was added to the dialyzing medium to prevent Donnan distribution of nucleotide across the membrane. After 7.5 days and 14 dialysate changes there was no detectable radioactivity outside the membrane. An aliquot of the material from inside the membrane was then assayed for radioactivity.

**Glucose-6-phosphatase—**The following procedure was used to determine whether water oxygen is incorporated into glucose-6-P.

An aqueous solution of the following compounds was lyophilized to dryness: glucose-6-P, 120 µmoles; citrate, 600 µmoles (titrated to pH 6.2 with NaOH); glucose-1-C$^{14}$, 120 µmoles (5.30 x 10$^4$ c.p.m. per µmole); and carrier glucose, 1794 µmoles. The residue was dissolved in 6.9 ml of H$_2$O containing 2.72 atom % excess O$^{18}$, and the glucose-6-phosphatase reaction was initiated by adding 0.6 ml of microsomes (in H$_2$O$^{18}$) after allowing for temperature equilibration at 37°C. After 20 minutes, the reaction was terminated by quick freezing in a Dry-Ice-acetone bath and the H$_2$O$^{18}$ was recovered by lyophilization. The remaining residue was taken up in 0.3 M perchloric acid and the microsomes were removed by centrifugation. After neutralization of the supernatant fluid with KOH and removal of insoluble KCIO$_4$ by centrifugation, an incomplete separation of glucose-6-P from P was obtained by the column chromatographic procedure of Hass and Byrne (4) when an 8% cross-linked Dowex 1(C1) resin was used. The glucose-6 P and P, containing fractions from the first column were pooled, neutralized with IOH, and added to a Dowex 1-XS (200 to 400 mesh) column (1 x 14 cm) in the formate form. After the addition of one drop of carrier-free P$^{32}$ (used as a marker) to the column, glucose-6-P and P were separated by the formic acid gradient elution system recommended by Bartlett (35). Pooled fractions of the separated compounds were then reduced to approximately 200 ml in a rotary evaporator and extracted with ether to remove formic acid (35). After a further reduction in volume, the fractions were lyophilized to dryness. The glucose-6-P residue was dissolved in 5.7 ml of H$_2$O and hydrolyzed (approximately 95 to 96%) by incubating with 1.2 ml of microsomes and 0.6 ml of citrate (1 M, pH 6.0) for 150 minutes at 37°C. The hydrolysis was terminated by the addition of perchloric acid, and the microsomes and CIO$_4$ were removed by the methods described above. P was isolated and its O$^{18}$ content was determined essentially as described by Harrison et al. (36).

Parallel glucose 6-phosphatase experiments were conducted with and without inhibitory concentrations of glucose to determine the extent of glucose incorporation into glucose-6-P and to estimate the amount of O$^{18}$ expected in hexose phosphate if H$_2$O were incorporated.

**RESULTS**

**Attempted Isolation of Phosphorylated Pyruvate Kinase and Hexokinase Proteins—**To determine whether purified hexokinase and pyruvate kinase formed phosphoryl proteins sufficiently stable to allow their isolation under conditions giving enzyme denaturation or inactivation, stoichiometric amounts of each of these enzymes were incubated with ATP$^{32}$ under conditions which would be expected to favor enzyme phosphorylation. The enzymes were then isolated by the repeated precipitation or dialysis techniques outlined in “Experimental Procedure” and assayed for radioactivity. The results from these experiments, and from controls in which ATP$^{32}$ was present in the precipitating medium, are given in Tables II and III.

The results illustrate that each precipitated enzyme retains only an exceptionally small and variable amount of radioactivity which, when calculated on an equivalent basis, is less than 1/500 of that expected for the stoichiometric formation of a phosphorylated intermediate. The control experiments given in Table II show that a large fraction and possibly all of the precipitable radioactivity can be accounted for as occluded ATP$^{32}$ or some radioactive impurity not separable from the protein by the techniques used. In addition, the possibility of nonspecific binding or occlusion might be greater in most of the incubated samples due to the presence of other P compounds, particularly glucose-6-P$^{32}$ and P-enolpyruvate-P$^{32}$. Also, in the case of hexokinase, the presence of glucose-6-P$^{32}$ could lead to labeling of any small amount of P-glucuronate present.

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1 Hass and Byrne recommend 2% cross-linked resin, which is apparently critical for the separation of glucose-6-P and P, by their method.
If any phosphoryl enzyme intermediate were extremely labile, it could have escaped detection in the above procedures by decomposition yielding $P_i$. However, the same amount of $P_i$ was present in the precipitating or dialyzing medium (24) from control and experimental samples; presence of additional $P_i$ equivalent to 5 to 10% of the active sites would have been readily detectable.

Also considered was the possibility that glucose or structurally related compounds might be necessary to impart the proper conformation to the hexokinase molecule before its phosphorylation by ATP. However, when 6-deoxy-6-fluoro-D-glucose, a competitive inhibitor (33) incapable of becoming phosphorylated, was substituted for glucose in the reaction medium, no increased phosphorylation of hexokinase was obtained as indicated by the results shown for Experiment 3 in Table II.

Tests for Phosphorylation of Stoichiometric Amounts of Pyruvate Kinase and Hexokinase—The possibility existed that in the experiments described above, any phosphoryl enzymes formed from pyruvate kinase and hexokinase rapidly transferred their phosphoryl group to substrates before isolation of the proteins. Such a reaction could occur during the transitory changes in pH value and ionic strength accompanying the protein isolation and before enzyme inactivation. Experiments were thus designed to test for possible phosphorylation of the enzymes under conditions where all substrates were present, although some in low amounts, and rapid net catalysis was possible.

Experiments with pyruvate kinase were made in the presence of DPNH, ATP, and $P$-enolpyruvate under conditions as given in Fig. 1. Lactate dehydrogenase and pyruvate kinase were first added in amounts sufficient to cause rapid catalytic reaction; the absorbancy of DPNH at 340 m$\mu$ fell to a constant value. At this stage the system provides a sensitive optical test for pyruvate or ADP formation. A relatively large amount of pyruvate kinase was then added as indicated in Fig. 1. Formation of a phosphoryl enzyme should result in ADP and pyruvate formation, with a rapid decrease in $A_{420}$ of approximately 0.04 on the basis of two binding sites per mole of pyruvate kinase (26). No detectable decrease was noted. The continued decline of the $A_{420}$ results from a slow dephosphorylation of p-enolpyruvate which occurs in the presence of high concentrations of pyruvate kinase (26). Subsequent addition of ADP as a control gave the expected decrease in $A_{420}$ and demonstrates the adequacy of the test system.

Preliminary experiments with hexokinase similar to those just described for pyruvate kinase showed that the crystalline hexokinase additions did allow ADP formation and thus that either the protein or some compound bound to the protein was being phosphorylated. These results led to the experiments reported in Fig. 2. Part A shows the extent of phosphorylation accompanying addition of relatively large amounts of hexokinase to a test system containing lactate dehydrogenase, pyruvate kinase, ATP, DPNH, and approximately $10^{-4}$ $M$ hexokinase. The drop in $A_{420}$ indicates the presence of roughly 1.6 phosphoryl acceptor sites per mole of hexokinase.\(^3\) The hexokinase molarities are based on the use of the $A_{280}$ given by McDonald (21). Values for the mole ratio of the bound acceptor to hexokinase can be taken only as rough approximations because of the uncertainty of the ultraviolet absorption for pure hexokinase and the probable presence of some protein impurities in the hexokinase preparation.

**Table II**

*Extent of incorporation of radioactivity from ATP\(^{32}\) into hexokinase*

The reaction mixture for Experiment 1 contained 1.1 $\mu$moles of ATP\(^{32}\), 3.94 $\mu$moles of hexokinase, and 0.1 $\mu$moles of glucose in a total volume of 2.0 ml. Conditions for Experiments 2 and 4 were the same as in Experiment 1 except that 3.44 $\mu$moles of hexokinase and 0.5 $\mu$moles of glucose were used. The reaction mixture for Experiment 3 contained 1.04 $\mu$moles of ATP\(^{32}\), 3.44 $\mu$moles of hexokinase, and 1.0 $\mu$moles of 6-deoxy-6-fluoro-D-glucose in a total volume of 1.0 ml. Conditions for Experiment 5 were the same as those in Experiment 3 except that 0.55 $\mu$moles of ATP\(^{32}\) was used and 0.2 $\mu$moles of glucose was substituted for 6-deoxy-6-fluoro-D-glucose. All reaction mixtures contained 0.065 $\mu$M Tris (pH 8.0) and 0.01 $M$ Mg\(^{2+}\). The ATP\(^{32}\) had specific activities of 1.2 to 4.5 $\times$ $10^{12}$ c.p.m. per $\mu$ mole. In all experiments, protein was isolated by the methods described in the text. In Experiment 1, precipitation was conducted at pH 7.0; all other ammonium sulfate precipitations were conducted at pH 3.9.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Method of enzyme isolation*</th>
<th>Radioactivity associated with protein</th>
<th>Expected for phosphoryl-enzyme formation(^{c.p.m.} \times 10^{-5})</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(NH(_4))(_2)SO(_4)</td>
<td></td>
<td>6.1</td>
<td>0.0028</td>
</tr>
<tr>
<td>2</td>
<td>(NH(_4))(_2)SO(_4)</td>
<td></td>
<td>2.8</td>
<td>0.0032</td>
</tr>
<tr>
<td>3</td>
<td>(NH(_4))(_2)SO(_4)</td>
<td></td>
<td>7.8</td>
<td>0.0113</td>
</tr>
<tr>
<td>3 (control)</td>
<td>(NH(_4))(_2)SO(_4)</td>
<td></td>
<td>0.0079</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Trichloroacetic acid</td>
<td></td>
<td>0.8</td>
<td>0.0081</td>
</tr>
<tr>
<td>4 (control)</td>
<td>Trichloroacetic acid</td>
<td></td>
<td>0.0021</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Dialysis</td>
<td></td>
<td>1.4</td>
<td>0.0028</td>
</tr>
</tbody>
</table>

* See "Experimental Procedure" for details of enzyme isolation and control experiments.

† Calculated on the basis of random labeling in ATP\(^{32}\), a molecular weight of 96,600 for hexokinase (21), and the assumption that one phosphoryl group of ATP is capable of being transferred to each molecule of hexokinase.

**Table III**

*Extent of incorporation of phosphate into pyruvate kinase*

The following incubation mixture was allowed to stand at room temperature for 5 minutes: 0.2 $\mu$l of ATP\(^{32}\), 10$^{-4}$ $M$, (5.0 $\times$ $10^{5}$ c.p.m. per ml); 0.2 $\mu$l of P-enolpyruvate, 10$^{-4}$ $M$; 0.4 $\mu$l of enzyme, 50 mg per ml; 0.2 $\mu$l of buffer; and 1.2 ml of H\(_2\)O. The buffer contained: MgSO\(_4\), 0.11 $M$; KCl, 1.10 $M$; and glycyglycine, 0.055 $M$ at pH 8.5. Two 1.0-ml aliquots were taken from the incubation mixture and precipitated with ammonium sulfate. One precipitate was dissolved in 0.25 $M$ (NH\(_4\))\(_2\)SO\(_4\), the other in 0.01 $M$ KOH, and the precipitation procedure was repeated as described in the text.

<table>
<thead>
<tr>
<th>Dissolving solution</th>
<th>Radioactivity associated with protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expected for phosphoryl-enzyme formation(^{c.p.m.} \times 10^{-4})</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4), 0.25 M</td>
<td>7.5</td>
</tr>
<tr>
<td>KOH, 0.01 M</td>
<td>7.5</td>
</tr>
</tbody>
</table>

* Calculated on the basis of random labeling in ATP\(^{32}\), a molecular weight of 237,000 for pyruvate kinase, and the assumption that two phosphoryl groups from ATP or P-enolpyruvate are capable of being transferred to each pyruvate kinase molecule (26).
Fig. 1. Lack of phosphorylation of pyruvate kinase by P-enolpyruvate. A cell of 1-cm light path in a Cary recording spectrophotometer contains, in a 1.5 ml total volume, pH 7, at 25°, 100 mM imidazole chloride buffer, 0.01 mM ATP, 2.5 mM P-enolpyruvate, 0.4 mM MgCl₂, 40 mM KCl, and 0.1 mM DPNH. At the time indicated, 10 μl of crystalline muscle lactate dehydrogenase solution (A₂₈₀ of solution = 7.6) and 5 μl of pyruvate kinase sufficient to give the final molarity as indicated were added. Subsequent additions of 50 μl of pyruvate kinase and of 25 μl of ADP were made to give final concentrations as indicated. Additions and mixing were made with the adder-mixer described previously (24) with the handle protruding through the cell compartment lid. Absorbancies as given in the figure are corrected for the slight dilution accompanying the additions, and for the small A₃₄₀ (0.011) given by the 3.2 × 10⁻⁸ M pyruvate kinase added.

decrease in A₂₈₀ after the second addition probably reflects inhibitory effects of residual (NH₄)₂SO₄ in the hexokinase solution on the enzyme catalytic rate; that the assay was operating satisfactorily is shown by the subsequent addition of glucose (Fig. 2A). As noted in Fig. 2A, the concentrated hexokinase allows a slow but detectable oxidation of DPNH in the assay system. This could reflect a very weak ATPase activity of the hexokinase.⁴

Nature of Phosphoryl Acceptor Present in Hexokinase—Three experimental results give pertinent information about the nature of the phosphoryl acceptor, namely, the ready dissociability of the phosphorylated acceptor from the hexokinase, the acid stability of the phosphoryl compound formed, and the ability of glucose to combine with the enzyme in a manner to restore its phosphoryl acceptor capacity.

Experiments with ATP²³ showed the dissociability and the acid stability of the phosphorylated acceptor. In the experiment reported in Fig. 2A, ATP²³ was present initially, and duplicate 10.0-μl samples were removed before and after the two additions of hexokinase. These samples were used to measure the amount of P²³ converted to P₁ by hydrolysis for 10 minutes in 1 M HCl. Additional duplicate 10-μl aliquots were mixed with a 10-μl drop of 6 mM glucose, allowed to stand for approximately 15 minutes, and then hydrolyzed in 1 M HCl. These samples gave a measure of the total P²³ transferrable to glucose in the reaction mixture; that is, the total P²³ present in the terminal phosphoryl group of ATP. The results showed that the two additions of hexokinase resulted in conversion of 34% of P²³ in the terminal phosphoryl of ATP to an acid-stable form. The total amount of ATP present was 8 × 10⁻⁵ M; thus, 2.7 × 10⁻⁶ M acid-stable phosphate was formed. This corresponds well with the total drop of 2.5 × 10⁻⁵ M DPNH as recorded in Fig. 2A. Thus, within an experimental error of 10 to 15%, all the phosphoryl acceptor is acid-stable and the number of phosphoryl sites per mole, as estimated from the radioactivity data, check well with that from the DPNH formation.

When the hexokinase, present after termination of the experiment reported in Fig. 2A, was precipitated with (NH₄)₂SO₄, less than 1% of the P²³ present accompanied the hexokinase, demonstrating that the phosphorylated acceptor did not precipitate with the protein. This corroborates the data presented in Table II which made it unlikely that any acid-stable P²³ compound was covalently bound to the protein. The lack of protein-bound P²³ was also demonstrated by direct counting of suspensions of the precipitated and washed hexokinase. In two experiments the radioactivity found was less than 3% of that expected for phosphorylation of 1.7 sites per hexokinase molecule.

⁴ K. A. Trayser and S. P. Colowick (37) have shown that in presence of higher ATP concentrations, hexokinase has a more pronounced ATPase activity.
The low amount of P\textsuperscript{32} found could have readily been present through occlusion or weak absorption of glucose-6-P\textsuperscript{32} and ATP\textsuperscript{32}.

The hexokinase isolated, after washing with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} solution showed poor ability to act as a phosphoryl acceptor, as shown in Fig. 2B. In another similar experiment, no acceptor ability was found.

The experiment recorded in Fig. 2C demonstrates that the hexokinase previously exposed to excess ATP and isolated, upon exposure to excess glucose, regains the ability to act as a phosphoryl acceptor. The supernatant solution from the final wash of the hexokinase used in the experiment reported in Fig. 2C, unlike glucose, did not show any acceptor ability; thus, as expected, free glucose had been reduced to a very low concentration by the wash procedure.

**Possible Incorporation of O\textsuperscript{18} into Glucose-6-P**—For these experiments, glucose 6-P was incubated with H\textsubscript{2}O\textsuperscript{18} and rat liver microsomes in the presence of a highly inhibitory concentration of glucose which gave incorporation of glucose-C\textsuperscript{14} into glucose-6-P. After isolation, the glucose-6-P was completely hydrolyzed by glucose 6-phosphatase and the liberated P\textsubscript{i} assayed for O\textsuperscript{18} content by the procedure described in the “Experimental Procedure.” The ability of microsomal glucose 6-phosphatase to catalyze exchange of the O of H\textsubscript{2}O with P\textsubscript{i} has not been measured directly, but analysis of the P\textsubscript{i} liberated in the incubation with H\textsubscript{2}O\textsuperscript{18} showed that it contained 0.57 atom % excess O\textsuperscript{18}. This value, although slightly lower than the predicted value of 0.63 for incorporation of one water oxygen into the P\textsubscript{i}, indicates that microsomal glucose 6-phosphatase does not catalyze any appreciable exchange of O\textsuperscript{18} in water with the oxygens of orthophosphate. Further, a mixture of the glucose-6-P and P\textsubscript{i} was stored at 0\textdegree C in several days and slight hydrolysis of the glucose-6-P could account for the low value.

The results of this experiment, presented in Table IV, show that even though glucose-C\textsuperscript{14} is transferred into glucose-6-P, the O\textsuperscript{18} from water is not incorporated into the phosphoryl group of glucose-6-P. If the basis for the glucose incorporation without P\textsubscript{i} incorporation was the formation of a noncovalent enzyme-P\textsubscript{i} complex with all of the phosphate oxygens in the enzyme phosphate complex equivalent, oxygen incorporation in the glucose 6-phosphate would have resulted.

**DISCUSSION**

The results make highly unlikely the participation of phosphoryl enzyme intermediates in the hexokinase and pyruvate kinase reactions. The experiments on isolation of enzyme protein after catalysis of phosphoryl transfer from ATP\textsuperscript{32} demon-
strate conclusively that no more than \( \frac{1}{2} \) and probably none of the catalytic sites form stable phosphoryl derivatives. The lack of formation of orthophosphate-P_{\text{a2}} during the isolation and washing of enzyme protein makes unlikely the initial presence of an extremely labile phosphoryl enzyme. The experiments with use of a sensitive coupled assay system to detect ADP formation (Figs. 1 and 2) show the lack of formation of detectable phosphoryl enzyme by pyruvate kinase in the presence of all substrates and fully active enzyme. Similar experiments with hexokinase (Fig. 2) show the presence of a phosphoryl acceptor with the hexokinase. All the acceptor is acid stable and readily dissociates from the protein. Thus, no detectable phosphorylation of any amino acid residue of the hexokinase occurs. The basis for the incorporation of P_{\text{a2}} into a hexokinase preparation as found by Ågren and Engström (15) is not clear, but may reflect the impure nature of the preparations. Details of their experiments are insufficient to make a thorough comparison with our findings.

The failure to detect phosphoryl enzyme formation in the experiments reported in Figs. 1 and 2 clearly does not result because the enzymes were already in the phosphoryl form. As discussed elsewhere, little or no phosphoryl residues derived from substrates become stably attached to the enzymes.

Thermodynamic considerations make it improbable that phosphoryl enzymes are formed as catalytic intermediates which escape detection because their concentration is low. For example, in the experiments with the coupled lactate dehydrogenase assay for detection of ADP formation (Figs. 1 and 2), no net reaction was occurring, and any phosphoryl intermediate should be present in an equilibrium concentration. The possible phosphorylation of the pyruvate kinase, as represented by Equation 1, and lactate dehydrogenase reaction, Equation 2, may be summed to give the net reaction that would accompany any phosphoryl enzyme formation (Equation 3) as follows:

\[
P_{\text{enolpyruvate}} + \text{enzyme} \rightleftharpoons \text{P-enzyme} + \text{pyruvate} \quad \text{(1)}
\]

\[
\text{Pyruvate} + \text{DPNH} \rightleftharpoons \text{lactate} + \text{DPN}^+ \quad \text{(2)}
\]

\[
P_{\text{enolpyruvate}} + \text{enzyme} + \text{DPNH} \rightleftharpoons \text{P-enzyme} + \text{lactate} + \text{DPN}^+ \quad \text{(3)}
\]

The experiments reported in Fig. 1 are sufficiently sensitive for detection of phosphorylation of approximately one-tenth of the enzyme, and thus it is highly improbable that phosphorylation of more than two tenths of the enzyme could have escaped detection. The equilibrium constant for Equation 3 based on phosphorylation of two-tenths of the enzyme and with the concentrations of other reactants as present, would be approximately 0.02, and the apparent \( \Delta F = 2,300 \) calories. The equilibrium of the lactate dehydrogenase reaction at \( \text{pH} \) 7, as estimated from the data of Winer and Schwert (38), gives a value of the apparent \( \Delta F \) for Equation 2, as written, of about \(-6,000 \) calories. From this it follows that for presence of two-tenths of the enzyme in the phosphorylated state at equilibrium, the apparent \( \Delta F \) value for Equation 1 would be \( 8,300 \) calories; phosphorylation of less than two-tenths of the enzyme would make the apparent \( \Delta F \) value even higher. Such calculations show that the minimal apparent \(-\Delta F \) value of hydrolysis of any postulated phosphoryl enzyme which escaped detection because it was present in low concentration would be \( 8,300 \) calories greater than that for P-enolpyruvate. Similar calculations apply to the hexokinase results. Thus, the possibility that a phosphoryl enzyme participates in the catalyses by these enzymes is small.

The presence of a bound phosphoryl acceptor with the crystalline hexokinase was somewhat unexpected. The Michaelis constant for glucose for yeast hexokinase has been reported as \( 1.5 \times 10^{-4} \text{ M} \) (38). Even if the dissociation constant were an order of magnitude less, bound glucose would not be expected to carry through the various steps in the isolation and recrystallization of the hexokinase. After phosphorylation the acceptor has a much smaller affinity for the enzyme, as shown by its appearance in the supernatant solution when the enzyme is precipitated with ammonium sulfate. That the bound acceptor could be glucose is demonstrated by the ability of addition of excess glucose to the acceptor-free hexokinase and subsequent precipitation and washing of the hexokinase to give a preparation with restored acceptor ability. The phosphorylated acceptor which dissociates from the enzyme is acid-stable, like glucose 6-phosphate. These results together with the known specificity of the hexokinase infer but do not prove that the acceptor present with the original hexokinase is glucose or a closely related sugar.

The occurrence of glucose as the bound acceptor is also consistent with the interesting findings of Najjar and McCoy (12) which show that yeast hexokinase readily acquired radioactivity when exposed to glucose-C_{\text{14}}. Our results strongly indicate that the acceptor present on the native enzyme is not covalently bound, and thus, contrary to the suggestion of Najjar and McCoy (12), the phosphorylation of bound glucose and subsequent nonenzymic phosphoryl transfer to a serine residue could not account for the findings of Ågren.

The various findings with hexokinase are consistent with the reaction which occurs by a mechanism similar to that suggested for pyruvate kinase, namely, by direct transfer of the phosphoryl group from ATP to the hydroxyl of glucose. Unlike pyruvate kinase, however, the Michaelis constant for at least one substrate, namely glucose, is not approximately equal to the dissociation constant.

The results of 0° studies with glucose 6-phosphatase rule out the possibility that glucose-C_{\text{14}} at inhibitory concentrations, reacts with a noncovalently enzyme-bound phosphate with a random elimination of phosphate oxygens as water. An enzyme-bound phosphate might, however, have the positions of 2 or more oxygens spatially fixed. This would make highly probable an enzymic distinction among the oxygens of the phosphoryl group. If this were so, the oxygen which entered from water would be eliminated as water when glucose was incorporated into glucose-6-P, and thus no oxygen from water would be found in the phosphoryl group of the unhydrolyzed glucose-6-P. Means of distinguishing between this possibility and that of formation of a phosphoryl enzyme, as suggested by Hase and Byrne (4), are not at present apparent.

K. A. Trayser and S. P. Colowick (37) have found that bound glucose present in initial crystals of hexokinase is lost on repeated recrystallization, and that no detectable glucose is bound (K_{d} > 10^{-3} \text{ M}) by hexokinase as measured by equilibrium dialysis. Also, they have estimated the K_{d} value for enzyme-glucose to be approximately 2.5 \times 10^{-4} as based on glucose protection from proteolytic digestion. The retention of bound glucose noted in our experiments may reflect an increased binding of glucose by the protein in presence of sufficient ammonium sulfate for precipitation of the protein.
SUMMARY

Relatively large amounts of highly purified muscle pyruvate kinase and yeast hexokinase were allowed to catalyze phosphoryl transfer from an excess of radioactively labeled adenosine triphosphate, followed by isolation of enzyme proteins by precipitation and dialysis techniques. The specific activity of the isolated proteins was less than $\frac{1}{500}$ of that of the adenosine triphosphate, demonstrating that, within experimental error, no stable phosphoryl enzymes are formed in the catalyses.

The addition of a relatively large amount of pyruvate kinase to a sensitive assay system for formation of adenosine diphosphate or pyruvate from adenosine triphosphate or phosphoenolpyruvate showed no detectable formation of a phosphoryl enzyme. Twice-crystallized hexokinase in a similar assay system showed the presence of a bound phosphoryl acceptor. The phosphorylated acceptor was acid-stable, and readily dissociated from the enzyme to give a catalytically active hexokinase which no longer acted as a phosphoryl acceptor, but whose acceptor capacity was restored by exposure to glucose.

The activity of some phosphoryl enzymes to escape detection in the assay used would require their $\Delta F^\circ$ value of hydrolysis to be approximately 8,000 calories greater than that of phosphoenolpyruvate. The findings give strong evidence against phosphoryl enzyme formation even in the presence of all substrates and under conditions where rapid net catalysis is possible.

Liver microsomal glucose 6-phosphatase, when allowed to hydrolyze glucose 6-phosphate in H_2O, with an excess of glucose present, does not catalyze an incorporation of water oxygen into the glucose 6-phosphate concomitant with the glucose incorporation. The over-all hydrolytic cleavage occurs with split of the P-O bond. These findings are consistent with the hypothesis that glucose 6-phosphatase forms a phosphoryl enzyme as a catalytic intermediate, but do not rule out the possible participation of a noncovalent enzyme-inorganic phosphate complex with stereospecific participation of the phosphoryl oxygens.

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Studies on Possible Phosphoryl Enzyme Formation in Catalysis by Hexokinase, Pyruvate Kinase, and Glucose 6-Phosphatase

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