Phosphoglucomutase

IV. INACTIVATION BY BERYLLIUM IONS*

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SUMMARY

Rabbit muscle phosphoglucomutase is irreversibly inhibited by Be+++. In the presence of those chelators which, together with Mg++, serve to stimulate the enzyme when preincubated in the absence of substrate, 1 g atom of Be+++ is bound per mole of enzyme. Other procedures which were calculated to remove unknown inhibitory ions in the isolated enzyme (ethylenediaminetetraacetate, 8-hydroxyquinoline, passage over Dowex 50) also permit Be+++ inactivation. Beryllium binding, which exhibits an activation energy of 19.9 kcal, prevents phosphorylation of dephospho-enzyme and dephosphorylation of phospho-enzyme. Exposure to 4 mM urea facilitates Be+++ inactivation and also results in removal of bound Be+++ with partial recovery of activity.

Be+++ similarly affects phosphoglucomutases from shark and flounder muscle, rabbit liver, and Escherichia coli but not those from Micrococcus lysodeikiticus or Bacillus cereus. Flounder enzyme, which is generally unstable, is much more readily denatured by urea than is rabbit enzyme.

The observation by Harshman et al. (1) and Robinson, Harshman and Najjar (2) that rabbit muscle phosphoglucomutase is stimulated 2- to 5-fold by incubation with histidine or imidazole and Mg++ has been repeatedly confirmed. Milstein (3) suggested that such activation is best interpreted as the removal of inhibitory metal ions from the enzyme, facilitated by a chelating agent, with replacement by Mg++. Presumably, the inhibitory metal ions are bound to the freshly isolated enzyme which must, therefore, offer greater affinity for these ions than for Mg++. Relatively tight binding of Cu++ and Zn+++ to rabbit P-glucomutase in a final volume of 1.0 ml. Activity was assayed by the increase in absorbance at 340 nm.

Inhibition of phosphoglucomutase by beryllium was discovered independently in several laboratories in studies conducted in vivo and in vitro. Cochran, Zerwic, and Dubois (7) showed that the P-glucomutase activities of kidney, liver, and muscle were inhibited by parenteral administration of BeCl2 to rats and guinea pigs. Recently Aldridge and Thomas (8, 9) demonstrated that, at low concentrations, Be+++ inhibits rabbit muscle P-glucosumutase in the presence of either histidine or cysteine, and noted that Be+++ inhibition was competitively prevented by the presence of Mg++.

The present report extends these studies and describes more completely the conditions of beryllium inhibition and of magnesium activation of phosphoglucomutase as well as the use of this tool to discriminate among classes of phosphoglucomutases. Preliminary descriptions of these findings have previously been reported (10, 11).

EXPERIMENTAL PROCEDURE

Glucose 1-phosphate and glucose 1,6-diphosphate were purified by the ion exchange procedure previously described (6). Glucose-1-32P was prepared by a slight modification of the method of McCready and Hassid (12). Beryllium was employed as BeCl2 in all experiments and was neutralized with Tris when necessary. Radioisotopic beryllium and histidine-14C as well as all other reagents were commercial products. The various phosphoglucomutases employed were prepared by procedures which have been previously described (4-6, 13). Glucose-6-P dehydrogenase was obtained from Calbiochem.

Metal-free enzyme was prepared by passing enzyme in 1 mM Tris-chloride over a column of Dowex 50, Tris form, which had previously been washed with 1.0 mM Tris-chloride, pH 7.5, and equilibrated with 1 mM Tris, pH 7.5. Metal-free Tris buffer was similarly prepared.

Assay Procedures—The coupled assay method described previously (4) was used for convenience throughout this study. The standard reaction mixture contained 20 μmoles of buffer, pH 7.5, 2 μmoles of Mg++, 2 μmoles of glucose-1-P, 3.5 μmoles of glucose-1,6-P, 0.5 μ mole of TPN++, 1 unit of glucose-6-P dehydrogenase, and P-glucomutase in a final volume of 1.0 ml. Activity was assayed by the increase in absorbance at 340 nm at 25°. At the concentration employed, Be+++ did not inhibit glucose-6-P dehydrogenase.

Time Course of Beryllium Inhibition—Studies of beryllium inhibition of alkaline phosphatase (14, 15) have indicated that Mg+++ serves as a competitive antagonist of Be+++ inhibition and that such inhibition is reversible. With the use of intestinal
Inhibition of rabbit muscle phosphoglucomutase by beryllium. Enzyme (0.5 m mol) was incubated at 25°C in a total volume of 1 ml of Tris-Cl buffer, pH 7.6. BeCl₂ (10 m moles) and histidine (pH 7.6, 20 m moles) were added at the times shown by the arrows. O—O, controls to which no histidine was added. In a parallel run, a mixture containing 2 m moles of Mg++, 2 m moles of glucose-1-P, and 3.5 m moles of glucose-1,6-P were added at the time shown by the broken arrow, and the subsequent course of enzyme activity is shown as △—△. All activities were estimated by the coupled assay on 0.02-ml aliquots withdrawn from the incubation mixture.

Requirements for Beryllium Inhibition of Phosphoglucomutases—As shown in Fig. 2, beryllium inhibition of rabbit muscle phosphoglucomutase occurred in the presence of cysteine, histidine, mercaptoethanol, or imidazole, in that order of effectiveness. At the same Be++ concentration, no inhibition developed when Tris, glycine, glycylglycine, serine, glutamic acid, glutamine, or aspartic acid was used as the buffer. Thus, those reagents which, in the presence of Mg++, activate P-glucomutase when incubated before addition of substrate were also effective in the development of beryllium inhibition. Citrate, glucose phosphates, and P₁ at 10⁻³ M, were antagonistic to Be++, but salicylic acid, an effective chelating agent for Be++ and Mg++ under other circumstances, did not affect the development of inhibition in the presence of histidine.

Stoichiometry—The stoichiometry of the reaction between beryllium and the phospho form of rabbit muscle P-glucomutase is shown in Fig. 3. After incubation of the enzyme with Be++ for 1 hour, aliquots were either assayed directly or transferred to a solution containing Mg++ and histidine for 20 min and then assayed. In both instances, the enzyme was completely inhibited in the presence of 1 g atom of Be++ per mole of protein. Similar studies with rabbit muscle dephospho-enzyme, rabbit liver, flounder, and shark enzymes gave identical results.

This stoichiometric relationship was not affected by the presence of either bovine serum albumin or rabbit muscle extract, indicating rather specific and tight binding of Be++ to a significant locus on the enzyme. Moreover, Be++ so bound did not appear to dissociate from the enzyme upon heat denaturation. A sample of rabbit muscle phospho-enzyme which had been fully inhibited by previous incubation with a stoichiometric amount of Be++ was placed in a boiling water bath for 5 min at pH 7.9. After cooling, addition of this solution to a sample of active enzyme was without effect on the activity of the latter. Conversely, when a stoichiometric amount of beryllium was added to a solution of similarly heat-inactivated phospho-enzyme, binding also occurred since subsequently added active enzyme retained full activity under these circumstances.

Direct Demonstration of Beryllium Binding—Rabbit muscle phospho-enzyme was incubated with ⁷Be⁺⁺ and histidine-¹⁴C by the usual procedure and reisolated by passage through Sephadex. A sample of rabbit muscle phospho-enzyme which had been fully inhibited by previous incubation with a stoichiometric amount of Be++ was placed in a boiling water bath for 5 min at pH 7.9. After cooling, addition of this solution to a sample of active enzyme was without effect on the activity of the latter. Conversely, when a stoichiometric amount of beryllium was added to a solution of similarly heat-inactivated phospho-enzyme, binding also occurred since subsequently added active enzyme retained full activity under these circumstances.
In the presence of buffers varying in their content of Tris, histidine, and Mg++. As shown in Table I, in no case was a significant amount of histidine bound to the reisolated enzyme, whereas stoichiometric binding of Be++ was clearly evident after Be++ had been incubated with the enzyme in the presence of histidine but not in the presence of Tris. If the histidine-dependent beryllium inhibition involves intermediary formation of an enzyme-Be++-histidine complex analogous to the enzyme-Mg++-histidine complex at one time contemplated by Harshman et al. (1) and Robinson et al. (2), it is clear that the binding of histidine is not as permanent as that of Be++ under such circumstances. In view of the greater effectiveness of cysteine and mercaptoethanol than that of histidine and imidazole, respectively, it appears unlikely that such complexes, if formed, involve stable binding of the organic reagent to the metal in the complex.

Kinetics of Beryllium Inhibition and Competition by Magnesium—The data of Fig. 1 suggest that the inhibition of P-glucosamidase by Be++ may follow first order kinetics. Accordingly, this process may be assumed to follow the course shown in Equation 1, in which E is phosphoglucomutase, E - Be* is an intermediate Michaelis complex, and E - Be is the irreversibly inactivated complex.

\[
E + Be \xrightarrow{k_1} E^* - Be \xrightarrow{k_2} E - Be
\]  

(1)

The kinetic equations for this process are

\[
-\frac{d[E]}{dt} = \frac{k_1[E][Be]}{K_m + [Be]} 
\]  

(2)

\[
\log \left( \frac{[E]}{[E]_T} \right) = -\frac{kt}{2.3} 
\]  

(3)

\[
k = \frac{k_1[E][Be]}{K_m + [E][Be]} 
\]  

(4)

TABLE I

**Be**++ binding to rabbit muscle phosphoglucomutase

The enzyme (10 nmoles) was incubated at 25° for 1 hour in a total volume of 0.5 ml containing 0.5 n mole of Tris or 1C-histidine (10° cpm), pH 7.6, and 0.05 n mole of Mg++ or Be**++ (2 x 10° cpm). The mixture was passed through Sephadex G-25 with the use of the buffers indicated (10 mM Tris or histidine, pH 7.6, and 1 mM Mg++ when added), and an aliquot of the percolate was assayed for radioactivity by scintillation counting.

<table>
<thead>
<tr>
<th>Incubation components</th>
<th>Be**++</th>
<th>Histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine -1° C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg**++ + histidine</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tris or histidine ± Mg**++</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Be**++ + histidine</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tris or histidine ± Mg**++</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>Be**++ + Tris</td>
<td></td>
<td>1.12</td>
</tr>
<tr>
<td>Tris ± Mg**++</td>
<td></td>
<td>1.09</td>
</tr>
</tbody>
</table>

**FIG. 4.** Kinetics of beryllium inhibition of rabbit muscle phosphoglucomutase. First order rate constants (k) were obtained by following the time course of development of inhibition by Be**++, at the concentrations shown, in the presence of the concentrations of Mg**++ shown along each line. All incubations were conducted in 20 mM histidine, pH 7.6, at 25°.

**FIG. 5.** Competitive nature of protection by Mg**++ against Be**++ inhibition of rabbit muscle phosphoglucomutase. The apparent K_m values for Be**++ obtained in Fig. 4 are plotted here against concentration of Mg**++ permitting estimation of true K_m Be**++ and K_m Mg**++. where (E)_T is the total concentration of the enzyme added, K_m is the Michaelis constant (k_1 + k_2)/k_1, and k is the observed first order rate constant.

Fig. 4 shows a plot of the reciprocals of the first order rate constants (k) of the beryllium inhibition against the reciprocals of concentrations of Be**++ at various concentrations of Mg**++. These data were rearranged as shown in Fig 5, permitting an estimation of the K_m for Be**++ in this system and K_m for Mg**++ with respect to the Be**++ inhibition. These values, 2.5 x 10°<sup>-6</sup> M and 1.3 x 10°<sup>-6</sup> M, respectively, refer to the kinetics of the development of beryllium inhibition and do not imply reversible dissociation of the enzyme-Be**++ complex. However, the formally competitive nature of the Mg**++ effect strongly suggests that Be**++ binds at the enzymic site at which Mg**++ is normally operative in the catalytic process.

**Activation Energy**—In Fig. 6 is shown an Arrhenius plot of the effect of temperature on the rate of development of beryllium inhibition. These data permit calculation of the activation energy for the inhibition process as 19.9 kcal, which is of the order of the normal activation energy for catalysis, 19.3 kcal, in contrast to the activation energy for the stimulation of rabbit muscle phosphoglucomutase by Mg**++ + imidazole of 4.8 kcal (1). Since completion of these studies, a similar
TABLE II
Inhibition by beryllium of reaction between phosphorylated form of rabbit muscle phosphoglucomutase and glucose 1-phosphate

32P-Labeled phosphoenzyme was prepared as described in text. Labeled enzyme (0.1 µmole) was incubated in 40 mM histidine buffer with and without 0.12 µmole of BeCl₂ for 15 min, and then 2 µmoles of nonlabeled glucose 1-phosphate were added. Incubation was continued for 5 min at 30°C, and the solution was made 65% saturated with ammonium sulfate. The precipitate was collected by centrifugation, washed three times by solution in histidine buffer, and reprecipitated. Radioactivity was assayed by the standard procedure.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Before glucose-1-P</th>
<th>After glucose-1-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Be⁺⁺</td>
<td>1.82 x 10⁶ cpm/0.1 µmole</td>
<td>0.08</td>
</tr>
<tr>
<td>+Be⁺⁺</td>
<td>1.60</td>
<td>1.42</td>
</tr>
</tbody>
</table>

FIG. 6. Arrhenius plot of influence of temperature on the first order velocity constant for development of Be⁺⁺ inhibition of rabbit muscle phosphoglucomutase.

FIG. 7. Influence of pH on stimulation of rabbit muscle phosphoglucomutase by Mg⁺⁺ and histidine and inhibition by Be⁺⁺ and histidine. Phospho-enzyme (0.2 µmole) was incubated at room temperature for 10 min in 0.5 ml of 20 mM histidine containing BeCl₂ (10⁻⁴ M) or MgCl₂ (10⁻⁴ M). All aliquots of 0.01 ml were stored for estimation of enzyme activity. In a separate series, the rate of development of inhibition by Be⁺⁺ and histidine was followed and the figure shows the effect of pH on the rate constant for that inhibition.
stant, \( k \), was determined over the same pH range, a relatively sharp maximum about pH 7.2 to 7.4 was obtained. These data suggest that at pH < 6 Mg\(^{2+}\) and Be\(^{2+}\) do not bind at the usual binding site. The decrease in the rate of beryllium binding evident above pH 7.5 cannot as readily be explained; it may, in part, reflect the formation of beryllate ion (BeO\(_2^–\)). In any case, it is clear that the pH optimum for the enzymic reaction coincides with that for binding of Be\(^{2+}\) to the enzyme, likewise the lowest pH at which Mg\(^{2+}\) stimulation occurs coincides with the lowest pH for Be\(^{2+}\) inhibition.

**Beryllium Inhibition in Presence of EDTA and 8-Hydroxyquinoline**—Milstein (3) has reported that P-glucomutase is activated by incubation with 1 mM EDTA; the activation was time-dependent and complete within 10 min. 8-Hydroxyquinoline was relatively ineffective in this regard. As shown in Fig. 8, addition of Be\(^{2+}\) to enzyme incubated with EDTA or 8-hydroxyquinoline, in Tris, elicited a progressive inhibition which was complete in about 30 min. These findings are most readily explained by removal of an inhibitory metal from the enzyme. Milstein (3) had also shown that rabbit muscle P-glucomutase could be activated merely by passage over a column of Amberlite IRC-50 and that the resultant preparation had lost whatever metal or metals may be responsible for development of a chromophore in the presence of 8-hydroxyquinoline. Enzyme so treated could not be further activated by EDTA. In our hands, commercial rabbit P-glucomutase could occasionally be inhibited up to 20% by Be\(^{2+}\) in Tris and up to 40% if the enzyme had first been dialyzed against Tris in deionized water. In view of these observations it was deemed desirable to examine the effect of Be\(^{2+}\) on a metal-free enzyme in the absence of a chelating agent. Accordingly, about 500 pg of enzyme were passed over a column of Dowex 50 (1.0 X 7.5 cm) previously equilibrated with 1 mM Tris, pH 7.5. The resultant preparation exhibited only 20% of the absorbance at 253 m\(\mu\) in the presence of 8-hydroquinoline that is shown by untreated enzyme. When it was employed in the usual assay, the activity of this preparation appeared to have been approximately doubled. Addition of 0.01 mM Be\(^{2+}\) to an aliquot of this preparation, in the presence of EDTA, 8-hydroxyquinoline, and Be\(^{2+}\) on phosphoglucomutase from rabbit muscle. Aliquots of 83 pg of enzyme were incubated with 0.04 M Tris, pH 7.6, and either 1 mM EDTA (O), 1 mM 8-hydroxyquinoline (A), or 0.01 mM Be\(^{2+}\) (W) in final volume of 0.5 ml, at room temperature. Enzymic activity was measured in 5-\(\mu\)l aliquots at the time intervals indicated. At 40 min, samples of the incubation mixtures lacking Be\(^{2+}\) were brought to 0.01 mM Be\(^{2+}\) with BeCl\(_2\) (closed symbols).

![Fig. 8. Effect of EDTA, 8-hydroxyquinoline, and Be\(^{2+}\) on phosphoglucomutase from rabbit muscle.](http://www.jbc.org/)

**Fig. 9. Effect of urea on rabbit muscle phosphoglucomutase.** Enzyme (50 pg) was incubated in a volume of 0.5 ml at room temperature with 0.04 M Tris containing urea at the following concentrations: 0 (O), 3.3 M (O), 4.16 M (A), 4.98 M (A), and 6.6 M (A). At the times shown, activity was assayed in the standard manner with 10-\(\mu\)l aliquots.
4.16 mM Be++ (0). The points (0) and (0) are almost identical. Activity was assayed at times indicated on 10-μl aliquots. These were incubated at room temperature in 0.04 M Tris and the following: control (●), 4.16 M urea (△), 4.16 M urea + 0.1 mM Be++ (■), 6.6 M urea (○), 6.6 M urea + 0.1 mM Be++ ( ● ). The points (○) and ( ■ ) are almost identical. Activity was assayed at times indicated on 10-μl aliquots.

A sample of rabbit muscle enzyme which had thus been treated successively with imidazole + 7Be++ and then 4.16 M urea followed by passage over Sephadex G-25 and which exhibited 56% of original enzymic activity, while retaining less than 5% of previously bound Be++, was then labeled with 32P with the use of the standard procedure employing glucose-1-32P. Only 0.55 mole of phosphate was found to be bound per mole of enzyme whereas the control which had been used through the entire procedure, but without beryllium, accepted 0.91 mole of phosphate per mole of enzyme.

Beryllium Inhibition of Various Phosphoglucomutases—In extension of the comparison of phosphoglucomutases obtained from diverse biological sources described in previous publications, the effect of incubation with Be++ on enzymic activity was ascertained with the phosphoglucomutases from rabbit, flounder and shark muscle, rabbit liver, E. coli, M. lysodeikticus, and B. cereus. The results are summarized in Fig. 12. This comparison was limited to a study of the rate of the enzymatically catalyzed reaction after preincubation of each enzyme with beryllium, in either cysteine or Tris buffer, for varying times. The influence of beryllium, in the presence of cysteine, on the catalytic properties of the enzymes from rabbit, flounder, and shark muscle as well as from rabbit liver was identical. In all instances Be++ failed to influence the enzyme in Tris buffer. The enzyme from E. coli which, like those of the previous group, has been found to operate a catalytic cycle involving alternate phosphorylation and dephosphorylation, was also unaffected by Be++ in Tris buffer. In the presence of cysteine, inhibition by Be++ developed more slowly than with the enzymes of the previous group, even after a 36-minute pre-exposure to Be++, 20% of the original activity was retained in the subsequent enzymic assay. In contrast, the phosphoglucomutases from both M. lysodeikticus and B. cereus, which were generously donated by Dr. Harry Dougherty and Dr. K. Hanabusa, were slowly inhibited by Be++ even in Tris and only somewhat more

constant. Treatment with Mg++ plus imidazole failed to elicit any further increase in activity.

To gain additional insight into these effects of urea, batches of 1 or 2 mg of rabbit P-glucomutase were incubated with 1 μmole of 7Be++ (1.9 to 2.6 × 10⁶ cpm per μg of Be++) in 40 mM imidazole, pH 7.5, in a total volume of 1.0 ml at room temperature. Excess Be++ and imidazole were removed by dialysis against 1 mM Tris. From estimation of the radioactivity of an aliquot of this preparation in a scintillation counter, the protein had bound 0.98 to 1.13 g atoms of Be++ per mole in keeping with the data reported above. An aliquot of this preparation was then diluted with an equal volume of 8.33 M urea, and the solution then passed over a column of Sephadex G-25 (5 cm × 0.75 cm) at 4° which had previously been equilibrated with 4 mM imidazole, pH 7.5. Radioactivity and protein content were measured on aliquots of the breakthrough solution. Whereas 95 to 97% of the 7Be++ was removed from the protein by this procedure, only 40 to 56% of the original enzymic activity was restored. For comparison, it should be noted that a control sample of the same enzyme preparation run through the same procedure without inclusion of Be++ exhibited an absolute increase in enzymic activity.
In a total volume of 1.0 ml, 156 μg of enzyme were incubated at room temperature for the times shown in 0.04 M Tris containing the following: control (○), 1.66 M urea (hexagon), 0.04 M imidazole + 1.66 M urea (●), 3.32 M urea (■), 1 mM EDTA (▲), 1 mM EDTA + 1.66 M urea (■), 0.04 M imidazole (■), 4.4 M urea (△). Activity was assayed with 10-μl aliquots on the standard assay medium. ▲ denotes complete loss of activity of enzyme in 6.6 M urea in less than 30 sec.

**TABLE III**

*Effect of activation and Zn++ on phosphorylation of phosphoglucomutase*

For incubation, 1.0 mg of rabbit muscle enzyme was incubated with either 40 mM imidazole + 1 mM Mg++, pH 7.5, or 1 mM EDTA, or 1 mM ZnSO4 in 40 mM Tris, pH 7.5, in a total volume of 0.5 ml for 60 min. Phosphorylation was then accomplished by addition of 1 μmole of glucose-1-32P (specific activity, 1.98 × 10^6 cpm per μmole of glucose-1-32P), 10 μmole of glucose-1,6-P, in a final volume of 1 ml, for the indicated times. Final concentrations of imidazole, Mg++, EDTA and ZnSO4, and Tris were maintained constant by appropriate addition. Reaction was terminated and enzyme was reisolated as stated in the text. Aliquots were taken for assay of protein and radioactivity by the usual procedure.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>30 sec</th>
<th>2 min</th>
<th>10 hrs</th>
</tr>
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<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>0.696</td>
<td>0.650</td>
<td>1.064</td>
</tr>
<tr>
<td>Imidazole + Mg++</td>
<td>0.948</td>
<td>0.868</td>
<td>0.632</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.974</td>
<td>0.684</td>
<td>1.194</td>
</tr>
<tr>
<td>ZnSO4</td>
<td>0.706</td>
<td>1.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

rapiyly in cysteine buffer; in either case, higher concentrations of Be++ were required than with the animal enzymes.

**Inhibition of Flounder Muscle Phosphoglucomutase**—Flounder P-glucomutase is decidedly less stable than is the rabbit enzyme and exhibits glucose diphosphatase activity, apparently related to the intrinsic instability of the phosphorylated form of the enzyme (4). It appeared of interest to compare its behavior with that of rabbit enzyme in somewhat greater detail. As shown in Fig. 13, pretreatment with EDTA was found to yield a 4-fold increase in activity over the course of about 10 min. This enzyme proved much less stable to urea than rabbit P-glucomutase; irreversible denaturation began in less than 60 sec in 4.16 M urea.

However, exposure to 1.66 M urea in the presence of either 0.04 M Tris or imidazole for 15 min increased enzyme activity by 100 to 200%. Similar exposure to urea of enzyme previously activated by EDTA or Mg++ plus imidazole was without effect. About 50% of the enzyme activity of a preparation of flounder P-glucomutase which had been inactivated by treatment with 0.01 mM Be++, in the presence of 1 mM EDTA, was restored by exposure to 1.66 M urea for 90 sec. After 15 min, however, under similar circumstances all activity was lost.

**Phosphorylation of Activated and Unactivated Enzyme**—Since pretreatment of muscle P-glucomutase with a chelator plus Mg++ effects a marked increase in enzyme activity, it appeared necessary to correlate this with the phosphorylation of the enzyme by its substrate. To this end, the phosphorylation of enzyme by an 8-fold molar excess of glucose 1,6 diphosphate in the presence of a 50-fold molar excess of glucose-1-32P was compared after activation by Mg++ + imidazole, and by EDTA, as well as after an incubation with Zn++ as a model inhibitory ion. Reaction was halted by addition of 4 volumes of saturated ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 0.002 M Tris, pH 7.5, and dialyzed against three changes of 4 liters of this buffer over the course of 24 hours. The results are shown in Table III.

Attempts were made by several procedures to detect a change in the conformation of beryllium-inhibited or the activated forms of P-glucomutase without success. No difference in optical rotatory dispersion, ultraviolet absorption, or ultracentrifugal behavior was seen in rabbit muscle enzyme after treatment with imidazole + Mg++, imidazole + Mg++, + glucose-1-P, imidazole + Be++, or EDTA after passage over Dowex 50 followed by Be++ addition, or after exposure to 4 M urea.

**DISCUSSION**

Most studies of the mechanism of beryllium intoxication at low dosage levels have emphasized the possibility of inhibition of phoshatase by this ion. However, such inhibitions require relatively large concentrations of beryllium, and beryllium inhibition of intestinal alkaline phoshatase is both readily antagonized by magnesium and completely reversible. The sensitivity of animal phosphoglucomutases to very low concentrations of beryllium and the generally irreversible nature of this process are compatible with the possibility that inhibition of this enzyme may be significant in beryllium poisoning, a possibility for which evidence in vivo has already been adduced (7).

At very low concentrations of Be++, binding of this ion to animal phosphoglucomutases occurs stoichiometrically, 1 g atom of metal ion per mole of enzyme. The product, so formed, cannot be distinguished from native enzyme by its absorption spectrum, by ultracentrifugal analysis, by molecular sieving on Sephadex G-200, or by examination of its optical rotatory dispersion. In view of the great sensitivity to low beryllium conc...
• Histidine, in the usual fashion and was inactivated thereby. Evidence for Mg\(^{++}\) binding to the enzyme in the absence of substrate has been reported by Milstein (3). Surprisingly, however, rather tight Be\(^{++}\) binding was also observed with heat denatured phosphoglucomutase. The activation energy for binding of Be\(^{++}\) to native, freshly isolated enzyme is relatively high, 19.9 kcal, and once binding occurs, the beryllium cannot readily be removed by such reagents as EDTA, salicylate, citrate, and phosphate.

The mercuric ion, in the absence of any reagents, is not stably bound to the enzyme-metal complex and that its dissociation from the enzyme-Mg\(^{++}\) reflects the formation of an enzyme-Mg\(^{++}\)-histidine complex which is more active than the original enzyme.

Beryllium binding with enzymic inactivation was originally observed only when the incubation was conducted in the presence of one of the reagents which are also effective in the stimulation of phosphoglucomutase activity when incubated together with the magnesium prior to addition of substrate. These auxiliary reagents include histidine, imidazole, cysteine, and mercaptoethanol. It has been suggested (1, 2) that stimulation by histidine plus Mg\(^{++}\) reflects the formation of an enzyme-Mg\(^{++}\)-histidine complex which is more active than the original enzyme. If the enzyme-Be\(^{++}\) complex formed under similar conditions is a fair model, it is clear that the auxiliary reagent, e.g., histidine, is not stably bound to the enzyme-metal complex and that its continuing presence in such a complex is unnecessary for either beryllium inhibition or magnesium activation.

The requirement for one of this group of relatively weak chelating agents as well as the similar requirement for activation of the enzyme by Mg\(^{++}\) would appear most readily to be explained by the presence, in the isolated enzyme, of a metal ion or ions which are inhibitory. Milstein (3), who suggested this possibility, noted the presence of several metals in his preparations. This concept is strengthened by the fact that EDTA at low concentration, passage over Dowex 50 or Amberlite IRC-50 (3), prolonged dialysis against Tris in previously deionized water, and exposure to urea at a concentration at which irreversible denaturation does not occur, also both promote activation by Mg\(^{++}\) in varying degree and facilitate binding of Be\(^{++}\) with consequent inhibition. Studies currently in progress are designed to identify the inhibitory metal ions in the isolated enzyme. If this concept is correct, it will be clear that if the inhibitory ions are not removed by some prior treatment, Mg\(^{++}\) activation and Be\(^{++}\) binding are each contingent upon the presence of a chelator adequate to remove the offending metal ion while inadequate to prevent transfer of Mg\(^{++}\) or Be\(^{++}\) from the solution to the binding site on the enzyme surface.

Of particular interest also is the fact that addition of substrate abruptly terminates both Mg\(^{++}\) activation and Be\(^{++}\) inhibition. The simplest explanation of these observations is effective binding of substrate to the enzyme in such a manner as to render the original inhibiting metal ion inaccessible to the chelating agent on the assumption that the native inhibitory ion, activating Mg\(^{++}\), and inhibiting Be\(^{++}\) all bind at the active catalytic site of the enzyme.

Present information fails to reveal whether the effects of binding of Be\(^{++}\) to the enzyme differ qualitatively or only quantitatively from those of the presumed inhibitory ions on the enzyme as isolated. Whereas bound Be\(^{++}\) totally prevents phosphorylation of the enzyme by glucose-1,6-P, unactivated enzyme is phosphorylated albeit somewhat more slowly than is the enzyme which has been activated by treatment with imidazole and Mg\(^{++}\).

These findings clearly indicate that an unactivated enzyme preparation does not consist of a mixture of catalytically active, phosphorylatable enzyme molecules and catalytically inactive, nonphosphorylatable molecules. It is conceivable that unactivated enzyme to which an inhibitory ion such as Zn\(^{++}\) is bound cannot be phosphorylated, and it is the greater ease of dissociation of this metal which permits the observed phosphorylation, in keeping with the decrease in V\(_{\text{max}}\) to 20 to 50% of that of the activated enzyme.

Alternatively unactivated enzyme may consist of enzyme molecules to which is bound a cation which is not inhibitory but rather simply less effective than is Mg\(^{++}\) in the catalytic process. Elucidation of these problems requires demonstration of the nature of the postulated cation bound to freshly isolated enzyme as well as a comparison of the rate of phosphorylation of enzyme under these various circumstances measured on the time scale of a single catalytic turnover.

The influence of urea in the system here described is poorly understood. The activation of native enzyme by exposure to the maximal urea concentration which does not occasion irreversible denaturation, and the facilitation of beryllium inhibition under these circumstances suggests release to the medium of native inhibitory ion from partially unfolded enzyme. It is clear that essentially all of the Be\(^{++}\) is released from enzyme-Be\(^{++}\) complex by the same concentration of urea. But it is not obvious why considerable inactivation of enzyme occurs in the latter circumstance. The only other procedure which was found to reactivate Be\(^{++}\)-inhibited enzyme was brief exposure to pH 1., and this never resulted in reappearance of more than 5 to 10% of original activity. Of interest also is the observation that bovine muscle P-glucomutase, which was previously noted to be relatively unstable as compared to the rabbit enzyme, is also much more sensitive to urea.

It is perhaps particularly noteworthy that, of the three bacterial phosphoglucomutases tested, only the sensitivity to Be\(^{++}\) of P-glucomutase from E. coli, which appears to undergo phosphorylation and dephosphorylation during the catalytic process, was found to resemble somewhat the behavior of the animal phosphoglucomutases. In contrast, the enzymes from M. lysodeikticus and B. cereus, for which both direct and kinetic evidence has been adduced to indicate that their catalytic mechanisms do not involve cyclic phosphorylation and dephosphorylation are affected reversibly and only rather slowly by somewhat higher concentrations of beryllium. Moreover, in these instances, development of inhibition was independent of the presence of an auxiliary chelating agent. These observations strengthen the suggestion that these two bacterial P-glucomutases differ substantially in structure and mechanism of action from the other P-glucomutases studied to date.
The physiological implications of the activation of P-glucomutase by diverse metal chelating agents remain to be resolved. Whereas the various reports concerning this phenomenon have largely related to properties of the purified, indeed frequently crystalline enzyme, 2- or 3-fold activation of the enzyme by imidazole or cysteine is also observed in initial extracts prepared from rabbit liver or muscle. Accordingly, the activatable state appears to be the normal state of most of the P-glucomutase of these tissues. It will be of interest to ascertain whether this situation is altered with varying physiological circumstances and, hence, reflects imposition of physiological control over the phosphoglucomutase reaction.

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