THE ENZYMATIC CONVERSION OF GLUCOSE-1-PHOSPHORIC ESTER TO 6-ESTER IN TISSUE EXTRACTS*

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The formation of glucose-1-phosphoric acid (1-ester) and its conversion to glucose-6-phosphoric acid have been shown to be initial steps in the degradation of glycogen to lactic acid (1). The enzyme which brings about the conversion of the 1-ester has been found in extracts of rabbit muscle, brain, heart, liver, in dog and frog muscle extracts, and in extracts of yeast (2); it is presumably present in all cells which ferment glycogen.

The present report deals with a study of the enzyme which causes a migration of the phosphate group from carbon atom 1 to the spatially adjacent carbon atom 6; the enzyme will be referred to as phosphoglucomutase.\(^1\) Owing to the presence, in all extracts, of the extremely active equilibrium enzyme\(^2\) of Lohmann (5), part of the glucose-6-phosphate is converted almost instantaneously to fructose-6-phosphate. The equilibrium mixture formed is referred to as 6-ester. Phosphoglyceromutase catalyzes a reversible equilibrium reaction, while as yet no indication has been found for a reversibility of the reaction catalyzed by phosphoglucomutase.

EXPERIMENTAL

Rabbit muscle extracts were prepared as previously described (6); 1 cc. of extract corresponded to about 0.4 gm. of muscle. The

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\(^1\) The enzyme which causes a migration of the phosphate group from position 3 to position 2 in phosphoglyceric acid has been given the name of phosphoglyceromutase (3).

\(^2\) The name phosphohexokinase has been proposed (4), but Lohmann now suggests "oxo-isomerase" for this group of enzymes which includes phosphohexo- and phosphotrioseisomerase.

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Predialyzed extracts were electrodialyzed until the resistance approached that of distilled H₂O, which took about 8 hours. Protein which precipitated during dialysis could be filtered off without materially affecting the enzyme activity of the solution. Upon storage of the extracts in the refrigerator under toluene a further slow precipitation of protein took place. As a rule this precipitate was resuspended prior to an experiment.

The day to day loss of enzyme activity was small at first, but increased after some weeks; after 2 months about three-fourths of the activity had disappeared. Depending on the age of the extracts and on the addition of accelerating ions, the experiments were carried out with dilutions of the extracts of 1:2 to 1:80; usually the dilutions were 1:10 to 1:20. Even in freshly prepared extracts the enzymatic processes stopped with the formation of 6-ester.

Natural as well as synthetic (6) glucose-1-phosphate was used as substrate; the Ba salt was converted to either the K, NH₄, or Na salt by treatment with the respective sulfates. After incubation of the extract with a known amount of substrate the proteins were precipitated with trichloroacetic acid and the amount of substrate left unchanged was determined.

The determination of the 1-ester is based on the fact that it is completely hydrolyzed to glucose and inorganic phosphate in 5 minutes at 100° in NH₄SO₄ (6), while the reaction product, the 6-ester, is not appreciably hydrolyzed by this procedure (8). The phosphate determinations were carried out by means of the method of Fiske and Subbarow (9). Information on the period of incubation, temperature, addition of ions, and other details will be found in the legends of Figs. 1 to 5 and of Table I.

Influence of Mg⁺⁺ and Mn⁺⁺.—It has been shown previously (10) that Mg greatly accelerates the conversion of the 1- to 6-ester. Since then it has been found that Mn has an even stronger effect. Of other ions tested, Ni⁺⁺ had a trace of accelerating action, while Co⁺⁺ showed an action of a similar order to that of Mg and Mn (see Experiments 6 and 7, Table I), but a detailed study of its effect was not carried out. Cu⁺⁺, Zn⁺⁺, and Ca⁺⁺ did not accelerate in any and inhibited in higher concentrations.

* An apparatus similar to that described by Bernhart et al. (7) was used, 110 volts being applied across the platinum electrodes.
TABLE I

Effect of Various Salts on Phosphoglucomutase

The dilution of the extract was 1:10, except as noted. The initial 1-ester concentration was 3 to 5 mM and the period of incubation 30 minutes at 37°.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Accelerating ion</th>
<th>Other additions</th>
<th>Per cent of 1-ester converted to 6-ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 mM MgCl₂</td>
<td>20 mM NaCl</td>
<td>54.9</td>
</tr>
<tr>
<td></td>
<td>10 &quot; &quot;</td>
<td>20 &quot; KCl</td>
<td>36.1</td>
</tr>
<tr>
<td></td>
<td>10 &quot; &quot;</td>
<td>20 &quot; CaCl₂</td>
<td>37.6</td>
</tr>
<tr>
<td>2</td>
<td>1.3 &quot; MnCl₂</td>
<td>20 µM NaCl</td>
<td>51.6</td>
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<td>58.2</td>
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<td>20 &quot; CaCl₂</td>
<td>55.2</td>
</tr>
<tr>
<td></td>
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<td>20 &quot; glucose</td>
<td>51.0</td>
</tr>
<tr>
<td>3</td>
<td>1.3 &quot; &quot;</td>
<td>20 mM CaCl₂</td>
<td>45.1</td>
</tr>
<tr>
<td></td>
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<td>10 &quot; MgCl₂</td>
<td>48.2</td>
</tr>
<tr>
<td></td>
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<td>4.5 mM 6-ester</td>
<td>66.6</td>
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<tr>
<td></td>
<td>1.3 &quot; &quot;</td>
<td>4.5 &quot; α-glycerophosphate</td>
<td>61.8</td>
</tr>
<tr>
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<td>4.5 mM 6-ester</td>
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<tr>
<td></td>
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<td>4.5 &quot; α-glycerophosphate</td>
<td>55.8</td>
</tr>
<tr>
<td>5</td>
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<td>1.5 mM adenylic acid</td>
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<td>1.5 mM adenylic acid</td>
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<tr>
<td>6</td>
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<td>1.5 mM adenylic acid</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td>10 mM NiCl₂</td>
<td>4.5 &quot; PO₄ buffer</td>
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<tr>
<td></td>
<td>10 &quot; CoCl₂</td>
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<tr>
<td>7</td>
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<td>5.5 mM mannose-1-phosphate</td>
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<tr>
<td></td>
<td>1.3 &quot; &quot;</td>
<td>5.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.7 &quot; CoCl₂</td>
<td>74.0</td>
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</table>

* Dilution of extract 1:2.
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It may be seen in Fig. 1 that the enzyme displayed some activity when no Mg or Mn was added. Since this extract had been electrodialyzed and diluted 20 times and since special care had been taken in the preparation of the 1-ester to avoid contamination with Mg or Mn, it is assumed that the phosphoglucomutase can act without these ions. The pH-activity curve (Fig. 3) leads to the same assumption, since the enzyme without addition of Mg is still maximally active at a pH at which added Mg had lost its effect, owing to formation of the insoluble hydroxide.

Fig. 1. Effect of Mn and Mg on conversion of 1- to 6-ester. 1 cc. of a 10-fold diluted muscle extract which was 34 days old and to which varying amounts of MnCl₂ or MgCl₂ had been added was mixed with 1 cc. of glucose-1-phosphate (as the NH₄ salt) containing 0.24 mg. of P. The mixture was incubated for 15 minutes at 37°. All values are given per 2 cc. of reaction mixture.

Fig. 1 shows that addition of Mn produced a sharp rise in the enzyme activity which reached its maximum at about 0.5 mM and dropped off slowly at higher concentrations. With Mg addition the increase in activity was more gradual, but the same maximum was reached as with Mn. The optimal concentration for Mg was between 5 and 10 mM. The wide plateau shown with Mg was not

The Mg content of undialyzed extracts was about 2.6 mM as determined by the method of Hoffman (11). After electrodialysis, no Mg was found when 10 cc. of extract were used for analysis, while 0.01 mg. of Mg in 10 cc. (0.04 mM) could still be detected with this method.
an invariable finding; in other experiments the activity fell off more steeply with rising Mg concentrations.

With more dilute enzyme solutions than were used in Fig. 1, no Mg concentration was found at which the rate of enzyme activity was as high as at the optimal Mn concentration. Furthermore, with such dilute solutions difficulties were encountered, on repetition of the same experiment, in obtaining the same degree of activation with Mg. This was particularly the case when the extract was centrifuged (in order to remove insoluble protein) before it was diluted. The following observations may have some bearing on this finding. The protein precipitate, when washed with a small amount of H2O, was devoid of enzyme activity, but when the washed precipitate was added to the clear supernatant fluid in larger amounts than were originally present, the accelerating effect of Mg became greater. This was not the case when the precipitate, suspended in H2O, had been previously heated to 70° for 30 minutes. These observations suggest that a protein is present in muscle extracts which, by an as yet unknown mechanism, increases the Mg effect on the phosphoglucomutase.

The question as to the maximal acceleration of enzyme activity which can be obtained upon Mg or Mn addition has not been fully investigated. In Fig. 1 the highest acceleration was 6.5 times, but since 76 per cent of the substrate was used up, this must have been a limiting factor. In other experiments with Mn, accelerations up to 15 times were observed. Of the two ions Mn is more effective in increasing the activity of the phosphoglucomutase, but physiologically Mg is probably of greater importance, since its concentration in muscle, namely 10 mM (12), is approximately optimal for the mutase, whereas Mn is present in suboptimal concentration (13).

Ohlmeyer and Ochoa (14) studied a system in which Mn and Mg act as accelerating ions under certain conditions. They found that the transfer of phosphate from phosphopyruvic acid to glucose in yeast extract, for which reaction the presence of adenylic acid or cozymase (diphosphopyridine nucleotide) is necessary, takes place at the maximal rate provided no Na ions are present. Na has an inhibitory effect which can be completely overcome by other ions in the following molar ratios: Mn : Mg : NH₄ : K = 1 : 50 : 1000 : 2000. Mg, Mn, or Co ions accelerate the phospho-
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glucomutase when no Na ions have been added and their action can therefore not be due to an overcoming of Na inhibition.

Effect of Other Ions—The phosphoglucomutase is inhibited by various salts, Na salts being no more effective than others. This inhibition is more pronounced when Mg is added as the accelerating ion than when Mn is added. In Fig. 2 it is seen that in the presence of 20 mM Na$_2$SO$_4$ the highest enzyme activity reached with Mg was below that reached with Mn. Fig. 2, as well as Fig. 1, shows that the Mg and Mn salts themselves, when added beyond their optimal concentration, decrease the enzyme activity.

![Graph showing effect of Mn and Mg in presence of 20 mM Na$_2$SO$_4$.](http://www.jbc.org/)

Fig. 2. Effect of Mn and Mg in the presence of 20 mM Na$_2$SO$_4$. The conditions were the same as in Fig. 1, except that the substrate was added as the Na salt.

The effect of other salts is shown in Table I. NaCl, KCl, and CaCl$_2$ in 20 mM concentration, inhibited the Mg effect, while NaCl and KCl had no retarding action in the presence of Mn, and CaCl$_2$ even seemed to enhance the Mn effect.

In the presence of Mn, phosphate buffer had no effect, and 6-ester (the reaction product of the enzyme) caused a slight inhibition, while adenylic acid and $\alpha$-glycerophosphate (all added as the Na salts) had a definite inhibitory effect. Without the addition of accelerating ions, all the salts just enumerated had inhibitory effects. The inhibitory effect of phosphate buffer is made use of in the preparation of 1-ester by means of muscle extract (6), 0.1 M buffer of pH 6.8 being recommended for that purpose.
Simultaneous addition of Mn and Mg in their respective optimal concentrations hardly increased the enzyme activity beyond the rate obtained with Mn or Mg alone (Experiment 3, Table I).

Influence of pH—All pH measurements were made with a glass electrode. Addition of the alkaline K, NH₄, or Na salts of the 1-ester (to give a final concentration of 3 mmol) to the more acid extract brought the pH to about 7.4, at which pH the experiments so far reported have been carried out.

![Graph showing effect of pH on enzyme activity.](http://www.jbc.org/)

The dissociation constants of the 1- and 6-esters have been shown to be the same (6), so that a change in pH during the activity of the phosphoglucomutase was not to be expected. Addition of a strong buffer seemed therefore unnecessary, and, besides, even dilute buffer solutions (such as citrate, veronal, glycerophosphate, NaHCO₃-CO₂) were found, in common with other salts, to have an inhibitory effect on the enzyme when no accelerating ions were added. Phosphate buffers were found unsuitable, partly for analytical reasons, partly because they also exerted an inhibitory action.
The pH-activity curve in Fig. 3 was obtained by adding to the substrate solution dilute HCl or NaOH. After addition of the enzyme solution the pH was measured in an aliquot of the reaction mixture. In some cases pH measurements were made at the start and end of the incubation period and only insignificant differences were found. The buffering capacity of the added substrate and its reaction product was therefore sufficient to keep the pH constant.

The curve for the enzyme without addition of Mg or Mn shows a broad maximum between pH 7.5 and 9.2. At pH 6.5 and 10.0 the enzyme activity has dropped to very low values.5

The pH-activity curve in the presence of Mn or of Mg (indicated by X in Fig. 3) shows a sharp drop on the alkaline side because of the formation of the insoluble Mn(OH)₂ or Mg(OH)₂. Up to pH 7.6 it closely resembles that obtained in the absence of these ions.

Effect of Enzyme Concentration and Temperature—In Fig. 4 is shown the activity of three dilutions of the same extract. Within

Fig. 4. Effect of enzyme concentration. No accelerating ions were added. Temperature, 37°. The substrate concentration was 3 mM. The enzyme concentrations for Curves A, B, and C, expressed in arbitrary units, were 1, 2, and 4 respectively. A fresh muscle extract was used.

5 At pH 6.5 the activity of the enzyme which forms glucose-1-phosphate from glycogen and inorganic phosphate is much less inhibited than that of the phosphoglucomutase, so that 1-ester accumulates even in the presence of Mg (10).
these limits there exists a fairly good proportionality between enzyme concentration and activity, as shown by the following values for $K$, when 30 per cent of the substrate has been used up: Curve A 50.0, Curve B 49.2, Curve C 50.4 ($ET = K$, where $T$ is time in minutes and $E$ is enzyme concentration, the latter being arbitrarily designated as 1 in Curve A).

A comparison of Curve C in Figs. 4 and 5 shows that as the substrate is being exhausted, the rate of enzyme activity drops off more slowly in the presence than in the absence of Mn. This difference, however, is not large enough to explain the accelerating action of Mn.

The enzyme activity proceeded, with and without addition of accelerating ions, until practically all the substrate (98 per cent) was used up. Experiments in which the temperature was changed after the reaction had gone to completion gave no indication of a reversible equilibrium nor did addition of 6-ester lead to formation of 1-ester.

The temperature coefficient (for the range 18–38°), without addition of accelerating ions, was found to be 2.5. Keeping the enzyme solution for 10 minutes at 50° before testing its activity had no noticeable effect on it, while at 60° the enzyme was destroyed.
Specificity of Phosphoglucomutase—Mannose-1-phosphate and galactose-1-phosphate, synthetically prepared (15), were not converted to the respective 6-esters by muscle, liver, or yeast mutase, with or without the addition of accelerating ions. With the muscle enzyme a slight inhibitory effect on the conversion of glucose-1-phosphate was noted when mannose-1-phosphate was added (see Experiment 7, Table I), while addition of glucose (Experiment 2) had no effect.

Phosphatase and Phosphohexoisomerase—Muscle extracts as prepared in this work did not form inorganic phosphate from 1-ester or 6-ester, even after 3 hours of incubation; they did not contain an active phosphatase or any other enzyme which could act on the 6-ester. They did contain, however, a very active phosphohexoisomerase, the enzyme which catalyzes the reaction glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate. When fructose-6-phosphate (prepared from hexosediphosphate) was added to an extract which had been diluted 100 times, 68 per cent of it was converted to glucose-6-phosphate in 5 minutes at 37°C, which indicates a close approach to equilibrium conditions. The disappearance of the fructose-6-phosphate was measured by means of the Seliwanoff reaction as modified by Roe (16). The isomerase acted without addition of Mg, Mn, or Co or a coenzyme. It has so far been impossible to prepare a mutase free of isomerase and the isolation of the primary conversion product of glucose-1-phosphate (presumably glucose-6-phosphate) could therefore not be undertaken.

When glucose-1-phosphate was incubated for short periods of time with dialyzed liver extract to which Mg had been added, 6-ester and inorganic P were formed. For example, of 0.235 mg. of 1-ester P added to 2 cc. of dialyzed and 4-fold diluted liver extract, 2 per cent was left unchanged, 54 per cent was converted to 6-ester, and 44 per cent to inorganic P during 30 minutes incubation at 37°C. About the same amount of inorganic P was formed when 6-ester was added instead of 1-ester. These results show that dialyzed liver extract contains a phosphatase, but leave it undecided whether it acts on 1-ester, 6-ester, or on both. This phosphatase seems to possess a high degree of specificity, since it does not split galactose-1-phosphate under the conditions of these experiments.
DISCUSSION

According to Lohmann (17) Mg ions are necessary for lactic acid fermentation in muscle. He showed that for the fermentation of glycogen, hexosemonophosphate, and hexosediphosphate, Mg is needed in decreasing amounts. No other ion was found which could replace Mg. Recently Warburg and Christian (18) reported that Mn ions are of importance in alcoholic yeast fermentation. According to Lohmann and Schuster (19) Mn or Mg ions are necessary for the activity of the carboxylase-cocarboxylase system of yeast which forms acetaldehyde and CO₂ from pyruvic acid. In the reaction studied by Ohlmeyer and Ochoa (14) in yeast extract, which was described in a previous section, Mn and Mg ions by themselves had no accelerating activity; they merely acted by overcoming the inhibition exerted by a large (0.1 M) Na concentration.

The effect of these ions in muscle extract can now be correlated to certain steps on the path to lactic acid. The first step, namely the formation of 1-ester from glycogen and inorganic phosphate with adenylic acid as coenzyme (20), takes place in electrodialyzed muscle extracts to which no Mg (or Mn or Co) has been added. The accelerating effect of Mg on this reaction appears to be due to the removal of the inhibitory reaction product; i.e., the 1-ester (unpublished experiments). The next step, the conversion of glucose-1- to glucose-6-phosphate, is greatly accelerated, while the third step, the partial transformation of glucose-6- to fructose-6-phosphate, is probably not accelerated by the ions just mentioned. Of subsequent steps on the path to lactic acid, reactions involving a transfer of phosphate through the adenosinetriphosphate system have been reported to be accelerated by Mg ions (21).

In the reaction catalyzed by phosphoglucomutase an intramolecular phosphate transfer takes place without the mediation of a coenzyme. For this reaction it is clear that the specific ions react with the enzyme and not with a coenzyme and that their effect does not consist in overcoming an inhibition exerted by Na ions.

The contention of Kendal and Stickland (22) that hexosediphosphate acts as coenzyme and is essential for the reaction is not substantiated by our findings.
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SUMMARY

1. The enzyme phosphoglucomutase, which is found in extracts of mammalian and frog tissues and of yeast, causes a transfer of phosphoric acid from carbon atom 1 of glucose-1-phosphate to carbon atom 6.

2. The enzyme is active in electrodialyzed extracts of rabbit muscle. It does not require addition of a coenzyme but its rate of activity is greatly accelerated (up to 15 times) by added Mg, Mn, or Co ions.

3. The optimal concentration for Mn\(^{++}\) was found to be 0.5 mM; that of Mg\(^{++}\) between 5 and 10 mM. The same activity was obtained with the two ions at their optimal concentrations. In the presence of salts or in very dilute enzyme solutions the highest activity obtained with Mg fell below that obtained with Mn.

4. The enzyme is inhibited by addition of salts, but this inhibition is not attached to any particular cation or anion. The inhibition by salts is least marked in the presence of Mn ions.

5. The pH-activity curve shows that the enzyme has a broad plateau of optimal activity between pH 7.5 and 9.2. In the presence of Mg the curve drops off sharply on the alkaline side, owing to the loss of Mg ions as insoluble Mg(OH)\(_2\).

6. Within specified limits the enzyme activity is proportional to enzyme concentration, both without and with added Mn ions.

7. The reaction catalyzed by this mutase practically goes to completion. No indication was found that a reversible equilibrium exists.

8. The enzyme without addition of accelerating ions has a \(Q_{10}\) of 2.5 for the range 18–38°. It is destroyed in 10 minutes at 60°.

9. The enzyme does not form the respective 6-esters from galactose-1-phosphate or mannose-1-phosphate.

10. Dialyzed rabbit liver extracts contain besides phosphoglucomutase a phosphatase. After short periods of incubation part of the added glucose-1-phosphate is found as 6-ester and part as inorganic phosphate, while galactose-1-phosphate is not changed.

Addendum—After this paper was submitted for publication, Lehmann (23) reported that insulin has an inhibitory effect on the transformation of the 1- to the 6-ester. While a commercial insulin was found to exert a slight inhibitory effect, this was not the case when a Zn-free, water-soluble insulin powder was used (for which the authors are indebted to Dr. P.
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A. Shaffer). Insulin was also without effect on the formation of 1-ester from glycogen and inorganic phosphate in muscle extract.

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