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The Mechanism of Activation of Phosphoglucomutase by Chelating Agents

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Phosphoglucomutase, the enzyme that catalyses the interconversion of glucose 1-phosphate and glucose 6-phosphate, has a rather complex requirement for cofactors. The activation by metal ions was discussed in the preceding paper (Milstein, 1961a), and the effect of chelating agents is the subject of this one.

Several enzymes have been described as being activated by chelating agents. In some instances this activation is essential, i.e. the activity in the absence of the activator is negligible (see, for instance, Tabor & Mehler, 1955; Stoppani & Milstein, 1957; Najjar, 1948). Although removal of an inhibitory metal has been generally considered to be the mechanism of the activation, a binding of the chelating agent to the active site, as a necessary step in enzyme activation, has also been suggested (Mounter, Floyd & Chanutin, 1953; Sauer & Rapoport, 1959).

Lehmann (1939) first observed activation of phosphoglucomutase in crude extracts by glutathione, and Najjar (1948) found that the pure crystalline enzyme required cysteine for activity. Independently Jagannathan & Luck (1949) reported the activation of phosphoglucomutase by

sulphite and serum albumin, and Sutherland (1949) reported activation also with histidine, free insulin, 8-hydroxyquinoline, dithizone and histamine.

This paper deals with the nature of the activation by chelating agents of phosphoglucomutase of rabbit skeletal muscle. It is shown that the absolute requirement for a chelating agent can be explained by an extreme sensitivity of the enzyme towards heavy metals, the concentration of which must be kept very low.

On addition of the enzyme to the assay mixture containing the chelating agent, removal of the metals and activation of the enzyme occurs. It will be shown that a different type of activation, which is time-dependent, also occurs when the enzyme is preincubated with either ethylenediaminetetra-acetic acid or Mg^{2+} ion plus a chelating agent.

A preliminary account of part of this work has been published (Milstein, 1960a).

MATERIALS AND METHODS

These were the same as described by Milstein (1961a), except the following:

'Metal-free' phosphoglucomutase. This was prepared by the following procedure: the purified enzyme was dialysed against large volumes of glass-distilled water and then

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against 1 mm-2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl, pH 7.5. It was then passed through an Amberlite IRC-50 column (8 cm. long) in the tris form, equilibrated with 1 mm-tris-HCl, pH 7.5. The metal content of the preparation after this treatment was undetectable by the 8-hydroxyquinoline test (Milstein, 1960*b*). The specific activity of the enzyme did not change with the treatment.

Special purification of the reagents. Metal-free tris-HCl buffer, pH 7.5, was prepared by passing the solution in the desired concentration through an Amberlite IRC-50 column treated with concentrated tris solution and then equilibrated with the dilute buffer solution.

The glucose 1-phosphate was freed from metal contamination before use by passing a solution of about 10 mg./ml. through an Amberlite IRC-120(H) column in the sodium or the potassium form. The potassium salt was generally used.

'Purified' glass-distilled water was obtained by passage through an Amberlite IR-120(H) column (30 cm. $\times$ 2 cm.) previously washed with 1*N*-HCl and then exhaustively with water.

When stated, MgSO₄ (A.R.) was also passed through an Amberlite IR-120(H) column which had been washed with HCl and then converted into the magnesium form with several volumes of 2*M*-MgSO₄. After washing the column with glass-distilled water, 6 mm-MgSO₄ was passed through.

Alternatively, a mixture of glucose 1-phosphate and Mg²⁺ ion, of the commercial (British Drug Houses Ltd.) potassium salt of glucose 1-phosphate and A.R. MgSO₄, in the appropriate concentrations at pH 7.5, was shaken with a solution of 0.01 % of dithizone in CCl₄ (Zn²⁺-free, A.R.) twice to remove metals, and then (unless otherwise indicated) five times with CCl₄ to eliminate any trace of dithizone.

L-Cysteine-HCl was recrystallized from a constant-boiling HCl solution of the commercial (Roche) product. pH was adjusted to 7.5 with tris.

DL-Histidine-HCl was recrystallized from water and washed with acetone and ether.

RESULTS

That pure phosphoglucomutase from rabbit skeletal muscle is inactive when no metal-chelating agent is present in the assay mixture has already been shown (Najjar, 1948), and additional evidence is presented here. A comparison of the activating effect of different chelating agents is shown in Table 1. Qualitatively the activating effect can be related to the ratio of the binding capacity of the activator for heavy metals to that for magnesium. Thus cysteine, with a high affinity for heavy metals and a low one for magnesium, is the best activator. On the other hand, ethylenediaminetetra-acetic acid (EDTA), with a very high affinity for both, is a very poor activator, and at higher concentrations its inhibitory effect, due to removal of magnesium from the enzyme, becomes apparent.

Histidine is a good complexing reagent for heavy metals, but not for magnesium, and its activating effect approaches that of cysteine. EDTA and histidine activations are not additive (Table 1, Expts. C and D). The activation does not seem to

Table 1. *Effect of chelating agents on the activity of phosphoglucomutase*

The enzyme was incubated in the presence of 1.7 mm-MgSO₄, 7.5 mm-glucose 1-phosphate and the additions given.

Expt.	Enzyme (μ g.)	Additions (mm)	Activity (μ g. of P)	Relative activity (cysteine = 100)
A	1.4	28.6 Cysteine	56.8	100
A	1.4	0.074 EDTA	8.4	14.6
A	1.4	0.016 EDTA	12.8	22.5
A	1.4	0.0029 EDTA	10.8	19.0
A	1.4	0.0013 EDTA	7.4	13.0
A	1.4	0.0007 EDTA	2.6	4.6
A	1.4	None	1.3	2.3
B	2.0	5.7 Cysteine	39.4	—
B	2.0	2.8 EDTA	4.0	—
B	2.0	0.57 EDTA	15.8	—
B	2.0	0.057 EDTA	23.0	—
C	5.2	25.0 Histidine	40.3	—
C	5.2	25.0 Histidine + 0.12 EDTA	40.3	—
D	5.2	25.0 Histidine	46.5	—
D	5.2	25.0 Histidine + 0.25 EDTA	45.8	—
E	2.9	28.0 Cysteine	52.6	100
E	2.9	0.1 8-Hydroxyquinoline	23.4	44.5
F	1.7	19.0 Cysteine	40.2	100
F	1.7	4.2 8-Hydroxyquinoline	19.4	48.2
G	2.2	28.0 Cysteine	52.4	100
G	2.2	20.0 Na ₂ SO ₃ + 0.4 % of egg albumin	15.0	28.6
H	Undetermined	20.0 Cysteine	17.8	100
H	Undetermined	22.0 Histidine	10.7	62
I	1.7	28.0 Cysteine	43.6	100
I	4.4	20.0 Na ₂ SO ₃	4.6	4.2

depend on reduction, since sulphite either alone or with ovalbumin does not improve the activation by other chelating agents.

Metal content of phosphoglucomutase

Phosphoglucomutase dialysed against large volumes of ordinary glass-distilled water for 24 hr. contained metals. This was shown both by the 8-hydroxyquinoline test and by neutron-activation analysis.

Fig. 1 shows the effect of the addition of 8-hydroxyquinoline on the spectrum of the enzyme, with both water and 8-hydroxyquinoline as blanks (difference spectra). Subtraction of the protein spectrum from the difference spectrum yields the typical metal-8-hydroxyquinoline peak, showing the presence of metals in the preparation. The value of $\Delta E_{253}/\text{mg.}$ in this experiment is 0.560.

Neutron-activation analysis is an extremely sensitive method for metal determinations (Meinke, 1959; Loveridge & Smales, 1957). The results obtained after irradiation of phosphoglucomutase showed, besides the two characteristic peaks at 1.37 and 2.65 mev with a half-life of roughly 14.5 hr. corresponding to ^{24}Na , another peak at 0.85 mev which has a shorter half-life. It roughly corresponds with ^{56}Mn .

The radioactive material was allowed to stand over a period of 1 week until the short half-life radiation was insignificant, and the spectrum of radioactivity was again measured. Some radioactivity was still present, in the range 0.4–2.7 mev, but no sharp peaks could be detected.

Fig. 1. Effect of 8-hydroxyquinoline on the spectrum of phosphoglucomutase. Spectra: (A) 300 $\mu\text{g.}$ of enzyme/ml. (against water); (B) 300 $\mu\text{g.}$ of enzyme/ml. and 53 $\mu\text{M-8-hydroxyquinoline}$ (against water); (C) 300 $\mu\text{g.}$ of enzyme/ml. and 53 $\mu\text{M-8-hydroxyquinoline}$ (against 8-hydroxyquinoline); (D) 53 $\mu\text{M-8-hydroxyquinoline}$ (against water).

The results of the activation analysis thus indicate the presence of a mixture of metals, each in small quantities, of which ^{56}Mn is the only one which could be identified, although some with long half-life are also present. The amount of manganese originally present is probably less than 0.3 atom/molecule of phosphoglucomutase, but this is only a tentative value calculated from the radioactivity measured (Loveridge & Smales, 1957) and is subject to errors, since no provisions for quantitative measurements were made. This amount of Mn^{2+} ion would not give a significant value in the 8-hydroxyquinoline test. It seems probable that the unidentified metals responsible for the long half-life radioactivity are also responsible for the high value of the 8-hydroxyquinoline test.

A 'metal-free' phosphoglucomutase was prepared as previously described. The 8-hydroxyquinoline test on the phosphoglucomutase solution at a concentration of 430 $\mu\text{g./ml.}$ gave a reading at 253 $\text{m}\mu$ of 0.176. After the addition of the 8-hydroxyquinoline to both blank and sample the reading was 0.175. It is interesting that when this purified preparation with a $\Delta E_{253}/\text{mg.}$ value of 0.00 was dialysed for 24 hr. against glass-distilled water, the 8-hydroxyquinoline test rose again to a $\Delta E_{253}/\text{mg.}$ value of 0.810, suggesting that 'metal-free' phosphoglucomutase is able to bind the very low concentrations of metal impurities present in the glass-distilled water. The specific activity of the preparation did not change with the column treatment, showing that the removed metals are not necessary for activity.

When phosphoglucomutase was shaken with dithizone in CCl_4 the 8-hydroxyquinoline-test value was also lowered. The enzyme with an original $\Delta E_{253}/\text{mg.}$ value of 0.409 was treated as follows: a solution containing 264 $\mu\text{g./ml.}$ of enzyme was shaken very gently for 30 sec. at 0° , with a 0.01 % solution of dithizone in metal-free CCl_4 , and the aqueous layer was separated after standing for some minutes. The 8-hydroxyquinoline test was then found to be negative. During dithizone treatment some denaturation occurs. Before the treatment the activity was 22.0 units/mg., and afterwards it was 18.4 units/mg., which represents a loss of 16 % of the original value. This treatment also introduced traces of dithizone and CCl_4 , judged by u.v. absorption. For freeing phosphoglucomutase from metals therefore the ion-exchange method was preferred.

Activation in the absence of chelating agents

'Metal-free' phosphoglucomutase had essentially the same requirements as the original enzyme. Removal of metals did not alter its specific activity, nor its requirement for chelating agents.

It has already been shown that in the absence of chelating agents the activity never reached values higher than 3% of the activity observed in the presence of 25 mM-cysteine. To increase this value, special purification of all the reagents was necessary.

All the reagents were purified with resin columns, as indicated in the Materials and Methods section, and special care was taken in the handling of the glassware (Thiers, 1957). After these precautions a several-fold increase in the activity in the absence of chelating agents was observed (Table 2). However, the values were still low compared with those obtained when cysteine was present. Values obtained in the absence of cysteine were poorly reproducible owing to the difficulty of preparing an absolutely 'metal-free' system. In the absence of cysteine the activity is adversely affected by using glass-distilled water instead of the 'metal-free' water, and by the use of MgSO_4 (A.R.) which was not especially purified.

Thus after the system has been freed from metals, chelating agents are no longer essential for activity. Another attempt to obtain full activity in the absence of chelating agents was made by treating the reagents with dithizone as follows.

A solution containing glucose 1-phosphate and MgSO_4 was shaken with 0.01% of dithizone in CCl_4 and the aqueous layer was separated after standing (first treatment). This solution was then shaken with CCl_4 to remove traces of dithizone and the aqueous layer was again separated after standing (second treatment). The activity of 2.1 μg . of 'metal-free' phosphoglucumutase was then measured with both treated solutions. A control with 11 mM-histidine as chelating agent was also used. The activity was 30.8 μg . of P converted in the histidine control, 25.7 when the supernatant from the first treatment was used (84.5% of control) and 22.3 when the supernatant from the

second treatment was used (72.3% of control). The treatment with dithizone is thus very effective, although not as effective as histidine. Removal of the traces of dithizone slightly decreases the activity, but this difference is low, and indicates an inhibition due to the presence of minute amounts of metals in the CCl_4 rather than the diminution expected if the presence of dithizone in the reaction mixture was critical for activity.

When the mixture of glucose 1-phosphate and MgSO_4 was prepared as indicated above but then washed several times with CCl_4 , the activity dropped to much lower values. After five washings (which may be considered sufficient to remove any possible traces of dithizone) the activity was 17% of the activity when cysteine was present as

Fig. 2. Activation by cysteine in a 'metal-free' and a non-purified system. Activity is expressed as disappearance of acid-labile phosphate. ○, A mixture of glucose 1-phosphate and MgSO_4 purified by dithizone treatment, with cysteine added as indicated, together with metal-free tris-HCl to maintain ionic strength and pH. □, As before, but reagents were not 'metal-free'.

Table 2. Activation of phosphoglucumutase by removal of metals

The activity of 'metal-free' phosphoglucumutase was assayed in the usual way. Where indicated, 0.02M-tris-chloride, pH 7.5, purified as described (Materials and Methods section) was used. Glucose 1-phosphate was in the Na form and purified as described. Where special purification is indicated, MgSO_4 and water were purified as described. Otherwise glass-distilled water and MgSO_4 (A.R.) were used.

Expt.	Enzyme (μg.)	Additions	Purity of MgSO_4 and water	P (μg.)	Activity (% of the complete system)
A	2.5	Complete system	—	63.8	100.0
A	5.5	No cysteine	—	5.2	3.8
A	5.5	No cysteine + tris-Cl	Purified	13.8	10.0
A	7.7	No cysteine	—	13.0	6.5
A	7.7	No cysteine	Purified	40.0	20.7
B	0.88	Complete system	—	14.2	100.0
B	2.2	No cysteine + tris-Cl	Purified	4.4	12.4
B	4.4	No cysteine + tris-Cl	Purified	23.8	32.2
B	9.9	No cysteine + tris-Cl	Purified	61.6	39.5
B	11.0	No cysteine + tris-Cl	Purified	61.8	34.8

activator. The activation with cysteine in such a purified system was studied quantitatively. It was observed to follow a different curve from that seen in a non-purified system (Fig. 2). The fact that the purified system requires less cysteine to activate the enzyme fully shows that activation involves removal of metals and not formation of a chelating-agent-enzyme complex.

*Further activation by
ethylenediaminetetra-acetic acid*

In Table 1 it is shown that low concentrations of EDTA can partially replace cysteine, and that higher concentrations have an inhibitory effect. In these experiments the reaction was started by adding the enzyme to the reaction mixture containing all the components of the system, including the chelating agent. However, when the enzyme was previously incubated with EDTA and then assayed in the presence of cysteine by the usual procedure, a further increase in activity was observed, this being related to the time of preincubation (Fig. 3). The activation was complete after about 1 hr. of incubation. By this method, the specific activity of the enzyme increased to 60–64 units/mg. of protein.

Under these conditions the activation seems to be specific for EDTA, since other chelating agents such as cysteine, histidine, 8-hydroxyquinoline or tris-HCl buffer do not produce the same effect (Fig. 3). When cysteine was used in the assay mixture, the preincubation with EDTA usually

Fig. 3. Activation by preincubation with EDTA. Phosphoglucosmutase ($44.6 \mu\text{g.}$) was incubated at 30° in the presence of 1 mM concentrations of the substances: ●, ○, EDTA (separate experiments); □, histidine; ■, 8-hydroxyquinoline; △, cysteine; ▼, tris. In all cases pH was adjusted to 7.5 with tris and the final volume was 1 ml. After the stated times 0.02 ml. was assayed for activity as indicated in the Materials and Methods section, with cysteine as activator and an incubation period of 2.5 min. Point (A) was determined with enzyme which was not preincubated with EDTA, in an assay mixture containing $50 \mu\text{M}$ -EDTA in addition to the other components.

increased the activity by a factor of 2.0–2.5. However, in some cases, especially when aged preparations of phosphoglucosmutase were tested with histidine in the assay mixture, the activity was increased by as much as a factor of 6 (Table 3). The activation was also observed when 'metal-free' phosphoglucosmutase was used.

When Mg^{2+} ion is added together with the chelate agent during the preincubation with phosphoglucosmutase, activation with EDTA is still observed, but the effect is no longer specific for EDTA. Robinson & Najjar (1960) have recently reported preactivation by histidine or imidazole plus Mg^{2+} ion. That hydroxyquinoline is also effective is shown in Table 4.

The further activation by EDTA is reversible. Preactivated phosphoglucosmutase drops to its original activity after exhaustive dialysis against tris buffer. This is best shown in the following experiment: 2 ml. of a solution containing 1 mg. of phosphoglucosmutase was incubated for 75 min. at

Table 3. Activation by preincubation with ethylenediaminetetra-acetic acid of 'metal-free' and aged phosphoglucosmutase

'Metal-free' phosphoglucosmutase (Expt. A) or phosphoglucosmutase 5 months old (Expt. B) was preincubated at 30° in the presence of 1 mM-EDTA, 10 mM-histidine and 1 mM-tris. The pH in all cases was 7.5. At different times, samples containing $1.3 \mu\text{g.}$ of phosphoglucosmutase in Expt. A and $0.9 \mu\text{g.}$ in Expt. B were assayed for activity as indicated in the Materials and Methods section, with histidine present in the reaction mixture as activator.

Expt.	Time of preincubation (min.)	Specific activity of the enzyme (units/mg.) after preincubation with		
		EDTA	Histidine	Tris
A	2	30.6	15.2	13.6
A	15	43.3	14.7	9.7
A	45	49.2	12.0	7.8
A	120	50.2	12.7	7.2
B	40	23.1	—	3.5

Table 4. Activation of phosphoglucosmutase by preincubation with 8-hydroxyquinoline in the presence of magnesium

Phosphoglucosmutase ($30 \mu\text{g.}$) was incubated at room temperature with 3 mM- MgSO_4 and 6.6 mM-8-hydroxyquinoline in 0.8 ml. final volume. Samples taken at different times were assayed in the presence of histidine.

Time of incubation (min.)	Relative activity (untreated enzyme = 100)
0.5	139
5	172
17	244
30	285

30° in the presence of 1 mM-EDTA, pH 7.5. The preparation was then dialysed for 24 hr. against 700 ml. of 1 mM-tris-HCl, pH 7.5, and again dialysed against a further 700 ml. for 48 hr. This preparation was diluted at 0°, and the activity tested at different times after the dilution. Under those conditions the activity quickly dropped to reach a constant value (Table 5).

The apparent Michaelis constant for Mg^{2+} ion does not seem to be affected by the EDTA pre-activation. This is shown in Fig. 4, for which an activated preparation dialysed 24 hr. against a large volume of tris-HCl, pH 7.5, was used.

The effect of EDTA on some physical properties

Table 5. *Reversibility of the activation by preincubation with ethylenediaminetetra-acetic acid*

Phosphoglucumutase (496 μ g./ml. final concentration) was incubated for 75 min. at 30° in the presence of 1 mM-EDTA, pH 7.5. The enzyme was then dialysed against 350 times its volume of tris-HCl, pH 7.5; 0.04 ml. was then diluted 10 times with glass-distilled water, and 0.02 ml. samples were assayed for activity after different times of incubation at 0°. The assay was made as described in the Materials and Methods section with histidine as activator.

Time after dilution (min.)	Activity (μ g. of P)
Dialysed enzyme	
0.5	27.2
1.5	17.1
8	12.7
25	12.6
Undialysed enzyme	40.0

Fig. 4. Magnesium activation of phosphoglucumutase and EDTA-preactivated phosphoglucumutase. Conditions of assay were as described in the Materials and Methods section. Histidine was used as activator. Δ , 4.1 μ g. of phosphoglucumutase; $\circ$, 0.90 μ g. of EDTA-activated phosphoglucumutase (dialysed once against mM-tris, pH 7.5); $\square$, same as $\circ$ but with 2.1 μ g. of enzyme.

of the enzyme was also investigated. The sedimentation pattern of the enzyme in 1 mM-EDTA is the same as when other media are used. The absorption spectrum of EDTA-activated phosphoglucumutase is essentially the same as the spectrum of untreated phosphoglucumutase. The ratio of the absorption at 220 and 278 $m\mu$ are, however, slightly different and at appropriate concentrations the curves cross at about 236 $m\mu$.

DISCUSSION

Chelating agents have two distinct activating effects on phosphoglucumutase of rabbit muscle. The first or 'essential' activation is obtained by including a chelating agent in the assay mixture. Negligible activity is obtained in its absence. This activation is due to removal of heavy metals, which are strong inhibitors of the reaction (Milstein, 1961b). However, if magnesium is also removed from the enzyme by excess of chelation, inhibition takes place. The second activating effect is obtained by preincubating the enzyme with EDTA [or, according to Robinson & Najjar (1960), with histidine plus magnesium]. In this case the activation is time-dependent and may produce a 2.5-fold increase in the molecular activity of the enzyme.

The evidence presented here led to the conclusion that crystalline phosphoglucumutase was in fact present as a metal-protein complex, even after exhaustive dialysis against glass-distilled water. It was therefore thought necessary to determine whether the active enzyme was necessarily a metal-protein or whether the essential activation by chelating agents really involved removal of inhibitory metals. That 8-hydroxyquinoline and EDTA, with higher affinity for metals than histidine, are less effective activators suggested, at first, that some mechanism other than removal of metals from the protein, such as a combination of the enzyme with the chelating agent, might be a possible explanation for the activation.

The fact that 'metal-free' phosphoglucumutase was fully active was not conclusive evidence against the hypothesis of the presence of a heavy metal at the active site, because of the possibility of reactivation by traces of metals in the test reagents. In fact, it was shown that 'metal-free' phosphoglucumutase was able to bind metals present in the distilled water. However, the fact that considerable activity (when tested in the absence of chelating agents) can be obtained by careful purification of the reaction mixture indicates that removal of inhibitory metals is the actual mechanism of the essential activation. Thus a satisfactory explanation for both activation and inhibition by metals (Milstein, 1961a, b) is the presence at the protein surface of a group or

groups with strong chelating properties, binding both Mg^{2+} ion and heavy metals. The best chelating activators are thus those removing heavy metals but not magnesium.

The mechanism of the second or 'further' activation obtained by preincubating phosphoglucomutase with EDTA is not clear at present. Robinson & Najjar (1960) published a preliminary communication on a further activation of phosphoglucomutase by incubation with histidine (which can be replaced by 8-hydroxyquinoline, as shown in this paper) plus magnesium. The extent of the activation, and the more marked response of aged preparations, are similar to those reported here with EDTA preactivation. The main difference in the results is that the activation as described by Robinson & Najjar requires both magnesium and histidine or imidazole. In the present work no added metal was required in the EDTA preactivation, and in fact 'metal-free' enzyme can also be further activated by EDTA. However, the possibility of minute contaminations with metal cannot be entirely ruled out at present, and considering the higher affinity of EDTA for several metals might provide an explanation for the apparent specificity of EDTA.

The second type of activation may possibly be explained by some change in protein configuration, which might be responsible for the small alteration in the absorption spectrum in the u.v. region. However, this is only tentative as little is known about the nature of the absorption at $220\text{ m}\mu$. The absorption at $280\text{ m}\mu$, on the other hand, is known to be affected by changes in the ionic state of tyrosine, connected with the tertiary structure of the protein.

SUMMARY

1. An 8-hydroxyquinoline test for metals has been used to show the presence of metals in the highly purified preparations of phosphoglucomutase, and to follow the preparation of a 'metal-free' enzyme. The 'metal-free' enzyme shows a positive test for metals after dialysis against glass-distilled water.

2. The necessity of a chelating agent in the assay of phosphoglucomutase is shown to be due to removal of inhibitory-metal impurities in the

system. Considerable activity is obtained even in the absence of chelating agents if special precautions are taken to keep the system 'metal-free'. The extent of the activation by metal-chelating agents varies with the amount of the metal impurities present. It is concluded that the activation does not involve a complex of phosphoglucomutase and chelating agent.

3. If phosphoglucomutase is incubated with ethylenediaminetetra-acetic acid before the assay, a further activation of a different kind is obtained. This is time-dependent, reversible, not obtained with other chelating agents under the same conditions and does not affect the Michaelis constant for Mg^{2+} ion. If preincubation is made in the presence of magnesium, the further activation is also obtained with 8-hydroxyquinoline.

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