4. Tetrahydropteroylglutamate is a competitive inhibitor of the cofactor activity of extract of heated *E. coli*, and of tetrahydropteroyltriglutamate and its *N*⁵-formyl derivative. It also inhibits the activity of the *N*¹⁰-formyl compound. In all cases the inhibition is overcome by the addition of cobalamin.

5. The cofactor activity of tetrahydropteroylglutamate and tetrahydropteroyltriglutamate is unstable to exposure to air at 30° for 18 hr., whereas the activities of extract of heated *E. coli* and of *N*¹⁰- and *N*⁵-formyltetrahydropteroyltriglutamate are relatively stable under these conditions.

6. Cobalamin is required for optimum formation of methionine by ultrasonic extracts of *E. coli* 121/176 (a cobalamin- or methionine-requiring auxotroph) when the derivatives of pteroyltriglutamate, tetrahydropteroylglutamate or an extract of heated organisms supply the necessary folic acid cofactor.

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On the Mechanism of Activation of Phosphoglucomutase by Metal Ions

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Phosphoglucomutase is a widely distributed enzyme which catalyses the interconversion of glucose 1-phosphate and glucose 6-phosphate; it requires a coenzyme, glucose 1:6-diphosphate (Cardini, Paladini, Caputto, Leloir & Trucco, 1949). The enzyme has been purified from rabbit skeletal muscle (Najjar, 1948; Jagannathan & Luck, 1949).

Cori & Cori (1937) first showed that Mg²⁺ ion activates the enzymic conversion of glucose 1-phosphate into glucose 6-phosphate. With the extracted enzyme, both as crude extracts and purified preparations, different authors reported differing

effects of Mg²⁺ ion. According to Stickland (1949) and Jagannathan & Luck (1949) no significant activity is present in the absence of Mg²⁺ ion, Mn²⁺ ion or other bivalent metals. Cori, Colowick & Cori (1938) and Najjar (1948), on the other hand, reported that the enzyme is partially active in the absence of added metal, but that activation is obtained by adding Mg²⁺ ion in optimum concentrations (15-fold activation with electrodialysed crude extracts; fourfold activation with the purified enzyme).

The effect of several other metals has also been reported. In crude muscle extracts, Stickland (1949) claimed a further activating effect of a

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second metal (Cr^{3+} or Al^{3+} ions) in addition to Mg^{2+} ion. Recently McCoy & Najjar (1959) reported that in the yeast enzyme Zn^{2+} ion could partially replace Mg^{2+} ion as activator.

Phosphoglucomutase appears to be an extremely complex system. Activation of the enzyme has been obtained with Mg^{2+} ion (or other bivalent metals), 'second metals', chelating agents added with the test reagents (Najjar, 1948; Sutherland, 1949), and further by preincubation of the enzyme with ethylenediaminetetra-acetic acid or histidine and magnesium (Milstein, 1960*b*, 1961; Robinson & Najjar, 1960). Reinvestigation of some of these effects was undertaken to clarify some controversial results and to define their nature more precisely. In this paper some observations on the activation by metals are described.

Magnesium is found to be essential for activity, thus confirming the results of Stickland (1949) and Jagannathan & Luck (1949). Some of the metal complexes formed with the components of the system have been studied, and their implication in the mechanism of the activation is discussed. The activation by a 'second metal' was also found with pure phosphoglucomutase. It is suggested that its mechanism is similar to that of the activation by chelating agents.

MATERIALS AND METHODS

Abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl radical; tris, 2-amino-2-hydroxymethylpropane-1,3-diol; EDTA, ethylenediaminetetra-acetic acid.

Protein was estimated by absorption at 278 $\mu\mu$, the extinction being taken as 7.70 for a 1% solution (Najjar, 1948).

Glucose 1-phosphate and glucose 6-phosphate (British Drug Houses Ltd.) were freed from metals before use. The solution (about 10 mg./ml.) was passed through an Amberlite IR-120 column, 1 cm. wide and 12 cm. high, which was previously washed with acid and transformed into the Na^+ or K^+ form. The potassium salt was used unless otherwise indicated.

Commercial Roche L-cysteine-HCl was recrystallized from a constant-boiling HCl solution. The pH was adjusted to 7.5 with tris before use.

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

Enzyme preparations. Phosphoglucomutase was prepared from rabbit skeletal muscle according to Najjar (1948). In general the method was found to reproduce the original results of Najjar, but the amount of crystalline material obtained was usually very low; however, the specific activity after the second crystallization was 26–27 units/mg. of protein, 26 being recorded by Najjar. The preparations proved to be homogeneous in the ultracentrifuge under a variety of conditions. Before use the enzyme solution was dialysed against glass-distilled water.

In all calculations a molecular-weight value for phosphoglucomutase of 74 000 (Lowry, Keller & Taylor, 1956) was used.

'Metal-free' phosphoglucomutase was prepared by the following procedure. The purified enzyme was dialysed against large volumes of glass-distilled water and then against 1 mM-tris-HCl, pH 7.5. It was then passed through an Amberlite IRC-50 column in the tris form, equilibrated with 1 mM-tris-HCl, pH 7.5. The metal content of the preparation after this treatment was negligible according to the 8-hydroxyquinoline test (Milstein, 1960*a*). The specific activity of the enzyme did not change with this treatment.

Measurements of enzyme activity. Unless otherwise stated, the incubation mixture contained: glucose 1-phosphate (containing as an impurity the coenzyme glucose 1:6-diphosphate in sufficient amounts), at a concentration of 6.5 or 0.5 mM depending on the method used for acid-labile phosphate determination; 1.5 mM- MgSO_4 ; 25.0 mM-L-cysteine-HCl or DL-histidine-HCl adjusted to pH 7.5 with tris immediately before use; enzyme as indicated in each case. The final volume was 0.4 ml.

The reaction was started by adding the enzyme to the incubation mixture. After incubation for 5 min. at $30 \pm 0.1^\circ$ acid-labile phosphate was determined according to Najjar (1948).

When low concentrations of glucose 1-phosphate were used, a modification of the Fiske & Subbarow (1925) method was used for phosphate determinations (Bartlett, 1959). The modification involves colour development, after all the reagents have been added, in a boiling-water bath for 7 min. The sensitivity with this simple modification increases tenfold. Since the activity determinations assume stability of glucose 6-phosphate, its stability was tested under the conditions used for colour development. Glucose 6-phosphate has been reported to be very stable to acid hydrolysis (Robison, 1932). Even in the presence of 4.5N- H_2SO_4 at 100° the 6-ester hydrolyses very slowly (Table 1, Expt. A). Under Bartlett's conditions of colour development, i.e. in the presence of molybdate and reducing agent in acid at 100° for 7 min., the stability of the

Table 1. *Stability of glucose 6-phosphate*

Glucose 6-phosphate containing 35.8 μg . (Expt. A) or 17.9 μg . (Expt. B) of total phosphorus, with the additions stated, in a final volume of 1.1 ml. (Expt. A) or 5 ml. (Expt. B), was incubated in a boiling-water bath. Inorganic phosphate was determined in the hydrolysate according to Fiske & Subbarow (1925) (Expts. A, B_1) or according to Bartlett (1959) (Expt. B_2).

Expt.	Additions	Time of incubation (hr.)	Inorganic phosphate (μg . of P)	Hydrolysis (%)
A	Blank	0	1.9	0
	4.5N- H_2SO_4	1	3.6	4.7
	4.5N- H_2SO_4	2	5.0	8.6
	4.5N- H_2SO_4	3	7.6	15.9
	4.5N- H_2SO_4	5	9.8	22.0
		(min.)		
B_1	Blank	0	2.0	0
B_2	1N- H_2SO_4 + 0.25% $(\text{NH}_4)_2\text{MoO}_4$ + 0.2 ml. of reducing agent	7	2.1	0.56

ester is also satisfactory (Table 1, Expt. B). As a final check of the applicability of the method, duplicate assays of enzymic activity were made and phosphate was estimated according to Bartlett, colour development being carried out for 7 and 17 min. at 100°. The values differed by less than 3% (3.38 and 3.30 $\mu\text{g.}$ respectively).

The unit of enzymic activity used throughout was that used by Najjar, i.e. the amount of enzyme which under standard conditions of assay produces a decrease of 32 $\mu\text{g.}$ of acid-labile phosphorus in 5 min.

Equilibrium dialysis experiments. The binding capacity of phosphoglucumutase for Mg^{2+} ion was measured by equilibrium dialysis. The experiments were carried out in special cells (for which I am indebted to Dr M. Dixon). These consisted of two circular pieces of Perspex in which a cylindrical depression of 2.8 cm. diameter was cut. The two pieces could be held together tightly by three screws. In use, a cellophan membrane soaked with 8-hydroxyquinoline and glass-distilled water was fixed between both pieces, dividing the space into two compartments of 2.25 ml. each. Two small holes through the sides of the compartments allowed the introduction and removal of samples. The actual experiment consisted in equilibrating (at 4°) the enzyme, placed in one compartment, with a solution in the other compartment. Equilibration was complete after 3 days (occasionally the cells were gently shaken). Samples from the compartment not containing protein were taken and assayed for Mg^{2+} ion. A blank was run without protein, and was used to evaluate the binding capacity of the cellophan membrane, which was very small. Binding was expressed as ν , the number of Mg^{2+} ions bound to one molecule of phosphoglucumutase, as calculated from the total amount of metal minus the free metal, corrected for the blank.

Dissociation constants of glucose 1-phosphate-magnesium and glucose 6-phosphate-magnesium complexes. The estimation of the dissociation constant of these magnesium complexes was made by the method of Burton (1959) with some modifications. The maximum of the 8-hydroxyquinoline-Mg chelate at 253 $\text{m}\mu$ (Milstein, 1960a) was used instead of that at 360 $\text{m}\mu$. Mg^{2+} ion was added to a sample containing 8-hydroxyquinoline and the glucose phosphate, and increase in the extinction at 253 $\text{m}\mu$ was plotted against the amounts of Mg^{2+} ion added.

The dissociation constant is calculated (Burton, 1959) from the slopes of the resulting curves at the origin

$$K = \frac{A - B}{[G - P] \times B} \quad (1)$$

in which A is the initial slope when no glucose phosphate is present, B the initial slope in the presence of glucose phosphate and $[G - P]$ is the total glucose phosphate concentration.

Reaction of 1-fluoro-2,4-dinitrobenzene with phosphoglucumutase. The effect of FDNB on phosphoglucumutase was studied in the presence of EDTA to avoid any protective effect of metal impurities, since extremely stable metal complexes have been shown to be formed with the enzyme (Milstein, 1960b, 1961) and FDNB reacts with free groups. Before use, phosphoglucumutase was preactivated (Milstein, 1960b) by incubation with 0.7 mM-EDTA, pH 7.5, for 1 hr. at 30°.

Enzyme (300 $\mu\text{g.}$) was incubated in the presence of 0.3 mM-EDTA with 5.4 mM-FDNB at 30°. The final volume

was 0.94 ml. The addition of FDNB was made as a small volume of a concentrated ethanolic solution giving a final ethanol concentration of 4.5%. The stability of the enzyme in the absence of FDNB was tested and no fall in activity was observed. The effect of 1.9 mM- MgSO_4 on the reaction with FDNB was also studied.

The effect of FDNB was followed by measuring the residual enzyme activity at different times after the addition of the reagent. Samples (2.1 $\mu\text{g.}$) of enzyme were taken and assayed for activity in the presence of cysteine as activator.

Since the concentration of FDNB is much higher than the concentration of the enzyme, the velocity of the reaction should be approximately proportional to the concentration of phosphoglucumutase. If the reaction follows apparently first-order kinetics, its rate is a linear function of the active enzyme concentration

$$-\frac{d[E]}{dt} = k'[E]$$

E being the concentration of active enzyme. In the integrated form the equation becomes

$$\log \frac{[E]}{[E_0]} = -kt \quad (2)$$

where $[E]/[E_0]$ is the amount of active enzyme relative to the total at any chosen time and is thus equal to A/A_0 , A and A_0 being the enzyme activities at time t and initial time of the reaction respectively. The graph of the logarithm of the residual activity plotted as a function of time gives a straight line with a slope of $-k$. The comparison of the values of k in the presence and the absence of Mg^{2+} ion gives a quantitative estimate of the protective effect of the metal towards the inhibition by FDNB.

The partially inactive DNP-enzyme used in the experiment in Fig. 5 was prepared as follows: 9.5 mg. of phosphoglucumutase was incubated at 23° in the presence of 1 mM-EDTA at pH 5.8. At time 0, 5 ml. of saturated aqueous FDNB and 1 drop of liquid FDNB were added to make the final volume 6.1 ml. The solution was stirred very gently (8 turns/min.) for 5.5 hr. During the reaction a yellow precipitate was slowly formed. At 1 and 5.5 hr. samples of the supernatant were taken and used for determinations of α -amino nitrogen and activity. The precipitate was collected at the end of the reaction, and its α -amino nitrogen was also determined by the ninhydrin method (Chibnall, Mangan & Rees, 1958). For these determinations the samples were extracted with ether and hydrolysed (15 hr. with 6N-HCl in sealed tubes, at 105°). The relative amounts of protein in the fractions were calculated from the α -amino nitrogen and compared with the activity.

After reaction for 1 hr., 85% of the protein remained in solution, containing 69% of the original activity. After 5.5 hr., 24% of the enzyme remained in solution, containing 7.6% of the original activity. The precipitate was inactive. The fall in the activity is then slightly quicker than the formation of the insoluble material. This insoluble material, after hydrolysis, was extracted with ether and treated as for the determinations of N -terminal groups in proteins by the FDNB method (Fraenkel-Conrat, Harris & Levy, 1955). There was no DNP-amino acid in the ether. The aqueous layer containing the DNP-amino acids (except the N -terminal) was subjected to chromatography in butanol-acetic acid-water (4:1:5) with markers of DNP-lysine and

Table 2. Requirement for a metal in the phosphoglucumutase reaction

Enzyme activity was determined by the standard assay, except for the addition of Mg^{2+} ion and EDTA (indicated below). Cysteine was used as activator.

Expt.	Enzyme ($\mu g.$)	Additions	Activity ($\mu g.$ of P/5 min.)	Relative activity (% of activity with optimum [Mg^{2+}])
A	*	None	-2.4	-3.5
A	*	$MgSO_4$ (1.5 mm)	65.8	100.0
B	5.1	None	2.7	4.3
B	5.1	$MgCl_2$ (0.37 mm)	57.6	92.0
C	2.7	None	10.2	7.8
C	2.7	$MgCl_2$ (1.5 mm)	> 75.0	—
C	1.1	$MgCl_2$ (1.5 mm)	65.5	100.0
C	2.7	EDTA (1.0 mm)	1.0	0.8
D	2.1	None	5.4	10.1
D	2.1	$MgSO_4$ (1.5 mm)	53.5	100.0
D†	10.1	$MgSO_4$ (1.5 mm) + EDTA (0.05 mm)	37.6	100.0
D†	10.1	$MgSO_4$ (1.5 mm) + EDTA (0.15 mm)	37.1	98.5
D†	10.1	$MgSO_4$ (1.5 mm) + EDTA (0.50 mm)	34.6	92.0
D†	10.1	$MgSO_4$ (1.5 mm) + EDTA (4.0 mm)	0.4	1.6

* Amount not determined.

† No cysteine or histidine present.

histidine. The two DNP-amino acids DNP-lysine and DNP-tyrosine were determined by their absorption in the ultra-violet, and free histidine was determined according to Fraenkel-Conrat & Singer (1956). No free histidine was found, implying complete reaction of this amino acid with FDNB; 6 molecules of DNP-lysine and about 5 of DNP-tyrosine per molecule of original enzyme were found, although with a high degree of error in the determination of the latter, due to its much lower absorption coefficient.

Under these conditions then, the reaction is not specific for any one of the groups involved, and there is the possibility of reaction with more than one essential group, or with groups involved in the tertiary structure.

Portions of the supernatant of the above experiment were taken after 1 min., 1 hr. and 5.5 hr. and used for determinations of $K_{app.}$ (see Results section) for magnesium shown in Fig. 5.

RESULTS

Activation by magnesium

When no Mg^{2+} ion was added to the assay mixture, the activity was in general very low, ranging from 0 to 3% of its optimum in the presence of Mg^{2+} ion, values which are well within the error of the activity determinations. The result, in agreement with that of Stickland (1949), who used a crude preparation, and with that of Jagannathan & Luck (1949) using their own preparation of purified enzyme, contrasts with that of Najjar (1948), who, using a preparation similar to that used in this work, reported only a fourfold activation by Mg^{2+} ion.

In some instances, however, values as high as 10% of the optimum activity were obtained in the absence of magnesium. Neither purification of the enzyme ('metal-free' enzyme) nor purification of the substrate was effective in reducing the activity in the absence of magnesium to zero.

Since the important point was to show the absolute requirement of a metal for activity, the effect of EDTA was studied in these cases, to see whether the activity was due to residual traces of metals. EDTA is a well-known complexing agent of metals, including the bivalent metals. It is an activator of phosphoglucumutase when used in low concentrations (Milstein, 1961), but when the concentration of EDTA is increased to a value higher than the concentration of Mg^{2+} ion the activity drops to almost zero, owing to chelation and effective removal of the metal. This was found in all cases, including those where absence of Mg^{2+} ion from the assay mixture was not enough to reduce the activity to zero. Furthermore, the small activity observed in the absence of added metals in these few cases can be completely abolished by the addition of 1 mM-EDTA to the assay mixture. Table 2 summarizes these observations, which indicate that the activity in these cases is due to residual traces of metals. Assuming that Mg^{2+} ion is essential for the activity at low concentrations the reaction in the absence of EDTA should be expressible in the reciprocal form as

$$\frac{1}{v} = \frac{1}{V} + \frac{K_{app.}}{V} \cdot \frac{1}{a} \quad (3)$$

in which V is a constant dependent on the conditions of the reaction, $K_{app.}$ is the apparent Michaelis constant for magnesium, a constant whose significance depends on the mechanism of the activation, a the concentration of the activating metal and v the velocity. A plot of $1/v$ against $1/[Mg^{2+}]$ yields a straight line from which $K_{app.}$ can be obtained (Fig. 1). This shows again that the activation should be considered as an essential activation (Segal, Kachmar & Boyer, 1952), and

phosphoglucumutase should be included among the 'true metal-enzymes' as described by Malmström & Rosenberg (1959).

The value of K_{app} ($50 \mu\text{M}$) is some 1/200 of the dissociation constants of the magnesium complexes of the substrates glucose 1-phosphate and glucose 6-phosphate. These last values were determined by the method of Burton (1959), but with the use of the absorption peak of the 8-hydroxyquinoline chelate at $253 \text{ m}\mu$ because of its higher sensitivity (Fig. 2). The values calculated from equation (1), as averages of duplicate determinations, are:

$$K_{\text{for glucose 6-phosphate}}^{\text{Mg}} = 16 \text{ mM};$$

$$K_{\text{for glucose 1-phosphate}}^{\text{Mg}} = 11 \text{ mM}.$$

Fig. 1. Effect of Mg^{2+} ion concentration on the reaction rate (reciprocal plots). The enzyme was incubated for 5 min. at 30° in the presence of 25 mM-histidine and Mg^{2+} ion as indicated at pH 7.5. Velocity is expressed as the amount of acid-labile phosphate disappearing. (a) $3.7 \mu\text{g.}$ of phosphoglucumutase and 5.5 mM-glucose 1-phosphate; (b) $0.7 \mu\text{g.}$ of phosphoglucumutase and 0.5 mM-glucose 1-phosphate. Phosphate determinations were according to Bartlett (1959).

The similarity between the two constants suggests that Mg^{2+} ion should not significantly affect the overall equilibrium of the enzyme reaction.

That phosphoglucumutase binds Mg^{2+} ion was shown by equilibrium-dialysis methods. The enzyme, in the presence of about an equimolar concentration of Mg^{2+} ion, was dialysed for several days until equilibrium was established; determinations of Mg^{2+} ion in the diffusate gave the concentration of free metal in the enzyme solution (the Donnan effect being insignificant at this concentration of buffer) and, from the total initial Mg^{2+} ion, the amount of bound metal can be calculated by difference. In Fig. 3 is shown the amount of bound metal at various concentrations of free Mg^{2+} ion. It can be seen from the experiment that a phosphoglucumutase-magnesium complex exists, even in the absence of substrate. Its dissociation constant calculated from these data is 0.5 mM . This is only a tentative value, and errors may be expected, mainly due to the fact that Mg^{2+} ion binding is calculated from the differences between two relatively high values, each subject to individual error.

An independent indication that Mg^{2+} ion forms a complex with the protein was obtained from inhibition experiments. Phosphoglucumutase is inhibited by FDNB, and the kinetics of the reaction are shown in Fig. 4. In the presence of an adequate concentration of Mg^{2+} ion for maximum activity

Fig. 2. Determination of the dissociation constants of glucose 1-phosphate and glucose 6-phosphate complexes with Mg^{2+} ion. To a cuvette containing 33 mM-tris-HCl buffer, pH 7.5, $83 \mu\text{M}$ -8-hydroxyquinoline and the additions indicated below (final volume, 3.0 ml.) MgCl_2 was added in successive portions of $20 \mu\text{l.}$ ●, None; ○, 0.05M-tris-HCl, pH 7.5; +, 7.3 mM-glucose 6-phosphate (sodium salt), pH 7.5; □, 8.4 mM-glucose 1-phosphate (sodium salt), pH 7.5. Readings were made at $253 \text{ m}\mu$ after the addition of each portion of MgCl_2 , against a blank containing the same reagents with MgCl_2 omitted.

(1.2 mM-free Mg^{2+} ion, after allowing for the EDTA-binding) the reaction proceeds at a clearly different rate (Fig. 4), indicating that Mg^{2+} ion diminishes the reactivity of the protein towards FDNB, probably through the formation of a phosphoglucumutase-magnesium complex. The value of k (equation 2) is $7.1 \times 10^{-4} \text{ min.}^{-1}$ in the presence of Mg^{2+} ion and $1.2 \times 10^{-2} \text{ min.}^{-1}$ in its absence. This indicates that Mg^{2+} ion produces a 17-fold decrease in the rate constant of the inactivation by FDNB.

If the reaction with FDNB affects groups in the enzyme surface which influence the formation of the enzyme-magnesium complex, a change in the value of the apparent Michaelis constant for magnesium (K_{app}) would be expected. On the other hand, if the inactive DNP-enzyme does not form an enzyme-magnesium complex, or if inhibition does not influence the activation by Mg^{2+} ion, a change in V will occur. In fact only V is affected by the inhibition and the same value of K_{app} is observed for different degrees of FDNB-inactivated enzyme (Fig. 5). The decrease in V (shown in the legend of Fig. 5) shows that the soluble enzyme is partially inactive before precipitating, since the values

Fig. 3. Comparison of Mg^{2+} ion activation and Mg^{2+} ion binding. ●, Binding of Mg^{2+} ion. 0.17 μmole of 'metal-free' phosphoglucumutase in the presence of 0.01 M-tris-HCl, pH 7.5, and various amounts of MgCl_2 . Equilibrium dialysis was carried out for 3 days, as described in the Materials and Methods section, and Mg^{2+} ion was determined in the diffusate. A blank without enzyme was made to correct for the binding capacity of the membranes. O, Activation by Mg^{2+} ion. Phosphoglucumutase (2.5 $\mu\text{g.}$) was assayed for activity as indicated in the Materials and Methods section. The sodium salt of glucose 1-phosphate was used and each point was determined from a time curve of the disappearance of acid-labile phosphate. Cysteine was used as activator, and Mg^{2+} ion concentrations are corrected for cysteine-binding.

Fig. 4. Rate of inhibition of phosphoglucumutase by FDNB, in the absence and in the presence of Mg^{2+} ions. Phosphoglucumutase (300 $\mu\text{g.}$), preactivated (1 hr. in 0.7 mM-EDTA at 30°), in 0.3 mM-EDTA. Final volume, 0.94 ml. (containing 4.5% of ethanol). ●, 5.4 mM-FDNB; Δ, 5.4 mM-FDNB + 1.9 mM- MgSO_4 . Portions containing 2.1 $\mu\text{g.}$ of enzyme were taken at the indicated times and assayed for activity (activator, cysteine; incubation time for the assay, 4 min.). The original activity of the preparation was 58.5 $\mu\text{g.}$ of P/4 min. Relative activity is expressed as percentage of the activity in the absence of inhibitor.

Fig. 5. Effect of FDNB inhibition on K_{app} and V . Partially inactive DNP-enzyme prepared as described in the Materials and Methods section was diluted 20-fold and samples (0.05 ml.) were assayed for activity with histidine as activator. DNP-phosphoglucumutase obtained after treatment of the enzyme with FDNB, A, for $5\frac{1}{2}$ hr., B, for 1 hr., C, for 1 min. (For measurement of the activity the assay mixture was incubated for 24 min. for A, and 2.5 min. for B and C. Activity is expressed as $\mu\text{g.}$ of phosphate converted in these times.) Values of V (expressed as $\mu\text{g.}$ of phosphate converted in 5 min.) were:

	P ($\mu\text{g.}/5 \text{ min.}/\mu\text{g.}$ of original protein)	P ($\mu\text{g.}/5 \text{ min.}/\mu\text{g.}$ of soluble protein)
(A)	2.6	12.1
(B)	32.0	43.5
(C)	47.7	47.7

shown have been calculated per milligram of soluble enzyme.

Phosphoglucumutase contains 3 histidine residues, 34–35 lysine residues and 31–32 tyrosine residues per molecule (Anderson & Jollès, 1957).

FDNB readily reacts with histidine, and all the histidine residues are destroyed during FDNB reaction, together with at least 6 lysine and 5 tyrosine residues per molecule of enzyme. Since histidine occurs in the active site of phosphoglucumutase (Milstein & Sanger, 1960) it was of interest to see whether Mg^{2+} ion had any effect on inactivation by photo-oxidation of phosphoglucumutase. It has been suggested by Koshland, Ray & Erwin (1958) that the destruction of one histidine residue by photo-oxidation in the presence of methylene blue is responsible for the inactivation of phosphoglucumutase. In Fig. 6 it is shown that the rate constant of inhibition by photo-oxidation without Mg^{2+} ion is $1.7 \times 10^{-2} \text{ min.}^{-1}$ and with 2 mM free Mg^{2+} ion it is $1.2 \times 10^{-2} \text{ min.}^{-1}$. The effect of Mg^{2+} ion on the photo-oxidation certainly cannot be compared with the protection given by the ion against inhibition by FDNB. The experiment suggests that Mg^{2+} ion does not affect significantly the sensitivity of the histidine presumably involved in the inactivation.

Fig. 6. Effect of photo-oxidation on phosphoglucumutase. The enzyme (63 $\mu\text{g.}$), preincubated with 1 mM-EDTA, for 1 hr., and containing in addition 0.01 M-tris-HCl, pH 7.5, and 0.8 $\mu\text{g.}$ of methylene blue and the additions mentioned below, was placed under a 150 w tungsten-filament lamp at 30 cm. distance in a dark room. Portions (0.02 ml.) were removed at different times, diluted with 0.4 ml. of iced 0.1 M-histidine, pH 7.5, and the activity of 0.1 ml. of the dilution was immediately assayed, with histidine as activator. Activity is expressed as percentage of the activity of a blank containing in addition 0.02 ml. of tris-HCl, pH 7.5, and kept in the dark during the procedure. (A) Contains also 0.02 ml. of 0.1 M-tris-HCl; (B) contains also 0.02 ml. of 0.05 M- $MgCl_2$. 100% activity is equal to 33.0 $\mu\text{g.}$ of P/5 min.

Activation by a 'second metal'

Stickland (1949) reported that a second metal (Cr^{3+} , Al^{3+} ions etc.), in addition to Mg^{2+} ion, was essential for the full activity of phosphoglucumutase. The enzyme he used was not the purified preparation and the activation by cysteine was still unpublished when he submitted his paper. The possible relationship of these two apparently independent activations remained obscure.

In the first attempts with the purified phosphoglucumutase I failed to find any activation by either Cr^{3+} or Al^{3+} ion. However, when higher concentrations of enzyme were used the effect became apparent (thanks are due to Dr L. H. Stickland for his advice on this point). With these concentrations of enzyme, however, the velocity is much too high to measure when cysteine is added. Lower enzyme concentrations were therefore used when cysteine was present. Therefore the concentration of enzyme used was adjusted so as to give roughly the same velocity in all cases and the amount of enzyme required then gives an indication of the activity. Thus when only glucose 1-phosphate, Mg^{2+} ion and enzyme were present in the incubation mixture, 96 $\mu\text{g.}$ of the pure phosphoglucumutase was necessary to convert 40 $\mu\text{g.}$ of acid-labile phosphate in 5 min. When the incubation mixture included also 0.25 mM- $CrK(SO_4)_2$ only 9.3 $\mu\text{g.}$ of the enzyme was required for conversion of the same amount of substrate in the same time. Cysteine is much more

Fig. 7. Activation of phosphoglucumutase by a 'second metal'. Enzyme (8.2 $\mu\text{g.}$) was incubated in a volume of 0.4 ml. in the presence of 50 mM-glucose 1-phosphate, 1.0 mM- $MgSO_4$ and the additions indicated below. At different intervals the amount of acid-labile phosphate in portions (0.05 ml.) was determined. O, No additions; ●, 0.16 mM- $CrK(SO_4)_2$; Δ, 16 mM-cysteine; □, 0.16 mM- $CrK(SO_4)_2$ + 16 mM-cysteine.

Table 3. *Effect of chromium potassium sulphate on the activation of phosphoglucomutase*

The enzyme was incubated with 25 mM-glucose 1-phosphate, pH 7.5, for the indicated period at 30°, in the presence of 1.5 mM-MgSO₄ and the additions stated. The amount of acid-labile phosphate was determined and the activity expressed as indicated in the Materials and Methods section.

Expt.	Enzyme (μg.)	CrK(SO ₄) ₂ (0.25 mM)	Tris-HCl (pH 7.5)	Incubation period (min.)	Activity (μg. of P)
A	18	—	—	7.5	3.0
A	18	Fresh	—	7.5	10.0
A	18	4 months old	—	7.5	12.2
B	—	Fresh	—	10.0	0.0
B	18.5	—	—	10.0	4.0
B	18.5	Fresh	—	10.0	9.4
B	18.5	4 months old	—	10.0	14.2
C	17.0	—	—	10.0	2.5
C	17.0	Fresh	—	10.0	11.5
C	17.0	—	10 mM	10.0	5.2
C	17.0	Fresh	10 mM	10.0	10.0

Table 4. *8-Hydroxyquinoline test of chromium potassium sulphate solutions*

The 8-hydroxyquinoline test was as described by Milstein (1960*a*) except that tris-HCl was used as indicated. Final concentration of CrK(SO₄)₂ was 54 μM.

Age of the chromic solution	Tris-HCl	ΔE_{253}
Fresh	—	0.265
Fresh	3.5 mM	-0.002
4 months	—	0.272
4 months	3.5 mM	0.000

effective, and when a concentration of 25.0 mM is present instead of the Cr³⁺ ion the amount of enzyme necessary to give the same velocity was only 0.8 μg. If the activity in the presence of cysteine is taken as 100 %, the activity in the presence of Cr³⁺ ion is 8.5 % and in the absence of either (i.e. with Mg²⁺ ion only) is 0.8 %. Higher concentrations of Cr³⁺ ion are inhibitory.

Activation by Cr³⁺ ion and cysteine are not additive (Fig. 7). When cysteine is present, Cr³⁺ ion does not activate, but has a weak inhibitory effect. This suggests that although these two activators are of a very different nature, they are acting in a rather similar way.

Similar observations have been made in some other systems (Altman & Crook, 1953; Snell, 1957; Wada *et al.* 1957). It has been suggested that the trivalent metals are acting as trapping agents of inhibitory metals through their hydrates (Altman & Crook, 1953), or that the 'second metal' competes with the inhibitory metal on the enzyme surface, but the complex thus formed is active (Wada *et al.* 1957).

Solutions of chromium salts are known to undergo changes in hydration on standing. When an aged solution is tested for its activating effect, it

can be seen that it is higher than the activating effect of a fresh solution (Table 3, Expts. A and B).

When the 8-hydroxyquinoline test was applied to a solution of a chromic salt, no change in extinction at 253 mμ was observed. However, if the test is made at the same pH but in the absence of tris-HCl, the characteristic peak of the 8-hydroxyquinoline chelates becomes evident (Table 4). The reason for the effect of tris is not clear, but the observation suggests that in the presence of the buffer there are no free Cr³⁺ ions available. However, the activating effect of chromium is not suppressed in the presence of tris-HCl buffer, pH 7.5, in even higher concentrations than those producing the quenching of the 8-hydroxyquinoline chelate peak (Table 3, Expt. C). This would again indicate that the activation is produced not by the Cr³⁺ ions but by the chelating properties of some of its derivatives. However, it should be emphasized that the experiment is by no means conclusive, and the fact that tris itself seems to have some effect introduces a new complication.

It has been shown (Milstein, 1960*b*, 1961) that activation by chelating agents is due to the removal of inhibitory metals; the above-mentioned observations seem to indicate that the explanation offered by Altman & Crook (1953) for a different system (namely that the hydrates of the activating trivalent metals are in fact acting in the same way as the chelating agents) might be the correct one.

DISCUSSION

The results obtained with the equilibrium-dialysis experiments and the protective effect of Mg²⁺ ions during inactivation by FDNB indicate that Mg²⁺ ion forms a complex with the protein. Whether formation of such a complex is responsible for activation of the enzyme remains to be proved.

In fact about 80% of the total activity can be obtained at a concentration of Mg^{2+} ion at which only 30% of the enzyme is present as the magnesium complex, assuming that 1 atom of magnesium combines with 1 molecule of enzyme (see Fig. 3). This is a simple reflexion of a difference between the dissociation constant and the apparent Michaelis constant for Mg^{2+} ion (K_{app}). At first sight this would indicate that the formation of the complex is not responsible for the activation. However, the Michaelis constant is a true dissociation constant only in some cases, depending on the mechanism of the activation. Several authors (Segal *et al.* 1952; Alberty, 1956; Dixon & Webb, 1958) have discussed the kinetic models of activation by metal ions and shown that in many cases the apparent Michaelis constant for the metal is a kinetic expression depending on the enzyme-metal dissociation constant and other parameters. For instance, the kinetic scheme

corresponds to case II discussed by Dixon & Webb (1958, page 458) for the extreme case in which an ES complex is not formed. The derivation of the value of the apparent Michaelis constant for the metal is

$$K_{app} = \frac{K_A}{1 + (s/K_s)} \quad (5)$$

where K_A is the true dissociation constant of the enzyme-metal complex and K_s the dissociation constant of the enzyme-metal-substrate complex with respect to substrate. This possibility should be verifiable by determining the value of K_{app} at low substrate concentration. Under these conditions K_{app} would be expected to rise as the concentration of substrate diminishes, to reach the value of K_A at the limit (substrate concentration nil). An experiment to test this was made. The apparent Michaelis constant for magnesium was determined with the lowest concentration that can be assayed (0.5 mM). The value obtained for the constant is much the same as that obtained under standard conditions with a substrate concentration 10 times as high (Fig. 1). Since the value of the apparent Michaelis constant for substrate under the experimental conditions used has been reported to be of the order of 0.1 mM or lower (Najjar & McCoy, 1958), it would have been expected that, at the concentration of substrate used, the value of K_{app} would have risen several times, and the difference should have been easily detected.

The fact that the enzyme-metal complex has a dissociation constant lower in value than that of the apparent Michaelis constant for the metal suggested to Malmström, Vängård & Larsson (1958) that the above-mentioned mechanism was opera-

tive in the metal activation of enolase, despite the fact that Would & Ballou (1957), postulating a similar mechanism, found that K_s did not change with magnesium concentration.

With phosphoglucumutase, the results indicate a rather more complex mechanism. Thus if, in addition to equation (4), the following steps are also present

with a value of K'_A for the dissociation constant of ESM, different from K_A , the velocity of the reaction (given by Dixon & Webb, 1958, page 466) will be

$$v = \frac{ke}{1 + K_s K_A / as [1 + (a/K_A) + (s/K_s)]} \quad (7)$$

The value of K_{app} derived from this equation is

$$K_{app} = \frac{K_A [1 + (K_s/s)]}{(K_A/K'_A) + (K_s/s)} \quad (8)$$

For such a mechanism the value of K_{app} will depend on both K_A and K'_A , and also on K_s and s . However, when the concentration of the substrate is high in relation to K_s , and $K_A > K'_A$, the value of K_{app} becomes independent of the substrate concentration, and at the limit (infinite substrate concentration) K_{app} is the same as K'_A .

Returning to the conditions of measurement of K_{app} , from equation (8) at the lowest possible concentrations of glucose 1-phosphate (0.5 mM, see Fig. 1), even assuming this is three times the value of K_s , the value of K_{app} could differ by less than 30% from the value at ten times this substrate concentration (5 mM, see Fig. 1). Such a difference is within the experimental error. The simple mechanism, including the steps shown in equations (4) and (6), explains the experimental results satisfactorily, but more complex models are not excluded.

A decrease in the dissociation constant for magnesium on passing from the binary to the ternary complex (EMg to EMgS) means that the affinity for the metal has been increased owing to the presence of the substrate. This might be because the phosphate group of the substrate is being used as a new bonding group of magnesium

or because of a change in the enzyme configuration during substrate binding [this type of change has been discussed by Koshland (1959)].

A possible way to investigate this point further would be by the binding of substrate in the presence and the absence of magnesium by equilibrium dialysis. This technique was used by Malmström (1955) in model experiments with albumin, and on

enolase. Although the model experiment gave a positive answer, his experiments with enolase showed no effect of the presence of magnesium on substrate binding. In our experiments the amount of substrate bound to the enzyme was measured by the total phosphorus determined in the diffusate, and also by radioactive measurements with [^{14}C]-glucose phosphate. The enzyme in high concentrations was allowed to equilibrate in an equilibrium-dialysis cell with an equimolar concentration of substrate, in the presence and in the absence of Mg^{2+} ion. The results were poorly reproducible, so an attempt to identify the products at the end of the experiments was made by ionophoresis of the hydrolysed material, in the experiment in which labelled substrate was used. Substances other than glucose and glucose phosphate were found to be formed, but were not further identified. This invalidated the results. The possibilities of this technique seem, from this experience, to be very restricted. In fact, in this case the presence, at a level of 1 part in 10 000, of a contaminating enzyme acting on either substrate would suffice to invalidate the results.

The effect of Mg^{2+} ion on FDNB inhibition suggests some sort of nitrogenous-group involvement in Mg^{2+} -ion binding, provided that the inactivation by FDNB is actually due to combination with a group involved in the binding of the metal. The protection by Mg^{2+} ion may be due to a prior combination with the group which reacts with FDNB. The effect in this case might be valuable in the identification of one of the groups involved in Mg^{2+} -ion binding and the amino acids around it.

However, that is not the only possible explanation of the effect of Mg^{2+} ion on FDNB inhibition. Consider the reaction sequence

Here the combination of Mg^{2+} ion with the enzyme, at a completely different site from the one that reacts with FDNB, should also lead to protection by Mg^{2+} ion, because the form E' necessary for reaction with FDNB will be present in much smaller concentrations in the presence of Mg^{2+} ion.

On the other hand, the lack of an effective protection by magnesium during photo-oxidation does not exclude involvement of histidine, since the reactivity towards photo-oxidation of the imidazole ring may not be affected in such a way as to prevent the reaction, as is observed in the effect of Mg^{2+} ion on the inhibition by FDNB already discussed.

SUMMARY

1. Magnesium is an essential activator of phosphoglucumutase. However, in some cases some activity is found in its absence, which can be suppressed by ethylenediaminetetra-acetic acid and is probably due to residual traces of metals. The apparent Michaelis constant for magnesium is about $50 \mu\text{M}$, with a concentration of glucose 1-phosphate of either 5 or 0.5 mM.

2. Magnesium forms complexes with both glucose 1-phosphate and glucose 6-phosphate, with dissociation constants slightly higher than 10 mM, and with the enzyme with a dissociation constant of about 0.5 mM.

3. Phosphoglucumutase is inhibited by 1-fluoro-2:4-dinitrobenzene, and magnesium protects against this inhibition at concentrations of the order required for full activation. The 1-fluoro-2:4-dinitrobenzene-inhibited enzyme has the same Michaelis constant for magnesium as the uninhibited enzyme. From hydrolysates of fully inactive dinitrophenyl-phosphoglucumutase, 6 molecules of dinitrophenyllysine and about 5 of dinitrophenyltyrosine per molecule of enzyme can be detected. All histidine residues have also reacted with the 1-fluoro-2:4-dinitrobenzene.

4. The activation by a 'second metal' in addition to Mg^{2+} ion is also found with pure phosphoglucumutase. It is suggested that its mechanism is similar to the activation by chelating agents.

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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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