ACTA CHEMICA SCANDINAVICA 16 (1962) 1682–1694

The Activation of Muscle Hexokinase by Divalent Metal Ions

EVA WALAAS and OTTO WALAAS

Institute of Medical Biochemistry, University of Oslo, Norway

Muscle hexokinase which is rapidly inactivated in solution, can to some extent be stabilized by EDTA and pyrophosphate. A procedure involving fractionation with ethanol is described for the partly purification of muscle hexokinase. The activation of the enzyme by different divalent metal ions has been investigated. The highest maximal activity was obtained by magnesium and at a molar ratio of Mg^{2+}/ATP = 1. Sodium pyrophosphate inhibits hexokinase apparently by the formation of a Mg-pyrophosphate complex, which excludes Mg^{2+} from the activated enzyme substrate complex. In addition, an inhibition of hexokinase by a 1:1 magnesium pyrophosphate complex has been shown. The kinetics of this inhibition is in accordance with a competitive type with respect to the Mg–ATP complex. This gives support for the assumption that the Mg–ATP complex is the actual substrate for muscle hexokinase. At increased concentrations of ATP^{-4} and Mg^{2+} the enzyme activity is inhibited.

Muscle hexokinase is in addition activated by Ca^{2+}, Co^{2+}, Mn^{2+}, and to a small extent by Zn^{2+}. In comparison with the Mg^{2+} effect the activation by these metal ions is characterized as follows. Maximal enzyme activity is less. Activation does occur at lower concentration of these metals and at a molar ratio Me^{2+}/ATP considerable below 1. Half maximal activity for Co^{2+} and Mn^{2+} is observed at a concentration 10 times lower than for Mg^{2+}. At increased concentration of these metal ions, strong inhibition of the enzyme activity is observed.

Since the work by Cori *et al.*[1] the role of hexokinase activity in regulating glucose uptake by muscle has been much discussed. Recently it has been concluded that at least under certain hormonal conditions glucose phosphorylation may be an important limiting step for glucose uptake in the rat diaphragm [2] and in the perfused rat heart[3]. Although this field has attracted considerable interest, small progress has been made concerning the properties of muscle hexokinase following the demonstration of this enzyme in muscle extracts [4,5]. The great lability of muscle hexokinase may be the reason why only slight purification of the enzyme has been obtained [6]. In the present work the possibility of stabilizing the enzyme has been investigated and a partly purified muscle hexokinase has been prepared. The activation of the

enzyme by magnesium has been investigated. Some evidence is obtained that the enzyme is activated by a Mg—ATP complex. It has been shown that muscle hexokinase can also be activated by calcium, manganese, cobalt and zinc *.

MATERIAL AND METHODS

Preparation of muscle extract. Grown rats (weighing 250 g) were decapitated, bled, and skeletal muscle and diaphragm removed, chilled on ice, minced with scissors and transferred to 10 volumes (w/v) of 0.25 M sucrose containing 0.05 M Tris buffer pH 8.2 and EDTA at a final concentration of 0.0018 M. The muscle tissue was homogenized in an all glass Potter-Elvehjem homogenizer for 10 min. All the procedures during the preparation of hexokinase were preformed in the cold room at + 2°C. In some experiments hexokinase activity in a particulate and soluble fraction were investigated separately. The nuclear fraction was removed by centrifugation at 600 × *g* for 10 min, whereupon the supernatant was centrifuged in an International refrigerated centrifuge at 8000 × *g* for 20 min. The soluble fraction (cell sap + microsomes) was removed and the sediment (particulate fraction) containing sarcosomes was resuspended in the medium used for homogenization and centrifuged at 8000 × *g* for 20 min. This procedure was repeated and finally the washed particulate fraction suspended in the same medium. It has previously been shown [7] in experiments on rat diaphragm that 90 % of hexokinase activity is present in the soluble and 10 % in the particulate fraction. The same distribution has been found in rat skeletal muscle. Determination of hexokinase activity in the soluble fraction gave values of 2.8 (rat diaphragm), 2.35 (rat leg muscle) and 2.0 (rat heart muscle). These values are expressed as μmoles glucose phosphorylated in 20 min under standard conditions.

Preparation of acetone powder. Rat skeletal muscle (500 g) was chilled on ice and ground in a meat grinder and extracted with 4 volumes of 0.05 M Tris buffer pH 8.0 containing 0.0018 M EDTA. The extract was added slowly to 9 volumes of acetone at −20°C. A crude hexokinase was prepared by extraction of the acetone powder with Tris buffer pH 8.0 containing 0.0018 M EDTA. Hexokinase extracts of the same activity was obtained by extraction with acetate buffer pH 4.0 or phosphate buffer pH 6.0.

Fractionation with ammonium sulphate. 2 g acetone powder from rat leg muscle extract was ground with 10 volumes 0.03 M Tris buffer — 0.0012 M EDTA pH 7.5 and shaken in the cold for 30 min. The insoluble material was removed by centrifugation at 2000 × *g* for 10 min. The supernatant was fractionated with ammonium sulphate (pH 7.5) and the precipitate between 30 % and 40 % saturation collected and dissolved in 0.03 M Tris buffer — 0.0012 M EDTA pH 8.2. This solution was dialyzed against several shifts of the same buffer for 3 h before hexokinase activity was determined. By this procedure 2- to 4-fold purification of hexokinase per mg nitrogen was obtained. However, marked inactivation of hexokinase did occur and the recovery of the hexokinase initially present was only 30—50 %.

Fractionation with ethanol. 2 g acetone powder was ground with 10 volumes 0.03 M Tris buffer — 0.0012 M EDTA, pH 8.2 and extracted for 20 min in the cold followed by centrifugation at 8000 × *g* for 10 min. Magnesium acetate in the solid state was added to the supernatant to a final concentration of 0.05 M and pH adjusted to 7.5. Fractionation with ethanol was performed at −3°C to −7°C. Ethanol between 5 and 15 % saturation precipitated 50 % of the hexokinase activity. This precipitate was centrifuged at 10 000 × *g* for 15 min at −10°C in an International refrigerated centrifuge. The supernatant was removed and traces of ethanol were evaporated from the residue over night at −20°C. By this procedure 5-fold purification of hexokinase per mg nitrogen was obtained. The enzyme preparation after having been dried *in vacuo* could be stored at −20°C with a constant enzyme activity for two weeks. Attempts to purify this preparation further by fractionation with ammonium sulphate failed, due to extensive inactivation of hexokinase. In a modification of this procedure ethanol fractionation was per-

* *Abbreviations used:* ATP, Adenosine triphosphate; ITP, Inosine triphosphate; GTP, Guanosine triphosphate; UTP, Uridine triphosphate; EDTA, Ethylenediaminetetraacetic acid.

Table 1. Thermal inactivation of muscle hexokinase. The soluble fractions were used in these experiments. Dialysis was performed against 0.03 M Tris buffer pH 8.2 containing 0.0015 M EDTA.

Rat muscle tissue	Procedure	Hexokinase activity * Before	After	Inactivation %
Leg muscle	standing at + 20°C	1.72	0.63	64
» »	for 18 h at + 1°C	1.72	0.80	54
» »	at −20°C	1.72	0.81	54
Diaphragm	at −20°C	2.80	1.70	39
Leg muscle	dialysis for	1.96	0.78	60
Diaphragm	18 h	2.82	0.42	86
Heart	at + 1°C	1.71	0.61	65
Acetone powder from leg muscle	dialysis for 16 h at + 1°C	0.46 **	0.22 **	52

* Activity expressed as μmoles per 50 mg muscle tissue.
** Activity expressed as μmoles per mg protein.

formed without addition of magnesium. In this case hexokinase was precipitated between 15 and 25 % of ethanol and the purification was 3- to 4-fold.

Procedure for assay of hexokinase activity. As a standard procedure hexokinase was incubated with the following mixture (concentrations): 0.001 M glucose, 0.05 M ATP, 0.05 M $MgCl_2$, 0.05 M KCl, 0.03 M Tris buffer pH 8.2. To each incubation vessel was added 0.5 ml enzyme solution and a total volume of 3.0 ml was used. Incubation was performed at + 30°C for 20 min. Hexokinase activity was usually determined by the glucose disappearance method. When incubation was finished precipitation with $ZnSO_4$ + $Ba(OH)_2$

Table 2. Effect of glucose, ATP, $MgCl_2$ and anions on the thermal inactivation of hexokinase. Acetone powder from rat leg muscle extract was used as the enzyme preparation. Dialysis was performed under the conditions stated in Table 1, and the compounds added were present at equal concentractions inside and outside the dialysis bag.

Procedure	Addition	Hexokinase activity Before	After (μmoles per mg protein)	Inactivation %
Standing at + 1°C for 16 hours	0.001 M Glucose	0.68	0.48	30
	0.005 M ATP	0.68	0.48	30
Dialysis at + 1°C for 16 hours	None	0.71	0.30	58
	0.0022 M glucose	0.71	0.39	45
	0.05 M $MgCl_2$	0.71	0	100
Dialysis at + 1°C for 16 hours	None	0.86	0.18	79
	0.1 M KCl	0.71 *	0.15 *	79
	0.1 M K_2HPO_4	0.68 **	0.36 **	44
	None	1.25	0.09	93
	0.03 M Napyrophosphate	0.77 ***	0.68 ***	12

* 0.02 M KCl was present in the incubation medium.
** 0.02 M K_2HPO_4 was present in the incubation medium.
*** 0.005 M sodium pyrophosphate was present in the incubation medium.
Corrections for the inhibitory effect by pyrophosphate present during incubation has been made according to the results given in Table 3.

Table 3. The inhibitory effect by anions on the hexokinase activity. Acetone powder from rat leg muscle extract was used as the enzyme preparation.

Addition	Concentration Moles per l.	Hexokinase activity μmoles per mg protein	Activity per cent of control
Na-pyro-phosphate	0	1.25	100
	0.001	1.48	119
	0.005	0.77	62
	0.01	0.22	18
NaCl	0	0.86	
	0.1	0.71	83
Na_2HPO_4	0	0.86	
	0.1	0.68	79

was performed and residual glucose determined by the method of Nelson [8]. Protein determinations were done by the method of Lowry *et al.*[9]

In the experiments where other divalent metals were substituted for Mg^{2+} in the reaction mixture, some influence on the Nelson method was observed, particularly with Mn^{2+}. Therefore, blanks at zero time were performed at each individual concentration of the metals for appropriate corrections.

Materials. The Ba-salt of ATP from Sigma Chemical Company was converted to the Na-salt before use. The same was done with Ba-salts of ITP, GTP and UTP obtained from Sigma. Analytical grade chemicals from Merck were used in the experiments.

RESULTS

Stabilization of hexokinase by EDTA and anions against thermal inactivation. Muscle hexokinase is subjected to extensive inactivation in solution. As shown previosuly [10] hexokinase in a rat diaphragm muscle extract is protected by EDTA, indicating that trace heavy metals partly are responsible for the inactivation. The addition of EDTA to the solution used for homogenizing the muscle tissue was also found necessary. Hexokinase activity was decreased by 50 % in the soluble as well as in the particulate fraction if EDTA was omitted at this step of the purification procedure. However, other factors are also concerned in the thermal inactivation of muscle hexokinase. As shown in Table 1 crude hexokinase preparations in solution were extensively inactivated by standing, during dialysis or by freezing at −20°C and thawing.

Muscle hexokinase could not satisfactorily be stabilized by addition of reactants of the incubation system (Table 2). Glucose (0.002 M) and ATP (0.005) slightly stabilized the enzyme, while prolonged contact with 0.05 M $MgCl_2$ completely inactivated hexokinase.

The effect by some anions was also investigated. Chloride was without any effect and orthophosphate gave a slight protection. Sodium pyrophosphate, however, at a concentration of 0.03 M was effective in protecting the hexokinase from inactivation during dialysis. On the other hand, pyrophosphate when present at concentrations above 0.005 M during incubation, excerted strong inhibition of hexokinase activity (Table 3). Therefore the use of pyrophosphate as a stabilizing agent is of limited value in the purification procedure. Sodium or potassium chloride and orthophosphate slightly inhibited hexokinase, when present in the incubation system.

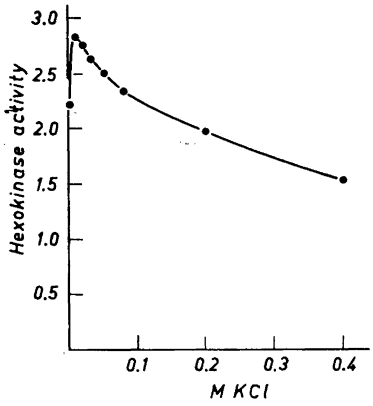

Fig. 1. The influence of KCl on hexokinase activity. Ethanol fractionated hexokinase was used as enzyme preparation.

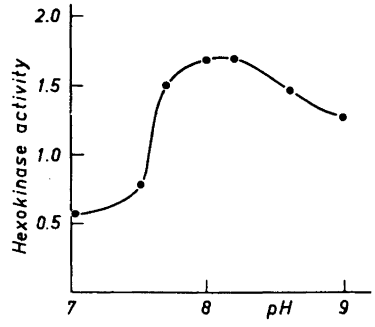

Fig. 2. The influence of pH on muscle hexokinase purified by ethanol fractionation. 0.03 M Tris buffer at the desired pH values was used under standard experimental conditions.

The effect of chloride was thoroughly investigated (Fig. 1). It was observed that KCl in a narrow range 0.02—0.05 M gave a slight activation, followed by inhibition at higher concentrations. Therefore, 0.05 M KCl routinely was added to the incubation mixture to get optimal conditions for hexokinase activity.

Effect of pH on activity. The pH profile of purified hexokinase from skeletal muscle was characterized by an optimum at pH 8.0—8.2 (Fig. 2). The activity rapidly declined at pH below 8 and reached one third maximal activity at pH 7.0. These results are similar to those obtained with crude hexokinase in fresh muscle extracts [7]. It has also been found that hexokinase in fresh homo-

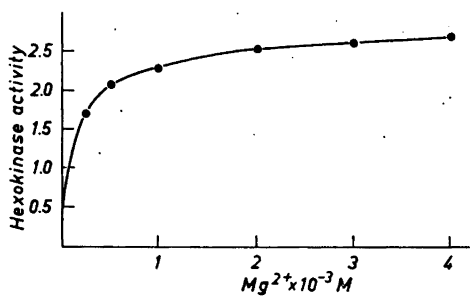

Fig. 3. The influence of $MgCl_2$ on muscle hexokinase at a constant level of ATP. Incubation mixture (concentrations): 0.001 M glucose, 0.001 M ATP, 0.05 M KCl, 0.03 M Tris buffer pH 8.2, ethanol fractionated enzyme preparation. 20 min incubation at 30°C.

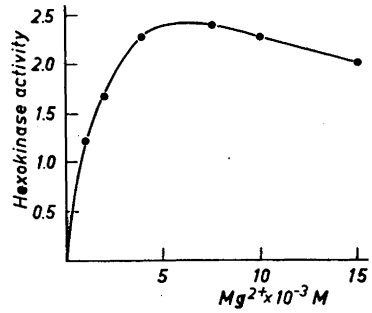

Fig. 4. Inhibition of hexokinase activity by $MgCl_2$ at a concentration above 10^{-2} M. ATP was present at a concentration of 4×10^{-3} M.

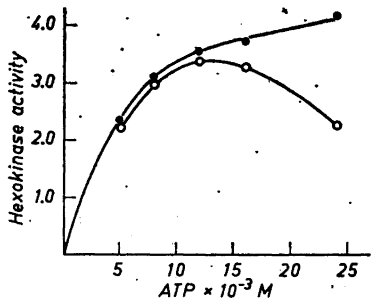

Fig. 5. Inhibition of hexokinase activity by ATP at a molar ratio $Mg^{+2}/ATP \ll 1$. ●: Mg and ATP present at molar ratio of 1. O: Mg present at a constant concentration of 4×10^{-3} M and ATP added at different concentrations. Hexokinase activity was measured by determination of the formation of ADP by an ion exchange resin method [19].

genate is most stable at pH 8, and is partly inactivated at the pH of 7 or below in accordance with previous work [4].

Effect of $MgCl_2$ and magnesium pyrophosphate complex on activity. If hexokinase is saturated with glucose, the activity increases with increasing concentration of Mg^{2+}, and maximum activity is obtained at a molar ratio $Mg^{2+}/ATP = 1$ (Fig. 3). This was found at several different concentrations of ATP. If Mg was added in excess over ATP no inhibitory effect was observed until rather high concentrations of Mg were present (Fig. 4). Similarly, if ATP was present at concentrations considerable higher than Mg ($Mg^{2+}/ATP < 4$), hexokinase activity was decreased (Fig. 5). This was in contrast to the situation when Mg and ATP were present at molar ratio of 1, where inhibition was never observed, even at high concentrations. In these experiments the activity of muscle hexokinase was half maximal at a concentration of ATP and Mg of 1.7×10^{-3} M (Fig. 6). The results are in accordance with those reported by Hers [11] and Liébecq [12] and may suggest that a ATP-magnesium complex is the actual substrate in hexokinase reaction. This was further indicated in experiments where the inhibition of hexokinase activity by inorganic pyrophosphate was studied. The inhibition of hexokinase by sodium pyro-

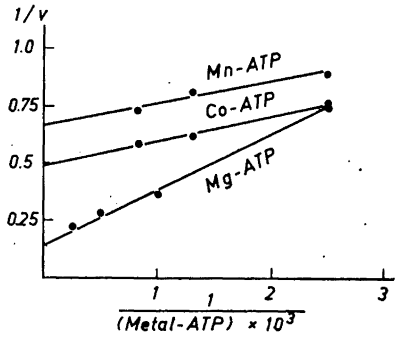

Fig. 6. The effect of different divalent metals on hexokinase activity. Metal and ATP was always added in equimolar concentrations (Mg^{2+} at a range 0.0004—0.004 M, Mn^{2+} and Co^{2+} at a range 0.0004—0.0012 M).

$$\frac{1}{v} = \frac{1}{\mu\text{moles glucose phosphorylated in 20 min at 30°C}}$$

phosphate is demonstrated in Fig. 7, where ATP and Mg were kept constant, and pyrophosphate inhibited the enzyme at higher concentrations. This can be explained by the formation of a Mg-pyrophosphate complex of great stability, which competes for the binding of Mg^{2+} to the enzyme molecule. In this system the molar ratio of Mg^{2+}/ATP was 5, and under these conditions a slight magnesium inhibition is present. Thus, if ATP was kept constant and Mg reduced to a level where the molar ratio Mg^{2+}/ATP = 1, the activity was somewhat increased (Fig. 7). The small activating effect by pyrophosphate at low concentrations therefore can be explained by a release of the Mg^{2+} inhibition of hexokinase. Further experiments indicated that in addition the Mg-pyrophosphate complex itself can act as an inhibitor of hexokinase. A soluble 1:1 complex of the type $MgP_2O_7^{-2}$ has been demonstrated [13]. Fig. 8 shows the inhibition of hexokinase by this complex when ATP was present at different concentrations at a molar ratio of Mg^{2+}/ATP = 1. The curve which is observed corresponds with that expected for a competitive type of inhibition. Accordingly, the results are consistent with the explanation that a 1:1 Mg-pyrophosphate complex can compete with a Mg—ATP complex for active sites on the surface of the enzyme.

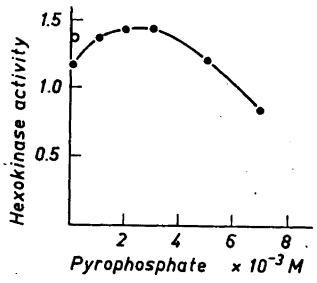

Fig. 7. The influence of sodium pyrophosphate on hexokinase activity. Hexokinase was purified by ethanol fractionation.
●: The system contained 0.002 M ATP and 0.01 M $MgCl_2$ (concentrations).
○: The system contained 0.002 M ATP and 0.002 M $MgCl_2$ (concentrations).

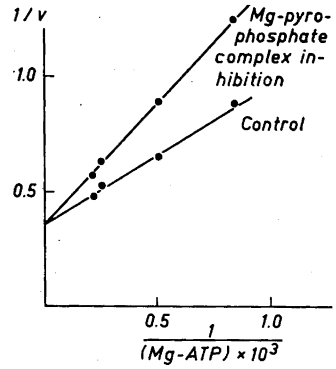

Fig. 8. Inhibition of hexokinase by a soluble magnesium pyrophosphate complex.
The enzyme was preincubated with sodium pyrophosphate and $MgCl_2$ at equimolar concentration (0.01 M) in Tris buffer pH 8.2 for 20 min at 10°C.
During the final incubation ATP and $MgCl_2$ were added at a constant molar ratio of 1:1 (The concentration range varied from 0.001 M to 0.005 M). The Mg pyrophosphate complex was present at a constant concentration in all experiments by dilution of the preincubation mixture 1:2. In unpublished experiments indication have been obtained that a soluble 1:1 complex of pyrophosphate with Mg is formed under these conditions. The final incubation period was 20 min at 30°C.

Table 4. Activation of muscle hexokinase by different divalent metal ions. A concentration of 0.001 M ATP was present with a molar ratio Metal/ATP = 1. Ethanol fractionated hexokinase was used as the enzyme preparation.

Metal ion	Hexokinase activity μmoles per mg protein
Mg^{2+}	2.25
Ca^{2+}	1.70
Co^{2+}	1.40
Mn^{2+}	1.30
Zn^{2+}	0.15
Cu^{2+}	0

Effect of other divalent metals on activity. Muscle hexokinase did not have an absolute requirement for Mg^{2+}. Thus, if magnesium was replaced by one of the divalent metal ions, calcium, cobalt or manganese, activation of the enzyme did occur (Table 4). Only a small activity was observed with Zn^{2+}, and Cu^{2+} was inactive in the system. In some respects the activation of hexokinase by these metal ions differed from that reported for magnesium. When ATP and metal ion were present at a molar ratio of 1, maximal activity with this series of metals was considerably less than with Mg^{2+}. However, half maximal activity

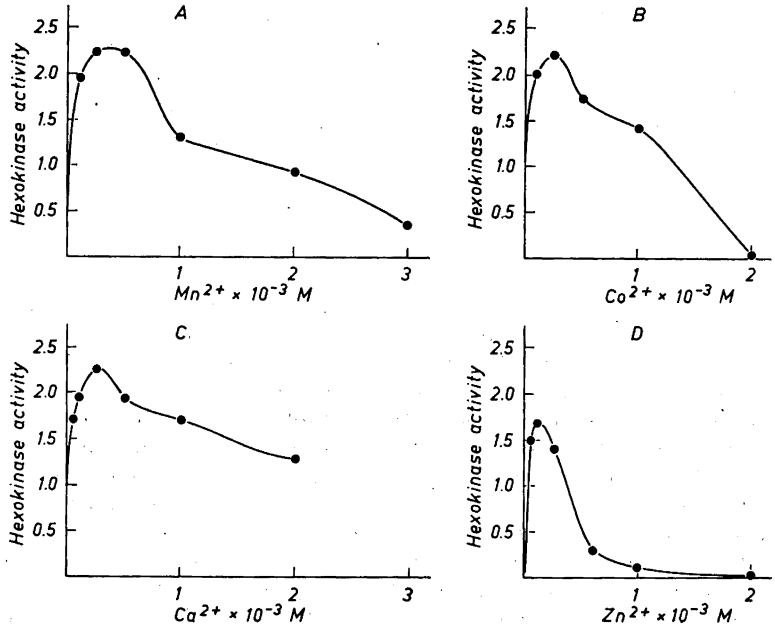

Fig. 9. The influence of $CaCl_2$, $MnCl_2$, $CoCl_2$ and $ZnCl_2$ on muscle hexokinase at a constant level of 0.001 M ATP. These experiments are comparable with those reported in Fig. 3.

with Co^{2+} was observed at 2.3×10^{-4} M and with Mn^{2+} at 1.7×10^{-4} M (Fig. 6) and thus lower than with Mg^{2+} (1.7×10^{-3} M) under the same experimental conditions, *i.e.* when ATP was added at concentrations equal with the metal ion. With these metal ions, hexokinase activity depended upon the concentration of the metal in a manner characteristically different from that described for magnesium. In the experiments, which are shown in Fig. 9, increasing concentrations of the metal ions were added while ATP was kept at a constant level. In contrast to the situation with Mg (Fig. 4), maximal activity was obtained at a ratio metal/ATP considerable below 1. Thus, with Ca^{2+}, Co^{2+}, and Mn^{2+}, the maximal activity appeared at a ratio of 0.5, whereupon strong inhibition did occur when the concentration of the metal was increased. Such an inhibitory effect was still more pronounced with Zn^{2+}, where maximal activity was present at a ratio $Zn^{2+}/ATP = 0.1$. Thus, for all these metal ions the activating effect disappears at higher concentrations. This must be explained by an inhibition of the enzyme at increased concentration of the free metal ions.

The effect of ITP, GTP and UTP on activity. A slow rate of glucose phosphorylation by UTP with muscle hexokinase extract [14] and a small activity of ITP on a partially purified phosphofruktokinase from brain [15] have been reported. This may be due to the contamination of these nucleotides by traces of ADP, since nucleoside diphosphokinase often occurs in these enzyme preparations [16]. However, in a purified preparation of plant hexokinase considerable activition by ITP was observed [17], and ITP and UTP were very active as phosphate donors with highly purified muscle phosphofructokinase [18]. Therefore, experiments on purified hexokinase were performed, where ITP, GTP or UTP purified by an ion exchange resin method [19], were substituted for ATP. However, no measurable glucose phosphorylation could be demonstrated with these nucleotides in the reaction mixture. So far, it must be concluded that muscle hexokinase specifically requires ATP as the phosphate donor.

DISCUSSION

It can be concluded that magnesium is the most effective cation in activating muscle hexokinase. Magnesium gives the highest maximal activity and no inhibitory action takes place until very high concentrations of Mg in excess of ATP are added. The mechanism of this cation activation can be considered as an equilibrium between Mg^{2+} and its complexes with ATP and the enzyme. Thus Najjar [20] has developed the hypothesis that in phosphate transferring enzymes, the function of the metal is to form a complex with the substrate which is the true substrate for the enzyme. Hers [11] working on the phosphofruktokinase and Liébecq [12] investigating muscle hexokinase observed maximal activity at a molar ratio $Mg^{2+}/ATP = 1$ and therefore proposed that the Mg—ATP complex was the actual substrate. On the other hand, Raaflaub and Leupin [21] came to the conclusion that $Mg-ATP^{-2}$ could not act as the substrate for yeast hexokinase. This was denied by Melchior and Melchior [22] who calculated the concentration of the molecular species, Mg^{2+}, $MgATP^{-2}$ and ATP^{-4} which exist in a solution containing ATP and $MgCl_2$. Their experi-

ments on yeast hexokinase could be interpreted by two possible reaction mechanisms.

$$\text{I. } E + MgATP^{-2} \rightleftharpoons E{-}MgATP^{-2}$$
$$\text{II. } E + ATP^{-4} \rightleftharpoons E{-}ATP^{-4}$$
$$E{-}ATP^{-4} + Mg^{2+} \rightleftharpoons E{-}ATP{-}Mg^{2+}$$

The Melchior's were not able to distinguish between the relative importance of the reaction mechanism I and II in the activation of the enzyme. Some information on the mechanism has been given in the experiments on the inhibition of muscle hexokinase by pyrophosphate. The inhibition of hexokinase by sodium pyrophosphate clearly indicates that pyrophosphate complexes Mg^{2+} and thereby competes with the activation of the enzyme. The same conclusion has been reached in studies on polyphosphate inhibition [23] and fluoride inhibition [24] of yeast hexokinase. On the other hand, in the present work the kinetics of the inhibition of muscle hexokinase by a Mg-pyrophosphate complex is in accordance with that of a competitive type with respect to a Mg—ATP complex. This gives some support to the assumption that the $Mg{-}ATP^{-2}$ complex can act as the substrate for the enzyme.

To throw light on this problem, the stability of ATP complexes with different metal cations has been investigated as reported earlier [25]. The formation of 1:1 complexes of the ligand ATP with different metals, Ca^{2+}, Mg^{2+}, Co^{2+}, and Mn^{2+}, were shown. The stability constant of the Mg—ATP complex was calculated to K_f 4.04 in accordance with the value reported by Martell and Schwarzenbach [26]. This does not correspond with the reaction kinetics of muscle hexokinase in activity experiments. As reported, half maximal activity was obtained at a concentration of 1.7×10^{-3} M of magnesium and ATP (Fig. 6). However, the conclusion which can be drawn by comparing activity studies and substrate complex dissociation constants are limited. First, the experimental conditions such as ionic strength, temperature *etc.* have not been completely identical. Further, as pointed out by Melchior [27], ATP at neutral pH in solution exist in several different configurations which can complex with the metal. The catalytic efficiency of a metal ion may therefore be restricted to a complex of a particular molecular shape of ATP which is specially favourable to the required combination with the enzyme.

Williams [28] has further pointed out that the stability of a mixed complex of magnesium with enzyme and substrate will be less than the corresponding Mg-substrate complex. This can be explained by the tendency of magnesium to coordinate with water molecules, especially if coordination through several ligand groups takes place. Therefore, in a mixed enzyme complex, coordination of magnesium with water molecules instead of ligand centres of the enzyme may take place. As a consequence, a mixed hexokinase-ATP complex with magnesium would be less stable than the Mg—ATP complex, which is in accordance with the experimental observations. The nature of the active ligand groups of hexokinase are not known. It is therefore of interest that Ågren and Engström [29] have demonstrated rapid ^{32}P labeling of phosphoserine of purified yeast hexokinase when the enzyme was incubated with ^{32}P labeled ATP. In this connection the hypothesis by Robison and Najjar [30] of the mag-

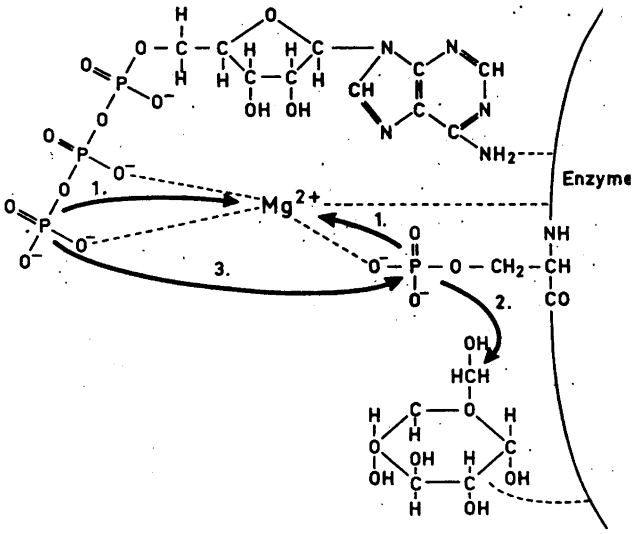

Fig. 10. This illustrates the postulated enzyme-substrate transition complex. By labilization of the phosphate bonds (1), transfer of the phosphate group of phosphorylserine in the enzyme to glucose may occur (2). ATP may serve as the donor for phosphorylation of the serine residue of the enzyme (3) and ADP is formed.

nesium activation of phosphoglucomutase is of interest. In Fig. 10 a tentative scheme for activation of hexokinase is proposed, where Mg^{2+} forms a bridge between ATP^{-2} and the active groups of the enzyme in a ternary transition complex. The positive charge of Mg^{2+} would lower the electron density at the serine phosphate bond of the enzyme and the pyrophosphate bond of ATP. Due to the labilization of these phosphate bonds, the transfer of phosphate from serine phosphate to form glucose-6-phosphate, succeeded by a transfer of phosphate from ATP to the dephosphorylated serine residue of the enzyme would be facilitated. The possible role of an intermediate serine phosphate group in the transphosphorylating process of hexokinase reaction requires further investigation, particularly in view of the results reported by Trayser and Colowick [31].

Compared with Mg^{2+} the activation of muscle hexokinase by the transition series metals Co^{2+} and Mn^{2+}, and by Zn^{2+} as well as by Ca^{2+} is characterized by (*a*) The maximal activity is less, (*b*) Half maximal activity occurs at a lower concentration and (*c*) Strong inhibitory action occurs by increased concentration of the metal ion. These results can be explained as a consequence of the different coordination complexes which is obtained with different metal ions. It has earlier been shown that Mn^{2+}, Co^{2+} (Ref.[25]) and Zn^{2+} (Ref.[32]) form rather stable complexes with ATP compared with the Mg—ATP complex. Different metal ions must also be expected to combine with different ligand centres of the enzyme. While Mg^{2+}, Ca^{2+} and Mn^{2+} mostly combine with oxy-anions under the experimental conditions, Co^{2+} can combine with mixed oxygen anion-nitrogen-ligands, and the Zn^{2+} is likely to be held by

nitrogen groups. The complexing properties of the transition series metals therefore may be the reason why the activation of hexokinase occurs at low concentration of these metal ions. The inhibition of muscle hexokinase at higher concentrations may be due to increased concentration of Me^{2+}-enzyme complex in the presence of excess cations. These complexes are likely to be more stable than the activated mixed complexes and may exert inhibitory action.

Muscle hexokinase is activated by calcium in a manner similar to that observed for Mn^{2+} and Co^{2+}. This occurs in spite of the lower stability of the Ca—ATP complex as reported earlier [25]. The activation of hexokinase by calcium at low concentration may therefore be explained by a high affinity of the enzyme for the Ca—ATP complex.

Finally, it should be pointed out that the metal activation of muscle hexokinase is similar to yeast hexokinase as far as activation by Mg^{2+} and the metals of the transition series is concerned [33]. The only exception is Ca^{2+} which is active in the muscle but inactive on the yeast enzyme.

Acknowledgment. This work has been supported by grants from the *Rockefeller Foundation*, the *Lilly Research Foundation* and from *The Norwegian Research Council for Science and the Humanities*.

Thanks are due to Mrs. Reidun Midthjell for expert technical assistance.

REFERENCES

1. Colowick, S. P., Cori, C. F. and Slein, M. W. *J. Biol. Chem.* **168** (1947) 583.
2. Kipnis, D. M., Helmreich, E. and Cori, C. F. *J. Biol. Chem.* **234** (1959) 177.
3. Park, C. R., Morgan, H. E., Henderson, M. J., Regen, D. M., Cadenas, E. and Post, R. L. *Recent Progr. in Hormone Research* **17** (1961) 493.
4. Colowick, S. P. and Price, W. H. *J. Biol. Chem.* **159** (1945) 563.
5. Cori, C. F. *Harvey Lectures* Ser. 41 (1945—46) 253.
6. Crane, R. K. and Sols, A. in Colowick, S. P. and Kaplan, N. O. (Editors) *Methods in Enzymology*, Academic Press, New York, Vol. 1, 1955, p. 282.
7. Jervell, K. F., Walaas, E. and Walaas, O. *Acta Physiol. Scand.* **35** (1956) 321.
8. Nelson, N. *J. Biol. Chem.* **153** (1944) 375.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
10. Walaas, E. and Walaas, O. *Biochim. et Biophys. Acta* **20** (1956) 77.
11. Hers, H. G. *Biochim. et Biophys. Acta* **8** (1952) 424.
12. Liébecq, C. *Biochem. J.* **54** (1953) p. xxii.
13. Rogers, L. B. and Reynolds, C. A. *J. Am. Chem. Soc.* **71** (1949) 2081.
14. Kleinzeller, A. *Biochem. J.* **36** (1942) 729.
15. Muntz, J. A. *Arch. Biochem. Biophys.* **42** (1953) 435.
16. Berg, P. and Joklik, W. K. *Nature* **172** (1953) 1008.
17. Saltman, P. *J. Biol. Chem.* **200** (1953) 145.
18. Ling, K.-H. and Lardy, H. A. *J. Am. Chem. Soc.* **76** (1954) 2842.
19. Alertsen, Aa. R., Walaas, O. and Walaas, E. *Acta Physiol. Scand.* **43** (1958) 105.
20. Najjar, V. A. *Phosphorus Metabolism* **1** (1951) 500.
21. Raaflaub, J. and Leupin, I. *Helv. Chim. Acta* **39** (1956) 832.
22. Melchior, N. C. and Melchior, J. B. *J. Biol. Chem.* **231** (1958) 609.
23. Dirheimer, G. and Ebel, J. P. *Bull. soc. chim. biol.* **58** (1956) 1337.
24. Melchior, N. C. and Melchior, J. B. *Science* **124** (1956) 402.
25. Walaas, E. *Acta Chem. Scand.* **12** (1958) 528.
26. Martell, A. E. and Schwarzenbach, G. *Helv. Chim. Acta* **76** (1956) 653.
27. Melchior, N. C. *J. Biol. Chem.* **208** (1954) 615.

28. Williams, R. J. P. in Boyer, P. D., Lardy, H. and Myrbäck, K. (Editors) *The Enzymes*, Academic Press, New York 1959, Vol. I, p. 397.
29. Ågren, G. and Engstrøm, L. *Acta Chem. Scand.* **10** (1956) 489.
30. Robinson, J. P. and Najjar, V. A. *Federation Proc.* **20** (1961) 90.
31. Trayser, K. A. and Colowick, S. P. *Arch. Biochem. Biophys.* **94** (1961) 161.
32. Walaas, E. *Unpublished observation.*
33. Brintzinger, H., Fallab, and Erlenmeyer, H. *Experienta* **15** (1959) 96.

Received April 5, 1962.