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Hexokinase of Human Erythrocytes

Purification, Kinetic Model and Its Application to the Conditions in the Cell

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Hexokinase was purified 680-fold from erythrocytes of man. The preparation was not contaminated by other glycolytic enzymes.

Initial reaction rate measurements were performed at pH 7.2, ionic strength 0.15 and 37 °C by use of coupled optical assays. The experimental data were used to construct a kinetic model of the enzyme, the kinetic parameters of which were estimated by means of a computer-fit program.

The hexokinase of erythrocytes operates by a rapid-equilibrium random mechanism. The dissociation constant of the E · glucose complex is 0.038 to 0.054 mM, of the E · MgATP complex 1.0 to 2.1 mM. Uncomplexed Mg^{2+} up to 4 mM is capable of activating the enzyme velocity twofold ($K_{Mg^{2+}} = 1.0$ mM). An inhibition was observed at higher Mg^{2+} concentrations. α-D-Glucose 6-phosphate and α-D-glucose 1,6-bisphosphate inhibit the hexokinase competitive to MgATP with dissociation constants of 0.069 mM. The enzyme is also inhibited by 2,3-bisphosphoglycerate competitive with respect to MgATP with a $K_{P_2G} = 2.7$ mM. An inhibition by uncomplexed ATP was not detected.

The parameters of the kinetic model of the enzyme in conjunction with the free intracellular concentrations of substrates and effectors were used to calculate the actual intracellular activity of the hexokinase in dependence on variations of pH, temperature and the state of haemoglobin oxygenation. The calculated hexokinase activities correspond to the glycolytic flux in all conditions considered, except at pH 8.

Great progress has been made in the kinetics and in the study of the reaction mechanisms of purified enzymes under artificial conditions by mathematical treatment of the experimental data. Numerous data on the metabolite concentrations have also been accumulated in diverse types of cells in various conditions *in vivo* and *in vitro*. There remains a deep gap between our knowledge of the response of a particular enzyme to various reactants *in vitro* and the factors determining its actual activity in the natural environment.

The mature red blood cell may be considered to be the best biological object to approach this problem on account of the simplicity of its metabolism which is practically restricted to glycolysis. The flux through this enzymatic chain is controlled only by the hexokinase-phosphofructokinase system [1].

The purpose of this paper is to present a model of the hexokinase of human erythrocytes based on initial rate measurements. Care was taken in the measurements to simulate the intracellular conditions regarding pH, temperature and ionic strength. The concentrations of the reactants were chosen so as to cover the range of their variations in erythrocytes under various experimental conditions. The kinetic parameters of the enzyme in conjunction with the free intracellular concentrations of substrates and effectors [2] were used to calculate its actual intracellular activity. The results are compared with the glycolytic rate in various conditions.

Abbreviations. P_2G, 2,3-bisphosphoglycerate; Glc*P*, α-D-glucose 6-phosphate; GlcP_2, α-D-glucose 1,6-bisphosphate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate.

Enzymes. Adenylate kinase (EC 2.7.4.3); fructose bisphosphate aldolase (EC 4.1.2.13); enolase (EC 4.2.1.11); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12); glucose phosphate isomerase (EC 5.3.1.9); hexokinase (EC 2.7.1.1); lactate dehydrogenase (EC 1.1.1.27); phosphofructokinase (EC 2.7.1.11); phosphoglycerate kinase (EC 2.7.2.3); pyruvate kinase (EC 2.7.1.40).

MATERIALS AND METHODS

Chemicals and Biochemicals

ATP, ADP, 2,3-bisphosphoglycerate, α-D-glucose 6-phosphate, α-D-glucose 1,6-bisphosphate and phospho*enol*pyruvate were products of Boehringer Mann-

heim GmbH (Mannheim, FRG). NADP and $NADH_2$ were obtained from VEB Arzneimittelwerk (Dresden, GDR). D-Glucose was from Polfa (Poland) and TES buffer from Calbiochem (Los Angeles, California, U.S.A.). Pyruvate kinase was obtained from Reanal (Budapest, Hungary) and glucose-6-phosphate dehydrogenase, lactate dehydrogenase and bovine serum albumin were purchased from Boehringer Mannheim GmbH. Mercaptoethanol was from Ferak (Berlin). The Sephadex gel types DEAE-A50, CM-C50 and G-100 were products of Pharmacia (Uppsala, Sweden). Hydroxyapatite suspended in 1 mM phosphate buffer pH 6 to 8 was from Serva (Heidelberg, FRG).

Measurements of the Hexokinase Activity

Initial reaction rate measurements were performed in a Unicam SP-800 recording spectrophotometer at 340 nm or in a recording photometer Eppendorf at 366 nm (light path 10 mm). The temperature of the cell housings was maintained at 37 °C by circulating water from a temperature-controlled bath through thermospacers.

The standard reaction mixture consisted of 75 mM TES buffer pH 7.2, 75 mM KCl, 5 mM glucose and ATP respectively and 10 mM $MgCl_2$. The velocity of glucose-6-phosphate formation was assayed with a coupled enzyme system, containing in addition to the other components, 0.6 mM NADP and 0.25 IU glucose-6-phosphate dehydrogenase. When glucose 6-phosphate was employed as inhibitor, the initial rates of ADP production were determined by use of a coupled enzyme assay, containing additionally 3 mM phospho*enol*pyruvate, 0.6 mM NADH and 3 IU both of pyruvate kinase and lactate dehydrogenase. The final volume of the reaction mixture samples was always 1.0 ml. 2,3-Bisphosphoglycerate and glucose 1,6-bisphosphate in the molarity employed were checked to have no effect on the auxiliary enzymes. With either type of measurement the amount of hexokinase preparation added was adjusted so that the reaction velocity was linear for at least 10 min at 37 °C. The reaction velocity was expressed in arbitrary units which are explained in the section "Effect of the Free Magnesium Ion".

Preparation of Hexokinase from Human Erythrocytes

The gels were prepared for use by previous equilibration in NaOH, water and HCl following the instructions of the manufacturers. DEAE-Sephadex was equilibrated with 3 mM potassium phosphate pH 7.0, 10 mM glucose, 5 mM mercaptoethanol, and 5 mM EDTA (buffer P), CM-Sephadex with 3 mM buffer P pH 5.8. The protein concentration was determined by the biuret procedure [3]. Blood in amounts of 3000 ml preserved in acid-citrate-dextrose solution was kindly supplied from the local blood preservation center. The erythrocytes were kept at 4 °C and used within 72 h after the collection. The red cells were washed twice suspending them in isotonic sodium chloride and by centrifugation at 4000 rev./min. The buffy coat was removed by suction.

Step I. Preparation of the Haemolysate. 1250 ml packed cells were haemolyzed by addition of two volumes of buffer P.

Step II. Removal of Haemoglobin. 3750 ml haemolysate was poured on a 400 ml DEAE-Sephadex gel sediment in a porous glass filter (Fritte G-2, VEB Schott & Gen., Jena, GDR). The suspension was rinsed with 5 l buffer P until the effluent was nearly colorless. Further protein was desorbed by rinsing with 1000 ml buffer P + 0.1 M KCl and the hexokinase was eluted with 900 ml buffer P + 0.5 M KCl. The eluate containing the hexokinase activity was concentrated by ultrafiltration in a 200-ml ultrafiltration chamber with a Diaflo membrane PM 30. It was dialyzed overnight against buffer P to remove KCl.

Step III. First Batch-Wise Treatment with CM-Sephadex. 300 ml of the dialyzed material were centrifuged and the supernatant poured on 50 ml CM-Sephadex sediment pH 5.8. The gel suspension on a Fritte G-2 was rinsed with 2 l buffer P pH 5.8 and the elution of the hexokinase was carried out with 400 ml 10 mM sodium pyrophosphate pH 7.5 containing the same amount of mercaptoethanol, EDTA and glucose as buffer P. The eluate was concentrated by ultrafiltration to 80 ml.

Step IV. Gel Filtration on Sephadex G-100. A 40-ml aliquot was transferred on a Sephadex G-100 column (3.25 × 80.0 cm) equilibrated with buffer P without EDTA. The rate of flow was 20 drops per min and 5-ml fractions were collected.

Step V. Chromatography on Hydroxyapatite. The fractions containing peak activities of hexokinase were concentrated to 15 ml and aliquots of 5 ml were transferred to an hydroxyapatite column (2.0 × 20 cm) preequilibrated with buffer P without EDTA. The column was washed with 500 ml buffer P without EDTA and the enzyme was eluted with 46 ml 0.3 M potassium phosphate buffer pH 7.0 (flow rate: 20 drops per min). The eluate was dialyzed against buffer P pH 7.0.

Step VI. Second Batch-Wise Treatment with CM-Sephadex. This procedure was performed like that in Step III. 46 ml enzyme solution was poured on 10 ml CM-Sephadex sediment pH 5.8 and the elution of the enzyme was carried out with 100 ml pyrophosphate solution as before. The eluate was dialyzed against buffer P pH 7.0.

An example of the preparation procedure is presented in Table 1. The preparation showed one

Table 1. *Preparation of the hexokinase from human erythrocytes*
The representative purification scheme is that from a 1:3 haemolysate of 1250 ml packed erythrocytes. The activity of the hexokinase was measured with the standard assay coupled with glucose-6-phosphate dehydrogenase. The protein concentration was determined with the biuret method [3], in the haemolysate haemoglobin was determined as cyan-methaemoglobin

Fraction	Hexokinase activity	Protein	Specific activity	Recovery	Purification factor
	μmol/min	mg	μmol $\times min^{-1}$ $\times mg^{-1}$	%	-fold
Haemolysate	142	338000	0.00042	100	1
DEAE-Sephadex	117.5	6230	0.019	83	45
CM-Sephadex	81.2	3115	0.026	87	62
Sephadex-C 100	46.6	613	0.076	33	181
Hydroxyapatite	35.5	298	0.119	25	285
CM-Sephadex	29.1	101	0.288	20.5	684

single band in agarose gel or cellogel electrophoresis [4]. The hexokinase preparation retained its activity for several months when stored at −40 °C in buffer P pH 7.0. 90% of the activity was still present after 6 months. The fractions of contaminant enzyme activities were less than 1% of the activity of hexokinase (phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, phosphofructokinase, aldolase, phosphoglycerate kinase, pyruvate kinase, lactate dehydrogenase, adenylate kinase, glyceraldehyde-3-phosphate dehydrogenase). The samples were thawed on the day of the measurements and prepared by desalting on a Sephadex G-25 column (1.0×20 cm) in 0.15 M KCl.

Computations

With the association constants of $K_{ass} = 1.2 \times 10^4$ M^{-1} for the MgATP complex and $K_{ass} = 6 \times 10^2$ M^{-1} for the MgP_2G complex, the ionic species were computed on a Hewlett-Packard 8910A calculator. These constants are "conditional" for pH 7.2, ionic strength 0.15 and 37 °C (for detailed information see [5]). According to Good *et al.* [6], the TES buffer does no interact with Mg^{2+}.

The experimental data for the activity of the hexokinase were used to build up a kinetic model of this enzyme. The kinetic equations were written under the assumption that the formation of the products from the active enzyme · ligand complexes is the rate-limiting step of the reactions, *i.e.* only "rapid-equilibrium mechanisms" were considered. This means that only models with random addition of the ligands to the enzyme were taken into account in keeping with all the available evidence. It has been pointed out by Dalziel [7] that the rapid equilibrium assumption cannot be valid for compulsory ordered mechanisms. The kinetic parameters were estimated by means of a computer-fit program presented by Reich *et al.* [8]. The calculations were performed on a BESM 6 computer. Input to the computer are the substrate and effector concentrations, the corresponding enzyme activities, a set of models to be checked, and the starting points of the parameters. The computer output consists of the sum of the least squares of the fitted model, the parameter values giving the best fit and their range of variations in percent (r.v.), a variance-covariance matrix giving the statistical interdependence of the parameters and tables of the fit obtained. For each model the mean deviation $\overline{\Delta v}$ of the theoretical from the experimental values of the enzyme activities was calculated by the formula

$$\overline{\Delta v} = \frac{100}{\bar{v}} \sqrt{\frac{\text{Sum of least squares}}{N-1}} \qquad (1)$$

N is the number of the experimental points and $\bar{v}$ is the mean value of the observed enzyme activities.

All figures show the experimental values as points and the theoretical curves calculated with the computer-generated parameters which are given in Tables 2—5. The models were fitted by non-linear regression of the original experimental values of enzyme activity *vs* substrate concentration. Owing to the distortion of the weights of the experimental values in a double-reciprocal plot the theoretical curves appear to deviate from the experimental values.

RESULTS

Kinetics of Substrates

The dependence of the hexokinase activity on glucose and MgATP was studied with a set of combinations of both substrates in the concentration ranges of 0.05 to 5.0 mM of glucose and 0.5 to 5.0 mM MgATP. The concentration of free magnesium was held constant at 5.0 mM. The data are presented in a double-reciprocal plot [9] in Fig. 1 and 2. They fit straight lines indicating a hyperbolic responses of the enzyme activity to glucose as well as to MgATP. The lines intersect near to the abscissa. Therefore a ping-pong mechanism appears to be ruled out [10].

Scheme I shows the rapid equilibrium mechanism for which the best fit to the experimental data could be obtained.

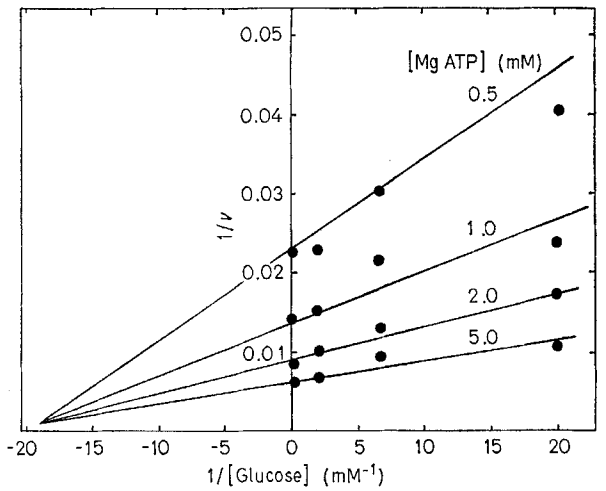


Fig. 1. *Plot of reciprocal of the reaction velocity* versus *reciprocal of glucose concentration.* The concentration of glucose was 0.05, 0.15, 0.5, and 5.0 mM, that of the MgATP complex is indicated on the curves. The concentration of uncomplexed Mg^{2+} was maintained at 5.0 mM. pH 7.2, 37 °C. Final volume of the incubation mixture 1.0 ml. Glucose 6-phosphate dehydrogenase coupled assay. The measured data of change in absorbance per min were plotted as percentage of the enzyme activity at saturating concentrations of glucose and MgATP and at a zero concentration of free Mg^{2+}

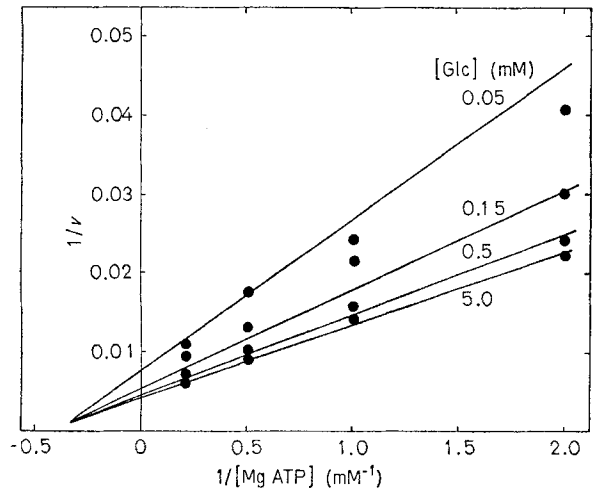
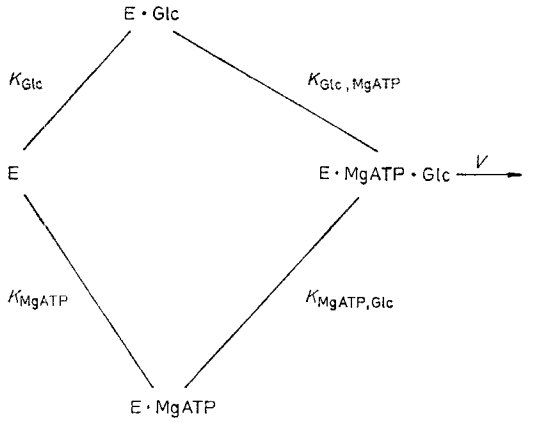


Fig. 2. *Plot of reciprocal of the reaction velocity* versus *reciprocal of concentration of MgATP.* The concentration of the MgATP complex was 0.5, 1.0, 2.0, and 5.0 mM, that of glucose is indicated on the curves. The concentration of uncomplexed Mg^{2+} was maintained at 5.0 mM. pH 7.2, 37 °C. Glucose 6-phosphate dehydrogenase coupled assay. Units of reaction velocity see Fig. 1

According to this model MgATP and glucose are added in a random manner to form a ternary complex with the enzyme which then will be converted to the products in the rate-limiting step. K_{Glc} and K_{MgATP} are the dissociation constants of the complexes



Scheme I

Table 2. *Kinetic parameters of hexokinase for the substrates* Uncomplexed Mg^{2+} was kept constant at 5.0 mM. V_1, the enzyme activity at saturating concentrations of MgATP and glucose, and $[Mg^{2+}] = 0$, was taken as 100. The range of variation is that of the particular parameter. $\overline{\Delta v}$, the mean deviation of the theoretical from the experimental values = 6.8%

Parameter	Value	Range of variation
	% V_1	%
V	194	7.1
	mM	
K_{MgATP}	2.06	15.6
K_{Glc}	0.038	33.0
$K_{MgATP,Glc}$	0.054	11.7

E · Glc and E · MgATP respectively. $K_{MgATP,Glc}$ and $K_{Glc,MgATP}$ are the dissociation constants of the ternary complex E · MgATP · Glc. The corresponding kinetic rate equation can be written as

$$v = \frac{V \dfrac{[MgATP]\,[Glc]}{K_{MgATP}\,K_{MgATP,Glc}}}{1 + \dfrac{[MgATP]}{K_{MgATP}} + \dfrac{[Glc]}{K_{Glc}} + \dfrac{[MgATP]\,[Glc]}{K_{MgATP}\,K_{MgATP,Glc}}} \quad (2)$$

From thermodynamic considerations it follows that:

$$K_{MgATP} \times K_{MgATP,Glc} = K_{Glc} \times K_{Glc,MgATP} \quad (3)$$

Thus the reaction can be fully described by three independent dissociation constants and the maximal activity V of the enzyme saturated with both substrates. Table 2 shows the values of the kinetic constants and their ranges of variation. The mean deviation $\overline{\Delta v}$ of each experimental value from that postulated by the model amounts to 6.8% indicating that this combination of dissociation constants gives a good fit. The value of $K_{MgATP,Glc}$ is somewhat

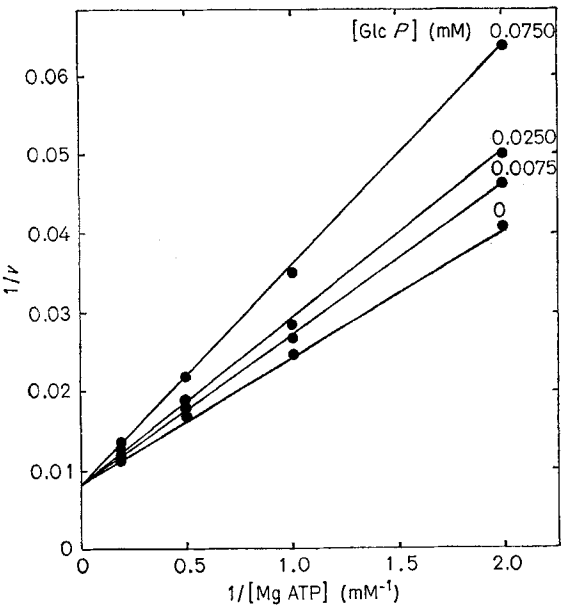


Fig. 3. *Inhibition by glucose 6-phosphate in a double-reciprocal plot 1/*v versus *1/[MgATP]*. The concentration of glucose 6-phosphate was 0, 0.0075, 0.025, and 0.075 mM. The concentration of the MgATP complex was 0.5, 1.0, 2.0 and 5.0 mM. Uncomplexed Mg^{2+} and glucose were kept at 5.0 mM each. pH 7.2, 37 °C. Pyruvate kinase—lactate dehydrogenase coupled assay. Units of reaction velocity see Fig. 1

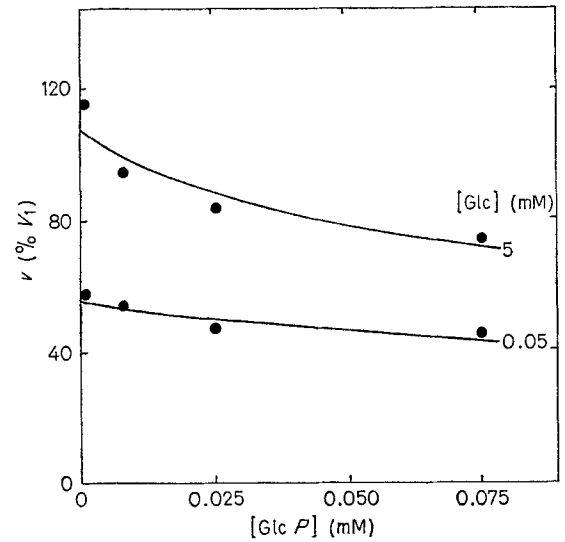
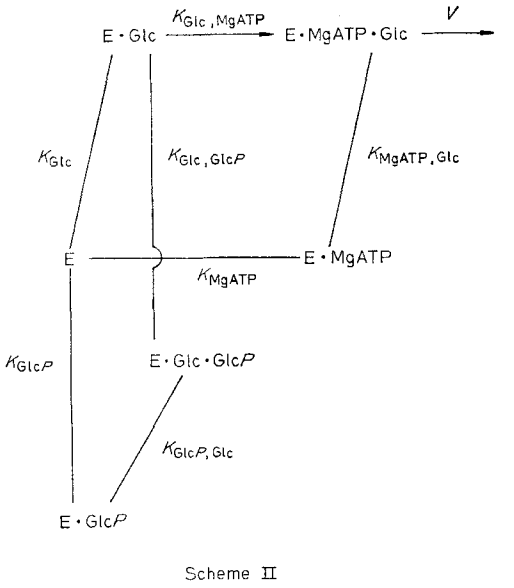


Fig. 4. *Inhibition of hexokinase by glucose 6-phosphate at low and saturating glucose concentration.* The concentration of glucose 6-phosphate was 0, 0.0075, 0.025 and 0.075 mM. The concentrations of MgATP and uncomplexed Mg^{2+} were maintained at 5 mM reach. The concentration of glucose was 0.05 and 5.0 mM. pH 7.2, 37 °C. Pyruvate kinase—lactate dehydrogenase coupled assay. Units of reaction velocity see Fig. 1



Scheme II

greater than K_{Glc}. Therefore, it seems that MgATP hinders slightly the binding of glucose and *vice versa.*

Effect of Hexose Phosphates

The inhibitory action of glucose 6-phosphate on animal hexokinases was first shown on the enzyme from brain [11] and has been confirmed since for hexokinases of a variety of animal tissues [12]. We investigated its effect on the erythrocyte enzyme at 5 mM free Mg^{2+} as a function of glucose and MgATP. The double-reciprocal plot $1/v$ *versus* 1/[MgATP] at 0, 0.0075, 0.025 and 0.075 mM glucose 6-phosphate shows that the inhibition of glucose 6-phosphate is competitive with MgATP (Fig. 3) in agreement with other reports [13—16]. A plot not presented here, where glucose was the substrate varied, shows non-competitive inhibition kinetics. Fig. 4 shows the glucose 6-phosphate inhibition at 0.05 mM and 5.0 mM glucose ([MgATP] = 2 mM). At the higher glucose concentration, the glucose 6-phosphate inhibition is much more pronounced than at the lower one.

The experimental results presented here can be fitted best by the model shown in Scheme II. It is based on the model derived in the foregoing paragraphs (Scheme I). According to the competitive inhibition kinetics with respect to glucose 6-phosphate and MgATP, the complexes E · MgATP · Glc*P* and E · MgATP · Glc · Glc*P* were not taken into account in Scheme II. By inclusion of such complexes in the model the approximation is not improved. The rate equation for this mechanism is:

$$v = \frac{V \dfrac{[\mathrm{MgATP}]\,[\mathrm{Glc}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Glc}}}}{1 + \dfrac{[\mathrm{MgATP}]}{K_{\mathrm{MgATP}}} + \dfrac{[\mathrm{Glc}]}{K_{\mathrm{Glc}}} + \dfrac{[\mathrm{MgATP}]\,[\mathrm{Glc}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Glc}}} + \dfrac{[\mathrm{GlcP}]}{K_{\mathrm{GlcP}}} + \dfrac{[\mathrm{Glc}]\,[\mathrm{GlcP}]}{K_{\mathrm{Glc}}\,K_{\mathrm{Glc,GlcP}}}} \cdot \qquad (4)$$

Table 3. *Kinetic parameters for the dependence of hexokinase on glucose 6-phosphate, glucose and MgATP*
For details see Table 2. $\overline{\Delta v} = 5.8\%$

Parameter	Value	Range of variation
	% V_1	%
V	193	4.5
	mM	
K_{MgATP}	1.76	45.7
K_{Glc}	0.040	34.7
$K_{MgATP,Glc}$	0.051	9.4
K_{GlcP}	0.334	327
$K_{Glc,GlcP}$	0.069	31.8

K_{GlcP} and $K_{Glc,GlcP}$ denote the dissociation constants of the complexes E · Glc*P* and E · Glc · Glc*P*, respectively. In addition to Eqn (3), the thermodynamic relation holds:

$$K_{Glc} \times K_{Glc,GlcP} = K_{GlcP} \times K_{GlcP,Glc}. \quad (5)$$

The results of the fitting are summarized in Table 3. The mean deviation $\overline{\Delta v}$ amounts to 5.8%. The values K_{MgATP}, K_{Glc}, $K_{MgATP,Glc}$ and V are in good agreement with those obtained from Scheme I. One should note that K_{GlcP} is significantly greater than $K_{Glc,Glcp}$. Therefore, the substrate glucose enhances the binding of the inhibitor.

The influence of glucose 1,6-bisphosphate on hexokinase at 5 mM free Mg^{2+} and saturating glucose concentrations was also studied. A plot of $1/v$ *versus* 1/[MgATP] provides clear evidence for competitive inhibition (Fig. 5). The comparison of this plot with Fig. 3 shows good agreement. Therefore, a detailed mathematical analysis was not performed and the binding parameters were taken to be identical for glucose 6-phosphate and glucose 1,6-bisphosphate.

10 mM inorganic phosphate [17] as well as fructose 6-phosphate, fructose 1,6-bisphosphate, 6-phosphogluconate, ribose 5-phosphate, 3-phosphoglyceric acid and phospho*enol*pyruvate at 1 mM concentrations neither inhibited the enzyme nor altered the inhibition by glucose 6-phosphate.

Effect of the Free Magnesium Ion

Experiments were performed to explore the dependence of the hexokinase reaction on uncomplexed magnesium and ATP. The concentrations of Mg^{2+}, ATP, and MgATP were calculated for each measurement as described in Materials and Methods. Solutions of Mg^{2+} and ATP were mixed to yield final concentrations of 0.5, 1.0, 2.0 and 5.0 mM MgATP and concentrations of 0.05, 0.5, 2.0 and 10.0 mM free Mg^{2+}. Due to the equilibrium the free ATP varies from 1.0 to 10.0 mM at 0.05 mM free Mg^{2+}, from 0.1 to 1.0 mM at 0.5 mM Mg^{2+}, from 0.025 to 0.25 mM

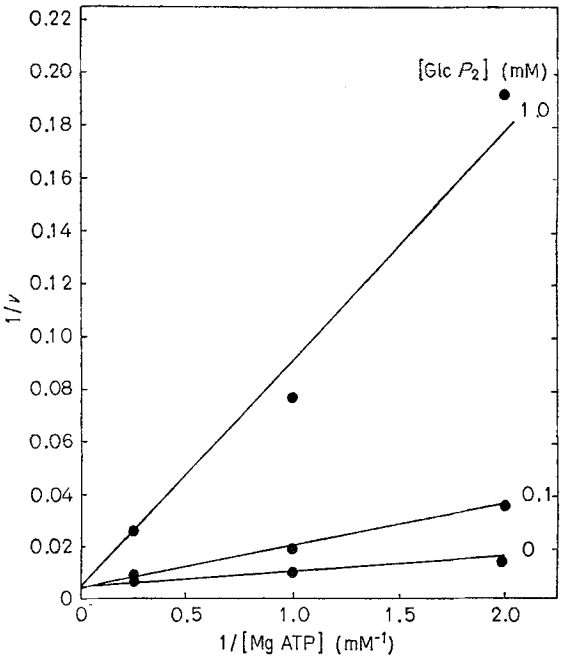


Fig. 5. *Inhibition by glucose 1,6-bisphosphate.* The concentration of glucose 1,6-bisphosphate was 0, 0.1 and 1.0 mM. The concentration of the MgATP complex was 0.5, 1.0 and 5.0 mM. Uncomplexed Mg^{2+} and the glucose concentration was 5.0 mM each. pH 7.2, 37 °C. Pyruvate kinase—lactate dehydrogenase coupled assay. Units of reaction velocity see Fig. 1

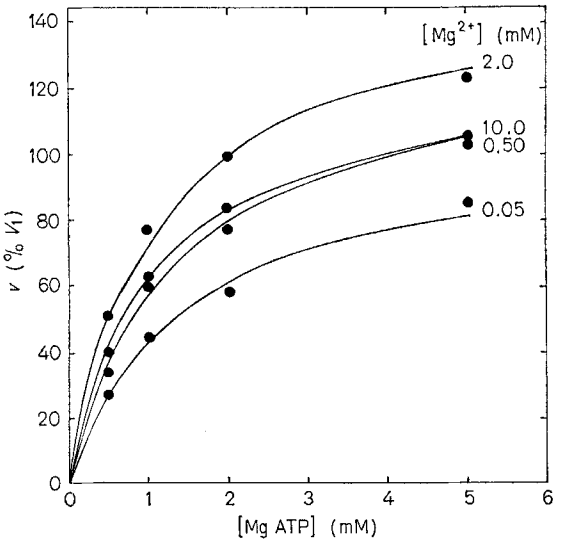


Fig. 6. *Dependence of hexokinase activity on MgATP at various concentrations of uncomplexed Mg^{2+}.* The concentration of the MgATP complex was 0.5, 1.0, 2.0 and 5.0 mM, the concentration of uncomplexed Mg^{2+} was 0.05, 0.5, 2.0 and 10.0 mM. Glucose concentration was 5.0 mM. pH 7.2, 37 °C. Glucose 6-phosphate dehydrogenase coupled assay. Units of reaction velocity see Fig. 1

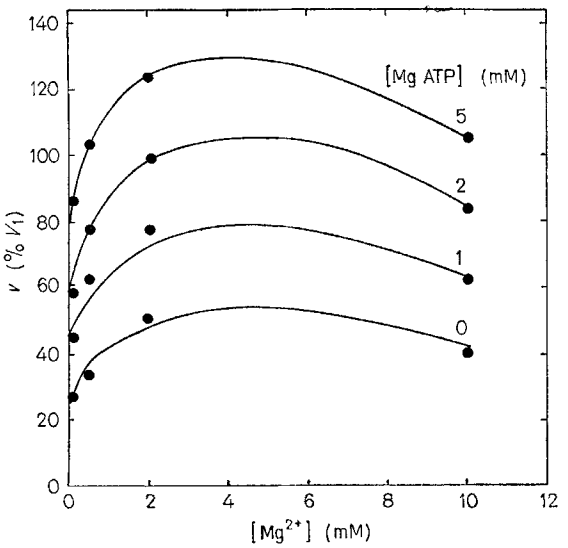
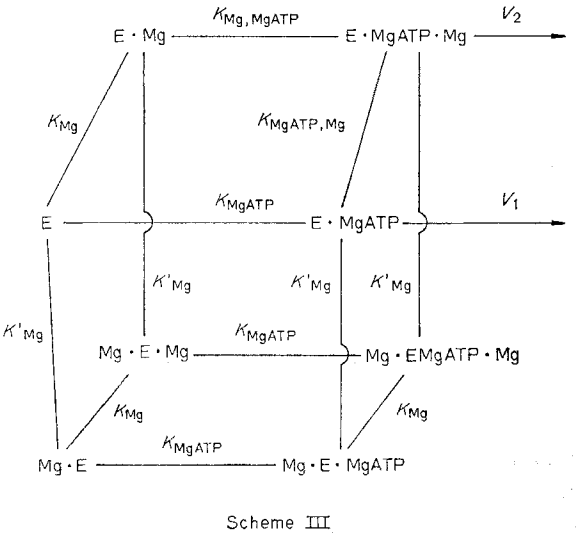


Fig. 7. *Activation and inhibition of hexokinase by uncomplexed Mg^{2+}.* The concentration of uncomplexed Mg^{2+} was 0.05, 0.5, 2.0 and 10.0 mM, the MgATP concentration was 0.5, 1.0, 2.0, and 5.0 mM. The concentration of glucose was 5.0 mM. pH 7.2, 37 °C. Glucose 6-phosphate dehydrogenase coupled assay. Units of reaction velocity see Fig. 1

at 5.0 mM Mg^{2+}, and from 0.005 to 0.05 mM at 10.0 mM Mg^{2+}.

In Fig. 6 and 7 are depicted data on the enzyme activity at the concentrations of Mg^{2+} and MgATP indicated. Elevation of Mg^{2+} up to 4 mM increases nearly two-fold the reaction rate; further elevation is accompanied by an inhibition of the enzymic rate. An excellent fit (mean deviation $\overline{\Delta v} = 3.9\%$) to these experimental data was obtained by the model represented in Scheme III.

Since the enzyme is saturated with glucose E stands here for the enzyme · glucose complex. Two binding sites are suggested for magnesium, one for its activating and one for the inhibitory effect. The model contains two active complexes: E · MgATP and E · MgATP · Mg. The rate equation may be written in the following form:



Scheme III

the inhibitory effect of Mg^{2+} by only one constant K'_{Mg}. It means that the dissociation constant of Mg^{2+} as inhibitor is assumed to be independent of whether or not the binding sites for MgATP and Mg^{2+} as activator are occupied. The parameters giving the best fit are seen in Table 4. V_1 designates the maximal reaction rate with saturating concentrations of MgATP and glucose and at zero concentration of free Mg^{2+}. It is arbitrarily taken as 100 and all other enzyme activities in the kinetic studies of this paper refer to it. V_2 indicates the extrapolated maximal activity of the hexokinase fully activated by Mg^{2+}, Mg^{2+} exerts its inhibitory action at concentrations 16 times higher than those of its activating effect.

Several authors found an inhibition of hexokinase from other tissues by uncomplexed ATP [11, 13, 18]. An excellent fit was obtained without assuming inhibition by uncomplexed ATP. A fit of the experimental data to a kinetic equation in which the assumption of a separate inhibitory site for uncomplexed ATP was made yielded identical parameters.

$$v = \frac{V_1 \frac{[\mathrm{MgATP}]}{K_{\mathrm{MgATP}}} + V_2 \frac{[\mathrm{MgATP}]\,[\mathrm{Mg}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Mg}}}}{\left(1 + \frac{[\mathrm{MgATP}]}{K_{\mathrm{MgATP}}} + \frac{[\mathrm{Mg}]}{K_{\mathrm{Mg}}} + \frac{[\mathrm{MgATP}]}{K_{\mathrm{MgATP}}}\,\frac{[\mathrm{Mg}]}{K_{\mathrm{MgATP,Mg}}}\right)\left(1 + \frac{[\mathrm{Mg}]}{K'_{\mathrm{Mg}}}\right)} \cdot \qquad (6)$$

K_{Mg} is the dissociation constant of the E · Mg complex, (Mg^{2+} as activator), $K_{MgATP,Mg}$ and $K_{Mg,MgATP}$ are the dissociation constants of the complex E · MgATP · Mg. The relation holds

$$K_{Mg} \times K_{Mg,MgATP} = K_{MgATP} \times K_{MgATP,Mg} \cdot \qquad (7)$$

Since there are just a few experimental data at high Mg^{2+} concentrations it is reasonable to characterize

Inhibition by 2,3-Bisphosphoglycerate

The effect of 2,3-bisphosphoglycerate on hexokinase was studied at 5.0 mM glucose. The 2,3-bisphosphoglycerate concentration was varied in the range of 0 to 10.0 mM, Mg^{2+} between 0.05 and 5.0 mM and MgATP between 0.5 and 5.0 mM; altogether the enzyme activity was tested at 64 combinations of reactants. It may be seen from the double-reciprocal

Table 4. *Kinetic parameters for the effect of Mg^{2+} on the hexokinase*
For explanation see Table 2. Glucose concentration = 5.0 mM. K_{Mg} and $K_{Mg,MgATP}$ denote the dissociation constants for activation by Mg^{2+}, K'_{Mg} for inhibition by Mg^{2+}. V_1 is the maximal activity at zero Mg^{2+} and saturating concentrations of MgATP and glucose; V_2 is the maximal activity of the enzyme fully activated by Mg^{2+} at saturating concentrations of both substrates. $\overline{\Delta v} = 3.9\%$

Parameter	Value	Range of variation
	% V_1	%
V_1	100	5.9
V_2	212	16.3
	mM	
K_{MgATP}	1.44	13.9
K_{Mg}	1.03	51.3
$K_{MgATP,Mg}$	1.14	44.7
K'_{Mg}	15.8	35.3

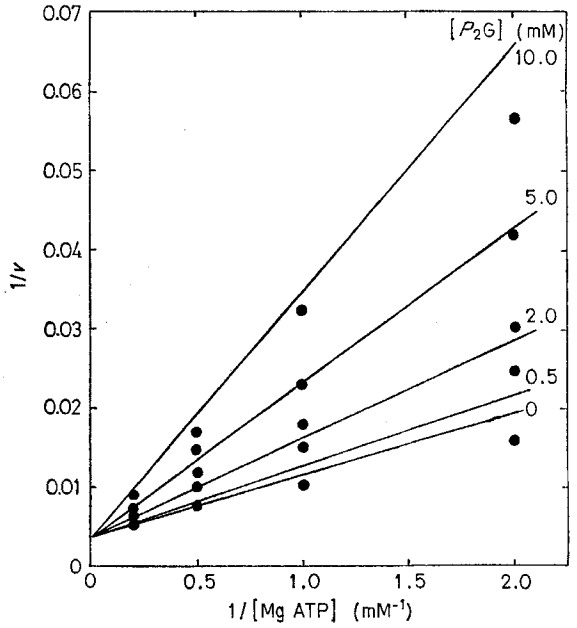
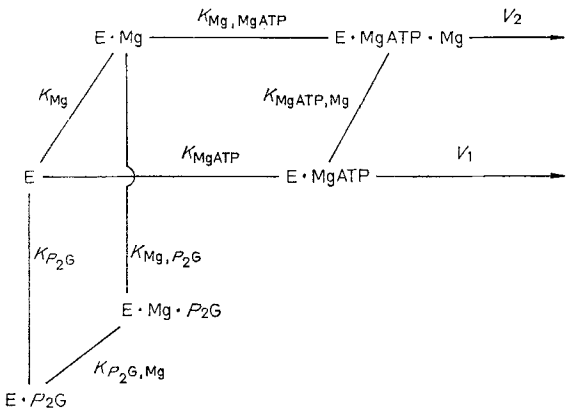


Fig. 8. *Inhibition of hexokinase by 2,3-bisphosphoglycerate in a double-reciprocal plot 1/v versus 1/[MgATP].* The concentration of 2,3-bisphosphoglycerate was 0, 0.5, 2.0, 5.0, and 10.0 mM, MgATP concentration was 0.5, 1.0, 2.0 and 5.0 mM. Uncomplexed Mg^{2+} and glucose were kept at 5.0 mM. pH 7.2, 37 °C. Glucose 6-phosphate dehydrogenase coupled assay. Units of reaction velocity see Fig. 1



Scheme IV

Table 5. *Kinetic parameters for the dependence of hexokinase on 2,3-bisphosphoglycerate, Mg^{2+} and MgATP*
For explanation see Tables 2 and 4. P_2G is the sum of free P_2G and its magnesium complexes. $\overline{\Delta v} = 9.3\%$

Parameter	Value	Range of variation
	% V_1	%
V_1	100	9.2
V_2	245	9.9
	mM	
K_{MgATP}	1.03	24.4
K_{Mg}	0.77	39.6
$K_{MgATP,Mg}$	1.72	38.0
K_{P_2G}	2.70	34.1
K_{Mg,P_2G}	3.44	36.1

plot (Fig. 8) that 2,3-bisphosphoglycerate inhibits competitively with respect to MgATP. The concentration of free Mg^{2+} in the experiment presented was 5.0 mM. The curves at other Mg^{2+} concentrations were similar. The experimental data could be fitted by a model seen in Scheme IV.

It is an extension of Scheme III for the activation of the enzyme by Mg^{2+}, where as before E means the enzyme · glucose complex. The inhibitory effect of Mg^{2+} at high concentrations is neglected, since its concentration did not exceed 5.0 mM. For the dissociation constants of Scheme IV the following equation holds

$$K_{Mg} \times K_{Mg,P_2G} = K_{P_2G} \times K_{P_2G,Mg}. \qquad (8)$$

For the kinetic equation we get:

$$v = \frac{V_1 \frac{[MgATP]}{K_{MgATP}} + V_2 \frac{[MgATP]\,[Mg]}{K_{MgATP}\,K_{MgATP,Mg}}}{1 + \frac{[MgATP]}{K_{MgATP}} + \frac{[Mg]}{K_{Mg}} + \frac{[MgATP]\,[Mg]}{K_{MgATP}\,K_{MgATP,Mg}} + \frac{[P_2G]}{K_{P_2G}} + \frac{[P_2G]\,[Mg]}{K_{MgP_2G}\,K_{Mg}}}. \qquad (9)$$

The dissociation constants (Table 5) correspond well enough with those in Scheme III. The constants for 2,3-bisphosphoglycerate inhibition amount to $K_{P_2G} = 2.70$ mM, $K_{Mg,P_2G} = 3.44$ mM. It follows that magnesium ions not only weaken the binding of MgATP to the enzyme molecule but also the binding of 2,3-bisphosphoglycerate. Additionally it was checked whether or not there are differences in the inhibitory action of MgP_2G and uncomplexed 2,3-bisphosphoglycerate. Such a model containing two further parameters gives no better fit than Scheme IV.

DISCUSSION

Detailed initial velocity measurements and inhibitor studies on the kinetics of various animal hexokinases confirmed the proposition [19] that these enzymes operate by a rapid-equilibrium random mechanism [13,20,21]. The ping-pong mechanism proposed for brain hexokinase [14] was meanwhile shown to be incorrect [13]. Toews [22] suggested for the rat muscle enzyme an ordered sequential mechanism where the addition of substrates and release of products is in the order: MgATP, glucose, glucose 6-phosphate and ADP. However, the competitive inhibition of this enzyme by mannose in relation to glucose, and the noncompetitive inhibition to MgATP [23] are inconsistent with an ordered mechanism. A detailed kinetic model for the hexokinase of the red blood cell has not been given so far. The conclusion drawn in this communication that all substrates must be bound on the enzyme before product formation can occur is in harmony with the mechanism of hexokinases of other animal tissues.

The Michaelis constant for glucose is known to lie at about 0.1 mM for the human erythrocyte hexokinase [24—26]. By a pH-stat assay we found previously in stroma-free haemolysates of rabbit erythrocytes and reticulocytes a K_m for 2-deoxyglucose of 0.18 mM [25]. Since the affinity to this glucose analogue is about one-fourth than to glucose [27] there appears to be a good accord between the value of the purified enzyme presented here ($K_{Glc} = 0.038$ to 0.054 mM) and that for the fresh haemolysate.

There exist four isozymes of animal hexokinase (types I to IV) of which type I accounts for about 90% in erythrocytes. This type also predominates in brain [15]. Therefore, it is justifiable to compare the kinetic data estimated here with those quoted in the literature on hexokinase of red blood cells and brain.

There is general agreement that the MgATP complex is the true substrate in the reaction. Discrepancies exist with respect to the action of uncomplexed Mg^{2+} and ATP on the enzyme. The kinetic measurements in the literature were mostly carried out with a constant ratio of the total Mg^{2+} and total ATP concentrations or with varying total ATP concentrations at a constant total Mg^{2+} concentration, Both procedures are unsatisfactory because the ionic species are continously changing. It is preferable to calculate the total concentrations of Mg^{2+} and ATP necessary to achieve the desired concentrations of one species at various fixed concentrations of the other while taking into account that the concentration of the third species varies proportionally to the MgATP complex. Association constants which are valid for the particular conditions must be taken for the computations. The K_{ass} for the MgATP complex is 1.2×10^4 M^{-1} for pH 7.2; $I = 0.15$ at 37 °C [5], as can be derived from the data given by Phillips *et al.* [28]. If the conditions regarding pH, ionic strength and temperature are not to be changed the use of an overall-K_{ass} might be preferable, since all the individual species ($MgATP^{2-}$, $MgHATP^{1-}$, ATP^{4-}, $HATP^{3-}$, $KATP^{3-}$, $NaATP^{3-}$) vary proportionally to each other. In the experiments with 2,3-bisphosphoglycerate five ionic species (Mg^{2+}, ATP, P_2G, MgATP, MgP_2G) were taken into account. In each case three species can be varied by variation of the total concentrations of Mg^{2+}, ATP and 2,3-bisphosphoglycerate. The $MgATP^{2-}$ complex amounts to 99% of the total MgATP complex under our conditions.

The $K_{MgATP} = 1.0—2.1$ mM corresponds well with the K_m-values of MgATP given for human red cell haemolysate [25] and partly purified hexokinase preparations [26,29,30]. The K_m-value for MgATP remains unchanged by variation of the pH between 6.5 and 8.1 and is independent of temperature between 37 °C and 0 °C (Gerber, G. and Elsner, R.: unpublished observations). For the brain enzyme values for K_{MgATP} between 0.5 and 1.7 mM were reported [13,16,19,31].

The present data clearly show that free magnesium ions at cellular concentrations activate the hexokinase of human erythrocytes. The best fit was obtained with the assumption of an activatory site for Mg^{2+} separate from the MgATP site. A similar conclusion was proposed for the hexokinases of brain [32] and muscle [33] without detailed kinetic analysis. On the other hand Bachelard's interpretation [32] of sigmoidal MgATP-saturation curves in terms of a homotropic cooperative binding of MgATP to the brain enzyme does not appear to be cogent. Sigmoidicity of the curves with inflection points at about 0.1 mM MgATP were observed with an [Mg]/[ATP] ratio of 1:1, but with a 5:1 ratio hyperbolic curves were seen. The sigmoidal saturation curves can be transformed into hyperbolic curves by application of a K_{ass} of 1×10^4 M^{-1} for the MgATP complex (instead of 7×10^4 M^{-1}) as was reported by Purich *et al.* [34]. However, according to our data it is not possible in this way to account for the activating effect of free Mg^{2+}.

The inhibitory action of high Mg^{2+} concentrations already shown by other authors [18,32,35] is confirmed by our investigation. Our conclusion that uncomplexed ATP^{4-} does not inhibit the hexokinase is at variance with reports on the brain enzyme [13,18,36]. The reasons for this discrepancy are not evident. In any case the inhibitory effect, if it exists, would be of no biological account, since the concentration of uncomplexed ATP^{4-} is only 0.1 mM in the erythrocyte [2].

There are some inconsistencies with respect to the type of inhibition as well as the inhibitory constant for glucose 6-phosphate. An inhibition competitive with MgATP has been shown previously [13—16,26] as well as by us, while in other reports [29,37—39] the inhibition was found to be competitive at low concentrations of MgATP, but of a mixed or noncompetitive type at high concentrations of glucose 6-phosphate. All reports agree that the inhibition was non-competitive with respect to glucose.

The values for the inhibitory constant of glucose 6-phosphate in the literature range from 2.5 to 68 μM [26,29,30,40]. This may be due partly to variations in the free Mg^{2+} concentration with varying ATP and glucose 6-phosphate concentrations. Our former value determined with a pH-stat assay was 30 μM [30]. During the course of the present study we repeated the determination by means of a coupled optical assay with the enzyme preparation used for the other kinetic measurements and we found an inhibition constant of 40 μM. The total Mg^{2+} concentration was kept constant at 12 mM and ATP and glucose 6-phosphate concentrations were varied. On the other hand a higher value of $K_{GlcP} = 69$ μM was found if the free Mg^{2+} was maintained at 5 mM which is in the lower range of the former determinations. The range of 12 to 66 μM for the K_i of glucose 6-phosphate determined on brain hexokinase preparations [14—16] corresponds to the values reported for the erythrocyte enzyme.

Rose *et al.* [29] and Graubner *et al.* [41] correlated the rate of glucose consumption by intact erythrocytes with the glucose 6-phosphate concentration at various experimental conditions. Half-maximal rates of glucose consumption were observed at 35 and 39 μmol glucose 6-phosphate per l cells respectively. It would correspond to about 50 to 60 μmol glucose 6-phosphate per l cell water which is close to the inhibition constant of the purified enzyme.

The inhibitory effect of glucose 1,6-bisphosphate was described once [37] on a crude hexokinase preparation from brain and was found to be identical to that of glucose 6-phosphate. The inhibitory constant estimated lies in the range of the intracellular glucose 1,6-bisphosphate concentration in erythrocytes and other cells [42].

The dependence of red cell hexokinase on 2,3-bisphosphoglycerate has been the subject of controversy. This effect was shown by Dische in 1941 on a crude haemolysate of human erythrocytes . De Verdier *et al.* [26] could not confirm such an effect in an incubation mixture containing 20 mM Mg^{2+}, 1.6 mM ATP and 16 mM 2,3-bisphosphoglycerate. Dische's finding was explained by a decrease of the MgATP concentration owing to formation of MgP_2G. Recently several workers [43—45] found that 2,3-bisphosphoglycerate is capable of inhibiting hexokinase and that the inhibition is relieved by increasing concentrations of ATP and Mg^{2+}.

From the competitive interaction of MgATP and 2,3-bisphosphoglycerate one may suppose that both species are bound to the same site on the hexokinase molecule. The distance between the most remote negative charges within the ATP molecule amounts to 0.97 nm and is identical with that in 2,3-bisphosphoglycerate [46]. In favour of this suggestion are the nearly identical kinetic constants for MgATP and 2,3-bisphosphoglycerate.

Application of the Kinetic Data to the Action of Hexokinase in the Cell

The mathematical model of the erythrocyte glycolysis, recently presented [1], allows the calculation of the glycolytic flux and the steady-state concentrations of the metabolites for various experimental conditions. It turned out that the kinetic parameters of the hexokinase are very important from the regulatory point of view. The glycolytic flux was shown to be controlled only by the hexokinase-phosphofructokinase system. This is mathematically expressed by the control strengths [47] of the glycolytic enzymes which at pH 7.2 is about 0.7 for the hexokinase, 0.3 for the phosphofructokinase and zero for all other enzymes. For all conditions the control strength of the hexokinase exceeds that of the phosphofructokinase. Both enzymes influence the concentrations of all metabolites of glycolysis.

The hexokinase of human erythrocytes is particularly well suited for calculations of the flux *in vivo* for the following reasons.

a) Owing to the high permeability of the cell membrane to glucose the enzyme operates under conditions of saturation with respect to this substrate.

b) Hexokinase has the lowest capacity among the glycolytic enzymes (12 μmol/ml $cells^{-1} \times h^{-1}$), and operates at a higher fraction of its maximal activity than all other enzymes of the glycolytic chain.

c) There occurs practically only one isoenzyme [48].

d) The reaction catalyzed is quasi-irreversible and any other glucose 6-phosphate splitting process was excluded [29]. Thus the glycolytic rate does represent the intracellular hexokinase activity.

e) The enzyme exhibits Michaelis-Menten kinetics. The estimation of the kinetic parameters and calculations are simpler than with allosteric enzymes.

f) The kinetic constants for the particular reactants lie near the range of their intracellular levels.

g) The free concentrations of the metabolites not bound to haemoglobin are available [2].

h) The enzyme molecules are apparently homogeneously distributed within the cell. The small percentage of activity reported to be centrifuged with the stroma [49] may well be due to absorption.

i) The prepared enzyme solution is stable enough in activity to carry out a sufficient number of measurements.

Summarizing the kinetic equations obtained for the various conditions we can write an equation taking into account simultaneously the action of both substrates and all effectors.

$$v = \frac{1}{N_1}\left[V_1\left(\frac{[\mathrm{MgATP}]\,[\mathrm{Glc}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Glc}}}\right) + V_2\left(\frac{[\mathrm{MgATP}]\,[\mathrm{Glc}]\,[\mathrm{Mg}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Glc}}\,K_{\mathrm{MgATP,Mg}}}\right)\right] \quad (10)$$

where

$$N_1 = 1 + \frac{[\mathrm{MgATP}]}{K_{\mathrm{MgATP}}} + \frac{[\mathrm{Glc}]}{K_{\mathrm{Glc}}} + \frac{[\mathrm{Mg}]}{K_{\mathrm{Mg}}} + \frac{[\mathrm{MgATP}]\,[\mathrm{Mg}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Mg}}} + \frac{[\mathrm{MgATP}]\,[\mathrm{Glc}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Glc}}} + \frac{[\mathrm{Glc}]\,[\mathrm{Mg}]}{K_{\mathrm{Glc}}\,K_{\mathrm{Mg}}} + \frac{[\mathrm{MgATP}]\,[\mathrm{Glc}]\,[\mathrm{Mg}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Glc}}\,K_{\mathrm{MgATP,Mg}}} + \left(\frac{[\mathrm{Glc}P]}{K_{\mathrm{Glc}P}} + \frac{[\mathrm{Glc}]\,[\mathrm{Glc}P]}{K_{\mathrm{Glc}}\,K_{\mathrm{Glc,Glc}P}} + \frac{[\mathrm{Glc}]\,[\mathrm{Glc}P_2]}{K_{\mathrm{Glc}}\,K_{\mathrm{Glc,Glc}P_2}} + \frac{[\mathrm{Glc}P_2]}{K_{\mathrm{Glc}P_2}}\right)\left(1 + \frac{[\mathrm{Mg}]}{K_{\mathrm{Mg}}}\right) + \left(1 + \frac{[\mathrm{Glc}]}{K_{\mathrm{Glc}}}\right)\left(\frac{[P_2\mathrm{G}]}{K_{P_2\mathrm{G}}} + \frac{[\mathrm{Mg}]\,[P_2\mathrm{G}]}{K_{\mathrm{Mg}}\,K_{\mathrm{Mg},P_2\mathrm{G}}}\right). \quad (11)$$

The inhibitory effect of Mg^{2+} was neglected since Eqn (10) applies to intracellular conditions. No experiments were made to study the effect of Mg^{2+} at varying concentrations of glucose, glucose 6-phosphate and glucose 1,6-bisphosphate. Therefore, it was assumed in Eqn (10) that the dissociation constants of Mg^{2+} do not depend on the concentrations of these species. Similarly the possible influence of glucose on the binding parameters of 2,3-bisphosphoglycerate was not taken into consideration. Owing to the saturation of the enzyme with glucose for conditions *in vivo* the kinetic Eqn (10) is simplified to

$$v = \frac{1}{N}\left[V_1\left(\frac{[\mathrm{MgATP}]}{K_{\mathrm{MgATP}}}\right) + V_2\left(\frac{[\mathrm{MgATP}]\,[\mathrm{Mg}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Mg}}}\right)\right] \quad (12)$$

$$N = 1 + \frac{[\mathrm{MgATP}]}{K_{\mathrm{MgATP}}} + \frac{[\mathrm{Mg}]}{K_{\mathrm{Mg}}} + \frac{[\mathrm{MgATP}]\,[\mathrm{Mg}]}{K_{\mathrm{MgATP}}\,K_{\mathrm{MgATP,Mg}}} + \left(\frac{[\mathrm{Glc}P]}{K_{\mathrm{Glc,Glc}P}} + \frac{[\mathrm{Glc}P_2]}{K_{\mathrm{Glc,Glc}P_2}}\right)\left(1 + \frac{[\mathrm{Mg}]}{K_{\mathrm{Mg}}}\right) + \frac{[P_2\mathrm{G}]}{K_{P_2\mathrm{G}}} + \frac{[\mathrm{Mg}]\,[P_2\mathrm{G}]}{K_{\mathrm{Mg}}\,K_{\mathrm{Mg},P_2\mathrm{G}}}. \quad (13)$$

The estimated values of the kinetic parameters differ slightly for the various conditions investigated (compare Tables 2 to 5). Therefore, the glycolytic flux was calculated by the use of mean values of the parameters summarized in the legend of the Table 6. In this table are summed up the results of the calculations of the actual hexokinase activity in dependence on pH, change of the oxygenation state of haemoglobin and on temperature.

Effect of pH. By changing the pH from 7.2 to 6.8 the actual hexokinase activity will be decreased for three reasons. Firstly, the capacity of the enzyme diminishes by 10%. Secondly, the distribution of the metabolites is changed owing to stronger binding of the phosphocompounds to haemoglobin. Thirdly, the glucose 6-phosphate concentration is markedly elevated because of the inhibitory influence of hydrogen ions on the phosphofructokinase [54]. There remains a considerable discrepancy (by a factor of 2) between the calculated hexokinase activity and the glycolytic flux at pH 8. Apparently the kinetic constants estimated at pH 7.2 are not valid at the more alkaline pH value. The inhibition by glucose 6-phosphate and the K_m-value of MgATP at saturating Mg^{2+} concentrations were observed to be identical at both pH values (Gerber, G. and Elsner, R.: unpublished observations).

Effect of Deoxygenation. Asakura *et al.* [55] demonstrated an elevated lactate formation rate in human erythrocyte suspensions following deoxygenation of haemoglobin. The authors assumed an activation of the phosphofructokinase by the intracellular alkalinization. The intracellular pH-shift of 0.07 units by deoxygenation [56, 57] would contribute only a minor part to the change in the glycolytic rate. The main contribution obviously comes from the profound changes in the free concentrations of metabolites: upon deoxygenation the activator Mg^{2+} is enhanced nearly two-fold and the inhibitor 2,3-bisphosphoglycerate is decreased to one-third whereas MgATP remains constant. Consequently the hexokinase activity increases substantially. The pH-shift alone is of no significance for the hexokinase. The phosphofructokinase is also inhibited by 2,3-bisphosphoglycerate [54]. Therefore the changes in metabolites and in pH may increase the actual phosphofructokinase activity. The glucose 6-phosphate concentration is then the result of the concerted action of these factors on the hexokinase-phosphofructokinase system. In line with this notion is the observation of a higher glycolytic rate of erythrocytes of

Table 6. *Comparison of the calculated hexokinase activity and the experimentally observed glycolytic flux of human erythrocytes in various conditions*

The concentrations of the reactants not bound to haemoglobin are given as mmoles per liter cell water. They were calculated from data of the literature [42,50—52] as follows: For pH 7.2, 37 °C, ionic strength 0.15 and haemoglobin concentrations 1.5 mM association constants (K'_{ass}) for the interactions among Mg^{2+}, ATP, 2,3-bisphosphoglycerate and oxygenated as well as deoxygenated human haemoglobin were recently published from this laboratory [5]. With these association constants and total concentrations of 2.0 mM ATP, 3.5 mM Mg^{2+}, 7.2 mM 2,3-bisphosphoglycerate and 6.7 mM haemoglobin the concentrations of unbound Mg-ATP, Mg^{2+} and 2,3-bisphosphoglycerate were computed [2]. A value of $1.0\times10^4\ M^{-1}$ was estimated from Phillips work [28] for the MgATP complexation at pH 6.8, 37 °C and $I = 0.15$ and $1.4\times10^4\ M^{-1}$ at pH 8.0. The K'_{ass} of $4.9\times10^2\ M^{-1}$ was chosen for the MgP_2G complex at pH 6.8 and $K'_{ass} = 6.9\times10^2\ M^{-1}$ at pH 8.0. For the binding of ATP and 2,3-bisphosphoglycerate to haemoglobin at pH 6.8 and 37 °C three times greater association constants in comparison to those at pH 7.2 and 37 °C were used [5]. The K'_{ass} of the Mg^{2+} · phospho-compound complexes at 4 °C are $0.4\times K'_{ass}$ (37 °C) and of the phospho-compound · haemoglobin complexes $3.5\times K'_{ass}$ (37 °C). For computation of the metabolite distribution at pH 8 a total concentration of ATP = 1.0 mM was introduced and the assumption was made that haemoglobin does not bind phospho-compounds at this pH-values [53]. Concentrations of glucose 6-phosphate and glucose 1,6-bisphosphate were converted on the water basis assuming no binding to haemoglobin. V_1 was measured at 37 °C and 4 °C at the pH-values indicated and V_2 was obtained by dividing V_1 by the factor 2.1 (see also Table 4). The following kinetic constants of the hexokinase were chosen to calculate its actual intracellular activity: $K_{MgATP} = 1.44$ mM, $K_{Mg} = 1.03$ mM, $K_{MgATP,Mg} = 1.14$ mM, $K_{Glc,GlcP} = K_{Glc,GlcP_2} = 0.069$ mM, $K_{P_2G} = 2.7$ mM, and $K_{Mg,P_2G} = 3.44$ mM. These parameters were assumed to be independent of the pH and temperature indicated, except $K_{Glc,GlcP} = K_{Glc,GlcP} = 0.019$ mM (Gerber, G. and Elsner, R.: unpublished data) was selected for the calculations at 4 °C

Reactant	Concentration of reactant at					
	37 °C				4 °C	
	O_2			N_2	O_2	
	pH 6.8	7.2	8.0	7.2	6.8	7.2
	mmol/l					
MgATP	1.23	1.44	0.88	1.43	0.97	1.10
Mg^{2+}	0.92	0.67	0.51	1.10	1.60	1.31
Glc*P*	0.18	0.06	0.015	0.026	0.18	0.04
$GlcP_2$	0.16	0.16	0.08	0.16	0.16	0.16
P_2G	3.35	4.43	7.20	1.95	2.50	3.30
Reaction velocities						
	μmol/min					
V_1	10.8	12.0	14.3	12.0	2.1	2.3
V_2	5.1	5.7	6.7	5.7	1.0	1.1
$v_{calc.}$	0.79	1.15	1.0	1.55	0.05	0.09
$v_{obs.}$	0.8	1.1	2.3	1.5		0.08

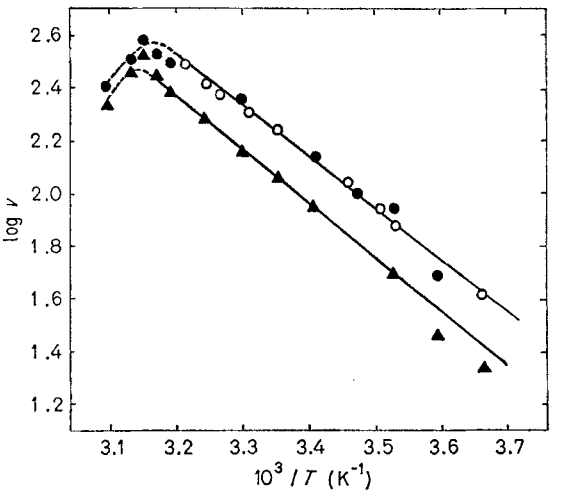


Fig. 9. *Temperature dependence of the hexokinase reaction (Arrhenius plot).* The measurements were performed with 1.3 mM ATP, 3.0 mM Mg^{2+} and 7.0 mM 2-deoxy-D-glucose in a final volume of 1.5 ml. Two methods were applied to determine the hexokinase activity: a pH-static assay at pH 8.1 (○——○), and the chemical determination of the 2-deoxy-D-glucose 6-phosphate formed (pH 8.1, ●——●, pH 7.2, ▲——▲) following separation from the non-phosphorylated sugar by $Ba(OH_2)$ and $ZnSO_4$. The details of the procedures were given in previous papers [25,60]

hypoxic persons with an increased glucose 6-phosphate concentration [58]. In that case the hexokinase activity was increased more than that of the phosphofructokinase. Phosphofructokinase seemed to be more activated than the hexokinase in Minakami's experiments [59] since the glucose 6-phosphate level was lower in deoxygenated cells.

Effect of Temperature. Decrease of temperature from 37 °C to 4 °C diminishes the hexokinase capacity by the factor 5.2, while the glycolytic rate declines by the factor 13.4. The Q_{10}-values was found to be 1.66 for the hexokinase (Fig. 9) and 2.1 for the overall glycolytic flux [61]. The lower activity of hexokinase at 4 °C is chiefly caused by the stronger inhibition by glucose 6-phosphate (K_{GlcP} at 4 °C = 0.019 mM). The lowered MgATP concentration at 4 °C is counteracted by an increase of Mg^{2+} and a decline of 2,3-bisphosphoglycerate.

From the present data one may draw conclusions with respect to the conditions within the intact erythrocyte. The basic glycolytic flux is adjusted to about one-tenth of the hexokinase capacity by the concentrations of MgATP, glucose 1,6-bisphosphate, 2,3-bisphosphoglycerate and Mg^{2+}. A fine control of the flux rate is exerted by the concentration of glucose 6-phosphate which is determined by the interplay of hexokinase and phosphofructokinase. Variations in the glycolytic flux may be produced by the action of effectors on the hexokinase, the phosphofructokinase or on both enzymes.

In line with our view of the importance of the hexokinase are observations on erythrocytes with hexokinase deficiency [62]. The reduction of the hexokinase capacity to about half of the normal value was accompanied by an equal decrease of the rate of glucose consumption.

All in all, the data on hexokinase *in vitro* represent a satisfactory basis for the assessment of the enzyme activity in the intact erythrocyte. An equally satisfactory correspondence between the calculated net flux and the experimentally measured glycolytic rate was obtained for the enolase of rabbit muscle [63] and the phosphoglucose isomerase of yeast [64]. The calculated activity of pyruvate kinase of yeast was found to be in agreement with the rate of ethanol formation at pH 6; at pH 7 there remained a difference by a factor of 2 [65]. The K_m-value of the hexokinase for 2-deoxyglucose was almost the same in intact Ehrlich ascites carcinoma cells and in the homogenate, whereas the maximal activity was two-fold higher in the cells [66]. In a study on yeast cells made permeable to metabolites by treatment with toluene identical values for maximal enzyme activity and K_m-values for ATP and glucose were found as for the isolated hexokinase [67]. In all these studies on muscle, ascites cells and yeast a number of assumptions with regard to the intracellular environment of the particular enzyme had to be made.

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