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KINETIC STUDIES ON THE INHIBITION OF GLYCOLYTIC KINASES OF HUMAN ERYTHROCYTES BY 2,3-DIPHOSPHOGLYCERIC ACID

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SUMMARY

1. In addition to its primary role as regulator of hemoglobin oxygenation, 2,3-diphosphoglyceric acid has been cited as an inhibitor of several enzymes in the erythrocyte. In this paper four enzymes of glycolysis [hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1.), phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11), phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3), and pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40)] were partially purified from the human red blood cell and the effect of 2,3-diphosphoglycerate upon their activities was measured.

2. It was demonstrated that in addition to hexokinase, about which there had been conflicting previous reports, the other three kinases are inhibited at normal intracellular levels of 2,3-diphosphoglycerate, but that a marked variation in sensitivity exists.

3. Kinetic studies of this inhibition reveal that generally 2,3-diphosphoglycerate is competitive with ATP, ADP, or the ATP–2 Mg^{2+} complex. A tabular summary of the K_i and K_m values for these enzymes is given.

4. It is concluded from these experiments together with studies on glucose utilization by cells containing varying amounts of 2,3-diphosphoglycerate that inhibition of hexokinase is probably the most significant step in regulation of glycolysis by 2,3-diphosphoglycerate.

5. The competitive effect between 2,3-diphosphoglycerate and ATP on these four kinases and its possible significance in mammalian erythrocytes is discussed briefly.

INTRODUCTION

2,3-Diphosphoglyceric acid was isolated from pig blood and identified by GREENWALD[1] in 1925, and the uniquely high concentration of this substance in the erythrocytes of most mammalian species was reported in 1941 by RAPOPORT AND GUEST[2]. In 1949 SUTHERLAND *et al.*[3] discovered that 2,3-diphosphoglycerate function-

Abbreviation: PEP, phosphoenolpyruvate.

ed as a cofactor for the enzyme phosphoglyceric acid mutase. Although this explained the occurrence of small amounts of 2,3-diphosphoglycerate in all tissues, the relatively enormous quantity of this compound in erythrocytes remained an enigma until the discovery of its significant effect upon the binding of oxygen by the hemoglobin molecule[1,5]. There remains no doubt that this is its major and unique function within the erythrocyte.

Others have tried to assign to 2,3-diphosphoglycerate a regulatory function over the activity of several different red cell enzymes. Included among these enzymes are hexokinase[6,7], transaldolase and transketolase[8], adenylate deaminase[9], and phosphoribosyl pyrophosphate synthetase[10]. The most convincing regulatory effect of 2,3-diphosphoglycerate upon an enzyme is its inhibition of the enzyme responsible for its synthesis, diphosphoglycerate mutase[11]. This inhibition has been suggested to be important in the regulation of intracellular 2,3-diphosphoglycerate concentration by governing its rate of synthesis.

In this paper we report the results of studies on the effects of 2,3-diphosphoglycerate upon the activities of the four glycolytic kinases which have been partially purified from human erythrocytes. All four enzymes, hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11), phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3), and pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), are inhibited by 2,3-diphosphoglycerate in the concentration normally prevailing within the red blood cell. Kinetic studies of these inhibitions are reported, and their possible significance is discussed.

EXPERIMENTAL

Materials

The following were obtained from Calbiochem: the sodium salts of ADP, ATP, $NADP^+$, NADH, NADPH, 3-phosphoglyceric acid, fructose 6-phosphate, phosphoenolpyruvate (PEP); the cyclohexylammonium salt of 2,3-diphosphoglycerate; and the purified enzymes glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aldolase, triose isomerase, α-glycerophosphate dehydrogenase, and lactate dehydrogenase. Dithioerythritol, glucose, DEAE-cellulose, coarse mesh, 0.84 mequiv/g; Tris, calcium phosphate gel and ethylene diaminetetraacetic acid were purchased from Sigma Chemical Company. Hydroxylapatite (Bio-Gel HTP) was a product of Bio-Rad Laboratories, and $(NH_4)_2SO_4$, enzyme grade, came from Nutritional Biochemicals Corporation. Mercaptoethanol was purchased from Eastman Organic Chemicals.

Protein was determined by the method of LOWRY *et al.*[12] using bovine serum albumin as standard.

Enzyme assays

The four enzymes were assayed by coupling with the appropriate enzyme systems and following the oxidation or reduction of pyridine nucleotides at 340 nm in a Cary 15 Recording Spectrophotometer at ambient temperature (23–24°). The compositions of the routine assay mixtures used to follow purification are given below.

The concentrations of reactants used in the kinetic studies are listed either in the legends to the figures or below.

Hexokinase was measured by coupling its reaction to that of glucose 6-phosphate dehydrogenase and measuring NADPH formation[13]. The reaction mixtures contained: Tris chloride buffer, pH 8.0, 50 mM; ATP, 1 mM; glucose, 1 mM; $NADP^+$, 1 mM; $MgCl_2$, 1 mM; and glucose 6-phosphate dehydrogenase, 0.1 mg.

Phosphoglycerate kinase was measured as by CHAPMAN *et al.*[13] by the reverse reaction coupling with glyceraldehyde 3-phosphate dehydrogenase. Reaction mixtures contained: Tris chloride buffer, pH 8.0, 50 mM; 3-phosphoglycerate, 1 mM; ATP, 1 mM; NADH, 0.3 mM; $MgCl_2$, 2 mM; dithioerythritol, 1 mM; and glyceraldehyde 3-phosphate dehydrogenase, 0.1 mg.

Pyruvate kinase was measured by the reduction of its product to lactate by lactate dehydrogenase[14]. Reaction mixtures contained: Tris chloride buffer, pH 8.0, 50 mM; PEP, 0.5 mM; NADH, 0.25 mM; $MgCl_2$, 2 mM; ADP, 1.5 mM; and lactate dehydrogenase, 0.1 mg.

Phosphofructokinase was measured by conversion of product to α-glycerophosphate with the NADH-dependent α-glycerophosphate dehydrogenase[15]. Reaction mixtures contained: Tris chloride buffer, pH 8.0, 50 mM; $MgCl_2$, 0.3 mM; dithioerythritol, 1 mM; NADH, 0.25 mM; fructose 6-phosphate, 0.4 mM; ATP, 1 mM; and aldolase, 0.1 mg; triose isomerase, 0.01 mg; and α-glycerophosphate dehydrogenase, 0.1 mg.

The effect of 5, 10 and 20 mM 2,3-diphosphoglycerate on phosphofructokinase was studied by varying ATP or ATP–2 Mg^{2+} from 0.015 to 0.15 mM, Mg^{2+} from 0.03 to 0.6 mM and fructose 6-phosphate from 0.02 to 0.4 mM. The concentrations of these reactants when constant were ATP, 0.09 mM; Mg^{2+}, 0.3 mM; and fructose 6-phosphate, 0.06 mM. Each reaction mixture contained 2.8 mg phosphofructokinase.

Enzyme purification

Phosphoglycerate kinase was partially purified by the method of HASHIMOTO AND YOSHIKAWA[16] employing ethanol to denature the hemoglobin, a calcium phosphate gel step, and finally column chromatography on DEAE-cellulose. The preparation used in these studies had a specific activity of 1.75 μmoles/min per mg protein.

Hexokinase was partially purified by removal of hemoglobin by batch adsorption and elution from DEAE-cellulose as described by CHAPMAN *et al.*[13], using 1 mM mercaptoethanol and 1 mM EDTA in all solutions. The hemoglobin-free material was then further purified by column chromatography on hydroxylapatite. The enzyme used in these studies had an activity of 0.08 μmoles/min per mg protein.

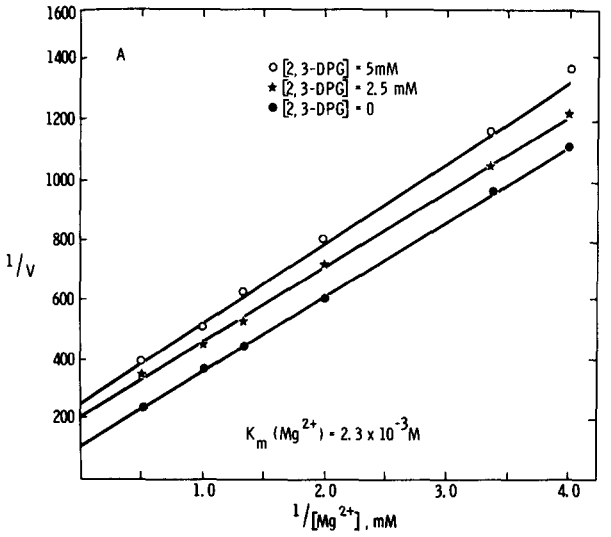
Phosphofructokinase and pyruvate kinase were freed of hemoglobin by the same method employed for hexokinase, and then the enzymes were precipitated with $(NH_4)_2SO_4$ (40% saturation) at pH 8.0 in the presence of 0.2 mM AMP and 0.1 mM dithioerythritol as by LAYZER *et al.*[17]. The pyruvate kinase had an activity of 0.1 μmole/min per mg protein. Phosphofructokinase was further purified by being heated at 65° for 5 min, which resulted in a preparation having an activity of 0.5 μmole/min per mg protein.

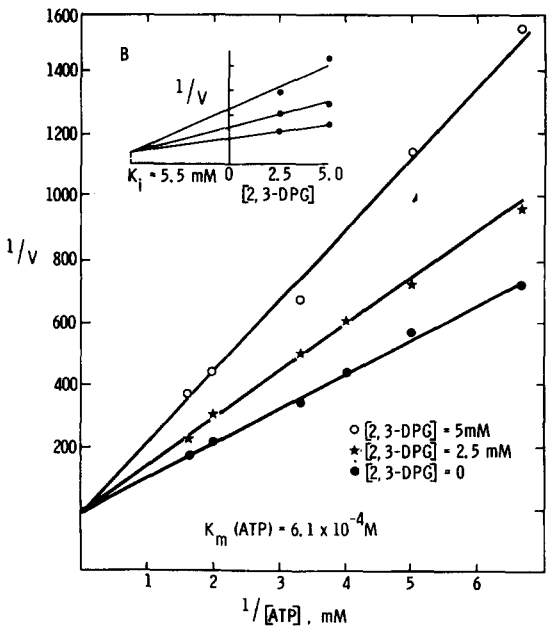
In preliminary studies it was demonstrated that 2,3-diphosphoglycerate had no significant inhibitory effect upon the activities of any of the coupling enzymes used. It was further demonstrated that the Tris salt, the sodium salt, and the cyclohexyl-

ammonium salt of 2,3-diphosphoglycerate produced the same inhibitory effects, and that therefore none of the effects reported is due to inhibition by the cyclohexylammonium ion.

RESULTS

Our studies with partially purified hexokinase from human red blood cells confirm the reports of others[6,7] that this enzyme is inhibited by 2,3-diphospho-





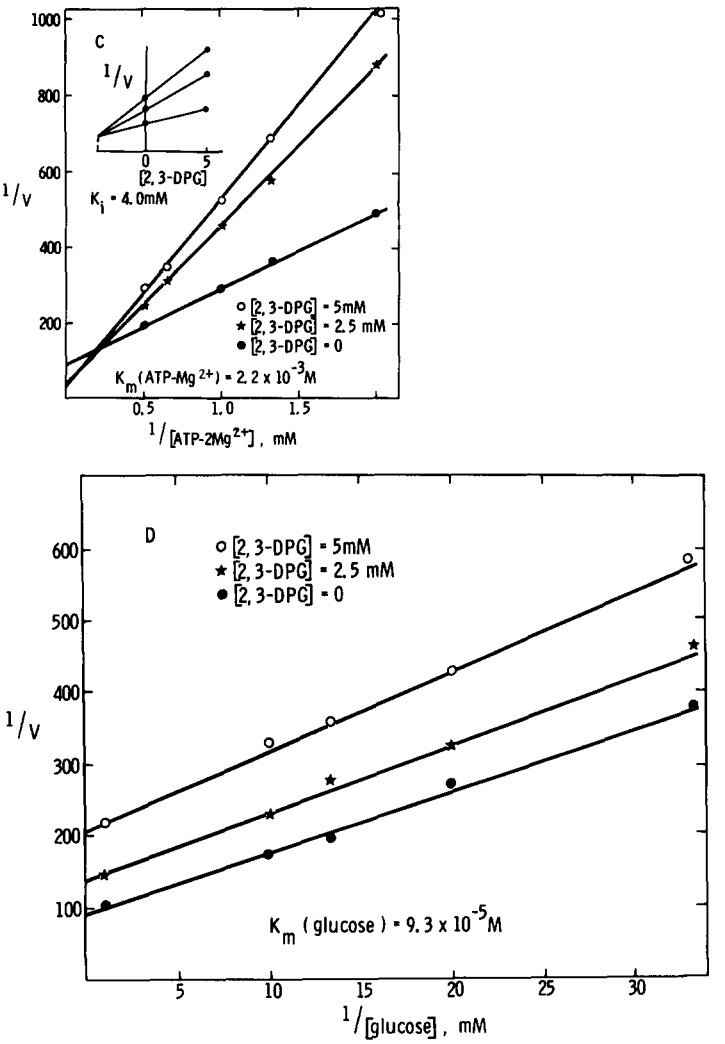


Fig. 1. Kinetic studies of the inhibitions of red cell hexokinase by 2,3-diphosphoglycerate (2,3-DPG). Graphed are double reciprocal plots of the effects of 0 (●), 2.5 (★) and 5.0 (○) mM 2,3-diphosphoglycerate upon the activity of hexokinase while varying ATP, Mg^{2+}, glucose, or ATP–2 Mg^{2+} concentrations. Reaction mixtures in addition to 40 μg hexokinase contained: (A) Mg^{2+}, 0.25 to 2.0 mM; glucose, 1 mM; ATP, 1 mM. (B) ATP, 0.15 to 0.75 mM; glucose, 1 mM; Mg^{2+}, 3 mM. (C) ATP–2 Mg^{2+}, 0.3 to 2.0 mM; Mg^{2+}, 3 mM; glucose, 1 mM. (D) Glucose, 0.3 to 1 mM; ATP, 1 mM; Mg^{2+}, 3 mM.

glycerate. The kinetics of this inhibition are depicted in the double reciprocal plots[18] in Fig. 1. The inhibition by 2,3-diphosphoglycerate is competitive with ATP and uncompetitive with respect to Mg^{2+} and glucose. When the concentration of a 1:2 mixture of ATP and Mg^{2+} (ATP–2 Mg^{2+}) was varied, the lines intersected to the right of the ordinate. Although it appears that at the higher ATP concentrations 2,3-diphosphoglycerate might actually enhance the enzyme's activity, it was not possible to demonstrate this because of the inhibitory effect of ATP at those concentrations.

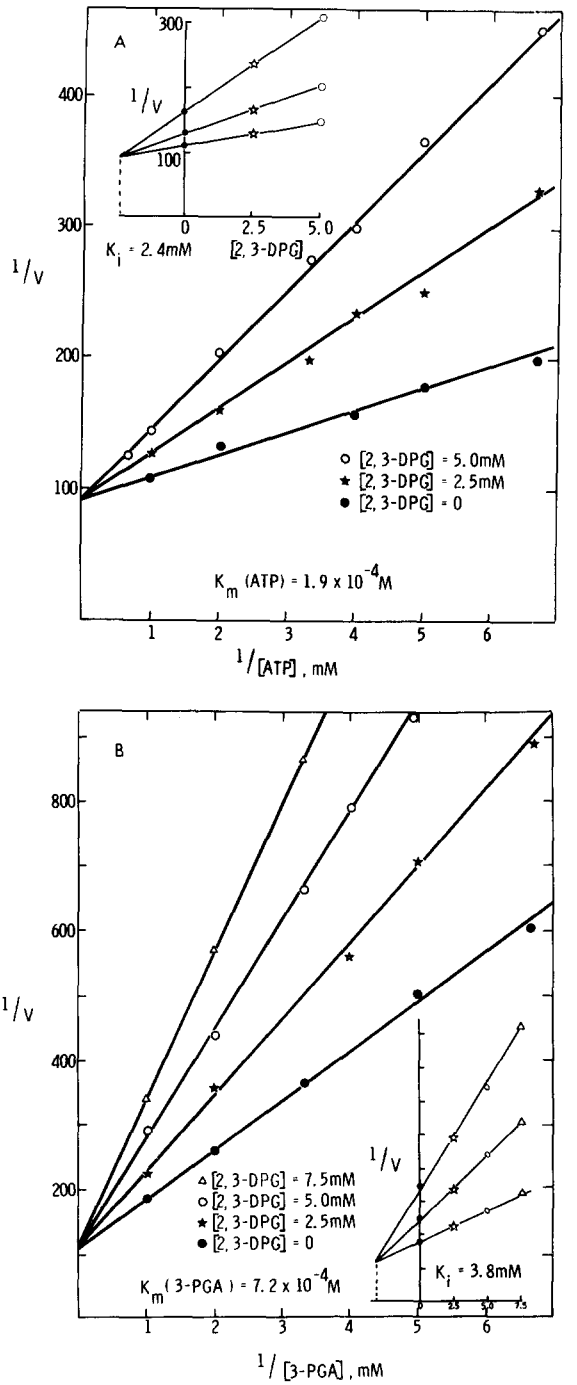


Fig. 2. Kinetic studies on the inhibition of red cell phosphoglycerate kinase by 2,3-diphosphoglycerate. Lineweaver–Burk plots of velocity against concentrations of Mg^{2+}, ATP, 3-phosphoglycerate (3-PGA) or ATP–2 Mg^{2+} in the presence and absence of 2,3-diphosphoglycerate (2,3-DPG). In addition to 1 μg phosphoglycerate kinase purified from human erythrocytes, reaction mixtures included: (A) ATP concentration was varied from 0.15 to 2.0 mM; Mg^{2+}, 1.0 mM; 3-phosphoglycerate, 1.0 mM. (B) 3-phosphoglycerate, 0.15 to 1.0 mM; ATP, 1.0 mM; Mg^{2+}, 1.0 mM. 2,3-Diphosphoglycerate concentrations were as follows: △, 7.5 mM; ○, 5 mM; ★, 2.5 mM; ●, 0 mM.

We consider this to represent competitive inhibition. With hexokinase the K_i for 2,3-diphosphoglycerate varied from 4 to 5.5 mM which is the amount present within normal erythrocytes.

Several other anions were tested for possible inhibitory effects upon hexokinase activity. When assayed in the presence of 0.75 mM ATP, 1 mM glucose, and 1 mM Mg^{2+} the concentrations of these anions which resulted in 50% inhibition of enzyme activity were: P_i, 30 mM; 3-phosphoglycerate, 17.5 mM; 2-phosphoglycerate, 12.5 mM; and ADP, 2.5 mM. ATP is also inhibitory and under these same assay conditions 50% inhibition was achieved at a concentration of 6 mM, whereas this degree of inhibition was produced by 7 mM 2,3-diphosphoglycerate. The inhibition by 2,3-diphosphoglycerate and 3-phosphoglycerate was additive.

Similar kinetic studies of the effect of 2,3-diphosphoglycerate upon purified yeast hexokinase revealed that it too was inhibited. With the yeast enzyme, 2,3-diphosphoglycerate was non-competitive with glucose, uncompetitive with Mg^{2+}, competitive with ATP–2 Mg^{2+} and mixed with respect to ATP. The degree of inhibition by 2,3-diphosphoglycerate was approximately the same for the hexokinase from both sources.

Phosphofructokinase from human erythrocytes is also inhibited by 2,3-diphosphoglycerate. The inhibition, which is not as marked as that of hexokinase, is non-competitive with fructose 6-phosphate and mixed with respect to Mg^{2+}, ATP and ATP–2 Mg^{2+}. Since competitive inhibition was not observed, the K_i for 2,3-diphosphoglycerate could only be estimated but is more than 4 times the normal intracellular level.

Inhibition of the red cell enzyme phosphoglycerate kinase by 2,3-diphosphoglycerate is considerably greater than that of the two above-mentioned enzymes. At normal intracellular concentrations of 2,3-diphosphoglycerate the enzyme is nearly 80 percent inhibited. The inhibition is competitive with respect to ATP, ATP–2 Mg^{2+}, and 3-phosphoglycerate (Fig. 2). Mixed inhibition is observed when the Mg^{2+} concentration alone is varied. The K_i's varied from 2.3 to 3.8 mM. Similar studies employing yeast phosphoglycerate kinase also revealed susceptibility to inhibition by 2,3-diphosphoglycerate. In this case, however, inhibition was found to be competitive with respect to Mg^{2+}, ATP, and ATP–2 Mg^{2+} and mixed with 3-phosphoglycerate.

The fourth red cell glycolytic kinase, pyruvate kinase, is nearly as sensitive to inhibition by 2,3-diphosphoglycerate as is phosphoglycerate kinase. Inhibition is competitive with respect to Mg^{2+} and ADP with a K_i of 1.25 and 2 mM respectively. Inhibition is non-competitive with PEP (Fig. 3). Purified red cell pyruvate kinase is also inhibited by ATP and the inhibitions by ATP and 2,3-diphosphoglycerate are additive. Increasing concentrations of 2,3-diphosphoglycerate resulted in the same percent inhibition with and without ATP. Pyruvate kinase from rabbit muscle is also markedly inhibited by 2,3-diphosphoglycerate. The inhibition is of the mixed type with respect to ADP and PEP and competitive with Mg^{2+}.

The activity of each of the four enzymes was measured with concentrations of reactants at normal intracellular levels in the presence of increasing concentrations of 2,3-diphosphoglycerate. These data are presented in Fig. 4. When represented in this way, the rather marked differences in sensitivity to inhibition are obvious. At 5 mM 2,3-diphosphoglycerate, the normal intracellular concentration, phosphofructokinase, hexokinase, pyruvate kinase, and phosphoglycerate kinase are inhibited 12,

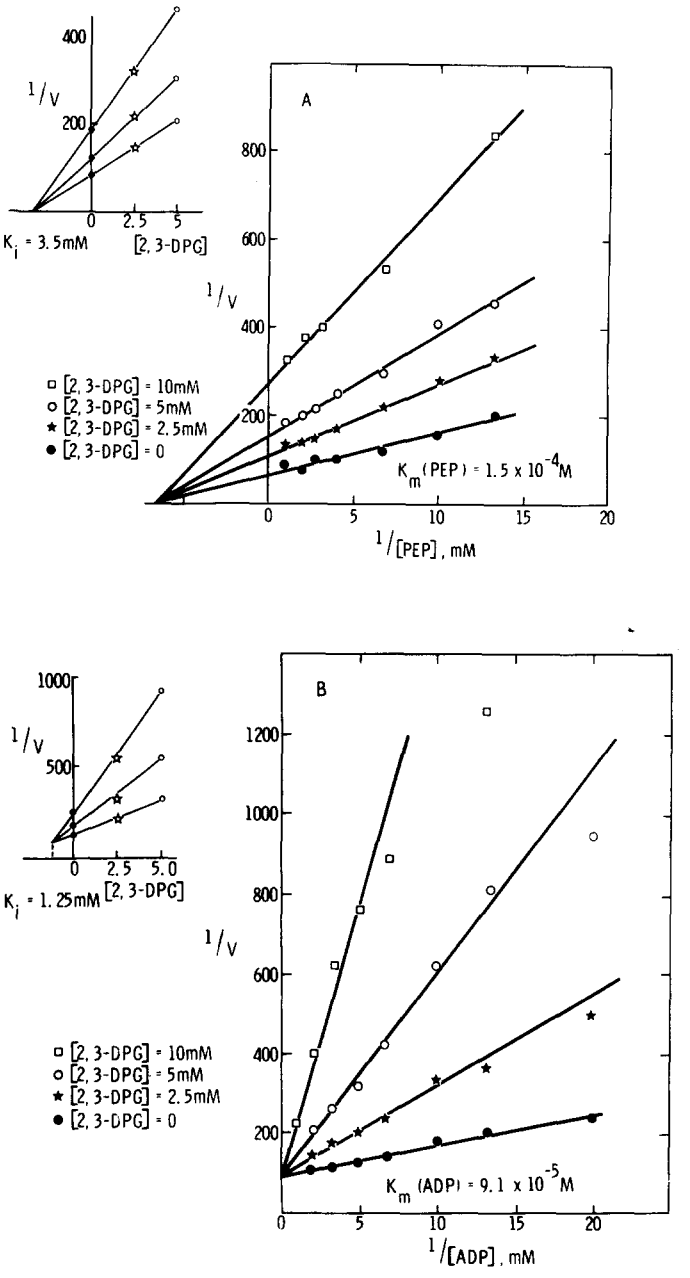


Fig. 3. The effects of 2,3-diphosphoglycerate (2,3-DPG) upon pyruvate kinase. Double reciprocal plots of the activity of pyruvate kinase from the human erythrocyte in the presence of 2.5 (★) 5.0 (○), and 10(□) mM 2,3-diphosphoglycerate and no 2,3-diphosphoglycerate (●) with varying PEP, and ADP concentrations. All incubations contained 50 μg enzyme. (A) Substrate PEP was varied from 0.1 to 1.0 mM; Mg^{2+}, 1 mM; ADP, 0.15 mM. (B) ADP, 0.05 to 1.0 mM; Mg^{2+}, 1 mM; PEP, 0.3 mM.

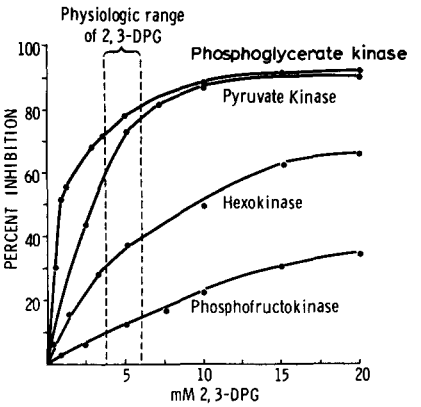


Fig. 4. The effect of 2,3-diphosphoglycerate concentration upon the activity of glycolytic cycle kinases of human erythrocytes. The percent inhibition of the activity of each enzyme is plotted against 2,3-diphosphoglycerate (2,3-DPG) concentration. The conditions for assay, chosen to approximate normal intracellular concentrations, were: (1) phosphoglycerate kinase: ATP, 1 mM; Mg^{2+}, 1 mM; 3-phosphoglycerate, 1 mM; (2) pyruvate kinase: Mg^{2+}, 1.5 mM; PEP, 0.3 mM; ADP, 0.2 mM; (3) hexokinase: Mg^{2+}, 2 mM; ATP, 1 mM; glucose, 1 mM; (4) phosphofructokinase: Mg^{2+}, 2,0 mM; fructose-6-phosphate, 0.05 mM; ATP, 1 mM.

TABLE I

SUMMARY OF KINETIC DATA ON RED CELL GLYCOLYTIC KINASES AND THEIR INHIBITION BY 2,3-DIPHOSPHOGLYCERATE

Enzyme	K_m *(M)**	K_i *(2,3-diphosphoglycerate) (mM)*	*Type*
Hexokinase			
ATP	$6.0 \cdot 10^{-4}$	5.5	Competitive
ATP–2 Mg^{2+}	$2.2 \cdot 10^{-3}$	4.0	Competitive
Glucose	$9.3 \cdot 10^{-5}$	—	Uncompetitive
Mg^{2+}	$2.3 \cdot 10^{-3}$	—	Uncompetitive
Phosphoglycerate kinase			
3-Phosphoglycerate	$7.2 \cdot 10^{-4}$	3.8	Competitive
ATP	$1.9 \cdot 10^{-4}$	2.4	Competitive
ATP–2 Mg^{2+}**	$4.4 \cdot 10^{-4}$	2.3	Competitive
Mg^{2+}***	$3.0 \cdot 10^{-4}$	—	Mixed
Phosphofructokinase			
Fructose 6-phosphate	$5.0 \cdot 10^{-4}$	—	Noncompetitive
ATP	$6.7 \cdot 10^{-6}$	—	Mixed
ATP–2 Mg^{2+}	$1.0 \cdot 10^{-5}$	—	Mixed
Mg^{2+}	$2.5 \cdot 10^{-5}$	—	Mixed
Pyruvate kinase			
PEP	$1.5 \cdot 10^{-4}$	—	Noncompetitive
ADP	$9.1 \cdot 10^{-5}$	1.25	Competitive
Mg^{2+}§	$1.0 \cdot 10^{-3}$	2.0	Competitive

* The specifics of the assay conditions may be found in the legends to Figs. 1–3.
** ATP–2 Mg^{2+}, 0.15 to 0.5 mM; 3-phosphoglycerate, 1.0 mM; ATP, 1.0 mM.
*** Mg^{2+}, 0.25 to 1.0 mM; 3-phosphoglycerate, 1.0 mM; ATP, 1.0 mM.
§ Mg^{2+}, 0.4 to 4.0 mM; ADP, 0.15 mM; PEP, 0.3 mM.

37, 73 and 78% respectively. The kinetic data obtained from the studies on these four enzymes from the human erythrocyte are summarized in Table I.

DISCUSSION

In this paper we have reported the results of experiments which show that not only hexokinase but also phosphofructokinase, pyruvate kinase, and phosphoglycerate kinase from human erythrocytes are inhibited by 2,3-diphosphoglycerate and that the degree of inhibition at normal intracellular levels appears to be significant. In 1941, DISCHE[6] reported that 2,3-diphosphoglycerate inhibited the phosphorylation of glucose by red cell hemolysates. He commented that inhibition of an early step in a sequence of reactions by a distant product might be a useful type of control for a metabolic pathway, a concept which only recently has been fully appreciated. GARBY AND DEVERDIER[19] failed to detect inhibition of hexokinase by 2,3-diphosphoglycerate, but BREWER[7] more recently confirmed Dische's findings and demonstrated that this inhibition in hemolysates was partially reversed by additional ATP or Mg^{2+}. Our studies, using partially purified enzyme, are in agreement with BREWER's results and show that the inhibition is competitive with ATP and ATP–2 Mg^{2+} complex.

To our knowledge the effects of 2,3-diphosphoglycerate upon the activity of the other glycolytic kinases have not been reported. In a recent study on a patient whose erythrocytes were deficient in diphosphoglycerate mutase and contained only one-third of the normal level of 2,3-diphosphoglycerate, LABIE *et al.*[20] found increased red cell fructose 1,6-diphosphate and triose phosphates. They concluded that at normal intracellular levels, 2,3-diphosphoglycerate exerts an inhibitory effect on phosphofructokinase. These investigators, however, failed to report the results of measurements on normal controls. We have found that the concentrations of fructose diphosphate and triose phosphates increase dramatically and linearly with time when cells are placed at 3°*. Spuriously high values are therefore obtained if the blood is first cooled; without parallel studies on normal cells, the significance of this report by Labie and coworkers is questioned. We have failed to find any relationship between the concentration of fructose diphosphate or the hexose monophosphates and 2,3-diphosphoglycerate in cells which have been artificially enriched with or depleted of 2,3-diphosphoglycerate (see below). A. KEITT (personal communication) has made similar observations.

We have performed a few preliminary experiments designed to at least partially clarify whether or not the inhibitory effects of 2,3-diphosphoglycerate upon these enzymes has any physiological significance for the intact erythrocyte. It is possible to artificially elevate intracellular 2,3-diphosphoglycerate by incubating cells with pyruvate, P_i, inosine, and glucose[21] and to deplete cells of 2,3-diphosphoglycerate by incubating with bisulfite[22]. We measured glucose consumption by cells which contained from 0 to 30 mM 2,3-diphosphoglycerate and found a reciprocal relationship between 2,3-diphosphoglycerate content and glucose utilization (D. R. HARKNESS AND S. ROTH, unpublished results). These findings are consistent with inhibition by 2,3-diphosphoglycerate at one or more steps in glycolysis.

The findings that the same four enzymes from other tissues are also inhibited by 2,3-diphosphoglycerate makes one hesitate in placing too much significance on the inhibition of the enzymes from the red blood cell. The red cell is, of course, somewhat

unique in its dependence upon glycolysis and might therefore have different regulatory mechanisms. We are left with the observation that red cells with high 2,3-diphosphoglycerate have low glycolytic rates and the demonstration that the four enzymes studied are inhibited to varying degrees by 2,3-diphosphoglycerate. Measurements of fructose diphosphate and hexose phosphates in cells containing variable amounts of 2,3-diphosphoglycerate do not indicate a significant block at phosphofructokinase. We have not yet measured the phosphorylated intermediates of the latter part of glycolysis. Since the other enzymes are present in considerable excess in the erythrocyte, at the moment we conclude that the effect of 2,3-diphosphoglycerate upon glycolysis most likely results from its effect upon hexokinase.

Under normal circumstances the total concentration of 2,3-diphosphoglycerate within the red blood cell has been thought to be fairly constant. Recently HAMASAKI *et al.*[25] have reported rather striking differences in 2,3-diphosphoglycerate content of venous and arterial blood but these studies have not yet been confirmed. Certainly the amount of unbound 2,3-diphosphoglycerate, that portion which is available to effect enzyme activity, does vary with the state of oxygenation of the hemoglobin. Furthermore, since the inhibitory effect of 2,3-diphosphoglycerate upon these enzymes is competitive with ATP, the concentration of the latter compound influences enzyme activity even without changes in 2,3-diphosphoglycerate. We do not know whether these fluctuations in 2,3-diphosphoglycerate or ATP are sufficient within normal erythrocytes to allow some control over glycolysis by the mechanisms described in this paper.

The competitive effect between 2,3-diphosphoglycerate and ATP on these enzymes is reminiscent of the competition of these compounds for the 2,3-diphosphoglycerate-binding site of hemoglobin. It is possible that, by virtue of their similar size and shape[24], competition between ATP and 2,3-diphosphoglycerate is a general phenomenon. The effects of this mutual interference might be significant only in mammalian erythrocytes where the high concentration of 2,3-diphosphoglycerate is so unique.

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