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ACTIVATION AND INHIBITION OF HUMAN ERYTHROCYTE PYRUVATE KINASE BY ORGANIC PHOSPHATES, AMINO ACIDS, DIPEPTIDES AND ANIONS

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SUMMARY

1. Human erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) has been proposed to conform to the two state conformational model for allosteric enzymes of Monod, Wyman and Changeux. We have examined the kinetic properties of the enzyme in this light and where appropriate made comparative studies on the non-allosteric isozyme from rabbit muscle.

2. In the pH range from 7 to 8 at low phosphoenolpyruvate concentration the erythrocyte enzyme activity has a strong pH dependence. This behaviour is of practical concern in the conditions used for blood banking.

3. A number of intracellular organic phosphates were tested. The most likely physiological activators of the erythrocyte enzyme are glucose 6-phosphate and fructose 1,6-diphosphate. Conflicting reports on the effect of 2,3-diphosphoglycerate on the enzyme appear due to differences in the magnesium concentration used in the assays.

4. L-Amino acids inhibit human erythrocyte pyruvic kinase in a pH and substrate-concentration dependent manner. Various alanine dipeptides activate the erythrocyte enzyme, others inhibit. Dipeptides give only inhibition with the muscle enzyme.

5. Anions inhibit both enzymes. Inhibition of the erythrocyte enzyme is pH and substrate-concentration dependent.

6. All of these observations are consistent with alterations in the R $\rightleftharpoons$ T equilibrium proposed for human erythrocyte pyruvate kinase.

INTRODUCTION

The mature human erythrocyte depends entirely on glycolysis for ATP production. The energy levels of the glycolytic intermediates in the human erythrocyte indi-

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Abbreviation: PEP, phosphoenolpyruvate.

cate three non-equilibrium steps which may control the rate 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) and pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40).[1] Analysis of changes in glycolytic intermediates following transition of red cells from the deoxygenated to oxygenated state by the cross-over method of Chance *et al.*[2] gives phosphofructokinase and pyruvate kinase as cross-over points[3]. Human erythrocyte pyruvate kinase has the properties necessary of a control enzyme since it is kinetically similar to the allosteric form of pyruvate kinase found in liver[4,5]. The enzyme has a sigmoidal velocity curve with respect to its substrate, phosphoenolpyruvate (PEP), and is positively modulated by Fru-1,6-P_2, the product of the phosphofructokinase reaction[5].

Control of ATP levels within the cell by regulation of pyruvate kinase activity will affect cell viability and indirect control of 2,3-diphosphoglycerate levels by the same enzyme will affect the oxygen-transporting capacity of the cell through the direct influence of 2,3-diphosphoglycerate on the oxygen-dissociation curve of hemoglobin[6]. In pyruvate kinase deficiency, the most common genetic defect found in human red cell enzymes[7], there is shortened red cell survival, increased 2,3-diphosphoglycerate and a hemoglobin oxygen-dissociation curve shifted to the right[8]. The properties of pyruvate kinase as a control enzyme are of potential importance in blood banking where the need to maintain 2,3-diphosphoglycerate levels as well as ATP production has recently become evident[9].

In this paper we have studied some of the factors influencing human erythrocyte pyruvate kinase activity and by comparative studies on the rabbit muscle enzyme, which is not under allosteric control, attempted to confirm the proposed basis for the allosteric properties of the erythrocyte enzyme[10].

METHODS

Chemicals

NADH, the disodium hydrate of Glc-6-*P*, the pentacyclohexylammonium salt of 2,3-diphosphoglycerate, the sodium salt of 3-phosphoglyceric acid, the dipotassium salt of Glc-1-*P*, the disodium salt of Fru-6-*P*, the tetracyclohexylammonium salt of Fru-1,6-P_2, the tetracyclohexylammonium salt of Glc-1,6-P_2, the disodium salt of Fru-1-*P*, D-alanine, L-alanine, L-isoleucine, L-phenylalanine and rabbit muscle lactate dehydrogenase, Type II, with an activity of 650 units/mg were obtained from Sigma Chemical Co., St. Louis, Mo. The crystalline tricyclohexylammonium salt of PEP, A grade, and the dihydrate of monosodium ADP, A grade, were obtained from Calbiochem, Los Angeles. L-Proline was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. The dipeptide L-Ala–L-Ala was obtained from Miles Laboratories, Kankakee, Ill. Gly–D-Ala, Gly–L-Ala, L-Ala–Gly, D-Ala–Gly, L-Ala–D-Ala, D-Ala–L-Ala and the D-Ala–D-Ala hemihydrate were obtained from Cyclo Chemical Co., Los Angeles D-Leucine and D-phenylalanine were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. L-Valine was obtained from Mann Research Laboratories Orangeburg, New York and isovaleric acid ,isobutylamine and 1,2-diaminopropane from the Aldrich Chemical Co., Milwaukie, Wisc. Glycine, 40% methylamine in water and 70% ethylamine in water were products of Matheson, Coleman and Bell, Norwood, Ohio. Propionic acid was obtained from Mallinckrodt Chemical Works, St

Louis, Mo. and Sephadex G-25 Fine from Pharmacia, Uppsala. All other reagents were the best quality commercially available. Double glass distilled water was used in making up all solutions.

The alanine dipeptides were checked for purity and freedom from contamination with alanine by chromatography on the long column of a Beckman 120 C amino acid analyzer using the normal first buffer (pH 3.25[11]) readjusted to pH 3.75 and single elution with this buffer without a buffer change[12]. Under these conditions alanine emerges at 48 min, D-Ala–L-Ala and L-Ala–D-Ala at 92 min and L-Ala–L-Ala and D-Ala–D-Ala at 109 min. By this criterion the dipeptides were pure and free of alanine.

Enzymes

Human erythrocyte pyruvate kinase was purified 215-fold from outdated blood by the method described by Jacobson and Black[13]. While the product was not homogeneous there was no evidence of contaminating activities contributing to the enzymatic assays. Ibsen *et al.*[14] have provided evidence for multiple polymeric forms of human erythrocyte pyruvate kinase. No attempt has been made to identify the form present in this preparation, however, the kinetic properties are those of the allosteric tetramer which was the only component consistently found in their preparations.

Rabbit muscle pyruvate kinase, A grade, with a specific activity of 340 I.U./mg at 30 °C was obtained from Calbiochem, Los Angeles as a crystalline suspension in 2.4 M $(NH_4)_2SO_4$.

Enzymatic assay

The pH-stat assay for pyruvate kinase[15] was used for the studies of the effect of pH on erythrocyte pyruvate kinase activity. The reaction was run at 37 °C in a final volume of 1.5 ml on a Radiometer pH-stat. The reaction mixture contained 2 mM ADP, 0.2 M KCl, 5 mM $MgCl_2$ and either 1 mM or 0.2 mM PEP. The reaction was started by addition of the enzyme and the uptake of 0.003 M HCl to maintain the desired pH was followed for 10 min.

In all other studies enzymatic activity was detected by the coupled assay with lactate dehydrogenase[16] following the decrease in absorption of NADH at 340 nm in a Gilford 2400 recording spectrophotometer. All assays were performed at room temperature in a final volume of 1 ml using 1-cm path-length microcuvettes (Precision Cells Inc., Hicksville, N.Y.). Unless otherwise noted the final concentrations in the cuvette were 0.15 mM NADH, 0.4 mM ADP, 1.5 mM PEP, 75 mM KCl, 8 mM $MgSO_4$, 6.5 units lactate dehydrogenase, 8 mM triethanolamine–HCl buffer (pH 7.4) and the appropriate amount of enzyme. The pH of all solutions, including any compound being tested for an effect on the enzyme, was adjusted to pH 7.4 before use. The PEP was added last to start the reaction after prior mixing of the other reagents. The change in absorbance during 10 min was measured. For studies on the effect of ion concentration on enzyme activity both the pyruvate kinase and the lactate dehydrogenase were desalted into distilled water on a 1.5 cm × 25 cm column of Sephadex G-25 Fine. In assays where inhibition of activity was observed, the possibility that the observed inhibition was of lactate dehydrogenase was eliminated by repeating the assay using half the quantity of lactate dehydrogenase. In the experiments on the activating effects of organic phosphates, control assays were performed with the organic phosphate being tested but eliminating PEP. In all cases there was no NADH

absorption change. Assays were run in triplicate and the results expressed here are the average value. After completion of the assay the contents of the three cuvettes were pooled and the pH checked on a Radiometer Model 26 pH meter.

RESULTS

pH

The effect of pH on the activity of human erythrocyte pyruvate kinase at high (1 mM) and low (0.2 mM) PEP concentration determined by the pH-stat assay gave the results shown in Fig. 1. At both PEP concentrations the pH optimum is 6.6. The relationship between the two curves differs from that of curves obtained with the rabbit muscle enzyme at 1.5 mM and 0.03 mM PEP shown in Fig. 2. The 0.03 mM

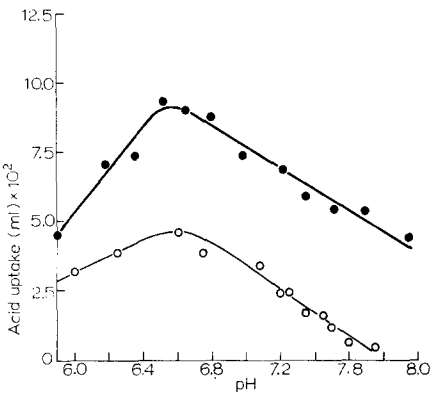


Fig. 1. Effect of pH on the activity of erythrocyte pyruvate kinase at two PEP concentrations. Enzymatic activity was determined by the pH-stat method[15] as described in Methods. ○—○, 0.2 mM PEP, ●—●, 1 mM PEP.

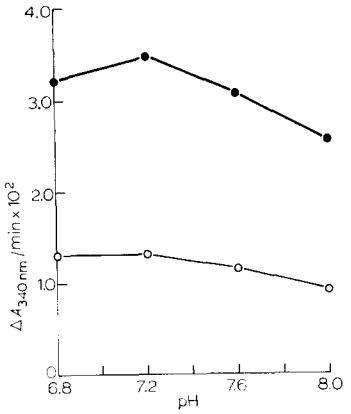


Fig. 2. Effect of pH on the activity of muscle pyruvate kinase at two PEP concentrations. Enzymatic activity was determined by the coupled assay with lactate dehydrogenase[16] as described in Methods. ○—○, 0.03 mM PEP, ●—●, 1.5 mM PEP.

PEP concentration was used to obtain approximately the same activity difference as with the erythrocyte enzyme and required the use of the spectrophotometric assay with triethanolamine–HCl buffers adjusted to the appropriate pH since the pH-stat assay became inaccurate at this PEP concentration. The pH optimum for the rabbit muscle enzyme is higher at pH 7.2, however, the main difference between the two enzymes is in the pH range between 7.0 and 7.6 at low PEP concentration where there is great variation of activity with pH for the human erythrocyte enzyme and relatively little variation for the rabbit muscle enzyme. From the data in Fig. 1, it would be expected that pH would affect the K_m for PEP of the erythrocyte enzyme. Under the conditions given for the pH-stat assay the values obtained were 0.24 mM at pH 6.6 and 0.6 mM at pH 7.5. Hill plots[17] of the experimental data gave n values of 1.13 at pH 6.6 and 1.42 at pH 7.5. These findings agree with the work of Staal[18] and Staal *et al.*[10]. These authors interpret the effect as due to a pH dependent conformational change in the enzyme between an active R form and an inactive T form according to

the model of Monod *et al.*[19]. The same mechanism was earlier postulated by Rozengurt *et al.*[20] to account for the effect of pH on the kinetics of rat liver pyruvate kinase, considered to be kinetically similar to the erythrocyte isozyme[4,5]. Since the concentration of PEP within the erythrocyte[21,22] is in the order of 10 μM, considerably lower than the low concentration studied here, it is likely that changes in erythrocyte intracellular pH will have a controlling effect on pyruvate kinase activity.

The relationship between pH and activity for erythrocyte pyruvate kinase is different from that for phosphofructokinase and hexokinase, the other postulated rate controlling enzymes of glycolysis in the human erythrocyte. At substrate concentrations approximating those found under physiological conditions, pyruvate kinase has maximal activity below pH 7 and little activity above pH 8 whereas the other two enzymes[23,24] have maximal activity above pH 8 and little activity at pH 7. If all three enzymes are controlling glycolysis it would be expected that the pH optimum for glycolysis would be around pH 7.5, however, from experiments with intact cells it is found that the pH optimum for lactate production[25] from glucose is in fact pH 8.1 which corresponds closely with the pH optimum of hexokinase and phosphofructokinase. It is evident that there must be modulating factors within the red cell which activate pyruvate kinase at higher pH values. The effect of Fru-1,6-P_2 on red cell pyruvic kinase has already been noted[5] as have some of the other phosphorylated intermediates of glucose metabolism[10]. It is not clear whether the concentrations required are within the probable physiological range.

Organic phosphates

The effect of various hexose and triose phosphates on the activity of red cell pyruvate kinase at pH 7.4 and 0.3 mM PEP is shown in Fig. 3. In agreement with the previous report[10] all of the compounds tested, including inorganic phosphate, have an activating effect on the enzyme. In the range of probable physiological concentra-

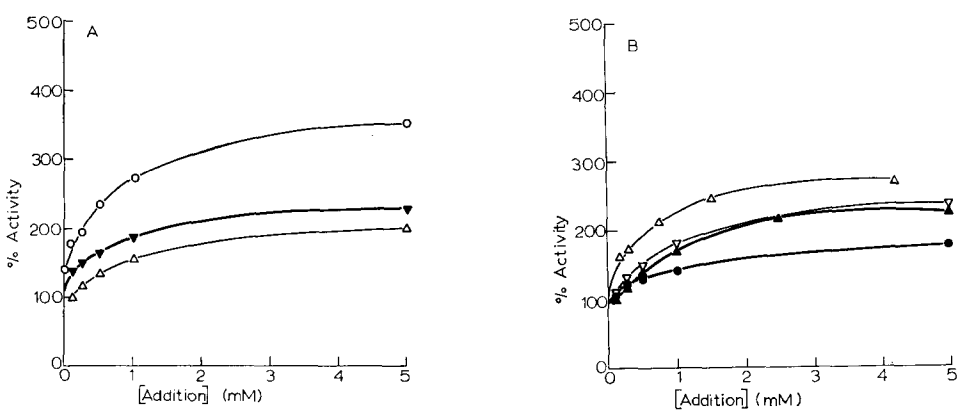


Fig. 3. Activation of human erythrocyte pyruvate kinase by organic phosphates. Enzymatic activity was measured at pH 7.4 by the coupled assay with lactate dehydrogenase[16] as described in Methods. The PEP concentration was 0.3 mM and the reaction velocity without any addition was taken as 100%. The various organic phosphates were added to the reaction mixture as volumes of a 50 mM stock solution, previously adjusted to pH 7.4. A. ○—○, Fru-1,6-P_2; ▲—▲, Glc-6-P; ▽—▽, 3-phosphoglyceric acid. B. △—△, PEP; ▽—▽, Fru-6-P; ▲—▲, 2,3-diphosphoglycerate; ●—●, inorganic phosphate.

tion[21,22] which varies from 5 μM for Fru-1,6-P_2 to 26 μM for Glc-6-P and is represented by the extreme left-hand side of the graphs, the order of effectiveness is Fru-1,6-P_2 > PEP > Glc-6-P $\gg$ Fru-6-P > 3-phosphoglyceric acid. Thus Fru-1,6-P_2, PEP and Glc-6-P are the most probable physiological modulators of red cell pyruvate kinase activity. Intracellular modulation will depend on the concentrations of these metabolites and their variation with changes in the cellular environment and is also likely to be of a different magnitude than reported here due to the lower intracellular concentration of PEP. Glc-6-P and Fru-1,6-P_2 are the products of the other two key glycolytic enzymes in the red cell, thus the metabolic network appears to be ideally suited to feed-forward activation of pyruvate kinase, the final controlling enzyme in the glycolytic pathway, and the apparently anomalous effects of pH on glycolysis can be explained on this basis. The effects observed with 2,3-diphosphoglycerate will be discussed later. The physiological concentrations of the other organic phosphates tested and inorganic phosphate in the human erythrocyte are such that their activating influence on pyruvate kinase will be slight or non-existent.

We investigated the features of the hexose phosphates which are necessary for activation of erythrocyte pyruvate kinase by examining the relative activation obtained at pH 7.4 and 0.3 mM PEP by addition of either 0.5 or 5 mM quantities of the three fructose phosphates and the three glucose phosphates using the coupled assay[16]. The results are shown in Table I. At the concentration of 0.5 mM all of the fructose

TABLE I

THE RELATIVE PERCENTAGE ACTIVATION OF ERYTHROCYTE PYRUVATE KINASE AT pH 7.4, 0.3 mM PEP OBTAINED ON ADDITION OF THE FRUCTOSE AND GLUCOSE PHOSPHATES LISTED

The coupled assay with lactate dehydrogenase was used[16] as described in Methods. The appropriate volume of a 50 mM stock solution of the hexose phosphate was added to the cuvette. Results are expressed as a percentage of the control value obtained in the absence of any hexose phosphate.

Addition	*Concentration of hexose phosphate in the cuvette:*	
	0.5 mM	*5 mM*
Fru-1,6-P_2	279	311
Fru-6-P	131	272
Fru-1-P	199	279
Glc-1,6-P_2	177	260
Glc-6-P	224	322
Glc-1-P	107	109

phosphates have an activating effect with Fru-1,6-P_2 being most effective while the phosphate on the 1-position has a greater effect than the phosphate on the 6-position. At the higher concentration there is less difference in the activating effect of the two fructose mono-phosphates. At both concentrations tested Glc-1,6-P_2 is less effective than Glc-6-P, and Glc-1-P has little, if any, activating effect. Previous reports indicated that for the red cell enzyme both Fru-1-P and Fru-6-P had equal but small activating effects[10] while for the rat liver enzyme Fru-6-P was more effective than Fru-1-P[26]. The results for Glc-6-P and Glc-1-P for both enzymes[10,26] were similar to those reported here. Glc-1,6-P_2 has not previously been tested.

Amino acids

There are a number of reports on the inhibitory effect of amino acids on the allosteric liver pyruvate kinase where L-alanine has been shown to inhibit at concentrations close to the estimated intracellular concentration[27–30]. Neither L-alanine nor L-proline has any inhibitory effect on the muscle enzyme[31] although they both inhibit the non-allosteric, muscle-like form of liver pyruvate kinase[32]. In Table II are listed

TABLE II

THE EFFECTS OF VARIOUS CONCENTRATIONS OF AMINO ACIDS AND RELATED COMPOUNDS ON THE ACTIVITY OF ERYTHROCYTE PYRUVATE KINASE AT pH 7.4, 0.3 mM PEP

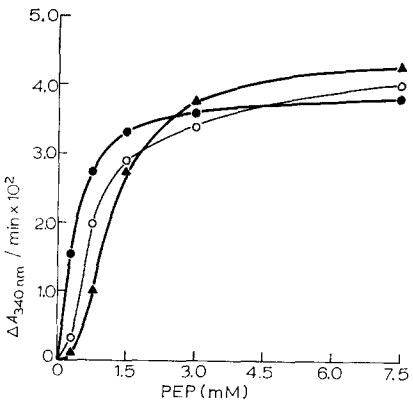
Conditions were as described for Table I except that 150 mM stock solutions were used.

Compound added	*Concentration in cuvette:*			
	7.5 mM	*15 mM*	*30 mM*	*75 mM*
Glycine	94	81	73	
L-Alanine	27	22	19	
L-Valine	16	6	3	
L-Proline	15	7	8	
L-Isoleucine	64	36	15	
L-Leucine	97	99	97	
L-Phenylalanine	21	10	5	
D-Phenylalanine			103	
Propionate	125	122	120	115
Methylamine		102	104	84
Ethylamine	95	82	71	38
1,2-Diaminopropane	73	54	30	

the results of studies on the effect of a number of L-amino acids on the activity of human erythrocyte pyruvate kinase at pH 7.4 and 0.3 mM PEP. There is clearly a relationship between the inhibition obtained and the size of the carbon side chain since L-valine and L-proline are both more effective than L-alanine which in turn is more effective than glycine. With larger side chains there is a decrease in inhibition, L-leucine being completely ineffective while isoleucine, which has certain structural similarities to L-valine, does inhibit at the higher concentrations. L-Phenylalanine, which also has a large side chain, is an effective inhibitor of the red cell enzyme. The inhibition is stereospecific since neither D-alanine nor D-phenylalanine give any inhibition. Of the amino acids listed only L-phenylalanine has been shown to have an inhibitory effect on muscle pyruvate kinase[31] which suggests that the mechanism of inhibition by this amino acid is different.

Table II also contains results obtained with compounds which are structurally related to the amino acids. Methylamine inhibits only at high concentrations while ethylamine with a larger carbon chain is a better inhibitor. 1,2-Diaminopropane with two ionic groups is a more potent inhibitor than ethylamine. The diaminopropane used was an unresolved mixture of the two stereoisomers and presumably better inhibition would be obtained with one of the isomers. Propionate gave a small but consistent activation of the red cell enzyme.

The effects of L-alanine and L-valine on erythrocyte pyruvate kinase as a function of PEP concentration at pH 7.4 are shown in Fig. 4. Both amino acids inhibit at the lower PEP concentrations and inhibition is overcome with increasing PEP con-



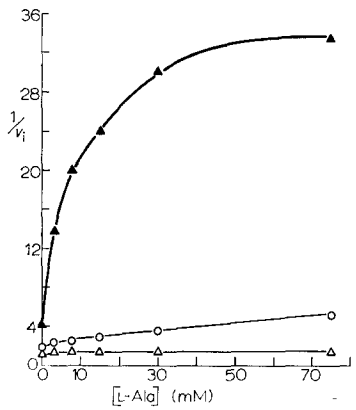


Fig. 4. The effects of L-alanine and L-valine on the activity of erythrocyte pyruvate kinase as a function of PEP concentration. The amino acid concentration was 15 mM and enzymatic activity was measured at pH 7.4 by the coupled assay with lactate dehydrogenase[16] as described in Methods. ●—●, no amino acid; ○—○, 15 mM L-alanine; ▲—▲, 15 mM L-valine.

Fig. 5. A Dixon plot[33] of the influence of pH and PEP concentration on inhibition of erythrocyte pyruvate kinase by L-alanine. Enzyme activity was determined using triethanolamine–HCl buffers of the appropriate pH by the coupled assay with lactate dehydrogenase[16] as described in Methods. ▲—▲, 0.3 mM PEP, pH 7.4; ○—○, 0.3 mM PEP, pH 6.8; △—△, 1.5 mM PEP, pH 7.4.

centration. The overall result is a shift of the activity curve to the right with L-valine, the more effective inhibitor, producing a larger shift than L-alanine and a corresponding increase in the sigmoidicity of the curve which is reflected in increased values for the Hill coefficient, n. The K_m values for PEP obtained under these experimental conditions are 0.45 mM with no addition, 0.75 mM in the presence of 15 mM L-alanine and 1.27 in the presence of 15 mM L-valine while the corresponding n values are 1.17, 1.79 and 2.4, respectively.

We have also studied the effect of various L-alanine concentrations at two different pH values and at two PEP concentrations on the activity of the erythrocyte enzyme. The results are shown as a Dixon plot[33] in Fig. 5. There is extensive inhibition at pH 7.4 with 0.3 mM PEP whereas at pH 6.8 with the same concentration of PEP the inhibition is minimal. At pH 7.4, the inhibition by L-alanine is negligible when the concentration of PEP is increased to 1.5 mM.

All of these findings are consistent with the idea that the amino acid either competes for the PEP binding site or occupies a separate allosteric site when the enzyme is in the T or inactive configuration. When either by increasing the PEP concentration or decreasing the pH, the enzyme is changed to the active R form the affinity of the enzyme for PEP is increased and the inhibitory effects of the L-amino acids are reduced.

We have expanded the amino acid studies to include a comparative study of the effects of various alanine dipeptides on the erythrocyte and muscle enzymes. The results obtained with 30 mM additions of the various dipeptides at 0.3 mM PEP and 0.1 M triethanolamine–HCl (pH 7.8), conditions chosen to emphasize the allosteric properties of the erythrocyte enzyme, are shown in Table III. The erythrocyte en-

TABLE III

THE EFFECT OF VARIOUS DIPEPTIDES ON THE ACTIVITY OF ERYTHROCYTE AND MUSCLE PYRUVATE KINASE

The enzymes were assayed in 0.1 M triethanolamine–HCl acid buffer (pH 7.8) and 0.3 mM PEP using the coupled assay with lactate dehydrogenase[16] as described in Methods. The dipeptide solutions were adjusted to pH 7.8 with 1 M NaOH prior to addition to the cuvette. The concentration of the dipeptides in the cuvette was 30 mM. The results are expressed as a percentage of the control containing no dipeptide.

Dipeptide	*Erythrocyte pyruvate kinase*	*Muscle pyruvate kinase*
L-Ala–L-Ala	84	95
L-Ala–D-Ala	20	75
L-Ala–Gly	10	65
D-Ala–L-Ala	198	84
D-Ala–D-Ala	166	84
D-Ala–Gly	176	62
Gly–L-Ala	107	102
Gly–D-Ala	5	11

zyme is both activated and inhibited whereas again only inhibition is observed with the muscle enzyme.

Anions

Rose and Warms[34] described a variation in K_m of human erythrocyte pyruvate kinase for PEP depending on the concentration of triethanolamine–HCl buffer used. They attributed the effect to the concentration of monovalent cation present. In an examination of the optimum potassium concentration for the red cell enzyme we observed that at low (0.3 mM) PEP concentration, pH 7.8, activity increased with KCl increase up to a maximum at 60 mM KCl and then decreased with further increases of KCl (Fig. 6). Inhibition over the KCl concentration range studied is considerably less either at low PEP concentration, pH 6.8, or high PEP concentration, pH 7.8, conditions which will favor the postulated R, active, configuration of the enzyme. In an attempt to determine whether the inhibition was due to the cation or the anion we added 60 mM KCl and then increased the salt concentration by addition of NaCl. As shown in Fig. 6B, the decrease in activity was the same as that obtained with further addition of KCl. Since sodium is not an activator of the enzyme this suggests that the inhibition is due to the anion, not the cation. This was confirmed by repeating the experiment with addition of $NaHCO_3$ instead of NaCl when a greater inhibition was obtained as shown. As in the case of inhibition by amino acids this suggests that anions are either competing for the PEP binding site or interacting with a separate allosteric site of the enzyme when it is in the inactive, T, configuration thus hindering the transition to the active, R, form.

An effect of ionic strength on the activity of rabbit muscle pyruvate kinase[35] has been reported and in Table IV we present a comparison of the effects of different monovalent anions on human erythrocyte pyruvate kinase and rabbit muscle pyruvate kinase at two different PEP concentrations in 0.1 M triethanolamine–HCl (pH 7.8). With the exception of acetate which, like propionate, activates the enzyme, the effect of anions on the human erythrocyte enzyme is a function of PEP concentration while

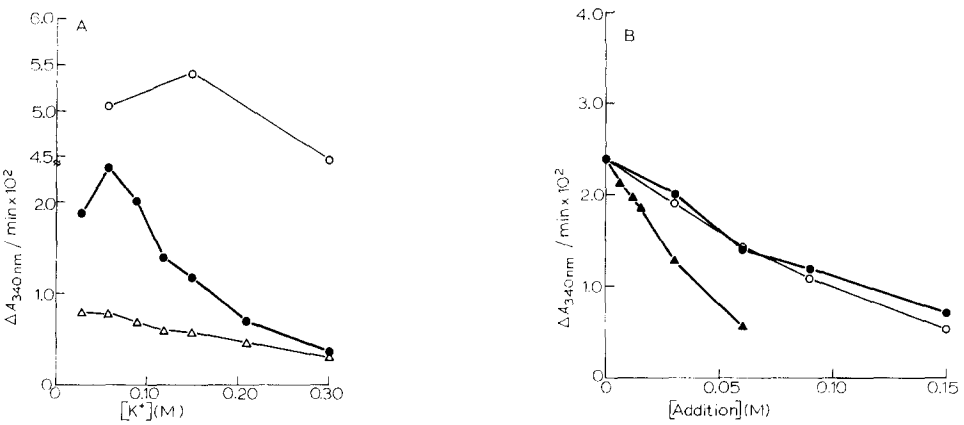


Fig. 6. A. The effect of pH and PEP concentration on the inhibition of erythrocyte pyruvate kinase by KCl. Enzyme activity was determined in 0.1 M triethanolamine–HCl buffers of the appropriate pH by the coupled assay with lactate dehydrogenase[16] as described in Methods with the exception that the KCl concentration was varied as shown. Less enzyme was used at pH 6.8 and no correction has been applied. ○—○, 1.5 mM PEP, pH 7.8; ●—●, 0.3 mM PEP, pH 7.8; △—△, 0.3 mM PEP, pH 6.8. B. The effect of cations and anions on the inhibition of erythrocyte pyruvate kinase. Enzyme activity was determined as in A at pH 7.8 and 0.3 mM PEP. KCl was initially present at 60 mM and gave the activity expressed as 0 addition. Inhibition was observed with the subsequent addition of the salts indicated. ●—●, KCl; ○—○, NaCl; ▲—▲, $NaHCO_3$.

TABLE IV

THE EFFECT OF ANIONS ON THE ACTIVITY OF ERYTHROCYTE AND MUSCLE PYRUVATE KINASE

The enzymes were assayed in 0.1 M triethanolamine–HCl acid (pH 7.8) at the PEP concentrations shown using the coupled assay with lactate dehydrogenase[16] as described in Methods. The anions were added as the sodium salts and the final concentration in the cuvette was 0.1 M, neglecting the anion concentrations in the assay mixture itself. The results are expressed as a percentage of the control value.

Anion added	*Erythrocyte pyruvate kinase*		*Muscle pyruvate kinase*	
	0.3 mM PEP	*1.5 mM PEP*	*0.3 mM PEP*	*1.5 mM PEP*
Chloride	77	109	54	54
Bromide	62	86	38	40
Nitrate	33	66	26	33
Acetate	147	135	55	68

all of the anions within experimental error give a PEP-independent inhibition of the rabbit muscle enzyme.

DISCUSSION

This *in vitro* study of activation and inhibition of human erythrocyte pyruvate kinase suggests that the *in vivo* regulation of the enzyme is likely to be complex and will depend on the cellular environment. It is generally agreed that the central point of glycolytic control in the human erythrocyte is at the phosphofructokinase step. This neglects the role of later enzymes in the pathway in regulating the levels of inter-

mediate metabolites of functional significance, in particular of 2,3-diphosphoglycerate. The effects of pH on pyruvate kinase at low substrate levels offer an explanation of the "storage lesion"[36] observed when blood is stored in acid–citrate–dextrose. The intracellular pH in this solution is initially 7.0 and drops to 6.8 over a period of three weeks. Under these conditions, hexokinase and phosphofructokinase will be effectively inhibited while pyruvate kinase will be activated in relation to enzymatic function at pH 7.4. This activation will allow ATP production, necessary for cellular integrity even under storage conditions, to continue, however, at the expense of 2,3-diphosphoglycerate content which is the only glycolytic intermediate between Fru-1,6-P_2, the product of phosphofructokinase, and PEP, the substrate for pyruvate kinase, present in appreciable quantities[21,22]. Storage of blood in acid–citrate–dextrose allows the cell to survive under the storage conditions but compromises its viability following subsequent transfusion. Beneficial effects of increased pH of acid-citrate-dextrose on the physiological properties of stored human erythrocytes have been reported[37].

Under the experimental conditions used in this study all of the phosphate derivatives tested, including inorganic phosphate, had some activating effect on the human erythrocyte enzyme. As noted above the most probable activators in the cell are Glu-6-P and Fru-1,6-P_2 which would fit in well with the requirements for overall control of glycolysis. There are conflicting reports on the effect of 2,3-diphosphoglycerate on human erythrocyte pyruvate kinase. Staal *et al.*[10] found that 2,3-diphosphoglycerate activated pyruvate kinase at pH 7.2, 0.25 mM PEP, and 20 mM $MgSO_4$, Beutler[38] reported no effect at pH 8.0, 0.75 mM PEP and 10 mM $MgSO_4$ while Ponce *et al.*[39] at pH 8.0, 0.5 mM PEP and 2 mM $MgCl_2$ showed inhibition which was non-competitive with respect to PEP but competitive with respect to both Mg^{2+} and ADP. The results we present above at pH 7.4, 0.3 mM PEP and 8 mM $MgSO_4$ agree with the observations of Staal *et al.*[10]. The lack of effect reported by Beutler[38] may be due to the relatively high PEP concentration which would favour the postulated, R, form of the enzyme. The other divergent results can be explained on the basis of the magnesium levels used in the assay mixtures. When the magnesium level is low, 2,3-diphosphoglycerate inhibits pyruvate kinase, whereas in the presence of high magnesium concentrations 2,3-diphosphoglycerate activates. This raises the question of whether 2,3-diphosphoglycerate in the first case inhibits by direct interaction with the enzyme or by chelation of the magnesium necessary for enzymatic activity. The work of Bunn *et al.*[40] taken with the reported competitive nature of 2,3-diphosphoglycerate inhibition with respect to magnesium and ADP[39] suggests that the latter explanation is correct. Bunn *et al.*[40] calculate intracellular variation of free magnesium concentration from 0.57 mM in oxygenated to 1.9 mM in deoxygenated red blood cells due to the interaction of unliganded 2,3-diphosphoglycerate with deoxyhemoglobin and release of 2,3-diphosphoglycerate and its subsequent chelation with magnesium in the oxygenated state. This again emphasizes the importance of variation in magnesium levels in the control of red cell glycolysis as has been pointed out by Bunn *et al.*[40] and Brewer[41]. The experiments reported in this paper do not differentiate between 2,3-diphosphoglycerate and its magnesium complex as the activator of red cell pyruvate kinase and in the absence of this information it is not possible to decide if such activation is of physiological significance.

It should be noted that we have not attempted to analyse any of the inhibitions we observed of erythrocyte pyruvate kinase activity in terms of the nature of the in-

hibition or of inhibition constants. None of the available methods for handling experimental data are adequate for studies of the complex kinetics of allosteric enzymes. An illustration is provided by Fig. 5 for 0.3 mM PEP, pH 7.4. A simple enzyme will give a straight line in such a Dixon plot[33].

It is doubtful if the inhibitory effects of amino acids on the human erythrocyte enzyme are of physiological importance since the intracellular concentration, in the range 0.1 to 0.3 mM for most amino acids[42], is considerably lower than the effective concentration used here. The results do suggest, however, that there is a hydrophobic binding site of limited size on the enzyme. The structural similarity between the amino acids and PEP or pyruvate favours interaction of the amino acids with the PEP binding site. As noted above, experimental verification of the competitive inhibition which would result is not possible and interaction with a separate allosteric site must remain a possibility.

The effects of the hexose phosphates and the dipeptides are difficult to interpret on a stereochemical basis with the available knowledge of their structures. Physicochemical studies on the purified enzyme should provide information on the sites of interaction on the enzyme which is necessary before useful interpretation can be approached. From model building studies there are similarities in terms of ionic and hydrophobic groups within the compounds involved. Of the dipeptides tested those with N-terminal L-alanine inhibit the human erythrocyte enzyme while those with N-terminal D-alanine activate. The inhibitory effects on the rabbit muscle enzyme are more confused although the peptides with N-terminal L-alanine follow the same rank order of effect as with the erythrocyte enzyme. Gly–D-Ala is exceptional in being the most effective inhibitor of both the red cell and muscle enzymes.

Monovalent anions can have two different inhibitory effects on enzymes. There is first a general disruptive effect on macromolecular structure which is independent of substrate concentration[43] and for certain enzymes there is a specific interaction with the active site, usually where the substrate is anionic, and inhibition is inversely proportional to substrate concentration[44]. The effects of anions on rabbit muscle pyruvate kinase, in the substrate concentration range studied, appear to be due to the former mechanism. At first inspection the effects on human erythrocyte pyruvate kinase seem due to the latter mechanism, however, if in the postulated R $\rightleftharpoons$ T conformational pair, the R form has greater structural stability than the T form, the same results could be obtained. The influence of pH on inhibition by potassium chloride (Fig. 6) support a differential effect on the R $\rightleftharpoons$ T pair. Irrespective of the exact mechanism, the K_m value for PEP of the erythrocyte enzyme will depend on the type of anions and their concentration in the assay mixture. No explanation is offered for the activating effects of acetate and propionate on the erythrocyte enzyme. Variations in bicarbonate concentrations occur in the red cell *in vivo* with blood pH variations and during CO_2 transport and are possibly of physiological importance since the concentrations involved are in the range studied in Fig. 6.

In conclusion, the studies reported above of the influence of pH, PEP concentration, amino acids, dipeptides and anions on the kinetics of human erythrocyte pyruvate kinase are consistent with the two state R $\rightleftharpoons$ T model of Monod *et al.*[19] applied originally to the allosteric liver pyruvate kinase isozyme by Rozengurt *et al.*[20] and extended to human erythrocyte pyruvate kinase by Staal *et al.*[10].

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