STUDIES ON THE ACTIVATION OF PYRUVATE KINASE BY MONOVALENT CATIONS

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ABSTRACT

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by Rivers Singleton, Jr.

Since the initial establishment of a potassium requirement for pyruvate kinase by Boyer in 1943, little knowledge has been gained as to the action of monovalent cations on enzyme systems. It is the objective of this study to establish the nature of the potassium activation of pyruvate kinase. Experiments used to achieve this end included, attempts to replace potassium by other charged species, attempts to induce a configurational change which might mimic the potassium action, differential reactivity of specific groups, and ultraviolet difference spectra.

Attempts to replace potassium and to induce an active configurational change were unsuccessful. The rate of reaction of the enzyme with parahydroxy mercuribenzoate and 5,5'-dithiobis(2-nitrobenzoic acid), both sulfhydryl reagents, was the same regardless of the presence or absence of activator. Ultraviolet difference spectra, in the range of 250 to 350 mμ, show two peaks occurring at 285 and 295 mμ, at pH 7.5. These peaks which are linear with protein concentration may be due to alterations of the spectra of tryptophyl residues in the protein molecule.

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By

Rivers Singleton, Jr.

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Dedicated

To My Parents

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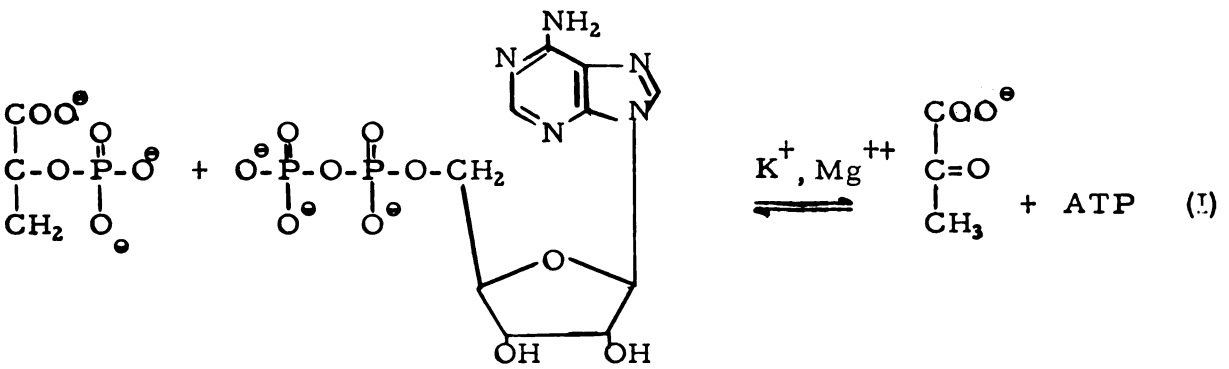
I. INTRODUCTION

A. Historical

Pyruvate kinase is a ubiquitous metabolic enzyme occurring in the Embden-Meyerhof-Parnas glycolytic scheme. The primary metabolic reaction catalyzed by the enzyme is the transfer of the phosphoryl group from phospho(enol) pyruvic acid (PEP)[1] to ADP with the formation of pyruvate and ATP, as shown in equation I.



(I)

The enzyme was first reported separately by Lohmann and Meyerhof (1) and Parnas and his co-workers (2, 3) in 1934. However, they did not recognize ADP as a substrate for the enzyme, and it was in 1935 that Mann (4) was able to demonstrate the products of the reaction were ATP and pyruvate.

In 1942, Boyer et al. (5) demonstrated the necessity of potassium in the transfer of phosphate from either 3-phosphoglycerate or PEP.

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[1]Abbreviations used in this paper are as specified in "IUPAC tentative rules. Abbreviations and symbols for chemical names of special interest in biological chemistry," J. Biol. Chem., 237, 1381 (1962). Abbreviations not specified in this source are indicated in the text by parenthesis.

Since they were working with a crude system, they could not distinguish which reaction was potassium dependent. The following year they established beyond a doubt that it was the pyruvate kinase reaction which required potassium in 0.1 M amounts for optimum activity (6). This was the first clear demonstration of a cofactor role for potassium in an enzyme system. Ten years later, Kachmar and Boyer (7) extended these observations by showing that ammonium and rubidium could replace potassium as activators for the enzyme with almost equal effectiveness (relative maximum velocity, 1:0.84:0.72). They found the K_A (association constant) for the three activating ions to be around 1.1×10^{-2} M, and the optimum concentration for ammonium and rubidium to be somewhat lower than that of potassium. Lithium and sodium counteract the potassium activation. However in the complete absence of potassium, sodium was found to give a weak but significant activation; lithium, on the other hand, resulted in little or no activation. Calcium was also found to be inhibitory, however the exact nature of this inhibition was not clear, as it could be overcome by increases in the concentration of potassium. In a qualitative sense, many of their findings have been found to hold true for other monovalent cation activated enzymes.

That the enzyme is ubiquitious in nature is demonstrated by its having been found in a variety of organisms ranging from the protozoa to humans (8, 9, 10, 11). In all of these preparations a definite requirement for potassium has been demonstrated. One also observes the activation by ammonium and rubidium, as well as the sodium/lithium antagonism. However, the optimum concentration of cation necessary for full activation, as well as the association constant for the activators varies from species to species. Thus, it appears that a common molecular configuration is active in all of these systems.

An interesting aspect of the pyruvate kinase molecule is its catalysis of two "side" reactions which apparently are unrelated to the usual metabolic reaction. The first of these reactions is the "fluorokinase" reaction, as demonstrated by Tietz and Ochoa (12) in 1958. The reaction involves the transfer of the phosphoryl group from ATP to fluoride as shown in equation II.

$$ATP + F^{\ominus} \xrightarrow[CO_2]{K^{+}, Mg^{++}} ADP + F\text{-}P(=O)(O^{\ominus})\text{-}OH \qquad \text{(II)}$$

Magnesium, potassium, and carbon dioxide are all demonstrated co-factors for the reaction. Data, such as:

a) Constancy of the ratio of pyruvate kinase to "fluorokinase" activity throughout purification and repeated crystallization;

b) Similar ratio of the two activities for preparation purified by different methods, either for pyruvate kinase or for "fluorokinase" activity;

c) Demonstration of a potassium requirement for both reactions;

d) Same nucleotide specificity;

e) Inhibition of "fluorokinase" activity by PEP or pyruvate;

lead to the conclusion that both reactions are catalyzed by the same protein molecule.

The second of these "side" reactions is the "hydroxylamine kinase" as demonstrated by Kupiecki and Coon (13) in 1960. The reaction involves the transfer of the phosphoryl group from ATP to hydroxylamine, as shown in equation III.

$$ATP + NH_2\text{-}OH \xrightarrow[CO_2]{Zn^{++}, K^{+}} ADP + NH_2\text{-}O\text{-}P(=O)(O^{\ominus})\text{-}OH \qquad \text{(III)}$$

Data similar to that given above for the "fluorokinase" reaction suggests that the protein molecule catalyzing the pyruvate kinase reaction is the same as that catalyzing the "hydroxylamine kinase" reaction. The one point of contrast in the data exists in the divalent metal requirement. Both pyruvate kinase and "fluorokinase" show a dependency on magnesium concentration. "Hydroxylamine kinase," however, is dependent on zinc (14) rather than magnesium. This proves to be of interest, since the pyruvate kinase isolated from erythrocytes has been shown to be inhibited by even trace amounts of zinc (10).

Although the above reactions appear to be entirely unrelated at first glance, they do have a common basis. They all involve the transfer of a phosphoryl group from ATP to a relatively active nucleophile. As has been proposed by Westheimer (15) and elaborated upon by Jencks (16), phosphate ester cleavage may involve the formation of a metaphosphate (pentavalent phosphorus) as an intermediate. If this mechanism is correct in the case of pyruvate kinase, then perhaps the "side" reactions are merely due to the nucleophilic attack of fluoride (or hydroxylamine) on the metaphosphate intermediate occurring on the enzyme surface.

Since the initial demonstration of a potassium requirement for pyruvate kinase by Boyer (6), many other enzymes have been shown to have a dependency on monovalent cation concentration. Several of these enzymes are shown in Table I, along with some of their properties. Although an absolute requirement has not been demonstrated in all cases, monovalent cations do cause stimulation of activity. It may well be that these enzymes in reality have an absolute requirement, but it has not been demonstrated due to residual cations in the assay procedure, as was the case for fructokinase (17). It is interesting that many of these enzymes exhibit a divalent metal requirement, usually magnesium, in addition to the monovalent cation requirement. Lowenstein (18) studied the activation of non-enzymatic transphosphorylation

Table I. List of Enzymes Requiring or Stimulated by Monovalent Cations

Enzyme	Source	Activators		Substrates	Na/Li Antagonism	Divalent Cation	Reference
		Primary	Secondary				
Pyruvate kinase	variable	K^+	NH_4^+, Rb^+	PEP, ADP	yes	Mg	5,6
Choline acetylase	rat brain	K^+	none	Acetate, Choline ATP	no	none	19
Aldehyde dehydrogenase	yeast	K^+	Rb^+, NH_4^+ Na^+, Cs^+	Acetaldehyde NAD	no	none	20
Fructokinase	liver	K^+	Rb^+, Na^+, NH_4^+	Fructose, ATP	no	Mg	17, 21
Phosphotransacetylase	C. kluyveri	K^+	NH_4^+	Acetylphosphate Co A	yes	data does not eliminate Mg	22
Glutathione synthetase	pigeon liver	K^+	none	L-glutamyl-L cysteine, glycine, ATP	no	Mg	23
Acetate activating enzyme	pig or rabbit heart	K^+	NH_4^+, Rb^+	Acetate, ATP, Co A	yes	none	24
Tryptophanase	E. coli	NH_4^+	K^+, Rb^+	Tryptophane	yes	none	25
Myosin ATPase	muscle	in presence of .005 M $CaCl_2$ all in presence of .001 M EDTA NH_4^+	none K^+, Rb^+	ATP	no	Mg	26, 27
Aldolase	yeast	K^+	none	Fructose-1,6-diphosphate	no	none	28

Continued

Table I - Continued

Enzyme	Source	Activators		Substrates	Na/Li Antagonism	Divalent Cation	Reference
		Primary	Secondary				
ATPase[1]	cardiac muscle	K^+, Na^+	none	ATP	no	Mg	29
5, 10-methylene-tetrahydrofolic dehydrogenase	yeast	K^+, Na^+	NH_4^+, Rb^+ Li^+	5, 10-methylene tetrahydrofolate, NADP	no	none	30
L-threonine dehydrogenase	S. aureus	K^+	Rb^+, Cs^+	L-threonine, NAD	yes	none	31

[1]Apparently different from the myosin enzyme.

by alkali metal ions and he found that their action is not simply a replacement or substitution for the divalent cation. The simplest hypothesis is that the monovalent cation neutralizes one negative charge on the ATP after it has been chelated with the divalent cation. However Lowenstein feels that for this to be true, lithium should exert a greater effect than potassium, which it does not. Lowenstein states that the relative specificity of potassium may be due to a charge-ionic relationship which is such as to make its chelate with ATP more active than the other monovalent cations. This could be true for pyruvate kinase, for as pointed out by Kachmar and Boyer (7) the activating ions have values of ionic radii, ionic mobility, and estimated size of hydrated ion which are approximately alike, and significantly distinct from the nonactivating ions. This is shown in Table II.

B. Molecular Properties and Mechanism of Pyruvate Kinase

Several methods exist for the isolation and purification of pyruvate kinase. The best of these is the preparation of Tietz and Ochoa (12). When assayed by means of the spectrophotometric procedure of Kubowitz and Ott (9) (equation IV) they obtained specific activities of 240-280 units per mg of protein. (Their definition of the unit of activity is that amount of enzyme catalyzing the formation of 1.0 μ mole of pyruvate per minute at pH 7.0 and 25° C.) Other schemes of purification have been given by Bucher and Pfleider (32), Kornberg and Pricer (33), Kupiecki and Coon (13), and Kachmar and Boyer (7).

$$\begin{array}{ccc} \text{PEP} + \text{ADP} & \rightleftharpoons & \text{Pyruvate} + \text{ATP} \\ & & \Big\downarrow \text{NADH} \quad \Big\uparrow \text{NAD} \\ & & \text{Lactate} \end{array} \qquad \text{(IV)}$$

Table II. Values of Certain Physical Properties of Alkali Metal Ions and Ammonium

Ion	Estimated radius of hydrated ion (Å) (34)	Relative number of water[1] molecules in hydrated ion (35)	Ionic radius from crystal data (Å) (36)	Relative ion mobility (37)	Heats of solvation (Kcal) (38)
Li^+	10.03	14.0	0.60	33.3	145
Na^+	7.90	8.4	0.95	43.4	119
K^+	5.32	5.4	1.33	64.6	94
NH_4^+	5.37	9.7	1.48	64.7	
Rb^+	5.09	4.8	1.48	67.5	85

[1]Data are corrected for a hydration value of the Cl ion of 4.0. Values for Rb and NH_4 are interpolated from a plot of atomic or molecular weight vs hydration number.

To date, the amino acid composition of the enzyme has not been determined. However, absorption data indicates a low content of the aromatic amino acids, tyrosine and tryptophane (39). At 280 mμ $A^{0.1\%}_{1\ cm}$ = 0.54 for the rabbit muscle preparation (40). Kubowitz and Ott (9) have reported an elemental analysis for the enzyme as isolated from human thigh muscle. Their data are given in Table III. This is the only data of the type currently in the literature.

Table III. Elemental Composition of Human Pyruvate Kinase (9)

Element	Percent
Carbon	53.35
Hydrogen	7.30
Nitrogen	17.40
Sulfur	1.60
Phosphorus	0.06

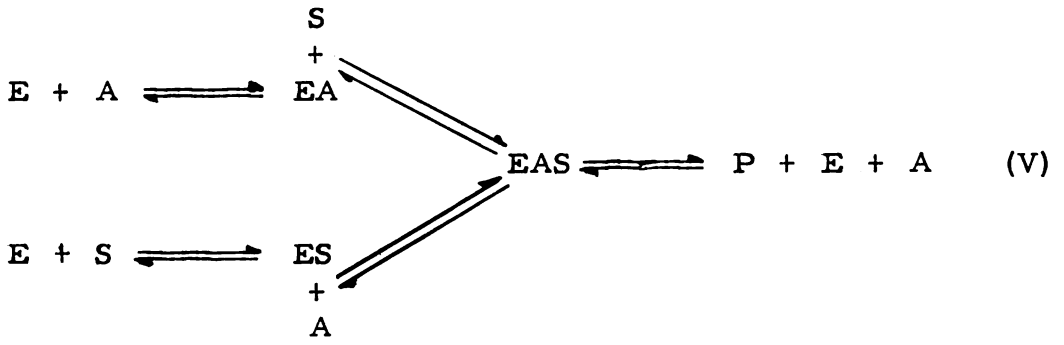
The best physical measurements on the enzyme are those of Warner (41). His data show $s^{o}_{20,w}$ = 10.45 S and $D^{o}_{20,w}$ = 3.96 x 10^{-7} sq cm/sec (both corrected to standard conditions). Sedimentation at 59,780 rpm for 48 minutes shows a single symmetric peak. The sedimentation data, with a partial specific volume of 0.74 yields a molecular weight of 237,000, the now accepted value for the enzyme. Electrophoretic patterns at pH 5.0, 6.02, and 7.76 indicate a single peak with a slight asymmetry. Electrophoresis at pH 6.9 for ten hours resolved this asymmetry into three components. Mobility calculations for the main component indicate an isoelectric point of 6.60.

The equilibrium lies in favor of ATP, with several values for K_{App} reported (42, 43, 44). The equilibrium is also subject to changes in pH and magnesium concentration, as demonstrated by McQuate and Utter (43) and Krimsky (44). The former authors report that the

increased concentration of magnesium shifts the equilibrium toward the pyruvate side, which would be expected due to the greater affinity of ATP for magnesium over that of ADP.

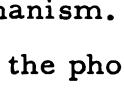
Morowiecki (45) has shown that the enzyme will dissociate in the presence of 6 M urea. Sedimentation studies on the dissociated enzyme indicate it is split into two subunits, having a molecular weight of around 150,000. There is no data to show the effect of potassium or other monovalent cations on this dissociation. Structural and functional changes may also be induced by certain steroids (46,47). Diethylstilbestrol (DES) causes a 60% inhibition at levels of 7.8×10^{-5} M. This inhibition is non-competitive with ADP, PEP, or potassium and is reversible upon dilution of the DES. The steroid causes a lowering of the limiting viscosity number and increases the electrophoretic mobility of the enzyme. The molecular weight as determined by sucrose gradient centrifugation, analytical ultracentrifugation, and light scattering, remains unchanged.

Enough research has been done on the mechanism of phosphoryl transfer reactions, so that we are in a position to draw some conclusions concerning a possible mechanism for pyruvate kinase. The first data of this nature, pertaining to pyruvate kinase, was the kinetic analysis by Kachmar and Boyer (7). Their data indicates the reaction proceeds through the combined pathway shown in equation V.



(V)

Since the velocity of the reaction was first order with respect to potassium concentration at various levels of PEP, they concluded that one potassium ion must be bound per molecule of PEP bound. Later data (48) indicated that there were two binding sites for PEP per molecule of protein, therefore two ions of activator per molecule of protein are necessary for full activation. The following conclusions may also be drawn concerning the mechanism.

a) Direct transfer of the phosphoryl group,  , from the donor to the acceptor molecule (49).

b) Participation of one catalytic site which can bind either PEP or pyruvate and a second site which can bind either ADP or ATP (48).

c) Competition of PEP and ATP for binding on the protein because of overlap of their transferable phosphoryl groups (48).

d) Random combination of PEP and ADP with the enzyme, governed by "equilibrium" kinetics (7, 48).

e) Enolization of pyruvate is dependent on both enzyme and activators (50).

f) Divalent cation-enzyme complex which may act as a bridge between the enzyme and ATP (51).

Schematically, the active site may thus look like that shown in Figure 1, as pictured by Reynard, et al.(48).

Additional information is gained regarding the mechanism from a study of the substrate specificity. Davidson (52) has shown a marked specificity for the purine di- and triphosphates. GDP and IDP have 60 and 75% of the activity obtainable with ADP as substrate. When ADP is replaced with 2'-deoxy ADP, the rate is approximately 11% of the maximum obtainable (53). Therefore it appears that the hydroxyl in the 2' position of the sugar is necessary for the binding of this substrate. PEP specificity is somewhat more difficult to ascertain, due to the paucity of the α-phospho(enol) acids available. Rose (50) has studied the detritiation (measure of the enolization of α-keto acids) of

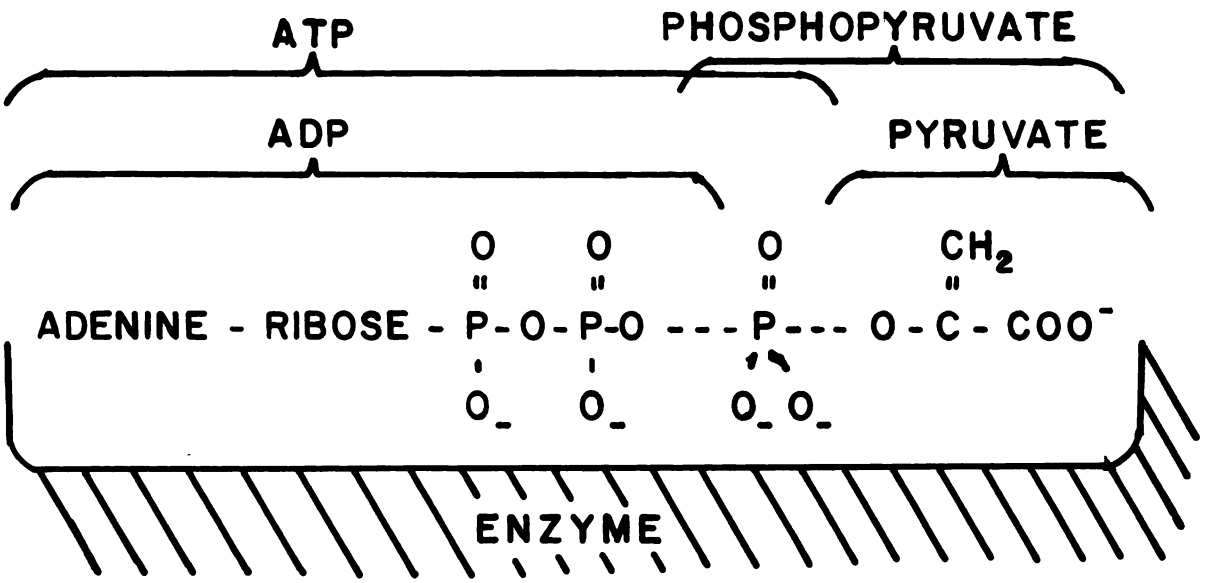


Figure 1. Schematic diagram of the active site of pyruvate kinase (after Reynard et al. (42), p. 2281).

α-ketoglutarate and α-ketobutyrate. In a system known to catalyze the enolization of pyruvate, he could not demonstrate the enolization of either of these acids. Thus, it appears that the pyruvate binding site is somewhat restricted in size and relatively specific for pyruvate and PEP.

Although the general mechanism appears to be clear at this time, there is a paucity of data to indicate the role of potassium in it. As has been mentioned, Rose (50) demonstrated the necessity for potassium and the enzyme before the enolization of pyruvate could occur. Also, the Michaelis-Menten constant for PEP is not affected by potassium concentration and the association constant of potassium is not affected by PEP concentration (7). Therefore, they appear to have different

binding sites. Similar data for ADP has not been obtained due to adenylate kinase contamination of the preparations. This is currently the sum of the knowledge of the role of monovalent cations in the enzyme catalyzed reaction.

C. Nature of the Problem

The ultimate goal of this problem is the elucidation of the action of inorganic monovalent cations in the pyruvate kinase reaction.

Several values for the potassium concentration of muscle have been reported. Gaudine and Levitt (54) report a value of 0.115 M for the in vivo potassium concentration of dog muscle. This value is within the requirement of the enzyme for full activation (0.1 M). However, other conflicting reports for various species of mammalian muscle have been given (55, 56). All of these values are below the requirement for optimal operation of the enzyme. We considered that some other compound of physiological importance may be the actual in vivo activator of the enzyme. Therefore a study of the effects of the polyamines was undertaken to see if they could possibly activate the enzyme.

Although attempts were made to find a physiological activator, we felt that a possible role of potassium was in causing a configurational change in the protein molecule thereby resulting in an active enzyme. Experiments were designed to detect this change and determine its nature. These included:

a) Use of specific reagents to mimic the activator action.

b) Reactivity with specific group reagents in presence and absence of activator.

c) Ultraviolet difference spectra.

II. EXPERIMENTAL

A. Materials and Methods

PEP was obtained from Sigma Biochemical Corporation as either the tricyclohexylammonium, sodium, or barium crystalline salt. The tricyclohexylammonium and sodium salts were used directly. The barium salt was converted to the sodium form by means of a Dowex exchange resin. Dowex-50W-X8, sodium form, was placed in an eyedropper column (5 cm x 0.7 cm). A solution of the barium PEP salt was mixed with a small amount of the dry resin to facilitate its going into solution. This was then placed on the column and eluted with water. Assay of the final solution by the method of Reynard, et al. (48) showed it to be 99% pure.

ADP was obtained from Pabst Laboratories as the crystalline sodium salt and was used directly. Paper chromatography in isobutyric acid:conc. NH_4OH:water (66:1:33) indicated trace amounts of AMP and ATP, however these constituted less than 1% of the total ADP.

Spermine and spermidine were obtained as the phosphate salts from General Biochemicals Corporation. Agamatine and cadaverine were obtained as the sulfate and hydrochloride respectively from Nutritional Biochemicals Corporation. All of these materials were used directly.

Para-hydroxymercuribenzoate (PMB) was obtained from Sigma Biochemical Corporation and 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNBA) was obtained from Aldrich Chemical Company. Both were used as obtained from these sources.

Tetramethylammonium chloride (TMA Cl) and 2, 4-dinitro phenylhydrazine (DNPH) were obtained from Eastman Organic Chemicals and were used directly.

Pyruvic acid was obtained from Matheson, Coleman, and Bell as the free acid. This was once distilled by vacuum distillation. Electrometric titration indicated this redistilled material was essentially 100% pure.

All other reagents were reagent grade and were used as obtained from the manufacturer.

Pyruvate kinase was either obtained from Sigma Biochemical Corporation (crystalline, Type II) or was isolated from rabbit muscle by the method of Tietz and Ochoa (12). Tissue used in this procedure was obtained from Pel-Freez Biologicals, Incorporated and was kept in the frozen state until use. Specific activities of preparation from both of these sources ranged from 150-200 units per mg of protein.

All protein concentrations were routinely determined by the absorbancy at 280 mμ. Pyruvate kinase has an $A^{0.1\%}_{1\ cm}$ of 0.54 (at 280 mμ) (4) and this value was used in calculation of protein concentration.

There are several published methods for the assay of pyruvate kinase activity (1, 9, 32, 48). The routine method used in this work was the dinitrophenylhydrazine (DNPH) method of Friedemann and Haugen (57) as modified by Reynard, et al. (48). The procedure was as follows: 0.10 ml of pyruvate kinase (0.1 to 1.0 μg of protein) was added to 1.90 ml of a reaction mix containing (at a final volume of 2.00 ml) $MgCl_2$, 1.6 μ moles; KCl, 100 μ moles; ADP, 2 μ moles; and Imidazole buffer, pH 7.5, 100 μ moles. The reaction was allowed to proceed in a controlled temperature water bath at 25° C (all assays were conducted at this temperature, unless otherwise specified) for five minutes. At this time, 0.1 ml of DNPH (0.025 M in 3 N HCl) was added and incubation continued for an additional ten minutes. Then, 0.5 ml NaOH (2.5 M with 0.2 M EDTA) was added and after an additional ten minutes the color read at 460 mμ on the Beckman DU Spectrophotometer. This "wait" period was instituted when it was found that the color developed on the addition of base was time dependent.

All determinations were made against a control tube which contained 0.10 ml of water in place of enzyme. Optical densities were converted to concentrations of pyruvic acid by means of a previously determined standard curve. The standard curve was determined by incubating known amounts of pyruvic acid under the above conditions. Pyruvic acid solutions used in obtaining the standard curve were of known concentration as determined by duplicate electrometric titrations. Occasionally optical densities were obtained which were too high to be read at 460 mμ; in these instances readings were obtained at 515 mμ. Concentration was linear with optical density over the entire range covered at both wavelengths.

In this study, a unit of pyruvate kinase activity is defined as that amount of enzyme which catalyzes the formation of 1.0 μ mole pyruvate per minute at pH 7.5 and 25° C.

On occasion, the coupled assay method of Kubowitz and Ott (9) was used. In this method, pyruvate is reduced to lactate by lactic dehydrogenase (equation IV) with the concomitant oxidation of NADH. With this assay additions were the same as listed above, with the exception that lactic dehydrogenase and 1.6 μ moles of NADH were added. A background rate was determined to allow for pyruvate kinase activity present in the lactic dehydrogenase, then 0.10 ml of the pyruvate kinase was added to give a total volume of 1.0 ml. The reaction was then followed by the optical density change at 340 mμ in a Beckman DU Spectrophotometer equipped with a Gilford cuvette positioner and multiple sample absorbancy recorder. However, since Kimberg and Yielding (46, 47) have shown that pyruvate kinase is activated by NADH this assay was not used to any great extent.

Both assays were linear with time and protein concentration, under the conditions described.

A procedure routinely used to obtain enzyme free of trace amounts of monovalent cations involved gel filtration on Sephadex. Sephadex G-25, medium mesh (Pharmacia, lot No. 9536) was packed in an ice jacketed column (20 cm x 2 cm). The column was equilibrated with Tris buffer, pH 8.2, 5×10^{-3} M containing 10^{-4} M EDTA, then 2-3 ml of pyruvate kinase (containing 150-300 mg of protein) was placed on the column. Elution was carried out with the above buffer. Protein was determined with a Gilford flow cell in the Beckman DU spectrophotometer by following the change in absorbancy at 280 mμ. (On occasion, fractions were collected and read by hand.) Salt was determined by following the resistance with a conductivity bridge (Industrial Instruments, Model RC-16 B2). Fractions of 1 ml volume were collected and those having the highest protein concentration and giving resistances approximating the original buffer were combined. The combined fractions were then assayed by the DNPH method. By this procedure, protein free of salt was recovered in approximately 80% yields with no loss of specific activity.

B. Experimental Procedures and Results

1. Attempts to replace potassium.

Several polyamines were examined for their effect on pyruvate kinase activity. In addition to the polyamines, choline, which exists as a positively charged species was also tested. DNPH assays were run as described under the methods section. A control tube containing potassium was run in each determination. In addition, a control tube, containing the compound tested but no enzyme, was also run to evaluate the effect of the compound on the assay procedure. As seen in Table IV, none of the compounds tested had any effect on the activation of the enzyme.

Table IV. Effect of Polyamines and Choline on Pyruvate Kinase Activity

Compound	Concentration (M)	% of Control
Spermidine	0.025	0
Spermine	0.025	0
Agamatine	0.025	0
Cadaverine	0.010	0
Cadaverine	0.050	0
Choline	0.010	0

2. Effects of organic compounds.

Several organic compounds, which are thought to induce configurational changes in protein structure, were examined for their effect on pyruvate kinase activity. We felt that perhaps an induced configurational change might mimic the effect caused by potassium. DNPH assays were run as described in the methods section. As in the previously described experiments, controls were run in all determinations. The data are shown in Table V. It is obvious that the compounds studied had no effect on the activation of the enzyme.

Table V. Effect of Organic Compounds on Pyruvate Kinase Activity

Compound	Concentration (M)	% of Control
2-Chloroethanol	0.377	0
2-Chloroethanol	2.260	0
2-Chloroethanol	3.770	0
Ethylene glycol	0.090	0
Ethylene glycol	0.179	0
Dioxane	0.057	0
Dioxane	0.103	0
Urea	0.500	0
Urea	1.000	0

3. Effect of sulfhydryl reagents on pyruvate kinase activity in the presence and absence of potassium.

If the pyruvate kinase molecule is undergoing a configurational change upon activation, then one might expect certain groups to be more susceptible to attack by specific group reagents in one configuration than in the other. To explore this possibility, two reagents which are relatively specific for sulfhydryl groups, were tested for their effect on pyruvate kinase in the presence of potassium and TMA Cl. The TMA Cl was used as a replacement for potassium to maintain constant ionic strength. Control experiments were run to demonstrate that the TMA Cl had no effect on the enzyme.

Reaction tubes were set up to contain: PMB, 0.01-0.05 μ moles; Imidazole, pH 7.5, 100 μ moles; KCl or TMA Cl, 100 μ moles; and pyruvate kinase (previously sephadexed, and known to be salt free), 0.9-1.0 mg; in a total volume of 1.0 ml. The tubes, minus the enzyme, were incubated at 25° C for about ten minutes before the addition of enzyme. At time zero the enzyme was added. At 5, 10, and 20 minutes, 0.10 ml aliquots were removed and diluted to assay levels of protein concentration. Assays were run by the DNPH method. Controls were run in the absence of PMB to establish loss of activity from means other than the inhibitor. The data are shown in Figure 2.

At 10^{-5} M, PMB causes little or no inhibition, either in the presence or absence of activator. At a five-fold increase in PMB concentration, however, approximately 40% inhibition occurred. This inhibition was not affected by the presence or absence of potassium.

The other sulfhydryl reagent used was DTNBA. The action of this reagent is shown in equation VI.[1]

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[1]Paul A. Srere, Personal Communication.

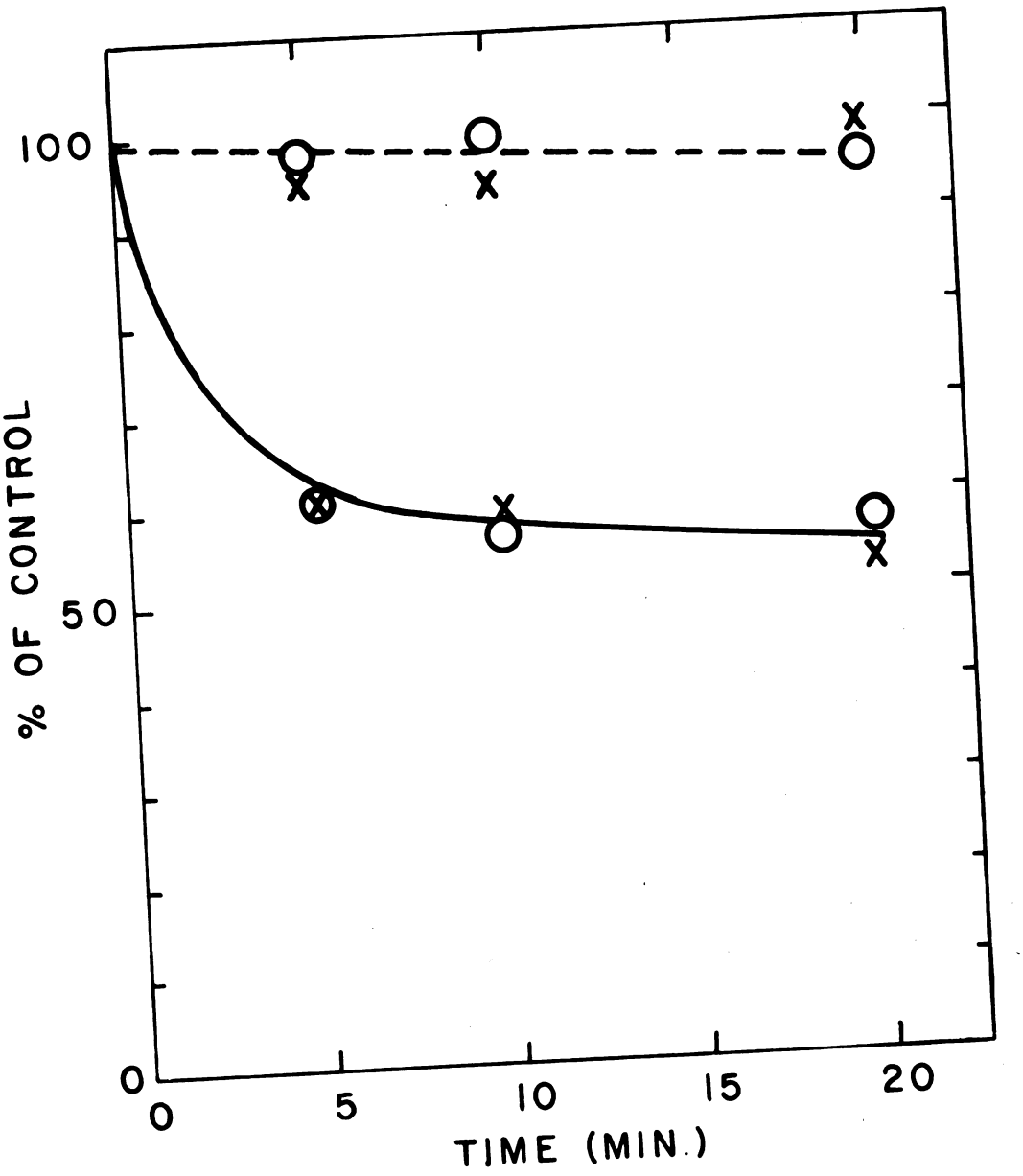
Figure 2. Effect of PMB on pyruvate kinase activity in the presence and absence of potassium (0.10 M).

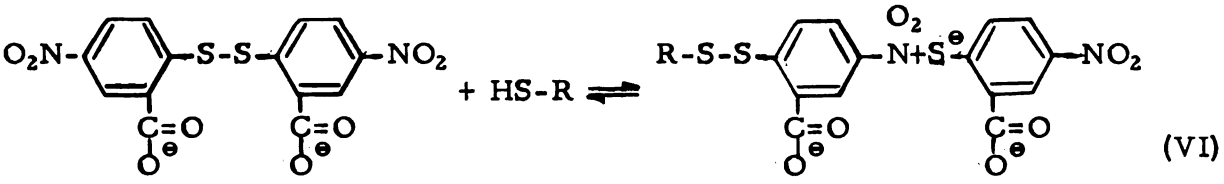
O, Enzyme in KCl

X, Enzyme in TMA Cl

---, 10^{-5} M PMB

——, 5 x 10^{-5} M PMB


100
50
0
% OF CONTROL
0
5
10
15
20
TIME (MIN.)



(VI)

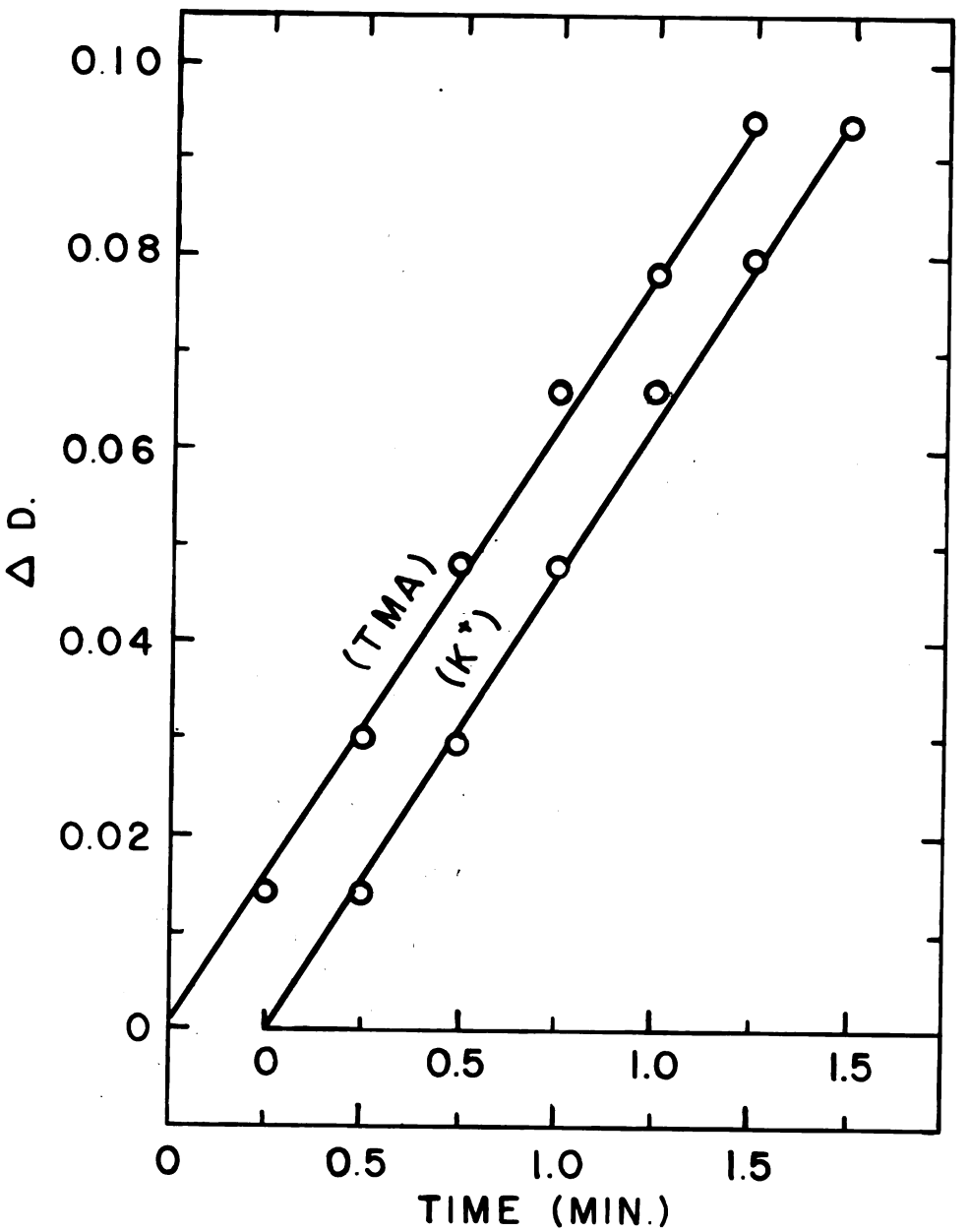
The thionitrobenzoic acid formed absorbs strongly at 410 mμ and the reaction may be followed by optical density change at this wavelength. The procedure used was as follows. A cuvette was set up to contain the following: Imidazole, pH 7.5, 100 μ moles; KCl or TMA Cl, 100 μ moles; DTNBA, 1.0 μ mole; and pyruvate kinase, 4 mg; in a total volume of 1.0 ml. Before the addition of the enzyme, a background rate due to non-enzymatic breakdown of the DTNBA was checked for and none found. At time zero, the enzyme was added and the reaction followed at 410 mμ in the Beckman DU spectrophotometer equipped with the Gilford attachments. The data are given in Figure 3 and show no difference in the rate of sulfhydryl exchange due to the presence or absence of potassium.

4. Ultraviolet difference spectra of pyruvate kinase.

Changes in the environment of the phenylalanyl, tyrosyl, and tryptophyl chromophoric groups in proteins can lead to a perturbation of the absorption spectrum of the protein in the ultraviolet range. As has been pointed out by Leach and Scheraga (58) these differences may be used as a reflection of changes in the internal structure of the protein. However, these differences are usually small and the most convenient method to detect them is by obtaining a difference spectrum.

Since perturbations in the difference spectrum reflect changes in the internal structure of proteins due to configurational changes, this became an important tool in the study of pyruvate kinase. To determine the difference spectrum at various levels of enzyme concentration, the following procedure was used. Cuvettes of 2.0 ml

Figure 3. Effect of DTNBA on pyruvate kinase activity in the presence and absence of potassium (0.10 M).


0.10
0.08
0.06
0.04
0.02
0
Δ D.
(TMA)
(K+)
0
0.5
1.0
1.5
0
0.5
1.0
1.5
TIME (MIN.)

size (light path = 1.0 cm) were matched, and the best two, out of the set of four, were used in these experiments. Although the cuvettes matched very well one of them was always used as the sample cell in all runs, and the other always used as the reference cell. To determine the base line, the cuvettes were filled with a dilution mix which was 0.10 M with respect to Imidazole and KCl. The cuvettes were then placed in the respective compartments to the Cary model 15 spectrophotometer and the base line adjusted by means of the multipot adjustments on the instrument. This was done on the "direct" sensitivity scale. Attempts to adjust the instrument on the 0.1 (full scale = 0.240 optical density) sensitivity scale were unsuccessful. To account for the base line deviations which occurred at this sensitivity, several traces were made, and these values averaged. The average values were used to correct all spectra determined at this sensitivity.

Enzyme, at a concentration of about 6 mg/ml, in 0.10 M KCl or TMA Cl and 0.10 M Imidazole, pH 7.5, was placed in each cuvette, and the spectrum determined from 350 to 250 mμ, at a scan speed of about 0.5 Å per second. The KCl-enzyme dilution was in the sample cell; the TMA Cl dilution in the reference. Spectra at lower levels of protein concentration were made from this original sample. Aliquots were removed and diluted to 1.0 ml with the dilution mix mentioned above. A TMA Cl dilution mix was used for the sample from the reference cell. No attempt was made to maintain a constant slit width; however, at no time during the scan did it exceed the maximum of the instrument (3.0 mm).

Since the spectra did not exactly reproduce themselves at any given protein concentration, several traces were made at each protein concentration. Optical density values at each wavelength were then averaged, corrected for base line deviations, and plotted. The data are shown in Figure 4. The deviations from the average optical density value at 285 and 295 mμ are shown in Figure 5.

Figure 4. Difference spectra of pyruvate kinase in the presence and absence of potassium (0.10 M) at different levels of enzyme concentration. Enzyme and KCl in sample cell; Enzyme and TMA Cl in reference cell. pH 7.5 (Imidazole buffer, 0.10 M).

▲, 6.36 mg/ml.

□, 3.13 mg/ml.

X, 2.12 mg/ml.

O, 1.57 mg/ml.

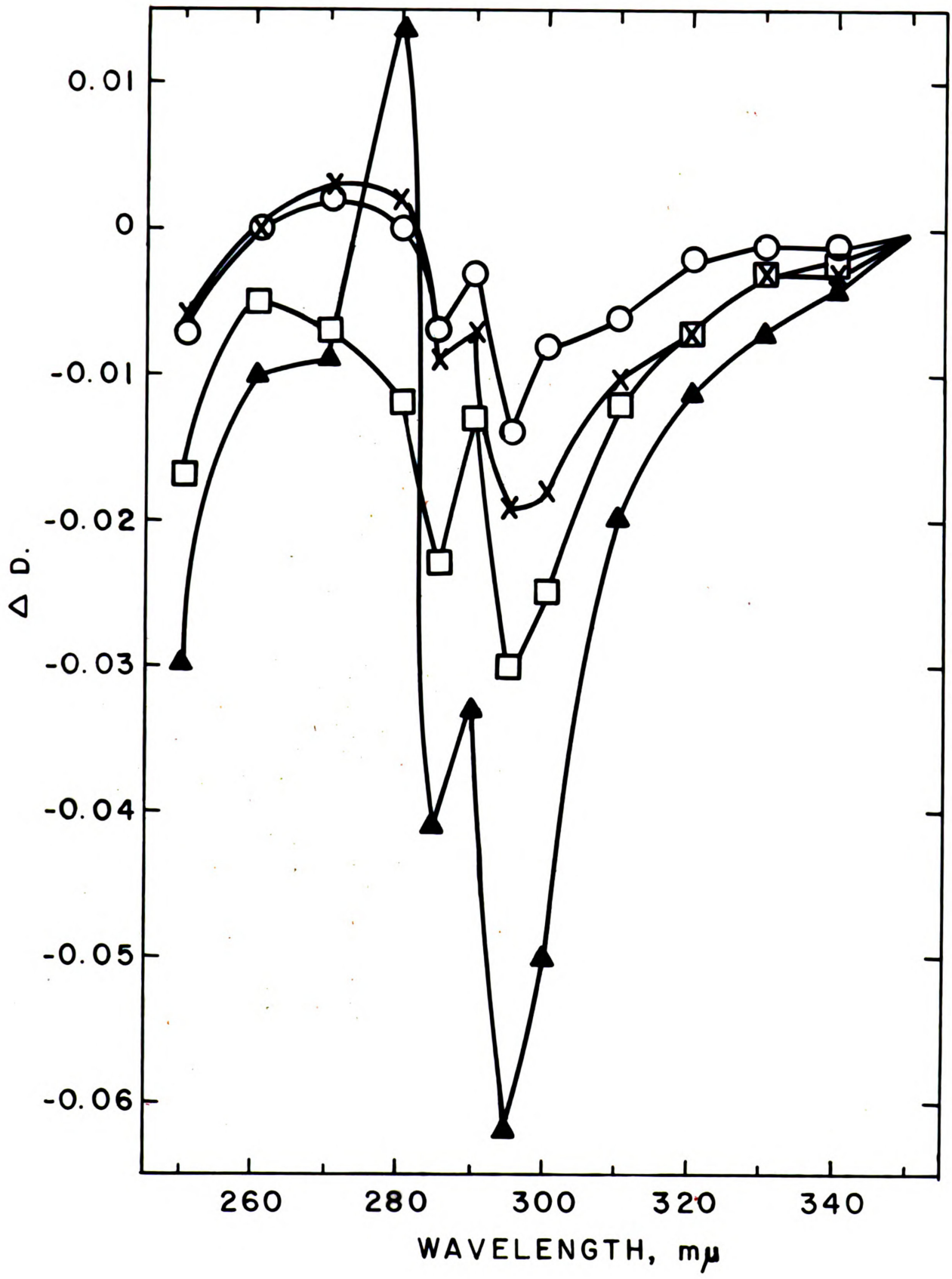
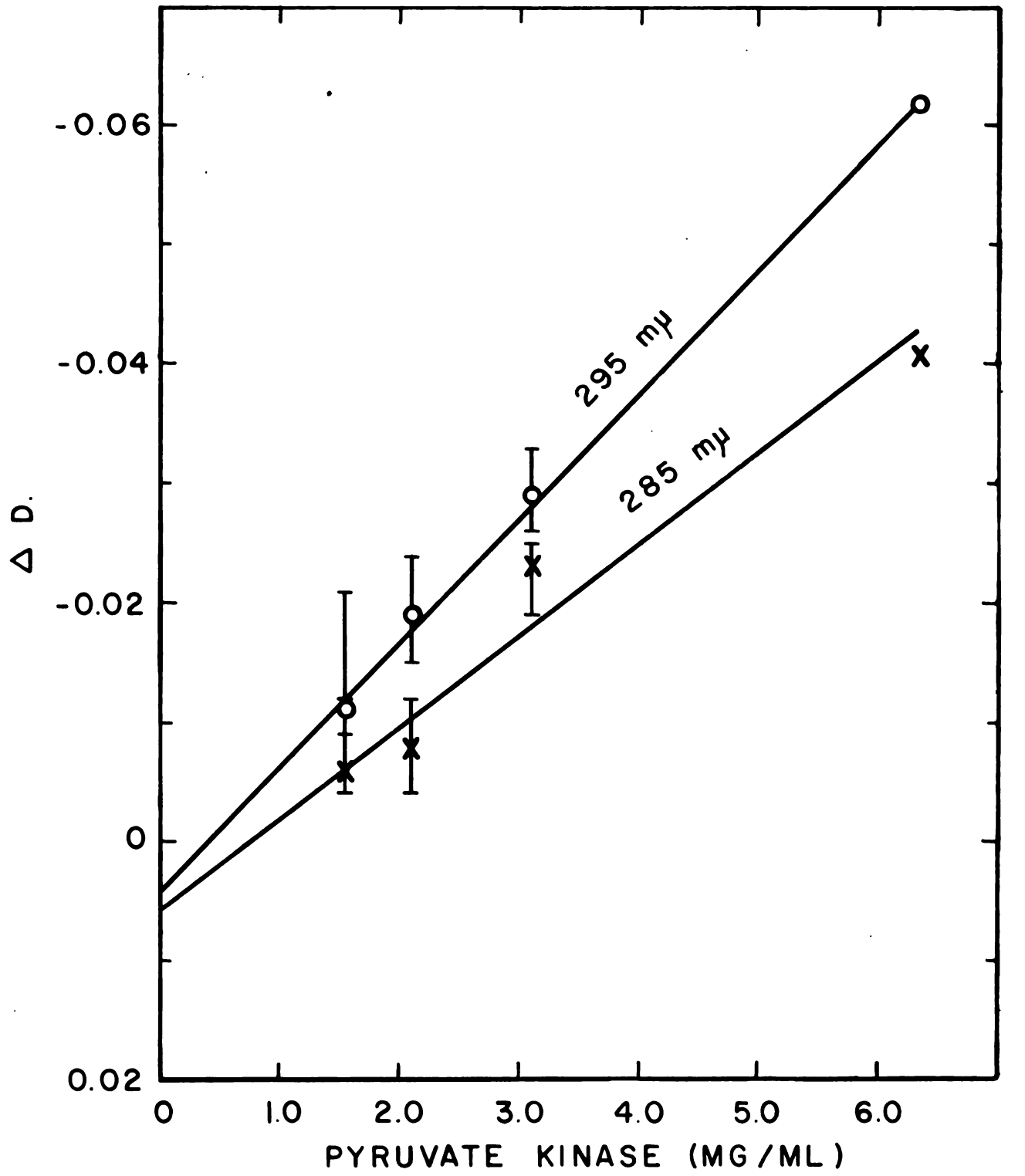

0.01
0
-0.01
-0.02
-0.03
-0.04
-0.05
-0.06
Δ D.
260
280
300
320
340
WAVELENGTH, mμ

Figure 5. Plot of density change vs. protein concentration for the 285 and 295 mμ peaks of pyruvate kinase difference spectra.

O, Density difference at 295 mμ.

X, Density difference at 285 mμ.


-0.06
-0.04
-0.02
0
0.02
Δ D.
295 mμ
285 mμ
0
1.0
2.0
3.0
4.0
5.0
6.0
PYRUVATE KINASE (MG/ML)

One of the most apparent characteristics of the difference spectra is the appearance of a double peak occurring at 285 and 295 mμ. To eliminate the possibility that this was an artifact caused by stray light (59, 60, 61, 62) a plot of protein concentration vs. optical density at the two peaks was made. If Beer's law is assumed to apply, then a plot of this sort should be linear with protein concentration. As shown in Figure 5, the perturbation is linear with concentration thereby eliminating the above mentioned effects. However, this does not eliminate the possibility of fluorescence errors (61), but if errors of this type were occurring it would be due to the presence of potassium. The sharp spike peak occurring at 280 mμ in the spectra at 6.36 mg/ml cannot be explained. The curve represents only one trace at that concentration, and therefore does not represent an average of several values. It may well be that the plateau observed at 260-270 mμ is the true error, and these points should perhaps be much higher. This would be indicated by the general shape of the other curves in this region.

III. DISCUSSION

The data for the attempts to replace potassium, speak for themselves. It is obvious that none of the compounds tested will activate the enzyme. No attempt was made to see if these compounds will inhibit the enzyme, as this would shed no light on the current problem.

The same may be said for the attempts to induce configurational changes with organic reagents. None of the compounds tested resulted in an active enzyme. If the compounds were indeed inducing a change, then it was not due to a configuration such as to give the protein catalytic activity.

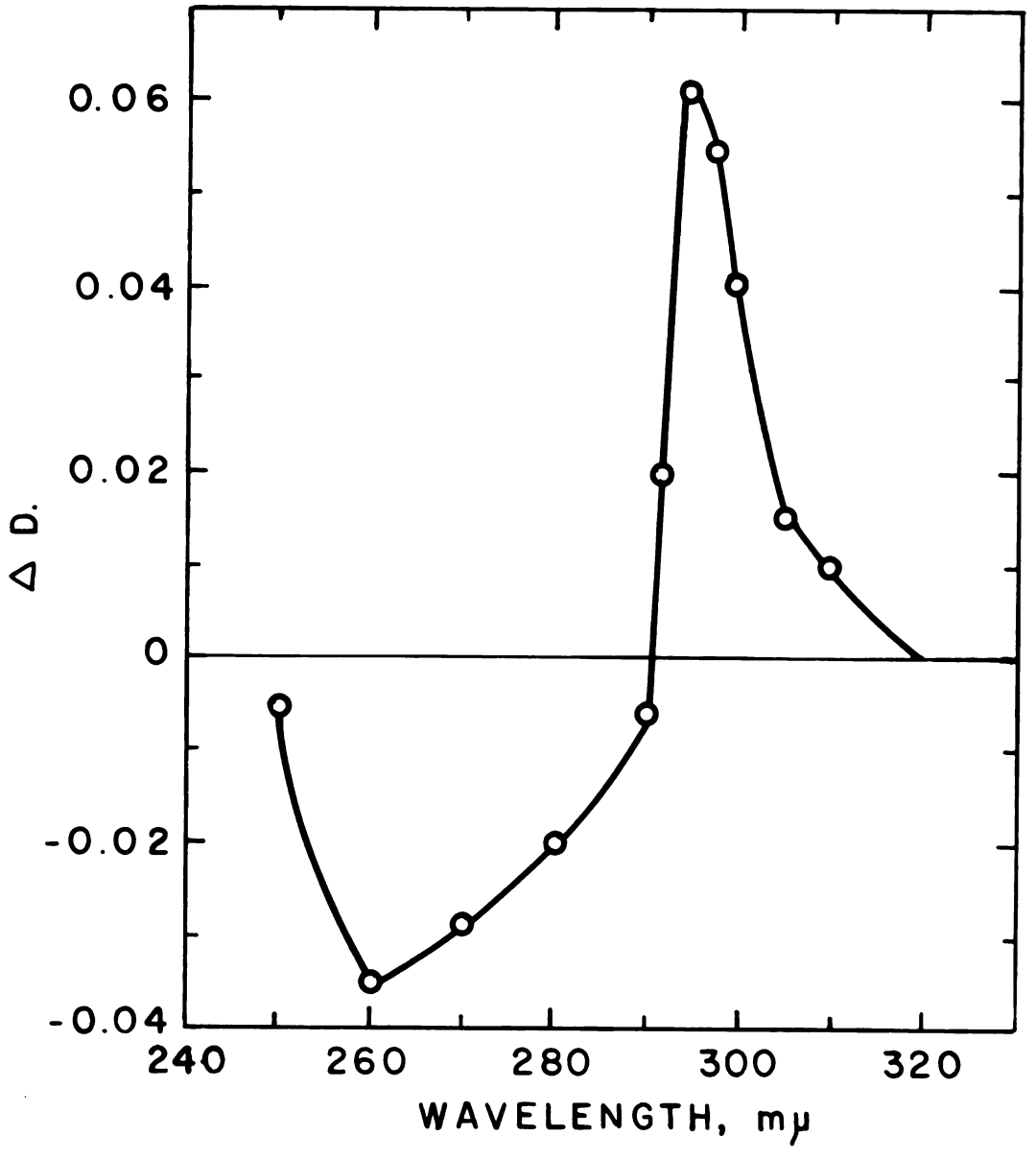
In the inhibitor studies, it is obvious that potassium has no effect on the reactivity of sulfhydryl groups detectable by these techniques. Therefore, one may conclude that these groups are in the same spatial configuration in both the native and the activated enzyme.

The significant data in this study is the ultraviolet difference spectra. The prominant peaks are at 285 and 295 mμ. The peak at 295 mμ appears to correspond to a perturbation caused by differences in the absorption of a tryptophyl residue. Donovan et al. (62) have demonstrated alterations of the spectrum of lysozyme, presumably due to the presence of tryptophyl residues, resulting in a characteristic absorption maximum at 295 mμ. Their data are reproduced in Figure 6.

The secondary peak occurring at 285 mμ is somewhat more difficult to explain; however, there are two possible explanations. Hermans et al. (63) have shown a difference spectrum similar to that shown for pyruvate kinase, for alkaline solutions of tryptophane. In their work, the reference cell is at pH 6 and the sample cell at pH 13.

Figure 6. Difference spectra of lysozyme (after Donovan, et al. (62), p. 2691).

Concentration = 0.23%; temperature = 25° C; μ = 0.15. Sample cell at pH 5.04; Reference at pH 1.15.


0.06
0.04
0.02
0
-0.02
-0.04
Δ D.
240
260
280
300
320
WAVELENGTH, mμ

The position of the peaks is temperature dependent, but at 25° C, they occur at 285 and 295 mμ. A second explanation is that the 285 mμ perturbation is due to the presence of another group. Changes in the ionization of the amino group of 0-methyltyrosine can result in perturbations of the spectrum of that compound at 285 mμ (64).

The difference spectrum of pyruvate kinase is also extremely subject to changes in pH. Preliminary studies in the course of this work indicate that the double peak at 285 and 295 mμ is completely lost below pH 6.5 and above pH 8.0. This behavior may well be a reflection of the active site of the enzyme, since the optimum pH range of the enzyme is from 7.0 to 8.0.[1]

The primary cause of the perturbations occurring at 285 and 295 mμ in the pyruvate kinase difference spectrum is difficult to explain at this time, due to inadequacies in the knowledge of these effects. The perturbations may be due to a direct interaction of the monovalent cation with the π-electrons of an aromatic nucleus, thereby altering the absorption of that group. It is also possible that the cation is complexing with some other group in the enzyme (e.g., a free carboxyl) and altering the absorption of an adjacent aromatic group through an inductive effect. Finally, it is possible that the cation is causing a major configurational change in the protein molecule resulting in the movement of a chromophoric group to a different environment (e.g., from a hydrophobic area into an aqueous environment). However, it is impossible to distinguish between these effects based on the current data.

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[1]C. H. Suelter, unpublished experiments.

IV. SUMMARY

1. The effects of various polyamines on pyruvate kinase activity are presented. It is shown that none of the compounds tested will cause activation of the enzyme.

2. Several organic reagents, thought to induce configurational changes in proteins, were studied in an attempt to mimic the monovalent cation effect. None of the compounds tested would activate the enzyme.

3. PMB and DTNBA, both sulfhydryl reagents, were used to examine the reactivity of sulfhydryl groups in the presence and absence of activator. There was no difference in the rate of reaction with either of these inhibitors.

4. Difference spectra, in the region 250-350 mμ, of the enzyme in the presence and absence of activator were determined. These data indicated alterations of the spectra of pyruvate kinase due to the presence of activator (0.10 M potassium). Two peaks were observed occurring at 295 mμ and 285 mμ. The change in optical density at these wavelengths is linear with protein concentration. It is felt that the perturbations represent alterations of the spectra of tryptophyl residues in the protein.

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