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Title: THE EFFECT OF UNIVALENT CATION SALTS ON PROPERTIES OF PYRUVATE KINASE

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Experiments described to determine the effect of a series of univalent cation salts on the physical properties of crystalline pyruvate kinase, an enzyme that has an absolute requirement for univalent cations for activity, are reported. An investigation of the stability of the enzyme in a series of different salt solutions revealed marked effects of different univalent cation species and of different species of anions. A strong positive correlation was shown between the capacity of univalent cations to protect against inactivation of the enzyme and to activate pyruvate kinase. In addition, potassium, an activator cation, restored to a considerable extent enzyme activity that was lost due to incubation of the enzyme in solutions of the non-activator cations, lithium and Tris. The enzyme consistently showed more stability in buffer solutions containing multivalent anions such as pyrophosphate, sulfate, phosphate and arsenate than in buffer solutions containing the univalent anions acetate, bromide, nitrate

and chloride.

Ultraviolet difference spectra were determined for pyruvate kinase in 0.1 M solutions of activator univalent cations (sample cuvette) versus enzyme in 0.1 M solutions of non-activator univalent cations (reference cuvette). Under these conditions it was found essential to add the non-activator univalent cation, Tris, to the reference cuvette in order to observe a difference spectrum with peaks at 286 mμ and 295 mμ. This difference spectrum was considered to be due to the perturbation of the tryptophan residues. The perturbation of aromatic residues has been interpreted in the literature to result in a small change in the extinction coefficient and a small shift in the wavelength of maximum absorption. A difference spectrum of pyruvate kinase in 0.1 M KCl versus enzyme in 0.1 M LiCl showed essentially no deviation from the base line. The addition of various substrates and magnesium to both cuvettes had no effect on the difference spectrum of enzyme in 0.1 M KCl versus enzyme in 0.1 M LiCl.

The perturbation of the tryptophan residues of pyruvate kinase in the presence of Tris was inhibited by the addition of the multivalent anions phosphate, arsenate and sulfate. In contrast, the tryptophan residues were perturbed in the presence of the Tris salts of the univalent anions, chloride, nitrate and acetate. Thus, a correlation was found between the capacity of anions to protect

the enzyme from inactivation and their capacity to perturb tryptophan residues. Furthermore, the valency of the anion appeared to be important in determining whether the anion contributed toward stability and inhibited perturbation.

At concentrations of univalent cation chlorides of 0.5 M or higher, the capacity to perturb the tryptophan residues as measured by difference spectrophotometry was inversely correlated with the capacity of univalent cations to activate. That is, those univalent cations which activate the enzyme do not perturb the tryptophan residues, wheareas, those univalent cations which do not activate perturb the tryptophan residues.

Analytical ultracentrifugation studies of the enzyme in different univalent cation phosphate salts showed that the enzyme sedimented as one homogeneous peak in three different univalent cation environments. A $s^{o}_{20,w}$ value for pyruvate kinase in 0.05 M lithium phosphate was 9.99. This value was statistically significantly different from the $s^{o}_{20,w}$ value of 10.15 for the enzyme in potassium phosphate and 10.17 for the enzyme in Tris phosphate.

The results of the stability and different spectral studies were interpreted to support the hypothesis that the enzyme can exist in at least two conformations depending on the species of univalent cation present. Activator univalent cations were postulated to poise the protein in a catalytically active conformation, which is

relatively stable and which exhibits little if any perturbation of the tryptophan residues at high salt concentrations. In contrast, non-activator univalent cations were postulated to poise the protein molecule in a catalytically non-active conformation, which is relatively unstable and at appropriate concentrations exhibits perturbation of tryptophan residues.

THE EFFECT OF UNIVALENT CATION SALTS ON PROPERTIES OF PYRUVATE KINASE

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INTRODUCTION

The essentiality of potassium for all forms of life is well known. Normally, potassium is the most abundant metal and principal univalent cation within the cell. A summary of the analysis of 15 plant species shows the average potassium contents ranging from 1.66 to 2.75% of the dry matter (Evans and Sorger, 1966).

Although required in considerable quantity, the specific function of potassium in cellular metabolism has largely remained unclear. The role of potassium has been implicated in a general way by suggestions that the ion participates in maintaining cell organization, cell membrane permeability (Sutcliffe, 1962; Nason and McElroy, 1963), and in the regulation of osmotic pressure (Sutcliffe, 1962). More recently, Evans and Sorger (1966) have proposed that the capacity of potassium to activate certain enzymes is its most important function. The authors list several enzyme systems in which potassium has been shown to stimulate or be required for activity. They pointed out that the concentration of potassium needed to obtain optimal activity of univalent-cation-activated enzymes is similar to the concentration of potassium found in the living cell. In addition, certain metabolic lesions which result from potassium deficiency may be accounted for by a block in one or more

univalent-cation-requiring enzyme systems in pathways considered essential for normal plant metabolism. Further, rubidium and ammonium generally have been shown capable of substituting for potassium as activators while sodium and lithium in many systems are inhibitory or do not substitute. It has been reported that under certain conditions rubidium can exert a sparing effect on potassium deficient plants (Richards, 1941, 1944; Scharrer and Schropp, 1933). The addition of sodium to potassium deficient plants is variable, and appears to depend on the plant species and experimental conditions (Harmer and Benne, 1945; Harmer et al., 1953; Lehr, 1953; Wybenga, 1957). Finally, lithium appears to be unable to effectively substitute for potassium as a necessary cation for plant growth (Scharrer and Schropp, 1933). These considerations support the hypothesis that potassium and similar univalent cations play a major role in cellular metabolism as a co-factor of certain enzyme systems.

The primary objective of this research is to study the function of univalent cations in the mechanism of enzyme activation. The enzyme selected for study was pyruvate kinase (E. C. 2. 7. 1. 40) which is a typical univalent-cation-requiring enzyme that can be obtained in high purity. In this study the effect of a series of univalent cations on certain physical properties of pyruvate kinase has been examined. In addition, a correlation has been made of the

effects of univalent cations on the physical properties of the enzyme and the capacities of univalent cations to function as activators. The anions associated with the univalent cations also have been observed to affect certain physical properties of the enzyme. The effect, therefore, of anions on the physical properties of the enzyme has been investigated and related to the activity of the enzyme.

REVIEW OF LITERATURE

Role of Univalent Cations in the Activation of Pyruvate Kinase

Kinetic Studies

Pyruvate kinase was the first enzyme reported to have a requirement for univalent cations (Boyer, Lardy and Phillips, 1942, 1943). A stimulation or absolute dependence for univalent cations has been shown for the enzyme from plants (Miller and Evans, 1957; Evans, 1963), animals (Boyer, 1953), and microorganisms (Seitz, 1949). In 1953 Kachmar and Boyer (1953) reported a detailed kinetic study of pyruvate kinase from rabbit muscle. In addition to the essentiality of magnesium for catalysis, they showed the indispensability of potassium, rubidium, or ammonium for activity. Sodium exhibited a weak capacity to activate the enzyme whereas lithium was completely ineffective. The Michaelis constant (K_m) for potassium was reported to be 0.011 M and was shown to be independent of the concentration of the substrate phosphoenol-pyruvate (PEP). Similarly, the concentration of potassium in the assay medium had essentially no effect on the Michaelis constant for PEP. The K_m for this substrate was found to be 8.6×10^{-5} M. From these results they considered a series of possible mechanisms by which the activator,

substrate and enzyme could react to form a ternary complex from which reaction products were formed. They concluded that the results supported a reaction mechanism probably involving an independent combination of substrate, PEP, and potassium, under equilibrium reaction conditions, to form an active ternary complex. From a straight line relationship of a plot of concentration of activator/velocity versus activator they suggested that full enzyme activity could be obtained when only one potassium was bound for each PEP molecule bound.

To explain the difference in the effectiveness of univalent cations as activators, the authors considered the size of the hydrated ionic radii as reported by Jenny (1932) and Nachod and Wood (1945). According to these workers, potassium, rubidium and ammonium, all activators, have hydrated ionic radii of 5.32 Å, 5.09 Å, and 5.37 Å respectively. Sodium, which showed a much lower capacity to activate, has a hydrated ionic radius of 7.09 Å. Lithium, which is a non-activator, has a hydrated ionic radius of 10.03 Å. They concluded that the effectiveness of these cations to function as activators depended on their capacity to bind at a negative site (sites) and to displace adjoining structures of the enzyme by some critical amount.

Reynard et al. (1961) have reported kinetic studies with highly purified pyruvate kinase from rabbit muscle. Their results showed

that the K_m for either PEP or adenosine diphosphate (ADP) was independent of the concentration of the other substrate. They interpreted these results to indicate an independent binding of PEP and ADP to the enzyme with "equilibrium kinetics" governing the transfer of the phosphoryl group from donor to acceptor molecule. No experimental results were reported on the role of potassium but these workers speculated that the univalent cation is necessary for the protein to assume the proper configuration. They did show that the enzyme in dilute solutions lost activity unless KCl was added.

Recently Melchior (1965) has studied the effect of a wide range of concentrations of activator metals and ADP on the reaction kinetics of pyruvate kinase. Using a digital computer for calculations, Melchior has estimated the concentrations of hypothetical intermediate reactants present at various stages of the reaction and evaluated their relative significance. She showed that the reaction velocity did not vary over a wide range of concentrations of the complex ($KADP^{-2}$) and that this species was probably not important in the reaction mechanism as was previously suggested (Melchior, 1954). She concluded that the complex ($MgADP^{-1}$) is the only ADP species which interacts appreciably with the enzyme and that the concentration of ($MgADP^{-1}$) complex and potassium were critical factors that determined the reaction velocity. Furthermore, when the concentration of magnesium is increased to a high level relative to the

concentration of potassium, the reaction is inhibited. Also, a large excess of potassium in relation to magnesium inhibits the activity of the enzyme. From these results she suggested that the enzyme has two sites capable of binding positive ions and that enzyme activity is affected by the species bound. When one site is occupied by potassium, the enzyme is active, but the occupation of both sites either by magnesium or by potassium results in reduced activity or no activity. Melchior's hypothetical mechanism fails to explain why potassium is superior to either sodium or lithium as enzyme activators. Betts (1966) also obtained kinetic data showing that magnesium and potassium compete with each other for binding sites on the enzyme.

Spectrophotometric Studies

The technique of difference spectrophotometry has been used by Suelter and co-workers to investigate the effect of a series of univalent and divalent cations on the ultraviolet absorption properties of crystalline pyruvate kinase (Suelter and Melander, 1963; Kayne and Suelter, 1965; Suelter et al., 1966). A comparison of pyruvate kinase in TrisCl buffer containing a divalent cation, magnesium or manganese, versus an equal concentration of enzyme in TrisCl buffer and tetramethylammonium chloride (TMACl) resulted in an ultraviolet difference spectrum characteristic of the perturbation of tryptophan residues (Suelter and Melander, 1963).

Qualitatively similar difference spectra were observed with pyruvate kinase in TrisCl buffer containing one of a series of different univalent cations versus a solution of enzyme in TrisCl buffer and TMACl. If enzyme in TrisCl buffer solution containing TMACl was compared with enzyme in a similar environment containing PEP a difference spectrum characteristic of the perturbation of the tryptophan residues was obtained (Kayne and Suelter, 1965). Finally, a difference spectrum characteristic of the perturbation of tryptophan residues also was observed with enzyme in cacodylate buffer at low temperatures (-10C) versus enzyme in the same cacodylate buffer solution at high temperatures (40C). It should be pointed out in all studies in which univalent or divalent cations were used as perturbants, the background buffer was TrisCl.

When any one of a series of inorganic univalent or divalent cations was used as the perturbant, the absorption difference at 295 mμ depended on the concentration of the cation present. The negative logarithm of the concentration of any one of a series of univalent or divalent cations plotted against the value resulting from dividing absorption difference at 295 mμ by the maximum difference in absorption at 295 mμ when an excess of salt was present ($\triangle A/\triangle A_{max}$) produced a sigmoid curve which could be described by the following equation:

$$n(pK_{2v}-pM) = \log \frac{a}{1-a}$$

In the equation n equals the number of moles of metal ion bound per catalytic site, K equals the dissociation constant for enzyme-metal complex, M equals the metal ion concentration and a equals $\Delta A/\Delta A_{max}$ (the fraction of enzyme-metal complex). By using the above equation and plotting the appropriate data the dissociation constants for both univalent and divalent cations were determined (Suelter et al., 1966). The results agreed with the dissociation constants that were determined kinetically by the authors or determined by nuclear magnetic resonance techniques (Mildvan and Cohn, 1963a) if the following assumptions were made: n = 2 for magnesium, manganese, potassium or sodium; n = 3 for rubidium and cesium; n = 4 for ammonium (Suelter et al., 1966). The authors point out that no direct measurement has been made of moles of univalent cation bound per mole of protein. For the case where n = 2 the value was justified on the basis of the nuclear studies of Mildvan and Cohn (1964) which indicated two active sites per molecule. No experimental justification was presented for the value of n assumed to be greater than two.

Considering those cations for which n values of 2 were assumed, the authors point out that their data do not support the

conclusion that the dissociation constants for the two separate cations are identical at each site. Yet, the sigmoid curve described by the above equation where $n = 2$ is the curve expected for the dissociation of a diprotic acid in which the two protons dissociate simultaneously (Schwarzenbach and Sulzberger, 1943; Maier and Metzler, 1957). The authors resolve this apparent dicotomy by suggesting that the dissociation constant for the second mole of cation is less than that for the first mole of cation. This means that the dissociation constant measured from the difference spectral studies is equal to a geometric mean of the dissociation constants at the individual sites. They conclude that the data can be interpreted as indicating a preferred order of binding in which one univalent cation binds at one site followed by a rapid binding at a second site. They point out, however, that a detailed kinetic and stoichiometric analysis of the individual steps of metal binding is required to provide a more critical test of their preferred-order hypothesis (Suelter et al., 1966). In a detailed kinetic study of the role of metal ions in the pyruvate kinase reaction, Melchior (1965) did not mention a preferred-order mechanism of metal binding.

Suelter et al. (1966) proposed that the univalent and divalent activating cations mutually influence the affinities of each other for the enzyme. They explain this by assuming that at each catalytic site there is a site for univalent cation binding and a site for divalent

cation binding. In the absence of a divalent cation, a univalent cation may bind to both sites and in the absence of a univalent cation, divalent cations may bind to both sites. This interpretation is in agreement with that of Melchior (1965). There is no indication in Suelter's studies of two separate dissociation constants for potassium or magnesium, that is, one describing interaction at the divalent sites and one describing interaction at the univalent site.

Kayne and Suelter (1965) interpreted the difference spectra produced by the interaction of cations or PEP with the enzyme or by temperature differences in the sample and reference cuvette to be caused by changes in the solvating environment of protein chromophores and arising from a blue shift in the spectrum of the enzyme. They suggested that after the cations bind or the temperature of the enzyme is lowered, hydrophobic forces that are responsible for maintaining certain residues of the enzyme in a nonaqueous environment are broken by some other force, resulting in the exposure of residues to an aqueous environment. The nature of this other force was not discussed.

The authors point out that if the tryptophan residues are initially perturbed by the addition of cations, further perturbation is not observed when PEP is added. Yet PEP binds to the enzyme and in the presence of non-activator cations can cause the perturbation of the tryptophan residues. They conclude, therefore, that the

tryptophan residues do not appear to be associated directly with the enzyme's catalytic site but are perturbed indirectly as a result of changes in the protein conformation (Kayne and Suelter, 1965).

In addition to the ultraviolet difference spectra, Kayne and Suelter (1965) reported some preliminary physical studies with pyruvate kinase in different cation environments. From a study of the movement of the enzyme in an analytical ultracentrifuge they reported an S value of 9.33 for pyruvate kinase in a solution containing 0.1 M potassium chloride and 0.001 M magnesium chloride. In comparison, the S value for pyruvate kinase in 0.103 M TMA chloride was 9.22. No significant difference was observed in the optical rotatory properties of the enzyme under conditions essentially the same as those uses for the analytical ultracentrifugation experiments. From these results they concluded that the portion of the enzyme molecule involved in the univalent-cation-induced conformation change is a relatively small fraction of the entire molecule.

Immunoelectrophoretic Investigations

Sorger, Ford and Evans (1965) have studied the effect of a series of univalent cations on the immunoelectrophoretic behavior of pyruvate kinase. When the immunoelectrophoresis was conducted in a medium containing activator cations, potassium, rubidium or

sodium, a simple immunoelectrophoretic pattern was obtained. In the presence of non-activator cations, lithium or Tris, a more complex pattern was observed consisting of a series of additional bands not seen in the pattern with activator cations. By changing the cationic environment in the buffer or agar gel the immunoelectrophoretic pattern of the enzyme could be altered from one form to the other. Additional experiments with catalase, an enzyme that does not have a requirement for univalent cations, showed no difference in the immunoelectrophoretic patterns due to different cationic environments. Furthermore, experiments in which pyruvate kinase was centrifuged through sucrose density gradients, some of which contained activator cations and others non-activator cations, revealed no differences in sedimentation due to the cation environment. These results indicate that the univalent cations affect the protein conformation and not its state of aggregation. The studies are in agreement with the kinetic results of Melchior (1965) indicating that univalent cations change the enzyme conformation and that divalent cations or substrates are not necessary for this effect.

Nuclear Magnetic Resonance Studies

Mildvan and Cohn (1964, 1966) and Cohn (1963) have examined the interaction of metal ions with pyruvate kinase using nuclear magnetic resonance. In the presence of the paramagnetic ion,

manganese, the longitudinal nuclear magnetic relaxation rate of the protons of water is increased. In the presence of the enzyme the relaxation rate is further enhanced. The enhancement in the presence of the enzyme is ascribed to a restriction in the relative rotational motion of the hydration shell of a paramagnetic ion when the ion is bound to an external site on the protein molecule. This leads to an increase in the rotational correlation time of the magnetic interaction between the nuclear spin of the protons of the hydrated shell, and the electron spin of the paramagnetic ion (Mildvan and Cohn, 1963). While Mildvan and Cohn's studies have been concerned primarily with the interactions of divalent cations with the enzyme, they have reported, in an abstract (Mildvan and Cohn, 1964), an effect of potassium ion on the relaxation rate of water protons. The effect of potassium was associated with the ternary complex of enzyme-Mn^{++}-PEP. This was interpreted to mean that potassium in the environment of the enzyme changes the conformation of the protein around the metal binding site, only when the substrate is present. The chemical basis for this change was not considered. Their results suggest that the conformation at the active site is altered by potassium in such a way as to influence strongly the binding of the substrate, PEP (Cohn, 1963).

Studies with Other Enzyme Systems

Evans and Sorger (1966) have listed over 40 enzyme systems which have been shown to be stimulated by or have an absolute requirement for univalent cations. The role of univalent cations in the mechanism of enzyme action has been considered in a very few of these enzyme catalyzed reactions. Happold and Beechey (1958) have studied the requirement of typtophanase from E. coli for univalent cations. The enzyme is active in the presence of potassium, ammonium and rubidium but is inactive in a reaction medium containing appreciable concentrations of sodium or lithium. On the basis of kinetic data they concluded that activator univalent cations are affecting the organization of the enzyme molecule possibly by a critical displacement of certain components of the enzyme molecule. Similar to the conclusion of Kachmar and Boyer (1953), they suggested that the difference in the capacity of univalent cations to function as activators may be explained by a consideration of the hydrated radii of the univalent cations. They listed the hydrated radii of univalent cations, in accordance with the calculations of Latimer (1955). Based on Latimer's studies, Happold and Beechey added 0.85 Å to the ionic radii values listed by Pauling (1953) to obtain a nearer approximation of the hydrated radii in aqueous solution. While the list of values for the hydrated ionic radii used by

Happold and Beechey, and by Kachmar and Boyer both may be correlated with the capacity of univalent cations to activate, the comparable values for the same ions in the two lists differ considerably.

Evans, Clark and Russell (1964) working with acetic thiokinase from yeast have shown that dialyzed enzyme requires potassium, ammonium or rubidium for catalysis of the reaction in which acetyl-CoA and AMP are formed from adenyl acetate and CoA. Cesium ions resulted in intermediate activity, while sodium and lithium were almost completely ineffective. From a logical consideration of the kinetic data it was concluded that the univalent cation must be influencing the conformation of the protein. These workers as well as Hiatt (1964) showed that potassium affected the V_{max} of the reaction but not the K_m for the substrates, CoA and adenyl acetate. For an interpretation of their results Evans, Clark and Russell used the following well known equation:

$$V_{max} = k_3 E_t$$

In this equation V_{max} is the maximum velocity of the reaction, k_3 is the rate constant for the formation of product from substrate-enzyme intermediate, and E_t is the total number of active sites on the enzyme. Since $K_m = (k_2 + k_3)/k_1$ it would be probable that a change in k_3 would also affect the K_m. They showed however that K_m is not affected by the univalent cation. Thus, the alternative

possibility is that V_{max} changes due to changes in E_t, the total amount of enzyme. They concluded that the most probable explanation is that univalent cations are affecting the number of active sites on the enzyme by inducing a conformation change.

Similar kinetic results have been observed with the potassium-activated acetaldehyde dehydrogenase from yeast (Stoppani and Milstein, 1957; Sorger and Evans, 1966). This enzyme is activated by potassium and rubidium, whereas, sodium and lithium antagonize the activation, apparently by competitive inhibition with potassium. Stoppani and Milstein (1957) demonstrated that the enzyme was activated by certain thiol compounds but the sensitivity varied according to the univalent cation present. Activator cations, potassium and rubidium diminished the effect of thiol reagents, whereas, non-activator cations increased the effectiveness. Sorger and Evans (1966) showed that potassium and rubidium protect acetaldehyde dehydrogenase from heat inactivation, but sodium and lithium were less effective as protectants. In addition, after prolonged dialysis in Tris, which caused the enzyme activity to decrease, the enzyme could be reactivated by incubation in appropriate solutions containing univalent cations. The order of effectiveness as reactivators is potassium, rubidium, sodium and lithium. As an explanation for their results the authors suggested that the conformation of the enzyme molecule in activator cations is different from that in

the presence of non-activator univalent cations, and that the enzyme is more stable in the presence of the activator than in the non-activator cations.

MATERIALS AND METHODS

Source and Purity of Chemicals

Crystalline pyruvate kinase was obtained commercially (California Biochemical Company and Sigma Chemical Company) as a suspension in 2.1 M $(NH_4)_2SO_4$. Salts used in all studies were reagent grade and in certain experiments were further purified by extraction with dithizone (Stout and Arnon, 1939). Tricine was obtained from California Biochemical Company, disodium ethylenediaminetetraacetate (EDTA) from Baker Chemical Company, mercaptoethanol (MCE) from Eastman Kodak Corporation, phosphoenol-pyruvate (PEP) from Nutritional Biochemical Company, and the barium salt of adenosine diphosphate (ADP) from Sigma Chemical Company. Tris ADP was prepared from the barium salt of ADP as described by Miller and Evans (1957).

Standard Assay Procedure

Pyruvate kinase activity was assayed by the method of Miller and Evans (1957) as modified from Kachmar and Boyer (1953). The assay involved the spectrophotometric determination of the 2,4-dinitrophenylhydrazine derivative of pyruvate. A final reaction mixture of one ml contained the following constituents in micromoles: potassium phosphate buffer pH 7.4, 50; cyclohexylamine PEP, 1.5;

Tris ADP, 2.5; $MgSO_4$, 8. A volume of 0.1 ml of enzyme solution with a protein concentration typically between 0.3-0.5 μg/ml was added. Under these assay conditions, the enzyme activity was linear with time and enzyme concentration.

Stability in Different Salts

In order to minimize the effect of endogenous salts in these experiments the enzyme was dialyzed for 3 hours against 2 liters of a solution containing 0.01 M potassium phosphate buffer at pH 7.4 and 0.001 M EDTA. At the end of 3 hours the buffer solution was changed and the dialysis continued for 3 additional hours. In the investigations of the effect of cations on the stability of pyruvate kinase the dialyzed enzyme was incubated in solutions of 0.05 M phosphate salts at pH 7.4 of the various univalent cations. Aliquots of the enzyme solution were removed at intervals and assayed for activity. The incubation mixtures were kept in an ice bath during the course of the experiment.

Ultraviolet Difference Spectra

Unless otherwise stated, pyruvate kinase, suspended in 2.1 M $(NH_4)_2SO_4$ was collected by centrifugation at 37,000 g for 10 min., dissolved in 0.05 M Tricine buffer, pH 7.4 containing 0.001 M MCE and passed through a column (20 cm by $1\frac{1}{2}$ cm) of Bio-Gel P-2,

50-150 mesh, equilibrated with 0.05 M Tricine buffer pH 7.4 containing 0.001 M MCE. The final protein concentration in each cuvette was 2 mg/ml. The specific activity of the enzyme ranged from 125-200 μmoles pyruvate/min/mg protein. Difference spectra were measured in matched cylindrical silica cuvettes of 1 cm path using a Cary model 11 spectrophotometer. A special slide wire was utilized that showed full scale deflection of the instrument at an absorbancy of 0.1 O. D. A scan speed of 10 Å per second was employed. Cuvettes were cleaned with a solution containing 50% alcohol and 1.0 N HCl and were rinsed thoroughly with glass-distilled water. Spectra were corrected for small absorbancy changes in solvent blanks which could not be eliminated by multipot adjustment.

In a typical experiment 2 ml of enzyme solution containing 3 mg/ml of pyruvate kinase and 0.05 M Tricine buffer pH 7.4 was added to two 3 ml cuvettes. In the sample cuvette 1 ml of 0.3 M activator univalent cation solution was added and in the reference cuvette 1 ml of a 0.3 M non-activator univalent cation solution was added, the cuvettes were shaken well, and a spectral scan was made between 280 mμ and 320 mμ. In those experiments where higher concentrations of salts were studied, microliter quantities of concentrated salts were added to the sample and reference cuvettes, the solutions mixed thoroughly, and a spectral scan made between 280 mμ and 320 mμ. In control experiments the same procedure

was followed with the exception that 2 ml of 0.05 Tricine buffer replaced the enzyme solution.

Analytical Ultracentrifugation Studies

For the preparation of the enzyme for the analytical ultracentrifugation experiments, pyruvate kinase was dialyzed against 2 liters of 0.05 M univalent cation phosphate buffer pH 7.4 for 3 hours; then the buffer discarded and the dialysis continued in 2 liters of new buffer for 3 hours. Sedimentation coefficients of the enzyme were determined with a Spinco model E analytical ultracentrifuge. In these experiments schlieren optics were utilized for the determination of the protein movement in standard and in wedge cells. The rotor speed was 59, 780 rpm at 2 to 5 °C, and a bar angle of 60°. The sedimentation coefficient values were corrected to standard conditions at 20° in water ($s_{20,w}$). Protein concentrations were determined by measuring absorbancy at 280 mμ using the following equation:

$$E = \frac{A}{cl} M.$$

In this equation E equals the molecular extinction coefficient, A equals the absorbancy, c is the concentration of the absorbing material in mg/ml, the letter l is the distance traveled by the light through the solution, and M is the molecular weight. For

pyruvate kinase the molar extinction coefficient is 1.28×10^{-5} (Bücher and Pfleiderer, 1955) and the molecular weight is 237,000 (Warner, 1958). The slope and the y intercept for the sedimentation coefficient at zero protein concentration were determined by the least squares method.

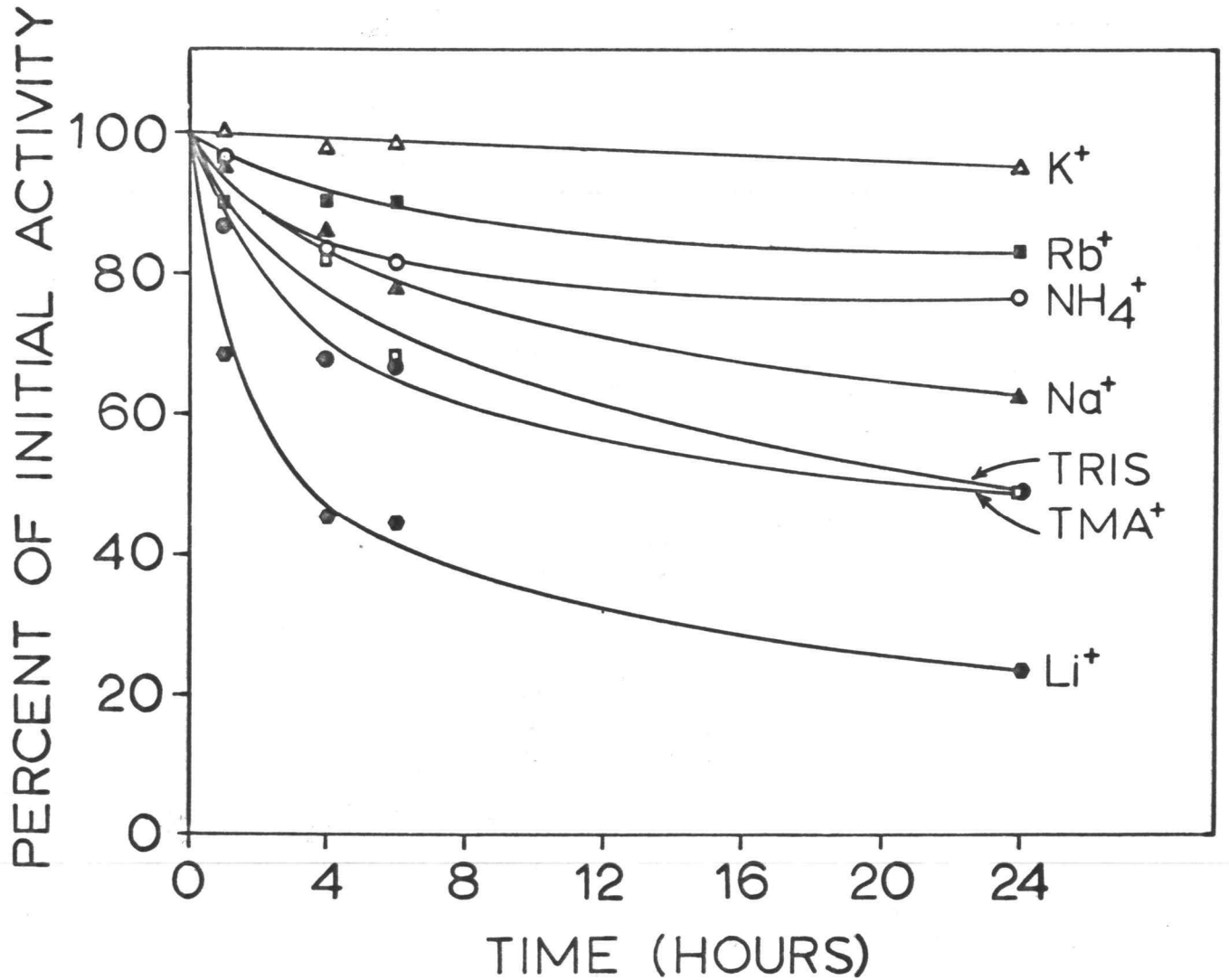
RESULTS

Stability in Solutions of Different Salts

Effect of Univalent Cations

Before the effect of a series of univalent cations on various physical properties of pyruvate kinase could be studied, it was necessary to determine their effect on the stability of the enzyme. Pyruvate kinase was incubated in a series of solutions containing different univalent cations and 0.001 M MCE. The activity of the enzyme in each solution was measured at intervals during a 24 hr period. As the results in Figure 1 show, the stability of the enzyme is markedly influenced by the species of univalent cation present in the incubation mixture. When incubated in solutions containing univalent cations which activate pyruvate kinase, the activity of the enzyme remained relatively high. The univalent cations in this group include potassium, rubidium and ammonium. In contrast to the effect of activator cations, incubation of the enzyme in solutions containing non-activator cations, resulted in a loss within 24 hrs of over 50% of the initial activity. The univalent cations in this group include Tris, TMA and lithium. Sodium has been reported (Kachmar and Boyer, 1953) to weakly activate pyruvate kinase. It is of interest, therefore, that the capacity of sodium salts to stabilize the enzyme

Figure 1. The effect of different univalent cation phosphate salts on the stability of pyruvate kinase. Dialyzed pyruvate kinase (3.25 mg/ml) in a solution of 0.01 M MCE was diluted 7500-fold in the above buffer solution. Univalent cation phosphate salts, pH 7.4, were added to aliquots of the enzyme solution to make a final concentration of 0.05 M with regard to the univalent cation. Samples of the enzyme were withdrawn at the indicated intervals and assayed for activity by the standard procedure.


PERCENT OF INITIAL ACTIVITY
100
80
60
40
20
0
0
4
8
12
16
20
24
TIME (HOURS)
K+
Rb+
NH4+
Na+
TRIS
TMA+
Li+

during incubation, is intermediate between that of activator and non-activator. Furthermore, it was observed that the addition of 0.001 M MCE to each univalent cation incubation mixture resulted in increased stability of the enzyme. As can be seen from Table 1, incubation mixtures containing a series of different univalent cations and 0.001 M MCE showed between 12% to 28% greater activity than a comparable series of incubation mixtures without MCE.

An experiment was conducted to determine the effect of potassium on the reactivation of pyruvate kinase incubated in Tris and lithium (Table 2). When pyruvate kinase was incubated for 24 hr in the phosphate salts of Tris and lithium, the activity of the enzyme decreased to 38% and 8%, respectively, of the initial activity. After the enzyme had been incubated for 24 hr in Tris and in lithium, a final concentration of 0.05 M potassium phosphate was added to each of the two mixtures. After this treatment, the incubation was continued for an additional 24 hr. A partial restoration of the enzyme activity resulted. The addition of potassium to the Tris incubation mixture resulted in an increase from 38% to 59% of the original activity. When potassium was not added to the Tris or lithium incubation mixtures after 24 hr, enzyme activity continued to decrease. After 48 hr there was 33% of the initial activity in the Tris incubation mixture, and 5% of the initial activity in the lithium incubation mixture. In comparison to these findings, the addition

Table 1. The effect of mercaptoethanol on the stability of pyruvate kinase in different univalent cation salts.

Dialyzed pyruvate kinase (3.25 mg/ml) in a solution of 0.01 M potassium phosphate buffer pH 7.4 containing 0.001 M EDTA was diluted 7500-fold in the above buffer solution. Univalent cation phosphate salts pH 7.4 were added to aliquots of the enzyme solution to make a final concentration of 0.05 M with regard to the univalent cation. In the mercaptoethanol treatment the enzyme was maintained in 0.001 M MCE throughout the procedure. Samples of enzyme were withdrawn after 24 hr and assayed for activity by the standard procedure.

Univalent cation	Enzyme activity after 24 hr[a]		Increased activity due to MCE[b] (%)
	-MCE (%)	+MCE (%)	
K^+	64	92	28
NH_4^+	50	77	27
N_a^+	42	62	20
$Tris^+$	31	49	18
TMA^+	34	49	16
Li^+	11	23	12

[a] Percent enzyme activity based on initial activity as 100%.

[b] The increase in activity due to MCE is the difference between the percent activity after 24 hr in the presence of MCE versus its absences.

Table 2. Effect of univalent cations on the stability of pyruvate kinase.

Dialyzed pyruvate kinase (2.1 mg/ml) in a solution of 0.01 M potassium phosphate buffer pH 7.4 containing 0.001 EDTA was diluted 500-fold in the above buffer solutions. Univalent cation phosphates pH 7.4 were added to the enzyme solution to make a final concentration of 0.05 M. After 24 hrs incubation samples of the enzyme were withdrawn, and assayed for activity by the standard procedure. Each univalent cation solution was then divided into two aliquots, and to one was added a final concentration of 0.05 M potassium phosphate pH 7.4. After an additional 24 hrs incubation, a sample of enzyme was withdrawn from each solution and assayed for activity by the standard procedure.

Cation phosphate in enzyme solution during incubation		Enzyme activity remaining	
0-24 hr	24-48 hr	after 24 hr (%)[a]	after 48 hr (%)
0.05 M K^+	0.05 M K^+	61	59
0.05 M K^+	0.10 M K^+	61	58
0.05 M $Tris^+$	0.05 M $Tris^+$	38	33
0.05 M $Tris^+$	0.05 M $Tris^+$ + 0.05 M K^+	38	59
0.05 M Li^+	0.05 M Li^+	8	5
0.05 M Li^+	0.05 M Li^+ + 0.05 M K^+	8	37

[a] Expressed as % of initial activity.

of 0.05 M potassium phosphate to the enzyme solution, which had previously been incubated for 24 hr in potassium phosphate, had little effect on the enzyme activity (Table 2). These results show that potassium has the capacity to restore partially the loss of pyruvate kinase activity due to incubation in solutions containing Tris or lithium cations.

Effect of Anions

In the course of the studies on the effectiveness of different univalent cations in stabilizing pyruvate kinase, it became apparent that the associated anionic species also influenced the stability. To test this effect pyruvate kinase was incubated in a series of solutions containing different potassium salts, and the activity of the enzyme was determined at intervals over a period of 24 hr. In addition, the effect of the presence of MCE in each incubation mixture also was examined. Enzyme activities remaining in solutions after 24 hr incubation are presented in Table 3. The results show that the anionic species markedly affects the stability of the enzyme. In the absence of MCE the activity of the enzyme after 24 hr incubation in different anionic environments ranged from 21% to 78% of the initial activity. With the exception of the incubation mixture containing pyrophosphate the presence of MCE in each solution tested, resulted in increased stability of the enzyme. The results

Table 3. Stability of pyruvate kinase in solutions containing different anions with and without mercaptoethanol.

Dialyzed pyruvate kinase (3.25 mg/ml) in a solution of 0.01 M potassium phosphate buffer pH 7.4 containing 0.001 M EDTA was diluted 7500-fold in the above buffer solution. Potassium salts of the various anions indicated were added to the enzyme solution to make a final concentration of 0.05 M with regard to the anion. In the MCE treatment the enzyme was maintained in 0.001 M MCE throughout the procedure. After incubation for 24 hr in an ice bath, samples of the enzyme were withdrawn and assayed for activity by the standard procedure. The results presented are an average of those obtained in two separate experiments.

Anion added to incubation mixture[a]	Enzyme activity after 24 hr[b]		Increased activity due to MCE[c]
	-MCE (%)	+MCE (%)	(%)
Pyrophosphate	78	77	-1
Sulfate	68	75	7
Phosphate	65	88	23
Arsenate	58	-	-
Acetate	45	61	16
Bromide	37	50	13
Nitrate	23	45	22
Chloride	21	50	29

[a] In a control experiment it was determined that the concentration of potassium, which necessarily varied in the different potassium anion solutions, did not appreciably effect the stability of the enzyme.

[b] Percent enzyme activity based on an initial activity as 100%.

[c] The increase in activity due to MCE is the difference between the percent activity after 24 hr in the presences of MCE versus its absences.

show a striking correlation between the valency of the anionic species and the capacity of the anion to stabilize the enzyme. The enzyme in all cases was more stable in each of the solutions containing multivalent anions than the solutions containing univalent anions. The activity of the enzyme remaining after 24 hr in the multivalent anion solutions ranged from 58% to 78% of the initial activity. The activity remaining after 24 hr in the univalent anion solutions ranged from 21% to 45% of the initial activity.

Difference Spectra Resulting from Salt Environments

Effect of Univalent Cations at 0.1 M Concentration

The technique of ultraviolet difference spectrophotometry has been found to be an extremely sensitive and useful means of studying protein structure (Wetlaufer, 1962; Schellman and Schellman, 1964). Certain analytical ultracentrifugation experiments, to be described later, as well as studies reported by Kayne and Suelter (1965) provide no evidence that univalent cations induce a gross conformation change in pyruvate kinase. If the role of univalent cations in enzyme activation is associated with a conformation change, it seems likely that a relatively small portion of the molecule is influenced. The sensitive technique of difference spectrophotometry was utilized therefore, to examine pyruvate kinase for evidence of

structural changes caused by univalent cation environments.

A series of experiments were conducted to determine the effect of different univalent cation salts on the difference spectrum of pyruvate kinase (Figures 2 to 6). For these experiments and for all other experiments, unless otherwise indicated, Tricine buffer (pH 7.4) at a final concentration of 0.033 M was added to both cuvettes to maintain a constant pH. A comparison was made of pyruvate kinase in a solution of 0.1 M KCl in the sample cuvette versus enzyme in 0.1 M TrisCl in the reference cuvette. The difference spectrum obtained is shown in Figure 2. The absorption maximum at 286 mμ and 295 mμ can be attributed to the perturbation of tryptophan residues (Yanari and Bovey, 1960; Wetlaufer, 1962) and are similar to those reported by Suelter et al. (1966), Kayne and Suelter (1965) and Suelter and Melander (1963) with pyruvate kinase. In this experiment the addition of up to 0.001 M $MgCl_2$ to both cuvettes had no apparent effect on the difference spectrum. In other studies, the concentration of KCl in the sample cuvette and TrisCl in the reference cuvette was increased in increments, and the absorbancy difference at 295 mμ at each concentration was measured. The results are presented in Table 4. As the concentration of salts was increased in both cuvettes, the magnitude of the absorbancy difference at 295 mμ increased correspondingly. Figures 3 to 5 present difference spectra of pyruvate

Figure 2. A difference spectrum of pyruvate kinase in KCl versus the enzyme in TrisCl. Each 3 ml cuvette mixture contained 0.033 M Tricine buffer pH 7.4, 6.7×10^{-4} M MCE, and 6 mg of pyruvate kinase. The final concentration of KCl in the sample cuvette and TrisCl in the reference cuvette was 0.1 M. Similar results were obtained in a duplicate experiment. The pH at the conclusion of the experiment was 7.4 ± 0.05.

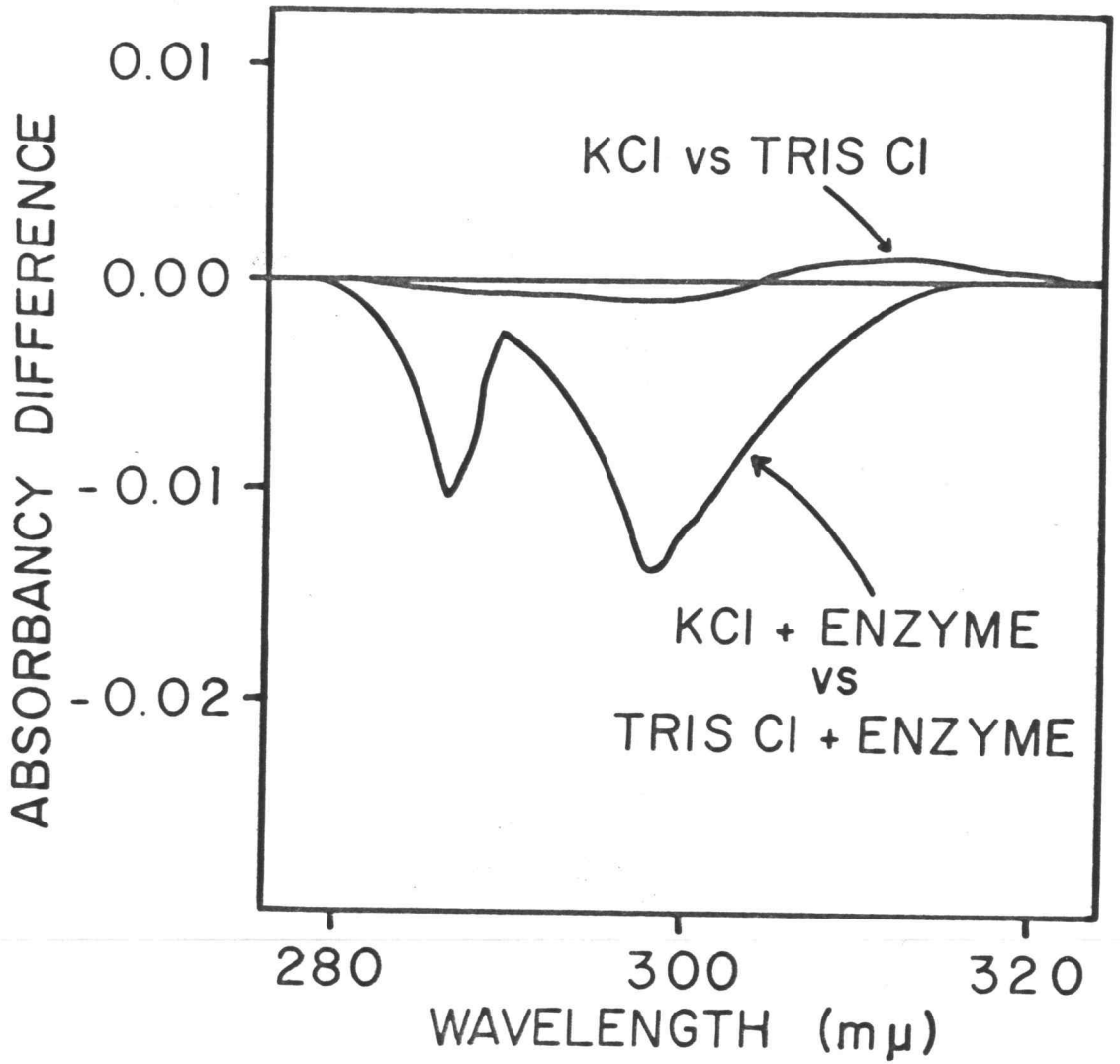

0.01
0.00
-0.01
-0.02
ABSORBANCY DIFFERENCE
KCl vs TRIS Cl
KCl + ENZYME
vs
TRIS Cl + ENZYME
280
300
320
WAVELENGTH (mμ)

Figure 3. A difference spectrum of pyruvate kinase in NH_4Cl versus enzyme in TrisCl. The procedure was identical with that described in Figure 2 with the exception that ammonium replaced potassium in the sample cuvette.

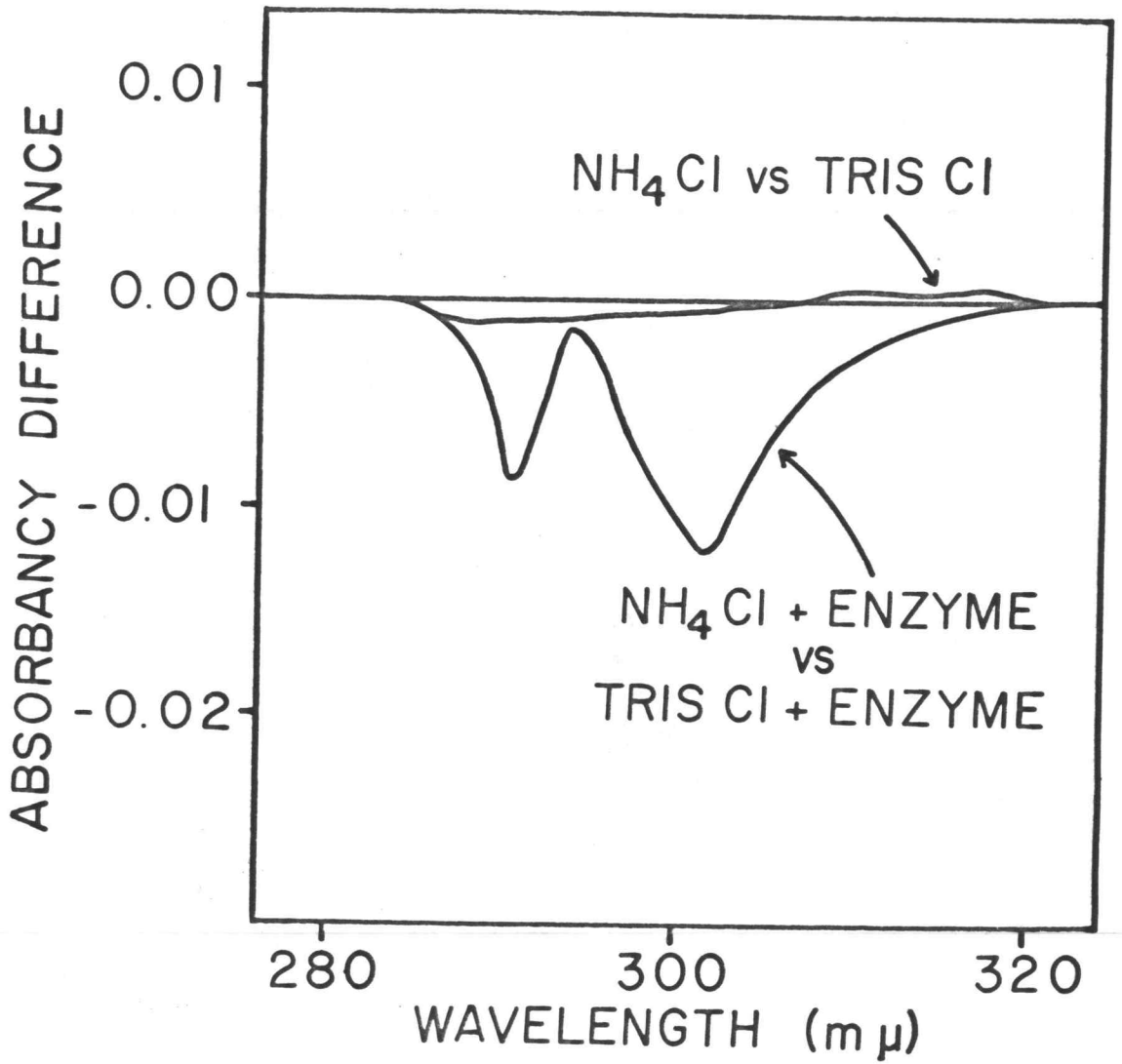

0.01
0.00
-0.01
-0.02
ABSORBANCY DIFFERENCE
NH4 Cl vs TRIS Cl
NH4 Cl + ENZYME
vs
TRIS Cl + ENZYME
280
300
320
WAVELENGTH (mμ)

Figure 4. A difference spectrum of pyruvate kinase in NaCl versus enzyme in TrisCl. The procedure was identical with that described in Figure 2 with the exception that sodium replaced potassium in the sample cuvette.

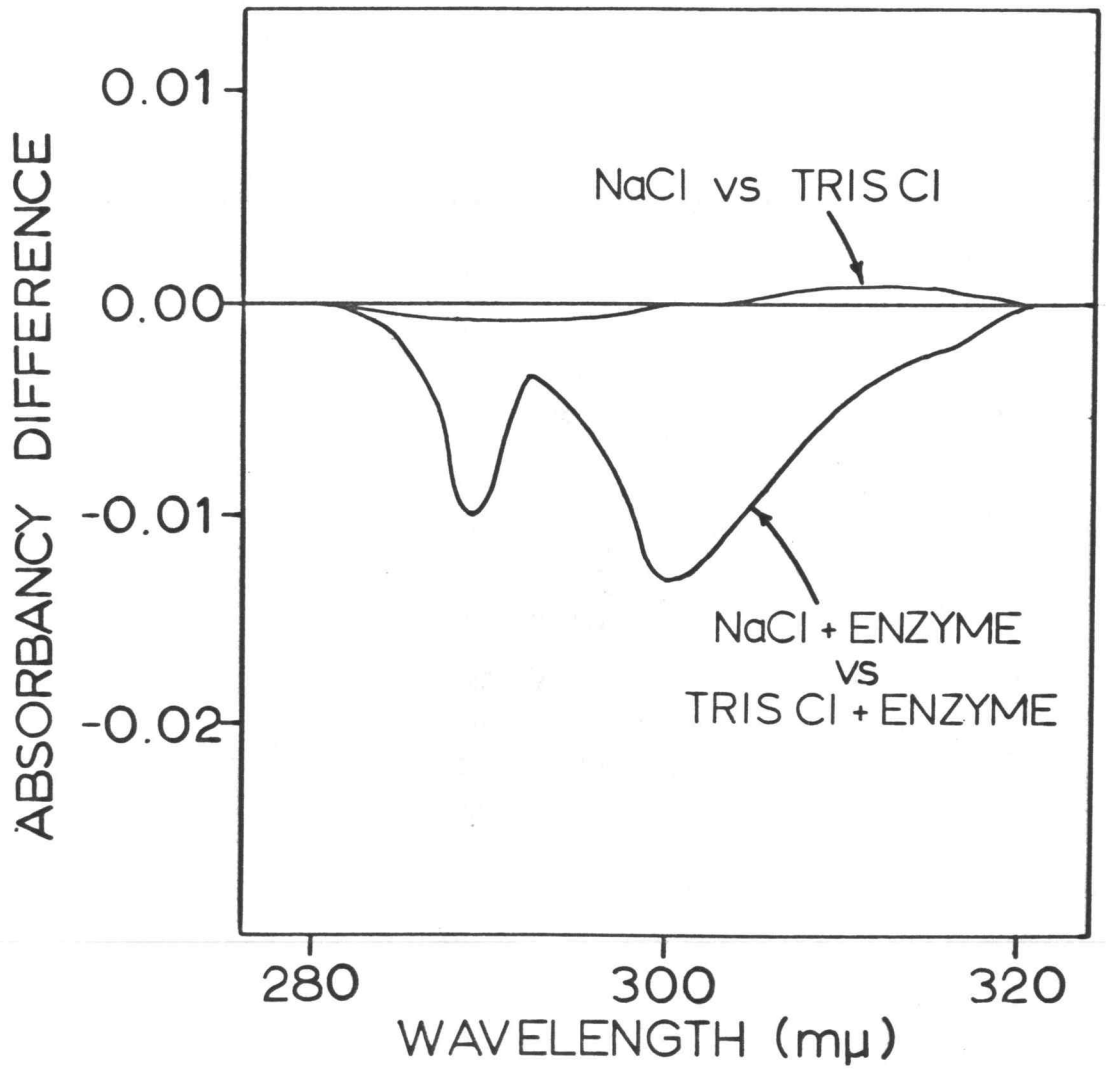

0.01
0.00
-0.01
-0.02
ABSORBANCY DIFFERENCE
NaCl vs TRIS Cl
NaCl + ENZYME
vs
TRIS Cl + ENZYME
280
300
320
WAVELENGTH (mμ)

Figure 5. A difference spectrum of pyruvate kinase in LiCl versus enzyme in TrisCl. The procedure was identical with that described in Figure 2 with the exception that lithium replaced potassium in the sample cuvette.

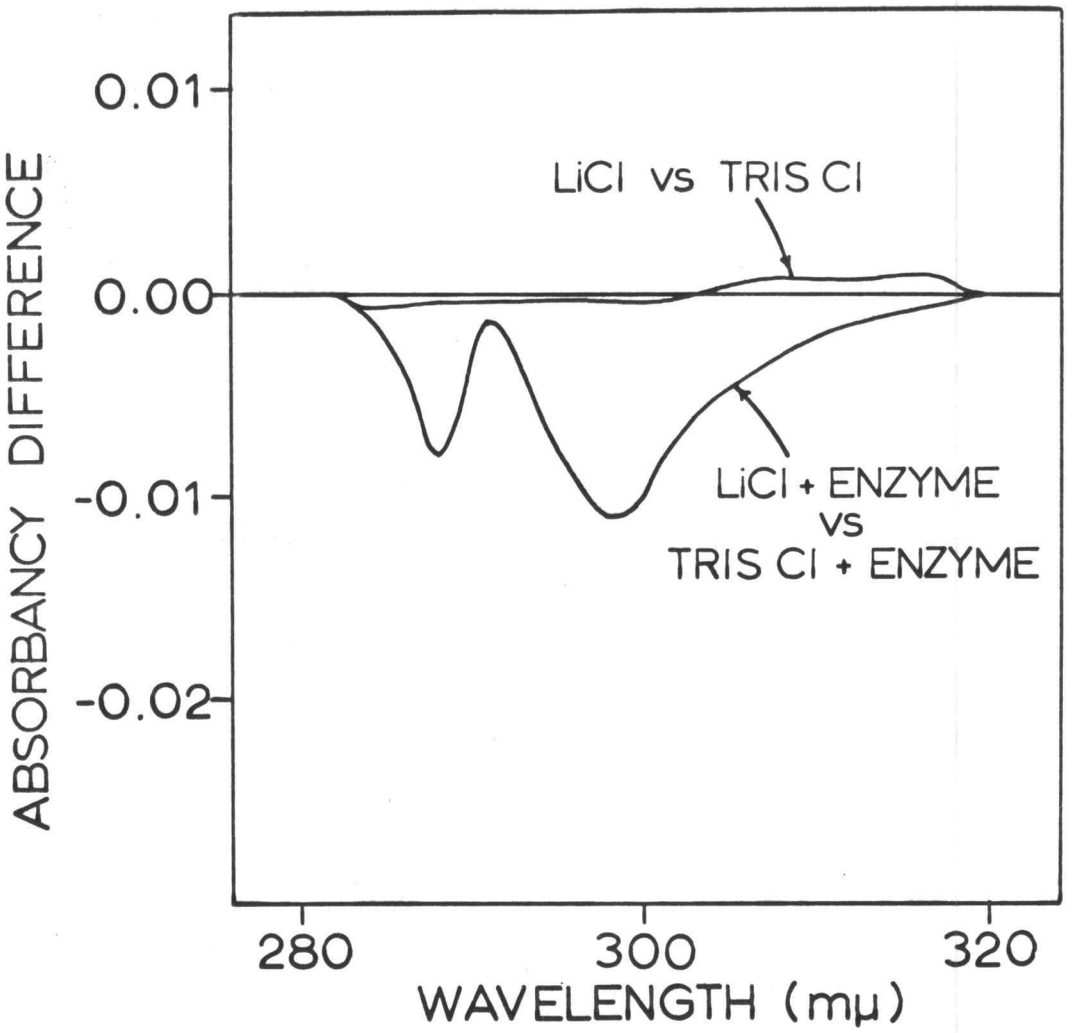

0.01
0.00
-0.01
-0.02
ABSORBANCY DIFFERENCE
LiCl vs TRIS Cl
LiCl + ENZYME
vs
TRIS Cl + ENZYME
280
300
320
WAVELENGTH (mμ)

Figure 6. A difference spectrum of pyruvate kinase in KCl versus enzyme in LiCl. The procedure was identical with that described in Figure 2 with the exception that lithium replaced Tris in the reference cuvette.

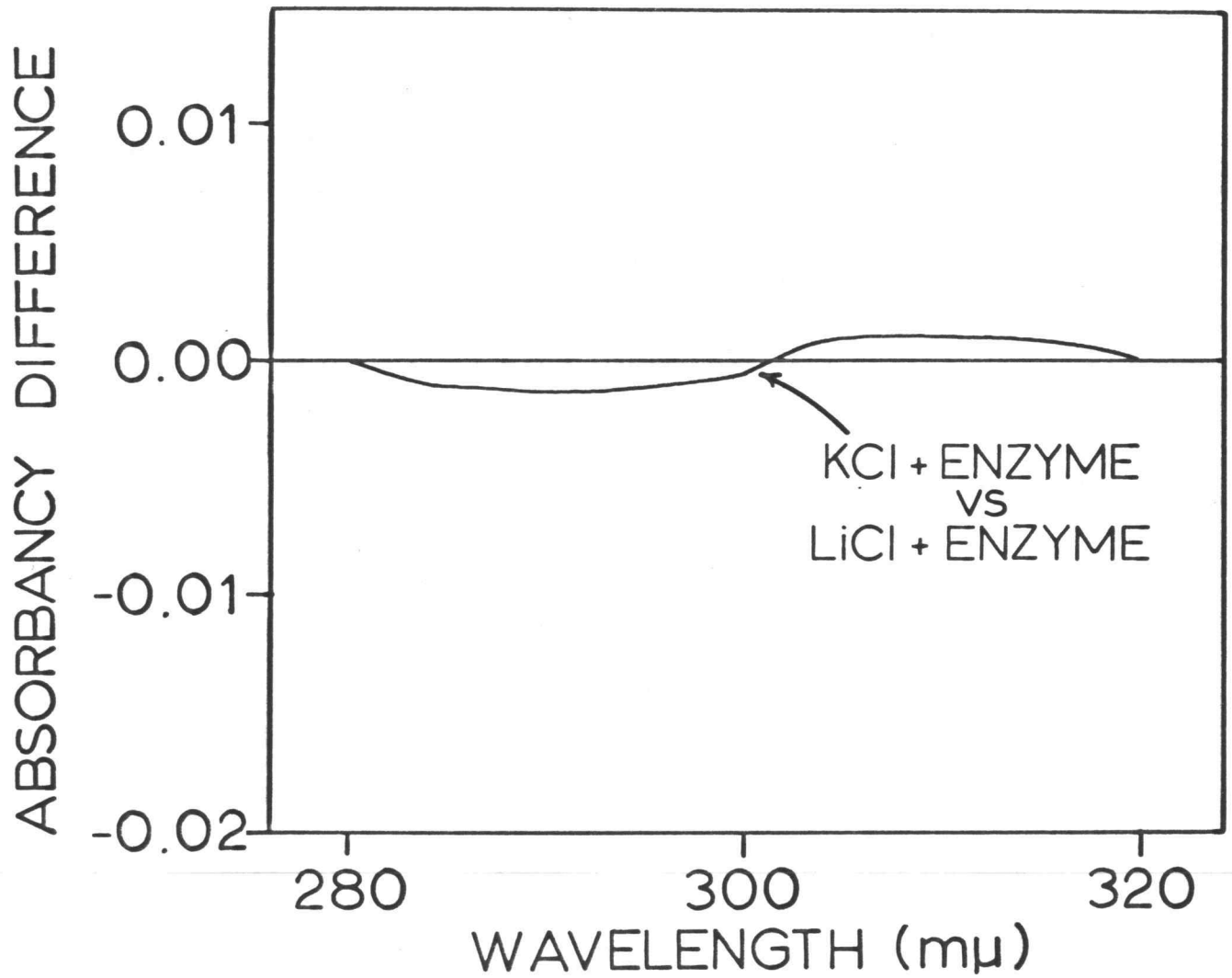

ABSORBANCY DIFFERENCE
0.01
0.00
-0.01
-0.02
280
300
320
WAVELENGTH (mμ)
KCl + ENZYME
vs
LiCl + ENZYME

Table 4. The effect of increasing concentrations of KCl and TrisCl on the absorbancy difference of pyruvate kinase at 295 mμ.

The procedure was identical with figure 2 with the exception that the concentration of KCl in the sample cuvette and TrisCl in the reference cuvette varied as indicated.

Concentration of KCl in the sample cuvette (M)	Concentration of TrisCl in reference cuvette (M)	Absorbancy difference at 295 mμ ($-\Delta$ O. D. $\times 10^3$)
0.000	0.000	0
0.030	0.030	5
0.060	0.060	8
0.090	0.090	11
0.120	0.120	15
0.150	0.150	18
0.170	0.170	19

kinase in a series of solutions of univalent cation chlorides at 0.1 M concentration in the sample cuvette versus solutions of enzyme in TrisCl at 0.1 M concentration in the reference cuvette. The difference spectra represent enzyme perturbations resulting from the presence of ammonium, sodium (activator cations) or lithium (a non-activator cation) in the sample cuvette versus Tris in the reference cuvette. In each comparison a difference spectrum characteristic of the perturbation of the tryptophan residues was obtained. The species of univalent cation present in the sample cuvette, regardless of whether it was an activator or non-activator, did not appear to affect the magnitude of the absorbancy differences at 286 mμ and 295 mμ. In contrast, a difference spectrum is shown in Figure 6 of pyruvate kinase in 0.1 M KCl versus enzyme in 0.1 M LiCl. At a 0.1 M concentration of these two univalent cation salts, no appreciable absorbancy difference was observed.

A study was made of the effect of different univalent cation salts on the difference spectra of pyruvate kinase prepared in phosphate buffer instead of Tricine buffer. The enzyme was dialyzed against 0.01 M potassium phosphate at pH 7.4 and phosphate buffer (final concentration 1.7×10^{-3} M) was used to maintain the pH in both cuvettes. A summary of the absorbancy difference at 295 mμ resulting from the addition of 0.1 M concentrations of salts in solutions of the enzyme is presented in Table 5. The data show

Table 5. The effect of different univalent cations at 0.1 M concentration on the absorbancy difference at 295 mμ of pyruvate kinase in potassium phosphate buffer.

The enzyme was prepared by dialysis for 3 hr against 2 liters of 0.01 M potassium phosphate buffer pH 7.4. After 3 hr the buffer solution was changed and the enzyme was dialyzed for an additional 3 hr against buffer of the same concentration. Each 3 ml cuvette mixture contained a final concentration of 1.7×10^{-3} M potassium phosphate buffer pH 7.4 and 6 mg of pyruvate kinase. A difference spectrum of the 0.1 M salt in the sample cuvette versus 0.1 M salt in the reference cuvette without enzyme was essentially no different from the base line. The values of the absorbancy difference are an average of two readings.

Ion comparison		Concentration of cation (M)	Absorbancy difference at 295 mμ ($-\triangle$ O.D. $\times 10^3$)
Sample cuvette	Reference cuvette		
KCl	TrisCl	0.1	12
NH_4Cl	TrisCl	0.1	12
NaCl	TrisCl	0.1	11
LiCl	TrisCl	0.1	11
TrisCl	TrisCl	0.1	0
KCl	LiCl	0.1	0
KCl	NaCl	0.1	0
KCl	NH_4Cl	0.1	0

absorbancy differences at 295 mμ of pyruvate kinase in a series of solutions of different univalent cations in the sample cuvette versus enzyme in a solution of TrisCl in the reference cuvette. No absorbancy differences at 295 mμ were observed in comparisons of pyruvate kinase in a solution of KCl in the sample cuvette versus enzyme in solutions of LiCl, NaCl or NH_4Cl in the reference cuvette. Furthermore, the magnitude of the absorbancy differences at 295 mμ in the difference spectra in which Tricine buffer was used (Figures 2 to 6), are comparable with the magnitudes of the absorbancy differences at 295 mμ in the difference spectra in which Tricine buffer was used (Figures 2 to 6), are comparable with the magnitudes of the absorbancy differences at 295 mμ in the difference spectra obtained in experiments in which potassium phosphate buffer was used (Table 5).

The absorbancy differences resulting from addition of 0.1 M concentration of univalent cation salts to the enzyme can not be correlated with the capacity of univalent cations to activate. For example, pyruvate kinase in a solution containing 0.1 M potassium chloride, an activator cation salt versus enzyme in a solution of an equal concentration of lithium chloride, a non-activator cation salt, gave no absorbancy difference at 295 mμ, whereas, enzyme in a solution of lithium chloride at 0.1 M versus enzyme in a solution of 0.1 M Tris chloride, both non-activator salts, resulted in a tryptophanyl

difference spectrum. The results indicate, therefore, that 0.1 M Tris, in contrast with other univalent cations tested at the same concentration, is affecting the enzyme's structure resulting in the perturbation of tryptophan residues.

Effect of Different Concentrations of Univalent Cations

As shown in Figure 6, a difference spectrum of pyruvate kinase in 0.1 M KCl versus enzyme in 0.1 M LiCl produced no absorbancy difference at 295 mμ. If the concentration of KCl in the sample cuvette and LiCl in the reference cuvette was increased above 0.1 M, the results shown in Figure 7 were obtained. As the salt concentration in both cuvettes was increased in increments from 0.1 M to 0.86 M, the absorbancy difference at 286 mμ and 295 mμ also increased. In Figure 8 difference spectra are shown of pyruvate kinase in solutions of increasing concentrations of NaCl versus enzyme in solutions of increasing concentrations of LiCl. Similar to results presented in Figure 7, the magnitude of the absorbancy difference at 286 mμ and 295 mμ increased with increasing concentrations of salts. The difference spectra shown in Figures 7 and 8 are qualitatively identical to those in Figures 2 to 5, and may be attributed to the perturbation of tryptophan residues.

In other experiments, pyruvate kinase in solutions containing

Figure 7. The effect of KCl versus LiCl at a series of concentrations on the difference spectra of pyruvate kinase. Each 3 ml mixture contained 0.033 M Tricine pH 7.4, 6.7×10^{-4} M MCE, 6 mg of pyruvate kinase and a concentration of KCl in the sample cuvette and LiCl in the reference cuvette as indicated. A difference spectrum of KCl versus LiCl without enzyme at each of the indicated concentrations was essentially no different from the base line. The experiment was repeated and the results were essentially identical with those presented. The pH of the reaction mixture at the conclusion of the experiment was 7.4 ± 0.05.

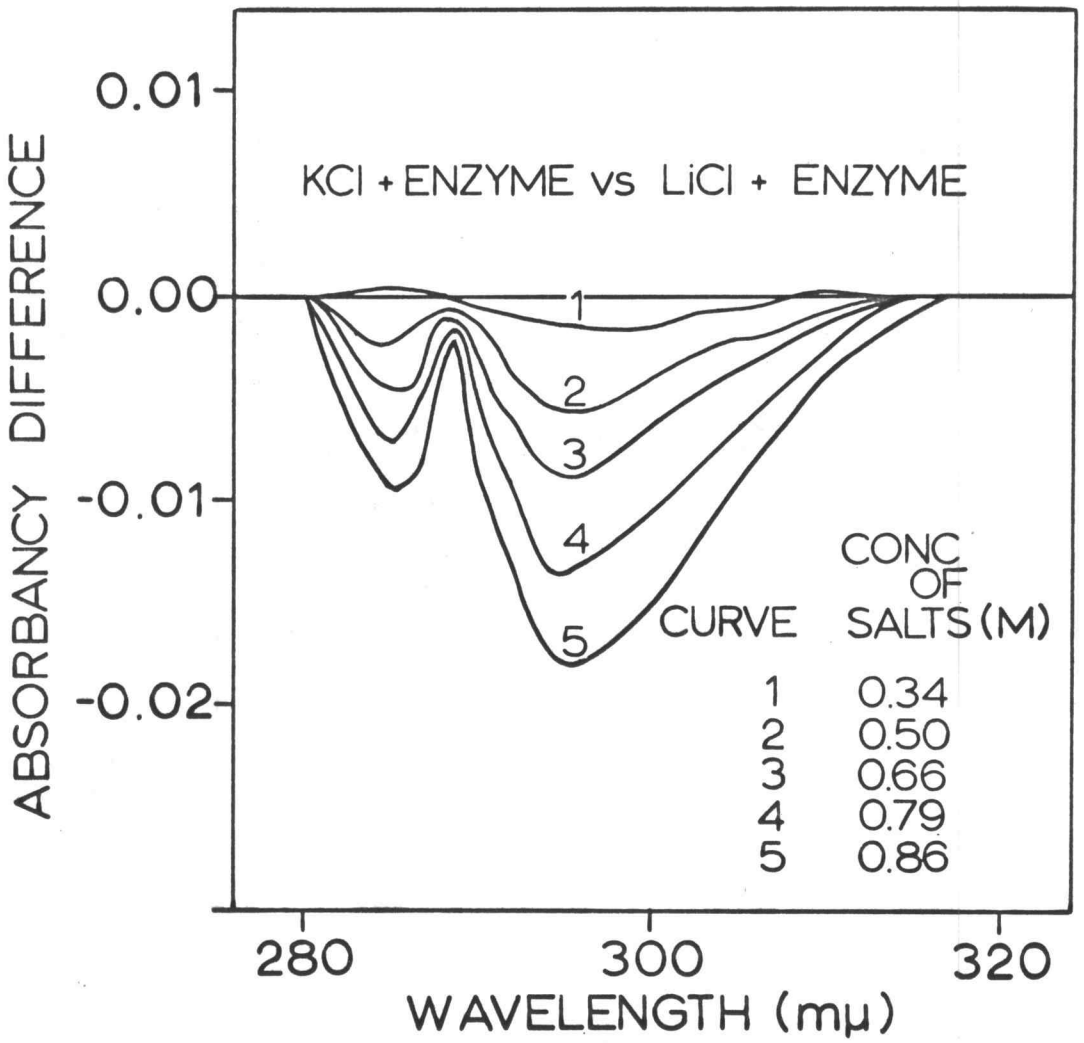
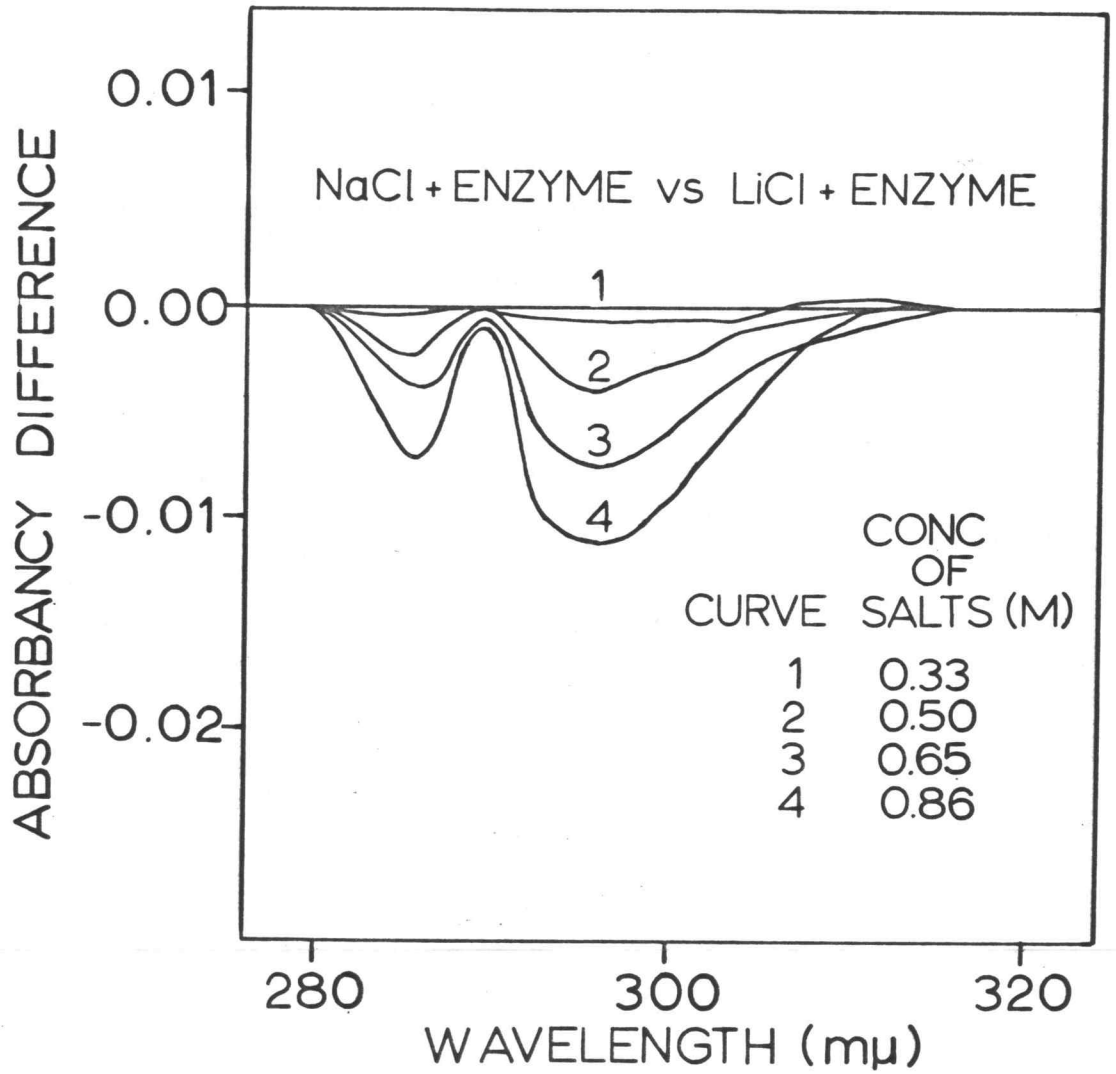

KCl + ENZYME vs LiCl + ENZYME
ABSORBANCY DIFFERENCE
0.01
0.00
-0.01
-0.02
1
2
3
4
5
CONC OF SALTS (M)
CURVE
1 0.34
2 0.50
3 0.66
4 0.79
5 0.86
280
300
320
WAVELENGTH (mμ)

Figure 8. The effect of NaCl versus LiCl at different concentrations on the difference spectra of pyruvate kinase. The procedure was identical with that described in Figure 7 with the exception that sodium replaced potassium in the sample cuvette.


NaCl + ENZYME vs LiCl + ENZYME
0.01
0.00
-0.01
-0.02
ABSORBANCY DIFFERENCE
1
2
3
4
CONC OF SALTS (M)
CURVE
1 0.33
2 0.50
3 0.65
4 0.86
280
300
320
WAVELENGTH (mμ)

different univalent cations at increasing concentrations were compared for their capacity to produce the tryptophanyl difference spectra. As shown in Table 6, absorbancy differences for all ion comparisons except potassium versus rubidium, both activators, increased with increasing concentrations of salts in the two cuvettes. Furthermore, at any one concentration of salt, it appears that the magnitude of the absorbancy difference was greatest when the enzyme was compared in a solution of univalent cation which results in maximum activity versus enzyme in a solution of univalent cation which completely fails to activate. For example, a difference spectrum of pyruvate kinase in a solution containing potassium, an activator, versus enzyme in solution containing lithium, a non-activator, consistently showed a relatively large absorbancy difference that increased with increasing concentrations of salt tested. In comparison, difference spectra of pyruvate kinase in solutions of potassium versus enzyme in solutions of sodium, a cation with a slight capacity to activate (Kachmar and Boyer, 1953), produced absorbancy differences at each concentration of salt tested that were smaller than those produced by enzyme in KCl versus enzyme in LiCl. Finally, a comparison of pyruvate kinase in a solution containing potassium versus enzyme in a solution containing rubidium showed no differences in absorbancy at all concentrations tested. These results obtained by use of salt solutions at relatively high concentrations

Table 6. A comparison of the effect of different univalent cations at a series of concentrations on the difference in absorbancy at 295 mμ of pyruvate kinase.

The procedure was identical with that described for figure 7 with the exception that the univalent cations were varied as indicated. The absorption differences at 295 mμ resulting from a comparison of the different cations at each concentration without enzyme were within experimental error. The results presented are an average of at least two separate experiments. In each experiment two special scans were recorded at each concentration and these absorbancy differences were averaged for each experiment.

Ion comparison		Absorbancy difference at 295 mμ ($-\Delta$ O. D. $\times 10^3$)					
Sample cuvette	Reference cuvette	Concentration of cation chloride (M)					
		0.10	0.33	0.50	0.66	0.79	0.86
K^+	Li^+	0	2	5	8	12	17
Rb^+	Li^+	0	1	7	11	13	18
K^+	TMA^+	0	2	4	7	11	16
K^+	Na^+	0	0	2	5	6	7
Na^+	Li^+	0	0	3	7	11	13
K^+	Rb^+	0	0	0	0	0	0

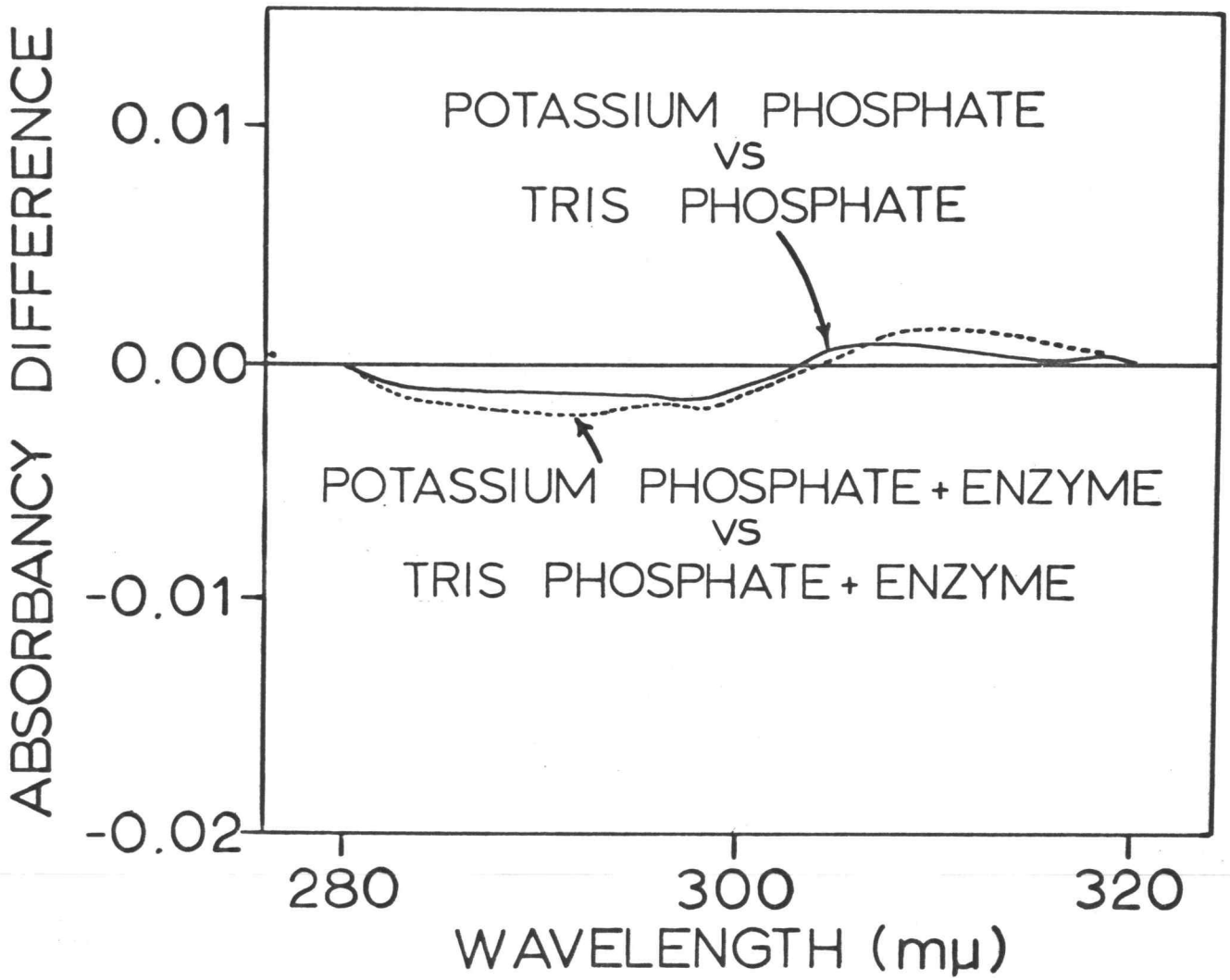
indicate an inverse correlation between the capacity of univalent cations to activate pyruvate kinase and the capacity to perturb the tryptophan residues.

The Effect of Anions on the Difference Spectrum in Solutions of Potassium Versus Solutions of Tris

Figure 9 shows a difference spectrum of pyruvate kinase in 0.1 M potassium phosphate versus enzyme in 0.1 M Tris phosphate. The spectrum shows no significant absorbancy differences. When the concentrations of potassium phosphate in the sample cuvette and Tris phosphate in the reference cuvette were increased in increments over a range from 0.05 M to 0.40 M in both cuvettes, a series of difference spectra essentially the same as the spectrum presented in Figure 9 were obtained. These results are in contrast with the previously mentioned difference spectra obtained with pyruvate kinase in a solution of potassium chloride versus enzyme in Tris chloride (Table 4). Apparently the presence of relatively high concentrations of phosphate prevents the absorbancy differences. The concentration of phosphate in these experiments is over 10-fold greater than the concentration of phosphate in the experiments summarized in Table 5, where phosphate buffer was used to maintain the pH in both cuvettes.

Since the anionic species associated with univalent cations appeared to influence the extent of perturbation of pyruvate kinase,

Figure 9. The effect of potassium phosphate versus Tris phosphate on the difference spectrum of pyruvate kinase. The procedure was identical with that described in Figure 2 with the exception that the phosphate salts of potassium and Tris were used.


ABSORBANCY DIFFERENCE
0.01
0.00
-0.01
-0.02
POTASSIUM PHOSPHATE
VS
TRIS PHOSPHATE
POTASSIUM PHOSPHATE + ENZYME
VS
TRIS PHOSPHATE + ENZYME
280
300
320
WAVELENGTH (mμ)

a series of difference spectra of enzyme were recorded in different anionic salts of potassium and Tris. The results, presented as absorbancy differences at 295 mμ, are summarized in Table 7. It is obvious that the anionic species have a striking effect on the perturbation of tryptophan residues of pyruvate kinase. When the multivalent anions, phosphate, sulfate and arsenate were associated with potassium in the sample cuvette and compared with the enzyme in Tris salts of the same group of multivalent anions in the reference cuvette, no absorbancy difference at 295 mμ was observed. When the univalent anions, chloride, nitrate or acetate were associated with potassium in solutions of the enzyme in the sample cuvette and compared with enzyme in Tris salts of the same group of anions in the reference cuvette, absorbancy differences at 295 mμ were observed consistently. The results show that perturbation of tryptophan residues on the enzyme by univalent cations is dependent upon the presence of univalent anions in the environment of the enzyme.

Although it has been shown that the anionic environment of pyruvate kinase strongly influences the perturbation of tryptophan residues in the experiments reported thus far, the anion in the sample cuvette has been the same as the anion in the reference cuvette. The experiments summarized in Table 8 demonstrate the effect of different combinations of chloride and phosphate salts of potassium in the sample cuvette and Tris in the reference cuvette

Table 7. The effect of anions on the absorbancy difference of pyruvate kinase due to potassium versus Tris.

The procedure was identical with that described in figure 2 with the exception that the anionic species of potassium in the sample cuvette and Tris in the reference cuvette varied as indicated.

Anion	Cation added at 0.1 M		Absorbancy difference at 295 mμ ($-\triangle$ O.D. $\times 10^3$)
	Sample cuvette	Reference cuvette	
Sulfate	K^+	$Tris^+$	0.0
Phosphate	K^+	$Tris^+$	0.0
Arsenate	K^+	$Tris^+$	0.0
Acetate	K^+	$Tris^+$	4.0
Nitrate[a]	K^+	$Tris^+$	9.5
Chloride	K^+	$Tris^+$	11.0

[a] Because nitrate absorbs at 295 mμ the experiment was conducted with 0.1 N nitrate in both cuvettes. Nitrate was added as Tris nitrate in the reference cuvette in order to maintain a constant pH. The final concentration of Tris was 0.12 N. The absorbancy difference at 295 mμ was determined at 0.12 N Tris and corrected to 0.1 N Tris.

Table 8. The effect of different combinations of potassium and Tris chlorides and phosphates on the absorbancy difference at 295 mμ of pyruvate kinase.

The procedure was identical with that described in figure 2 with the exception that the anionic species of potassium in the sample cuvette and of Tris in the reference cuvette were varied as indicated. The absorbancy difference at 295 mμ resulting from a comparison of the different salts without enzyme was subtracted from the values obtained with enzyme when it represented a significant amount. The results are an average of two separate experiments. The pH at the conclusion of the experiment was 7.4 ± 0.05.

Ion comparison		
Sample cuvette	Reference cuvette	Absorbancy difference at 295 mμ ($-\Delta$ O. D. $\times 10^3$)
0.1 M potassium chloride	0.1 M Tris chloride	12
0.1 M potassium phosphate	0.1 M Tris phosphate	0
0.1 M potassium phosphate	0.1 M Tris chloride	15
0.2 M potassium phosphate	0.1 M Tris chloride + 0.1 M Tris phosphate	6
0.1 M potassium chloride	0.1 M potassium phosphate	3

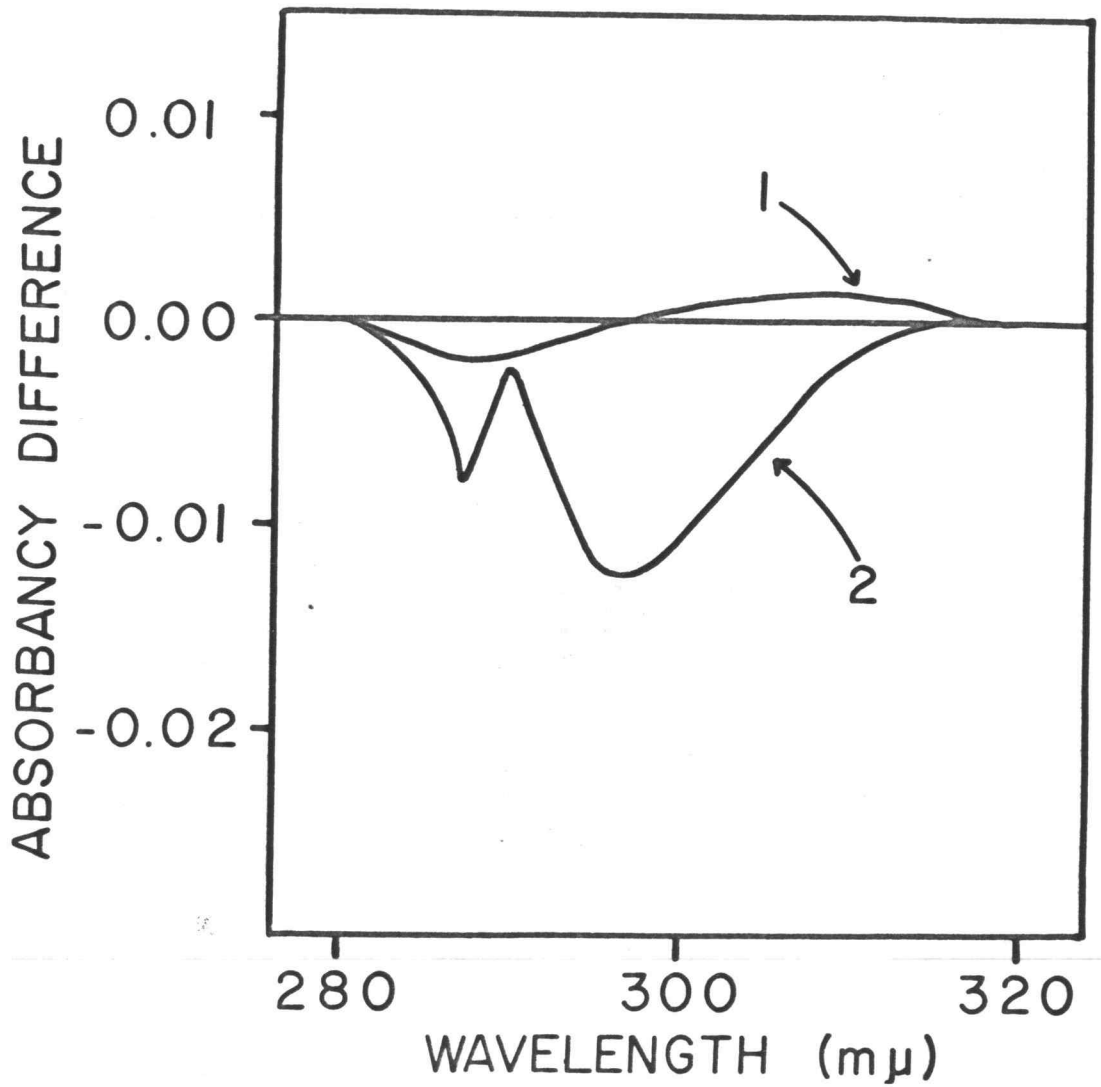
on the absorbancy difference at 295 mμ of pyruvate kinase. As previously mentioned, the difference spectrum of pyruvate kinase in a solution of potassium chloride versus enzyme in a solution of Tris chloride resulted in an absorbancy difference at 295 mμ. The substitution of potassium phosphate for potassium chloride in the sample cuvette and Tris phosphate for Tris chloride in the reference cuvette abolished the absorbancy peak at 295 mμ. A comparison, however, of pyruvate kinase in 0.1 M potassium phosphate in the sample cuvette versus enzyme in 0.1 M Tris chloride in the reference cuvette results in an absorbancy difference at 295 mμ. This difference was diminished by the addition of potassium phosphate to the sample cuvette and Tris phosphate to the reference cuvette. Thus the major conclusion from these results is that the perturbation of the tryptophan residues of pyruvate kinase occurs in a 0.1 M Tris environment containing a univalent anion and that phosphate or other multivalent anions of Tris inhibit the perturbation. Finally, a comparison of pyruvate kinase in 0.1 M potassium chloride versus enzyme is 0.1 M potassium phosphate resulted in a small but significant absorbancy difference at 295 mμ. The meaning of this relatively small difference is not apparent and requires further investigation.

Effect of Background Buffer
on the Difference Spectra

In a previous experiment (Table 6) where Tricine buffer was utilized to maintain a constant pH in the reference and sample cuvettes, the addition of 0.1 M KCl to a solution of enzyme in the sample cuvette and 0.1 M TMACl to the enzyme solution in the reference cuvette resulted in no absorbancy difference at 295 mμ. In contrast Suelter et al. (1966) reported that the addition of KCl versus TMACl to the enzyme solutions resulted in difference spectra characteristic of the perturbation of the tryptophan residues. In Suelter's experiments however the pH of the enzyme preparation was maintained with TrisCl buffer. A study was made therefore, to determine the effect of TrisCl background buffer on the difference spectra of pyruvate kinase in solutions of KCl and TMACl. The enzyme was prepared by passing it through a column of Bio-Gel equilibrated with 0.01 M potassium phosphate buffer pH 7.4. In these experiments potassium phosphate buffer replaced Tricine buffer that had previously been used (Materials and Methods).

A difference spectrum resulting from a comparison of pyruvate kinase in KCl in the sample cuvette versus enzyme in TMACl in the reference cuvette is shown in Figure 10, curve 1. In this experiment potassium phosphate was used as the background buffer. The spectrum shows no significant absorbancy differences. If TrisCl at

Figure 10. The effect of background buffer on the difference spectrum of pyruvate kinase. Curve 1) Each 3 ml cuvette contained 6 mg pyruvate kinase and 6.7×10^{-3} M potassium phosphate buffer pH 7.4. The sample cuvette contained a final concentration of 0.12 M KCl and the reference cuvette contained a final concentration of 0.12 M TMACl. Curve 2) The same conditions as curve 1 with the exception that a final concentration of 0.08 M TrisCl was added to both cuvettes. Essentially identical results were obtained in a duplicate experiment. The pH determined at the conclusion of the experiment was 7.4 ± 0.1.


0.01
0.00
-0.01
-0.02
ABSORBANCY DIFFERENCE
1
2
280
300
320
WAVELENGTH (mμ)

a final concentration of 0.08 M and at pH 7.4 was added to the enzyme in KCl solution in the sample cuvette and the enzyme in TMACl in the reference cuvette, the difference spectrum shown in Figure 10, curve 2, was obtained. A significant absorbancy difference is apparent at 286 mμ and 295 mμ. Furthermore, the data in Table 9 show that the concentration of TrisCl added to both cuvettes influenced the magnitude of the absorbancy difference at 295 mμ. Tryptophan residues apparently are perturbed as a result of the addition of TrisCl to both cuvettes. The perturbation may be due to a differential capacity of Tris to compete with potassium bound to the protein in the sample cuvette and TMA bound to the protein in the reference cuvette. When 0.48 M TrisCl was added to both cuvettes the effect of this salt seemed to dominate, and thus any differential effect of potassium or TMA at 0.12 M was not apparent.

Effect of Substrates on the Difference Spectra in the Presence of Potassium Versus Lithium

It has been shown that no absorbancy differences were obtained with pyruvate kinase in a solution containing 0.1 M KCl versus enzyme in a solution of 0.1 M LiCl (Table 6). One possible explanation is that univalent cation activators affect the enzyme conformation only when a substrate or magnesium, or a substrate and magnesium, are present (Mildvan and Cohn, 1964). To examine these

Table 9. Absorbancy differences resulting from comparisons of pyruvate kinase in solutions of KCl versus the enzyme in solutions of TMACl at different concentrations of TrisCl.

Each 3 ml cuvette contained 6 mg pyruvate kinase and 6.7×10^{-3} M potassium phosphate buffer pH 7.4. The sample cuvette contained a final concentration of 0.12 M KCl and the reference cuvette a final concentration of 0.12 M TMACl. TrisCl was added to both cuvettes at the concentrations indicated. The pH at the termination of the experiment was 7.4 + 0.1.

Concentration of TrisCl in both sample and reference cuvettes	Absorbancy difference at 295 mμ ($-\triangle$ O.D. $\times 10^3$)
0.000	0
0.015	4
0.030	7
0.045	9
0.080	12
0.110	7
0.200	5
0.310	3
0.480	1

possibilities, pyruvate kinase was compared in 0.1 M KCl versus enzyme in 0.1 M LiCl with substrates and divalent cation activators present in both cuvettes. The results of the difference spectra are shown in Figures 11 to 14. As shown in Figure 11, a difference spectrum of pyruvate kinase in 0.1 M KCl in the sample cuvette versus enzyme in 0.1 M LiCl in the reference cuvette and 2×10^{-3} M PEP in both cuvettes shows no absorbancy differences. The presence of PEP at concentrations of 5×10^{-4} M and 1×10^{-3} M in both cuvettes, similarly, resulted in no difference in absorbancy. The addition of 0.001 M $MgCl_2$ to both cuvettes containing PEP had no measurable effect on the difference spectrum of the enzyme. In another experiment sodium pyruvate was substituted for PEP in both cuvettes. In separate experiments the concentrations of pyruvate used in the sample and reference cuvettes were 5×10^{-4} M, 1×10^{-3} M and 2×10^{-3} M. The results, presented in Figure 12, show no appreciable absorbancy differences resulting from a concentration of 2×10^{-3} M pyruvate, in the two cuvettes. Furthermore, the addition of pyruvate at 5×10^{-4} M and 1×10^{-3} M also produced negative results.

As shown in Figure 13, a difference spectrum resulting from a comparison of 0.1 M KCl in the sample cuvette versus 0.1 M LiCl in the reference cuvette with enzyme, 0.001 M $MgCl_2$, and 5×10^{-4} M ATP in both cuvettes shows no absorbancy difference.

Figure 11. The effect of PEP on the difference spectrum of pyruvate kinase in KCl versus enzyme in LiCl. The enzyme was prepared by passing it through a column of Bio-Gel equilibrated with 0.01 M potassium phosphate and 0.001 M MCE. The potassium phosphate buffer replaced the Tricine buffer used in the procedure outlined in Materials and Methods. Each 3 ml mixture contained 6.7×10^{-3} M potassium phosphate buffer pH 7.4, 6.7×10^{-4} M MCE, 6 mg of pyruvate kinase and PEP. The concentration of KCl in the sample cuvette and LiCl in the reference cuvette was 0.1 M. The pH at the conclusion of the experiment was 7.4 ± 0.05.

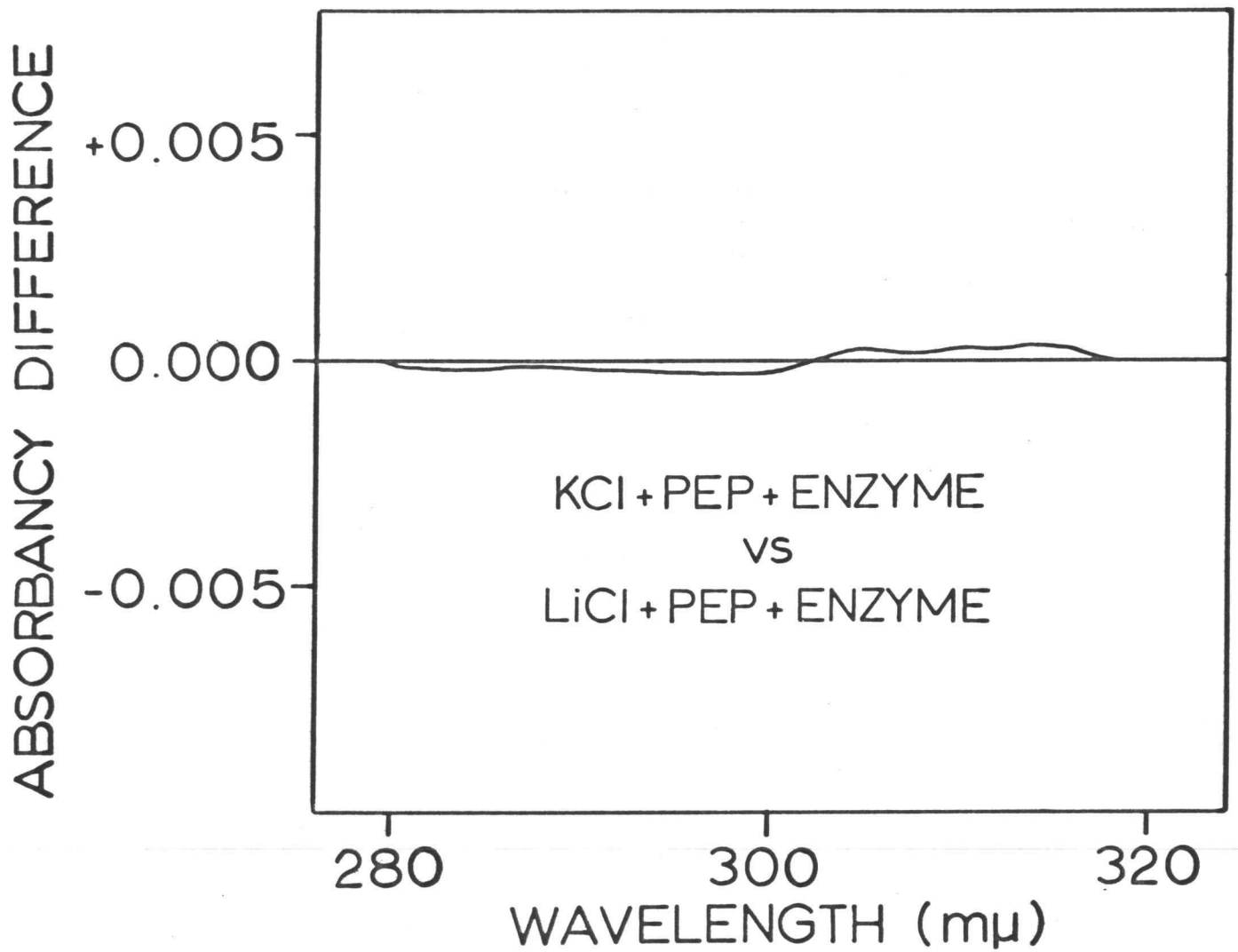

ABSORBANCY DIFFERENCE
+0.005
0.000
-0.005
KCl + PEP + ENZYME
vs
LiCl + PEP + ENZYME
280
300
320
WAVELENGTH (mμ)

Figure 12. The effect of pyruvate on the difference spectrum of pyruvate kinase in KCl versus enzyme in LiCl. The procedure was identical with that described in Figure 11 with the exception that sodium pyruvate replaced PEP in both cuvettes.

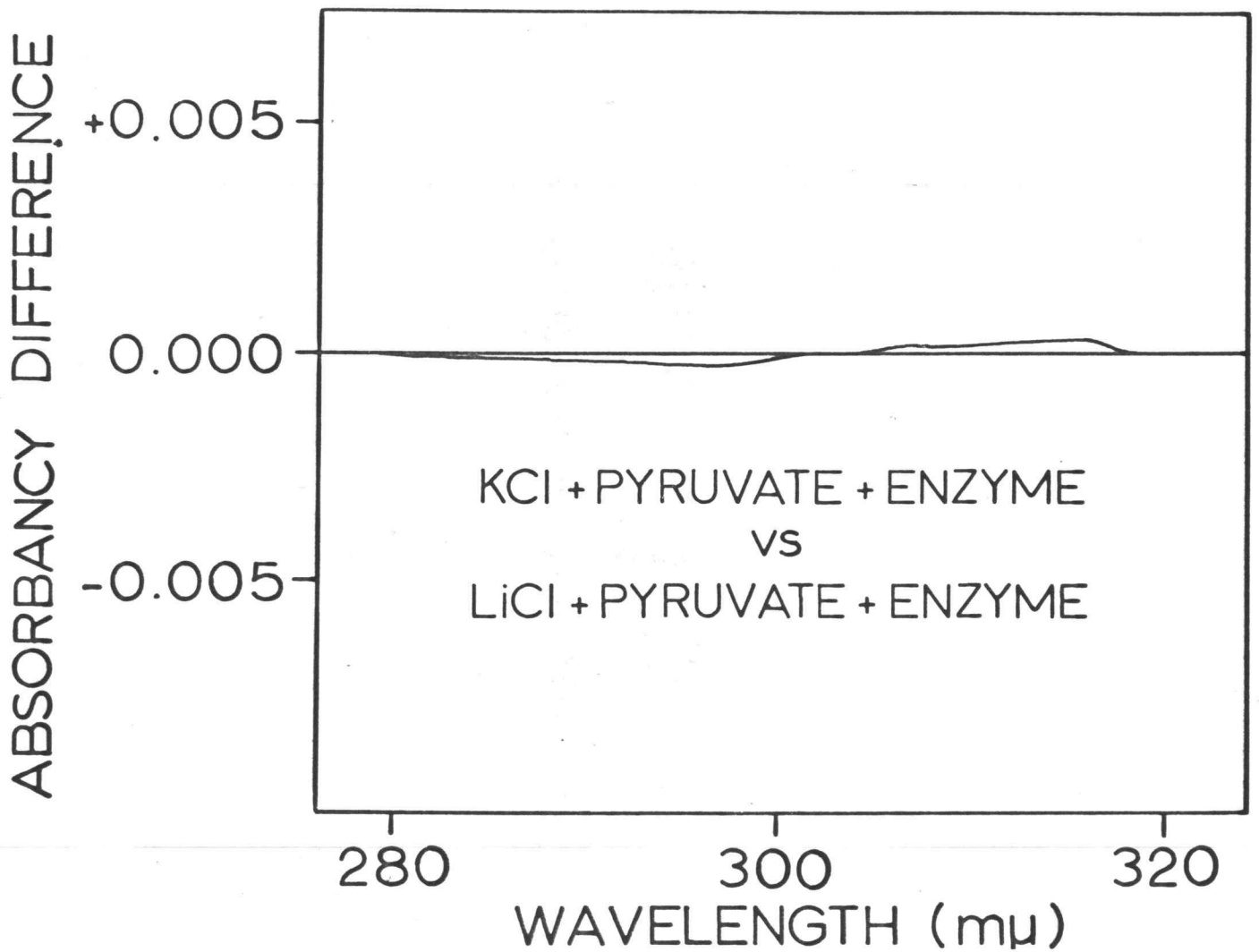

ABSORBANCY DIFFERENCE
+0.005
0.000
-0.005
KCl + PYRUVATE + ENZYME
vs
LiCl + PYRUVATE + ENZYME
280
300
320
WAVELENGTH (mμ)

Figure 13. The effect of ATP on the difference spectrum of pyruvate kinase in KCl versus enzyme in LiCl. The procedure was identical with that described in Figure 11 with the exception each cuvette contained 0.001 M $MgCl_2$. In addition sodium ATP at a concentration of 5×10^{-4} replaced PEP in both cuvettes.

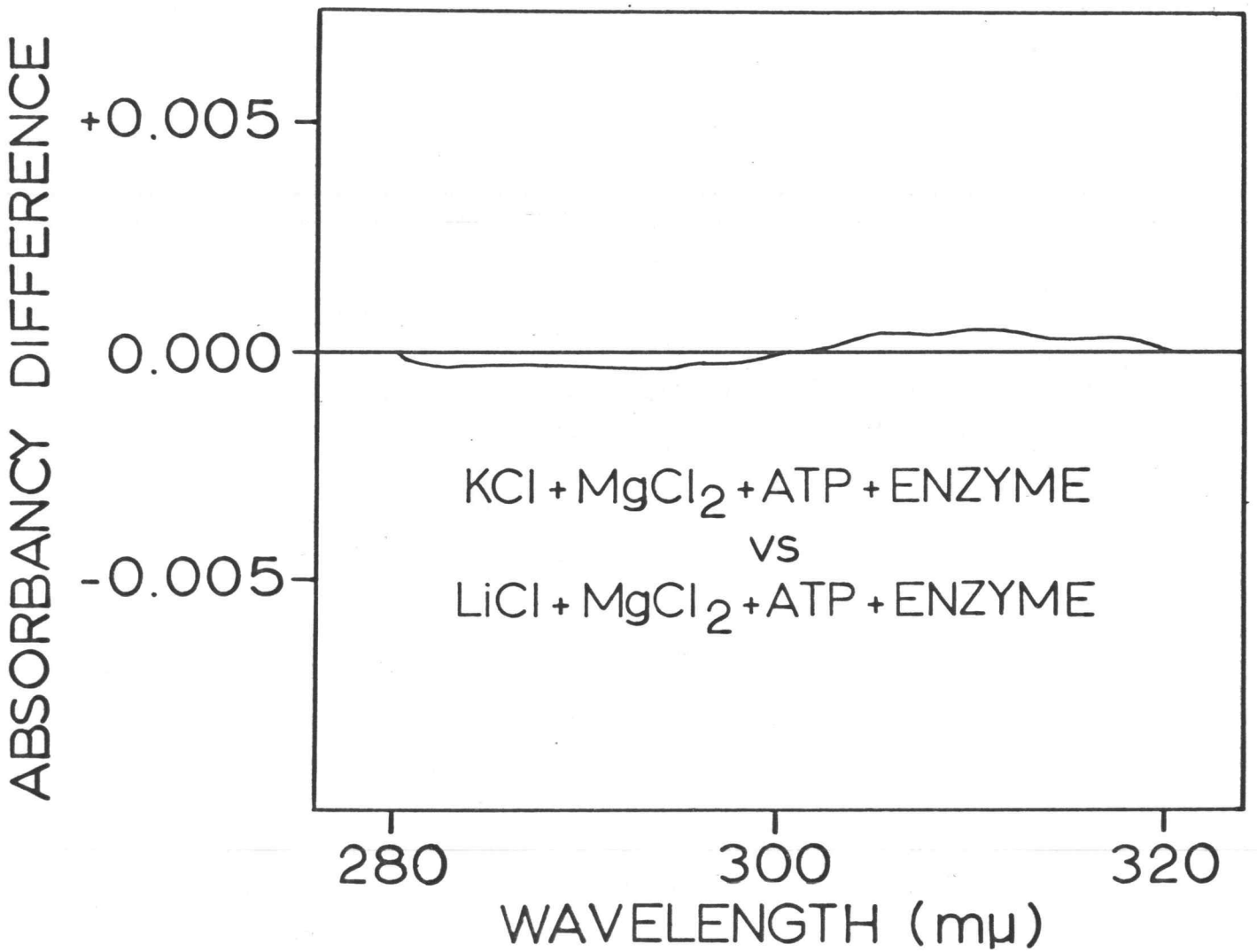
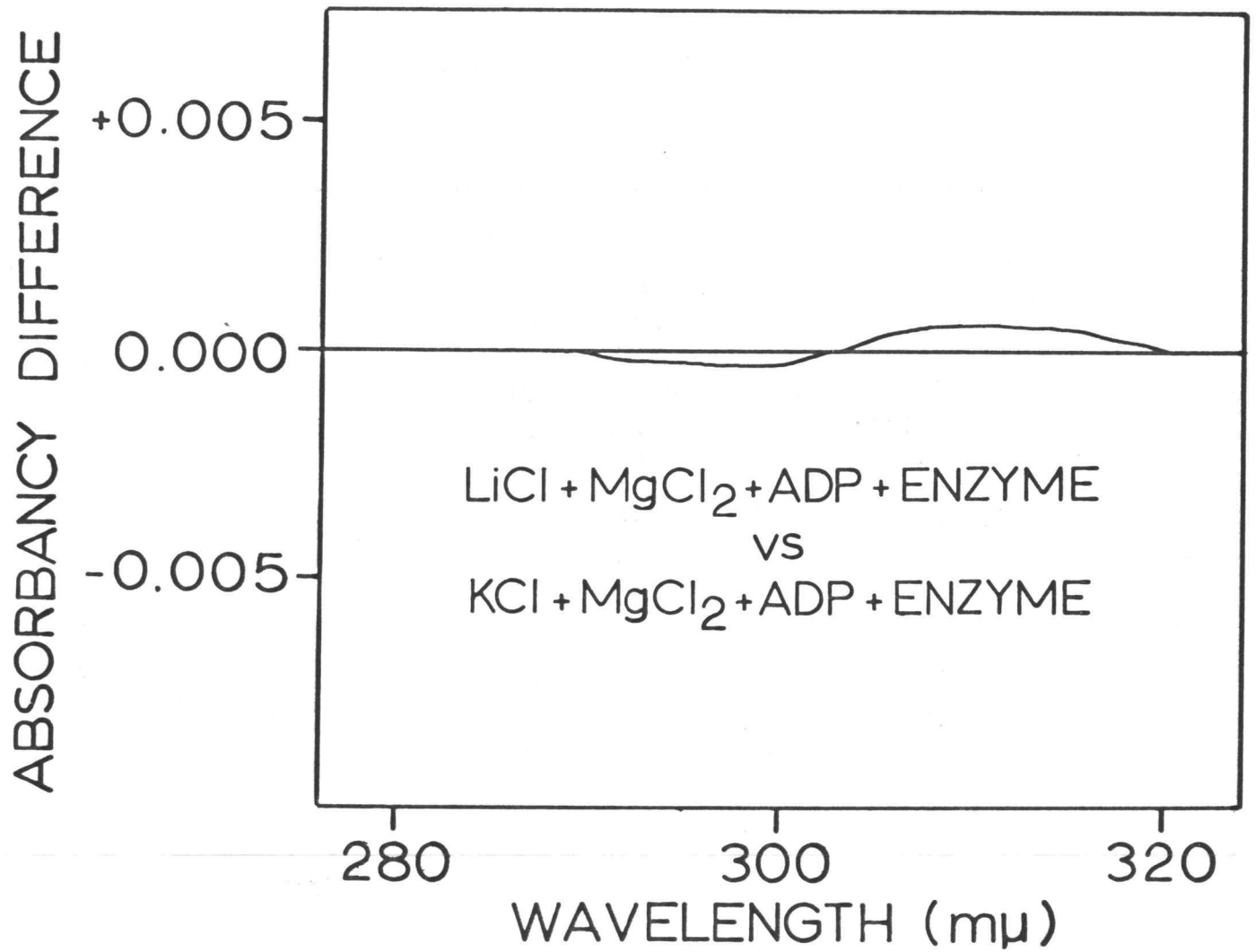

ABSORBANCY DIFFERENCE
+0.005
0.000
-0.005
KCl + MgCl2 + ATP + ENZYME
vs
LiCl + MgCl2 + ATP + ENZYME
280
300
320
WAVELENGTH (mμ)

Figure 14. The effect of ADP on the difference spectrum of pyruvate kinase in KCl versus enzyme in LiCl. The procedure was identical with that described in Figure 13 with the exception sodium ADP replaced sodium ATP in both cuvettes.

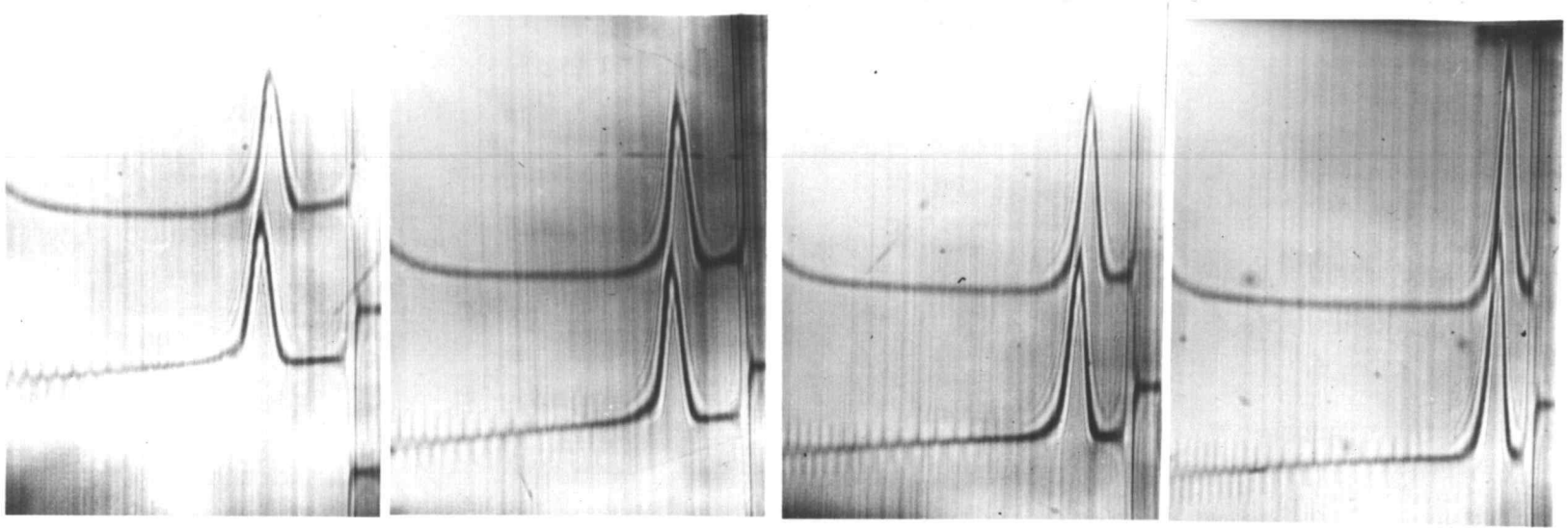

ABSORBANCY DIFFERENCE
+0.005
0.000
-0.005
LiCl + MgCl2 + ADP + ENZYME
vs
KCl + MgCl2 + ADP + ENZYME
280
300
320
WAVELENGTH (mμ)

Similar results were obtained in an experiment in which the ATP concentration was 7.3×10^{-5} M. The deletion of $MgCl_2$ in both cuvettes failed to influence the difference spectrum. In another study, difference spectra were made of 0.1 M KCl, enzyme, 0.001 M $MgCl_2$, and 5×10^{-4} M ADP in the sample cuvette versus a reference cuvette containing the same amounts of the components except 0.1 M LiCl replaced 0.1 M KCl. The results in Figure 14 show no effect from these additions. Also in other experiments where ADP concentrations of 7.3×10^{-5} M and 1×10^{-4} M were utilized with potassium and lithium, no appreciable absorbancy differences were observed. These results indicate that the addition of substrates or magnesium to the enzyme in activator univalent cation versus non-activator univalent cation environments at concentrations of 0.1 M failed to perturb either tyrosine or tryptophan residues.

Ultracentrifugation in Different Univalent Cation Solutions

A study was made of the sedimentation behavior of pyruvate kinase in phosphate salts of potassium, lithium and Tris. In Figure 15 the results are presented of typical Schlieren patterns for pyruvate kinase in potassium phosphate and Tris phosphate. The enzyme appears to move as one homogeneous peak in both salts. A similar pattern was obtained for the enzyme in lithium phosphate.

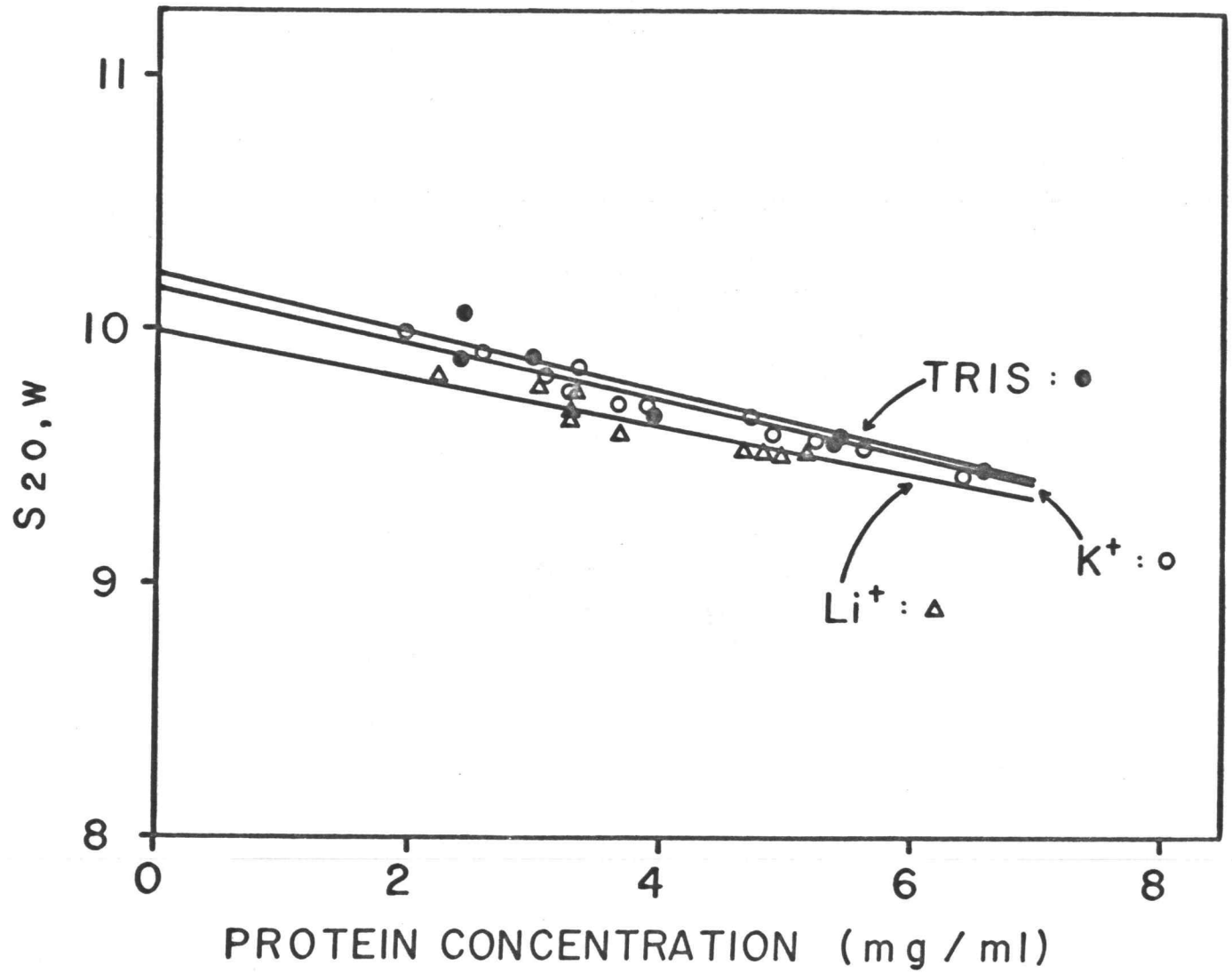
Figure 15. Schlieren pattern of the sedimentation of pyruvate kinase in a Spinco Model E analytical ultracentrifuge. The top pattern was obtained from a wedge cell containing 4.90 mg/ml enzyme in 0.05 M Tris phosphate pH 7.4. The bottom pattern was obtained in a standard cell containing 4.94 mg/ml enzyme in 0.05 M potassium phosphate pH 7.4. The rotor speed was 59,780 rpm at 2 to 5° and the bar angle was 60°. Sedimentation is from right to left. The photographs were taken at 8 min. intervals.



24 16 8 0

A plot of the sedimentation coefficients at different protein concentrations is shown in Figure 16. The extrapolated S values for enzyme solutions of lithium phosphate, potassium phosphate and Tris phosphate were 9.99, 10.15 and 10.17 respectively. The sedimentation value for the enzyme in a solution of potassium phosphate is not significantly different from the value obtained for the enzyme in a solution of Tris phosphate. The sedimentation value of pyruvate kinase in a solution of lithium phosphate, however, was statistically significantly different from the values for the enzyme in solutions of potassium phosphate or Tris phosphate. An investigation of the effect of univalent cation chloride environments on the sedimentation behaviour of pyruvate kinase is of interest, but such experiments have not been conducted to date.

Figure 16. Sedimentation values for pyruvate kinase at different protein concentrations in potassium, lithium and Tris phosphate buffers pH 7.4. For experimental procedure see Materials and Methods.


11
10
9
8
S20,W
0
2
4
6
8
PROTEIN CONCENTRATION (mg/ml)
TRIS : ●
K+ : o
Li+ : Δ

DISCUSSION

Stability, Perturbation and Activity as Influenced by Univalent Cations

The results of investigations of the effect of univalent cations on the stability of pyruvate kinase show a strong correlation between the capacity of univalent cations to stabilize and to activate pyruvate kinase (Figure 1). Sorger and Evans (1966) have reported a similar correlation with the univalent-cation-requiring-enzyme acetaldehyde dehydrogenase from yeast. Potassium and rubidium protect this enzyme from inactivation by heat, whereas sodium and lithium were less effective. Moreover, a correlation was shown between the capacity of univalent cations to activate acetaldehyde dehydrogenase and the capacity to restore enzyme activity after extensive dialysis against Tris buffer, a treatment which leads to loss of activity. With pyruvate kinase the presence of potassium was shown to restore to a considerable extent the activity of the enzyme which had been lost as a result of incubation in solutions of lithium or Tris (Table 2). Sorger and Evans interpreted their results with acetaldehyde dehydrogenase to indicate that univalent cations affect the physical structure of the enzyme molecule. The results with pyruvate kinase are consistent with this interpretation and suggest a similar effect of univalent cations on this enzyme. If one accepts the hypothesis that

different univalent cation species differentially affect the conformation of the enzyme as a plausible explanation for these results, then potassium not only stabilizes the protein conformation, thus preventing loss of activity, but it also reverses the conformation from the unstable form in the presence of lithium or Tris to the stable form in the presence of potassium.

At 0.5 M and higher concentrations of univalent cation chlorides, the capacity to perturb tryptophan residues as measured by difference spectrophotometry was inversely correlated with the capacity of univalent cations to activate the enzyme (Table 6). Those univalent cations which activate the enzyme do not perturb the tryptophan residues, whereas, those univalent cations which fail to activate perturb the tryptophan residues. This correlation is consistent with the theory that the enzyme can exist in at least two different conformations, one which is induced by the presence of activator univalent cations and another which is induced by the presence of non-activator univalent cations. Yankeelov and Kosland (1965) have observed a tryptophanyl difference spectrum of phosphoglucomutase in the presence of the substrate, glucose-6-phosphate, compared with the enzyme without substrate. They interpreted their difference spectrum to indicate a conformation change of the protein induced by the presence of the substrate. Sorger, Ford and Evans (1965) reported that pyruvate kinase exhibited an

immunoelectrophoretic pattern in the environment of non-activator univalent cations that was visibly more complex than the immunoelectrophoretic pattern in the environment of activator univalent cations. They concluded that the enzyme must exist in at least two conformations, one of which is catalytically active and the other of which is catalytically inactive. The difference spectral results presented here are consistent with the conclusions of these workers and of Melchior (1965) and do not indicate a requirement for substrates for the cation induced conformation change (Figures 11 to 14).

It should be pointed out that the concentration of salts, other than those of Tris, needed to perturb the tryptophan residues of pyruvate kinase is relatively high compared with the concentration of univalent cations necessary to activate the enzyme. Although it is not immediately evident why the higher salt concentrations are required for perturbation, it may be that at physiological concentrations of salts, in which the enzyme shows the maximum reaction velocity, the protein molecule is poised by activator univalent cations in an active conformation. In comparison, at the same concentrations of non-activator univalent cations the protein may be poised in a non-active conformation. When the concentration of salts is increased in either environment, changes in the medium are postulated to lead to additional effects on the physical structure of the protein. At least one effect of the higher salt concentrations on the non-active conformation of the protein is the perturbation of the tryptophanol

chromaphore. With the active conformation of the protein, which is postulated to be poised differently, the higher concentrations of salts result in less or no perturbation of the tryptophanyl chromophore (Table 6).

Bigelow and Geschwind (1960) have reported difference spectra of ribonuclease in high but non-denaturing concentrations of lithium bromide (3.0 M) or sodium acetate (3.6 M) versus enzyme in water. The spectra were characteristic of the perturbation of tyrosine residues. In addition, they reported that a proteolytic enzyme from B. subtilis (Novo enzyme) produced difference spectra in 4.2 M sodium bromide and 4.1 M sodium chloride which were characteristic of the perturbation of tryptophan residues. Although the concentrations of salts used in these experiments were considerably higher than those utilized in the experiments reported here, the factors responsible for the difference spectra at high salt concentrations in Bigelow and Geschwind's study may be similar to the factors responsible for the difference spectra of pyruvate kinase at 0.5 M and greater salt concentrations in this study.

In comparison with the 0.5 M or higher concentrations of non-activator univalent cations necessary for the perturbation of the tryptophan residues of pyruvate kinase, the presence of TrisCl at 0.03 M and higher in the environment of the enzyme resulted in the perturbation of the tryptophan residues. Support for this

conclusion can be seen from several experiments (Table 4, 5, 7, 8, 9, and Figures 2 to 6 and 10). Except for Tris, both activator and non-activator univalent cations at 0.1 M concentrations apparently fail to perturb the tryptophan residues (Table 5 and 6 and Figure 6). The results indicate that the tryptophanyl difference spectrum occurs as a result of a long wavelength shift in the absorption of the tryptophan residues due to the presence of Tris at relatively low concentrations or other non-activator univalent cations at 0.5 M or higher concentrations.

Kayne and Suelter (1965) and Suelter et al. (1966) have reported similar tryptophanyl difference spectra of pyruvate kinase in univalent and divalent activator cation salts versus enzyme in TMA, a non-activator cation. They attributed their difference spectra to the perturbation of tryptophan residues by the activator cation in the sample cuvette. The results presented here do not exclude the possibility that potassium perturbs the tryptophan residues, particularly at salt concentrations higher than 0.5 M, but they support the conclusion that the non-activator univalent cations are responsible for the perturbation. On the basis of observations made here, the presence of Tris buffer in both cuvettes complicated the difference spectral studies of Suelter et al. (1966) Kayne and Suelter (1965) and Suelter and Melander (1963). It has been shown in this study that the addition of TrisCl to both cuvettes can result in a

tryptophanyl difference spectra of pyruvate kinase in a solution containing TMA chloride (Figure 10, Table 9). In addition, Betts (1966) has shown that Tris can affect the K_m value for potassium in the pyruvate kinase reaction. Thus, in the studies of Suelter et al. (1966) and Kayne and Suelter (1965) it appears that the presence of TrisCl buffer in the enzyme environment in both cuvettes was differentially competing with the univalent cations in the reference and sample cuvettes and resulting in the perturbation of the tryptophan residues. This conclusion is supported by the fact that experiments identical with those of Suelter et al. (1966) with the exception that TrisCl was omitted in both cuvettes and replaced by low concentrations of phosphate buffer failed to show a difference spectrum (Figure 10).

Perturbation and Stability as Influenced by Anions

Difference spectra of pyruvate kinase in TrisCl versus enzyme in KCl (Table 5) indicated a perturbation of tryptophan residues but enzyme in Tris phosphate versus enzyme in potassium phosphate (Figure 9) failed to show the perturbation. A similar inhibition of the perturbation was observed with the multivalent anion salts, Tris sulfate and Tris arsenate (Table 7). In comparison, enzyme in Tris salts of the univalent anions chloride and nitrate, versus enzyme in potassium salts of these same anions

resulted in the perturbation of tryptophan residues (Table 7). In addition to the effect of multivalent anions in preventing the perturbation of tryptophan residues of pyruvate kinase, it was shown that multivalent anions effectively protect the enzyme against inactivation (Table 3). In contrast, enzyme activity generally deteriorated in the presence of univalent anions (Table 3). It is interesting that acetate was the most effective univalent anion from the standpoint of enzyme stability and that enzyme in potassium acetate versus enzyme in Tris acetate produced the least absorbancy difference at 295 mμ of any of the univalent anions of Tris (Table 7). Thus, the evidence indicates a positive correlation between the capacity of anions to protect pyruvate kinase from inactivation and the capacity to perturb tryptophan residues.

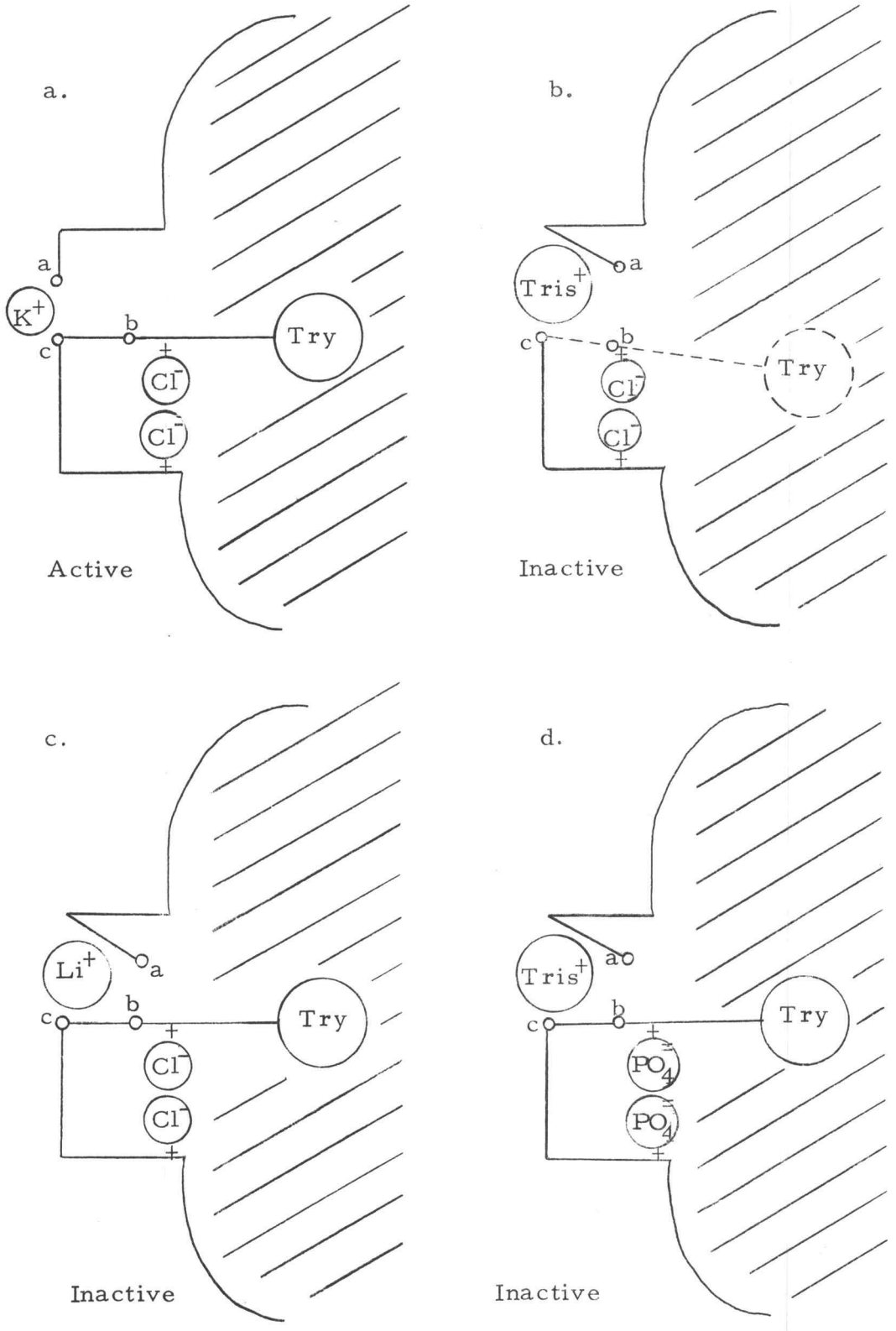
In connection with the anion studies, it is interesting that Sela and Anfinsen (1957) and Sela, Anfinsen and Harrington (1957) reported that the molar extinction coefficient at 286 mμ of ribonuclease in 8 M urea was influenced by the anionic environment of the enzyme. In the presence of 8 M urea the ribonuclease protein is reported to unfold and exhibit a shift of the tyrosyl absorption maximum to shorter wavelengths (Harrington and Schellman, 1956). Sela et al. (1957) showed a complete inhibition of the spectral shift of ribonuclease in 8 M urea by the multivalent anions, phosphate and arsenate, and a partial inhibition by suflate. Univalent anions

chloride, bromide and acetate had essentially no effect on the perturbation. Similarly, Sela and Anfinsen (1959) and Barnard (1961) have reported an inhibition by phosphate of the perturbation of tyrosine residues of ribonuclease in 8 M urea. These observations are consistent with the observed effects of anions on the perturbation of tryptophan residues of pyruvate kinase.

An Interpretation of the Effect of Salts

An interpretation of the effect of different univalent cation salts on certain properties of pyruvate kinase can be made on the basis of illustrative models of the enzyme shown in Figure 17. The models show a possible mechanism of interaction of potassium, an activator, and lithium and Tris, both non-activators, with the enzyme. In these models the enzyme is considered to be activated when points a, b, and c are arranged as shown in Figure 17a. This is the form the enzyme is postulated to assume in the presence of an activator such as potassium. The tryptophan chromophore, designated by Try, is not perturbed. Figure 17b shows the same portion of the protein molecule in a Tris environment. Not only does Tris result in an alteration of points a and b of the activation site, but this cation also causes the perturbation of the tryptophan chromophore, designated in the model by the movement of the tryptophan chromophore. In this form the enzyme is catalytically

Figure 17. Schematic models of the effect of different cations and anions on the conformation of pyruvate kinase. a) The presence of 0.1 M potassium chloride results in a conformation which is catalytically active as shown by the positioning of points a, b and c. The tryptophan chromophore is unperturbed as seen by the position of the Try symbol. b) The presence of 0.1 M Tris chloride results in an alteration of the activation site and the perturbation of the Try chromophore. c) The presence of 0.1 M lithium chloride results in an alteration of the activation site but does not perturb the Try chromophore. d) The presence of 0.1 M Tris phosphate also results in an alteration of the activation site but does not perturb the Try chromophore.


a.
a
K+
b
c
Try
Cl-
Cl-
Active
b.
Tris+
a
c
b
Try
Cl-
Cl-
Inactive
c.
Li+
a
b
c
Try
Cl-
Cl-
Inactive
d.
Tris+
a
b
c
Try
PO4
PO4
Inactive

inactive. The presence of lithium in the environment of pyruvate kinase is postulated to result in the configuration of the enzyme shown in Figure 17c. In an environment of lithium at 0.1 M concentration the enzyme is neither catalytically active nor is the tryptophan chromophore perturbed. Thus, the effect of univalent cations on the enzyme may be due to their capacity to induce conformation changes in the enzyme illustrated by those shown in the models in Figure 17a to c. The difference in the effectiveness of univalent cations may be due to the smaller hydrated ionic radii of the activator univalent cations than the non-activator univalent cations (Kachmar and Boyer, 1953).

Multivalent anions were shown to inhibit the perturbation of the tryptophan residues by Tris (Figure 9). A model of the possible mechanism of inhibition by phosphate is presented in Figure 17d. The divalent phosphate anion, which is the most abundant species of phosphate present at pH 7.4, is shown to inhibit the movement of the tryptophan chromophore due to repulsion of like charges on the phosphate anions bound to the enzyme. In addition, the presence of Tris has altered the position of point "a" of the activation site. Thus, not only is the enzyme inactive in a solution of Tris phosphate, but also the tryptophan chromophore is not perturbed (Figure 17d). A similar model might explain the inhibition of the perturbation of the tryptophan residues by Tris sulfate and Tris

arsenate (Table 7).

Rose (1960) has studied the enzymatic stimulation of the rate of enolization of pyruvate by pyruvate kinase. Since different cations and anions were shown to affect the enolization reaction, his results were particularly pertinent to this study. Using the detritiation of beta-tritiated pyruvate as a measure of the enolization reaction, he showed that all the components of the net over-all reaction of pyruvate kinase were essential for the enolization. These factors included ATP, magnesium, potassium and enzyme. The essentiality of ATP for the enzymatic catalyzed detritiation reaction, could be replaced by a number of multivalent anions including phosphate, arsenate and pyrophosphate. Sulfate failed to replace ATP. When phosphate was used in place of ATP in the detritiation reaction, the rate of the reaction in the presence of potassium was 3-fold greater than that in the absence of the cation. The rate of the reaction in the presence of lithium was slightly lower than that of a reaction lacking a univalent cation. Rose concluded from the results of kinetic studies that the multivalent anion activators of the enolization reaction bind at a site on the enzyme common to both ADP and PEP, namely, the position adjacent to the site which carries the carbonyl group of pyruvate.

In considering the role played by the multivalent anion activators and ATP in affecting the enolization of pyruvate, Rose

suggested that an electrophilic group situated near the α-carbonyl oxygen of pyruvate might influence the flow of electrons from the β-C-H bond of pyruvate toward the α-carbonyl oxygen. Furthermore, he suggested that the activators, magnesium or potassium, in a suitable configuration might promote this electron flow and consequently deprotonation. He suggested that the phosphorus or arsenic atoms of phosphate and arsenate, respectively, may act as electrophilic centers, due to their positive residual charge (Rose 1960).

Although Rose's studies were confined to the enolization reaction of pyruvate, it appears that this reaction has the same general requirements as the net over-all reaction of pyruvate kinase. It is interesting, therefore, to correlate the results of the interaction of cations and anions in Rose's study with the results reported in this investigation. In this study it has been shown that univalent-cation activators of the net over-all reaction, protect the enzyme from inactivation and do not appear to perturb the tryptophan residues. These effects of activator univalent cations may be due to their capacity to induce a conformation change in the enzyme near the active site analogous to the mechanism proposed by Rose for the enolization reaction. In contrast, non-activator univalent cations, such as lithium or Tris, fail to replace potassium as an activator and accordingly they do not effectively protect the enzyme from inactivation. Also the observation that multivalent

anions such as phosphate and sulfate inhibit perturbation of tryptophan residues and contribute toward stability of pyruvate kinase in general is consistent with Rose's observations of the effects of these anions on the enolization reaction.

SUMMARY

Experiments are reported of the effect of a series of univalent cations on some physical properties of crystalline pyruvate kinase, an enzyme with an absolute requirement for univalent cations for activity. In addition, the anionic species associated with the univalent cations have been observed to affect certain physical properties of the enzyme. The results of these investigations are summarized as follows:

1) A study of the stability of the enzyme in a series of different solutions containing univalent cation phosphates revealed a strong positive correlation between the capacity of univalent cations to protect against inactivation of the enzyme and capacities to activate pyruvate kinase. The addition of potassium, an activator cation, restored to a considerable extent enzyme activity that was lost due to incubation of the enzyme in solutions of the non-activator cations, lithium and Tris.
2) The enzyme was consistently more stable in buffer solutions containing multivalent anions pyrophosphate, sulfate, phosphate and arsenate than in buffer solutions containing the univalent anions acetate, bromide, nitrate and chloride.
3) Ultraviolet difference spectra were determined for pyruvate kinase in 0.1 M solutions of activator univalent cations

(sample cuvette) versus enzyme in 0.1 M solutions of non-activator univalent cations (reference cuvette). Under these conditions it was found essential to add the non-activator univalent cation, Tris, to one cuvette in order to observe a difference spectrum with peaks at 286 mμ and 295 mμ. The spectrum was considered to be due to the perturbation of the tryptophan residues.

4) The perturbation of the tryptophan residues of pyruvate kinase in the presence of Tris was inhibited by the addition of the multivalent anions phosphate, aresenate and sulfate. In contrast, the tryptophan residues were perturbed in the presence of the Tris salts of the univalent anions chloride, nitrate and acetate. Thus, a correlation was found between the capacity of anions to protect the enzyme from inactivation and their capacity to perturb tryptophan residues. Furthermore, the valency of the anion appeared to be important in determining whether the anion contributed toward stability and inhibited perturbation.

5) Difference spectra of pyruvate kinase in a solution of 0.1 M KCl in the sample cuvette versus a solution of 0.1 M LiCl in the reference cuvette failed to produce difference spectra characteristic of the perturbation of tyrosine or tryptophan residues. The addition of the various substrates of the

pyruvate kinase reaction and magnesium to both cuvettes also failed to produce a difference spectra.

6) At concentrations of univalent cation chlorides of 0.5 M or higher, the capacity to perturb the tryptophan residues as measured by difference spectrophotometry was inversely correlated with the capacity of univalent cations to activate. For example, those univalent cations which activate the enzyme do not perturb the tryptophan residues, whereas, those univalent cations which do not activate, perturb the tryptophan residues.

7) Analytical ultracentrifugation studies of the enzyme in different univalent cation phosphate salts showed that the enzyme sedimented as one homogeneous peak in the three different univalent cation environments. An $s^{o}_{20,w}$ value for pyruvate kinase in 0.05 M lithium phosphate was 9.99. This value was statistically significantly different from the $s^{o}_{20,w}$ values of 10.15 and 10.17 of the enzyme in buffers of potassium phosphate and Tris phosphate respectively.

8) The results of the stability and difference spectral studies were interpreted to support the hypothesis that the enzyme can exist in at least two conformations depending on the species of univalent cation present. Activator univalent cations were postulated to poise the protein in a catalytically active

conformation, which is relatively stable and which exhibits little if any perturbation of the tryptophan residues at salt concentrations from 0.1 to 0.86 M. In contrast, non-activator univalent cations were postulated to poise the protein molecule in a catalytically non-active conformation, which is relatively unstable and exhibits perturbation of tryptophan residues at appropriate salt concentrations.

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