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ACTIVATION OF PIG HEART PROPIONYL-CoA CARBOXYLASE BY POTASSIUM IONS

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SUMMARY

The potassium stimulation of pig heart propionyl-CoA carboxylase (propionyl-CoA:CO_2 ligase (ADP), EC 6.4.1.3) has been investigated. The stimulation process was instantaneous and under the conditions used, reached a maximum in 2 min. An absolute requirement for univalent cations could not be demonstrated. The most significant kinetic change induced by K^+ was a decrease in the apparent K_m value for HCO_3^- (from $8.0 \cdot 10^{-3}$ M to $3.0 \cdot 10^{-3}$ M) while the apparent K_m value for the other substrates was unchanged. The change in the binding constant for HCO_3^- suggested that K^+ caused a conformational change on the enzyme, a result which was supported by determining the entropy change (47.8 entropic units) induced in the protein by this univalent cation. Further support for this hypothesis was obtained by demonstrating that both the rate of binding of *N*-[^{14}C]ethylmaleimide and loss of enzymic activity caused by *N*-ethylmaleimide increased in the presence of K^+.

INTRODUCTION

Studies by NEUJAHR[1] and NEUJAHR AND MISTRY[2] showed that the activity of pig heart propionyl-CoA carboxylase (propionyl-CoA:CO_2 ligase (ADP), EC 6.4.1.3) was stimulated by monovalent cations. Cesium and rubidium were the most efficient activators although ammonium and potassium ions were quite effective. However, since other workers have performed their assays in the presence of either sodium or potassium bicarbonate, it has not been possible hitherto to ascertain whether the enzyme possesses an absolute requirement for monovalent cations, and hence the true extent of the stimulation process has not been assessed. Furthermore, the locus of the K^+ stimulation has not been determined nor has the mechanism by which the cation exerts its effect been studied.

The investigation reported in this communication shows that there is not an absolute requirement for the alkali metal. In addition to a stimulation in the overall reaction velocity, the addition of potassium ions to the reaction mixture causes a change in the apparent K_m value for HCO_3^- and an entropy change which can only be explained by a protein conformation change.

METHODS AND MATERIALS

Pig heart propionyl-CoA carboxylase was prepared using modifications of the method of TIETZ AND OCHOA[3] The enzyme (specific activity, 3 2) was dialysed in 0 02 M *N*-ethylmorpholine–HCl, pH 7 0, containing $1 \cdot 10^{-3}$ M EDTA, 5 10^{-4} M glutathione and $1 \cdot 10^{-4}$ M dithiothreitol The pH values of all acidic reagents was adjusted with *N*-ethylmorpholine which had been purified by distilling under reduced pressure Propionyl-CoA was prepared by the method of SIMON AND SHEMIN[4], treated chromatographically by the method of ZETTERSTROM AND LJUNGGREN[5] and eluted with 10^{-6} M EDTA, pH 7 6 *N*-[^{14}C]Ethylmaleimide bicarbonate was prepared from $Ba^{14}CO_3$ by distilling the $^{14}CO_2$ from perchloric acid into an equivalent amount of *N*-ethylmorpholine base *N*-Ethylmorpholine ATP was prepared by ion exchange chromatography using ZeoKarb 225 (*N*-ethylmorpholine-form) Potassium-free $MgCl_2$ was prepared from spectroscopically pure magnesium metal The magnesium was dissolved in freshly distilled constant boiling HCl then evaporated to dryness Solutions of the magnesium salt were standardized by compleximetric titration[6] using solochrome black as indicator.

Enzymic activity was measured using the isotopic assay method of TIETZ AND OCHOA[3]. Each assay mixture contained (total volume, 0 50 ml) in μmoles, 50, *N*-ethylmorpholine–HCl, pH 8 0, 5, *N*-[^{14}C]ethylmaleimide bicarbonate ($2.5 \cdot 10^5$ counts/min per μmole), 1 5, *N*-ethylmorpholine ATP, 3 0, $MgCl_2$, 0 5, propionyl-CoA, 0 25, glutathione. When K^+ were added, 4 μmoles of KCl were used. The reaction mixtures were incubated at 30° for 10 min and stopped by the addition of 10% trichloroacetic acid. After centrifuging, aliquots of the supernatants were dried on Whatman 3MM paper discs[7] and counted in a Packard Tricarb Scintillation Spectrometer. Each assay was counted in triplicate

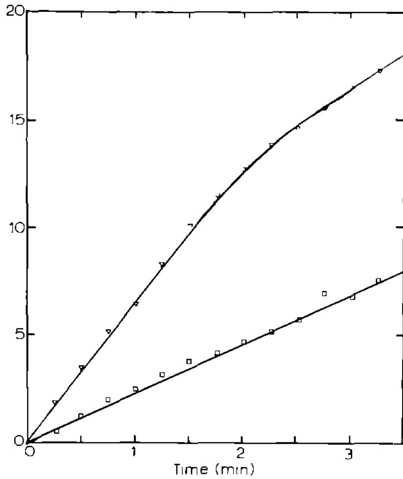
RESULTS

K^+ stimulation

A reexamination of the K^+ stimulation reported by NEUJAHR AND MISTRY[2] was accomplished with the reaction mixture described in the METHODS AND MATERIALS section Although every effort was made to exclude univalent cations from the reagents, it was not possible to demonstrate an absolute requirement for K^+ Addition of K^+ (8 mM) induced a 2 5-fold stimulation, furthermore, there was very little difference in enzymic activity when the anion added with the K^+ was Cl^-, Br^-, I^-, ClO_4^-, or CH_3COO^- although SO_4^{2-} caused a 50% loss in activity

Time course of activation

The activation of some enzymes by metal ions has been shown to be time dependent[8–11]. Since any postulated mechanism of K^+ stimulation of propionyl-CoA carboxylase would require a knowledge of the time course of the process, this aspect was investigated The enzyme was placed in two separate reaction mixtures containing high specific activity $H^{14}CO_3^-$, one solution containing K^+ and the other without univalent metal ions At 15-sec intervals, aliquots were removed, the enzymic reaction stopped by the addition of the standard aliquot to measured volumes of 10% cold aqueous trichloroacetic acid and the fixed $^{14}CO_2$ determined



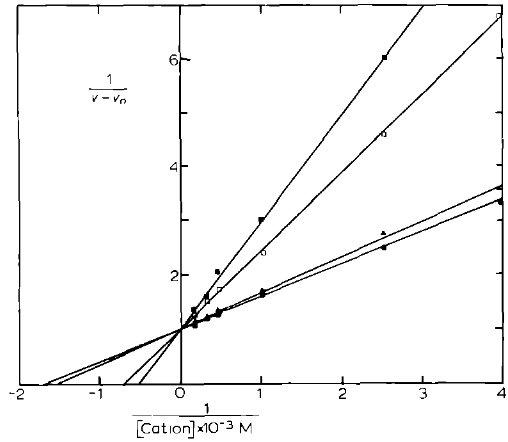


Fig 1 Time course of activation The velocity of the propionyl-CoA carboxylase reaction in the presence (▽—▽) of $8 \cdot 10^{-3}$ M K^+ and absence of K^+ (□—□) plotted as a function of time Aliquots of the assay mixture were removed at 15-sec intervals and the reaction stopped with 0 5 volume of 5% trichloroacetic acid

Fig 2 Determination of the apparent K_m and v_{max} values for various monovalent cations The velocity of the reaction in the absence (v_0) and presence (v) of the activating cations was determined The reciprocal $1/(v_0 - v)$ was plotted against the reciprocal of the cation concentration Rb^+, (●—●), K^+, (▲—▲); NH_4^+, (□—□) and Cs^+, (■—■) The reaction mixture (final volume 0 5 ml) contained in µmoles N-ethylmorpholine–HCl buffer, pH 8 0, 100, N-[^{14}C]-ethylmorpholine bicarbonate ($2\ 2 \cdot 10^5$ counts/min per µmole), 5, propionyl-CoA, 0 5, N-ethylmorpholine glutathione, 0 25, Mg^{2+}, 3.0, and N-ethylmorpholine ATP^{4-}, 1 5

The results (Fig 1) indicate that the initiation of the activation process proceeded without a lag phase, *i e*, the K^+ activated enzyme reaction rate curve when extrapolated back intersected the time scale at zero time Furthermore, activation was complete within 2 min under the conditions used, and after this time the ratio of the rates of the stimulated to unstimulated systems remained constant

Effect of other monovalent cations

In addition to K^+, other monovalent cations were shown to stimulate propionyl-CoA carboxylase activity[2] The kinetic constants for each of the activating cations were determined and from Fig. 2 it can be seen that, although the apparent K_m value

TABLE I

KINETIC CONSTANTS OF ACTIVATING CATIONS

Metal	*Apparent K_m (10^{-4} M)*	*Affinity constant*
Rb^+	5 9	1695
K^+	6 6	1515
NH_4^+	14 6	685
Cs^+	19 6	510

for each cation was different, the v_{max} was identical. The apparent K_m value and affinity constant, *i e*, the reciprocal of the apparent K_m value for each of the cations, is summarised in Table I The order of affinity for the binding of the cations was Rb^+, K^+, NH_4^+ and Cs^+. Na^+ did not stimulate the enzyme in the experiments where it was used

Effect of K^+ on the kinetic constants of the reaction components

The addition of K^+ to various enzyme systems has resulted in a number of different effects. In some cases the v_{max} value was changed while in others, the cation caused an alteration in the apparent K_m value for one or more of the substrates[12]. Therefore, the investigation into the mode of K^+ stimulation on propionyl-CoA carboxylase was extended to include the effects of the activation on the kinetic constants of the substrates

Double reciprocal plots were obtained using propionyl-CoA, $MgATP^{2-}$ and HCO_2^- as the variable substrates in the presence of K^+—Figs 3, 4 and 5, respectively The apparent K_m value for propionyl-CoA—2 7 10^{-4} M in the presence of K^+ and

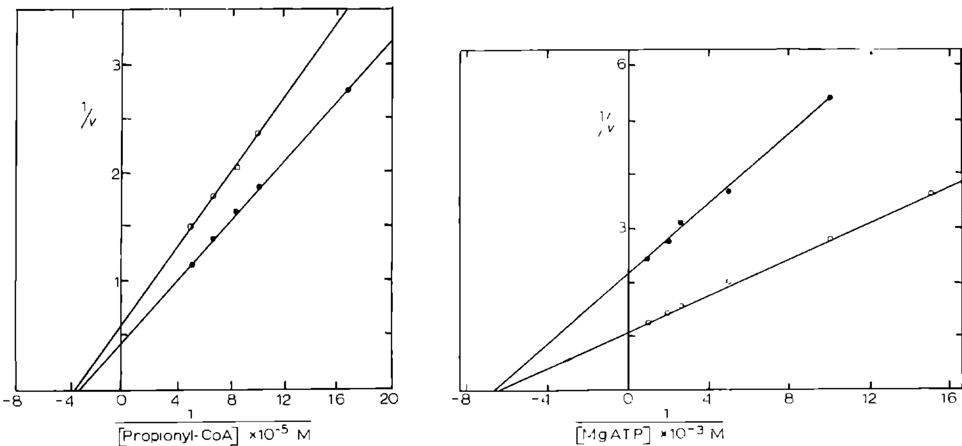


Fig 3 Determination of the apparent K_m and v_{max} for propionyl-CoA in the presence and absence of K^+ The reciprocal of the velocity plotted as a function of the reciprocal of the propionyl-CoA concentration in the absence (○—○) and presence (●—●) of 8 10^{-3} M K^+ The reaction mixture is described in Fig 1

Fig 4 Determination of the apparent K_m value and v_{max} for $MgATP^{2-}$ in the presence and absence of K^+ Conditions were the same as those described in Fig 3 (○—○) in the presence of 8 10^{-3} M K^+ and (●—●) in the absence of K^+

$2.5 \cdot 10^{-4}$ M in the absence of K^+—was similar to the value obtained by TIETZ AND OCHOA[3] using the spectrophotometric assay method and of similar magnitude to that obtained by HALENZ, FENG, HEGRE AND LANE[13] using the enzyme isolated from beef liver The same type of result was obtained when $MgATP^{2-}$ was the variable substrate, *i e*, the apparent K_m value was not altered although the apparent v_{max} value almost doubled. It should be noted that the apparent K_m value for $MgATP^{2-}$ was different from that obtained by TIETZ AND OCHOA[3] The difference could be due

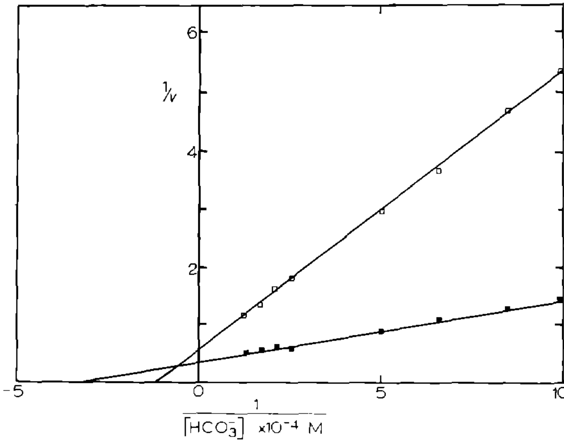
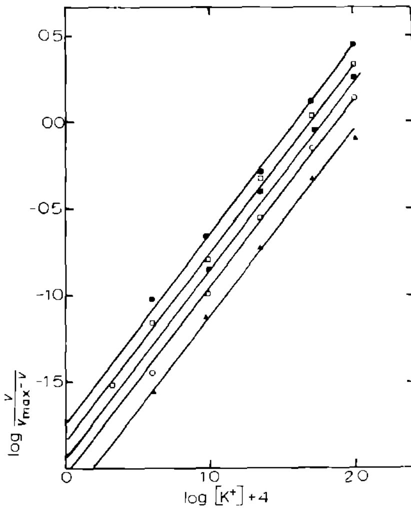


Fig 5 Determination of the apparent K_m value and v_{max} for HCO_3^- in the presence and absence of K^+ Conditions were the same as those described in Fig 3 (■—■) in the presence of 8 10^{-3} M K^+ and (□—□) in the absence of K^+

to the fact that these investigators used excess Mg^{2+} in the reaction mixture whereas in these experiments equimolar concentrations of Mg^{2+} and ATP^{4-} were used KEECH AND BARRITT[14] using sheep kidney pyruvate carboxylase showed that excess Mg^{2+} resulted in a halving of the apparent K_m value for $MgATP^{2-}$

In contrast to the zero effect on the affinities of the other substrates, the presence of K^+ markedly lowered the apparent K_m value for HCO_3^- The value changed from $8.0 \cdot 10^{-3}$ M to $3.0 \cdot 10^{-3}$ M.



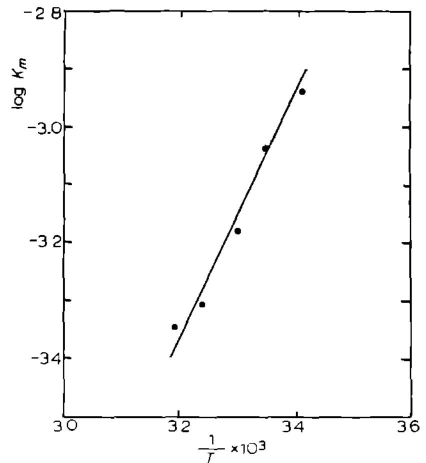


Fig 6 Hill plots for K^+ at various temperatures. Temperatures used were 40° (●—●), 35° (□—□), 30° (■—■), 25° (○—○), and 20° (▲—▲).

Fig 7 Determination of the ΔH of the activation of propionyl-CoA carboxylase by K^+ The slope of the line was determined by the method of least squares

Effect of temperature on the K^+ stimulation

A change in K_m for HCO_3^- of the magnitude described above has been taken to indicate a protein conformation change For example, COOPER AND BENEDICT[15] using yeast pyruvate carboxylase showed that acetyl-CoA lowered the apparent K_m value for HCO_3^- and concluded that the allosteric modifier caused a change in the tertiary structure of the protein. However, kinetic evidence for conformational changes can only be circumstantial More definitive evidence can be obtained from thermodynamic data and therefore the entropy change due to the presence of K^+ was measured

Hill plots for K^+ over the temperature range from 20° to 40° are presented in Fig 6. Since the slopes of the lines never exceeded a value of 1 0, it was assumed that there was only one binding site for K^+. This conclusion was supported by the fact that when K^+ was the variable ligand, the enzyme exhibited classical Michaelis–Menten kinetics In systems exhibiting Michaelis–Menten kinetics, the apparent K_m value is equal to $(k_2 + k_3)/k_1$ in Eqn 1,

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E + \text{products} \quad (1)$$

However, since the enzyme–potassium complex cannot break down to enzyme and products, then $k_3 = 0$, that is, the apparent K_m value for potassium is k_2/k_1, which is also the dissociation constant for the enzyme–potassium complex Therefore, by determining the apparent K_m or K_s value for K^+ at various temperatures, thermodynamic values can be determined

The entropy change (ΔS) can be determined from the equation

$$\Delta S = \frac{\Delta H - \Delta F}{T} \quad (2)$$

where ΔH is the standard enthalpy change, ΔF the free energy change and T is the change in absolute temperature ΔH can be evaluated from measurements of the dissociation constant as a function of the absolute temperature according to Eqn 3

$$\ln K = \frac{\Delta H}{RT} + \text{constant} \quad (3)$$

where K is the apparent dissociation constant (or apparent K_m value) and R is the gas constant A plot of $\log_{10}K$ as a function of the reciprocal of the absolute temperature

TABLE II

THERMODYNAMIC PROPERTIES OF THE K^+ ACTIVATION

ΔH = 10 070 cal/mole

Temperature	*Apparent K_m ($\cdot 10^{-4}$ M)*	*ΔF (cal/mole)*	*ΔS (entropic units)*
20°	11 5	−3930	47 76
25°	9 2	−4300	48 23
30°	6 6	−4390	47 73
35°	4 9	−4650	47 78
40°	4 5	−4780	47 42

(Fig. 7) yielded a straight line with slope, $\Delta H/2.3\ R$, from which ΔH was calculated to be 10.1 kcal/mole The change in free energy, ΔF, is derived from the following equation:

$$\Delta F = -RT \ln K \quad (4)$$

This value, ΔF, together with ΔH can be substituted into Eqn. 2 to determine ΔS Table II summarises the data obtained from a series of experiments. In particular it should be noted that the standard entropy change, ΔS, was 47.8 entropic units per mole Since the range of values for most chemical reactions is in the range +10 to −30 entropic units per mole, the value of 47.8 presented in Table II would predict that K^+ caused a significant conformational change in the enzyme.

Supporting evidence for a K^+-induced conformation change

Conformational changes in proteins induced by reaction with small molecules can be investigated by a variety of techniques However, physical methods have the disadvantage that although they may indicate that changes have occurred, these changes cannot necessarily be related to biological activity. In the case of propionyl-CoA carboxylase, spectral changes in the ultraviolet region in the presence and absence of K^+ were not observed Several explanations to account for the inability to observe a change are possible: (a) spectral changes can only be expected if the environment around aromatic residues is altered, (b) since the molecular weight of the enzyme is so

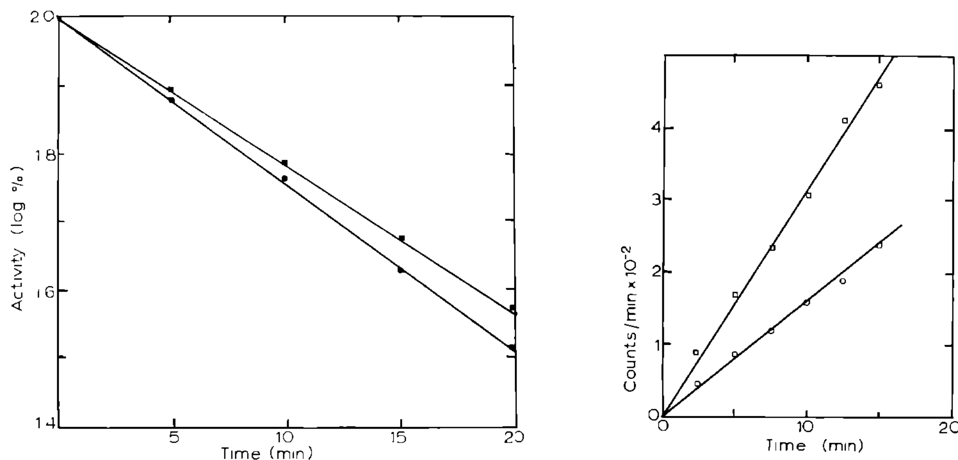


Fig 8. Effect of K^+ on the rate of inhibition of propionyl-CoA carboxylase by *N*-ethylmaleimide Propionyl-CoA carboxylase (0.2 unit) was incubated at 22° with $2 \cdot 10^{-4}$ M *N*-ethylmaleimide in $4 \cdot 10^{-2}$ M Tris–HCl buffer, pH 8.2, with and without $8 \cdot 10^{-3}$ M K^+ Aliquots of the solution were removed at various time intervals and assayed for enzymic activity (●—●) in the presence of K^+, (■—■) in the absence of K^+

Fig 9. Effect of K^+ on the uptake of *N*-[^{14}C]ethylmaleimide by propionyl-CoA carboxylase. Propionyl-CoA carboxylase (0.5 unit) was incubated with $2 \cdot 10^{-4}$ M *N*-[^{14}C]ethylmaleimide contained in 0.04 M Tris–HCl, pH 8.2, at 22° with (□—□), and without $8 \cdot 10^{-3}$ M K^+ (○—○) Aliquots of the incubation mixture were taken at various time intervals and the reaction stopped quickly by dilution in 10% trichloroacetic acid containing $1 \cdot 10^{-2}$ M *N*-ethylmaleimide The denatured protein was isolated by filtration on a membrane filter apparatus, washed thoroughly with *N*-ethylmaleimide and 1% acetic acid The membranes were dried and the radioactivity determined

high (700 000)[16] it is possible that changes affecting one or two aromatic residues would represent a very small percentage of the total ultraviolet absorption so that any changes occurring would be beyond the sensitivity of the instruments available.

Therefore, confirmatory evidence for a change in tertiary structure due to K^+ was obtained by measuring the rate of inactivation of the enzyme by *N*-ethylmaleimide in the presence and absence of K^+. The data obtained are presented in Fig 8. It can be seen that the presence of K^+ increased the rate of inactivation Although the difference in the two inhibition rates was not very great, the inhibition was primarily a reflection of the environment at the propionyl-CoA binding site[17]. To confirm this result, the rate of incorporation of radioactivity from *N*-[^{14}C]ethylmaleimide was determined in the presence and absence of K^+ in the hope that the difference between the two systems would be greater than that obtained in Fig 8 Fig 9 shows that the presence of K^+ changed the rate at which *N*-[^{14}C]ethylmaleimide was bound to the protein

DISCUSSION

The original aims of this investigation were (a) to determine whether the enzyme exhibited an absolute requirement for monovalent cations or whether the cation simply stimulated a basal activity, (b) to locate in the reaction sequence the locus of univalent cation influence, and (c) if possible, to obtain some information which could elucidate the mechanism by which the cation exerts its effect.

With regard to the first objective, it was not possible to demonstrate an absolute requirement for K^+ even after complete removal of alkali metals from both the enzyme preparation and the reagents used in the assay mixture.

The locus of the K^+ stimulation, as deduced from a kinetic analysis of the enzyme in the presence and absence of the univalent cation, indicated that the point of action is at or near the HCO_3^- binding site, resulting in a significant increase in the affinity of the enzyme for HCO_3^-. Studies by GIORGIO AND PLAUT[18] using bovine liver propionyl-CoA carboxylase, which is stimulated 7-9-fold in the presence of K^+, showed that the ATP–P_i, ADP–ATP exchange reactions and the transcarboxylating activity catalysed by the enzyme were stimulated by K^+. Two possible explanations could be advanced to account for the apparently different roles of K^+ in the two enzymes First of all it is possible that the liver and heart enzymes have different reaction mechanisms. Alternatively, the increase in the nucleotide exchange rates observed by GIORGIO AND PLAUT[18] could be due to the influence of K^+ at or near the HCO_3^- binding site since HCO_3^- is essential for the ATP–P_i exchange reaction It would be expected that to be most effective, K^+ and, for that matter, all other activating ligands would have to exert their effect at the slowest step in the reaction sequence.

Although any postulated mechanism of K^+ action must remain speculative at this stage, the evidence presented here would suggest that a substantial conformation change is induced by potassium Supporting the kinetic evidence is the fact that K^+ induces a ΔS of 47 8 entropic units per mole. TAKETA AND POGELL[19] showed that the allosteric inhibitor of fructose-1,6-diphosphatase, AMP, induced a ΔS of -99 entropic units per mole. WORCEL[20] investigated the activation of NADH dehydrogenase by AMP and showed that the interaction between the enzyme and the activator resulted in a ΔS of $+57$ entropic units per mole. In each case the authors concluded that the

allosteric ligand produced a significant change in the tertiary structure of the enzyme. In the case of propionyl-CoA carboxylase, further supporting evidence for a K^+-induced conformation change was the fact that K^+ changed the rate at which *N*-ethylmaleimide reacted with the enzyme. The conclusion reached here is similar to that reported by MELCHIOR[21] who suggested that the role of K^+ in the pyruvate kinase reaction was to affect the enzyme conformation and thus influence the binding of substrates. By use of a nuclear magnetic resonance method, MILDVAN AND COHN[22] extended the work of MELCHIOR and concluded that the activator univalent cation K^+ enhanced the relaxation rate of water protons in the presence of the ternary complex pyruvate kinase–manganese–phosphoenolpyruvate. This was interpreted to mean that K^+ affects the conformation of the enzyme only in the presence of phosphoenolpyruvate and Mn^{2+}.

The K^+ stimulation process requires a mechanism whereby the activator changes the conformation of the protein instantaneously so that the modified protein is stimulated as soon as the cation reacts with the enzyme. It is difficult to visualise how K^+ could induce a conformational change except by influencing the ionic environment at the active site Therefore, a detailed description of the stimulation process will have to wait until more information about the chemical environment at the active centre is available.

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