KINETIC ANALYSIS OF ENZYME REACTIONS

II. THE POTASSIUM ACTIVATION AND CALCIUM INHIBITION OF PYRUVIC PHOSPHOFERASE*

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Muscle pyruvic phosphoferase,[1] catalyzing the reaction PEPA + ADP $\rightleftharpoons$ pyruvate + ATP,[2] has been shown to require both Mg^{++} (3) and K^+ or NH_4^+ ions (4–6) for maximum activity and to be strongly inhibited by Ca^{++} (4, 5). The mechanism of action of these ions is not known. The principal objective of the research reported in this paper was to acquire information about the activation by K^+ ions through the application of kinetic studies. Recent extensions of the theory of enzyme inhibition and activation (7) serve as a basis for the experimental approach applied in this paper.

A second objective of this research was to determine whether the activation of rabbit muscle pyruvic phosphoferase was essential or only stimulatory. In previous work (4, 5) the possible indispensable requirement of K^+ for rat muscle pyruvic phosphoferase was left open to question.

[1] We prefer this designation for the enzyme. The enzyme has also been variously referred to as a transphosphorylase, a phosphotransferase, a phosphokinase, pyruvopherase, and a transphosphatase. Some standardization in the nomenclature of the phosphate-transferring enzymes would be welcome. Dixon (1) and Hoffmann-Ostenhof (2) have advanced proposals in this direction.

[2] The following abbreviations and symbols are used throughout: PEPA, phospho-(enol)pyruvate; ATP, ADP, adenosinetri- and adenosinediphosphate, respectively; THAM, tris(hydroxymethyl)aminomethane; v, velocity; A, enzyme activator, for these studies, K^+, Rb^+, or NH_4^+.

Materials and Methods

Phospho(enol)pyruvate—The sodium salt was prepared from the once recrystallized silver-barium double salt[3] by trituration with a 5 to 10 per cent excess of N HCl in an ice bath, followed by addition of a bare excess of 0.5 M Na_2SO_4. The $BaSO_4$-AgCl residue was thoroughly washed, the solution of PEPA neutralized with dilute NaOH to a pH of 7.2 to 7.3, frozen overnight, and thawed, and any traces of $BaSO_4$ removed by filtration. The clear, colorless solution was diluted to an estimated 0.02 M. Small amounts of free pyruvate (0.3 to 0.4 μM per ml.) were present in all preparations.

Adenosinetriphosphate and Adenosinediphosphate—Solutions of the sodium salts, pH 7.2 to 7.3, were prepared in the usual manner from the barium salts (Sigma Chemical Company) and diluted to 0.01 M.

Tris(hydroxymethyl)aminomethane-Hydrochloride Buffer—An 0.8 M solution of THAM, m.p. 169°, recrystallized twice from aqueous methanol, was acidified at 37° with concentrated HCl to a pH of 7.40, and then diluted to 0.5 M. THAM-HCl buffers have appreciable temperature coefficients and separate buffer solutions were prepared for use at cold room temperatures. Both amine and buffer solutions deteriorate on standing at room temperature and were refrigerated when not in use.

Yeast Hexokinase—The hexokinase used was Fraction 3a prepared according to Berger *et al.* (9) by Dr. Marco Rabinovitz and Mr. M. P. Stulberg in this laboratory. For the experiments, fresh dilutions (1:40 in 2 per cent glucose) of this stock solution were made daily.

Pyruvate—The sodium salt was prepared from redistilled Eastman Kodak pyruvic acid according to the procedure of Robertson (10). Stock standard solutions were assayed by the alkaline hypoiodite method.

Phosphate and Iodine-Labile Phosphate Determinations—Inorganic phosphate was determined essentially as described by Sumner (11), and iodine-labile phosphate by an adaptation of the procedure of Meyerhof and Kiessling (12).

Pyruvate Determination—Pyruvate was assayed by a simplification of the 2,4-dinitrophenylhydrazine method (13). Except for Mg^{++} and NH_4^+, none of the components in the enzyme reaction system was found to interfere. Mg^{++} interfered by forming a precipitate if more than 7 to 8 μM were present in the assay aliquot; this amount was encountered only when the entire contents of the reaction tube were used for assay. The NH_4^+ interference occurred only if the assay sample contained more than 0.5 μM. PEPA was stable and did not react with the reagents under the conditions of the assay.

[3] We are deeply indebted to Dr. E. Baer, University of Toronto, for a gift of the crystalline double salt, prepared by the procedure of Baer and Fischer (8).

When 0.01 to 0.2 μM of pyruvate was to be determined, the entire 2.0 ml. reaction mixture was treated with 1.0 ml. of cold 0.0125 per cent dinitrophenylhydrazine in 2 N HCl, the reagent serving also to stop the reaction. After 10 minutes incubation at 37°, the tubes were removed from the bath, 2.0 ml. of water and 5.0 ml. of 0.6 N NaOH added, and the tubes allowed to stand for 10 minutes for color development. The tubes were centrifuged briefly to remove any small amount of $Mg(OH)_2$ present and the optical density at 510 mμ was read in 5 cm. horizontal cuvettes with the Coleman model 10 spectrophotometer.

When larger amounts of pyruvate were present, the reaction was stopped by addition of 1.0 ml. of cold 15 per cent trichloroacetic acid to the 1.50 ml. reaction mixture and the solutions chilled, neutralized, and diluted to 5.0 ml. Aliquots containing 0.03 to 0.45 μM were diluted to 3.0 ml. and treated with 1.0 ml. of 0.025 per cent dinitrophenylhydrazine in 2 N HCl and with 6 ml. of 0.5 N NaOH under the conditions described above. The optical density was read in 19 mm. tubes in the Coleman junior spectrophotometer.

The measurement of pyruvate formed gave a considerably more reproducible and sensitive determination of the extent of the enzymatic reaction than that of PEPA disappearance, particularly at low substrate concentrations. Initial experiments were performed by measurement of the change in iodine-labile phosphate; the limitations of this procedure led to the development and use of the assay based on pyruvate formation.

Potassium Determination—Potassium was determined by means of a Perkin-Elmer flame photometer, by use of the lithium internal standard technique and potassium standards in the range 0 to 0.5 mM per liter.

Protein Determination—Protein was estimated by the trichloroacetic acid turbidity method, according to a procedure based on that of Bücher (14) and Schwimmer and Balls (15). Turbidity was measured by light absorption at 400 mμ. The readings were calibrated against a bovine serum albumin solution, standardized by micro-Kjeldahl nitrogen determination.

Enzyme Reaction System—The K^+ activation of pyruvic phosphoferase could be studied in relation to either of the two substrates, ADP or PEPA. The studies reported herein were concerned with the relation between K^+ and PEPA, and it was thus necessary to have the ADP present in excess or unchanged concentration during the course of the reaction. This was achieved by use of an excess of hexokinase and glucose, the equilibrium of the hexokinase reaction being such as to assure rapid regeneration of ADP from any ATP formed as the reaction proceeded. In most of the experiments, the concentration of the ATP-ADP system was above 0.002 M, sufficient for nearly maximum activity. In experiments in which ATP-ADP concentrations were less than that necessary for maximum activity of

the pyruvic phosphoferase, the only effect on the velocity measurements was that they were lower by a constant factor than the maximum otherwise attainable.

A typical enzyme reaction mixture had the following composition: 0.04 M THAM-HCl buffer, pH 7.4, 0.005 M MgSO$_4$, 0.0021 M ATP, 0.15 M KCl, 0.00136 M PEPA, 0.052 ml. of hexokinase solution in 2 per cent glucose, and 0.154 ml. of pyruvic phosphoferase (1:2000 dilution of a stock solution) in a final total volume of 1.50 ml. The buffer, MgSO$_4$, hexokinase-glucose, and ATP were first incubated at 37° for 5 to 10 minutes; then water, K$^+$, PEPA, and enzyme were added and the tubes incubated at 37° for the desired reaction interval. All solutions except enzyme were brought to 37° before addition and mixing. Additions were made from appropriate solutions with carefully calibrated Levy-Lang constriction micro pipettes.

The THAM-HCl buffer had no effect on the rate of phosphate transfer in the range 0.01 to 0.08 M. At high buffer concentrations there occurred an inhibition of the reaction similar to that observed with higher concentrations of K$^+$ and NH$_4$$^+$. Approximately maximum activity was reached when Mg^{++} was present in concentrations of 0.002 to 0.003 M in trials with ADP added directly in place of ATP, hexokinase, and glucose.

The reaction velocity under the conditions given above was linear with enzyme concentration and linear with time within experimental error when less than 40 per cent of the total PEPA present had reacted, except at PEPA concentrations below about 1.5×10^{-4} M. At the lower phospho-pyruvate concentrations the initial velocities were approximated by extrapolation to zero time of plots of amount of reaction during a time period against the mid-point of the time period. Requisite corrections were not over 10 per cent and accurate velocity measurements with substrate concentration well below the K_m were obtained; thus accurate evaluation of the K_m is allowed.

Rabbit Muscle Pyruvic Phosphoferase—The enzyme used was partially purified by a combination of (NH$_4$)$_2$SO$_4$ and alcohol fractionation. 750 gm. of fresh rabbit muscle were promptly ground and extracted in the cold with 3 liters of water, the pH during the extraction being maintained near 6.0 by the addition of phosphate buffer. After low speed centrifugation, the pink, opalescent extract was brought to pH 5.1 with dilute acetic acid and to 0.3 saturation with (NH$_4$)$_2$SO$_4$ by addition of (NH$_4$)$_2$SO$_4$ solution saturated at 2°. After 3 days the precipitate was centrifuged and eventually discarded, and the supernatant brought to approximately 0.65 saturation (2°) by the addition of 222 gm. of solid (NH$_4$)$_2$SO$_4$ per liter. After 3 days the precipitate was collected by centrifugation, dissolved in 0.5 M THAM-HCl buffer, pH 7.2, and dialyzed for 4 days at 2° against frequent changes of 0.005 M THAM-HCl buffer, pH 7.2. Insoluble material was

removed by centrifugation, the pH adjusted to 6.3, the solution cooled to near 0°, and 52 per cent ethanol (volume per volume) added carefully with stirring. The fractions which precipitated at 10 per cent ethanol and $-4°$ and at 20 per cent ethanol and $-9°$ were discarded after assay. The fraction that precipitated at 30 per cent ethanol and $-10.5°$ was collected by centrifugation at 12,000 r.p.m. in a Servall high speed centrifuge at $-5°$, dissolved in 0.5 M THAM-HCl buffer, pH 7.2, 0°, and dialyzed against frequent changes of 0.005 M THAM-HCl buffer, pH 7.2. The insoluble material was removed by centrifugation and the solution stored in the refrigerator as a stock solution.

The final fraction used for assay contained approximately 26 per cent of the activity of the original extract and represented an 8-fold purification over the original turbid extract, based on activity per unit of protein.[4] The fraction which precipitated at 20 per cent ethanol and $-9°$ contained approximately 28 per cent of the original activity and represented a 4-fold purification. The stock solution used for assay was sufficiently free from interfering enzymatic activities to be satisfactory for the studies; most important was that enolase activity was less than 1 per cent of the phosphoferase activity. The stock solution was relatively stable over a period of 6 months in the cold; 1:200 dilutions were stable for 2 weeks in the cold; very dilute solutions deteriorated rapidly. Potassium ion exhibited a slight capacity to retard deterioration in dilute solutions, but cysteine none. The stock solution was found to be approximately 5×10^{-4} M with respect to both K^+ and NH_4^+; hence the dilute solutions used in the kinetic studies were essentially free of these ions.

EXPERIMENTAL

Indispensability of Potassium Activation—The relation between K^+ concentration and enzyme activity has been determined in several trials; some of the experimental results are given later in this paper. In the absence of added K^+ the activity was never over 1.5 per cent of the maximum attainable. By direct assay the complete reaction system contained about 1.5 $\times 10^{-4}$ M K^+. In view of the difficulty of estimating K^+ at such low concentration in the presence of a 100-fold excess of Na^+, the K^+ assay may be regarded as an approximation only. Determination of ammonia by a micro diffusion procedure showed that the NH_4^+ ion was present at about 10^{-4} M; thus the complete reaction system contained roughly 2×10^{-4} M

[4] Kornberg and Pricer (16) have recently reported a procedure for the extraction of pyruvic phosphoferase from rabbit muscle in which is obtained a preparation roughly $2\frac{1}{2}$ times the activity of that used in these experiments and approximately equal in activity to the crystalline material of Kubowitz and Ott (17) prepared from human muscle.

total K^+ and NH_4^+. This amount could readily account for the residual 1.5 per cent activity observed in the absence of added K^+. The conclusion thus seems justified that K^+ functions as an indispensable activator of rabbit muscle pyruvic phosphoferase.

NH_4^+ and Rb^+ Activation—Both Rb^+ and NH_4^+ are able to replace K^+ as activators of the enzyme. In Figs. 1 and 2 are presented the velocity-concentration curves and the plots of A/v *versus* A for these ions. Essentially identical results were obtained with several experiments with each ion. In the case of NH_4^+ there is observed a decrease in velocity beyond
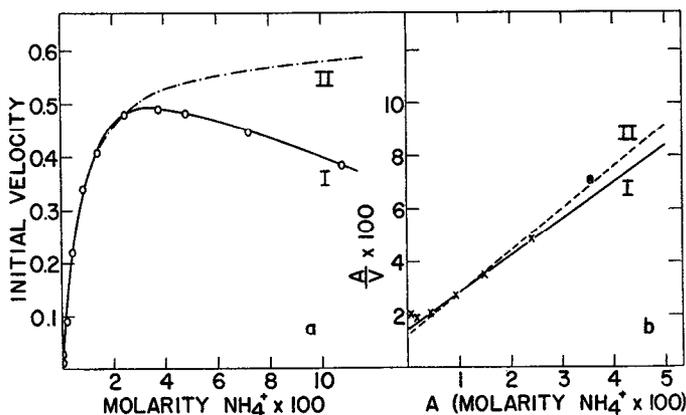

FIG. 1. The activation of pyruvic phosphoferase by NH_4^+. The initial velocity is expressed as micromoles of pyruvate formed per 10 minutes. The assay conditions were as described in the text with 1.2×10^{-3} M PEPA and 1.4×10^{-3} M ATP. (a) Velocity *versus* NH_4^+ concentration. Curve I, the experimental data; Curve II, the theoretical curve based on $K_A = 0.008$ M. (b) A/v *versus* A plots. Curve I, the line based on the first seven points; Curve II, the least squares fitted line based on the first eight points. K_A for Curve I = 0.010; K_A for Curve II = 0.008.

approximately 0.035 M NH_4^+. This decrease at relatively low NH_4^+ concentrations makes difficult accurate evaluation of the Michaelis constant, K_A. By excluding points at which inhibition was evident, calculations gave a value of 0.011 M for K_A.

In the case of Rb^+ a decrease from the maximum velocity was noted with Rb^+ concentrations of 0.065 M or greater (Fig. 2), and K_A, calculated by using points free of evident inhibition, was approximately 0.01 M. All three ions, K^+, Rb^+, and NH_4^+, were thus observed to have values of K_A close to 0.011 M.

By comparing the reaction velocities obtainable for a given enzyme concentration with each of the three ions at those concentrations at which the velocity-concentration curves passed through their maximum values, there are obtained the following relative values: $K^+ : Rb^+ : NH_4^+ = 1.00 : 0.84 : 0.72$.

The potential enzymic activity obtainable with K⁺ is thus greater than that for the other cations.

The inhibition of the reaction velocity at the higher concentrations of K^+, NH_4^+, and Rb^+ may be the result of ionic strength effects or of combination of the enzyme with ions present to give inactive forms. The occurrence of inhibition by excess activator at lower NH_4^+ and Rb^+ concentrations than K^+ concentrations suggests that combination of the enzyme with additional monovalent cations is responsible for the inhibition. Theoretical curves based on such considerations are in harmony with the experimental data.
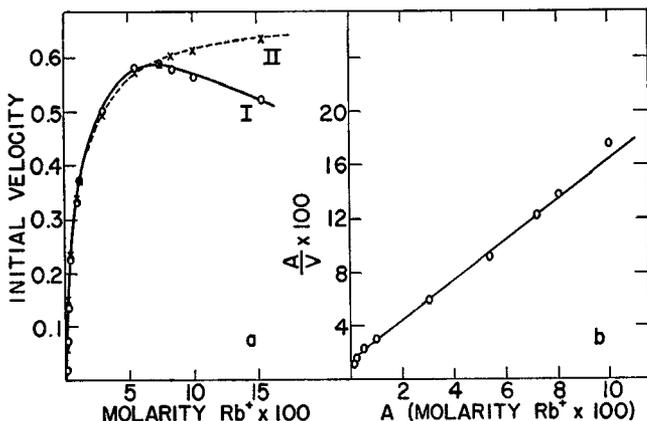

Fig. 2. Rubidium activation of pyruvic phosphoferase. The initial velocity is expressed as micromoles of pyruvate formed per 10 minutes. The assay conditions were as described in the text with 1.3×10^{-3} M PEPA and 1.8×10^{-3} M ATP. (a) v versus Rb^+ curves. Curve I, experimental; Curve II, theoretical curve based on $K_A = 0.0094$. (b) A/v versus A plot for the first nine points.

Action of Ca^{++}, Li^+, and Na^+—No pyruvate was formed when several concentrations of Ca^{++} were added in place of K^+. Low concentrations of Ca^{++} inhibited activation of the enzyme by K^+; with K^+ at 0.05 M and 0.002 M Ca^{++} ($K^+/Ca^{++} =$ 25), approximately 50 per cent inhibition was observed. Addition of further K^+ reversed the Ca^{++} inhibition appreciably, but not completely. For a more detailed analysis of the inhibition, the effect of variation of K^+ at different concentrations of Ca^{++} was studied. Plots of A/v against A at three different concentrations of Ca^{++} are shown in Fig. 3. The possible nature of Ca^{++} inhibition is discussed later in this paper.

Na^+ was found to possess a real but weak activating capacity. Li^+ gave little or no activation. Both these ions were found to counteract K^+, as shown by the data of Fig. 4, but the nature of Li^+ and Na^+ inhibition was not subjected to a more detailed study.
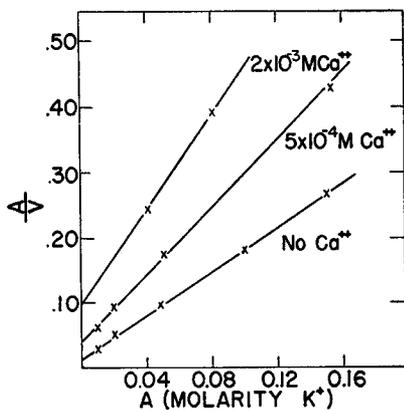
Fig. 3   Fig. 4

Fig. 3. The relation between K^+ activation and Ca^{++} inhibition of pyruvic phosphoferase. The assay conditions were essentially as described in the text. Enzyme and Ca^{++} were incubated together for 40 minutes before being added to enzyme reaction tubes containing K^+ and other components.
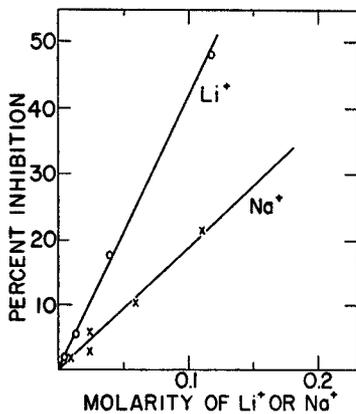
Fig. 4. The inhibition of K^+ activation of pyruvic phosphoferase by Li^+ and Na^+. The assay conditions were as described in the text with 0.055 M K^+ and 1.2×10^{-3} M PEPA.

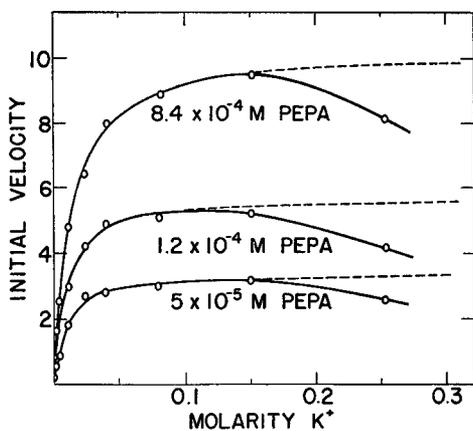Fig. 5   Fig. 6

Fig. 5. The relation between K^+ concentration and reaction velocity at three fixed concentrations of PEPA. The initial velocity is expressed as micromoles of pyruvate formed per 10 minutes $\times$ 100. The assay conditions were as described in the text. The dotted lines represent theoretical curves in the absence of inhibition by excess K^+.
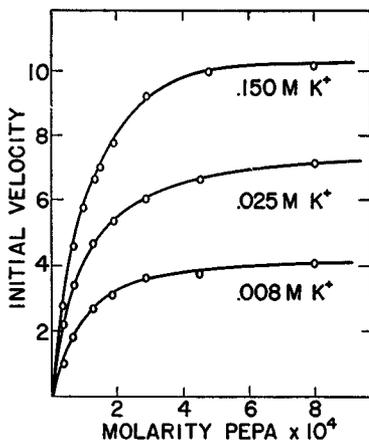
Fig. 6. The relation between PEPA concentration and reaction velocity at three fixed concentrations of K^+. The assay conditions were as described in the text. The initial velocity is expressed as micromoles of pyruvate formed per 10 minutes $\times$ 100.

Kinetic Studies of K+ -PEPA Interaction—For the desired kinetic analyses, the velocities of phosphoferase reaction were measured over a range of PEPA concentrations at three different values of K+ and over a range of K+ concentrations at three different values of PEPA. In different experiments the velocities obtainable under optimum conditions (0.15 M K+, 8 × 10⁻⁴ M PEPA) were determined in order to obtain a measure of the respective enzyme concentrations. This allowed calculation of all results on a basis of equivalent concentrations of enzyme. The data in Fig. 5
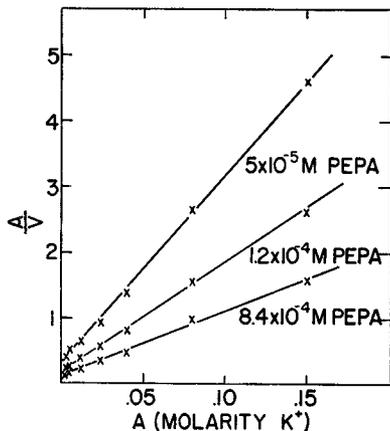

FIG. 7

FIG. 8

FIG. 7. Plots of A/v versus A for the data of Fig. 5 relating reaction velocity and K+ concentration for three fixed concentrations of PEPA. The lines are drawn on the basis of least squares treatment of the data.
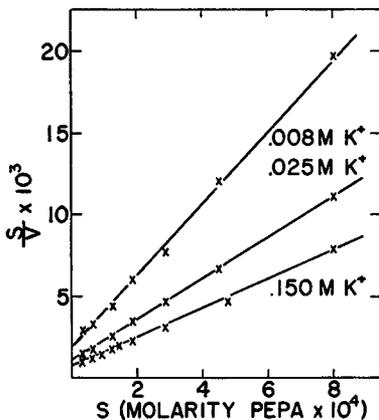
FIG. 8. Plots of S/v versus S for the data of Fig. 6 relating reaction velocity and PEPA concentration. The lines are drawn on the basis of least squares treatment of the data.

show that at all three fixed concentrations of PEPA no activity was observed, within experimental error, in the absence of potassium. Also, at all three values of PEPA the velocity decreased at K+ concentrations greater than 0.15 M. The curves of Figs. 5 and 6 show typical Michaelis-Menten relationships at the various concentrations tested. Reasonably good straight lines were obtained in plots of A/v against A and S/v against S (substrate) as shown in Figs. 7 and 8.

The values of the slopes, intercepts, K_S, and K_A (corresponding to Michaelis constants and equivalent to the ratios of intercepts to slopes) obtained from the data are listed in Table I. These results show that the values of K_s and K_A were constant within reasonable experimental error at the three different concentrations studied in the respective experiments.

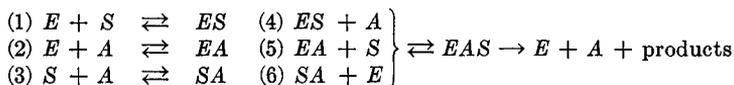For similar conditions other measurements of K_A gave values of 0.0108, 0.0113, 0.0179, and 0.0107 M.

TABLE I

Values of Slopes, Intercepts, K_S, and K_A Obtained from Plots of S/v versus S and A/v versus A of Velocity-Concentration Data

	S/v vs. S plot			A/v vs. A plot			
K^+	Intercept × 10^3	Slope	$K_S \times 10^5$	PEPA × 10^5	Intercept	Slope	K_A
M				M			
0.008	1.93	22.09	8.72	5	0.312	28.50	0.0109
0.025	1.08	12.62	8.54	12	0.187	16.87	0.0111
0.147	0.754	8.76	8.61	84	0.121	9.83	0.0123
Average....			8.62				0.0114

DISCUSSION

Explanations of the activation by K^+ in terms of present concepts of enzyme action are most logically based on the assumption of formation of a complex of enzyme, activator, and substrate. The six possible reactions between enzyme (E), activator (A), and substrate (S), leading to the formation of a ternary complex, EAS, which undergoes reaction to give products, are as follows:

$$
\begin{array}{ll}
(1) \ E + S \ \rightleftarrows \ ES & (4) \ ES \ + A \\
(2) \ E + A \ \rightleftarrows \ EA & (5) \ EA \ + S \\
(3) \ S + A \ \rightleftarrows \ SA & (6) \ SA \ + E
\end{array} \right\} \rightleftarrows EAS \rightarrow E + A + \text{products}
$$

The dissociation constants for the various Reactions 1 to 6 may be denoted by $K_1, K_2 \ldots K_6$. The ternary complex could be formed by one or more of the following pathways: I, Reactions 1 and 4; II, Reactions 2 and 5; or, III, Reactions 3 and 6. The reactions may proceed in such a manner that the concentrations of the various components present are essentially the equilibrium concentrations as determined by the various dissociation constants involved (equilibrium conditions). Alternatively, the concentrations of the components, although remaining nearly constant over a given time period, may deviate considerably from the equilibrium values (steady state conditions). The derivation and form of the velocity equations for enzyme activation reactions have been discussed elsewhere (7).

The results of the kinetic studies of K^+ activation lead to elimination of some of the possible reaction pathways and allow a likely but not final choice between the remaining alternate possibilities. Compulsory reaction by Pathway III requires that the velocity-substrate concentration

curve for a given suboptimum fixed value of A be superimposable on the velocity-activator concentration curve for the same fixed value of S. This is not in accord with the experimental results and hence this mechanism can be eliminated from further consideration.

Compulsory reaction by Pathway I under equilibrium conditions is ruled out because this path requires a constant slope in the plots of A/v versus A for all concentrations of S, whereas actually the plots given in Fig. 7 show pronounced changes in both intercepts and slopes.[5] Analogously, Pathway II can be eliminated. If equilibrium conditions prevail, the change in both the slopes and intercepts is consonant with a combination of Pathways I and II describing the enzyme reaction. This possibility is regarded as the most probable description of the enzyme reaction mechanism. If such is the case, the constancy of the intercept-slope ratio (values of K_S and K_A) could only occur if $K_1 = K_5$ and $K_2 = K_4$;[6] i.e., if the affinity of the enzyme for the substrate is unaffected by a previous bonding with activator and vice versa (7). Under these conditions the combination of reaction Pathways I and II leads to a relation between velocity and concentration of A and S as follows:

$$v = \frac{\phi AS}{K_A K_S + K_A S + K_S A + AS}$$

where ϕ is the maximum attainable velocity with excess A and S. A combination of Pathways I and II under steady state conditions leads to non-linear plots of A/v versus A and S/v versus S and thus, within experimental error, this possibility may be eliminated from consideration.

If steady state conditions prevail, the assumption that Pathway I alone or Pathway II alone describes the enzyme reaction would give results in conformity with the experimental data. The differentiation between such steady state and equilibrium conditions with independent combination of A and S requires an independent means of measuring K_1 or K_2. From previous considerations (7) it may be deduced that the observed constancy of the intercept-slope ratio for the S/v versus S and A/v versus A plots would be observed under steady state conditions only if certain relationships existed between some of the specific reaction rate constants. Such relationships appear less probable than the existence of equilibrium condi-

[5] The values of the slopes and intercepts would be interchanged in these plots if $1/v$ versus $1/A$ and $1/v$ versus $1/S$ plots were used, in correspondence with the equations given by Segal et al. (7).

[6] The experimentally obtained values for the ratio of intercept to slope appear constant within about 10 per cent; the experimental error was of about this same order of magnitude. Therefore, the results would not permit demonstration of a real difference between K_1 and K_4 (or K_2 and K_5), if they differed roughly by less than 15 to 20 per cent.

tions, and therefore it appears that the pyruvic phosphoferase reaction mechanism is best described by assuming independent combination of K^+ and PEPA with the enzyme under equilibrium conditions.

Some deductions may be made as to the number of essential potassium ions bound per enzyme molecule. If, for each reactive site for phosphopyruvate, more than 1 K^+ has to be bound for full activation of the enzyme, the velocity should be related to a higher power of the K^+ concentration. As shown in Fig. 7, a straight line relationship was obtained

TABLE II

Values of Certain Physical Properties of Alkali Ions and NH_4^+

Ion	Estimated radius of hydrated ion			Relative No. of H₂O molecules in hydrated ion		Ionic radius from crystal data	Relative ion mobility
	A	A	A			A	
Li$^+$	10.03*	3.09†	2.30‡	13.9§	6.5‖	0.60¶	33.3**
Na$^+$	7.90	2.56	1.79	8.4	3.5	0.95	43.4
K$^+$	5.32	1.98	1.22	5.4	1.9	1.33	64.6
NH$_4^+$	5.37					1.48	64.7
Rb$^+$	5.09		1.17	(5.1)	1.25	1.48	67.5
Cs$^+$	5.03		1.17	4.8		1.69	68.0
Ca^{++}			3.05			0.99	51.9 ($\frac{1}{2}$ Ca^{++})
Mg^{++}			3.44			0.65	45.9 ($\frac{1}{2}$ Mg^{++})

  * Jenny (18); *cf.* also Nachod and Wood (19).

  † Gorin (20).

  ‡ Moelwyn-Hughes (21).

  § Based on International Critical Tables (22), assuming 4 for hydration number of Cl$^-$ as given by Taylor and Taylor (23). The value of Rb$^+$ is interpolated.

  ‖ Robinson and Stokes (24).

  ¶ Pauling (25).

  ** Taylor and Taylor (23).

with a plot of A/v *versus* A, indicating that only 1 K^+ needs to be bound per phosphopyruvate bound for full activation.

The observed activation of the enzyme by K^+, NH_4^+, and Rb^+, together with its inhibition by Na^+ and Li^+, strongly suggests that some distinctive characteristic which K^+, NH_4^+, and Rb^+ have in common is essential for the activation. Consideration of available data suggests that perhaps those properties of the ions associated with their hydration (summarized in Table II) allow explanation of the experimental observations. The three active ions, K^+, NH_4^+, and Rb^+, have values for ionic radii in crystals, ion mobility, and estimated size of hydrated ion which are approximately alike and significantly distinct from those for the inactive ions.

It seems probable that the active and inactive ions both combine with the same negatively charged site (or sites) on the enzyme. Such combination could logically involve some displacement of adjoining structures, and catalytic activity may be associated with a critical amount of such displacement. Whether the combination would be accompanied by a displacement governed by the dimensions of the hydrated or the unhydrated ion is uncertain.

Nature of Ca^{++} Inhibition—This is of interest because of the well recognized biological antagonism between Ca^{++} and K^+. The results clearly show that inhibition by low Ca^{++} concentrations can be partially overcome by increase in the K^+ concentrations; *i.e.* that the inhibition is, in part, competitive. However, the observed effects of Ca^{++} inhibition (Fig. 3) appear complex and are not in agreement with equations describing various commonly considered types of inhibition (7). As shown in Fig. 3, the plots of A/v *versus* A at different Ca^{++} concentrations do not have a common slope. Further, the ratios of slope to intercept decrease with increasing Ca^{++} concentration and plots of $1/v$ *versus* Ca^{++} at different K^+ concentrations do not yield straight lines. Consideration of these results suggests that Ca^{++} has both "apparent competitive" and "non-competitive" effects in relation to K^+. More definite conclusions would require study of possible antagonistic effects between Ca^{++} and Mg^{++}, the other activating ion for the enzyme.

SUMMARY

A detailed kinetic analysis of the interaction of K^+ and phosphopyruvate with rabbit muscle pyruvic phosphoferase has been made. The indispensability of K^+, NH_4^+, or Rb^+ for the activity of the enzyme has been demonstrated and the Michaelis constants for these ions have all been found to be close to 0.011 M. Li^+ and Na^+ counteract K^+ activation.

Measurements of the Michaelis constants for K^+ at different concentrations of phosphopyruvate and for phosphopyruvate at different concentrations of K^+ have shown that the respective Michaelis constants remain essentially constant. The average values were $K_A = 0.0114$ M for K^+ and $K_S = 8.6 \times 10^{-5}$ M for phosphopyruvate.

From kinetic considerations various possible mechanisms of the K^+ activation have been eliminated. The data point to independent combination of the K^+ and the phosphopyruvate with the enzyme under equilibrium conditions to form an active ternary complex. However, final choice between this reaction pathway and a compulsory combination of K^+ with either free or substrate-bound enzyme under "steady state" conditions is not at present feasible.

The inhibition of pyruvic phosphoferase by Ca^{++} appears complex. In

part the Ca^{++} inhibition can be overcome by increase in the K^+ concentration; this portion appears to be of the "apparent competitive" type. In addition Ca^{++} exerts an inhibitory effect which cannot be removed by increasing K^+ concentration.

A simple procedure for partial purification of rabbit muscle pyruvic phosphoferase and a convenient method of enzyme assay based on measurement of the amount of pyruvic acid formed are described.

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