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KINETIC STUDIES WITH PHOSPHOTRANSACETYLASE

V. THE MECHANISM OF ACTIVATION BY UNIVALENT CATIONS*

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SUMMARY

1. We have examined the kinetic effects of monovalent cations, M^+, on the phosphotransacetylase (acetyl-CoA: orthophosphate acetyltransferase, EC 2.3.1.8) catalysed reactions 1a and 1b. All our experiments were conducted at 25 °C and used Tris buffers (pH 8.32).

$$\text{acetyl phosphate} + \text{CoA} \underset{1b}{\overset{1a}{\rightleftharpoons}} \text{acetyl-CoA} + \text{phosphate}$$

2. In the absence of monovalent cations the enzyme is probably entirely ineffective. It is activated by low concentrations of NH_4^+, K^+ and Na^+, their sequence of effectiveness, at a given concentration, being $NH_4^+ \gtrsim K^+ \gg Na^+$. Li^+ has little effect. At high concentrations of M^+ their activating power declines owing to increasing formation of the species $CH_3CO \cdot OPO_3M^-$ and $MHPO_4^-$ which are not adsorbed by the enzyme. (The true substrates being $CH_3CO \cdot OPO_3^{2-}$ and HPO_4^{2-}.) As the concentration of M^+ rises the activation therefore passes through a maximum value, which occurs when $[M^+] = 0.1–0.2$ M.

3. Kinetic analysis of both the forward and reverse reactions shows that the adsorption of nucleotide species is unaffected by M^+ and that the activation involves an interaction between M^+ and the enzyme which controls the adsorption of phosphate species. Our findings strongly suggest that the adsorption of M^+ leads to a conformational change which makes available the (separate) site at which phosphate species are adsorbed. The activation involves no sigmatropic effects.

4. Na^+, a much less effective activator than either NH_4^+ or K^+, acts as an inhibitor in the presence of these latter ions. Kinetic analysis of the reverse reaction 1b shows that Na^+ is probably a competitive inhibitor with respect to NH_4^+ (or K^+) and with respect to phosphate ions, but inhibits non-competitively with respect to acetyl-CoA. These facts are all in agreement with out findings for NH_4^+ and K^+ activation in the absence of Na^+ if it is assumed that all these M^+ compete for the same metal binding site, but that, once adsorbed, Na^+ is much less effective in inducing the important conformational change.

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* A preliminary report of this work appeared in *Biochem. J.* 129, (1972) 1163.

5. Values of K_{AP}, K_P and K_M, the Michaelis constants for acetyl phosphate, phosphate and M^+, respectively, have been calculated both from the activation, and from the Na^+ inhibition of activation, experiments. The values are everywhere self-consistent.

6. We are able to explain some discrepancies observed by other authors between K_{AP} values determined under different conditions. Certain preliminary work on the M^+ activation of the enzyme is also shown to be faulty in detail.

7. Our general conclusions about M^+ activation support one of two previous theories of its mechanism but are incompatible with the other.

INTRODUCTION

In previous papers[1-4] we have identified the general kinetic form of the phosphotransacetylase (acetyl-CoA: orthophosphate acetyltransferase, EC 2.3.1.8) catalysis of Equilibrium 1 as random bimolecular in type, there being independent adsorption of substrates by the enzyme. We have also described, for reaction in both directions, the very unusual effects of inhibition by products. The enzyme had been shown in earlier work[5,6] to be activated by univalent cations, such as K^+ or NH_4^+, and it had been claimed[6] that a certain minimum concentration of either NH_4^+ or K^+ was necessary to achieve maximum catalytic efficiency, although no detailed study of metal ion activation had been made. In our own previous experiments we accordingly used ionic concentrations in excess of those apparently needed for maximum efficiency and we also, so far as possible, employed conditions of constant ionic concentration. We now report a detailed examination of the effects of univalent cations. In our studies the source of the enzyme has been *Clostridium kluyveri*.

$$\text{acetyl phosphate} + \text{CoA} \underset{1b}{\overset{1a}{\rightleftharpoons}} \text{acetyl-CoA} + \text{phosphate} \qquad (1)$$

MATERIALS AND METHODS

Phosphotransacetylase (from Boehringer Corp.) was supplied as a suspension in 3 M $(NH_4)_2SO_4$. To test the effect of various metal ions it was necessary to produce a metal ion-free enzyme preparation. Accordingly aliquot samples of the suspension in $(NH_4)_2SO_4$ were centrifuged in a narrow tube until negligible activity remained in the supernatant liquid (20 min at 2000 rev./min for a 50-μl sample). As much as possible of the supernatant liquid was then removed and the enzyme taken up in the same volume of Tris buffer containing dithiothreitol (37.6 mM). These solutions were stored at 0 °C. An NH_4^+-free solution in buffer alone rapidly (about 10 min) loses effectively all its actual and potential activity, presumably owing to thiol oxidation. The metal ion-free solutions containing dithiothreitol had an activity approx. 3% of that observed when the optimum concentration of NH_4^+ had been added. This residual 3% almost certainly arises from the fact that the centrifuged enzyme suspension remains damp after removal of the supernatant liquid (tests of the buffer solutions of the enzyme for SO_4^{2-} were indeed positive). It was not worthwhile to attempt to free the enzyme more completely of $(NH_4)_2SO_4$ by a lengthier process since it was found that, even in the presence of dithiothreitol, the solutions at 0 °C lost actual or potential activity at the rate of about 6% per h. (The metal ion-free enzyme solutions were never used for the main kinetic experiments after 2 h.) Deliberate addition of $(NH_4)_2SO_4$ to the metal ion-free solutions led to the recovery of approx. 95% of the activity displayed by the original suspension, as shown by standard assays using either Reaction 1a or 1b. In making up the foregoing, and all other solutions used in the present work, carefully purified and ion-free water was employed. This water was thrice distilled and twice extracted with a 0.001% solution of dithiazone in CCl_4. All solutions were stored in polythene bottles fitted with screw caps.

Previous work[5], confirmed by us, has shown that Li^+ has a very small effect on phosphotransacetylase activity compared with Na^+, K^+ or NH_4^+. When $[Li^+] < 1$ mM its effect is negligible. Accordingly, when the presence of metal ions, other than those whose effect was being studied, was not easily avoided, we chose the lithium salts. Thus acetyl-CoA and acetyl phosphate were used as their tri- and di-lithium salts, respectively. These compounds were never present in concentrations exceeding 0.1 mM and 20 mM, respectively. CoA was used as the free acid and phosphate was added as H_3PO_4 neutralised with Tris. Tris–HCl buffers (pH 8.32, total concentration 0.20–0.28 M) were used throughout, and a constant ionic strength of 0.75 M was maintained by the addition of tetramethylammonium chloride. This salt has very little effect on the enzyme's reactivity, even at high concentrations. The effects of chosen metal ions were studied by adding known concentrations of their A.R. chlorides. The sources of the various chemicals are given in our previous papers[1–4].

All the kinetic experiments were conducted at 25 °C. Reactions 1a and 1b were examined using our previous methods[1–4] based on monitoring the absorption of acetyl-CoA at 235 nm. As explained above, reaction in either direction is very slow in the absence of deliberately added Na^+, K^+ or NH_4^+. Preliminary experiments on the effects of these ions showed that they all lead to activation (*e.g.* Figs 1–3), the sequence of effectiveness being $NH_4^+ \gtrsim K^+ \gg Na^+$. However, as the concentration of the added ion, M^+, rises the velocity passes through a maximum value; this value occurs at $[M^+] = 0.1–0.2$ M. The acceleratory effect of Na^+ is relatively small and this ion was not studied in detail as an activator since the residual activity of the metal ion-free enzyme preparation was not negligible compared with the activity brought about by the addition of Na^+*. Detailed studies of activation were made using NH_4^+ and K^+. These are recorded in Figs 1–8. Since Na^+ is a less effective activator than either NH_4^+ or K^+, when present together with them it would, if occupying the same site, be expected to act as a competitive inhibitor of these other ions. Experiments on Na^+ inhibition are given for the reverse Reaction 1b in Figs 11–14. Similar effects are observed for the forward reaction, but were not studied in detail. The significance of the various figures is discussed in detail below.

RESULTS AND DISCUSSION

(1) *Activation by NH_4^+ and K^+*

The region where the velocity is rising with addition of M^+ (see Figs 1–2) can be subjected to kinetic activation analysis. There exist three generally recognised and

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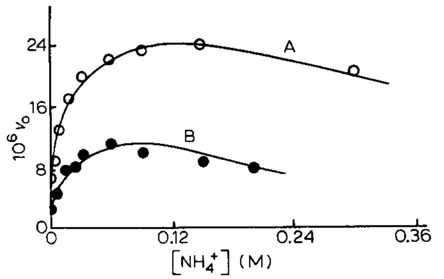
* Our results for Na^+ differ from Stadtman's[5]; he concluded that Na^+ only produces deactivation. Stadtman was not using a sufficiently metal ion-free enzyme sample.



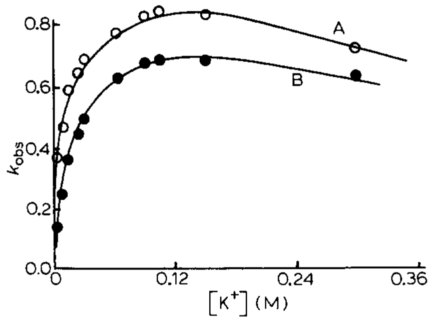
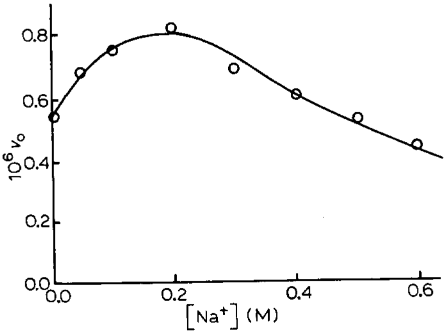


Fig. 1. Typical effects of NH_4^+ on Reaction 1b. v_0 = initial rate (mole·l^{-1}·min^{-1}); $[\text{acetyl-CoA}]_0$ = 63 μM; (A), $[\text{phosphate}]_0$ = 18.9 mM and (B) 4.7 mM; $[E_0]$ = 0.00275 μM.

Fig. 2. Typical effects of K^+ on Reaction 1a. k_{obs} = observed first-order rate constant[1] (min^{-1}); $[\text{CoA}]_0 \simeq$ 60 μM; (A), $[\text{acetyl phosphate}]_0$ = 1.02 mM and (B), 0.53 mM; $[E_0]$ = 0.00275 μM.

formally distinct ways in which an activator can function. (i) It may combine with the enzyme to facilitate either substrate adsorption or the surface reaction of the adsorbed substrate, or both. (ii) It may combine with a substrate to give the enzyme's true (or a more satisfactory) substrate. (iii) It may, by combining with an already formed enzyme–substrate complex, facilitate this complex's further reaction. These three possibilities have been treated mathematically for one-substrate systems[7,8]. For two-substrate systems, like the present, in which there exists independent, random adsorption of the substrates (A, B) under Michaelis–Menten conditions Pre-equilibria 2–17 are possible. In these equations E represents enzyme, M is a metal ion activator (M^+)



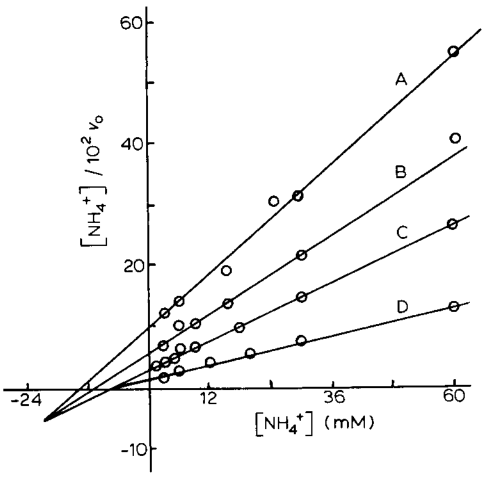


Fig. 3. Effect of Na^+ on Reaction 1b in the absence of other added metal ions. For v_0 see Fig. 1; $[\text{acetyl-CoA}]_0$ = 66 μM; $[\text{phosphate}]_0$ = 18.9 mM; $[E_0]$ = 0.00275 μM.

Fig. 4. Reciprocal plots for NH_4^+ in Reaction 1b. Plots based on data like those in Fig. 1. A, $[\text{phosphate}]_0$ = 4.7 mM and $[\text{acetyl-CoA}]_0$ = 63 μM; B, $[\text{phosphate}]_0$ = 9.4 mM and $[\text{acetyl-CoA}]_0$ = 63 μM; C, $[\text{phosphate}]_0$ = 18.9 mM and $[\text{acetyl-CoA}]_0$ = 63 μM; D, $[\text{phosphate}]_0$ = 18.9 mM and $[\text{acetyl-CoA}]_0$ = 160 μM.

$$A + \mathrm{M} \rightleftharpoons A\mathrm{M} \qquad K^{\mathrm{A}}{}_{\mathrm{M}} \tag{2}$$

$$B + \mathrm{M} \rightleftharpoons B\mathrm{M} \qquad K^{\mathrm{B}}{}_{\mathrm{M}} \tag{3}$$

$$E + A \rightleftharpoons EA \qquad K_{\mathrm{A}} \tag{4}$$

$$E + B \rightleftharpoons EB \qquad K_{\mathrm{B}} \tag{5}$$

$$E + \mathrm{M} \rightleftharpoons E\mathrm{M} \qquad K_{\mathrm{M}} \tag{6}$$

$$E + A\mathrm{M} \rightleftharpoons EA\mathrm{M} \qquad K_{\mathrm{AM}} \tag{7}$$

$$E + B\mathrm{M} \rightleftharpoons EB\mathrm{M} \qquad K_{\mathrm{BM}} \tag{8}$$

$$EA + B \rightleftharpoons EAB \qquad K_{\mathrm{B}} \tag{9}$$

$$EB + A \rightleftharpoons EAB \qquad K_{\mathrm{A}} \tag{10}$$

$$EA + \mathrm{M} \rightleftharpoons EA\mathrm{M} \qquad K_{\mathrm{M}}' \tag{11}$$

$$EB + \mathrm{M} \rightleftharpoons EB\mathrm{M} \qquad K_{\mathrm{M}} \tag{12}$$

$$E\mathrm{M} + A \rightleftharpoons EA\mathrm{M} \qquad K_{\mathrm{A}}' \tag{13}$$

$$E\mathrm{M} + B \rightleftharpoons EB\mathrm{M} \qquad K_{\mathrm{B}} \tag{14}$$

$$EAB + \mathrm{M} \rightleftharpoons EAB\mathrm{M} \qquad K_{\mathrm{M}}' \tag{15}$$

$$EA\mathrm{M} + B \rightleftharpoons EAB\mathrm{M} \qquad K_{\mathrm{B}} \tag{16}$$

$$EB\mathrm{M} + A \rightleftharpoons EAB\mathrm{M} \qquad K_{\mathrm{A}}' \tag{17}$$

and the various enzyme–substrate complexes (EAB, $EA\mathrm{M}$, $EAB\mathrm{M}$, *etc.*) have, at this stage of the argument, no particular structural significance. In treating case (i), for which Eqns 2, 3, 7 and 8 can be neglected, we assume, for the moment, that the metal ion affects the adsorption of only one of the substrates (A). K_{A}, K_{A}', K_{M}, K_{M}' and K_{B} are the various Michaelis dissociation constants, with $K_{\mathrm{A}} > K_{\mathrm{A}}'$. If the presence of M^{+} is essential for effective reaction, as appears to be so for the phosphotransacetylase system (since the small residual activity shown by M^{+}-free preparations is almost certainly due to residual $\mathrm{NH_4}^{+}$), then the overall rate is controlled by Eqn 18. The initial rate, v_0, can then be shown by an extension of the treatment given in ref. 7 to be given by Eqn 19,

$$EAB\mathrm{M} \xrightarrow{k_2} E + \mathrm{M} + \text{products} \tag{18}$$

where $[E_0]$ is the total enzyme concentration.* Eqn 19 can be

$$\begin{aligned} v_0 &= k_2\,[EAB\mathrm{M}] \\ &= \frac{k_2[E_0]}{\left(1 + \dfrac{K_{\mathrm{A}}'}{[A]}\right)\left\{1 + \dfrac{K_{\mathrm{M}}}{[\mathrm{M}]}\,\dfrac{\left(1 + \dfrac{[A]}{K_{\mathrm{A}}}\right)}{\left(1 + \dfrac{[A]}{K_{\mathrm{A}}'}\right)}\right\}} \cdot \frac{[B]}{([B] + K_{\mathrm{B}})} \end{aligned} \tag{19}$$

rearranged to give Eqn 20. This equation shows that if $[\mathrm{M}]/v_0$ is plotted against $[\mathrm{M}]$, at constant values of $[A]$ and $[B]$, straight lines will result

$$\frac{[\mathrm{M}]}{v_0} = \left\{[\mathrm{M}]\left(1 + \frac{K_{\mathrm{A}}'}{[A]}\right) + \frac{K_{\mathrm{A}}'K_{\mathrm{M}}}{K_{\mathrm{A}}} + \frac{K_{\mathrm{A}}'K_{\mathrm{M}}}{[\mathrm{A}]}\right\} \frac{([B] + K_{\mathrm{B}})}{k_2[E_0][B]} \tag{20}$$

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* It follows from Eqns 4, 6, 11 and 13 that $K_{\mathrm{A}}K_{\mathrm{M}}' = K_{\mathrm{A}}'K_{\mathrm{M}}$. Hence Eqn 19 can also be expressed in terms of K_{M}'. Eqn 19 can also be derived from a general equation for three substrate systems given by Dalziel.[9] However, Dalziel's equation was not designed with metal ion activation in mind and does not cover, for example, case (ii) discussed below.

Such lines constructed at different fixed values of $[A]$, but at a constant value of $[B]$, will intersect at the point $[\mathrm{M}]/v_0 = K_\mathrm{M}(K_\mathrm{A}'/K_\mathrm{A} - 1)\,([B] + K_\mathrm{B}/k_2[E_0][B])$ and $[\mathrm{M}] = -K_\mathrm{M}$. Since $K_\mathrm{A} > K_\mathrm{A}'$ the point of intersection will lie in the third quadrant of the graph, where both coordinates have negative values. If A can only combine effectively with the enzyme in the presence of M (*i.e.* $K_\mathrm{A} = \infty$ or, at least, $K_\mathrm{A} \gg K_\mathrm{A}'$) then Eqn 20 simplifies to 21.

$$\frac{[\mathrm{M}]}{v_0} = \left\{[\mathrm{M}]\left(1 + \frac{K_\mathrm{A}'}{[A]}\right) + \frac{K_\mathrm{A}'K_\mathrm{M}}{[A]}\right\}\cdot\frac{([B] + K_\mathrm{B})}{k_2[E_0][B]} \tag{21}$$

The point of intersection still lies in the same quadrant, but is now $[\mathrm{M}]v_0 = -K_\mathrm{M}$ $([B] + K_\mathrm{P})/k_2[E_0][B]$, $[\mathrm{M}] = -K_\mathrm{M}$.

If $[\mathrm{M}]/v_0$ is plotted against $[\mathrm{M}]$ at different fixed values of $[B]$, but at a constant value of $[\mathrm{A}]$, then Eqn 20 predicts that the resulting straight lines will meet on the M axis at $[\mathrm{M}] = -(K_\mathrm{M}K_\mathrm{A}')/K_\mathrm{A}\cdot([A] + K_\mathrm{A})/[A] + K_\mathrm{A}')$ or, if $K_\mathrm{A} = \infty$ (or, at least $K_\mathrm{A} \gg [A]$), at $[\mathrm{M}] = -K_\mathrm{M}K_\mathrm{A}'/([A] + K_\mathrm{A}')$.

For case (ii), where the activator combines with a substrate before adsorption to produce a superior substrate, then if only substrate A (and not B) is involved in this process the relevant pre-equilibria are Eqns 2, 4, 5, 7, 9, 10 and 16. The rate is again controlled by a step(s) like Eqn 18 involving some complex EABM. The mathematical treatment for case (ii) differs from that for case (i) in that in the latter the effective concentrations of A, B and M^+ are always equal to their stoicheiometric concentrations since little material is removed in forming complexes with the relatively very small amount of enzyme (or enzyme complex) present. In case (ii) however, A and M^+ may be present in comparable stoicheiometric concentrations and, by forming AM, may significantly reduce these stoicheiometric concentrations. Hence a rigorous treatment requires the use of the actual (or free) concentrations of A and M^+. The equation for v_0 expressed in terms of the free concentrations can be shown to be Eqn 22. If it is assumed that the free and stoicheiometric concentrations are approximately equal,

$$v_0 = \frac{k_2[E_0]}{\left(1 - \frac{K_\mathrm{AM}}{K_\mathrm{A}}\right) + \left(1 + \frac{[A]}{K_\mathrm{A}}\right)\left\{\frac{2K_\mathrm{AM}}{K_\mathrm{M} + [\mathrm{M}] + [A] - \{(K_\mathrm{M} + [\mathrm{M}] + [A])^2 - 4[\mathrm{M}][A]\}^{\frac{1}{2}}}\right\}}\cdot\frac{[B]}{([B] + K_\mathrm{B})} \tag{22}$$

then v_0 is given by Eqn 23 which rearranges to Eqn 24.

$$v_0 = \frac{k_2[E_0]}{\left\{1 + \frac{K_\mathrm{M}K_\mathrm{AM}}{[\mathrm{M}][A]}\left(1 + \frac{[A]}{K_\mathrm{A}}\right)\right\}}\cdot\frac{[B]}{[B] + K_\mathrm{B}} \tag{23}$$

$$\frac{\mathrm{M}}{v_0} = \left([\mathrm{M}] + \frac{K_\mathrm{M}K_\mathrm{AM}}{[A]} + \frac{K_\mathrm{M}K_\mathrm{AM}}{K_\mathrm{A}}\right)\cdot\frac{([B] + K_\mathrm{B})}{k_2[E_0][B]} \tag{24}$$

It is evident that Eqn 22 cannot lead to rectilinear plots of $[\mathrm{M}]/v_0$ against $[\mathrm{M}]$, while for the conditions applying for Eqn 24, such plots at different constant values of $[A]$, but at a constant value of $[B]$, will be parallel straight lines.

For case (iii), where M^+ only combines with an enzyme–substrate complex containing A, the relevant equations are Eqns 4, 5, 9, 10, 11, 15, 16 and 18. The resulting equation for v_0 can now be shown to be Eqn 25. Rearrangement to Eqn 26 shows that plots of $[\mathrm{M}]/v_0$ against $[\mathrm{M}]$ at different fixed values of $[A]$, but at a constant value of

$$v_0 = \frac{k_2[E_0]}{\left(1 + \frac{K_{\mathrm{M}}'}{[\mathrm{M}]} + \frac{K_{\mathrm{M}}'K_{\mathrm{A}}}{[\mathrm{M}][A]}\right)} \cdot \frac{[B]}{([B] + K_{\mathrm{B}})} \tag{25}$$

$$\frac{[\mathrm{M}]}{v_0} = \left([\mathrm{M}] + K_{\mathrm{M}}' + \frac{K_{\mathrm{M}}'K_{\mathrm{A}}}{[A]}\right)\left(\frac{[B] + K_{\mathrm{B}}}{k_2[E_0][\mathrm{B}]}\right) \tag{26}$$

$[B]$, will again be parallel straight lines. In the special sub-case of (iii) where the activator combines only with the ternary complex *EAB*, the resulting rate equation is symmetrical in $[A]$ and $[B]$. The behaviour of a series of plots of $[\mathrm{M}]/v_0$ against $[\mathrm{M}]$ at different constant values of $[A]$, but at a constant value of $[B]$, will therefore be analogous to that of a series constructed at different fixed values of $[B]$, but at a constant value of $[A]$. This behaviour is unique among the cases considered. A further conclusion from the foregoing discussion is that case (i) can, at least formally, be distinguished by our treatment from cases (ii) and (iii).

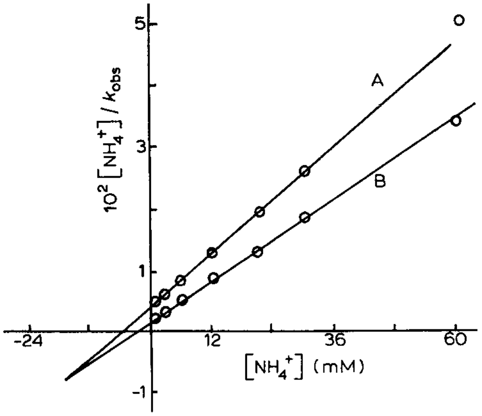


Fig. 5. Reciprocal plots for $\mathrm{NH_4^+}$ in Reaction 1a. Plots based on data like those in Fig. 2. $[\mathrm{CoA}]_0 \simeq 60\ \mu\mathrm{M}$; (A), [acetyl phosphate]$_0$ = 0.53 mM and (B), 1.02 mM.

For the present system plots of $[\mathrm{M}]/v_0$ against $[\mathrm{M}]$, where $\mathrm{M} = \mathrm{K^+}$ or $\mathrm{NH_4^+}$, are shown for both the forward and reverse reactions in Figs 4–7. We can conclude:

(a) That since the plots were constructed using stoicheiometric concentrations yet are rectilinear, therefore no significant interaction between $\mathrm{M^+}$ and either substrate occurs in the concentration ranges involved.

(b) That since plots at various fixed acetyl-CoA concentrations, but at a constant phosphate concentration, meet on the horizontal axis, whereas plots at various fixed phosphate (or acetyl phosphate) concentrations, but at a constant nucleotide concentration, pass through a common point in the third quadrant, therefore the activator does not react only with the ternary complex *EAB* and case (i), rather than cases (ii) or (iii), obtains. The plots also suggest that the adsorption of the nucleotide species is not affected by the activator. Thus the nucleotide species have

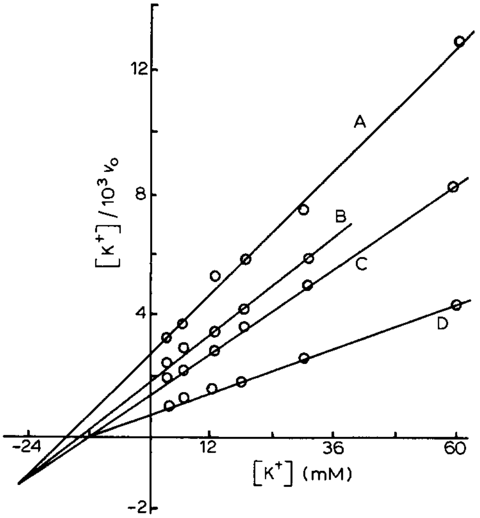


Fig. 6. Reciprocal plots for K^+ in Reaction 1b. Plots based on data like those in Fig. 1. A, $[\text{phosphate}]_0 = 4.7$ mM and $[\text{acetyl-CoA}]_0 = 63\ \mu M$; B, $[\text{phosphate}]_0 = 9.4$ mM and $[\text{acetyl-CoA}]_0 = 63\ \mu M$; C, $[\text{phosphate}]_0 = 18.9$ mM and $[\text{acetyl-CoA}]_0 = 63\ \mu M$; D, $[\text{phosphate}]_0 = 18.9$ mM and $[\text{acetyl-CoA}]_0 = 162\ \mu M$.

the behaviour expected of *B*, whereas phosphate species have the behaviour expected of *A*, in Eqns 20 or 21 and either of these equations appears to represent satisfactorily the observed behaviour.

It remains to distinguish between Eqns 20 and 21. These equations show that

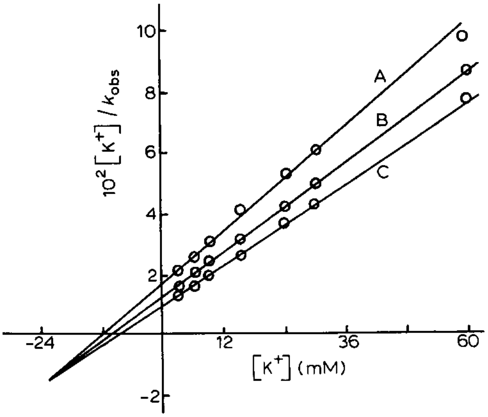


Fig. 7. Reciprocal plots for K^+ in Reaction 1a. Plots based on data like those in Fig. 2. [CoA] $\simeq$ 60 μM; (A), $[\text{acetyl phosphate}]_0 = 0.53$ mM, (B), 0.87 mM and (C), 1.02 mM.

the intercepts of plots of $[M]/v_0$ against $[M]$ on the $[M]/v_0$ axis will be given by Eqns 27 and 28, respectively. Hence a plot of these intercepts against $1/[A]$ will pass through the origin only for the conditions of Eqn 21. Fig. 8 therefore suggests that Eqn 21 is a

$$\frac{[\mathrm{M}]}{v_0} = \frac{K_\mathrm{A}'K_\mathrm{M}([B] + K_\mathrm{B})}{k_2[E_0][B]}\left(\frac{1}{[A]} + \frac{1}{K_\mathrm{A}}\right) \tag{27}$$

$$\frac{[\mathrm{M}]}{v_0} = \frac{K_\mathrm{A}'K_\mathrm{M}([B] + K_\mathrm{B})}{k_2[E_0][B]} \cdot \frac{1}{[A]} \tag{28}$$

closer approximation than is Eqn 20 in the present system. Were K_A at all comparable in magnitude with the observed Michaelis constants for phosphate species (K_A') the intercept ($^1/K_\mathrm{A}$) from Eqn 27 would have been easily detectable. It is clear that $K_\mathrm{A} \gg K_\mathrm{A}'$ and that $K_\mathrm{A} \gg [\mathrm{A}]$; these are the essential conditions for the application of Eqn 21. We may conclude that the presence of the activator is effectively essential for significant binding of the phosphate species to phosphotransacetylase.

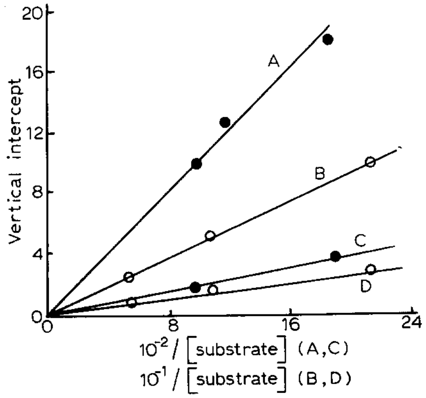


Fig. 8. Test of Eqns 27 and 28.

The arguments above, together with our previous studies, point to the following outline mechanism for phosphotransacetylase catalysis of Reaction 1a. In Eqns 29–36 the representations of the various enzyme complexes still have no particular structural significance. Accepting steps 29–36, it can be shown that v_0 is given by Eqn 37.

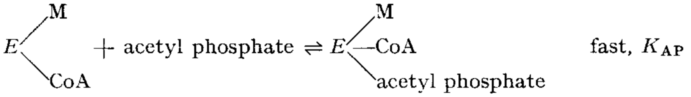
$$E + \mathrm{M} \rightleftharpoons E\mathrm{M} \qquad \text{fast, } K_\mathrm{M} \tag{29}$$

$$E + \mathrm{CoA} \rightleftharpoons E\mathrm{CoA} \qquad \text{fast, } K_\mathrm{CA} \tag{30}$$

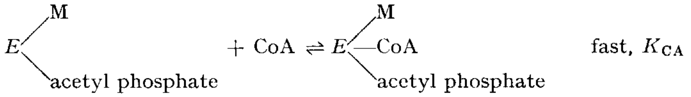
$$E\mathrm{M} + \mathrm{CoA} \rightleftharpoons E\langle\begin{matrix}\mathrm{M}\\ \mathrm{CoA}\end{matrix} \qquad \text{fast, } K_\mathrm{CA} \tag{31}$$

$$E\mathrm{M} + \text{acetyl phosphate} \rightleftharpoons E\langle\begin{matrix}\mathrm{M}\\ \text{acetyl phosphate}\end{matrix} \qquad \text{fast, } K_\mathrm{AP} \tag{32}$$

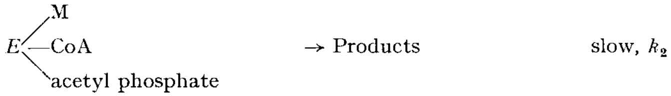
$$E\mathrm{CoA} + \mathrm{M} \rightleftharpoons E\langle\begin{matrix}\mathrm{M}\\ \mathrm{CoA}\end{matrix} \qquad \text{fast, } K_\mathrm{M} \tag{33}$$



$$E\begin{array}{l}\diagup \text{M}\\ \diagdown \text{CoA}\end{array} + \text{acetyl phosphate} \rightleftharpoons E\begin{array}{l}\diagup \text{M}\\ \text{—CoA}\\ \diagdown \text{acetyl phosphate}\end{array} \qquad \text{fast, } K_{\text{AP}} \qquad (34)$$



$$E\begin{array}{l}\diagup \text{M}\\ \diagdown \text{acetyl phosphate}\end{array} + \text{CoA} \rightleftharpoons E\begin{array}{l}\diagup \text{M}\\ \text{—CoA}\\ \diagdown \text{acetyl phosphate}\end{array} \qquad \text{fast, } K_{\text{CA}} \qquad (35)$$



$$E\begin{array}{l}\diagup \text{M}\\ \text{—CoA}\\ \diagdown \text{acetyl phosphate}\end{array} \rightarrow \text{Products} \qquad \text{slow, } k_2 \qquad (36)$$

$$v_0 = \frac{k_2[E_0][\text{acetyl phosphate}][\text{CoA}]}{([\text{acetyl phosphate}] + K_{\text{AP}})([\text{CoA}] + K_{\text{CA}})\left\{1 + \dfrac{K^{AP}K_{\text{M}}}{[\text{M}]([\text{acetyl phosphate}] + K^{AP})}\right\}} \qquad (37)$$

Thus a series of plots of $[\text{M}]/v_0$ against $[\text{M}]$ at different fixed values of [acetyl phosphate], but at a constant value of [CoA], will be rectilinear, as found, and intersect at the point $(-K_{\text{M}}, -K_{\text{M}}([\text{CoA}] + K_{\text{CA}})/k_2[E_0][\text{CoA}])$. From the observed point of intersection and the known[1–3] value of K_{CA}, the values of K_{M} and k_2 can be calculated. Analogous equations and calculations apply for Reaction 1b and lead to K_{M} and k_2 for the reverse reaction. Our results from such calculations are in Table I.

(2) *Inhibition by NH_4^+ and K^+*

When $[\text{M}^+] \gtrsim 0.1$ M, and the further addition of M^+ causes a fall in v_0 (see Figs 1–3), the results can be treated by the standard graphical procedures for analysing

TABLE I

MICHAELIS PARAMETERS FROM EXPERIMENTS ON THE EFFECTS OF MONOVALENT CATIONS

pH 8.32; temperature 25 °C; ionic strength 0.75; $K_{\text{CA}} = 1.20 \cdot 10^{-4}$ moles·l⁻¹; $K_{\text{ACA}} = 1.00 \cdot 10^{-3}$ mole·l⁻¹; CA = CoA and ACA = acetyl-CoA (values from Table II); P = phosphate and AP = acetyl phosphate.

M	*$10^3 \times K_M$ (moles·l⁻¹)*		*$10^2 \times K_P$ (moles·l⁻¹)*	*$10^4 \times K_{AP}$ (moles·l⁻¹)*	*$10^{-4} \times k_2$ (min⁻¹)*	
	Reaction 1a	*Reaction 1b*			*Reaction 1a*	*Reaction 1b*
NH_4^{+a}	17.4 ± 1.8	20.7 ± 1.8			1.2 ± 0.1	2.5 ± 0.3
NH_4^{+b}						5.3 ± 0.5
NH_4^{+c}			2.1 ± 0.2			5.2 ± 0.5
NH_4^{+d}			1.7 ± 0.2	7.8 ± 1.0	1.2 ± 0.1	5.2 ± 0.6
K^{+a}	25.0 ± 2.1	25.0 ± 2.1			0.78 ± 0.09	1.3 ± 0.2
K^{+e}			1.5 ± 0.2			1.4 ± 0.2
Na^{+b}		130 ± 10				
Na^{+c}		120 ± 10				
Na^{+e}		130 ± 10				

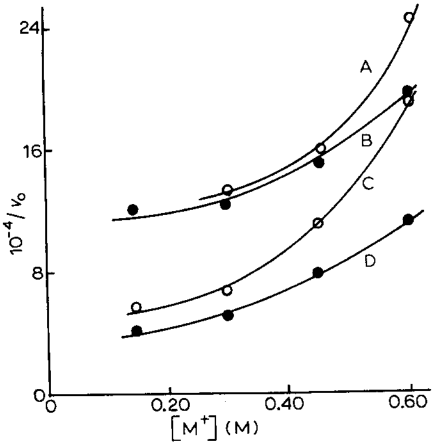
[a] Activation experiments.

[b] Competitive inhibition by Na^+ with respect to NH_4^+.

[c] Competitive inhibition by Na^+ with respect to phosphate in the presence of NH_4^+.

[d] Michaelis curves for substrates in presence of NH_4^+ (Table II).

[e] Competitive inhibition by Na^+ with respect to K^+.



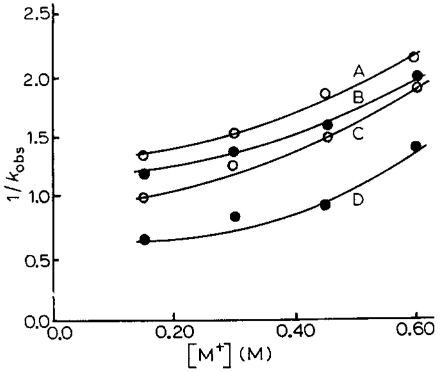


Fig. 9. Inhibition by NH_4^+ and by K^+ in Reaction 1b. From data like those in Fig. 1. $[\text{Acetyl-CoA}]_0 = 63\ \mu M$; A and B for K^+ with $[\text{phosphate}]_0 = 9.4$ mM and 18.9 mM, respectively; C and D for NH_4^+ with $[\text{phosphate}]_0 = 9.4$ mM and 18.9 mM, respectively.

Fig. 10. Inhibition by NH_4^+ and by K^+ in Reaction 1a. From data like those in Fig. 2. $[\text{CoA}]_0 \simeq 60\ \mu M$; A and B for K^+ with $[\text{acetyl phosphate}]_0 = 0.53$ mM and 1.02 mM, respectively; C and D for NH_4^+ with $[\text{acetyl phosphate}]_0 = 0.53$ mM and 1.02 mM ,respectively.

inhibition data[7]. Dixon plots of $^1/v_0$ against [M], at constant substrate concentrations, curve upwards (Figs 9 and 10). This behaviour is typical of inhibition caused by an inhibitor which combines with a free substrate to form an inactive complex[10]. Inorganic phosphate and acetyl phosphate are known to exist in solution at pH 8.3 predominantly in their dianionic forms (HPO_4^{2-}, $CH_3CO \cdot OPO_3^{2-}$). The complexes formed between K^+ and Na^+ and HPO_4^{2-} are known[11] to have dissociation constants $K = 0.34$ and $K = 0.26$ mole$\cdot l^{-1}$, respectively, at 25 °C. In our experiments these complexes will therefore begin to constitute a significant fraction of the total phosphate when $[M^+] \gtrsim 0.1$ M. Since it is known from our previous work[2,3] that only HPO_4^{2-} (and not $H_2PO_4^-$) is adsorbed by phosphotransacetylase, it appears very probable that the observed inhibition of the reverse reaction 1b by K^+ when $[K^+] \gtrsim 0.1$ arises from the formation of significant amounts of $KHPO_4^-$ which is not adsorbed by the enzyme. The equilibrium constants for K^+ and NH_4^+ are likely to be similar, so that the same effect doubtless applies for NH_4^+ inhibition. Since it is probable that the complexes between $CH_3CO \cdot OPO_3^{2-}$ and NH_4^+ and K^+ also have dissociation constants of approx. 0.3, the same type of explanation is available for the inhibition of the forward reaction at high M^+ concentrations, the monoanionic complexes not being adsorbed by the enzyme.

(3) The mechanism of activation by M^+

It follows from the foregoing sections that metal ion controls the adsorption of phosphate, and of acetyl phosphate, although metal ion–phosphate complexes are not themselves usefully adsorbed. These conclusions imply that the metal ion and phosphate species occupy separate sites on the enzyme and that that for phosphate, or acetylphosphate, only becomes available after adsorption of M^+. The reason that M^+ and phosphate are likely to occupy separate sites is that if adsorbed M^+ were to bind phosphate directly it would certainly be *via* the phosphate oxygen atom(s), so that a coordination site, or sites, on M^+, *plus* steric access, would have to be available after any occupation of any coordination sites on M^+ by the enzyme. If such a process could occur it is chemically very difficult to conceive how M^+–phosphate complexes could fail to be adsorbed by the enzyme, for M–O coordination is also involved in these complexes and space would be available on the surface. To find that M^+–phosphate complexes are unacceptable to the enzyme, as we do, is effectively to establish that any binding between adsorbed M^+ and free phosphate species will lead to the removal of M^+ from the surface rather than to binding of phosphate to the surface. The facilitation of phosphate binding by adsorbed M^+ therefore probably involves an indirect effect, such as a conformational change.

It is interesting that Shimuzu *et al.*[12] were led by results of protection studies to suggest that phosphotransacetylase undergoes a conformational change in the presence of $(NH_4)_2SO_4$. Shimuzu *et al.*[12] suggest that it is the SO_4^{2-} ion which is responsible for this change, but our results make it likely that it arises from the adsorption of NH_4^+.

That cations can produce conformational changes in proteins and lead them to adsorb small molecules not otherwise adsorbed was demonstrated by Klotz *et al.*[13]; that such effects underlie enzyme activation by monovalent cations has been suggested by Evans and Sorger[14]. The present work appears to be the first reasonably clearcut vindication of this suggestion. In contrast, our conclusions are incompatible with the roles assigned to univalent cations by Suelter[15] in his review of enzyme activation by such ions. Nor is there any evidence from more conventional catalysis of acylation that there is any benefit to be had from forming an enolic form of the acylating

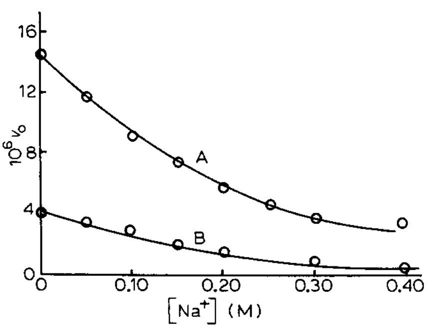


Fig. 11. Typical effects of Na^+ on Reaction 1b in the presence of other M^+. $[\text{phosphate}]_0$ = 18.9 mM; $[E_0]$ = 0.00275 μM; A, $[\text{acetyl-CoA}]_0$ = 90 μM, $[NH_4^+]$ = 6.0 mM; B, $[\text{acetyl-CoA}]_0$ = 74 μM, $[K^+]$ = 10 mM.

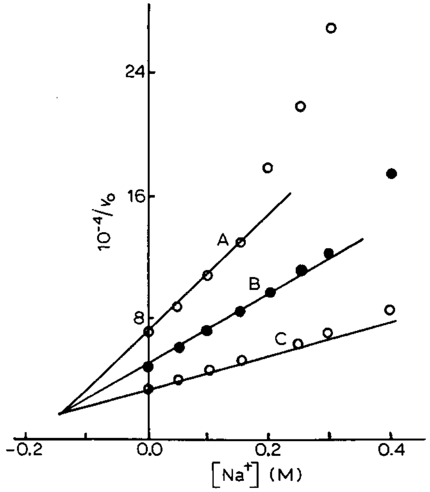


Fig. 12. Competitive inhibition by Na^+ with respect to NH_4^+ in Reaction 1b. v_0 = initial rate (mole·l⁻¹·min⁻¹); $[\text{acetyl-CoA}]_0$ = 90 μM; [phosphate] = 18.9 mM; $[E_0]$ = 0,00275 μM; (A), $[NH_4^+]$ = 6.0 mM, (B), 18 mM and (C), 60 mM.

agent[16]. (Suelter[15] suggests that, among other roles, the univalent cation somehow favours enol formation.)

(4) Inhibition by Na$^+$

Our results cover various concentration conditions for the substrates acetyl-CoA and phosphate and for the activators NH_4^+ and K^+. The presence of added Na^+ leads to inhibition (*e.g.* Fig. 11) and Dixon plots (Figs 12–14) of $^1/v_0$ against $[Na^+]$ are found to be rectilinear when $[Na^+] \leqslant 0.15$ M, but to curve upwards at higher concentrations. The region of upward curvature is similar to that found at high concentrations of K^+ and NH_4^+ (p. 136) and doubtless arises from the formation of significant amounts of $NaHPO_4^-$, in which form phosphate is not adsorbed by the enzyme. That is, in this concentration region, in addition to any other role it may have, the inhibitor is forming an inactive complex with a substrate.

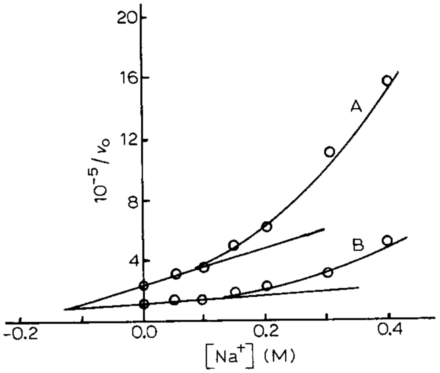


Fig. 13. Competitive inhibition by Na^+ with respect to K^+ in Reaction 1b. As for Fig. 12, but $[\text{acetyl-CoA}]_0 = 74\ \mu M$; (A), $[K^+] = 10$ mM, and (B), 50 mM.

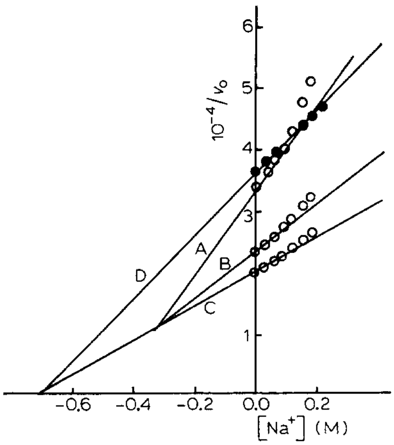


Fig. 14. Inhibition by Na^+ with respect to phosphate and to acetyl-CoA in Reaction 1b. v_0 = initial rate ($mole \cdot l^{-1} \cdot min^{-1}$); $[E_0] = 0.00137\ \mu M$; $[NH_4^+] = 40$ mM; A, $[\text{phosphate}]_0 = 30$ mM and $[\text{acetyl-CoA}]_0 = 0.2$ mM; B, $[\text{phosphate}]_0 = 50$ mM and $[\text{acetyl-CoA}]_0 = 0.2$ mM; C, $[\text{phosphate}]_0 = 80$ mM and $[\text{acetyl-CoA}]_0 = 0.2$ mM; D, $[\text{phosphate}]_0 = 80$ mM and $[\text{acetyl-CoA}]_0 = 0.1$ mM.

At $[Na^+] \lesssim 0.15$ M the rectilinear Dixon plots show that the inhibition by Na^+ may be competitive with respect to NH_4^+ (or K^+) and to phosphate, but is non-competitive with respect to acetyl-CoA. These results are expected in the light of the foregoing discussion if it is assumed that Na^+, K^+ and NH_4^+ all compete for the same enzyme site, but that, once adsorbed, Na^+ is less able to bring about the change leading to the adsorption of phosphate. The conclusions from the Dixon plots arise in the following way. The rate equation for a one-substrate system in which a modifier inhibits competitively with respect to an activator essential for substrate binding is given by Webb[10]. This equation can easily be adapted to represent a two-substrate system based on equations analogous to Eqns 29–36, in which the second (nucleotide) substrate enjoys independent adsorption. The appropriate equation is Eqn 38 in which M represents K^+ or NH_4^+, K_{Na^+} represents the dissociation constant of enzyme–Na^+ complexes, $K_M'' = K_P K_M/([\text{phosphate}] + K_P)$ and $k_2' = k_2[\text{phosphate}][\text{acetyl-CoA}]/([\text{phosphate}] + K_P)([\text{acetyl-CoA}] + K_{ACA})$.

$$v_0 = k_2'[E_0][M]/\left\{[M] + K_M''\left(1 + \frac{[Na^+]}{K_{Na^+}}\right)\right\} \tag{38}$$

Eqn 38 predicts, as found (Figs 12 and 13), that plots of $1/v_0$ against $[Na^+]$ at different fixed concentrations of M^+, but at constant concentrations of phosphate and of acetyl-CoA, will display competitive inhibition between Na^+ and M^+. These plots intersect according to Eqn 38 at the point $(-K_{Na^+}, 1/k_2'[E_0])$. Any one of these plots will have vertical and horizontal intercepts of $([M] + K_M'')/k_2'[E_0][M]$ and $-K_{Na^+}([M] + K_M'')/K_M''$, respectively. Hence the values of K_{Na^+}, K_M, K_P and k_2 can be calculated.

Eqn 38 also predicts, as found (Fig. 14), that plots constructed for various fixed acetyl-CoA concentrations, but at constant concentrations of phosphate and M^+, will indicate non-competitive inhibition between Na^+ and acetyl-CoA, meeting on Na^+ axis, whilst plots at constant M^+ and acetyl-CoA concentrations, but at various fixed phosphate concentrations, will indicate competitive inhibition between Na^+ and phosphate, intersecting in the fourth quadrant of the graph. The various intercepts and points of intersection can again be used to calculate the Michaelis parameters for Na^+, acetyl-CoA and phosphate. Our results are collected in Table I.

The inhibition by Na^+ therefore arises from two causes: at $[Na^+] \lesssim 0.15$ M the Na^+ competes with K^+ or NH_4^+ for a common enzyme site, while at $[Na^+] \gtrsim 0.15$ M, Na^+ also removes HPO_4^{2-} as the inactive $NaHPO_4^-$.

(5) *Consequences of metal ion activation and inhibition*

Apart from the inherent interest of our conclusions regarding the roles played by metal ions in reaction 1, the actual K_M values have certain important implications for our earlier work. Let us first, however, consider the K_M values generally. Table I shows that NH_4^+ is adsorbed slightly more strongly than is K^+, while the predomi-

TABLE II

COLLECTED MICHAELIS PARAMETERS FOR SUBSTRATES CORRECTED FOR THE EFFECTS OF MONOVALENT CATIONS

This table contains K_m and k_2 values from our earlier work[1–3] corrected (where necessary) for the effects of cation concentration (see text). All results refer to pH 8.32 and temperature 25 °C.

Substrate	*$10^3 \times K_m$ (moles·l⁻¹)*	*$10^{-4} \times k_2$ (min⁻¹)*
Acetyl phosphate	0.78 ± 0.10	1.2 ± 0.1
Propionyl phosphate	0.30 ± 0.10	0.21 ± 0.02
i-Butyryl phosphate	1.1 ± 0.2	0.0058 ± 0.0007
Chloroacetyl phosphate	1.0 ± 0.2	0.24 ± 0.02
Phosphate	17 ± 2	5.2 ± 0.6
Arsenate	11 ± 1	4.4 ± 0.7
CoA	0.12 ± 0.03	1.1 ± 0.1
Acetyl-CoA	1.2 ± 0.2*	4.4 ± 0.4*
	0.79 ± 0.11**	6.1 ± 0.9**

* In reaction 1b[3].
** In the arsenolysis reaction[2].

nantly inhibiting Na^+ is much less strongly adsorbed than either. The excellent agreement between the values for K_{Na^+} obtained in different inhibitory contexts is good support for our overall scheme. The reasonable agreement also between the k_2 and K_P values obtained in the present metal ion experiments and those obtained from our earlier experiments on substrate adsorption again argues for the overall self-consistency of our interpretation.

The slight superiority of NH_4^+ over K^+ as an activator can be seen in the former's smaller K_M value and its somewhat greater facilitation of the slow process k_2. NH_4^+ and K^+ are of similar size; Na^+ may fail as an activator owing to its relative smallness.

The magnitudes of the various K_M values in Table I reveal that, although in our earlier experiments we had, as pointed out in the Introduction, always used constant ionic concentrations and an NH_4^+ concentration in excess of that supposed[6] necessary to achieve maximum enzyme efficiency, nevertheless these concentrations cannot in every case have been sufficient for the purpose. Some of our previously quoted K_m and k_2 values are therefore in error, being only apparent values. The true values (see Tables I and II) can, however, readily be calculated using the earlier data and the fuller rate equation revealed in the present study. Thus earlier[1–3] calculations for reaction 1b were based on Eqn 39, whereas Eqn 38 can be written as Eqn 40. It can be seen that our earlier values for K_{ACA} will be unaffected, whereas our earlier K_P values

$$v_0 = \frac{k_2[E_0][\text{phosphate}][\text{acetyl-CoA}]}{([\text{phosphate}] + K_P)([\text{acetyl-CoA}] + K_{ACA})} \tag{39}$$

$$v_0 = \frac{k_2[E_0][\text{phosphate}][\text{acetyl-CoA}]}{\left([\text{phosphate}] + K_P\left\{1 + \frac{K_M}{[M]}\left(1 + \frac{[Na^+]}{K_{Na^+}}\right)\right\}\right)([\text{acetyl-CoA}] + K_{ACA})} \tag{40}$$

(hereafter K_P^{app}) will have been related to the true K_P values by Eqn 41, in which K_M, $[M]$, K_{Na^+} and $[Na^+]$ are all now known. Normally M^+ was NH_4^+. With K_P available, the true k_2 values can also be obtained from the earlier results. Similar considerations

$$K_P^{app} = K_P\left\{1 + \frac{K_M}{[M]}\left(1 + \frac{[Na^+]}{K_{Na^+}}\right)\right\} \tag{41}$$

apply for reaction 1a and for the related arsenolysis of acetyl-CoA[2]. Our revised substrate Michaelis constants and k_2 values at pH 8.32 are collected in Table II. The revisions also involve where required, a further (usually small) correction for the mutual removal of phosphate and M^+ species as inactive monoanionic complexes (see above). It has been assumed, as a first approximation that similar complexes of (i) NH_4^+ and K^+ and (ii) phosphate and acetyl phosphate, have similar dissociation constants; the values for Na^+ and K^+ with HPO_4^{2-} being known, all the remainder can then be obtained.

The revised K_m and k_2 values show that (i) our earlier general conclusions regarding the effect of substituents R in $RCO{\cdot}OPO_3^{2-}$ remain valid, and (ii) our earlier surprisingly large value of K_P arose from metal ion effects; the true value lies much closer to that of K_{As}, as is more reasonable.

Tables I and II refer to pH 8.32. K_M values are not known from the present work at other pH values studied earlier. However, since the adsorption of phosphate is controlled by M^+, yet is probably independent of pH over the range studied[3], it is likely that K_M is also pH-independent over this range also. With this assumption it can be shown, using Eqn 40, that (i) the variations of K_m and k_2 values with pH previously reported by us[1-3] are correct, and (ii) minor discrepancies between K_m values found in our previous work and there attributed to the very different ionic concentrations obtaining between certain comparisons, are indeed due to such differences. We also have little doubt that the discrepancies recorded by Shimuzu *et al.*[12] between K_{AP} values obtained in studies of phosphotransacetylase catalysis of reaction 1a and of the arsenolysis of acetyl phosphate, arose owing to the different cation concentrations used by these authors in the two systems. (The true value for their probable arsenolysis conditions is $K_{AP} \simeq 8.3 \cdot 10^{-4}$ moles·l^{-1}, in good agreement with their other value and with ours). There are thus no grounds for suggesting, as do Shimuzu *et al.*[12], that their discrepant K_{AP} values imply that K_{AP} is really a kinetic (*i.e.* Briggs–Haldane) constant.

A final issue affected by the present work is our earlier examination of substrate inhibition[4]. The complex inhibition patterns were rationalised in terms of three (or more) distinct sites for adsorption of phosphate (or arsenate) species, only two of which, sites A and C, lead directly to product formation. A mathematical requirement of the scheme was that the K_m values for these species determined directly, in the absence of inhibition, should lie numerically between the values calculated for the same species at sites A and C from our analysis of the inhibition patterns. This condition was then just met. Recalculation of the various K_m values in the light of our new knowledge leads to the results in Table III. The condition is well satisfied for K_P and K_{As}, but not for K_{AP}. To what extent this result casts doubt on our previous explanation is uncertain. We are, in any event, still unable to devise a simpler explanation of our unique results on substrate inhibition.

TABLE III

MICHAELIS CONSTANTS FROM SUBSTRATE INHIBITION EXPERIMENTS

This table contains K_m values quoted in ref. 4 corrected for the effects of univalent cation concentration. As = arsenate; pH 8.32; temperature 25 °C.

	$10^4 \times K_{AP}$	$10^3 \times K_P$	$10^3 \times K_{As}$
Inhibition experiments			
Site A	0.74 ± 0.10	6.6 ± 1.3	4.4 ± 0.5
Site C	2.9 ± 0.8	22.7 ± 4.5	22 ± 4
Direct determination in absence of inhibition	7.8 ± 1.0	17 ± 2	11 ± 1

The mechanism of phosphotransacetylase catalysis is clearly very complicated. Our present results reveal new facets and also increase the general self-consistency of our previous picture.

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