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KINETIC STUDIES WITH MUSCLE ACYLPHOSPHATASE

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SUMMARY

1. We have established that catalysis of acyl phosphate hydrolyses by muscle acylphosphatase (acyl phosphate phosphohydrolase, EC 3.6.1.7) is competitively inhibited by the orthophosphate product. There is negligible inhibition by the carboxylic acid product, but added buffer anions like chloride or perchlorate do inhibit.

2. Using acyl phosphatase extracted from chicken muscle, and benzoyl phosphate as substrate, a kinetic analysis employing the integrated rate equation and the pH range 3–12, has provided the values and pH dependencies of the Michaelis parameters at 25°.

3. The effect of pH on K_s (enzyme–substrate dissociation) reveals that $PhCO \cdot OPO_3H^-$ is less strongly bound than is $PhCO \cdot OPO_3^{2-}$, and that a group in the enzyme, $pK_a = 7.9$, when deprotonated, slightly weakens $PhCO \cdot OPO_3^{2-}$ binding. pK_a for the dissociation of $PhCO \cdot OPO_3H^-$ is 4.8 at 25°.

4. The effect of pH on K_p (enzyme–phosphate dissociation) shows that $H_2PO_4^-$ is also less strongly bound than is HPO_4^{2-}. Both these species, however, are more strongly bound to the enzyme than their benzoylated counterparts. The HPO_4^{2-} binding is also affected by the group of $pK_a = 7.9$. All the K_p and K_s values lie within a factor of about 10, centreing on 10^{-3} M.

5. The effect of pH on k (the rate constant of the surface reaction) shows that $PhCO \cdot OPO_3H^-$, although adsorbed, is not readily hydrolysed by the enzyme, and that a group in the enzyme, $pK_a = 11.0$, must be protonated for efficient enzymatic hydrolysis of $PhCO \cdot OPO_3^{2-}$.

6. A complete reaction scheme involving the various substrate, product, and enzyme acid–base ionisations and complexes, leads to a rate equation which correctly reproduces the observed, overall pH dependencies of K_s, K_p, and k, thus confirming the essential correctness of our analysis.

7. Our results, together with those of previous studies, lead to a chemical mechanism for the enzymatic catalysis which differs significantly from those of non-enzymatic hydrolyses of acyl phosphates. The substrate is held primarily by the phosphate group. During the surface reaction the phosphorus atom is uniquely located, by bonds to three of the phosphate oxygen atoms, and suffers a slow, nucleophilic attack by an adjacent water molecule. The mechanism explains the observed unsuitability of species $ROPO_3^{2-}$, $RCO \cdot OPO_3H^-$, and $RCO \cdot OPO_3R'^-$ as substrates for muscle acylphosphatase.

INTRODUCTION

An enzyme (acyl phosphate phosphohydrolase, EC 3.6.1.7, known as acylphosphatase) which catalyses the hydrolysis of acyl phosphates (1) was first identified by Lipmann[1,2].

$$\text{Acyl phosphate} + H_2O \rightarrow \text{carboxylic acid} + \text{orthophosphate} \quad (1)$$

A variety of animal tissues contain such a substance, but muscle has a particularly rich supply. Most work on acylphosphatase has employed muscle sources and enzyme of good purity can be obtained[3–6]. We have used chicken muscle. There is abundant evidence[3–6] that different muscle sources yield predominantly enzymes with similar stabilities, metal requirements, and molecular weights. It is also likely that the muscle acylphosphatases all have similar amino acid compositions. Acylphosphatases from other tissues appear to have different general properties and amino acid contents[7–10]. In short, conclusions from work using any muscle source of the enzyme are likely to be directly relevant to the present study, whereas conclusions from work using other sources may be misleading.

As enzymes, muscle acylphosphatases are unusually stable to heat and to acid, and they have apparently no metal ion requirement[11]. Quoted[5,6,12,13] isoelectric points are in poor agreement, but the enzyme is generally regarded as very basic. Recorded[3,5,6,13] molecular weights vary between 9000 and 23 000, centreing mostly upon about 10 000 and about 20 000; it is possible therefore that monomers and dimers exist, and tissue extracts often yield more than one active fraction[3–6]. It seems that the enzyme will efficiently hydrolyse almost any compound of formula $RCO \cdot OPO_3^{2-}$, but is much less effective with species $ROPO_3^{2-}$ and $RCO \cdot OPO_3R'^{-}$. Hydrolysis proceeds *via* P–O bond fission[14]. K_m values and relative reactivities are available for a limited number of substrates at arbitrary pH values (see below). The product, inorganic phosphate, and other deliberately added phosphates, powerfully inhibit the enzyme, but the nature of the inhibition is still debated[5,15,16]. Little is known about the active site, save that an accessible –SH group is not involved[6,7,16]. All previous kinetic work with acylphosphatases has involved initial velocity measurement only, and the effects of pH are incompletely known. We now report detailed kinetic and pH analyses of reaction (1) using benzoyl phosphate as substrate (2). We reach a number of conclusions about the reaction mechanism.

$$\text{Benzoyl phosphate} + H_2O \xrightarrow{\text{Enzyme}} \text{benzoic acid} + \text{orthophosphate} \quad (2)$$

MATERIALS AND METHODS

Chemicals

Acylphosphatase was isolated from chicken breast muscle using Pechère's[4] procedure, with minor modifications. We used acetone–water (7:3, v/v) mixtures in the initial fractionations since we find that a large fraction of the activity remains in the supernatant liquid if only a 3:7 (v/v) mixture is used. The purifications by Sephadex and Bio-Rex 70 ion-exchange chromatography followed Pechère's, except that in the former we used DEAE-Sephadex with an 0.01 M Tris–HCl buffer as eluant (pH 8.5 at the temperature of the elution, 5°). Tris buffer produces no inactivation

of the enzyme. During the purifications enzyme activity was assayed by the method of Ramponi *et al.*[17] based on the determination of the initial rate of hydrolysis of standard benzoyl phosphate solutions (10^{-3} M, pH 5.3, 0.1 M acetate buffer). It was verified that this rate was proportional to the enzyme concentration for chicken muscle enzyme. Measured initial velocities were reproducible to within $\pm 5\%$. Our elution diagrams and purification levels were similar to those of Pechère, except that we found only two active fractions. These fractions eluted close together on both columns, that eluting first comprising one-third of the total activity. Both fractions eluted before cytochrome *c*. In our main kinetic experiments we used enzyme from the first fraction. Stock enzyme, stored in Tris or cacodylate buffer at 4°, retained its activity unchanged over several months. When calculating enzyme molarities, we have assumed that the molecular weight is 10 000 and that the foreign protein content is small.

Dilithium benzoyl phosphate was prepared by the method of Ramponi *et al.*[18], except that the final conversion of the benzoyl phosphoric acid to its dilithium salt was accomplished by the careful addition of saturated, aqueous LiOH to pH = 7–8. The yield of crude salt was 12%. It was purified by dissolution in water at pH 5.5, followed by filtration to remove insoluble lithium phosphate, and reprecipitation of the dilithium benzoyl phosphate with ethanol. The purified, dried material was >98% pure, as shown by analyses for both phosphate and benzoate after complete hydrolysis, and by comparison of its ultraviolet spectrum with that of the pure sample of Ramponi *et al.*[18]. DEAE-Sephadex and Bio-Rex 70 ion-exchange resins were obtained from Pharmacia and Bio-Rad Laboratories, respectively. *p*-Chloromercuribenzoate was the Sigma product. All other chemicals were B.D.H. Analar or Reagent grade materials and were used as such.

Preliminary experiments on the stability and the inhibition of acylphosphatase

All kinetic and stability experiments were at 25°.

(*i*) *Effects of pH and buffer components.* Acylphosphatase is known[2–6] to be stable in solutions of intermediate pH and in acid solutions at least up to pH 1; its

TABLE I

EFFECT OF 1.0 M NaOH ON ACYLPHOSPHATASE AT 25°

[Enzyme] = $5 \cdot 10^{-6}$ M, activity of samples, taken at intervals shown, assayed by Ramponi's method.

Incubation time (h)	*Relative activity*
0	1
3.5	0.42
14.0	0.16
20.0	0.11

behaviour at high pH is less certain. The effect of 1.0 M NaOH is shown in Table I. Inactivation occurs, but relatively slowly.

The separate inhibiting effects, at pH 4–5, of acetic acid, acetate, Na^+ and Cl^- can be judged from Tables II–IV. Here we are dealing with instantaneous effects which are not time-dependent. It is evident that, of these species, only the Cl^- is an

TABLE II

EFFECT OF ACETATE BUFFER CONCENTRATION ON ACYLPHOSPHATASE AT 25°

[Enzyme] = $4 \cdot 10^{-8}$ M; [benzoyl phosphate]$_0$ = $4 \cdot 10^{-5}$ M; pH 4.1; ionic strength 0.1–0.09; initial rate of hydrolysis determined at 232 nm. Conclusion: acetic acid and acetate ions have a negligible effect on the enzyme.

[CH_3CO_2H]	*[$CH_3CO_2^-Na^+$]*	*Total buffer (M)*	*[NaCl]*	*[Na^+] total*	*Relative activity*
0.08	0.02	0.10	0.08	0.1	1.0
0.04	0.01	0.05	0.08	0.09	1.0

TABLE III

EFFECT OF NaCl CONCENTRATION ON ACYLPHOSPHATASE AT 25°

[Enzyme] = $4 \cdot 10^{-8}$ M; [benzoyl phosphate]$_0$ = $4 \cdot 10^{-5}$ M; pH 4.1 (0.1 M acetate buffer). Conclusion: increase in [NaCl] and/or in ionic strength deactivates the enzyme.

[NaCl]	*Ionic strength (M)*	*Relative activity*
0	0.02	1.00
0.08	0.10	0.24
0.16	0.18	0.08

TABLE IV

EFFECT OF Na^+ CONCENTRATION AND OF IONIC STRENGTH ON ACYLPHOSPHATASE AT 25°

[Enzyme] = $8 \cdot 10^{-8}$ M; [benzoyl phosphate]$_0$ = $4 \cdot 10^{-5}$ M; pH 5.3 (1:4 acetic acid–sodium acetate buffer). Conclusion: Increase in [Na^+] and in ionic strength have a negligible effect. Hence added Cl^- inhibits the enzyme.

Total buffer (M)	*[Na^+]*	*Ionic strength (M)*	*Relative activity*
0.02	0.016	0.016	1.00
0.06	0.048	0.048	1.05
0.10	0.08	0.08	1.00

TABLE V

BUFFERS USEFUL WITH MUSCLE ACYLPHOSPHATASE

Buffer acid	*pK_a (25°)*	*pH range used*
Formic acid	3.7	2.6
Acetic acid	4.7	3.4– 6.0
Cacodylic acid	6.2	6.2– 7.5
Glycine	9.8	8.5– 9.5
Glucose	12.3	11.2–12.0

inhibitor. This result means, however, that it is undesirable to use amine–HCl buffers. Inhibition by Cl^- appears previously to have been overlooked[15]. Experiments with added $NaClO_4$ showed that ClO_4^- also inhibits the enzyme, so that amine–$HClO_4$ buffers must also be rejected. To cover a wide pH range, we chose accordingly the buffers in Table V. A given buffer ratio was obtained by appropriate, partial neutralization of buffer acid (HA) with aqueous NaOH. In all cases $[Na^+ A^-]$ was held constant at 0.05 M, and [HA] varied to yield the required pH. Usually 1.0 M > [HA] > $5 \cdot 10^{-3}$ M. It was established that, except for glucose buffers, the buffer components had negligible inhibiting effects on the enzyme, both instantaneously and after incubation for 1 h. Incubation of enzyme in the glucose buffers (pH 11.2–12.0) led to its progressive inactivation, which was complete after 1 h. The inactivation, however, only amounts to 1–5% in the first 5 min.

These experiments showed that, for the pH range 2.6–9.5, it would be possible to follow, undisturbed, the kinetic course of Reaction 2 for an extended period using the chosen buffer, but that at pH approx. >11.0 it was essential to measure initial velocities only. This we have done. We, in fact, used initial velocities also at pH approx. <3.5 owing to the extreme slowness of the reaction at these pH values.

(*ii*) *Effects of products.* As noted in the Introduction, phosphate is known to inhibit acylphosphatase; the effect of the other product in Reaction 1 is less certain. The experiments in (*i*) above suggest, however, that carboxylic acids and their anions are generally not inhibitors. We have checked this for our substrate by deliberate additions of benzoate ions, and of benzoic acid, up to concentrations of $4 \cdot 10^{-3}$ M (the

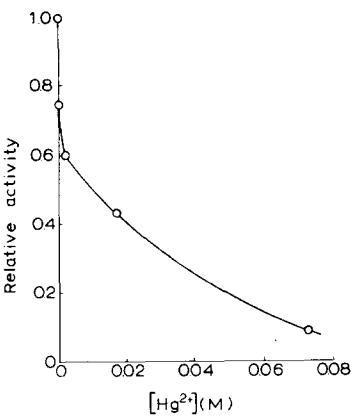


Fig. 1. Effect of Hg^{2+}. Enzyme (10^{-5} M) incubated with Hg^{2+} for 30 min followed by assay of activity.

maximum initial concentration of benzoyl phosphate). No effect on the initial rate of hydrolysis was observed.

(*iii*) *Effects of other compounds.* We find that 10^{-3} M *p*-chloromercuribenzoate has a negligible inhibiting effect on the enzyme after 1 h, but that 0.12 M sodium thioglycollate reduces the activity by 30% after 3 h. A similar reduction is produced in 30 min by 10^{-4} M Hg^{2+}. Further increase in $[Hg^{2+}]$ produces further inhibition, but is relatively less effective (Fig. 1). These results suggest that the activity is dependent on an S–S linkage, but not upon an –SH group.

Acid dissociation of $PhCO \cdot OPO_3H^-$

Interpretation of our results requires a knowledge of the pK_a value for the second ionisation of benzoyl phosphate.

$$PhCO \cdot OPO_3H^- + H_2O \rightleftharpoons PhCO \cdot OPO_3^{2-} + H_3O^+ \quad (3)$$

We have determined this by titration. A solution of dilithium benzoyl phosphate ($5 \cdot 10^{-3}$ M), adjusted to ionic strength 0.05 M with NaCl, was titrated against aqueous HCl (0.02 M), using a Radiometer automatic titration assembly. The titration was completed in 5–10 min to avoid significant hydrolysis of benzoyl phosphate. The (reproducible) titration curves led to $pK_a = 4.8 \pm 0.1$ for equilibrium 3 at 25°.

Kinetic procedure

The hydrolysis leads to a reduction in ultraviolet absorption in the region 250–300 nm, inorganic phosphate species absorbing negligibly, and benzoate ions and benzoic acid less strongly than benozyl phosphate species at all pH values studied. It can be shown that, for any given wavelength, λ, [benzoyl phosphate]$_t = (A_t - A_\infty)/\Delta\varepsilon_\lambda$, where A represents absorbance, and the subscripts t and ∞ refer to times t and infinity, respectively. $\Delta\varepsilon_\lambda$ is the difference between the effective extinction coefficients of the benzoyl phosphate and benzoate species at the wavelength involved. Although the relevant benzoyl phosphate species, $PhCO \cdot OPO_3H^-$ and $PhCO \cdot OPO_3^{2-}$ (also the pair $PhCO_2H$ and $PhCO_2^-$) have slightly different ε values, the ratios $[PhCO \cdot OPO_3H^-]/[PhCO \cdot OPO_3^{2-}]$ and $[PhCO_2^-]/[PhCO_2H]$ are constant at fixed pH; therefore $\Delta\varepsilon_\lambda$ is a determinable constant at each pH. It is convenient to use $\lambda = 283$ nm when [benzoyl phosphate]$_0$ approx. $< 10^{-3}$ M and $\lambda = 288$ nm at higher concentrations.

Reaction mixtures were made up in graduated flasks (5 ml) from aliquots of a stock benzoyl phosphate solution in buffer, the flask being filled to the mark with more buffer. Stock benzoyl phosphate solution was prepared fresh each day and stored at −20° until required, to minimize spontaneous hydrolysis. Five values of [benzoyl phosphate]$_0$ varying between approx. $0.2 \cdot 10^{-3}$ and $3 \cdot 10^{-3}$ M were used at each pH. Reaction mixture (2.5 ml) was quickly transferred to a thermostatted spectrophotometer cell (quartz, 1 cm path, approx. 3 ml capacity) and, as soon as thermal equilibrium was attained, reaction begun by adding a small (5–20 μl) volume of enzyme solution. The cell was shaken and measurements then taken using either an SP500

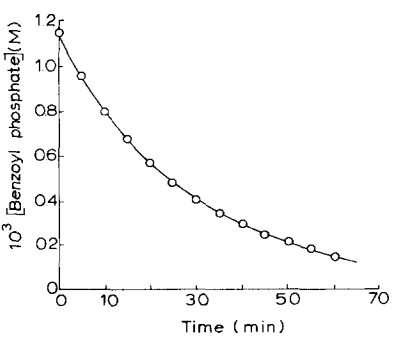


Fig. 2. Typical run. [Benzoyl phosphate]$_0 = 1.15 \cdot 10^{-3}$ M; [enzyme] $= 8.8 \cdot 10^{-8}$ M; acetate buffer, pH 5.3; ionic strength 0.05 M.

or an SP800 Unicam spectrophotometer. Except when initial rates were taken, when only the first 2–5% of reaction was monitored, runs were followed for about 3 half-lives, this usually occupying 30 min–2 h. A typical run is shown in Fig. 2. A number of runs were allowed to go to completion (>10 half-lives) and in every case the observed final absorbance was that expected for essentially complete hydrolysis. Under all conditions used, the rate of the non-enzymatic hydrolysis of benzoyl phosphate was negligible compared with that of the enzymatic hydrolysis.

Treatment of results

The overall reaction is Eqn. 2, which, under our conditions, proceeds far to the right, owing to the large excess of water over benzoyl phosphate. We know that of the products, only the phosphate inhibits. If we assume this inhibition is competitive, we have, ignoring ubiquitous water species, the simple scheme Eqns. 4–6 in which E, S, P, and B represent, respectively, enzyme, benzoyl phosphate, inorganic phosphate, and benzoic acid species.

$$E + S \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} ES \qquad K_s = \frac{k_{-s}}{k_s} \tag{4}$$

$$ES \xrightarrow{k} EP + \mathrm{B} \tag{5}$$

$$EP \underset{k_p}{\overset{k_{-p}}{\rightleftharpoons}} E + P \qquad K_p = \frac{k_{-p}}{k_p} \tag{6}$$

Under our conditions $[S]_0 \gg [E]$. Assuming Michaelis–Menten kinetics the appropriate differential rate equation at any fixed pH is Eqn. 7:

$$-\mathrm{d}[S]_t/\mathrm{d}t = k[E]_0 \Big/ \left(1 + \frac{K_s}{[S]_t}\left[1 + \frac{[P]_t}{K_p}\right]\right) \tag{7}$$

since $[P]_t = [S]_0 - [S]_t$, Eqn. 7 is equivalent to Eqn. 8:

$$-\mathrm{d}[S]_t/\mathrm{d}t = k[E]_0 \Big/ \left\{\left(1 - \frac{K_s}{K_p}\right) + \frac{K_s}{[S]_t}\left(1 + \frac{[S]_0}{K_p}\right)\right\} \tag{8}$$

The corresponding integrated equation is Eqn. 9.

$$\frac{([S]_0 - [S]_t)}{t} = \frac{k[E]_0}{(1 - K_s/K_p)} - K_s \frac{(1 + [S]_0/K_p)}{(1 - K_s/K_p)} \ln \frac{([S]_0/[S]_t)}{t} \tag{9}$$

Values of K_s, K_p and k can be evaluated from this equation by Foster and Niemann's[19] method if reaction curves like Fig. 2 are available for a series of $[S]_0$ values. We have just such data for a series of pH values from 3.5 to 9.5. Foster–Niemann plots at two of these pH values are shown in Figs. 3, 4, and 5 (Fig. 5 illustrates a convenient method for computing K_p). Analogous plots at the other pH values displayed comparably good Foster–Niemann behaviour, and there is therefore little doubt that Eqns. 4–6 adequately represent the enzymatic reaction.

When pH < 3.5 or > 9.5 we have only initial velocity data. For Eqns. 4–6 the appropriate differential rate equation at fixed pH is then Eqn. 10. Thus K_s and k can be determined, but not K_p. Lineweaver–Burk plots (*e.g.* Fig. 6) were used to calculate K_s and k. At pH values between 3.5 and 9.5 the initial velocity procedure

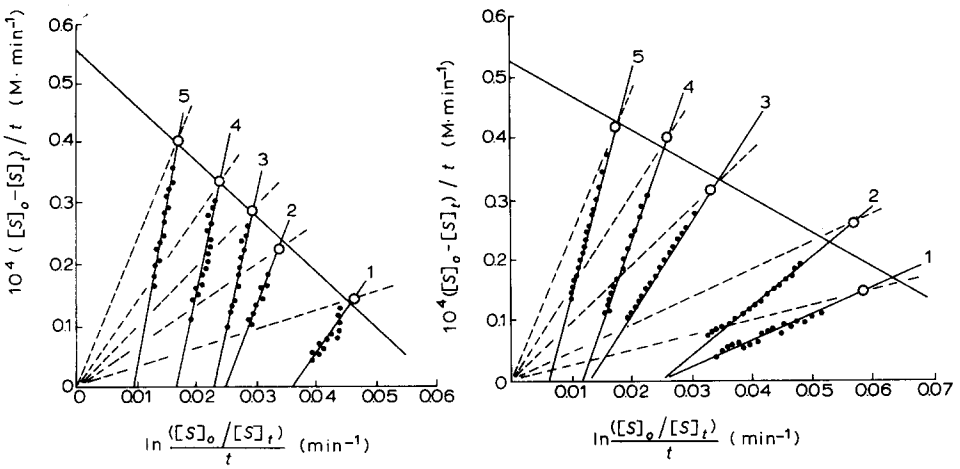


Fig. 3. Foster–Niemann plots of Eqn. 9 for pH = 4.65. $10^3[S]_0$ = 0.33 (1); 0.66 (2); 0.95 (3); 1.43 (4); 2.38 (5); [enzyme] = $8 \cdot 10^{-8}$ M; ionic strength 0.05 M. ....., experimental points; ---------, lines of slope $[S]_0$.

Fig. 4. Foster–Niemann plots of Eqn. 9 for pH 7.50. $10^3[S]_0$ = 0.24 (1); 0.48 (2); 0.95 (3); 1.55 (4); 2.38 (5); otherwise as for Fig. 3.

led to values for K_s and k in good agreement with those obtained from the Foster–Niemann analysis. Our different sets of rate data are therefore self-consistent. Our collected values of K_s, K_p and k are in Table VI.

$$d[S]_0/dt = v_0 = k[E]_0/\left(1 + \frac{K_s}{[S]_0}\right) \qquad (10)$$

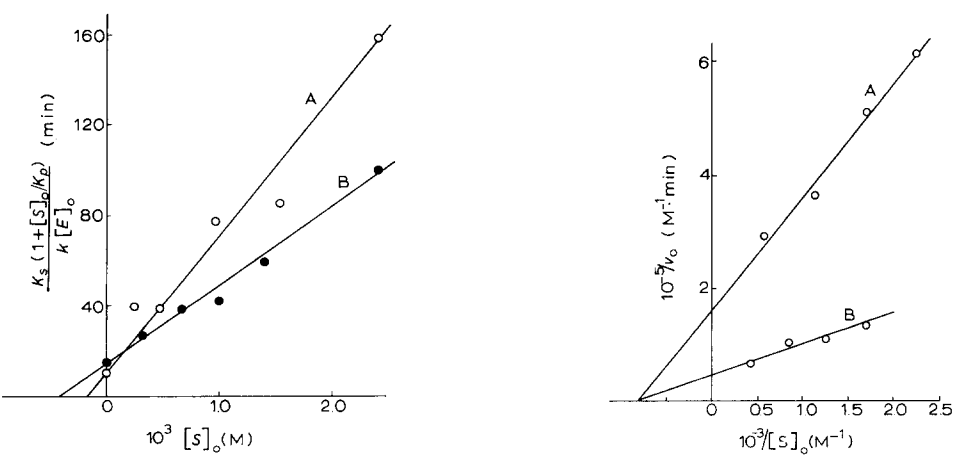


Fig. 5. Determination of K_p. Reciprocals of abscissa intercepts of experimental lines in Figs 3 and 4 plotted against $[S]_0$. A, pH 7.50; B, pH 4.65.

Fig. 6. Lineweaver–Burk plots at high pH. Glucose buffer; ionic strength 0.05 M; [enzyme] = $8 \cdot 10^{-8}$ M; A, pH 12.0; B, pH 11.2.

TABLE VI

COLLECTED VALUES OF K_s, K_p AND k

pH (± 0.05)	$10^3 K_s$ (*M*)	$10^3 K_p$ (*M*)	k (*min*$^{-1}$)
2.60*	3.3	—	75
3.40*	3.0	—	275
3.95	1.5	0.50	525
4.65	0.9	0.40	688
4.95	0.51	0.43	675
5.30	0.52	0.59	688
5.50	0.37	0.37	637
5.75	0.53	0.50	712
6.00	0.64	0.40	788
6.20	0.56	0.40	900
6.90	0.43	0.30	650
7.50	0.57	0.15	650
8.50	1.15	0.30	425
8.90	1.25	0.45	562
9.45	1.30	0.6	812
11.20*	1.10	—	262
12.00*	1.10	—	75

* Initial velocity experiments.

DISCUSSION

Implications of the observed kinetic form

Since the observed kinetic behaviour at all pH values agrees closely with that expected if Eqns. 4–6 operate, and since non-competitive inhibition by phosphate entails a quite different kinetic form not fitted by our data, it is evident that simple competitive inhibition is involved, and that phosphate therefore binds to the same (or part of the same) site as does benzoyl phosphate. Our results thus support Harary's[16] view, but not that given by Guerritone *et al.*[15].

Implications of the observed pH dependencies

(*i*) *Comparison with earlier work.* The results in Table VI are plotted as logarithmic functions in Fig. 7. Earlier work[15,16,20] on the reactivity of muscle acylphosphatase as a function of pH has suggested that reactivity increases sharply from pH approx. 3 to a maximum at pH 5–6, followed by a steady decline in reactivity in region pH 6–9. It is not easy to assess the implications of this work, all based on initial velocities, because the various concentration conditions to which it refers are not always specified. It is, however, fairly common practice to determine pH dependencies from initial velocities obtained under conditions when the enzyme is saturated with substrate, the effects then reflecting changes in the rate of decomposition of the enzyme–substrate complex. If we make this assumption about the previous work in this case, we must compare its findings with ours for k depicted in Fig. 7a. We concur with an increase from pH 3 to 5, but do not observe a steady fall in the range pH 6–9; log k only begins to fall at pH approx. 10. The discrepancy probably arises from one, or both, of two circumstances: (a) the earlier measurements really reflect both k and K_s, and since pK_s (Fig. 7b) does fall somewhat in the pH range 6–9, some appropriate superposition of Curves 7a and 7b could account for the previous

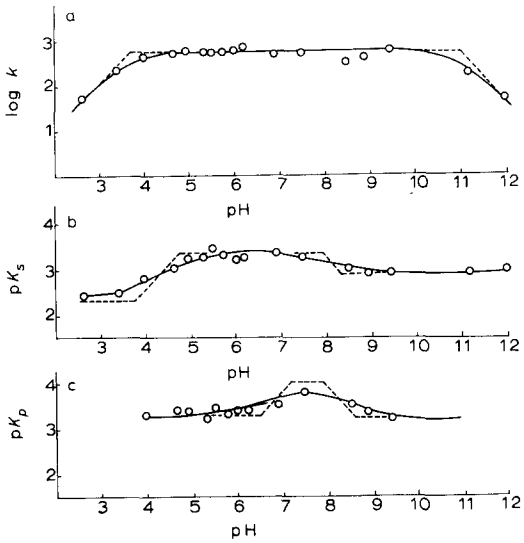


Fig. 7. pH dependence of K_s, K_p and k.

observations; (b) inhibition by phosphate is powerful and increases (Fig. 7c) in the region pH 6–9, so that "initial velocities"* in this region could be progressively too small owing to the presence of significant inhibition even in the early stages of reaction. An apparent fall in k could again be observed. The true details of the pH dependence are revealed by Fig. 7.

(*ii*) *The effect on* K_s. Fig. 7b, interpreted according to Dixon's[21] rules, shows that binding between benzoyl phosphate and enzyme is decreased by the protonation of a group with $pK_a = 4.75$ in either the free benzoyl phosphate or enzyme, and also by the deprotonation of a group with $pK_a = 7.9$ in either of these free species. Binding is slightly increased by the deprotonation of a group with $pK_a = 3.7$, and by the protonation of a group with $pK_a = 8.4$, both in the enzyme substrate complex. The ionisation with $pK_a = 4.75$ is clearly that of Eqn. 3. It follows that $PhCO \cdot OPO_3^{2-}$ is more strongly bound than is $PhCO \cdot OPO_3H^-$ (K_s is changed by a factor of about 10). There is every reason to suppose (see also below) that the group in the enzyme–substrate complex with pK_a approx. 3.7 also represents this ionisation in the bound benzoyl phosphate. Since an increase in pH above about 6 cannot further affect $PhCO \cdot OPO_3^{2-}$, the group with $pK_a = 7.9$ must be in the free enzyme. The same group probably has $pK_a = 8.4$ in the enzyme–substrate complex.

(*iii*) *The effect on* K_p. Our assignments in (ii) above are supported by the observations for K_p. The value of K_p is constant in the range pH 4–6. If the group with $pK_a = 4.75$ did not refer to benzoyl phosphate, but to the free enzyme, it should appear again for phosphate binding; but it does not. The curve for K_p is actually similar to that for K_s, except that the region of maximum binding is shortened owing to the shifting of the inflection on the left-hand side to higher pH. If our interpretation for benzoyl phosphate is correct, the position of this inflection is determined by the

* Often measured from only one, fixed incubation time.

pK_a value of the second ionisation of orthophosphate, Eqn. 11. And for Eqn. 11 pK_a

$$H_2PO_4^- + H_2O \rightleftharpoons HPO_4^{2-} + H_3O^+ \quad (11)$$

is known[22] to be 7.2, which is exactly in keeping with Fig. 7c. The value of pK_a for this ionisation in the enzyme–phosphate complex is 6.5. As with benzoyl phosphate, the phosphate dianion is more strongly bound to the enzyme than is the monoanion (K_p changes by a factor of about 3). On the higher pH side a group with pK_a = 7.9 is again implicated in the free enzyme[21]. This is clearly the same group which affects the binding of $PhCO \cdot OPO_3^{2-}$. In the enzyme–phosphate complex this group has pK_a = 8.7. It will be evident that our interpretation of the pH dependencies of K_s and K_p shows these dependencies to be self-consistent and in keeping with the concept of competitive inhibition.

(*iv*) *The effect on k.* Fig. 7a shows k to be effectively constant from pH 4 to 10. A group in the enzyme–substrate complex with pK_a = 3.7 leads, when protonated, to inactivation of the complex towards decomposition to products[21]. This ionisation is clearly that reflected in Fig. 7b, and represents the protonation of the bound $PhCO \cdot OPO_3^{2-}$. It follows that only the bound dianion can lead to reaction. At high pH a group in the enzyme–substrate complex (doubtless located in the enzyme) with pK_a = 11.0, leads when deprotonated, to inactivation of the complex towards decomposition. This group has apparently little effect on binding, not appearing in Fig. 7b or c.

A theoretical representation of the observed pH profiles

The various K_s, K_p, k and K_a values involved in, and implicated by, Fig. 7 have been used to construct a more detailed model of Eqns. 4–6. This is shown in Fig. 8, in which the horizontal equilibria represent proton ionisations, and the vertical equilibria binding. Charges and water species have been omitted for simplicity, but H represent the proton. In enzyme complexes H_1 represents the proton affecting binding (pK_a = 7.9) and H_2 that (pK_a = 11.0) influencing k. At pH values where

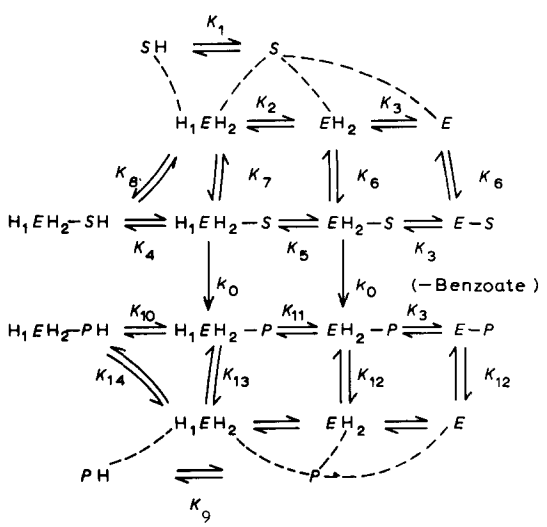


Fig. 8.

significant amounts of $PhCO\cdot OPO_3H^-$ (*SH*) exist, the enzyme is effectively all H_1EH_2; interaction between *SH* and EH_2 and *E* can therefore be neglected as a first approximation. The complex H_1EH_2–*SH* is inactive (see (*iv*) above). For $PhCO\cdot OPO_3^{2-}$ (*S*), formed at pH approx. 4, interaction with all three forms of enzyme needs consideration. Binding of *S* with EH_2 and *E* is equally strong (see (*ii*) above) and species H_1EH_2–*S* and EH_2–*S* are equally reactive towards product formation (see (*iv*) above). The complex *E*–*S* is unreactive. An exactly analogous situation obtains for product binding, although the approximation that for $H_2PO_4^-$ (*PH*) only the interaction with H_1EH_2 needs consideration is less satisfactory than for *SH*.

Application of Michaelis–Menten assumptions to the scheme in Fig. 8 leads to an equation identical in form with Eqn. 8, but where

$$K_s = \frac{K_6(1 + [H]/K_1)(1 + [H]/K_2 + K_3/[H])}{(1 + [H]/K_5 + [H]^2/K_4K_5 + K_3/[H])} \quad (12)$$

$$K_p = \frac{K_{12}(1 + [H]/K_9)(1 + [H]/K_2 + K_3/[H])}{(1 + [H]/K_{11} + [H]^2/K_{10}K_{11} + K_3/[H])} \quad (13)$$

and

$$k = k_0[E]_0\,(1 + [H]/K_5)/(1 + [H]/K_5 + [H]^2/K_4K_5 + K_3/[H]) \quad (14)$$

If Fig. 8 is a correct representation of the enzymatic hydrolysis Eqns. 12, 13, and 14 should describe the observed pH dependencies of K_s, K_p, and k respectively. Using the experimental values of pK_n given in Table VIII, and taking $k_0 = 700$ min^{-1}, K_s, K_p, and k were computed from Eqns. 12–14 at numerous pH values. The results are plotted as the continuous lines in Fig. 7; the agreement with the experimental points is excellent. This complete internal self-consistency greatly strengthens every aspect of our interpretation of the kinetics of this enzymatic reaction.

The chemical mechanism of catalysis

The fact that HPO_4^{2-} and $PhCO\cdot OPO_3^{2-}$ are about equally strongly adsorbed, and rather more so than the corresponding monoanions, together with the established facts that a wide range of phosphate-containing compounds act as inhibitors, and that the binding constants[18] of certain other, structurally dissimilar, acyl phosphates are not very different from that of benzoyl phosphate, strongly suggest that all the bound compounds are held primarily by their phosphate groups at an electrophilic centre(s) in the enzyme. Groups in the acyl portion of an acyl phosphate can probably slightly affect the overall strength of binding by electronic effects, but important steric inhibition of adsorption is clearly absent. The nature of the principal electrophilic site holding the phosphate group is unknown; it can bind at least two oxygen atoms and is insensitive to pH in the range 3–12. This suggests a metallic site, but muscle acylphosphatase has no known metal requirement[11]. It is perhaps significant also to compare the typical magnitude of the K_s values (approx. 10^{-3}–10^{-4} M) with the dissociation constants[22,23] of the complexes HPO_4^{2-}–Mg^{2+} and $CH_3CO\cdot OPO_3^{2-}$–Mg^{2+} (approx. 10^{-2} M); binding to the enzyme is thus appreciably stronger than to a divalent cation. The enzyme is unusually basic and stable to acids; perhaps therefore protonated nitrogen centres in the enzyme, with unusually large pK_a values (*i.e.* >12, guanidinium ?), form the site of phosphate binding.

That binding of phosphate dianions is reduced by the deprotonation a group

TABLE VII

RELATIVE REACTIVITIES OF SUBSTRATES TOWARDS ENZYMATIC HYDROLYSIS

Substrate $R \cdot OPO_3$	*Relative reactivity**	pK_a *of ROH***
p-$NO_2C_6H_4CO \cdot OPO_3$	1.0	3.4
$C_6H_5CO \cdot OPO_3$	0.3	4.2
$CH_3CO \cdot OPO_3$	0.05	4.7
$NH_2CO \cdot OPO_3$	0.005	6.4***
p-$NO_2C_6H_4 \cdot OPO_3$	$5 \cdot 10^{-6}$	7.1

* From ref. 3; conditions largely unspecified, but since K_s is insensitive to structure these values probably reflect k.

** Ref. 28.

*** Value for $HO \cdot CO_2H$.

with $pK_a = 7.9$, is best explained by assuming that this group is local to the principal phosphate binding site and exerts its influence purely electrostatisally. There are reasons (given below) for believing this group is not directly involved in covalent bonding to phosphate oxygen atoms. This group is also unidentified, but its pK_a suggests ammonium.

That the species $PhCO \cdot OPO_3H^-$ (and[3] $RCO \cdot OPO_3R'^-$) does not easily undergo enzymatic hydrolysis, and that the decomposition of $PhCO \cdot OPO_3^{2-}$ is dependent upon the protonation of a group with $pK_a = 11.0$, is explicable if we assume that

TABLE VIII

VALUES OF pK_n DEDUCED FROM Fig. 7

n	pK_n	n	pK_n
1	4.75	8	2.3
2	7.9	9	7.2
3	11.0	10	6.5
4	3.7	11	8.7
5	8.4	12	3.2
6	2.9	13	4.0
7	3.35	14	3.3

two of the phosphate oxygen atoms must be firmly held, and another (the double-bonded oxygen) positioned by weak hydrogen bonding, so that the phosphorus atom itself is uniquely located (Fig. 9). If the hydrogen bonding from group B is absent, the phosphorus atom has considerable freedom of movement, and is not held adjacent to the locally bound water molecule[24] which initiates hydrolysis by nucleophilic attack on phosphorus. This attack is considered to be the slow step of the enzymatic reaction. The dual catalytic role of the proton on group B will be evident from Fig. 9. The group X in the enzyme, to which the important water molecule is bound, does not respond to pH changes in the range 3–12. It is perhaps an alcohol residue. The reason that the group of $pK_a = 7.9$ is not considered to bind a phosphate oxygen atom covalently, is that when this group is in its basic form (and not binding) reaction

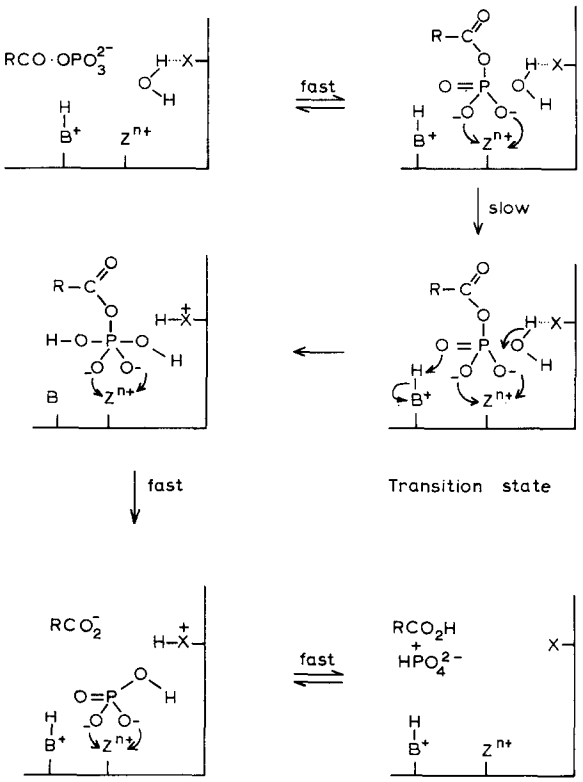
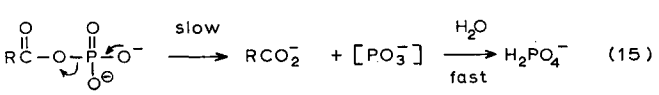


Fig. 9.

still proceeds, whereas compounds, like $PhCO \cdot OPO_3H^-$, in which one oxygen is not bonded, are not readily hydrolysed.

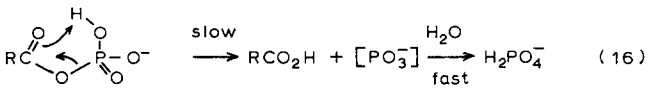
The mechanism of Fig. 9 is consistent with two other important facts. First, since the slow step involves nucleophilic attack on phosphorus, electron withdrawal by the acyl group should increase the value of k. Results in Table VII suggest that this is so. The evident reason that simple phosphate esters ($ROPO_3^{2-}$) make very poor substrates is that the electron withdrawal by RO– is much less powerful than by most acyl groups RCO–. Secondly, hydrolysis proceeds *via* P–O (not C–O) bond fission, in agreement with experiment[14].

It is instructive to compare the mechanism of enzymatic catalysis of acyl phosphate hydrolysis with those of the non-enzymatic hydrogen ion[25,26,27] and metal ion[23] catalysed reactions, and also with that of the spontaneous hydrolysis[25]. The latter reaction, important in the range pH 6–9, involves a unimolecular decomposition of the dianion, with P–O bond fission (Eqn. 15).



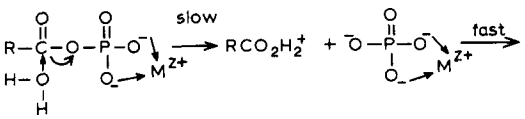
Reduction of the pH leads to the formation of the monoanion, but to little change in velocity. The mechanism (Eqn. 16) is essentially unaltered, the loss of electron

provision by the phosphate group being compensated by the intramolecular acid catalysis of carboxylate departure. This is the hydrogen ion catalysed reaction of the dianion.



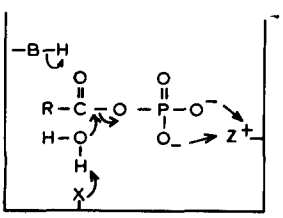
(16)

Metal ion catalysis involves assistance of phosphate departure *via* chelation (Eqn. 17). Since electrons are here drawn towards the phosphate group, P–O bond fission leading to RCO_2^- is disfavoured and the mechanism changes to C–O fission, with concurrent attack by the nucleophile at carbon. A similar mechanism applies for the hydrogen ion catalysed decomposition of the monoanion (*via* $RCOPO_4H_2$) in very acidic solutions.



$$RCO_2H + HOPO_3^{2-} - M^{z+} \qquad (17)$$

The enzymatic mechanism is a combination of these mechanisms, being perhaps closest to Eqn. 17. It is not at first obvious why the enzymatic hydrolysis did not develop as an intramolecular (*i.e.* surface) version of Eqn. 17, *e.g.* Eqn. 18.



Phosphotransacetylase[27] employs this type of scheme. The penalty is that the structure of the group R then assumes a much greater steric importance. Muscle acylphosphatase has avoided this by taking full advantage of the increased electrophilicity of the phosphorus atom resulting from its binding to an acid site.

The unimolecular slow step for the enzymatic hydrolysis has, at 25°, a rate constant $k = 700\ min^{-1}$. This is approx. 10^6-fold larger than that for the spontaneous, unimolecular hydrolysis (Eqn. 15). The scheme in Fig. 9 is not, however, a straightforward catalysis of Eqn. 15, since P–O bond formation is now rate-determining. For a true measure of the enzyme's acceleratory power the comparison should be with the (in practice negligible) rate of the spontaneous bimolecular attack by water on phosphorus. An acceleration of between 10^9–10^{12}-fold is probable. The proposed mechanism is, however, entirely compatible with such a factor for it involves not only conversion of a bimolecular to a unimolecular process, but also multiple, intramolecular acid and base catalysis. Each of these items alone can account for a factor of approx. 10^3.

Acylphosphatase provides an example where a rather complete knowledge of the non-enzymatic reaction mechanisms is of no direct value in unravelling the enzymatic mechanism.

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