Biochem. J. (1969) **111**, 187
Printed in Great Britain

Studies on Alkaline Phosphatase

TRANSIENT-STATE AND STEADY-STATE KINETICS OF *ESCHERICHIA COLI* ALKALINE PHOSPHATASE

BY H. N. FERNLEY AND P. G. WALKER
Department of Biochemistry, Institute of Orthopaedics, Stanmore, Middlesex

(*Received* 8 *July* 1968)

1. The transient-state and steady-state phases of the reaction between *Escherichia coli* alkaline phosphatase and 4-methylumbelliferyl phosphate were investigated by using a fluorimetric stopped-flow technique. 2. At low substrate concentration (5μM) in the pH range 3·8–6·3 there was an initial rapid liberation of up to 1 mole of 4-methylumbelliferone/mole of enzyme. 3. At very low substrate concentration (0·1μM) in the pH range 4·9–5·9 an initial lag in 4-methylumbelliferone production was observed, from which values for k_{+1} and k_{-1} could be obtained. 4. The pH profiles for the rates of phosphorylation and dephosphorylation are quite different, and it is postulated that an ionizing group which determines the conformation during the phosphorylation step is not involved in the dephosphorylation step. 5. The binding constants for substrate and P_i are similar throughout the pH range 4–8. The ionization of substrate or P_i appeared to have no marked effect on the binding.

In a preliminary publication (Fernley & Walker, 1966) we established that a phosphoryl-enzyme intermediate is formed during the hydrolysis of a phosphate ester (MUP*) by alkaline phosphatase. This result has been confirmed by several groups of workers using different substrates (Williams, 1966; Fife, 1967; Ko & Kezdy, 1967; Trentham & Gutfreund, 1968) and most agree there appears to be only one active site/unit dimer.

This paper is concerned with the determination of the phosphorylation–dephosphorylation rate parameters, with reference to MUP, insofar as they are accessible with a stopped-flow technique, and the binding parameters for MUP and P_i. For this purpose the transient-state data have been supplemented with steady-state data over the pH range 4–9. Other steady-state studies have been published by Garen & Levinthal (1960), Heppel, Harkness & Hilmoe (1962), Wilson, Dayan & Cyr (1964), Lazdunski & Lazdunski (1966) and Jenkins & D'Ari (1966).

MATERIALS AND METHODS

Materials. *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) (code no. BAPC) as a suspension (5mg. of protein/ml.) in 65%-saturated $(NH_4)_2SO_4$. Before use it was dialysed against four changes of 0·02M-tris–0·01M-acetic acid buffer, pH 8·75 at 2°, and stored as a 1 mg./ml. solution in the above buffer. The activity under standard conditions was about 50% of that quoted by Pigretti & Milstein (1965) for the pure enzyme.

MUP was synthesized and purified as previously described (Fernley & Walker, 1965). Tris was recrystallized once before use; other chemicals were analytical-grade reagents.

Transient-state investigations. These were carried out in a stopped-flow apparatus (Gibson & Milnes, 1964) modified for fluorescence measurement as described by Fernley & Bisaz (1968). All experiments were performed at 20° as follows. Equal volumes of enzyme (6–40μg. of enzyme protein/ml.), in 0·025% bovine plasma albumin (crystallized grade; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) and otherwise unbuffered, and MUP, in 0·025% albumin buffered at the pH under study, were rapidly mixed in the stopped-flow apparatus. The subsequent release of 4-methylumbelliferone was followed as described previously (Fernley & Bisaz, 1968).

Steady-state investigations: determination of K_m. In the pH range 3·8–5·4, where P_i is a very weak competitive inhibitor, K_m was evaluated by analysis of total hydrolysis curves obtained by mixing enzyme and MUP in the stopped-flow apparatus. The integral form of the Michaelis equation was used to derive a linear plot. For the pH range 5·9–7·4 the stopped-flow apparatus was employed to determine initial rates at various substrate concentrations. The approach suggested by Jenkins & D'Ari (1966), namely determination of total hydrolysis curves at various substrate concentrations, was also tried, and in our experience it was not as satisfactory as measuring initial rates. Above pH 8 a conventional start–stop assay technique previously described for the calf-intestinal enzyme (Fernley & Walker, 1965) was used.

Determination of K_i for P_i. As shown below, P_i behaves as a purely competitive inhibitor in the pH range 4–9 and it is possible to evaluate the K_i/K_m ratio by measuring initial rates at high substrate concentration in the presence and absence of various concentrations of P_i. Above pH 8 it is

* Abbreviation: MUP, 4-methylumbelliferyl phosphate monoester.

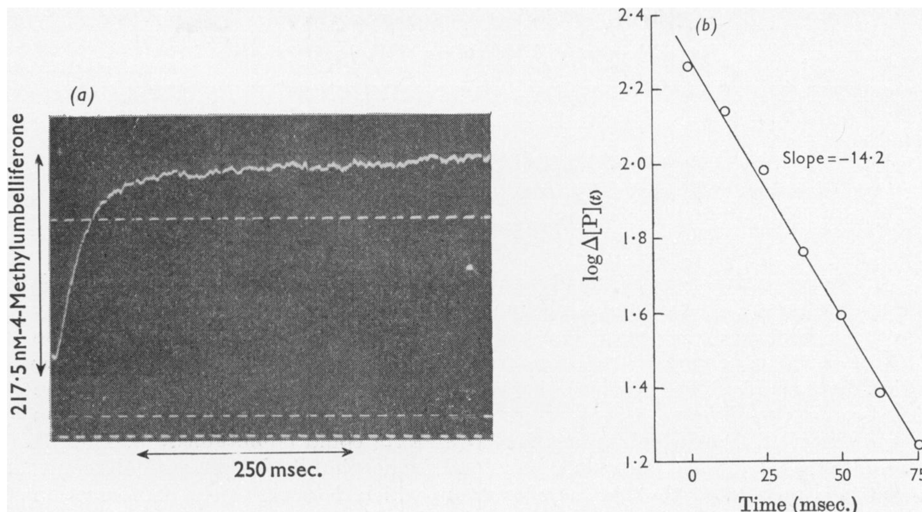
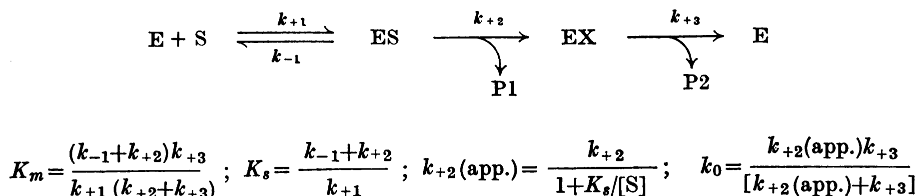


Fig. 1. (*a*) Reaction of alkaline phosphatase (20μg./ml.) with 5μM-MUP at pH 4·51. The stopped-flow procedure is described in the Materials and Methods section. The time-constant is 4 msec. (*b*) Plot of $\log \Delta[\mathrm{P}]_{(t)}$ against time (msec.). The slope of this plot is $-[k_{+2}(\mathrm{app.})+k_{+3}]/2{\cdot}303$ and the intercept on the ordinate at zero time is $\log[\mathrm{E}]_0 k_{+2}(\mathrm{app.})^2/[k_{+2}(\mathrm{app.})+k_{+3}]^2$. The steady-state rate, k_0, is 27·5 nM-methylumbelliferone/sec.



$$\mathrm{E}+\mathrm{S} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{ES} \xrightarrow[\downarrow \mathrm{P1}]{k_{+2}} \mathrm{EX} \xrightarrow[\downarrow \mathrm{P2}]{k_{+3}} \mathrm{E}$$

$$K_m=\frac{(k_{-1}+k_{+2})k_{+3}}{k_{+1}(k_{+2}+k_{+3})};\quad K_s=\frac{k_{-1}+k_{+2}}{k_{+1}};\quad k_{+2}(\mathrm{app.})=\frac{k_{+2}}{1+K_s/[\mathrm{S}]};\quad k_0=\frac{k_{+2}(\mathrm{app.})k_{+3}}{[k_{+2}(\mathrm{app.})+k_{+3}]}$$

Scheme 1.

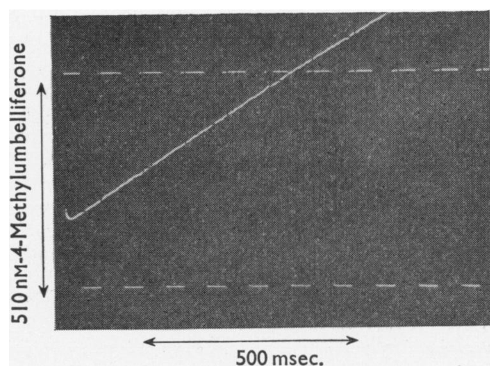


Fig. 2. Reaction of alkaline phosphatase (10μg./ml.) with 5μM-MUP at pH 7·94. The time-constant is 4 msec. The steady-state rate is 720 nM-product/sec.

possible to obtain K_i directly by determining K_m in the presence and absence of a fixed concentration of $\mathrm{P_i}$.

For all the steady-state investigations 0·02 *I* tris–acetic acid buffers, pH 3·8–8·9, were employed. Tris is known to affect the kinetics of the enzyme (Trentham & Gutfreund, 1968), but at the concentrations used the effect will be small. Assays were carried out at 20° in the presence of 0·01% albumin.

RESULTS

Transient-state data. Photographs of oscilloscope traces obtained by mixing enzyme with substrate at different pH values in the stopped-flow apparatus are given in Figs. 1 and 2. The derivative plot in Fig. 1(*b*) is used to evaluate the first-order exponential term and the magnitude of the burst (Fernley & Bisaz, 1968). Analysis of such traces at the various pH values gives values for the concentration of

Table 1. *Parameters determined from transient-state traces of the reaction between* E. coli *alkaline phosphatase and* 5 μM-*MUP*

The buffer system employed was 0·02 *I* tris–acetic acid. At pH 3·76 and 4·51 20 μg. of enzyme protein/ml. was used, and for the rest 10 μg./ml. All concentrations refer to the reaction mixture. Other details are given in the Materials and Methods section.

pH ...	3·76	4·51	4·93	5·45	5·91	6·34	6·95	7·38	7·94	8·90
$[E]_0$ (nM)	253	216	138	138	127	128	—	—	—	—
k_{+2}(app.) (sec.$^{-1}$)	61	32	21	15	10·2	6·1	—	—	—	—
k_{+3} (sec.$^{-1}$)	0·052	0·128	0·263	0·517	1·34	2·95	—	—	—	—
k_0 (sec.$^{-1}$)	—	—	—	0·50	1·2	2·0	4·4	4·4	7·9	11·0

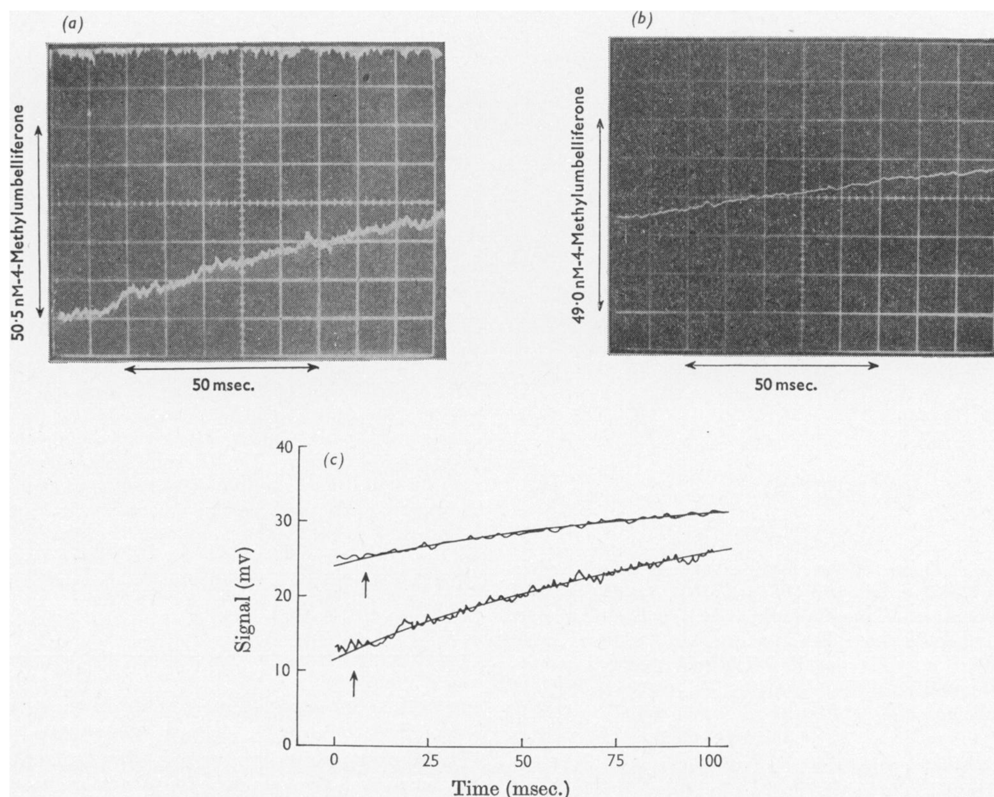


Fig. 3. (*a*) Reaction of alkaline phosphatase (3·3 μg./ml.) with MUP at pH 4·91 with an initial substrate concentration of 71 nM. The time-constant is 1 msec. (*b*) Reaction of alkaline phosphatase (3·3 μg./ml.) with MUP at pH 5·90 with an initial substrate concentration of 87 nM. The time-constant is 3 msec. (*c*) Tracings of (*a*) and (*b*) with extrapolated curves from which values for *T* (app.) are derived.

Table 2. *Parameters determined from the steady-state reaction between* E. coli *alkaline phosphatase, MUP and* P_i

Details are given in the Materials and Methods section.

pH ...	3·78	4·39	4·96	5·38	5·90	6·34	6·78	7·38	7·98	8·90
K_m (nM)	1·5	3·7	4·7	6·8	19	43	107	220	510	1700
K_i/K_m	770	405	200	78	23	6·7	3·5	1·18	0·74	0·77
K_i (nM)	1700	1500	820	530	430	290	380	260	380	1300

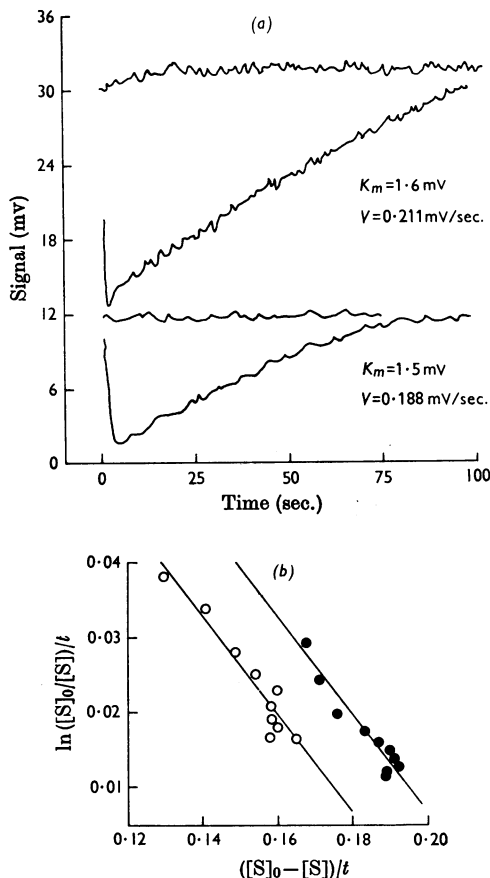


Fig. 4. (a) Traces of the reaction between alkaline phosphatase ($0{\cdot}33\,\mu$g./ml.) and MUP at pH 3·78 in the stopped-flow apparatus. A signal of 10 mv corresponds to 10·4 nM-methylumbelliferone. The time-constant is 1 sec. Other details are given in the Materials and Methods section. (b) Plots of $\ln([S]_0/[S])/t$ against $([S]_0-[S])/t$, the [S] and t values being obtained from the traces in (a). The slope of this plot is $-1/K_m$ and the intercept on the abscissa when $\ln([S]_0/[S])/t=0$ gives V.

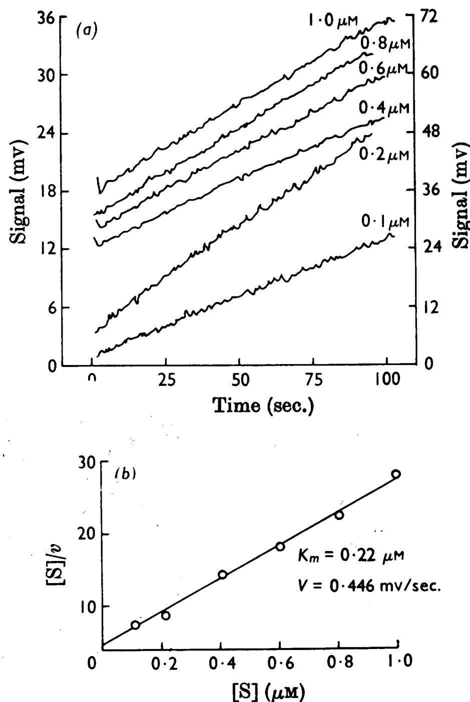


Fig. 5. (a) Traces of the reaction between alkaline phosphatase (6·7 ng./ml.) and MUP at pH 7·38 in the stopped-flow apparatus. A 10 mv signal corresponds to 10·9 nM-methylumbelliferone. The left-hand scale refers to $0{\cdot}1\,\mu$M- and $0{\cdot}2\,\mu$M-substrate and the right-hand scale to $0{\cdot}4$–$1{\cdot}0\,\mu$M-substrate. The time-constant is 1 sec. (b) Plot of $[S]/v$ against [S] where [S] and v are the substrate concentration and rate of change of signal (mv/sec.) respectively in (a). The line is derived statistically by the method of least squares.

active sites, $[E]_0$, the rate of phosphorylation, k_{+2}(app.), and the rate of dephosphorylation, k_{+3}, based on the kinetic scheme shown in Scheme 1. Here E and S denote free enzyme and substrate respectively, ES is the enzyme–substrate complex, EX the phosphoryl-enzyme, P1 and P2 the reaction products, k_{+2} the maximum phosphorylation rate and k_0 the turnover. It is implied that the substrate concentration remains constant during the transient-state phase of the reaction, a condition that is fulfilled when $[S]\gg[E]_0$. Values for the various parameters obtained are given in Table 1. At pH 6·95 and above, the initial burst of 4-methylumbelliferone production was either too small to be measured accurately or not observable (Fig. 2). The average number of active sites/molecule, based on a molecular weight of 86000 (Schlesinger & Barrett, 1965), is estimated to be 1·1. A different *E. coli* preparation, 90% pure by comparison with the activity of the Worthington enzyme, gave an active-site concentration of $266\,\mu$M and an enzyme concentration of $230\,\mu$M.

Transient state at low substrate concentration. Oscilloscope traces of the reaction between enzyme ($3\,\mu$g./ml.) and approx. $0{\cdot}1\,\mu$M-MUP at pH 4·91 and 5·90 are given in Fig. 3. Under these conditions the reaction is not first-order because [S] cannot be considered as constant. A second exponential term involving all the rate constants appears in the initial phase of the transient state as a lag in 4-methylumbelliferone production. It can be shown (Gutfreund, 1955) that extrapolation of the burst to zero

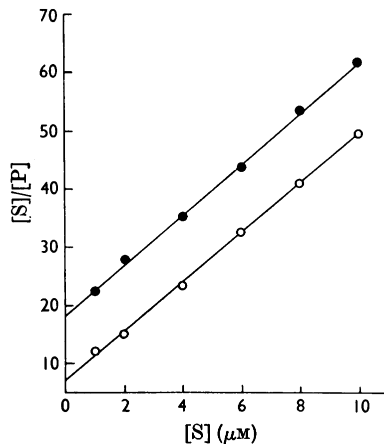


Fig. 6. Determination of K_m for MUP and K_i for P_i at pH 8·90. The substrate concentration was varied in the presence (●) and absence (○) of 2 μM-P_i with 0·67 ng. of alkaline phosphatase/ml. Values of [P] are micromolar concentrations of methylumbelliferone in the reaction mixture after 30 min. incubation at 20°. Other details are given in the Materials and Methods section. The slope of this plot is $1/V$, the parallel lines indicating that inhibition by P_i is competitive: $K_m = 1·70\,\mu$M; $K_i = 1·30\,\mu$M; $V = 0·236\,\mu$M-product/30 min.

product concentration cuts the time axis at a point T. To obtain T, one must subtract the electronic time-constant from the apparent value and add the 'dead time' of the apparatus, i.e. the time of flow between mixing of the reactants and the point of observation. The time-constant was controlled by varying the capacitance across the oscilloscope input (1 MΩ impedance) and an estimate of the dead time (3 msec.) was arrived at by measuring the flow time for the latter half of the flow (0·25 ml. in 20 msec.) and the dead volume between the outlet of the first mixing chamber and the mean observation point along the flow tube (2 mm. bore) (40 μl.). Gibson & Milnes (1964) quote 3·5 msec. for the same apparatus with a slightly larger dead volume. For the pH 4·91 trace, T(app.) is 5 msec. and the time-constant 1 msec., giving $T = 7$ msec. At pH 5·91, T(app.) $= T = 8$ msec., since here the time-constant is equal to the dead time.

Effect of P_i on the transient state. At pH 4·96 the addition of 20 μM-P_i to a reaction mixture containing 3 μg. of enzyme/ml. and 2 μM-MUP had no effect on the steady-state rate or on the magnitude of the burst, but decreased the exponential term k_{+2}(app.) + k_{+3} from 17·7 sec.$^{-1}$ to 4·6 sec.$^{-1}$. Under these conditions P_i increases K_s and is therefore acting as a competitive inhibitor.

Steady-state data. Values of K_m for MUP in the pH range 3·78–8·98 were determined and are given

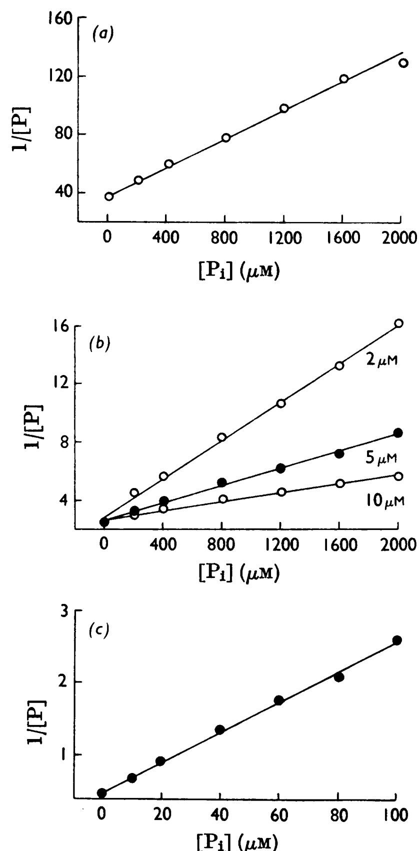


Fig. 7. Determination of K_i for P_i with alkaline phosphatase. P_i concentrations are plotted against 1/[P]. Values of [P] are micromolar concentrations of methylumbelliferone in the reaction mixture after 30 min. incubation at 20°. (*a*) pH 3·78 with 33 ng. of enzyme/ml. and 1 μM-MUP: $K_i/K_m = 770$. (*b*) pH 4·96 with 67 ng. of enzyme/ml. and the stated concentrations of MUP: mean value for $K_i/K_m = 200$. (*c*) pH 7·43 with 14 ng. of enzyme/ml. and 20 μM-MUP: $K_i/K_m = 1·18$.

in Table 2. The three methods employed are illustrated in Figs. 4–6. The experimental approach was dictated mainly by the changing values of K_m and K_i (P_i) with pH, and it was necessary to establish that the inhibition by P_i was indeed competitive in order that K_i could be determined. This was achieved (*a*) as described above and (*b*) by varying the concentration of P_i in the presence of an otherwise saturating concentration of substrate, as shown in Fig. 7. The linearity of the plot, the constancy of the intercept at different substrate concentrations and the fact that the K_i/K_m ratio is independent of the substrate concentration indicate that the inhibition is strictly competitive at

pH 4·95 as it is at pH 8·90 (Fig. 6). It was assumed that the inhibition is competitive at all the pH values investigated, and that K_i/K_m ratios can be used to derive K_i values. These are given in Table 2.

In the pH range 3·78–5·38, P_i is a weak inhibitor shown that $a^2 \gg 4b$ and the roots r_1 and r_2 approximate to $-a$ and $-b/a$ respectively. At sufficiently high substrate concentrations ([S] > 1 μM) the first exponential term is rapidly disappearing and eqn. (1) simplifies to:

$$[\mathrm{P1}] = [\mathrm{E}]_0 k_0 t + \frac{[\mathrm{E}]_0 k_{+2}(\mathrm{app.})^2}{[k_{+2}(\mathrm{app.}) + k_{+3}]^2} \cdot \{1 - \exp - [k_{+2}(\mathrm{app.}) + k_{+3}]t\} \quad (2)$$

and, provided that the enzyme is sufficiently stable under the reaction conditions, complete hydrolysis curves may be used to evaluate K_m as shown in Fig. 4. The pH range 5·90–7·38 presents some difficulties because K_m is too low to measure in the usual way and inhibition by P_i interferes with the analysis of total hydrolysis curves. The compromise adopted here, namely measurement of initial rates at different substrate concentrations (Fig. 5), appears to be reasonably satisfactory.

DISCUSSION

On the basis that the reaction between alkaline phosphatase and MUP is represented by Scheme 1 (which adequately describes the results presented in this paper) it is possible to evaluate all the rate constants involved. However, if the reaction ES→EX is more than one step, as suggested by Trentham & Gutfreund (1968), the validity of Scheme 1 requires further discussion. There is at present no evidence that more than one step is rate-determining and in many cases the reaction will then conform to Scheme 1 with altered significance of the rate constants. These are now operational constants describing the following reactions: k_{+1}, the association of E and S; k_{-1}, the net dissociation of S from all ES complexes; k_{+2}, the formation of EX from all ES complexes; k_{+3}, the formation of E from all EX complexes.

The equation describing the time-course of the transient state and early steady state may be written:

$$[\mathrm{P1}] = \frac{c}{b}\left(k_{+3}t - \frac{r_2(k_{+3}+r_1)(\exp r_1 t - 1)}{r_1(r_2 - r_1)} + \frac{r_1(k_{+3}+r_2)(\exp r_2 t - 1)}{r_2(r_2 - r_1)}\right) \quad (1)$$

with:

$$r_1 = \frac{-a - (a^2 - 4b)^{\frac{1}{2}}}{2}; \quad r_2 = \frac{-a + (a^2 - 4b)^{\frac{1}{2}}}{2}; \quad a = k_{+1}[\mathrm{S}] + k_{-1} + k_{+2} + k_{+3};$$

$$b = k_{+1}[\mathrm{S}](k_{+2} + k_{+3}) + k_{+3}(k_{-1} + k_{+2}); \quad c = [\mathrm{E}]_0[\mathrm{S}]k_{+1}k_{+2}$$

Eqn. (1) holds provided that the substrate concentration does not change appreciably during the transient state and that $a^2 > 4b$. Under all the conditions employed in this work, as made clear below from the magnitude of the rate constants, it can be

and evaluation of $[\mathrm{E}]_0$, k_{+2}(app.) and k_{+3} is straightforward. At low substrate concentration, the early phase of the transient state is given (provided that $r_1 \gg r_2 \gg k_{+3}$) approximately by:

$$[\mathrm{P1}] = [\mathrm{E}]_0 k_{+2}(\mathrm{app.})\left(t - \frac{(1 - \exp - at)}{a}\right) \quad (3)$$

and $T = 1/a$

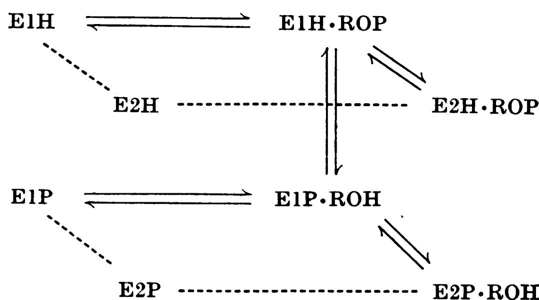
Evaluation of K_s and k_{+2}. From the values of K_m, k_{+2}(app.) and k_{+3} given in Tables 1 and 2 one can derive values for K_s and k_{+2} by using the relationships listed in Scheme 1. These values are given in Table 3.

In most cases K_s is so low that the alternative approach, i.e. determination of k_{+2}(app.) at different substrate concentrations, is too difficult with the present technique. The uncertainty at pH 3·76 is due to the K_m value being almost at the limit of resolution: the noise level with a 1 sec. time-constant is about 1 nM-methylumbelliferone. There can be no doubt, however, that k_{+2} approaches a minimum value at pH 7 where both k_{+2} and k_{+3} are about 6 sec.$^{-1}$. The maximum turnover at low ionic strength is about 20 sec.$^{-1}$ (pH 10). Since there is no measurable transient state in the pH range 8–10, it is likely that $k_{+2} = k_0$ here. The pH profile for k_{+2} therefore has a minimum at pH 7, rises to 20 sec.$^{-1}$ on the alkaline side and to at least 70–80 sec.$^{-1}$ on the acid side.

The variation of K_s with pH appears to reflect k_{+2}, as does K_i, in that both have minimum values at pH 7 and on the acid side rise more or less in step with k_{+2}. It follows (if k_{-1} and k_{+2} are affected similarly by pH) that the rate of association of enzyme with substrate or P_i (pK values 5·6 and 7·1 respectively) is substantially pH-independent in the pH range 4–8, where both MUP and P_i are

Table 3. *Values of K_s and k_{+2} derived from the data of Tables* 1 *and* 2

pH ...	3·76	4·51	4·93	5·45	5·91	6·34
K_s (nM)	(>1000)	1160	408	213	169	134
k_{+2} (sec.$^{-1}$)	(>70)	40	23	16	11	6·3



Scheme 2.

changing charge, from −1 to −2. It is implied that either the pK values do not change when bound to alkaline phosphatase, or, if they do, compensatory changes in the enzyme mask the effect. Though the latter appears unlikely, it is equally difficult to consider electrostatic interactions as not being of primary importance in the binding.

Evaluation of k_{+1} and k_{-1}. From the T values for the reaction at pH 4·91 and 5·90 (Fig. 3) and the respective values for K_s, k_{+2} and k_{+3}, one can derive approximate values for k_{+1} and k_{-1}. Thus for pH 4·91 $k_{+1}=3\cdot0\times10^{8}$M^{-1}sec.$^{-1}$ and $k_{-1}=100$sec.$^{-1}$, and at pH 5·90 $k_{+1}=4\cdot8\times10^{8}$M^{-1} sec.$^{-1}$ and $k_{-1}=71$sec.$^{-1}$. The upper limit for the rate of enzyme–substrate association is determined by the rate of diffusion of the reactants and is approx. 10^{9}M^{-1}sec.$^{-1}$ (Eigen & Hammes, 1963). The above k_{+1} values are of this order, suggesting that the reaction between alkaline phosphatase and substrate is mainly diffusion-controlled. The values for k_{-1} are considerably greater than k_{+2}, indicating that K_s is essentially an equilibrium constant and that the pH-dependence of k_{-1} is probably similar to that of k_{+2}.

Effect of pH *on k_{+2} and k_{+3}.* One can postulate (*a*) that k_{+3} is the microscopic reverse of k_{+2} and (*b*) that two forms of the enzyme–substrate complex exist as shown in Scheme 2. This depicts the half-reaction between alkaline phosphatase and substrate. For the dephosphorylation step read H for R. Only the relevant species are here connected by reversible arrows; for instance phosphoryl-enzyme in aqueous solution will rapidly form a complex with water and E2P can be neglected. E1H·ROP either dissociates or is transformed into E1P·ROH, whereas E2H·ROP does neither. The equilibrium between these two forms can account for the changes in k_{+2} with pH. A similar argument applied to the phosphoryl-enzyme accounts for the changes in k_{+3} with pH, but, whereas only one functional group, with an alkaline pK, need be invoked for the E1P·HOH–E2P·HOH equilibrium, two groups (one with an alkaline and one with an acid pK) are required for the E1H·ROP–E2H·ROP equilibrium. One can speculate that E1 and E2 represent different conformational states; thus E2P might be favoured when a particular amino group is in the charged form, whereas E2H·ROP might require both a charged amino group and an ionized carboxyl group. There is some evidence that the free enzyme and the phosphoryl-enzyme differ in conformation, since the former is markedly labile in weakly acid solution (Pigretti & Milstein, 1965) whereas the latter is not (Fernley & Walker, 1966). One could argue therefore that the free enzyme has the E1 conformation, whereas E2H·ROP and E2P·HOH are the predominant complex species under these conditions.

Number of active sites/molecule. Some controversy was introduced when Fife (1967) claimed, on the basis of stopped-flow observations with *o*-nitrophenyl phosphate, that there were two to three sites/molecule. Other workers, however, so far all agree there is only one active site/molecule. We have checked our original observation (Fernley & Walker, 1966) several times and conclude there is probably only one site/molecule. We suggested that at high ionic strength and pH two sites may be active, but this hypothesis offers no explanation for

the observed participation of only one site at lower pH. There are two possibilities: either the dimer is not initially symmetrical, or the binding of one substrate molecule is associated with a change in the enzyme whereby the second site becomes non-reactive. In terms of the discussion in the previous section one might say that, in the E2 conformation, the substrate is either 'locked in' or not bound at all, the enzyme still preserving spatial symmetry.

This work was supported by a grant from the National Fund for research into Poliomyelitis and other Crippling Diseases. The technical assistance of Mr D. A. Byers is gratefully acknowledged.

REFERENCES

Eigen, M. & Hammes, G. G. (1963). *Advanc. Enzymol.* **25**, 1.

Fernley, H. N. & Bisaz, S. (1968). *Biochem. J.* **107**, 279.

Fernley, H. N. & Walker, P. G. (1965). *Biochem. J.* **97**, 95.

Fernley, H. N. & Walker, P. G. (1966). *Nature, Lond.*, **212**, 1435.

Fife, W. K. (1967). *Biochem. biophys. Res. Commun.* **28**, 309.

Garen, A. & Levinthal, C. (1960). *Biochim. biophys. Acta*, **38**, 470.

Gibson, Q. H. & Milnes, L. (1964). *Biochem. J.* **91**, 161.

Gutfreund, H. (1955). *Disc. Faraday Soc.* **20**, 167.

Heppel, L. A., Harkness, D. R. & Hilmoe, R. J. (1962). *J. biol. Chem.* **237**, 841.

Jenkins, W. T. & D'Ari, L. (1966). *J. biol. Chem.* **241**, 295.

Ko, S. H. D. & Kezdy, F. J. (1967). *J. Amer. chem. Soc.* **89**, 7139.

Lazdunski, C. & Lazdunski, M. (1966). *Biochim. biophys. Acta*, **113**, 551.

Pigretti, M. M. & Milstein, C. (1965). *Biochem. J.* **94**, 106.

Schlesinger, M. J. & Barrett, K. (1965). *J. biol. Chem.* **240**, 4284.

Trentham, D. R. & Gutfreund, H. (1968). *Biochem. J.* **106**, 455.

Williams, A. (1966). *Chem. Commun.* no. 19, p. 676.

Wilson, I. B., Dayan, J. & Cyr, K. (1964). *J. biol. Chem.* **239**, 4182.