The Kinetics of Hydrolysis of Phenyl Phosphate by Alkaline Phosphatases

By R. K. MORTON
*Department of Biochemistry, University of Cambridge**

(*Received* 13 *August* 1956)

Alkaline phosphatases purified from cow's milk and calf intestinal mucosa have a similar substrate specificity. However, under optimum conditions, the rate of hydrolysis of typical substrates by intestinal phosphatase is about five times that of the milk enzyme (Morton, 1955*a*). A comparative study of the kinetic behaviour of the two enzymes therefore appeared desirable. Variation of such properties as maximum initial velocity of hydrolysis ($V_{max.}$) and Michaelis constant (K_m) with pH might be expected to indicate whether the same or a different mechanism was involved in the hydrolysis of substrate by these two enzymes. Moreover, determination of the kinetics of substrate hydrolysis was an essential preliminary to investigation of the kinetics of the transfer reactions catalysed by phosphatases (Morton, 1952, 1953*a*).

Milk phosphatase is derived from mammary-gland microsomes which are secreted with milk (Morton, 1953*b*, 1954*a*). This and other evidence has established that the milk and mammary-gland alkaline phosphatases are identical (Morton, 1955*a*). By using a crude preparation of alkaline phosphatase from guinea-pig mammary gland, Folley & Kay (1935) found that the optimum pH for hydrolysis of monophenyl phosphate varied with substrate concentration. Preliminary findings with purified cow's milk phosphatase were in reasonable agreement with those of Folley & Kay (1935). Hydrolysis of monophenyl phosphate by purified calf intestinal phosphatase was investigated in detail, extending the range of conditions used by Folley & Kay (1935) with mammary-gland (milk) enzyme. The results are described in this paper. This work was carried out in 1950 and, although not previously published, has been described elsewhere (Morton, 1952).

MATERIALS

Monophenyl phosphate. A commercial preparation of the disodium salt (British Drug Houses Ltd.) was dissolved in water, free phenol removed by extraction with diethyl ether, and inorganic phosphate precipitated with ammoniacal magnesia. The product was dried *in vacuo* over conc. H_2SO_4 at room temp.

For metal-activation studies, this product was dissolved in a minimum of hot glass-distilled water, and metal-free 6N-HCl added until the pH was about 2 (at 20°). The solution was cooled to about 2° and excess of redistilled ethanol added to precipitate about half the phenyl phosphate acid, which was washed with ethanol and with diethyl ether and dried *in vacuo* over conc. H_2SO_4 at room temp. The product was dissolved in redistilled ethanolamine (4M) to give a concentrated solution at the required pH value.

Buffers. These were purified and prepared as described previously (Morton, 1955*a*). Veronal (sodium diethylbarbiturate)–sodium acetate–HCl buffers (Michaelis, 1931) were used for pH values between 7 and 9, and ethanolamine–HCl buffers for values greater than 9. For metal-activation studies, twice-recrystallized diethylbarbituric acid was dissolved in ethanolamine so that the resultant solution was 0·25M with respect to both components. This veronal–ethanolamine buffer was adjusted to the required pH with either metal-free 6N-HCl or 2N-NaOH.

Enzymes. Alkaline phosphatases purified from calf intestinal mucosa (Morton, 1950, 1954*b*) and cow's milk (Morton, 1950, 1953*c*) were used throughout.

METHODS

pH *values.* Except where otherwise indicated, all values given refer to pH at 38°. They were determined with replicate tubes of the buffered substrates used for activity measurements.

Inorganic phosphate. This was estimated by the method of Fiske & Subbarow (1925) or, in metal-activation studies, by the method of Horwitt (1952).

Phenol. This was estimated by the method of King (1951), modified as previously described (Morton, 1955*b*).

Initial rate of hydrolysis (V_0). For the kinetic studies, this was determined by estimating both the inorganic phosphate and the phenol liberated from phenyl phosphate in 5 min. at 38°. There was generally good agreement between the two determinations, but values obtained with the more sensitive phenol method were preferred where very low substrate concentrations were used. The procedure was essentially as described previously (Morton, 1953*d*). The reaction was commenced by addition of 0·1 ml. of enzyme solution to 4·9 ml. of buffered substrate, and stopped by the addition of 0·2 ml. of 20% (w/v) trichloroacetic acid. Results are expressed as μg. of P liberated in 5 min. by 0·1 ml. of the standard solution of enzyme. This contained in 1 ml. approx. 55 enzyme units, as previously defined (Morton, 1953*d*), equivalent to approx. 4·1 μg. of purified intestinal phosphatase or to approx. 22 μg. of purified milk phosphatase.

* Present address: Department of Biochemistry, University of Melbourne.

It was confirmed that the initial velocity of hydrolysis was linear up to 10% hydrolysis of the substrate (Morton, 1953*d*). Except at the lowest substrate concentration ($2{\cdot}5 \times 10^{-5}$ M), the hydrolysis in no case exceeded 10%.

RESULTS

Phosphatase from calf intestinal mucosa

Influence of substrate concentration on the activity–pH curve. The initial velocity of hydrolysis of phenyl phosphate was determined at several pH values over the range 8·25–10·1 and at eight substrate concentrations from $2{\cdot}5 \times 10^{-5}$ M to $7{\cdot}5 \times 10^{-2}$ M. The results are shown in Fig. 1.

At low substrate concentration (Fig. 1, curves *A–C*), the optimum pH is not well defined. An observation at pH 7·6 with $2{\cdot}5 \times 10^{-5}$ M substrate showed little further change of V_0 from that shown for pH 8·2 (Fig. 1, curve *A*), so that V_0 is fairly constant over the pH range 7·6–9·0 at this very low substrate concentration. However, hydrolysis is very slow at pH values above 9·6 (Fig. 1, curve *A*). This has an important bearing on the measurement of phosphotransferase activity (Morton, 1952, 1953*a*). Observations made since this experiment was carried out show that, for the highest substrate concentration ($7{\cdot}5 \times 10^{-2}$ M; curve *H*), the optimum pH is about 10·0 and the activity at this pH is slightly greater than the maximum shown for curve *G*.

Fig. 1 shows that the optimum pH for hydrolysis varies with the substrate concentration and the optimum substrate concentration also varies with the pH. Fig. 2 shows that the relationship between the optimum pH for hydrolysis (as estimated from Fig. 1) and p[*S*] is linear over the substrate range $2{\cdot}5 \times 10^{-5}$ to $2{\cdot}5 \times 10^{-3}$ M, but deviates considerably from linearity at higher substrate concentrations. There is some uncertainty about the optimum pH values at very low substrate concentrations, since these are not well defined (Fig. 1).

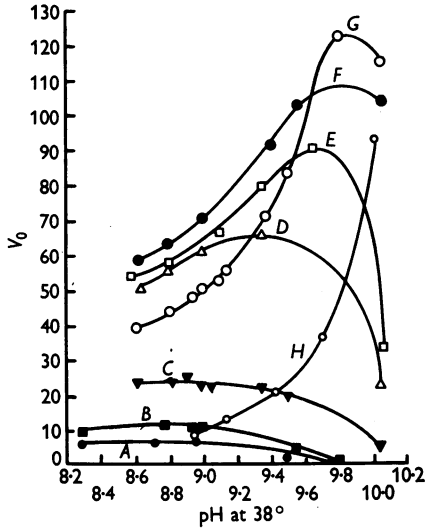

Fig. 1. Plot of initial rate of hydrolysis, V_0, against pH for hydrolysis of phenyl phosphate at various concentrations. The reaction mixture (5 ml.) contained either veronal–sodium acetate–HCl buffer (0·05 M), for pH values less than 9, or ethanolamine–HCl buffer (0·05 M) for pH values greater than 9, magnesium acetate (0·01 M), and approx. 5·5 units of purified calf intestinal alkaline phosphatase. The reaction was carried out for 5 min. at 38°, and enzymically liberated phenol and inorganic phosphate were estimated as described in the text. The curves are for the following substrate concentrations (M): *A*, $2{\cdot}5 \times 10^{-5}$; *B*, 5×10^{-5}; *C*, 10^{-4}; *D*, 5×10^{-4}; *E*, $7{\cdot}5 \times 10^{-4}$; *F*, $2{\cdot}5 \times 10^{-3}$; *G*, $2{\cdot}5 \times 10^{-2}$; *H*, $7{\cdot}5 \times 10^{-2}$.

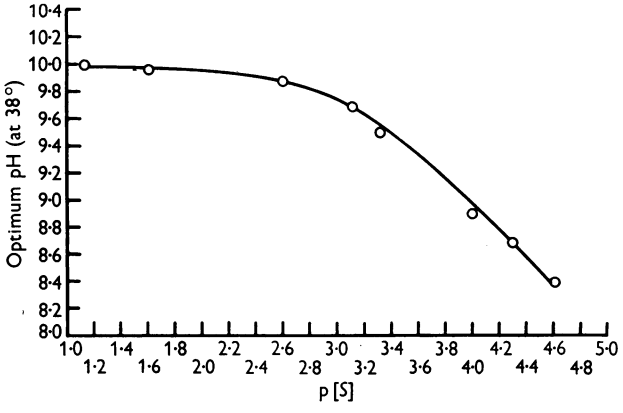

Fig. 2

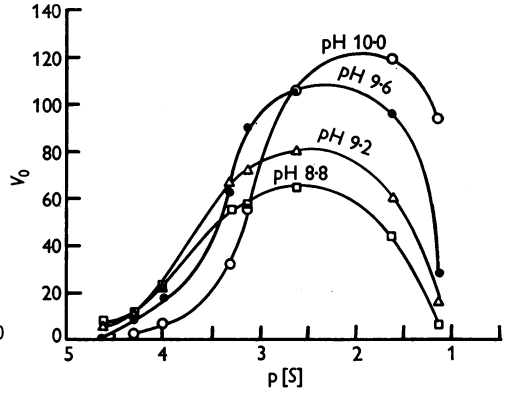

Fig. 3

Fig. 2. Dependence of optimum pH for hydrolysis of phenyl phosphate by alkaline phosphatase on substrate concentration. Values of optimum pH were obtained from Fig. 1.

Fig. 3. Influence of phenyl phosphate concentration on the initial rate of hydrolysis by intestinal alkaline phosphatase at several pH values. Values of V_0 were obtained from Fig. 1.

Values of V_0 for various substrate concentrations at regular pH increments of 0·2 unit from pH 8·6 to 10·0 were obtained from Fig. 1. Plots of V_0 against p[S] (Michaelis & Menten, 1913) for pH values 8·8, 9·2, 9·6 and 10·0 are shown in Fig. 3. The inhibition by high substrate concentrations is apparent at all pH values. Especially noteworthy is the relatively narrow range of substrate concentrations over which optimum activity is obtained.

Variation of K_m with pH. Plots of $1/V_0$ against $1/[S]$ (Lineweaver & Burk, 1934) are shown in Fig. 4 for pH values 9·0, 9·4 and 9·8. The non-linear relationship is apparent. This is even more pronounced if the results for the lowest substrate concentration ($2·5 \times 10^{-5}$ M) are included. They were omitted from Fig. 4 for the sake of clarity, as were the curves for other pH values investigated.

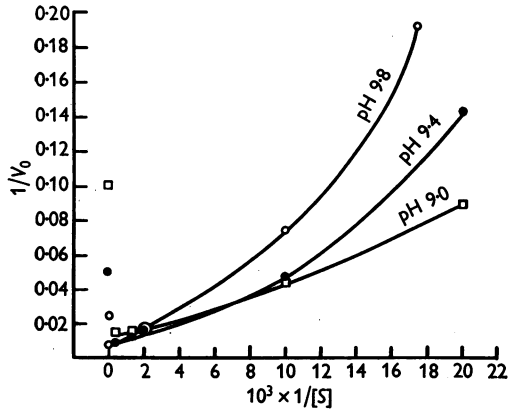

Fig. 4. Lineweaver–Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for intestinal alkaline phosphatase with phenyl phosphate at various pH values. Values of V_0 were obtained from Fig. 1.

Values for K_m were estimated from Michaelis–Menten plots, as the substrate concentration for one-half maximum velocity. The Dixon (1953) plot of pK_m against pH is shown in Fig. 5 (curve A). This reveals a -1 unit slope at higher pH values, changing to zero slope at lower values, the discontinuity appearing at about pH 9·2.

A similar plot for mammary gland (milk) alkaline phosphatase, based on the data of Folley & Kay (1935), is shown in Fig. 5 as curve B. Although the data are limited, here also there are indications of a -1 unit slope at higher pH values, with a discontinuity at about pH 9·2. Some values obtained with purified milk phosphatase are included in curve B in Fig. 5.

Turnover number of intestinal alkaline phosphatase. As shown by Fig. 4, the Lineweaver–Burk plots are non-linear at several pH values, especially when considered over a wide substrate range. Plots of $[S]/V_0$ against $[S]$, and of V_0 against $V_0/[S]$ (Hofstee, 1952), were also found to be non-linear, as expected. Hence it is difficult to obtain any reliable estimate of the variation with pH of $V_{max.}$, the maximum rate for infinite substrate concentration. However, from the plot of V_0 against pH (Fig. 1), a maximum observed velocity ($U_{max.}$) was obtained for each substrate concentration. Such values, of course, are obtained at different pH values according to the substrate concentration. A plot of $1/U_{max.}$ against $1/[S]$ is shown in Fig. 6. The plot is quite linear and gives a value for V_{max} of 250 μg. of P/5 min./0·1 ml. of enzyme. On this basis, the turnover number of purified calf intestinal phosphatase is about

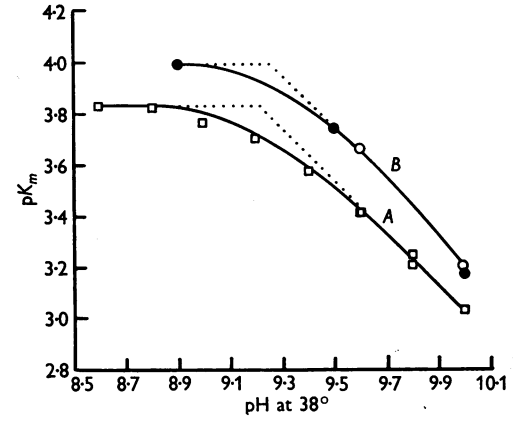

Fig. 5. Dixon plots (pK_m against pH) for hydrolysis of phenyl phosphate by alkaline phosphatases. Curve A is for intestinal phosphatase and curve B for milk phosphatase. Curve B includes results of Folley & Kay (1935) marked thus: ●.

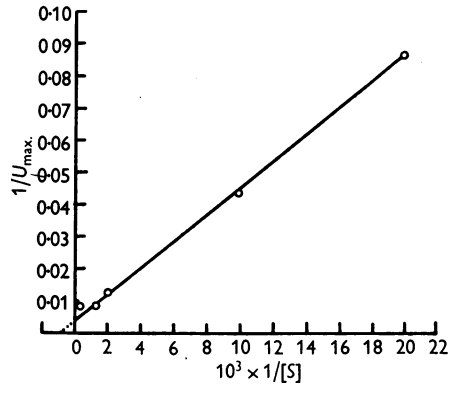

Fig. 6. Lineweaver–Burk plot (reciprocal of $U_{max.}$ against reciprocal of substrate concn.), where $U_{max.}$ is the maximum observed rate of hydrolysis of phenyl phosphate at each substrate concn. The values of $U_{max.}$ are for intestinal alkaline phosphatase as determined from Fig. 1.

394 000 moles of phenyl phosphate hydrolysed/min./100 000 g. of protein at 38°. Taking the molecular weight as 60 000 (Schramm & Armbruster, 1954), the turnover number is 236 000 moles/min./mole of enzyme at 38°.

Influence of metal salts on phosphatase activity. As shown previously (Morton, 1955*a*), purified intestinal phosphatase is activated by magnesium and manganese salts, but nevertheless shows considerable activity in the absence of such salts, even after extensive dialysis against water. Some aspects of metal activation were re-investigated with intestinal phosphatase which had been purified to stage 6 (Morton, 1954*b*, Table 4) and held in the dried state over $CaCl_2$ at room temp. for about 4 years. After dialysis of solutions of this preparation in Visking tubing against glass-distilled water, the behaviour in response to added $MgCl_2$ and $MnCl_2$ was generally similar to that already described (Morton, 1955*a*).

The enzyme was then treated with a metal-chelating agent in an attempt to remove metal firmly bound to the enzyme. About 10 mg. of the phosphatase was dissolved in 10 ml. of 0·1 M disodium ethylene diaminetetraacetate (EDTA), previously brought to pH 8·5 with M ethanolamine. The solution was dialysed in Visking tubing against 0·1 M-EDTA at pH 8·5, and subsequently against frequent changes of glass-distilled water for 6–16 hr. at 2–4°.

This treatment caused considerable loss of activity, which was partly restored by addition of $MgCl_2$ (5×10^{-3} M) and, to a lesser extent, by $MnCl_2$ (10^{-3} M). As shown in Table 1, the extent of activation by $MgCl_2$ depends on the concentration of substrate, the activation being relatively greater for low concentrations of phenyl phosphate. From Table 1 it is seen that the relationship between the reciprocal of V_0 and the reciprocal of the concentration of added metal salt ($[M]$) is grossly non-linear at both concentrations of substrate.

Fig. 7 shows that the optimum pH for hydrolysis of phenyl phosphate ($2{\cdot}5 \times 10^{-3}$ M) is more than one higher with $MgCl_2$ (5×10^{-3} M) than with $MnCl_2$ (10^{-3} M). There was no substantial difference in the pH optima obtained with high (10^{-2} M) and low (10^{-5} M) concentrations of $MgCl_2$, although the activity was much reduced at the lower concentration. Essentially the same opimum pH for hydrolysis of phenyl phosphate ($2{\cdot}5 \times 10^{-2}$ M) was obtained with large amounts of enzyme in the absence of added $MgCl_2$ as with about one-twentieth of the enzyme with $MgCl_2$ (10^{-2} M).

Preliminary observations with phosphatase from cow's milk

The activity–pH curve for hydrolysis of phenyl phosphate ($2{\cdot}5 \times 10^{-3}$ M) by the milk phosphatase showed an optimum pH of 10·05 (Morton, 1955*b*). The shape of the curve for V_0 against pH was generally similar to that obtained by Folley & Kay (1935), who obtained an optimum pH of 10·05 for

Table 1. *Influence of phenyl phosphate concentration on the activation of intestinal alkaline phosphatase by magnesium chloride*

The reaction mixture (5 ml.) contained veronal–ethanolamine buffer (0·05 M with respect to both components) at pH 9·6, $MgCl_2$ and phenyl phosphate at the concentrations indicated, and about 1·5 units of enzyme. The reaction was for 10 min. at 38°. Values of $MgCl_2$, $[M]$, are expressed as molar concentrations, and the initial rates of hydrolysis, V_0, as μg. of P liberated/10 min./0·2 ml. of enzyme solution. The results are given as reciprocals.

	$10^4/V_0$	
$1/[M]$	At $2{\cdot}5 \times 10^{-3}$ M phenyl phosphate	At $2{\cdot}5 \times 10^{-4}$ M phenyl phosphate
10^8	228	2500
10^7	193	1620
10^6	152	1600
10^5	135	1430
10^4	130	435
10^3	128	322
10^2	115	303

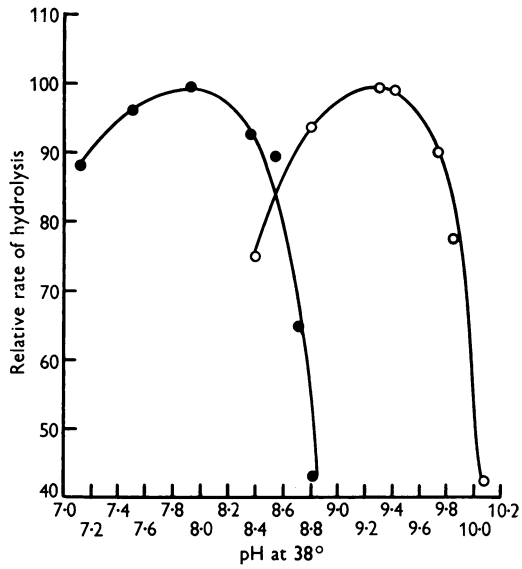

Fig. 7. Dependence of the optimum pH for hydrolysis of phenyl phosphate by intestinal alkaline phosphatase on the nature of the activating cation. The reaction mixture (2 ml.) contained phenyl phosphate ($2{\cdot}5 \times 10^{-4}$ M), veronal–ethanolamine buffer (0·05 M with respect to both components), and either $MgCl_2$ (0·005 M) with about 0·1 unit of enzyme (○), or $MnCl_2$ (0·001 M) with about 0·6 unit of enzyme (●). The reaction was for 10 min. at 38°. The results are expressed as relative rates of hydrolysis, the maximum value in each case being assigned a value of 100.

hydrolysis of phenyl phosphate ($4{\cdot}5 \times 10^{-3}$ M) by an extract of guinea-pig mammary gland. Moreover, as shown in Fig. 5 (curve *B*), the K_m values at pH 9·6 and 10·0 were also in close agreement with values obtained at or near the same pH by Folley & Kay (1935).

No further kinetic work was carried out with the milk phosphatase, but the observations suggest that the results of Folley & Kay (1935) may be tentatively accepted as applying to cow's milk phosphatase.

DISCUSSION

The marked dependence of the optimum pH for hydrolysis upon substrate concentration (Figs. 1, 2) appears to be characteristic of alkaline phosphatases from kidney (Asakawa, 1928; Jacobsen, 1932, 1933) and mammary gland (Folley & Kay, 1935) as well as from intestine. Moreover, with the intestinal enzyme this dependence is found with substrates other than phenyl phosphate, as is shown by the results of Ross, Ely & Archer (1951), which appeared sometime after the work described here had been completed (see Morton, 1952). Especially noteworthy is the broad pH optimum (extending towards pH 7) obtained with low concentrations of phenyl phosphate (Fig. 1). The optimum pH values obtained with phenyl phosphate are considerably higher than with β-glycerophosphate and most naturally occurring organic phosphates (Delory & King, 1943; Morton, 1955*b*). These observations and those of Ross *et al.* (1951) support Neumann's (1949) suggestion that alkaline phosphatases may be quite active at so-called 'physiological' pH values (assumed to be about pH 7). If intracellular pH values may be varied (as, for example, by endocrine control), this would offer an effective means for controlling alkaline phosphatase activity and possibly, therefore, the intracellular concentration of phosphate esters.

The optimum pH values shown in Fig. 1 are probably due to reversible effects and not due to irreversible enzyme denaturation, since the incubation period was short (5 min.) and since the phosphatase is protected against alkaline pH effects by its substrate (Folley & Kay, 1935; Morton, unpublished work). Other workers, such as Ross *et al.* (1951), have employed incubation periods of up to 1 hr. The values so obtained are frequently not good estimates of the true initial rates, and the pH optima are appreciably lower than the true values owing to irreversible enzyme denaturation, as has been demonstrated by Folley & Kay (1935).

Fig. 2 shows that with phenyl phosphate as substrate, the optimum pH increases by unity for unit decrease of p[*S*] over the range 4·6–3·3, whereas there is very little change over the p[*S*] range from 3 to 1·1. The discontinuity between the two parts of the curve occurs at a p[*S*] value of about 3, and the limiting optimum pH is approached at p[*S*] values of less than 3. A similar plot (not shown here) of the results of Ross *et al.* (1951) with crude rat intestinal alkaline phosphatase and with β-glycerophosphate as substrate shows a linear relation between optimum pH and p[*S*] over the range 4·9–1·3, with a change of about pH 0·5 for unit change of p[*S*]. It is possible that studies of the change of optimum pH with p[*S*] for several different substrates could provide additional information concerning the mechanism of action of alkaline phosphatase.

From Figs. 1 and 2, it is clear that the rate of hydrolysis of substrate will rapidly decline if carried out at a fixed pH value such as that which gives the maximal initial rate. Where complete hydrolysis of a substrate is desired therefore the reaction should be initiated at a pH value considerably below the optimum value for the initial substrate concentration. Not only will the optimum pH for substrate cleavage change during the course of hydrolysis, but inhibition due to liberated inorganic phosphate will also change. This inhibition varies with pH (Jacobsen, 1933). It is therefore apparent that time curves for hydrolysis of a substrate by alkaline phosphatase are quite complex and have limited value for kinetic purposes. This investigation has therefore been restricted to measurements of initial reaction rates.

Fig. 7 shows that the optimum pH for hydrolysis is dependent on the nature of the metal activator. Since the optimum pH obtained in the absence of added activator is substantially the same as with added magnesium chloride, it seems probable that the activity in the absence of activator is due to the retention by the enzyme of a small amount of magnesium, even after treatment with EDTA. This supports the view expressed previously (Morton, 1955*a*) that purified intestinal phosphatase binds magnesium so strongly that it is not removed by extensive dialysis against water.

Complex kinetic behaviour of alkaline phosphatases

In considering the interpretation of (*a*) change of optimum pH with substrate concentration and change of optimum substrate concentration with pH (Figs. 1, 2); (*b*) inhibition at high substrate concentrations (Fig. 3) (the inhibition changing with pH); and (*c*) metal activation, which varies with the concentration of the substrate, it is necessary to take account of the various equilibria likely to influence the rate of formation of a complex and its decomposition to products, according to the classical formulation

$$E + S \rightleftharpoons ES \rightarrow E + P, \qquad (1)$$

in which *E* represents enzyme, *S* substrate and *P* the products of the reaction.

It is assumed that phosphatase activity is

dependent on the presence of a metal (M), especially magnesium (see Morton, 1955*a* and Table 1). Thus in a reaction mixture containing E, S and M, and which yields P, the following equilibria must be considered:

$$E+M \rightleftharpoons EM, \quad (2)$$
$$EM+S \rightleftharpoons EMS, \quad (3)$$
$$M+S \rightleftharpoons MS, \quad (4)$$
$$E+MS \rightleftharpoons EMS, \quad (5)$$
$$E+S \rightleftharpoons ES, \quad (6)$$
$$ES+M \rightleftharpoons EMS, \quad (7)$$
$$EMS+S \rightleftharpoons EMS_2, \quad (8)$$
$$M+(OH)_2 \rightleftharpoons M(OH)_2, \quad (9)$$
$$EMS \rightarrow EM+P, \quad (10)$$
$$EMS+M \rightleftharpoons EM_2S. \quad (11)$$

EMS is considered to be the only complex which undergoes decomposition to form the products of the reaction (equation 8).

It is clear that equations 3, 5 and 7 represent competitive routes for formation of EMS. Under these conditions, plots of $1/V_0$ against $1/[S]$, and of $1/V_0$ against $1/[M]$ will both be non-linear (see Friedenwald & Maengwyn-Davies, 1954, p. 154). The non-linear plots obtained (Fig. 4 and Table 1) suggest that such competitive pathways are operative. Although this kinetic evidence supports the existence of a complex between metal and substrate (equation 4), this has not been directly demonstrated. However, it has been observed that a precipitate of magnesium hydroxide from magnesium chloride (10^{-2} M) appears at a higher pH value when phenyl phosphate ($2{\cdot}5 \times 10^{-3}$ M) is also present. Moreover, by measurement of paramagnetic-resonance absorption, Cohn (1954) has directly demonstrated complexes between phosphomonoesters (such as glucose 6-phosphate) and manganese.

Equation 9 shows that the concentration of hydroxyl ion will influence the concentration of effective activator, quite apart from the effect on the enzyme protein. If a complex is formed between S and M, then high substrate concentrations will, to some extent, counter the effect of high hydroxyl ion concentration. Thus equations 4 and 9 are competitive. At similar buffered substrate concentrations, the precipitate of manganese hydroxide appears at much lower pH values than does that of magnesium. This is in accordance with the known affinities of these two metals for hydroxyl ions (see Klotz, 1954). Hence the removal of activator as metal hydroxide (equation 9) will occur at lower pH values with manganese than with magnesium chloride. This would quite well account for the lowering of the pH optimum as well as for the considerably reduced activity when manganese is the activator as compared with magnesium salt (Fig. 7).

The curves shown in Fig. 3 reveal inhibition by excess of substrate. Haldane (1930) suggested that such inhibition may be due to the formation of an inactive complex of enzyme with two molecules of substrate (equation 8). For such a case, Haldane showed that

$$V_0 = \frac{k}{(1+K_1/[S]+[S]/K_2)},$$

where V_0 is the observed rate; $[S]$ is the substrate concentration; k is a constant; and K_1 and K_2 are the equilibrium constants for the reactions represented by equations 3 and 8 respectively. As discussed by Folley & Kay (1935), bell-shaped curves of the type shown in Fig. 3 are consistent with Haldane's formulation.

Although it is necessary to consider at least three routes to the formation of EMS (equations 3, 5 and 7), the same group of the enzyme is always involved in the binding of the substrate molecule in this active complex. It is reasonable to assume that a different group will be involved in the binding of a second substrate molecule to form the inactive complex EMS_2 (equation 8). Qualitatively it would be expected that, if the pK of the group involved in binding the first substrate molecule (in EMS) differs appropriately from the pK of the group binding the second molecule (in EMS_2), then there will be inhibition at high substrate concentrations, the extent of which will depend on pH. Moreover, the optimum pH will be expected to change with substrate concentration and the optimum substrate concentration with pH.

Friedenwald & Maengwyn-Davies (1954, p. 191) have shown that change of optimum pH with substrate concentration, together with change of optimum substrate concentration with pH, will be obtained if the substrate exists in two forms, one of which combines with the enzyme to form an active intermediate, the other of which acts as a non-competitive inhibitor. These workers derived an equation representing the relationship between V_0 and $V_{max.}$ under these conditions. They suggested that the results of Ross *et al.* (1951) for hydrolysis of β-glycerophosphate by rat intestinal phosphatase approximately fit such an equation, if the true substrate is S^- and the non-competitive inhibitor SH. The dissociation of SH must be substantially affected in the pH range under consideration. This is unlikely in the studies of Ross *et al.* (1951), which include the range pH 7·5–9·5, with β-glycerophosphate, pK 6·34 (Delory & King, 1943), as substrate; it is excluded in the present study over the pH range 8·25–10·0 with phenyl phosphate, pK 5·73 (Delory & King, 1943), as substrate.

Since with increasing concentrations of magnesium and manganese salts, the activity of purified phosphatase passes through a maximum, followed by a slight decline in activity (see Morton, 1955*a*,

Fig. 6), it is possible that more than one molecule of metal may be bound by the enzyme to form an inactive complex (EM_2S). This situation is represented by equation 11.

The results indicate that, over the range of substrate concentrations and pH conditions studied, all of the several equilibria represented by equations 2–11 may influence the rate of hydrolysis of phenyl phosphate by alkaline phosphatase. A general discussion of the kinetic situation where several equilibria are involved in enzymic catalysis has been given by Bull (1954), who has derived an equation expressing the rate of formation of the reaction products in terms of the concentrations of free enzyme and of the various enzyme complexes. Bull (1954) has applied this treatment to hydrolysis of urea by urease. The kinetic behaviour of alkaline phosphatase is rather similar to that of urease, as has been discussed elsewhere (Morton, 1952). However, whereas the optimum pH for alkaline phosphatase increases with increasing substrate concentration (Fig. 2), that for urease decreases (Van Slyke & Cullen, 1914; Van Slyke, 1942).

Interpretation of the Dixon plot for alkaline phosphatases

The -1 unit slope shown in the Dixon (1953) plot (Fig. 5, curve A), for pH values above 9·4, is found not only for intestinal and milk phosphatase with phenyl phosphate as substrate, but also for rat-kidney phosphatase with glycerophosphate as substrate (see Dixon, 1953). A similar plot (not shown here) of results of Roche & Sarles (1954) also gives approximately a -1 unit slope at pH values above 9 for dog intestinal, kidney and liver alkaline phosphatases with glycerophosphate as substrate. The results of Roche & Sarles (1954) for dog intestinal phosphatase also show a discontinuity at about pH 9·0, thus indicating that the discontinuity shown in Fig. 5 is probably independent of the nature of the substrate.

Interpretation of these results is dependent on the meaning of the experimentally determined K_m values. In view of the importance of the various equilibria previously discussed, it is clear that the K_m value is not identical with K_s, the dissociation constant of the enzyme–substrate complex. However, this need not necessarily invalidate the use of the Dixon plot (see Dixon, 1953). In view of the whole-number slopes (zero and -1) obtained (Fig. 5), it seems certain that the dissociation of a rate-determining enzyme complex is substantially affected in the pH range under consideration.

According to 'rule 9' of Dixon (1953), the -1 unit slope (Fig. 5, curve A) thus indicates that, at pH values above about 9·4, 'desubstration' of the rate-determining complex proceeds with gain of unit negative charge or with loss of unit positive charge, whereas there is no change of charge at pH values below 9·0. According to 'rule 10' of Dixon (1953), the discontinuity (Fig. 5, curve A) indicates the pK value of a group, either of the substrate or of the enzyme, which is involved in the reversible formation of the rate-determining complex. Since the pK value of the relevant group of phenyl phosphate is 5·73 (Delory & King, 1943), the discontinuity at about pH 9·2 must be the pK value either of an amino acid residue of the enzyme or of the hydroxide of the bound metal. Thus considering only amino acid residues of the enzyme, and neglecting the metal component and the ionized state of the substrate, the reversible formation of the active enzyme complex could be represented as involving either (a) a cationic or (b) an anionic group, as follows:

$$\left.\begin{array}{ll} (a) & E\mathrm{H}^+ + S \rightleftharpoons [E\mathrm{H}S]^+ \\ \text{or} \quad (b) & E\mathrm{H} + S \rightleftharpoons [E\mathrm{H}S] \end{array}\right\} \quad \text{below pH 9·0,}$$

and

$$\left.\begin{array}{ll} (a) & E + \mathrm{H}^+ + S \rightleftharpoons [E\mathrm{H}S]^+ \\ \text{or} \quad (b) & E^- + \mathrm{H}^+ + S \rightleftharpoons [E\mathrm{H}S] \end{array}\right\} \quad \text{above pH 9·4.}$$

The pK values of the ionizable groups of amino acids, when free and in some simple peptides, are fairly well established (see Cohn & Edsall, 1943, pp. 84 and 445). However, values listed by Steinhardt & Zaiser (1955) show that in proteins the pK value for any amino acid group may be shifted quite considerably, owing to interaction with neighbouring groups. Since either a cationic or an anionic group may be involved, as suggested by Dixon (1953), it is possible that the phenolic hydroxyl of tyrosine, the ϵ-amino of lysine, or the thiol group of cysteine may be the relevant group.

Since alkaline phosphatase is not inhibited by compounds which react more or less specifically with thiol groups (Barron & Singer, 1945; Roche, 1950), it is unlikely that the group is that of cysteine. The irreversible inhibition by keten (Gould, 1944) and the reversible inhibition by iodine in potassium iodide (Sizer, 1942; Morton, 1955*b*) indicate that the phenolic hydroxyl of a tyrosine residue is essential for enzymic activity, as first suggested by Sizer (1942). Hence the discontinuity at pH 9·2 probably represents the pK of a phenolic hydroxyl of a tyrosine residue, although the possibility that it is due to the ϵ-amino group of lysine (Morton, 1952) cannot be eliminated. Further identification of the relevant group could possibly be obtained by investigation of the apparent heat of ionization, since the values for the phenolic hydroxyl of tyrosine and for the ϵ-amino group of lysine are characteristically very different (Cohn & Edsall, 1943, p. 89; Alberty, 1953).

From Fig. 6 a K_m value of $9{\cdot}6 \times 10^{-4}$ M is obtained for calf intestinal phosphatase with phenyl phos-

phate. From a similar type of plot, Ross *et al.* (1951) obtained a value of 3×10^{-2}M for rat intestinal phosphatase with β-glycerophosphate. Figures obtained in this way could be most useful for expressing the affinity of alkaline phosphatase for different substrates. Since K_m values should always be expressed for a given pH, it is suggested that a figure obtained from a plot of $1/U_{max.}$ against $1/[S]$ (as in Fig. 3) should be termed the 'affinity index'. Such indices could be used to compare the affinities of alkaline phosphatase for different substrates. Indices obtained with the one substrate would be useful for comparing alkaline phosphatases of different origins.

Although the kinetic results for milk alkaline phosphatase are limited, the observations presented here and previously (Morton, 1955*a*), taken together with the classical studies of Folley & Kay (1935), suggest that the kinetic behaviour of milk phosphatase is not substantially different from that of intestinal phosphatase. Fig. 5, curve *B*, indicates a discontinuity in the Dixon plot at about pH 9·2. A similar interpretation to that already offered for intestinal phosphatase leads to the possibility that a tyrosine residue is concerned in the formation of a rate-limiting enzyme complex. The differences between intestinal and milk phosphatase, which have been outlined in a previous paper (Morton, 1955*a*), appear to reflect some difference in the enzyme–metal interaction.

SUMMARY

1. The hydrolysis of phenyl phosphate by purified alkaline phosphatase from calf intestinal mucosa has been investigated at substrate concentrations from $2{\cdot}5 \times 10^{-5}$M to $7{\cdot}5 \times 10^{-2}$M and at pH values from 8·25 to 10·1, the concentration of added magnesium chloride (enzyme activator) being maintained constant at 10^{-2}M.

2. There is marked inhibition by high concentrations of substrate, especially at lower pH values. The optimum pH for hydrolysis varies considerably with substrate concentration. The optimum substrate concentration also varies with pH. At a fixed substrate concentration, the optimum pH is influenced by the nature of the metal activator.

3. The existence of several equilibria involving enzyme, substrate and metal are postulated to explain these results. Evidence is presented which supports this hypothesis.

4. The Dixon (1953) plot of pK_m against pH reveals a discontinuity at about pH 9·2 (at 38°). This is interpreted as indicating that a phenolic hydroxyl group of a tyrosine residue of the enzyme is directly involved in the formation of a rate-limiting enzyme complex.

5. The turnover number of intestinal alkaline phosphatase is calculated to be about 394 000 moles of phenyl phosphate/min./100 000 g. of protein (236 000 moles/min./mole of enzyme) at 38°.

6. Results obtained with purified alkaline phosphatase from milk support earlier observations by Folley & Kay (1935) using alkaline phosphatase from mammary gland of the guinea pig. The behaviour of the milk (mammary-gland) phosphatase suggests that the several equilibria recognized for the intestinal phosphatase system are also important for the milk-phosphatase system.

I wish to thank Dr M. Dixon, F.R.S., for his valuable advice during the course of this work, which was mostly carried out during the tenure (1949–51) of a Travelling Scholarship awarded by the Gowrie Scholarship Trust of Australia.

REFERENCES

Alberty, R. A. (1953). In *The Proteins*, vol. 1, part A, p. 461. Ed. by Neurath, H. & Bailey, K. New York: Academic Press.

Asakawa, K. (1928). *J. Biochem., Tokyo*, **10**, 157.

Barron, E. S. G. & Singer, T. P. (1945). *J. biol. Chem.* **157**, 221.

Bull, H. B. (1954). In *The Mechanism of Enzyme Action*, p. 141. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Cohn, M. (1954). In *The Mechanism of Enzyme Action*, p. 246. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*. New York: Reinhold Publ. Corp.

Delory, G. E. & King, E. J. (1943). *Biochem. J.* **37**, 547.

Dixon, M. (1953). *Biochem. J.* **55**, 161.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Folley, S. J. & Kay, H. D. (1935). *Biochem. J.* **29**, 1837.

Friedenwald, J. S. & Maengwyn-Davies, G. D. (1954). In *The Mechanism of Enzyme Action*. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Gould, B. S. (1944). *J. biol. Chem.* **156**, 365.

Haldane, J. B. S. (1930). *Enzymes*. London: Longmans, Green and Co.

Hofstee, B. H. J. (1952). *Science*, **116**, 329.

Horwitt, B. N. (1952). *J. biol. Chem.* **199**, 537.

Jacobsen, E. (1932). *Biochem. Z.* **249**, 21.

Jacobsen, E. (1933). *Biochem. Z.* **267**, 89.

King, E. J. (1951). *Micro-Analysis in Medical Biochemistry*, 2nd ed. London: Churchill.

Klotz, I. M. (1954). In *The Mechanism of Enzyme Action*, p. 257. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Michaelis, L. (1931). *Biochem. Z.* **234**, 139.

Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.

Morton, R. K. (1950). *Nature, Lond.*, **166**, 1092.

Morton, R. K. (1952). Ph.D. Thesis: University of Cambridge.

Morton, R. K. (1953*a*). *Nature, Lond.*, **172**, 65.

Morton, R. K. (1953*b*). *Nature, Lond.*, **171**, 734.
Morton, R. K. (1953*c*). *Biochem. J.* **55**, 795.
Morton, R. K. (1953*d*). *Biochem. J.* **55**, 786.
Morton, R. K. (1954*a*). *Biochem. J.* **57**, 231.
Morton, R. K. (1954*b*). *Biochem. J.* **57**, 595.
Morton, R. K. (1955*a*). *Biochem. J.* **60**, 573.
Morton, R. K. (1955*b*). *Biochem. J.* **61**, 232.
Neumann, H. (1949). *Biochim. biophys. Acta*, **3**, 117.
Roche, J. (1950). In *The Enzymes*, vol. 1, part 1, p. 473. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press.
Roche, J. & Sarles, H. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 491.
Ross, M. H., Ely, J. O. & Archer, J. G. (1951). *J. biol. Chem.* **192**, 561.
Schramm, G. & Armbruster, D. (1954). *Z. Naturf.* **96**, 114.
Sizer, J. W. (1942). *J. biol. Chem.* **145**, 405.
Steinhardt, J. & Zaiser, E. M. (1955). *Advanc. Protein Chem.* **10**, 163.
Van Slyke, D. D. (1942). *Advanc. Enzymol.* **2**, 33.
Van Slyke, D. D. & Cullen, G. E. (1914). *J. biol. Chem.* **19**, 141.

Fungal Detoxication

2. THE METABOLISM OF SOME PHENOXY-*n*-ALKYLCARBOXYLIC ACIDS BY *ASPERGILLUS NIGER**

By R. J. W. Byrde and D. Woodcock
Long Ashton Research Station, Bristol University

(*Received* 2 *August* 1956)

The earlier paper in this series (Byrde, Harris & Woodcock, 1956) described the metabolism of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids by *Aspergillus niger* van Tiegh. By using a replacement culture technique it was found that 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids were hydroxylated in the 6-position; higher members of the homologous series underwent both nuclear hydroxylation and β-oxidation of the side chain.

Further evidence of β-oxidation by a microorganism has been presented by Webley, Duff & Farmer (1955), who studied the metabolism of ω-phenyl-substituted fatty acids by *Nocardia opaca* Waksman & Henrici. Microbiological nuclear hydroxylation of these acids was also reported by these workers, and of 4-chlorophenoxyacetic acid by Evans & Smith (1954).

The importance of β-oxidation in the plant-growth-regulating activity of the phenoxyalkylcarboxylic acids has been clearly demonstrated by Fawcett, Ingram & Wain (1954) and has led to the development of highly selective weed-killers (Wain, 1954). Though the side chains of these acids are β-oxidized by the flax plant (Fawcett *et al.* 1954), Fawcett, Spencer & Wain (1955) found no indication from fungitoxicity tests that this degradation could be effected by the fungi *Botrytis fabae* Sardiña and *Pythium ultimum* Trow.

In addition to their use as plant-growth regulators phenoxyalkylcarboxylic acids have been studied as possible systemic fungicides (Fawcett *et al.* 1955). The present paper describes the metabolism of four members of this series by *A. niger*.

* Part 1: Byrde, Harris & Woodcock (1956).

EXPERIMENTAL

Materials and reference compounds

Phenoxyacetic acid. This was prepared by the condensation of sodium phenoxide and ethyl bromoacetate. It crystallized from benzene–light petroleum (b.p. 60–80°) in prisms, m.p. 97–98°. Fritzsche (1879) found m.p. 96°.

β-Phenoxypropionic acid. β-Propiolactone (3·6 g.) was added dropwise with stirring during 5 min. to a boiling solution of phenol (4·7 g.) and NaOH (2 g.) in water (30 ml.). The solution was boiled for a further 20 min., cooled, acidified and extracted with ether. The acid crystallized from aqueous methanol in prisms, m.p. 94–95°. (Found: C, 64·9; H, 6·0. Calc. for $C_9H_{10}O_3$: C, 65·0; H, 6·0%.) Bischoff (1900) found m.p. 97·5–98°.

γ-Phenoxy-n-butyric acid. Crude γ-phenoxybutyronitrile (11·2 g.), obtained by refluxing a solution of γ-phenoxypropyl chloride (11·4 g.) (see below) and sodium cyanide (4·4 g.) in ethanol (70 ml.) overnight, was hydrolysed by refluxing for 18 hr. with a solution of KOH (5·4 g.) in water (5 ml.) and ethanol (70 ml.). The acid, crystallized from light petroleum (b.p. 40°), had m.p. 64–65°. (Found: C, 66·6; H, 6·7. Calc. for $C_{10}H_{12}O_3$: C, 66·7; H, 6·7%.) Lohmann (1891) found m.p. 60°.

γ-Phenoxypropylmalonic acid. A solution of sodium phenoxide, prepared from phenol (18·8 g.) and sodium (4·6 g.) in ethanol (50 ml.), was added dropwise over a period of 1 hr. to a boiling solution of 1-bromo-3-chloropropane (40 ml.) in ethanol (40 ml.). After heating under reflux for a further 4 hr., the mixture was cooled, diluted with water and extracted with ether. After removal of the solvent, distillation of the residue gave γ-phenoxypropyl chloride, b.p. 90–100°/0·5 mm. (30 g.). This was refluxed for 6 hr. with a solution of ethyl sodiomalonate, prepared from diethyl malonate (33·6 g.) and sodium (4·8 g.) in ethanol (120 ml.). After the addition of 40% NaOH (40 ml.) the mixture was heated for a further 30 min., cooled, and acidified with conc.