Studies on Alkaline Phosphatases

1. KINETICS OF PLASMA PHOSPHATASE OF NORMAL AND RACHITIC CHICKS

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In a previous paper some of the general characteristics of the plasma phosphatase of chicks have been described (Motzok & Wynne, 1950). It was established also that the phosphatase activities of plasma and of bone extracts increased markedly with decreasing dosages of vitamin D, but activities of extracts of liver, kidney and intestinal mucosa were not related to the vitamin D intake (Motzok, 1950). The increase in plasma phosphatase in rachitic conditions has been regarded as the result of leakage of the enzyme from the bones into the blood stream. It was of interest to determine whether or not vitamin D administered to the chick had an effect on the alkaline phosphatase of the plasma in a manner that would alter the affinity of the enzyme for a substrate when the activity was measured *in vitro*.

This paper is concerned with the applicability of the Michaelis & Menten (1913) theory of enzyme kinetics to the activities of the plasma phosphatases of normal and rachitic chicks and with the influence of the relationship between substrate concentration and the optimum pH for the enzyme activity on the K_m (Michaelis constant) of the enzyme–substrate complex. This study, using glycerol β-phosphate (β-glycerophosphate) as the substrate, was completed in 1944 and was the subject of a thesis (Motzok, 1945). Subsequently, the hydrolysis of phenyl phosphate by plasma phosphatase of mature fowls was investigated. Studies were made on the variation of optimum pH with substrate concentration, the influence of pH on K_m and the determination of K_m with the optimum pH for each concentration of substrate. Variations in pK_m with pH, when each value for K_m was determined at a constant pH, are also discussed.

METHODS AND MATERIALS

The rearing of normal and rachitic chicks and the methods for the preparation of plasma and the determination of phosphatase activity have been described previously (Motzok & Wynne, 1950). Plasma from mature fowls, fed on a commercial diet, was used in the studies with disodium phenyl phosphate [$Na_2(C_6H_5)PO_4$]. In the latter experiments the liberated phenol was determined as the measure of phosphatase activity by a modification (Kaplan & Narahara, 1953) of the Gomori method (1949).

EXPERIMENTAL AND RESULTS

Experiments with β-glycerophosphate

Influence of pH *and substrate concentration on phosphatase activity.* Data in Fig. 1 show that the initial concentration of substrate had a marked influence on the pH required for optimum activity of chick plasma phosphatase; the optimum shifted from pH 9·5 for mM-sodium β-glycerophosphate to pH 10·0 for 0·12 M-substrate in mixtures containing 5 mM-$MgCl_2$. The determination of pH optima with higher concentrations of this substrate is subject to an error caused by precipitation in the reaction mixtures at the high pH values, since these are close to the effective limits of the buffer system.

This change in optimum pH for alkaline phosphatases with change in initial concentration of substrate is in general agreement with the limited findings of other workers (see Motzok & Wynne, 1950), which have been confirmed for a wide range of substrate concentration by Ross, Ely & Archer (1951) and Morton (1957). A similar relationship

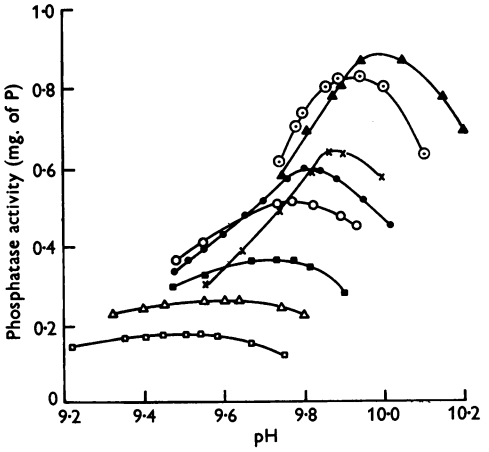

Fig. 1. Relationship between pH and the hydrolysis of different concentrations of sodium β-glycerophosphate (□, 1 mM; △, 2 mM; ■, 6 mM; ○, 0·01 M; ●, 0·02 M; ×, 0·03 M; ⊙, 0·08 M; ▲, 0·12 M) by chick plasma phosphatase in the presence of 5 mM-$MgCl_2$, during a reaction period of 10 min.

between optimum pH and substrate concentration was exhibited in the activity of the acid phosphatase of yeast (Dr A. M. Wynne, personal communication).

Fig. 1 also shows that with low concentrations of substrate maximum activity was obtained over a wider range of pH than with high concentrations of substrate. Moreover, when the activities were measured at a pH on the acid side of the optimum for a high concentration of substrate, the rate of hydrolysis was not as great as when the initial concentration was lower. This illustrates the phenomenon which many workers have attributed to 'inhibition' by excess of substrate and shows the effect of pH in the manifestation of this characteristic of phosphatases. For example, at pH 9·55 (the optimum pH for 2 mM-substrate), the rates of hydrolysis increased with increasing concentration of substrate up to 0·01 M. The rate with 0·02 M-substrate at pH 9·55 was slightly lower than that with 0·01 M; with 0·03 M-substrate the rate of hydrolysis at this pH was lower than the rate with 6 mM and considerably below that with 0·01 M-substrate. On the other hand, a comparison of activities at the optimum pH for each concentration of substrate shows that there was no diminution in rates of reaction with high concentrations of substrate up to 0·12 M.

The importance of the use of short periods of reaction in the determination of optimum pH has been emphasized (Motzok & Wynne, 1950), particularly for phosphatases, since variations in the initial concentration of substrate affects the optimum pH.

Influence of pH *on* K_m. Folley & Kay (1935) concluded from their studies that the relationship between the concentration of monophenyl phosphate and its hydrolysis by mammary-gland phosphatase conformed to the theory of Michaelis & Menten (1913). Similar observations have been made by Jacobsen (1933), Kutscher & Wörner (1936), Pfankuch (1936), Bailey & Webb (1944), Bodansky (1946) and Schønheyder (1951) in their investigations with other phosphatases.

Previous workers, in determining the K_m of phosphatases, have adjusted all reaction mixtures (containing increasing concentrations of substrate) to the same pH, namely, an 'optimum' pH value for an arbitrarily chosen concentration of substrate. Often a pH which is optimum for a relatively high concentration of substrate has been used. Fig. 1 shows that at a high pH the hydrolysis of the substrate in low concentration is inhibited by hydroxyl ions and the determination of K_m under these conditions would be influenced by this inhibition.

The nature of the inhibition, competitive or non-competitive, was studied by plotting data according to the method of Lineweaver & Burk (1934). Three concentrations of enzyme were used, namely, 0·375, 0·5 and 0·75 ml. of plasma/20 ml. of digest for each set of concentrations of substrate (1·2 mM to 0·08 M). The pH was maintained at 9·88 and the concentration of added Mg^{2+} ions was 5 mM in the digest; this amount of Mg^{2+} ions was found to be optimum for high concentrations as well as dilute solutions of substrate. Under these conditions the concentration of inhibitor (hydroxyl ions) remained constant, but the amount of enzyme, hence the inhibitor:enzyme ratio, was varied.

The line of best fit for each set of values in Fig. 2 was determined by the method of least squares (Treloar, 1939) and the values for K_m and $V_{max.}$ were derived from these calculations. These show that K_m values obtained from velocities at a constant pH, the optimum for a high concentration of substrate, decreased with increasing amounts of enzyme; with 0·375 and 0·75 ml. of plasma/20 ml. of digest the values for K_m were 37·9 and 19·5 mM respectively. Fig. 2 also shows that the inhibition by hydroxyl ions did not appear to be perfectly competitive in nature. $V_{max.}$ increased from 1·14 to 1·59 when the concentration of enzyme was increased twofold. There is no doubt that some of the discrepancies in the value for K_m for the phosphatase of the same source, which have been reported in the literature, are due in part to variations in the amount of enzyme employed and the inhibitory effect of hydroxyl ions when the hydrolysis of glycerophosphate, present in low concentration, was measured at a relatively high pH.

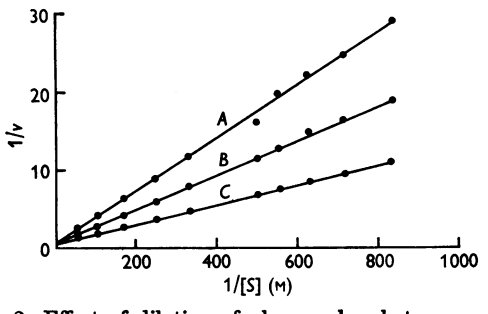

Fig. 2. Effect of dilution of plasma phosphatase on K_m when the velocities were measured at pH 9·88 for all concentrations of sodium glycerophosphate (mixture of α and β forms). Curves *A*, *B* and *C* were obtained with 0·375, 0·5 and 0·75 ml. of plasma/20 ml. of digest in the presence of 5 mM-$MgCl_2$, during a reaction period of 10 min. Data were plotted according to Lineweaver & Burk (1934).

Enzyme concentration	$V_{max.}$	K_m (M)
A	1·14	0·0379
B	1·24	0·0270
C	1·59	0·0195

The phosphatase activity of plasma of chicks fed on a vitamin D-free diet was found to be about twice that of plasma from chicks receiving 20 units of vitamin D/100 g. of diet. Fig. 3 shows that the slope of the $1/v:1/[S]$ line for the enzyme in plasma of chicks fed with 20 units of vitamin D/100 g. of ration is considerably greater and K_m smaller than those for the enzyme of plasma of chicks reared on the vitamin D-free diet. These relationships are analogous to the effect of dilution of the enzyme (Fig. 2).

The establishment of the relationship between optimum pH and substrate concentration for alkaline phosphatase, illustrated in the previous section, suggested the maintenance of optimum pH for each dilution of substrate in the determination of K_m of alkaline phosphatase of chick plasma.

Five preparations of plasma were used, one from 40 chicks fed on a vitamin D-free diet, three dilutions of plasma obtained from 120 chicks which received 10 units of vitamin D/100 g. of diet and one preparation of plasma from 40 chicks which received 20 units of vitamin D/100 g. of diet. The Mg^{2+} ion concentration was maintained at 5 mM in all reaction mixtures.

Plots of experimental values (Fig. 4) showed that with 1·2–4·0 mM-substrate the $1/v:1/[S]$ relationships were linear. The values for K_m and $V_{max.}$, designated as K_{m_1} and $V_{max.1}$, were derived from the calculations of the lines of best fit for this range of substrate concentration. With concentrations of substrate from 6 mM to 0·02 M, the $1/v:1/[S]$ relationships varied in a curvilinear manner. With substrate concentrations greater than 0·02 M the $1/v:1/[S]$ relationships were again linear, but the slopes of the lines of best fit were considerably greater than for those obtained with the low concentrations of substrate. These data were used to calculate the second set of values for K_m and $V_{max.}$, designated as K_{m_2} and $V_{max.2}$, for the hydrolysis of substrate present in high concentration (Fig. 5).

These data show that the inhibition by hydroxyl ions, causing variation in K_m with changes in enzyme concentration when all reactions were measured at a relatively high pH, e.g. pH 9·9, appeared to be eliminated when the optimum pH for each concentration of substrate was used.

At this stage of the investigation it was found that the glycerophosphate, which had been used as substrate, contained about 33 % of the α form and 67 % of the β form of the compound. A sample of pure β-glycerophosphate became available and experiments were conducted to confirm the findings

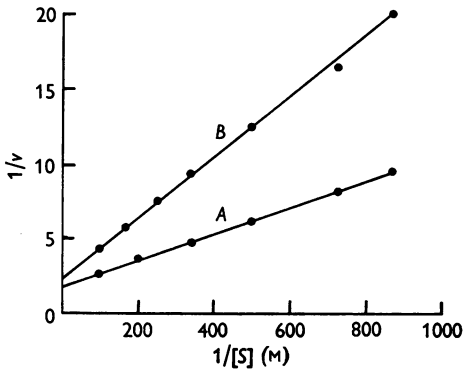

Fig. 3. K_m values for the hydrolysis of sodium glycerophosphate (mixture of α and β forms) by the phosphatase of plasma (0·5 ml./20 ml. of digest) obtained from chicks fed on a vitamin D-free diet (curve *A*) and from chicks fed with 20 units of vitamin D/100 g. of diet (curve *B*). Hydrolysis was performed at pH 9·9 for all concentrations of substrate in the presence of 5 mM-$MgCl_2$, during a reaction period of 10 min. Data were plotted as in Fig. 2.

Enzyme preparation	$V_{max.}$	K_m (mM)
A	0·72	6·2
B	0·42	9·6

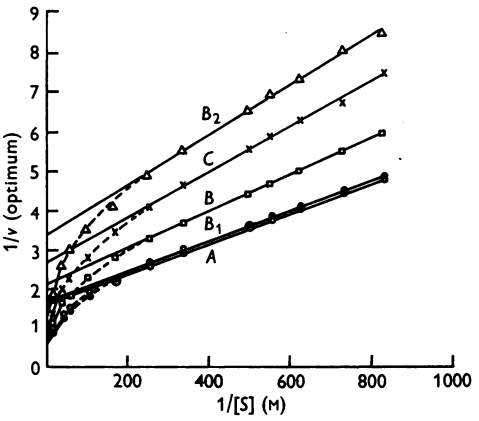

Fig. 4. K_m values (designated as K_{m_1}) for the hydrolysis of dilute solutions of sodium glycerophosphate (mixture of α and β forms) by chick plasma phosphatase at the optimum pH for each concentration of substrate in the presence of 5 mM-$MgCl_2$, during a reaction period of 10 min. Curves *A*, *B* and *C* were obtained with three samples of plasma (0·5 ml./20 ml. of digest) from three groups of chicks which had been fed with 0, 10 and 20 units of vitamin D/100 g. of diet respectively. Curves B_1 and B_2 were obtained with 0·75 and 0·375 ml. of plasma (used for curve *B*)/20 ml. of digest. Data were plotted as in Fig. 2.

Enzyme preparation	$V_{max.1}$	K_{m_1} (mM)
A	0·64	2·4
B	0·47	2·2
B_1	0·61	2·2
B_2	0·30	1·8
C	0·38	2·1

described above. The shift in optimum pH with changes in the initial concentration of pure β-glycerophosphate was the same as with the mixture of α and β salt (Motzok, 1945). When the optimum pH was employed for each concentration of substrate, the $1/v:1/[S]$ relationships were similar to those shown in Figs. 4 and 5. The calculated K_m values for two dilutions of the enzyme (0·375 and 0·5 ml. of plasma/20 ml. of digest) were 2·1 and 2·2 mM with the dilute solutions of pure β-glycerophosphate and 0·03 M with the high concentrations of this substrate.

Experiments with phenyl phosphate

Influence of pH *and substrate concentration on phosphatase activity.* The rate of hydrolysis of disodium phenyl phosphate by plasma phosphatase of fowls was determined in the presence of 5 mM-$MgCl_2$ at sufficient values of pH to establish the optimum pH for a wide range of substrate concentration. The data in Fig. 6 (*A*) show that the optimum shifted from pH 9·4 for the hydrolysis of mM-substrate to pH 10·36 for 0·03 M-substrate.

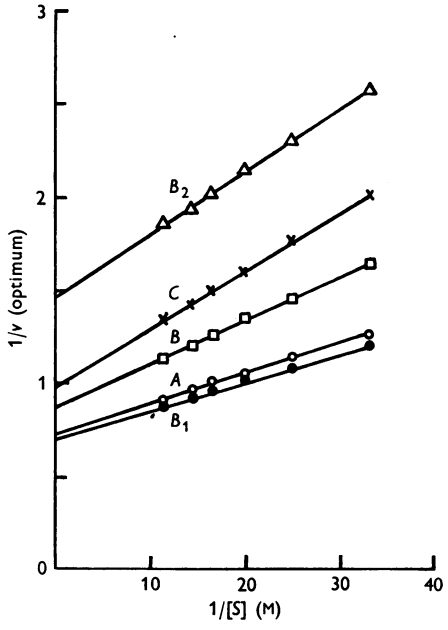

Fig. 5. Part of the data from Fig. 4 expanded to show clearly the $1/v:1/[S]$ relationship and K_m values, designated as K_{m_2}, for the hydrolysis of substrate present in high concentration.

Enzyme preparation	$V_{max.2}$	K_{m_2} (M)
A	1·38	0·021
B	1·15	0·026
B_1	1·45	0·023
B_2	0·69	0·023
C	1·01	0·030

The pH–activity curves shown in Fig. 6 (*B*) were derived from the data presented in Fig. 7. This selection of the data was made to show clearly the rates of reactions with concentrations of substrate from 2 mM to 0·03 M on the acid side of the pH optima and to illustrate the phenomenon described by other workers as inhibition by excess of substrate. At pH 8·9 the highest rate was obtained with 2 mM-substrate and the lowest rate with 0·03 M-substrate. With changes in pH toward more alkaline conditions the differences in the rates diminished. At pH 9·7 the rates were approximately equal with 0·01, 0·02 and 0·03 M-substrate and appreciably higher than the rates with 2 and 5 mM-substrate. At still higher pH values the greatest velocity was obtained with the highest concentration of substrate.

These data also show that with fowl plasma phosphatase the pH optima for the various concentrations of phenyl phosphate were appreciably

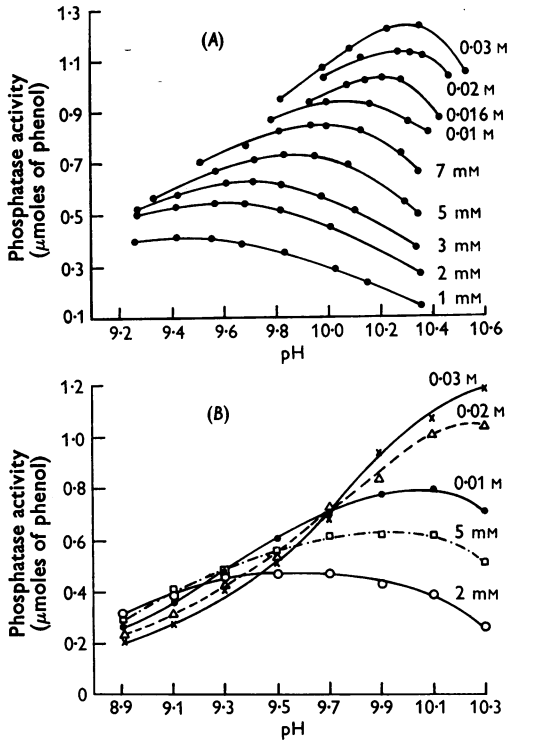

Fig. 6. (*A*) Relationship between pH and the hydrolysis of different concentrations of disodium phenyl phosphate by fowl plasma phosphatase. (*B*) Data derived from Fig. 7, showing the relationship between rates of hydrolysis and initial concentrations of substrate on the acid side of pH optima. Activities are expressed as phenol (μmoles) liberated by the enzyme in 1 ml. of plasma in the presence of 5 mM-$MgCl_2$, during a reaction period of 15 min.

higher than the pH optima for the corresponding concentrations of glycerophosphate (Fig. 1).

Influence of pH *on* K_m. The effect of pH on K_m of fowl plasma phosphatase was determined with disodium phenyl phosphate, 16 dilutions of the substrate being used, from mM to 0·03 M, at eight pH values from 8·9 to 10·3, and plotting the data (Fig. 7) according to the method of Lineweaver & Burk (1934). At pH 8·9 and 9·1 the reciprocals of velocities with high concentrations of substrate deviated markedly from linearity and only the data with 1–3 mM-substrate were used to calculate the lines of best fit; at other pH values the data for 1–4 mM-substrate were employed. The values for K_m (Fig. 7) increased with increasing alkalinity from 1·5 mM at pH 8·9 to 8·3 mM at pH 10·3.

The rates of hydrolysis of phenyl phosphate were also determined at the optimum pH for each concentration of substrate. Fig. 8 shows that with 1–3 mM-substrate the $1/v:1/[S]$ relationship was linear and these values were used in the calculation of the line of best fit and K_{m_1} for dilute substrate. The slope of the line (*A*) was 0·00125 and K_{m_1} was mM. The transition point in the $1/v:1/[S]$ relationship from the low to the high concentrations of substrate was at approximately 4 mM-substrate. With concentrations of substrate greater than 4 mM the $1/v:1/[S]$ relationship was also linear but the slope of the line (*B*) was 0·0033 and K_{m_2} derived from these data was 4·5 mM.

Influence of pH *on* pK_m. When the values for K_m for phenyl phosphate, determined previously (Fig. 7), were plotted as pK_m versus pH, they

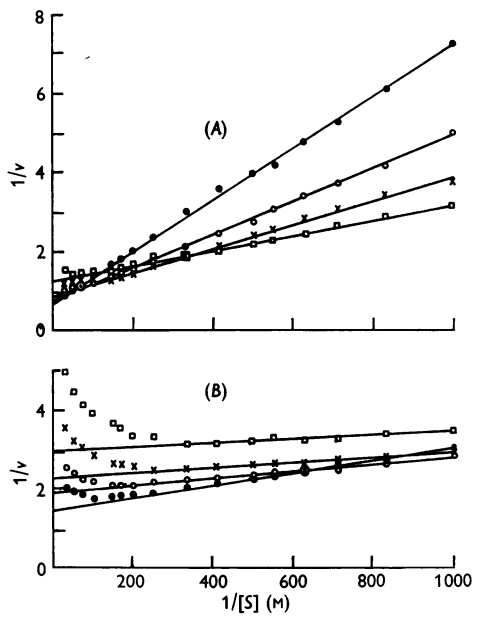

Fig. 7. The influence of pH on K_m values for the hydrolysis of disodium phenyl phosphate by fowl plasma phosphatase. Activities were expressed as in Fig. 6 and plotted as in Fig. 2. K_m values (mM): (*A*) ●, 8·3 (pH 10·3); ○, 5·8 (pH 10·1); ×, 3·0 (pH 9·9); □, 1·6 (pH 9·7). (*B*) ●, 0·95 (pH 9·5); ○, 0·50 (pH 9·3); ×, 0·27 (pH 9·1); □, 0·15 (pH 8·9).

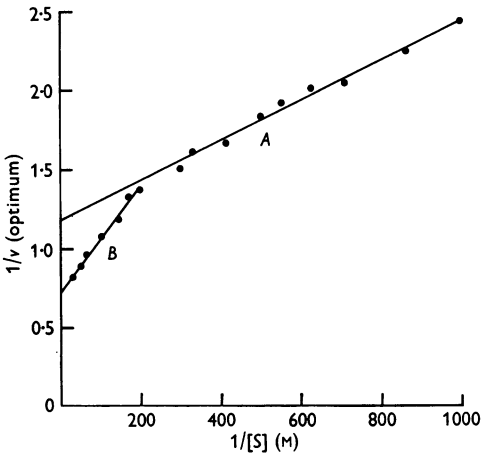

Fig. 8. K_m values (designated as K_{m_1} for dilute substrate and K_{m_2} for substrate present in high concentration) for the hydrolysis of disodium phenyl phosphate by fowl plasma phosphatase when the activities were determined at the optimum pH for each concentration of substrate. Activities were expressed as in Fig. 6 and plotted as in Fig. 2. Curve *A*, K_{m_1} 1·1 mM; curve *B*, K_{m_2} 4·5 mM.

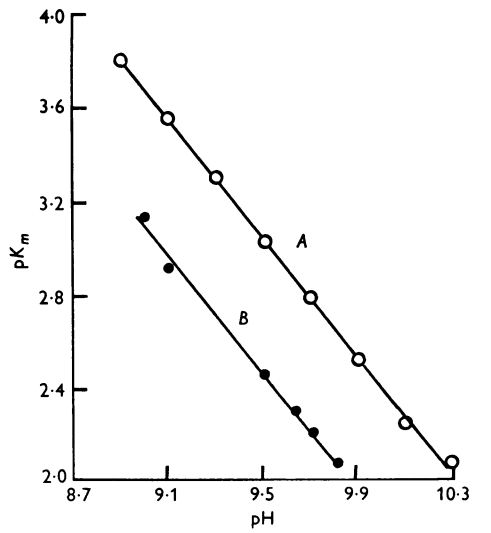

Fig. 9. Effect of pH on pK_m of alkaline phosphatases. Curve *A* is for fowl plasma phosphatase with phenyl phosphate (data from Fig. 7). Curve *B* is for bone phosphatase of rachitic chicks with β-glycerophosphate.

appeared to fall along a straight line with a slope of −1·3 (Fig. 9, curve *A*). A similar relationship between pK_m and pH (Fig. 9, curve *B*) was obtained with data (unpublished) from an earlier study on bone phosphatase of rachitic chicks with β-glycerophosphate as the substrate. In both cases the velocities were measured in the presence of the optimum concentration of Mg^{2+} ions (5 mM) in the reaction mixtures.

DISCUSSION

In the determination of rates of reaction for the calculation of K_m, the general practice has been to maintain a constant pH for all concentrations of substrate. This procedure is applicable to enzyme systems which exhibit pH–activity curves of similar shape, and have optimum activity at the same pH for all concentrations of substrate. Any effect of pH can be avoided by the simple expedient of measuring velocities at the optimum pH.

With phosphatases, however, the data in Figs. 1 and 8 and the findings of Motzok & Wynne (1950), Ross *et al.* (1951) and Morton (1957) show that the pH–activity curves are not similar in shape for all concentrations of substrate and that the optimum pH varies with changes in the initial concentration of substrate. Indeed, there appears to be no single value of pH at which hydrogen or hydroxyl ions do not have some inhibitory effect on phosphatase activity when the range of concentration of substrate is large enough for an accurate estimation of K_m.

Many workers (see Laidler, 1955) have considered the influence of pH on the rate of enzyme reactions and various types of equations have been devised for the analysis of specific conditions. With phosphatases, however, the influence of pH is quite complex and no one has proposed a solution of the problem.

With enzyme systems in which the optimum pH does not vary with changes in the initial concentration of substrate, the observed velocities are in fact 'optimum' velocities for each concentration of substrate when the activities are measured at the optimum pH. The only way in which comparable 'optimum' velocities can be obtained for phosphatases is by measuring activities at the optimum pH for each concentration of substrate. It is recognized that in the double-reciprocal plot of the data, shown in Figs. 4 and 8, pH is a variable factor. Nevertheless, the velocities were optimum for each concentration of substrate and data derived in this manner gave reasonably constant values for K_m regardless of the concentration of enzyme in the digestion mixtures.

No doubt some of the discrepancies in the value for K_m for the phosphatase of the same source, reported in the literature, are due in part to variations in the amount of enzyme employed and the inhibiting effect of hydroxyl ions when the hydrolysis of dilute substrate was measured at a relatively high pH. If the inhibition by hydroxyl ions had not been taken into consideration in these studies, the apparently erroneous conclusion might have been drawn that vitamin D, administered to chicks in the basal diet, affected the nature and properties of plasma phosphatase in a manner which altered the K_m of the enzyme–substrate complex (Fig. 3).

The double-reciprocal plot of velocities at the optimum pH for each substrate concentration resolved the data into two parts from which two values for K_m and $V_{max.}$ could be calculated, one set of values from data with dilute substrate and the second from velocities when the substrate was present in high concentrations. The change in the slope of the $1/v : 1/[S]$ lines from the low to the high concentrations of glycerophosphate occurred in a curvilinear manner over a range of 6 mM- to 0·02 M-substrate. With phenyl phosphate the change in the slope of the lines took place over a very small range of concentration of substrate, giving no marked curvilinear transition such as the one observed with glycerophosphate.

Neilands & Stumpf (1955) pointed out that, instead of straight lines in the double-reciprocal plot, deviations may be observed with high concentrations of substrate. They suggested that when the substrate concentration is high an enzyme may require two molecules of substrate, and the attachment of one facilitates the rate of reaction with the second molecule, resulting in abnormally high velocities. With phosphatases 'abnormally' high velocities with high concentrations of substrate were evident only when the velocities were measured at the optimum pH for each concentration of substrate (Figs. 4 and 8). If phosphatase has two sites for the substrate, one might expect that $V_{max.}$ calculated from the $1/v : 1/[S]$ plot of velocities with high concentrations of substrate (designated here as $V_{max._2}$) would be twice as large as $V_{max.}$ calculated from velocities with dilute substrate (designated as $V_{max._1}$). Values for $V_{max._1}$ and $V_{max._2}$ were calculated (unpublished data) in all cases when K_m was determined, with the optimum pH for each concentration of substrate, and the average of $V_{max._2}/V_{max._1}$ was found to be 1·84. Although this average value approximates the theoretical ratio of 2, there was considerable variation among these ratios, the range being 1·2–2·9. Unless there is an explanation for the wide variation in the degree to which one molecule aids in the rate of reaction with the second molecule, the present data do not support the suggestion that phosphatases can utilize two molecules of substrate at one time when

the substrate concentrations are high and the velocities are measured at the respective optimum pH.

Neilands & Stumpf (1955) also suggested that abnormally slow velocities (similar to those shown in Fig. 7*B*) may cause choking of the active site by two molecules of substrate, a phenomenon described by other workers as inhibition by excess of substrate. Figs. 1 and 6 (*B*) show that the inhibition by 'excess' of substrate occurred at pH values on the acid side of pH optima required for high concentrations of substrate. These data are similar to those of Morton (1957), who used phenyl phosphate with purified calf intestinal phosphatase, and those of Ross *et al.* (1951), who used β-glycerophosphate with a partially purified enzyme of intestinal mucosa of rats.

There have been several reports on the possible occurrence of two or more phosphatases in an organ or tissue. For example, Tsuboi, Wiener & Hudson (1957) isolated a highly purified acid phosphatase of yeast, which appeared to be homogeneous by ultracentrifuging but contained at least four catalytically active components distinguishable by electrophoretic analysis. Although these active components may have existed as such in the yeast, it is possible that they were derived as a consequence of the purification procedure. Gryder, Friendenwald & Carlson (1955) reported on the presence of two alkaline phosphatases in rat kidney based on inhibition by iodoacetate and activation by glycine and zinc, and Trubowitz, Feldman, Benante & Kirman (1957) suggested the occurrence of two alkaline phosphatases in human erythrocytes on the basis of inhibition by ethylenediamine and activation by zinc and magnesium. Both of the studies, however, did not exclude the possibility that treatment with iodoacetate or ethylenediamine altered the original properties of the enzyme with respect to activation by an amino acid or various metals.

The phenomenon, illustrated in Figs. 4 and 8, has been exhibited by the alkaline phosphatases of various tissues (bone, kidney, liver and intestinal mucosa) of several species of mammals and birds (Motzok & Branion, 1959). If the resolution of data into two parts by the double-reciprocal plot of 'optimum' velocities signifies the presence of two enzymes, it would have to be assumed that two phosphatases exist in a variety of tissues and sources, one being able to act with dilute substrate at low pH values extending to the physiological range (Ross *et al.* 1951), and the second characterized only by its requirements *in vitro* of high concentrations of substrate and high pH. The postulation of the second phosphatase cannot be based on an assumption that its properties *in vitro* are functional under physiological conditions. It would seem unlikely that there exists in various tissues an auxiliary enzyme system which would operate when the substrate was present in such high concentrations at an alkaline pH far removed from the physiological range. It is possible, however, that the natural substrate of the second enzyme and the conditions for normal activity may be quite different from those used in these studies.

Although the present data do not explain the 'abnormally' high velocities with high concentrations of substrate measured at each optimum pH, the relative uniformity of the values for K_{m_1} and K_{m_2} for the phosphatase of any one source suggests that constants derived in this manner may have some significance in the kinetics of these enzymes. Similar studies with other enzymes which exhibit relationships between substrate concentration and optimum pH, e.g. urease (Van Slyke, 1942) and β-glucosidase (Hofstee, 1955), may aid in solving the questions raised in the present work on phosphatases.

Van Slyke (1942) has discussed the characteristics of the two-phase reaction of hydrolytic enzymes and has suggested that, when the substrate is present in low concentration, the rate of enzyme action is determined by the velocity of combination of substrate with the enzyme. When the concentration of substrate is large, the rate of combination of substrate with the enzyme becomes negligible compared with that required for the decomposition of the substrate.

Motzok (1945) found that when the initial concentration of substrate was high the rate of reaction was of zero order during the reaction periods used. When the initial concentrations of substrate were low the rate of reaction was found to be unimolecular. Consequently, when the 'optimum' velocities were employed in the determination of K_m, the values for K_{m_1} were derived from velocities which appeared to conform to the unimolecular rate of reaction and the values for K_{m_2} were obtained with reaction rates which were of zero order or true initial velocities.

It may be that, when the velocities are measured at the optimum pH for each concentration of substrate, the value for K_{m_1}, obtained with dilute substrate, gives an estimate which approaches the true value of the dissociation constant of the enzyme–substrate compound (ES), i.e. k_2/k_1, where

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$$

according to Michaelis & Menten (1913).

On the other hand, K_{m_2}, obtained with high concentrations of substrate, may be governed largely by the velocity of decomposition of the enzyme–substrate complex into products of hydrolysis (P) and free enzyme, i.e. K_{m_2} equals $(k_2 + k_3)/k_1$, where

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_3}{\rightarrow} P + E$$

according to Briggs & Haldane (1925).

Variation of pK_m *with* pH. Dixon (1953) plotted K_m values, as pK_m versus pH, from the data of Jacobsen (1933) on kidney phosphatase with glycerophosphate and those of Morton (1952) on purified intestinal phosphatase with phenyl phosphate, and obtained straight lines with a slope of $-1{\cdot}0$. He suggested that the discontinuity in the slope of the line for Morton's data, as well as for the data of Folley & Kay (1935) on milk phosphatase with phenyl phosphate, may be due to an ionizing group in the enzyme concerned with the combination of phenyl phosphate but not with glycerophosphate.

In the present study there was no evidence of discontinuity of the slope of the line of pK_m–pH plots of data with either glycerophosphate or phenyl phosphate. The data in Fig. 9 also did not appear to fit precisely Dixon's theory of a -1 unit relationship between pK_m and pH, the slope of the lines being $-1{\cdot}3$. These K_m values were derived from velocities measured in the presence of the optimum concentration of magnesium chloride (5 mM). Preliminary studies of the role of Mg^{2+} ions on the reaction constant have shown that, when no magnesium chloride was added to the plasma phosphatase–phenyl phosphate system, the slope of the line of the pK_m–pH plot was only about $-0{\cdot}8$ (unpublished data). Indeed, Folley & Kay (1935) used mM-magnesium chloride in the reaction mixtures and Jacobsen (1933) used 2·4 mM-magnesium chloride. Although, Morton (1957) employed a concentration of 0·01 M-magnesium chloride for intestinal phosphatase, the enzyme was highly purified. Furthermore, Morton's data produced a non-linear relationship when plotted as $1/v:1/[S]$ and he estimated K_m values from Michaelis–Menten plots as the substrate concentration for one-half of maximum velocity (1957). Determination of K_m by the latter procedure may be subject to an appreciable error, particularly when the data do not conform to the Lineweaver & Burk derivation of the Michaelis–Menten equation (1913). Morton (1957) observed no substantial change in optimum pH with and without added magnesium chloride and he suggested that the deviation from linearity in the $1/v:1/[S]$ plots was due to competitive pathways in the formation of the enzyme–metal–substrate complex as postulated by Friedenwald & Maengwyn-Davies (1954).

The influence of Mg^{2+} ions on the pK_m–pH relationship for plasma phosphatase may be due at least in part to its effect on the pH–activity characteristics of this enzyme with different concentrations of substrate. It was found that, when no magnesium chloride was added, the pH optima shifted to lower values with β-glycerophosphate (Motzok, 1945) and phenyl phosphate (Motzok & Branion, 1959) from the pH optima in the presence of added magnesium chloride. In addition, without added magnesium chloride, optimum activity was observed over a wider range of pH, particularly with higher concentrations of substrate than with added magnesium chloride. K_m values, determined at constant pH, would be affected by variation in the inhibition by hydroxyl ions with different concentrations of substrate when the amount of added magnesium chloride was varied.

SUMMARY

1. The optimum pH for the hydrolysis of β-glycerophosphate and phenyl phosphate by fowl plasma phosphatase was found to change to higher values with increases in the initial concentration of substrate. The pH optima for various concentrations of phenyl phosphate were higher than the optima for the corresponding concentrations of β-glycerophosphate.

2. On the acid side of pH optima the rate of hydrolysis with dilute substrate was greater than with substrate present in high concentrations.

3. At relatively high pH values, hydroxyl ions inhibited the hydrolysis of dilute glycerophosphate in a manner which suggested competitive inhibition. When velocities were measured at pH 9·88 for all dilutions of glycerophosphate, K_m decreased with increases in the amount of enzyme in the digest, i.e. K_m varied with variations in the enzyme:inhibitor ratio. Under similar conditions of pH, K_m for plasma of rachitic chicks, having a high phosphatase activity, was lower than K_m for the plasma of chicks fed with vitamin D, possessing low phosphatase activity.

4. When velocities were measured at the optimum pH for each concentration of substrate, variations in K_m due to differences in enzyme concentration were greatly reduced. The $1/v:1/[S]$ plots of velocities obtained in this manner also resolved the data into two parts from which two values for K_m were calculated for each preparation of enzyme, namely K_{m_1} for data with dilute substrate and K_{m_2} for data with high concentrations of substrate.

5. The presence or absence of vitamin D in the diet of chicks had no effect on K_{m_1} and K_{m_2} for plasma phosphatase when the velocities were measured at the optimum pH for each concentration of substrate.

6. When velocities were measured at the same pH for all concentrations of phenyl phosphate, K_m values for fowl plasma phosphatase increased with increasing alkalinity from 0·15 mM at pH 8·9 to 8·3 mM at pH 10·3.

7. K_m values, plotted as pK_m versus pH, fell along a straight line with a slope of $-1{\cdot}3$ when the velocities were measured in the presence of opti-

mum amounts of magnesium chloride in the digest. The effect of activators on the relationship between pH and K_m is discussed.

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Studies on Alkaline Phosphatases

2. FACTORS INFLUENCING pH OPTIMA AND MICHAELIS CONSTANT

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It has become quite evident that the pH required for optimum activity of alkaline phosphatases, as well as for some other enzymes, is not a constant entity. For example, the optimum pH for phosphatases from various sources has been shown to vary with the initial concentration of substrate (Motzok & Wynne, 1950; Ross, Ely & Archer, 1951; Morton, 1957). This phenomenon has been observed with other enzymes, namely urease (Van Slyke & Cullen, 1914) and β-glucosidase (Hofstee, 1955). Other factors, such as buffers (Aebi & Abelin, 1948; Zittle & Della Monica, 1950), type of substrate (Delory & King, 1943; Walker & King, 1950), source of enzyme (Zittle & Della Monica, 1950; Motzok, 1950; Morton, 1955) and activators (Motzok, 1945; Aebi & Abelin, 1948; Sadasivan, 1952; Morton, 1957) also have been found to exert an influence on the optimum pH for phosphatase activity. In our studies on the kinetics of alkaline phosphatases of tissues and plasmas of different species of birds and mammals, the pH optima were determined for a wide range of concentration of substrate with each enzyme preparation. Several factors were found to influence the optimum pH and some of the data are recorded in this paper.

It was demonstrated (Motzok, 1959) that K_m values (Michaelis constant) for chick plasma phosphatase varied with the amount of enzyme in the reaction mixture when the activities were measured at a constant high pH for all dilutions of substrate and enzyme. However, when the activities were measured at the optimum pH for each concentration of substrate, the variation in K_m values due to variation in the concentration of enzyme was largely eliminated. In addition, the plotting of reciprocals of velocities and substrate concentrations, according to Lineweaver & Burk (1934), resolved the data into two parts from which two values for K_m were calculated, K_{m_1} from the data with low concentration of substrate and K_{m_2} from