The Rate of Enzymic Hydrolysis of Phosphoric Esters

2. RELATION OF STRUCTURE TO DISSOCIATION CONSTANT, MICHAELIS CONSTANT, AND RATE OF HYDROLYSIS

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Since Fischer [1898] suggested the possibility that a chemical combination took place between an enzyme and its substrate, the kinetics of many enzyme reactions have been explained by postulating such a combination, and applying the mass action laws. The classical example of this form of treatment is the equation of Michaelis & Menten [1913]. In their study of mammary gland phosphatase, Folley & Kay [1935] obtained results which were in good agreement with the requirements of this equation, when allowance was made by the method of Haldane [1930] for inhibition with high substrate concentrations. Martland & Robison [1927] found no variation of activity of phosphatase with concentration, but our results suggest that these workers were using concentrations of substrate too high to show the Michaelis-Menten effect.

Several workers have noted the different rates at which phosphoric esters are hydrolyzed by phosphatase, e.g. Martland, Hansman & Robison [1924], Kay [1926; 1932] and Asakawa [1928]. King & Armstrong [1934] showed that hydrolysis of phenyl phosphate by bone phosphatase is more than twice as fast as that of glycerophosphate and many times faster than that of ethyl phosphate. King & Delory [1939] studied other phosphoric esters and suggested that the hydrolysis rates might be related to the Michaelis-Menten constants. In the present paper we have made a study of the rates of hydrolysis, optimum pH, and Michaelis constants, together with determination of the degree of dissociation of the phosphoric esters employed. The following esters were used: α-glycero-, β-glycero-, ethyl-, phenyl-, *o*-bromophenyl-, *m*-bromophenyl-, *p*-bromophenyl-, 2:4-dibromophenyl-, 2:4:6-tribromophenyl-, *p*-nitrophenyl-, and *o*-cresyl phosphates.

METHODS

Preparation of a potent phosphatase. A potent preparation was desirable in order to minimize the effect of contaminants and to permit short (5 min.) periods of hydrolysis, during which the rates of enzymic hydrolysis were not very different from initial velocities. Dialyzed and precipitated kidney and intestinal preparations were not effective. The potent phosphatase derived from dog faeces by the method of Armstrong [1935] was used. The brown powder obtained by procedures 'A' and 'B' of this author was dried *in vacuo*. In the presence of an optimal amount of Mg it showed an activity of 10,000 King-Armstrong phosphatase units/g., and still retained its activity after 5 years in the refrigerator.

A solution of 1 unit/4 ml. was prepared as follows: 1 mg. of the preparation was dissolved in 30 ml. 0·9% NaCl containing enough $MgSO_4$ to make the final $MgSO_4$ concentration $M/100$. The phosphatase activity (about 30 units/100 ml.) was measured accurately by the method of King, Haslewood & Delory [1937]. The solution was then diluted so as to contain exactly 25 units/100 ml.

Preparation of phosphoric esters, see King & Nicholson [1939].

Determination of degree of dissociation of phosphoric esters. The apparatus used for the pH estimations was a hydrogen electrode with saturated calomel electrode and the Moloney [1921] platinum electrode. The latter was designed to hold a film of the solution, whose pH is to be determined, across the platinum electrode. Since it was only necessary for a small volume of solution to become saturated with H_2, ionic equilibrium was reached immediately. The potentiometer available was accurate to the nearest 0·01 pH. The apparatus was checked with a standard acetic acid-sodium acetate buffer of pH 4·63. The following procedure was adopted: 20 ml. of a $N/10$ solution of the salt of the phosphoric ester were pipetted into the reaction vessel. $N/10$ HCl was added in 0·2 ml. quantities, the pH being determined after each addition, and the pK determined graphically [cf. Pirie & Pinhey, 1929].

The graphs for *p*-bromophenyl-, *o*-bromophenyl-, phenyl- and α-glycero-phosphate are given as examples in Fig. 1. The pK's are included in Table 1.

Choice of a suitable buffer system. A buffer system covering the range of pH 7–11, and having the minimum effect on the rate of hydrolysis was obtained by combining the Michaelis [1930] veronal—HCl buffer and the Kolthoff [1925] Na_2CO_3-HCl buffer [King & Delory, 1940].

Determination of the optimum pH *for hydrolysis and rate of enzyme activity.* 4 ml. phosphatase solution (1 unit), 4 ml. buffer and 2 ml. $M/100$ substrate were incubated at 37° for exactly 5 min. The enzyme action was stopped by adding 2 ml. acid molybdate [King & Delory, 1939].

Determination of Michaelis constant:

$$\frac{v}{V}=\frac{x}{K_m+x}, \quad \text{or} \quad \log\frac{1}{x}=\log\frac{1}{K_m}+\log\left(\frac{V}{v}-1\right),$$

where x is the molar concentration of substrate, v is the velocity of hydrolysis, K_m is the dissociation constant of the enzyme-substrate compound (Michaelis constant) and V is the velocity when x is large compared with K_m. A curve of this equation will clearly be of the form of the dissociation of a weak acid and at the point at which $v/V=\frac{1}{2}$, i.e. where the velocity of hydrolysis is half the limiting velocity, K_m will be equal to the substrate concentration x.

Two ml. of the appropriate buffer solution together with an appropriate amount of water to make a final total volume of 10 ml. were pipetted into 15 ml. volumetric flasks. The flasks were placed in a water bath at 37° for 2 min. to allow the contents to attain the temperature of the bath. Two ml. of the enzyme solution were added, followed by the stated amount of $M/100$ substrate solution. The arrangement for the 7 flasks used was as follows:

Flask	ml. $M/100$ substrate	ml. water	log molarity of substrate
1	0·1	5·9	−4·00
2	0·2	5·8	−3·70
3	0·5	5·5	−3·30
4	1·0	5·0	−3·00
5	2·0	4·0	−2·70
6	4·0	2·0	−2·40
7	6·0	—	−2·23

After exactly 5 min., 2 ml. acid molybdate were added. An observer stood by with a stop-watch to note the time at which the substrate was added to each flask, and to signal when the enzyme activity should be stopped by the addition of the molybdate solution. 0·5 ml. aminonaphtholsulphonic acid was then added to each flask, and water to the mark [King, 1932]. The amount of inorganic P liberated was plotted against the logarithm of the substrate concentration. Inspection of the graph gave the substrate concentration at which half the limiting velocity was reached and the value was checked by substituting for several points in the Michaelis-Menten equation.

RESULTS

In Table 1 are given for each ester: (*a*) the negative logarithm of the dissociation constant (pK); (*b*) the pH at which hydrolysis by the faecal phosphatase

Table 1. *Rates of enzymic hydrolysis of phosphoric esters*

Phosphoric ester	pK	Optimum pH	Inorganic P enzymically liberated in 5 min. (mg./unit phosphatase)	K_m
p-bromophenyl	5·44	9·96	0·099	0·0003
2:4-dibromophenyl	5·60	9·95	0·085	0·0004
o-bromophenyl	5·60	9·80	0·086	0·0004
p-nitrophenyl	5·70	9·80	0·082	0·0005
phenyl	5·73	9·76	0·069	0·0006
o-cresyl	6·04	9·55	0·042	0·0008
2:4:6-tribromophenyl	6·10	8·94	0·036	0·0008
β-glycero	6·34	8·82	0·029	0·0012
α-glycero	6·44	8·62	0·019	0·0014
ethyl	6·45	8·08	0·008	0·0025
m-bromophenyl	Almost undissociated		Very little hydrolyzed	—

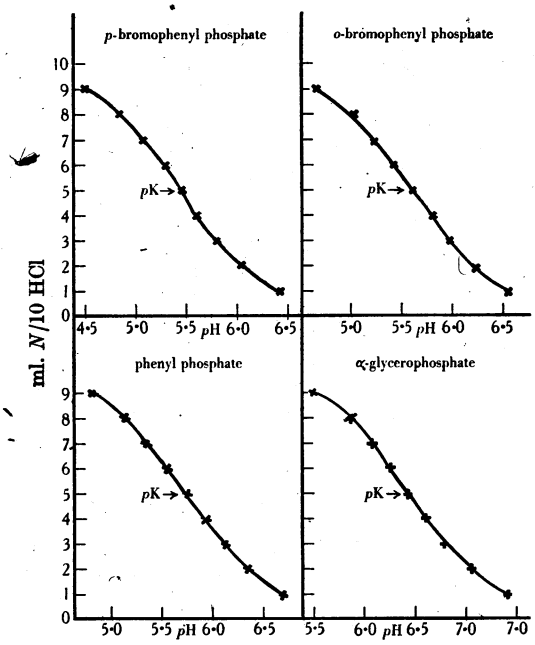

Fig. 1.

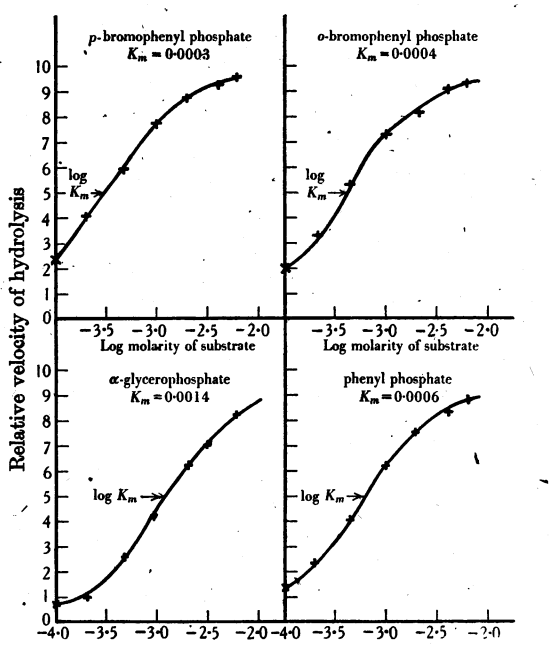

Fig. 2.

Fig. 1. Dissociation curves for phosphoric esters. 20 ml. of a $N/10$ solution of the salt of the ester titrated with $N/10$ HCl. Continuous lines were drawn from data derived from the equation $\mathrm{pH} = pK + \log \alpha/(1-\alpha)$, the value of the constant pK being chosen so as to give the best fit for the determined values.

Fig. 2. Curves showing Michaelis effect of phosphoric esters and faecal phosphatase at the optimum pH. The continuous lines were drawn from data derived from the equation $\log \frac{1}{x} = \log \frac{1}{K_m} + \log \left(\frac{V}{v} - 1\right)$, the values of the constant K_m being chosen so as to give the best fit for the determined values.

occurs at the maximum rate (optimum pH); and (c) the Michaelis constant (K_m) for the enzymic hydrolysis at the optimum pH. The pH-dissociation curves for *p*-bromophenyl-, *o*-bromophenyl-, phenyl- and α-glycero-phosphate are given in Fig. 1, while the curves showing the rate of enzymic hydrolysis at the optimum pH for the same four esters are illustrated in Fig. 2. In the latter case, the method adopted by Folley & Kay [1935] has been followed in the presentation of the graphs, the continuous line showing the theoretical curves for the Michaelis constant specified, and the experimental values being represented by crosses.

A preliminary experiment had shown that in the case of phenyl phosphate the theory of Michaelis was closely followed up to a concentration of 0·009 *M*, but that above this concentration, the velocity of hydrolysis decreased in accordance with the theory of Haldane [1930]. In the main series of experiments the higher concentrations, where deviations from the Michaelis-Menten equation might have been expected, were not investigated, since the main object was the determination of the Michaelis constants for the several esters.

The results indicate that with increasing acidity (i.e. decreasing pK) of the substrate there is an increase in the amount of hydrolysis, whilst the pH of optimum hydrolysis shifts to an increasingly alkaline reaction.

DISCUSSION

All the aromatic phosphoric esters (except *m*-bromophenyl) are more acidic and combine more strongly with the enzyme than the aliphatic esters. Of the latter ethyl phosphate is a weaker acid and is less strongly attacked enzymatically than the glycero-phosphates, which recalls the fact that ethyl chloride is much less chemically active than the chlorohydrins. The addition of an aliphatic group to an aromatic nucleus, as in *o*-cresyl phosphate, appreciably lowers activity.

The difference in behaviour of the *o*-, *m*-, and *p*-phenyl phosphates is noteworthy. The *p*-compound is the most rapidly hydrolyzed, whilst the *o*-compound occupies an intermediate position. These observations may be compared with the facts that *p*-methoxybenzyl bromide is hydrolyzed by acids much more rapidly than the benzyl bromide. The *o*-compound is also hydrolyzed more rapidly than the parent compound, whilst *m*-methoxybenzyl bromide is hydrolyzed less rapidly than the unsubstituted benzyl bromide [Waters, 1935]. It is also quite common to find a descending order of acidity in passing from *p*-compounds through *o*- to *m*-. An example may be given from the pK's of the nitrophenol isomers: *p*-nitrophenol 7·15, *o*-nitrophenol 7·17, *m*-nitrophenol 8·41.

Martland & Robison [1927] suggested two possibilities: (1) that phosphatase had the properties of a weak base whose undissociated molecules combine with a phosphoric ester to form a compound, which thereupon breaks down into the products of hydrolysis, or (2) that the enzyme combined with the ester to form a compound having the properties of a weak acid, whose anions suffer spontaneous decomposition. Our results seem to favour the first hypothesis, since an enzyme having basic properties might be expected to have the greatest affinity for that substrate with the most electro-negative grouping. Thus the greater the affinity between the substrate and the enzyme, the greater will be the rate of enzymic activity and the smaller the Michaelis constant.

Many workers, e.g. Martland & Robison [1927], have shown the gradual inactivation of phosphatase in alkaline solution. Kay [1932] has suggested that the rate of hydrolysis continues to increase with rise of alkalinity, and the apparent optimum is set by the increasing rate of enzyme destruction. This accords well with the further observation of Robison, who found that the enzymic hydrolysis of glycero-phosphate reached a maximum at pH 8·4 for an 18 hr. period of hydrolysis, and 9·4 for shorter periods. It is therefore suggested that the higher pH optimum values obtained here for the more acid esters are due to a greater protection of the enzyme against the inactivating effect of the hydroxyl ion. There are many examples of the protection of an enzyme by its substrate, e.g. trypsin is protected against heat inactivation by egg albumin, malt amylase by starch, and urea protects urease against the inactivating effect of certain dyestuffs.

The following explanation may, therefore, account for the results described in this communication. The enzyme has basic properties which enable it to combine with the substrate, the affinity between the two substances increasing with decreasing pK of the substrate. The greater this affinity, in other words, the smaller the Michaelis constant, the greater is the rate of hydrolysis, the greater is the degree of protection given to the enzyme against the inactivation effect of hydroxyl ions, and in consequence the higher the optimum pH.

SUMMARY

1. The rates of hydrolysis of several phosphoric esters by faecal phosphatase have been investigated, together with the Michaelis constants of the reactions and the dissociation constants of the esters.

2. The results show that with decreasing pK of the substrate the rate of hydrolysis increases progressively, and the enzyme is optimally active at a more alkaline pH. The Michaelis constants decrease with decreasing pK of the substrate.

3. These observations are consistent with the hypothesis of Martland & Robison [1927] that the enzyme possesses the properties of a weak base, whose undissociated molecule combines with the substrate to form a compound which breaks down into the products of hydrolysis, and with the postulate of Kay [1932] that the rate of hydrolysis continues to increase with increasing alkalinity and that the apparent optimum is set by the increasing rate of enzyme destruction.

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The Nutritive Value of Potato Protein for the Pig

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It has long been known that only about one-half of the nitrogen of the potato is in the form of protein, the remainder being in amino-acids and amides, chiefly asparagine. Neuberger & Sanger [1942] have recently shown that not only the total N of the potato, but also the relative proportions of the above three types of nitrogenous compounds, are widely different in different varieties, and that generally the amount of true protein N is rather less than half the total.

Although it is common convention in agricultural practice to reckon the protein value of the potato N as that obtained by adding together the true protein value and one-half of the value obtained by multiplying the non-protein N by 6·25, in this paper the proportion of the N in the different diets, rather than that of the protein, is considered, and the term crude protein is used to indicate the figure obtained by multiplying the total N by 6·25.

The maintenance of N equilibrium in adult man by potatoes was established by Hindhede [1913], Abderhalden, Ewald, Fodor & Rose [1915], Rose & Cooper [1917], Rubner & Thomas [1918] and by Kon & Klein [1928]. The last-named workers found that N equilibrium and good health could be maintained for several months in an adult man and woman on a diet of steamed potato, butter fat and fruit. The daily N intakes averaged 5·7 and 3·8 g. respectively.

The value of potato nitrogen for the support of growth. The value of potato N for the support of growth is not so clear, for it is difficult to obtain a sufficiency for young animals with potato alone since the protein is diluted with so much starch. McCollum, Simmonds & Parsons [1918] found that hardly any weight increase occurred in young rats when potatoes provided the only source of N. The diets contained but 7–8 % crude protein. However, growth was no better when cereal proteins were fed at the same level but was much improved on addition of 5 or 10 % of casein. Growth of rats on diets containing 9 % protein from oats, maize, peas or millet was much better and reproduction occurred when a proportion of these was substituted by potato, thus indicating a supplementary action of the N of the latter for that of the above grains [McCollum, Simmonds & Parsons, 1921]. Hartwell [1925; 1927] found that potato providing 7·6 % crude protein in the diet supported only very poor growth in rats and was quite insufficient for lactation.

Mitchell [1924*b*] determined the biological value of potato N for growth and maintenance in young rats by his modification [1924*a*] of Thomas's N balance technique. The biological value, when the diet contained 5 % crude protein, was 68·5, as compared with 72 for maize, 78·6 for oat, and 86·1 for rice protein. With 10 % crude protein in the diet, the biological values were 66·7 for potatoes, 59·6 for maize