CXLV. THE RATES OF ENZYMIC HYDROLYSIS OF PHOSPHORIC ESTERS

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THE markedly different rates at which esters of phosphoric acid are hydrolysed by both animal and vegetable phosphatases have been noted by several workers, e.g. Martland *et al.* [1924], Kay [1926], Roche [1931] and Asakawa [1929]. Most primary esters of phosphoric acid are optimally hydrolysed by animal phosphatases at an alkaline reaction, but both the *pH* optimum and the rates of hydrolysis with the same enzyme preparation differ greatly with different esters. This is the case even with such closely related substances as α - and β -glycerophosphate [cf. Rae *et al.* 1934]. With more pronounced variations in the nature of the esterified alcohol or phenol the differences become more marked: thus phenyl phosphate is split by bone phosphatase more than twice as fast as glycerophosphate and many times faster than ethyl phosphate [King & Armstrong, 1934]. The *pH* optima for secondary esters, all near neutrality, do not differ so greatly as in the case of primary esters, but the rates of hydrolysis show the same large variations.

With more complex phosphoric esters, i.e. the phospholipins, it was found [King, 1934] that similar changes could be brought about in *pH* optimum and rate of hydrolysis by introducing changes in the structure of the esterified grouping. Lysolecithin was hydrolysed by the enzymes of the intestinal mucosa and of bone at a much faster rate than the parent lecithin and optimally at a more alkaline reaction. By brominating lecithin the rate of enzymic hydrolysis was raised 10 to 100 times, and the optimum *pH* of hydrolysis rose from 7.4 to 8.4. An explanation was sought in the nature of the substituent groups of the alcohol residue, and the suggestion was advanced that the more negative these groupings the faster would be the rate of hydrolysis and the more alkaline the *pH* optimum. This hypothesis that the negativity of the alcohol part of the ester influences the rate of enzymic decomposition is tested further in the present study.

A comparison is made of the rates of hydrolysis of several simple phosphoric esters in relation to the degree of acidity of the ester and the optimum *pH* of hydrolysis. The following esters have been studied, ethyl, α - and β -glycero, cresyl, phenyl, nitrophenyl and bromophenyl phosphates.

EXPERIMENTAL

The experimental work fell naturally into three parts, determination of the titration curves and *pK* values, determination of the optimum *pH* values for enzymic hydrolysis, and comparison of the rates of hydrolysis of the various substrates each at its optimum *pH*.

The titration constants were determined, using the hydrogen electrode, and saturated calomel electrode, according to the general procedure followed by Pirie & Pinhey [1929]. The change of *pH* on addition of 0.1 *N* HCl to 20 ml. of the 0.01 *M* solution of the salt of the ester was plotted on a graph, and from this the *pK* value could be derived.

For the determination of the *pH* optima of hydrolysis it was necessary to choose a buffer capable of giving *pH* values from 7.9 to 11 in order to embrace the full range of *pH* optima covered by the esters. This was accomplished by using a combination of the Michaelis [1930] and Kolthoff [1925] buffers, i.e. a mixture of veronal, Na_2CO_3 and HCl . Each buffer contained the same amount of veronal, namely 25 ml. of 0.1 *M* sodium veronal per 100 ml., and the same amount of Na_2CO_3 . A range of *pH* 7.87–10.6 was obtained by including amounts of 0.1 *N* HCl varying from 2.5 to 40 ml.

The following technique was adopted to avoid loss of CO_2 during the preparation of the buffer: 25 ml. of 0.1 *M* sodium veronal were carefully pipetted into a 100 ml. graduated flask, so as to overlay the appropriate amount of 0.1 *N* HCl . 25 ml. of 0.1 *M* Na_2CO_3 were placed in the flask and the contents mixed by gentle rotation. Water was added to the mark and the contents of the flask thoroughly mixed.

The *pH* curve for this buffer system was determined with the hydrogen electrode¹ and the saturated calomel electrode both at 22° and at 37°. The standard of reference was a sodium acetate-acetic acid buffer of *pH* 4.63 [cf. Sendroy, 1938]. As a check, reference measurements were made on Michaelis veronal buffer mixtures, and Kolthoff mixtures. We were able to check the *pH* values recorded by these authors with a maximum difference of 1 mV. corresponding to *ca.* 0.02 *pH*.

The source of the phosphatase used in these experiments was a powder prepared from dogs' faeces by the method of Armstrong [1935]. This material was freely miscible in water and was of great potency. The amount of this phosphatase preparation necessary to liberate measurable amounts of phosphorus in the required time (5 min.) was so small and contained so little protein material as to give no clouding with the ordinary protein-precipitating reagents. This made unnecessary the usual use of trichloroacetic acid followed by filtration to remove precipitated protein material. It was necessary only to stop the enzyme action at the desired time, and this could be done by the addition of the acid molybdate reagent used for the colorimetric phosphate determination.

For each experiment, an amount of the phosphatase preparation was weighed and dissolved in water so that an activity of approximately 30 King-Armstrong [1934] units per 100 ml. was obtained. MgSO_4 was added in amount sufficient to give a concentration of 0.01 *M* [cf. Kay, 1930]. The phosphatase activity of this solution was then determined,² and an appropriate dilution made to give a final activity of 25 units per 100 ml. The diluted enzyme solution then contained 1 unit of phosphatase in 4 ml.

The substrate was prepared by dissolving the Na salt of the ester in water to make a 0.01 *M* solution. Ba salts of the esters were dissolved in water and treated with the requisite amount of H_2SO_4 ; the solution was neutralized with NaOH , made to volume, mixed and filtered. The strength of each substrate solution was confirmed by colorimetric analysis for total P by the perchloric acid digestion method [King, 1932].

¹ Since it was considered advisable to establish equilibrium as rapidly as possible the electrode described by Moloney [1921] was used.

² 0.2 ml. of the phosphatase solution, together with 4 ml. of *M*/200 disodium phenyl phosphate in *M*/20 sodium veronal, is incubated at 37° for 30 min. The enzyme action is stopped by the addition of 1.8 ml. of diluted (1 in 3) Folin-Ciocalteu [1927] phosphotungstic-phosphomolybdic acid reagent. 4 ml. of the mixture (filtered if necessary) are treated with 1 ml. of 25% Na_2CO_3 and the blue colour compared against an appropriate phenol standard. The increase in free phenol is taken as a measure of the phosphatase activity. A phosphatase unit is defined as the amount of enzyme necessary to liberate 1 mg. of phenol in 30 min., under the above conditions.

For the determination of the optimum pH for hydrolysis of each substrate, and the relative amount of hydrolysis, the following procedure was adopted. Into each of five 15 ml. flasks were pipetted 4 ml. (1 unit) of the enzyme preparation. The flasks were placed in a water bath at 37° . Five test tubes containing an appropriate range of buffers to cover the expected optimum pH of the substrate (determined by a rough preliminary experiment) were likewise placed in the water bath. A 4 ml. pipette was introduced into each tube of buffer ready for use. The substrate solution was also placed in the bath. When the temperature of the solutions to be used had reached 37° (2–4 min.) the experiment was started. 4 ml. of the buffers were pipetted into the flasks containing the 4 ml. of phosphatase. 2 ml. of the substrate were then added to each of the flasks by means of a graduated pipette. (It is advisable to have an observer standing by with a stop-watch to note the time when the substrate is added to each tube.) The contents of the flasks were rapidly mixed. After a lapse of 5 min., 2 ml. of 5% ammonium molybdate solution in 15% H_2SO_4 were added. This was pipetted into each flask at the same rate as the substrate had been previously added. The ester was thus in contact with the phosphatase exactly 5 min. in each flask before the destruction of the enzyme by the acid ammonium molybdate.

The flasks were immediately cooled and to each were added 0.5 ml. of aminonaphthol sulphonic acid solution and water to the 15 ml. mark. The blue colours thus obtained were read in a colorimeter against an appropriate standard.

The above procedure was used to determine the optimum pH of hydrolysis for each phosphoric ester. In Fig. 1 are shown four curves relating the degree

Fig. 1. Optimum pH for hydrolysis of phosphoric esters. A, ethyl phosphate; B, β -glycero-phosphate; C, phenyl phosphate; D, bromophenyl phosphate. Substrate 0.002 M , phosphatase 1 unit, veronal-sodium carbonate buffer, 5 min. 37° .

of hydrolysis to the pH . The extent of the enzymic hydrolysis of the ester is expressed in terms of the actual mg. of P liberated in each test, i.e. the P set free by 1 unit of phosphatase in 5 min. The difference in the extents to which the esters are attacked is remarkable: ethyl phosphate is much more slowly split than glycerophosphate, and the introduction of a bromine atom has greatly increased the rate at which the phenyl phosphoric ester is split. The great

variation from one ester to another in the *pH* optimum is likewise seen. The figures determined for the *pH* optima for the several esters studied are contained in Table I.

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

Substrate: phosphoric esters	<i>pK</i>	Optimum <i>pH</i> of hydrolysis	mg. P hydrolysed per phosphatase unit	Relative rate of hydrolysis (β -glycerophosphate = 1)
Bromophenyl	5.44	9.96	0.099	3.4
Nitrophenyl	5.70	9.80	0.082	2.8
Phenyl	5.73	9.76	0.069	2.4
<i>o</i> -Methylphenyl (<i>o</i> -cresyl)	6.04	9.55	0.042	1.5
β -Glycero	6.34	8.82	0.029	1.0
α -Glycero	6.44	8.62	0.019	0.7
Ethyl	6.45	8.08	0.008	0.3

The comparison of the relative rates of hydrolysis of the several esters was redetermined in an experiment in which all the esters were used simultaneously, each at its optimum *pH*. In this way the relative rates were determined with the identical enzyme solution and at the same time. This experiment was repeated several times, but with very little variation in the results obtained. The average figures are set out in Table I.

Table II. *Hydrolysis of phosphoric esters by several phosphatase preparations*

mg. P liberated by 1 ml. of phosphatase solution in 1 hr. at 37°

Substrate: phosphoric esters	Phosphatase preparation					
	Rabbit kidney	Pig kidney	Human intestine	Human kidney	Human bile	Dog faeces
Bromophenyl	0.19	0.24	0.99	0.78	1.37	3.3
Nitrophenyl	0.13	0.17	0.56	—	0.98	2.4
Phenyl	0.10	0.12	0.52	0.58	0.98	—
Glycero	0.05	—	—	—	0.30	1.7
Ethyl	0.02	—	—	—	0.25	0.58

Table III. *Hydrolysis of secondary phosphoric esters by phosphatase*

5 ml. 0.01 *M* NaR_2PO_4 + 5 ml. veronal buffer *pH* 7 + 0.5 ml. enzyme solution

	mg. phosphorus hydrolysed		
	24 hr.	48 hr.	72 hr.
Diphenyl phosphate			
Rat kidney	0.031	0.042	0.050
Cat kidney	0.009	0.024	0.059
Cat intestine	0.039	0.041	0.063
Rabbit intestine	0.083	0.113	0.136
Rat bone	—	0.007	—
Chicken bone	—	0.011	—
Dicresyl phosphate			
Rat kidney	0.003	0.013	0.019
Cat kidney	0.005	0.011	0.019
Cat intestine	0.010	0.023	0.027
Rabbit intestine	0.046	0.089	0.125
Diethyl phosphate			
Rat kidney	0.002	0.002	0.003
Cat kidney	0.004	0.006	0.008
Cat intestine	0.002	0.007	—
Rabbit intestine	0.006	0.014	—

The relative rates of hydrolysis of some of the esters have also been studied with other phosphatase preparations. The results for these experiments are contained in Table II, and are in substantial agreement with the findings obtained with the more highly purified phosphatase preparation. With the three secondary phosphoric esters which have been prepared the same phenomenon of different rates of hydrolysis is likewise seen. Diphenyl phosphate is attacked more quickly than dicresyl and dicresyl more quickly than diethyl phosphate. Some results for these esters are given in Table III.

DISCUSSION

All determinations of relative rates of hydrolysis for the different esters, and all *pH* activity curves, have been made with the same amount of enzyme and with the same final concentration of substrate, namely 0.002 *M* P ester. It is believed that the results are comparable amongst themselves, and that a true picture of relative velocities of hydrolysis is shown. Under the conditions of the experiments, i.e. 5 min. enzymic action at the optimum *pH*, it may be expected that the observed figures do not differ markedly from initial velocities.

Martland & Robison [1927] found no difference in the rates of hydrolysis of glycerophosphate between concentrations of 0.003 and 0.3 *M*. At lower concentrations both Asakawa [1928] and Jacobsen [1933] found that the *pH* optimum of glycerophosphate varied with the substrate concentration. Folley & Kay [1935] found a *pH* optimum of 10 for phenyl phosphate hydrolysed by mammary phosphatase in glycine buffer when the concentration of ester was 0.0045 *M*, while with a concentration of 0.00023 *M* the lower *pH* optimum of 9.3 was found. They concluded that the enzyme was fully combined with the ester at the higher substrate concentration but not at the much lower one represented by 0.0023 *M*. The *pH* optimum of 9.8 found in this experiment for phenyl phosphate is slightly lower than that recorded by Folley & Kay (10.0), as likewise that for β -glycerophosphate, i.e. 8.8, as compared with the usually accepted value of 8.9 [cf. Kay, 1932]. It was thought possible that, with the very potent enzyme preparation used, higher *pH* optimum values might have been observed with greater concentrations of the substrate. In control experiments with phenyl phosphate and with α -glycerophosphate the same *pH* optima were observed at 0.002 *M* concentration of ester as at 0.02 *M*, although there was a somewhat greater amount of total hydrolysis with the stronger substrate, i.e. with an increased substrate/enzyme ratio. With β -glycerophosphate the slightly higher value (*pH* 8.9) was found. It is not certain, of course, that the enzyme contained in the preparation from dog faeces is identical with the mammary gland phosphomonoesterase, or that the different buffers used were without effect. These aspects of the problem will be investigated further.

On the basis of the determined constants for the Michaelis-Menten [1913] equation, Folley & Kay pointed out that "the 'alkaline' phosphomonoesterase has a much greater affinity for the phenyl phosphate than for glycerophosphate, which accords well with the fact that the former substrate is hydrolysed by it more rapidly than the latter". The data here presented appear to support the hypothesis that the negativity of the esterified alcohol or phenol, as evidenced in the *pK* of the resulting substituted phosphoric acid, conditions the *pH* at which the enzyme optimally combines with the ester and the rate at which cleavage takes place. Although the Michaelis constants are not yet known for several of the esters used in the present investigation, the general behaviour fits well with the conception that the more strongly acid the phosphoric ester the greater will be the affinity of the enzyme for it.

SUMMARY

A study has been made of the rates of enzymic hydrolysis of several phosphoric esters. The rate of hydrolysis and the optimum pH for hydrolysis appear to increase with increasing acidity of the phosphoric ester.

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