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ENZYMATIC AND NONENZYMATIC HYDROLYSES OF THE HETERO-AROMATIC PHOSPHATES[1)]

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Enzymatic hydrolyses of 3-pyridyl, 8-quinolyl, 2-pyridylpropyl, and phenyl phosphates were studied in the presence of potato acid phosphatase at 37°C while an ionic strength of the aqueous reaction system was maintained at 0.10 with potassium chloride. The kinetic data were analyzed in terms of the Michaelis-Menten model. The hydrophobic interaction was found to prevail over the polar interaction in the ES-complex formation. A LFER trend observed in the enzymatic hydrolyses suggests that the electrostatic interaction between substrate and enzyme under hydrophobic atmosphere in the enzyme-recess contributes to the profound rate enhancement.

We have extensively investigated intramolecular catalysis system and a structure-reactivity relationship for the alkyl phosphates having hetero-aromatic moiety in their leaving groups.[2~5)] In the course of these studies, it became interesting to device a new enzyme model system which may work as an improved homogeneous catalyst for the hydrolysis. In order to obtain an additional clue to the design of such a phosphatase-like catalyst system which may work on the substrate of ionic character, nature of the recess of such an enzyme need to be clarified. As an initial step of this attempt, we studied in this work the hydrolyses of 3-pyridyl, 8-quinolyl, phenyl, and 2-pyridylpropyl phosphates in the presence of acid phosphatase from potato.

Nonspecific acid phosphatase have been shown to cleave their substrates at the P-O bond and usually have pH optima at around 5.0. Meanwhile, aryl and alkyl orthophosphates generally undergo acid dissociation of the second phosphate proton at pH 3-8. Thus, most of the orthophosphates usually exist as a monoanionic species at around pH 5, where this ionic species is generally the most reactive except those

having good leaving groups such as dinitrophenyl. Therefore, the enzymatic hydrolyses using potato acid phosphatase which has been regarded as one of the nonspecific phosphatases were compared with the corresponding nonenzymatic spontaneous hydrolyses previously investigated by us.

Assay of Phosphatase and Kinetic Procedures. Organic phosphates used in this work were the same as those used in nonenzymatic hydrolysis independently studied.[2~5] Potato acid phosphatase was commercially purchased from the Sigma Chemical Company, St. Louis, Missouri and used without further purification. Phosphomonoesterase activity was assayed with the use of the assay-kit for acid phosphatase (ACP-S) commercially obtained from Iatron Laboratories, Inc., Tokyo. The stock solution of potato acid phosphatase of the order of 0.2 mg/ml was stable for several weeks at 5~10°C. All runs were carried out at 37°C under nitrogen atmosphere. A 50.0-ml mixture containing all components except enzyme was preincubated for 15 or 20 min. To start the reaction, 10 ml of a dilute enzyme stock solution (0.2 mg/ml) was added as rapidly as possible with enough stirring. At this stage, the concentration of substrate in an experimental solution was $0.7\sim16\times10^{-3}$ M, and the ionic strength was maintained at 0.10 with potassium chloride. Thus, the sample solution was amounted to 60 ml in total at the start. During the course of reaction, pH was adjusted at a given value within an accuracy of ±0.02 using a pH-Stat with 0.1N perchloric acid or 0.1N sodium hydroxide. At an appropriate time interval an aliquot amount of sample solution was drawn out and analyzed for inorganic phosphate released according to the Allen's method as described in our previous paper.[2] All reactions followed first-order kinetics for at least two half-lives.

Optimum pH. For hydrolyses of 3-pyridyl, 8-quinolyl, and phenyl phosphates the same pH-optima (5.0) were observed. In the hydrolysis of 2-pyridylpropyl phosphate under the same conditions the optimum pH was not apparent due to its lower reaction rate relative to the aryl phosphates, though a broad rate maximum region around pH 4.8~5.2 was likely present. On the other hand, in the nonenzymatic hydrolyses of pyridylalkyl,[2,4] 3-pyridyl, and 8-quinolyl phosphates,[3] the optimum pH in their pH-rate profiles generally appeared around pH 2.5~3.5, where the major fraction of the phosphate is in the neutral zwitterion form and this ionic species is the most reactive due to the intramolecular catalysis or the usual electron-withdrawing effect caused by the pyridinium group. This difference in the pH-rate profile between enzymatic and nonenzymatic hydrolyses may suggest that the intramolecular catalysis may be no longer effective at the enzyme site.

K_m and k_{cat}. The linearlity of Lineweaver-Burk plots of $1/v$ *vs.* $1/[S]$ for all of the present phosphates indicates that the reaction follows the most simple rate law: $v = V_{max}[S]/(K_m + [S])$. The kinetic parameters for enzymatic reactions at optimum pH's are listed in Table 1. A noticeable point of results is the relationship

Table 1 Summary of the rate data obtained with acid phosphatase

Phosphate	pK_2 a)	Optimum pH	K_m (M)	V_{max} (M sec^{-1})	k_{cat} b) (M sec^{-1} ml mg^{-1})
3-Pyridyl	5.64	5.0	6.21×10^{-3}	2.43×10^{-7}	7.29×10^{-6}
Phenyl	5.88	5.0	1.01×10^{-3}	7.99×10^{-8}	2.40×10^{-6}
8-Quinolyl	6.42	5.0	1.46×10^{-3}	6.58×10^{-8}	1.97×10^{-6}
2-Pyridylpropyl	6.90	4.8~5.2	2.6×10^{-3}	1.2×10^{-8}	3.5×10^{-7}

a) The acid dissociation constant of the second phosphate proton, which was obtained by potentiometric titration at 25°C and an ionic strength of 0.10 (KNO_3). b) k_{cat} was calculated by the equation, $k_{cat} = V_{max}/[E]_0$, $[E]_0$ is the initial concentration of enzyme.

between pK_2 and K_m at the optimum pH. If one may consider that a K_m-value under present consideration corresponds to a reciprocal of the formation constant for an enzyme-substrate complex as the first approximation, it is clear that the hetero-aromatic moiety in the substrate such as pyridyl and quinolyl suppress the binding of the substrate to the enzyme, at least at the optimum pH. In addition, 8-quinolyl phosphate binds more tightly than 3-pyridyl phosphate by factor of 4.5 at the same pH-value. It is analogous to the fact the naphthyl group demonstrated larger hydrophobic function than the phenyl group.[6] Thus, the hydrophobic interaction seems to prevail over the polar interaction in the formation of ES-complexes.

Different from the trend of Michaelis constants, the decomposition rate of ES-complexes are certainly connected with the pK_2-values of substrate molecules, as shown by the upper line in Fig. 1. Those results, thus, tempt us to suggest that the mechanistic pathway in the hydrolysis of the orthophosphates at the reaction site of the enzyme molecule may be essentially the same as those in the nonenzymatic hydrolysis of them. There is no conclusive evidence whether the proton for the general acid catalyst is brought about intermolecularly (by the functional group of enzyme) or intramolecularly (by the phosphate proton). However, the acceleration of the hydrolysis rate by about 10^2-fold with enzyme could not be interpretated by an intramolecular mechanism.

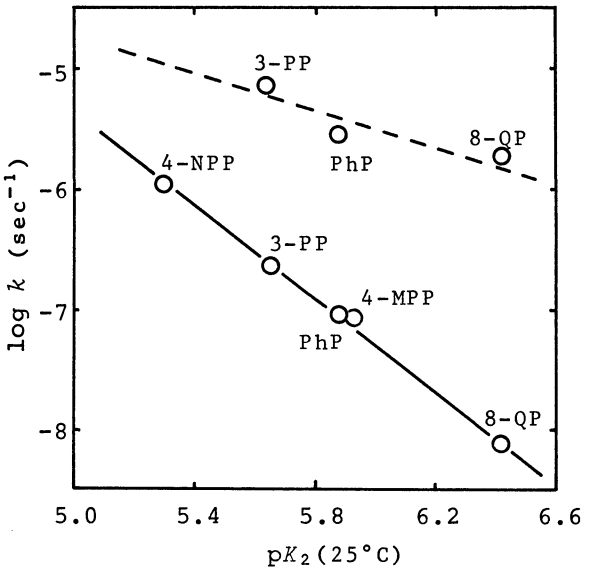
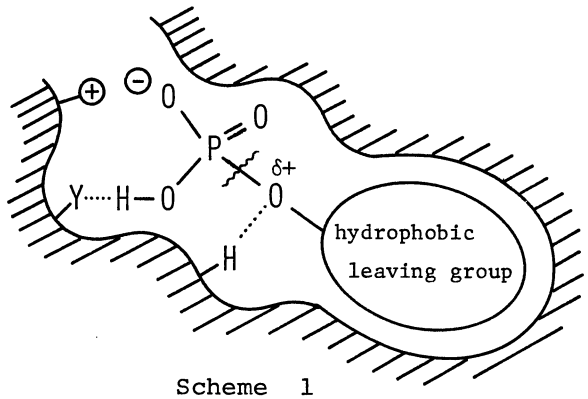


Fig. 1 Linear free energy relationships for the enzymatic hydrolyses (the upper line) in terms of log k_{cat} (at 37°C) *vs.* pK_2 (at 25°C) of the phosphates and for the nonenzymatic hydrolyses (the lower line) in terms of log $k_{monoanion}$ (at 39°C) *vs.* pK_2 (at 25°C). Abbreviations in the illustration are as follows: 4-NPP, 4-nitrophenyl phosphate[7]; 3-PP, 3-pyridyl phosphate; PhP, phenyl phosphate; 4-MPP, 4-methylphenyl phosphate[7]; 8-QP, 8-quinolyl phosphate.

A plausible geometry is schematically illustrated in Scheme 1 for the present enzymatic reaction. Since the pK_2 dependency of k_{cat} is less than that in the corresponding nonenzymatic reactions, the electrostatic interaction between substrate and enzyme under hydrophobic atmosphere in the enzyme-recess seems to contribute exclusively to the profound rate enhancement. Thus, this scheme may provide useful information for designing a phosphatase-like model.



Scheme 1

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