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Studies on Human Prostatic Acid Phosphatase. III.[1,2] The Effects of Alcohols on Human Erythrocyte Acid Phosphatase and on Human Prostatic Acid Phosphatase

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The activity of partially purified erythrocyte acid phosphatase (EAPase) was enhanced by alcohols, 7.6-fold by methanol, 4-fold by butanol, 3-fold by propanol, and 2.6-fold by ethanol and by pentanol. The carbon number of the aliphatic alcohols was proportional to the activation rate (defined as half the most activated value per the alcohol concentration) of EAPase, except in the case of ethanol. This rule was also applicable to human prostatic acid phosphatase (PAPase). However, the extent of PAPase activation by alcohols was lower than that of EAPase. In the presence of polyhydric alcohols, sorbitol, glycerin, and ethylene glycol, the extent of activation of EAPase was found to be proportional to the number of hydroxyl groups of the alcohols. The activation of EAPase was analyzed by means of the Lineweaver-Burk plot, and was found to be noncompetitive. PAPase activation by aliphatic alcohols was also noncompetitive.

Keywords——erythrocyte acid phosphatase; prostatic acid phosphatase; activation and inhibition by alcohols; aliphatic alcohols; polyhydric alcohols

Human erythrocyte acid phosphatase (EAPase) (EC 3.1.3.2) was reported to have more than 100 times greater activity than normal human serum acid phosphatase with phenyl phosphate as a substrate.[4] Human prostatic acid phosphatase (PAPase) and EAPase differ somewhat in substrate specificity, in the effects of metals, and in the effects of certain organic radicals. They are similar in some respects, including inhibition by methanol, ethanol, and butanol.[5]

In common with a number of other erythrocyte enzymes, EAPase exhibits a genetically controlled polymorphism. By means of starch-gel electrophoresis, six distinct phenotypes, A, BA, B, BC, C, and CA, have so far been distinguished,[6] each showing at least two major bands of enzymic activity. A, B, and BA have been reported to be most frequent among the Japanese.[7] Fenton and Richardson[8] have separated three homozygous phenotypes, AA, BB, and CC, of EAPase into their two major components. The six isozymes fell into two groups on the basis of pH optima, K_i for phosphate, and K_m for *p*-nitrophenyl phosphate disodium salt (*p*NPP). The isozymes have essentially the same molecular weight (from 15000 to 20000, depending on the method used for determination).

Although the irreversible inhibition of PAPase and EAPase by alcohols has been described, it has been reported that alcohols serve as accelerators of activation in the hydrolysis of phos-

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phate esters by human PAPase[9,10] and by human EAPase.[11,12] Luffman and Harris[12] reported that the phosphotransferase activity of EAPase appeared to be the same in the different phenotypes. The phosphotransferase activities were stimulated 3-fold by addition of glycerol or methanol at concentrations of 20—25%.

In the present report we describe that effects of various alcohols on EAPase and on PAPase.

Experimental

Materials——Separated red cell samples from normal human adults were obtained from Gifu Prefectural Red Cross Blood Center. Fresh prostate obtained from a patient with benign hypertrophy during surgical operation was frozen at −70° until use. *p*NPP, methanol, ethanol, *n*-butanol, *n*-propanol, benzyl alcohol, glycerin, ethylene glycol, 2-pyridineethanol, and 4-pyridinemethanol were purchased from Wako Pure Chemical Industries Co. (Osaka). Sorbitol was obtained from Nakarai Chemical Industries Co. (Kyoto). Hydrolyzed starch was from Connaught Medical Res. Labs. (Canada). Other chemicals were of analytical grade.

Purification of EAPase——All the preparations were carried out at 4°. Red cells were washed three times with an equal volume of cold 0.9% NaCl. The hemolysate was prepared from the washed red cells by adding 4 volumes of distilled water. The mixture was mechanically shaken for 30 min. After centrifuging the mixture, we used the clear supernatant. The EAPase patterns of the hemolysate were determined by starch-gel electrophoresis. Those showing phosphatase patterns of phenotype B were separately pooled. Two volumes of $Ca_3(PO_4)_2$ gel prepared by the method of Tsuboi and Hudson[13] was added. The gel containing the adsorbed enzyme was separated by centrifugation at 3000***g*** for 10 min and the supernatant was discarded. The gel was washed 4 times with distilled water. Finally, the enzyme was eluted from the gel by shaking with 0.3 M acetate–0.03 M citrate buffer (pH 4.5) followed by centrifugation at 3000***g*** for 10 min. The 30—70% $(NH_4)_2SO_4$ fraction was obtained and dialyzed against 0.01 M Tris-HCl (pH 7.5) overnight. The enzyme preparation was passed through a Sephadex G-75 column, and the peak was applied to a DE32 column with the same buffer. The enzyme was eluted with a linear 0.01—0.02 M concentration gradient of NaCl. The enzyme appeared as two activity peaks, fraction I and fraction II.

Purification of PAPase——Human PAPase was purified as reported previously.[14]

Enzyme Assay——Assays were carried out essentially by the method of Smith and Whitby.[15] Enzyme activity was measured by incubating diluted enzyme for 10 min at 37° with 2 mM *p*NPP in a total volume of 2 ml of 0.1 M sodium citrate (pH 5.5 for EAPase, and pH 6.0 for PAPase). The reaction was stopped by addition of 1 ml of 0.4 M NaOH. The absorbance of the liberated *p*-nitrophenol (*p*NP) was read at 410 nm. Specific activity was expressed as μmol *p*NP per min per mg protein. Protein was determined according to the method of Lowry *et al.*,[16] with crystalline bovine serum albumin as a standard.

Starch-gel Electrophoresis——The phenotype B of the hemolysate was determined by starch-gel electrophoresis. Eleven g of hydrolyzed starch was dissolved in 120 ml of 100-times-diluted tank buffer. The dimensions of the gel used were $15\times15\times0.4$ cm. The tank buffer was 0.245 M NaH_2PO_4–0.15 M trisodium citrate (pH 5.9). The hemolysate which was absorbed on a 2×4 mm filter paper placed at the starting point of the gel. After electrophoresis the gel with filter paper containing absorbed 10 mM phenolphthalein diphosphate pentasodium salt as a substrate was incubated for 3 hr, and the separated zones on the gel were visualized by the addition of 28% ammonia.

Results

Purification of Human EAPase

The Sephadex G-75 column yielded a single activity peak, and the yield was 7.5% with 120-fold purification. This partially purified EAPase was resolved into two components by

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DE32. The first eluted peak was named F I, and the second F II. The yield of F I was 0.4% and that of F II was 0.09% after 80-fold and 340-fold purification, respectively. Unfortunately, these purified enzymes were easily inactivated even during storage at −20°. Therefore the partially purified enzyme fraction up to the Sephadex G-75 stage was used in the following experiments.

The Effects of Various Alcohols on EAPase

The effects of aliphatic straight chain alcohols (monohydric and polyhydric), aromatic alcohol, and heterocyclic alcohols were tested. Figure 1A shows the relative activities of partially purified EAPase in the presence of various concentrations of monohydric alcohols, methanol, ethanol, and propanol. The amount of liberated *p*NP varied markedly with the different alcohols. With 7.2 M methanol, EAPase activity appeared to be as much as 760% of the "no addition" level. With ethanol or propanol, it was much less. With polyhydric alcohols such as sorbitol, glycerin, and ethylene glycol, the amount of liberated *p*NP was higher with increase of alcohol concentration (Fig. 2A). No inhibition was seen up to 1.4 M sorbitol, 3.4 M glycerol, 5.4 M ethylene glycol. However, inhibition was observed with pyridine alcohol and slight activation with benzyl alcohol (not shown).

Figure 1B shows the activation of EAPase by the addition of methanol as a double-reciprocal plot. The values plotted on the ordinate were subtracted control value (100%) from the relative activities. In Fig. 1B, the reciprocal of c_1 gives the most activated value and the reciprocal of a_1+b is the methanol concentration at half the most activated value. We defined activation rate as half the most activated value divided by the methanol concentration at which this activation was obtained. Activation rates of EAPase and PAPase with various alcohols were obtained in the same way and are listed in Table I. No inhibition of EAPase activity by polyhydric alcohols was seen. In Figure 2B, apparent most activated values were obtained by extrapolating the linear portion. They show the same value, c_2, and the reciprocal of c_2

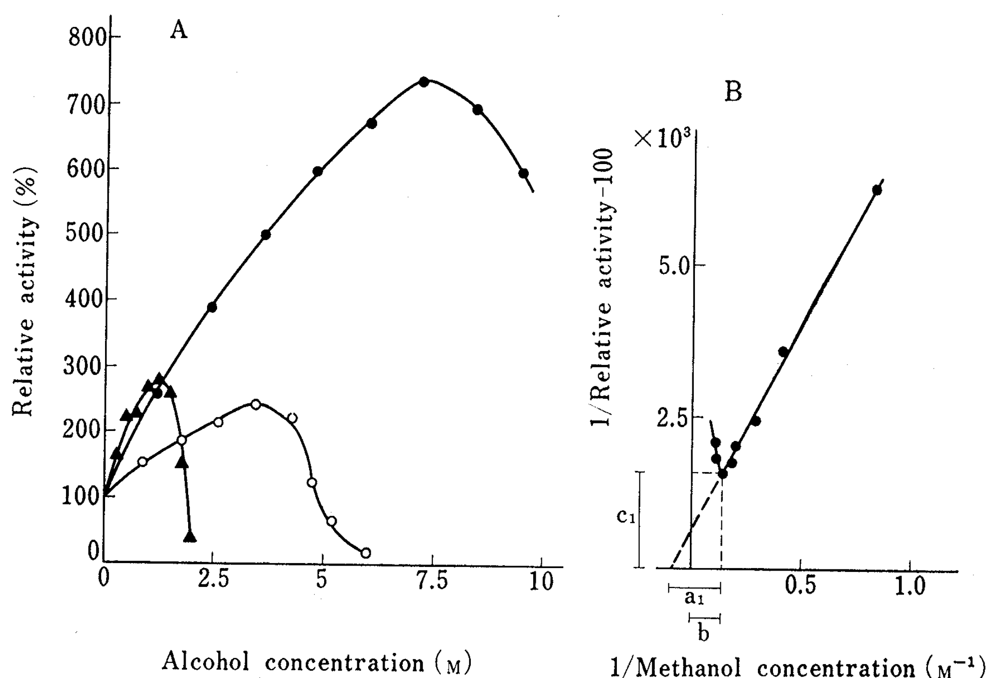

Fig. 1. Effects of Various Monohydric Alcohols on Human Erythrocyte Acid Phosphatase Activity

(A) Relative activity is expressed as a percentage of the control run with no addition of alcohol. ●—●, methanol; ○—○, ethanol; ▲—▲, propanol.
(B) Double reciprocal plot for methanol; data taken from Fig. 1A. The terms a_1, b and c_1 are explained in the footnote to Table I.

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is the apparent most activated value. The reciprocal of a_2 is the sorbitol concentration at half the most activated value, and the activation rate was expressed as the ratio of half $1/c_2$ to sorbitol concentration at half the most activated value. The activation rates on the addition of other polyhydric alcohols were also obtained in the same way and are listed in Table I. The "relative" most activated values in this table correspond to the most activated value plus the control value (100%).

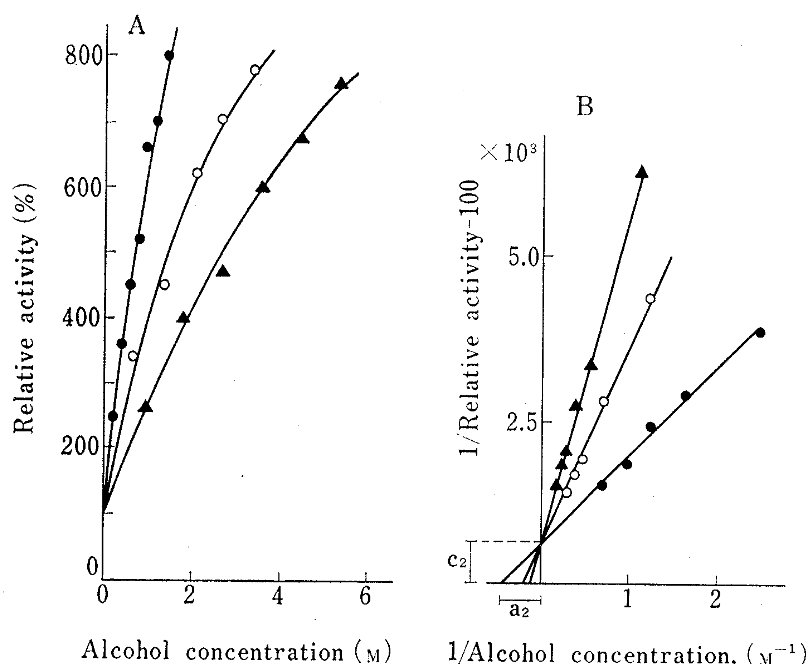

Fig. 2. Effects of Three Polyhydric Alcohols on Human Erythrocyte Acid Phosphatase Activity

(A) Relative activity is expressed as a percentage of the control run with no addition of alcohol. ●—●, sorbitol; ○—○, glycerin; ▲—▲, ethylene glycol.
(B) Double reciprocal plot of data taken from Fig. 2A. The terms a_2 and c_2 are explained in the footnote to Table I.

Table I. Activation of Human EAPase and PAPase by Monohydric and Polyhydric Alcohols

Compound	Relative most[a)] activated value (%)		Alcohol concentration[b)] at half the most activated value (M)		Activation rate[c)] $1/\text{M}\cdot 2c_1$ or $1/\text{M}\cdot 2c_2$	
	EAPase	PAPase	EAPase	PAPase	EAPase	PAPase
No addition	100	100				
Methanol	758	120	2.78	0.45	118	22
Ethanol	261	117	1.56	0.24	52	35
Propanol	306	146	0.40	0.14	258	164
Butanol	394	160	0.19	0.10	774	300
Pentanol	264	212	0.05	0.03	1640	1867
Ethylene glycol	1639		7.14		108	
Glycerin	1639		4.00		192	
Sorbitol	1639		1.81		425	

a) The "relative" most activated values (%) are calculated as $1/c_1+100$ and $1/c_2+100$; c_1 is shown in Fig. 1B in the case of methanol addition, and c_2 in Fig. 2B in the case of sorbitol addition.
b) The alcohol concentrations at half the most activated value (M) are expressed as the reciprocal of a_1+b of Fig. 1B for addition of methanol, and as the reciprocal of a_2 of Fig. 2B for addition of sorbitol, for example.
c) The activation rates are expressed as the ratio of $1/2c_1$ or $1/2c_2$ to alcohol concentration (M).

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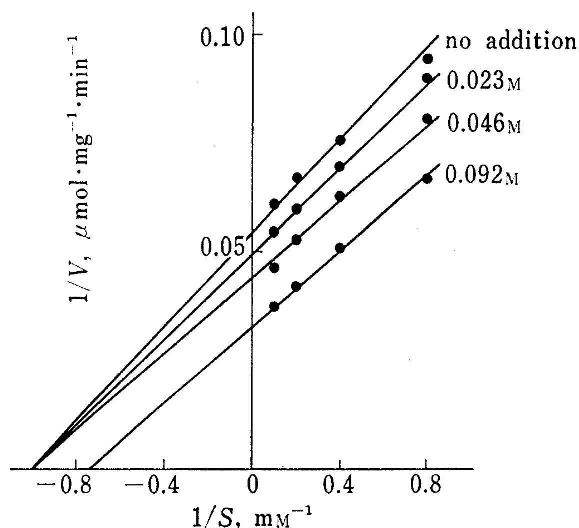

Fig. 3. Lineweaver–Burk Plot of Erythrocyte Acid Phosphatase Activity in the Presence of the Indicated Concentrations of Pentanol

Type of Activation upon Alcohol Addition

Figure 3 shows Lineweaver–Burk plots of EAPase activity with *p*NPP as a substrate in the presence of various concentrations of pentanol. The plots show noncompetitive-type activation having unaffected K_m and increased V_{max} with increase of pentanol concentration. Plots of 0.023 M addition and 0.046 M addition are shown. However, plots with pentanol concentrations above 0.046 M (0.092 M for example) were parallel to the slope of the 0.046 M addition plot and K_m as well as V_{max} of the former plot were higher than those of the latter. Changes of activation type were similarly observed in the activation of EAPase by other alcohols. The alcohol concentration at which the activation type changed was 0.30, 0.64, 0.25, 0.14 or 0.046 M in the addition of methanol, ethanol, propanol, butanol, or pentanol, respectively. Pyridine alcohols inhibit EAPase activity noncompetitively, with unaffected K_m, and decreased V_{max}. K_i for 2-pyridineethanol was 2.3×10^2 M, and K_i for 4-pyridinemethanol was 2.5×10^2 M.

Effects of Various Alcohols on PAPase

Figure 4 shows the effects of various alcohols on PAPase. In general, the activity was enhanced proportionally to the amount of monohydric alcohols added up to a certain concentration. The most activated value was enhanced and the alcohol concentration at which the

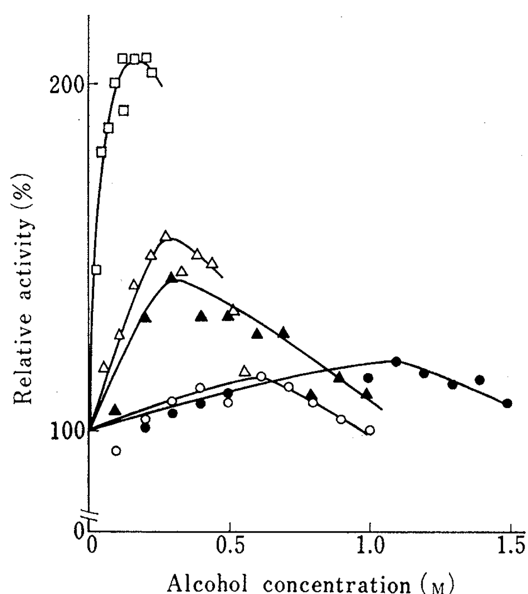

Fig. 4. Effects of Various Monohydric Alcohols on Human Prostatic Acid Phosphatase Activity

Relative activity is expressed as a percentage of the control run with no addition of alcohol. ●—●, methanol; ○—○, ethanol; ▲—▲, propanol; △—△, butanol; □—□, pentanol.

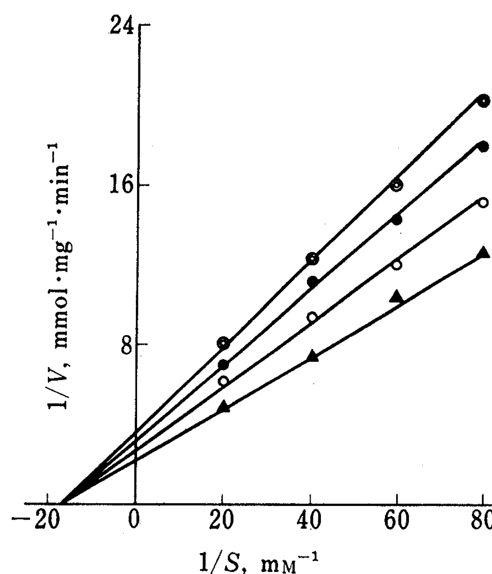

Fig. 5. Noncompetitive Activation of Prostatic Acid Phosphatase Activity by Various Concentrations of Pentanol

◎—◎, no addition; ●—●, 10 mM; ○—○, 40 mM; ▲—▲, 160 mM pentanol.

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most activated value occurred decreased as the carbon number of aliphatic alcohols increased. Methanol, a powerful activator of EAPase, activated PAPase to a small extent (Table I). Fig. 5 shows a Lineweaver–Burk plot for PAPase in the presence of pentanol, representing activation of noncompetitive type, with constant K_m and increased V_{max}. Activation of noncompetitive type was also seen with other aliphatic alcohols (not shown).

Products of the EAPase and PAPase Reactions in the Presence of Methanol

Figure 6 shows the time courses of the EAPase (Fig. 6A) and PAPase (Fig. 6B) reactions in the presence of 7.2 M and 1.1 M methanol. In Figure 6A, the yield of liberated inorganic phosphate in the supernatant of the EAPase reaction mixture was about 10% and that of *p*NP was 100% in the presence of methanol. However, the yield of free inorganic phosphate was 100% in the absence of methanol. In the case of PAPase (Fig. 6B), the yield of free inorganic phosphate in the supernatant was about 90% and that of *p*NP was 100%.

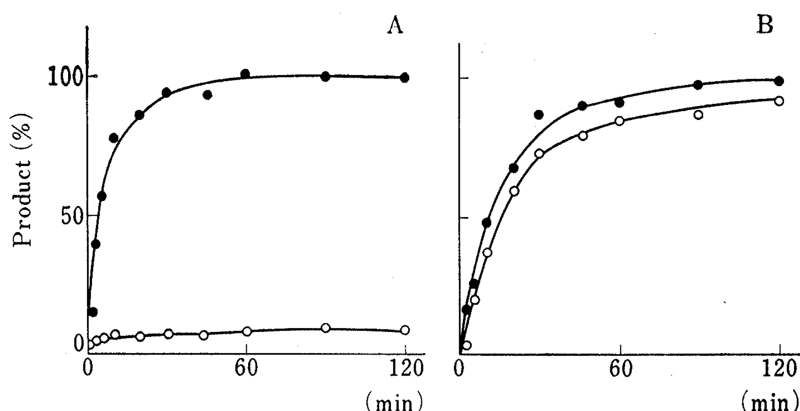

Fig. 6. Release of Orthophosphate and *p*-Nitrophenol during the Acid Phosphatase Reaction in the Presence of Methanol

Releases of orthophosphate and *p*-nitrophenol are shown for the reaction of human erythrocyte (A) and prostatic acid phosphatase (B) with *p*-nitrophenyl phosphate in the presence of methanol. ○—○, orthophosphate (Pi); ●—●, *p*-nitrophenol (*p*NP).

Discussion

It is known that PAPase is activated by alcohols.[9,10] In the case of EAPase, Iio *et al.*,[17] have reported that horse EAPase is activated 2-fold by methanol and 1.5-fold by ethanol or *n*-propanol with *p*NPP as a substrate. In the present study, the activity of human EAPase was enhanced 7.6-fold by methanol, 4-fold by butanol, 3-fold by propanol, and 2.6-fold by ethanol or by pentanol. The alcohol concentration at which the most activated value is obtained increases with decrease of the carbon number of the aliphatic alcohol.

We defined the activation rate as half the most activated value per the alcohol concentration at which this activation is obtained. The carbon number was proportional to this activation rate. In this rule, however, we found that the expected activations by ethanol and by methanol were actually reversed, but the reason for this is not known. This rule is consistent with the case of PAPase,[10] where the extent of activation by alcohols was generally lower than with EAPase. The alcohol concentration at which the most activated value of PAPase is obtained decreases with increase of the carbon number of the aliphatic alcohol. In particular, methanol addition enhanced PAPase activity 1.2-fold at 1.2 M, with an activation rate of 22, although it activated EAPase 7.6-fold at 7.2 M, with an

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activation rate of 118. By using sorbitol, glycerin and ethylene glycol, the extents of activation of EAPase in the presence of these polyhydric alcohols were found to be proportional to the number of hydroxyl groups in the polyhydric alcohols. These hydroxyl groups may accelerate dephosphorylation from *p*NPP (Table I).

In our experiments, liberated inorganic phosphate in the supernatant of the EAPase reaction mixture dropped to 10% in the presence of methanol, while the value in the absence of methanol was 100% (Fig. 6A). Iio *et al.*[17] have also observed liberated inorganic phosphate and methyl phosphate in a ratio of 10: 90 (with a total number of phosphates equal to that of *p*NP produced) using horse EAPase. This result is considered to show that the enzyme and substrate complex (EH–ROP) breaks down with the expulsion of the phosphate ester to yield the covalently bound intermediate (E–P). The two following reactions then take place.

$$\text{E-P} + \text{A-OH} \rightleftarrows \text{E-H} + \text{A-OP} \qquad (1)$$

$$\text{E-P} + \text{HOH} \rightleftarrows \text{E-H} + \text{P-OH} \qquad (2)$$

Since phosphate transfer from E–P to methanol (1) is much easier than that to the hydroxyl ion of water (2), and water competes with alcohol, this would result in accelerated dephosphorylation by acid phosphatase.

In the case of PAPase, however, we observed 90% free inorganic phosphate in the supernatant of reaction mixture in relation to 100% *p*NP produced (Fig. 6B). Seventy-five to 90% inorganic phosphate was produced relative to 100% *p*NP when ethanol, propanol, butanol, or pentanol was used. Ostrowski and Barnard[18] also reported that ethanol addition to PAPase resulted in 77% inorganic phosphate plus 23% ethyl phosphate. The reason for this difference between PAPase and EAPase is not known. τ-Phosphohistidine has already been isolated as a decomposition product of E–P in the cases of human prostate,[19,20] rat liver,[21] and wheat germ acid phosphatase.[22] This is evidence for the presence of the E–P intermediate. The final step of the hydrolysis, E–P+H–HO⟶E–H+P–OH is considered to be the rate-limiting step.[23] Besides the hydrolytic activity, this enzyme has transferase activity, E–P+A–OH⟶E–H+A–OP. This transferase activity is stimulated by alcohol addition, and the phosphotransferase activities of EAPase and of PAPase may be different. Boer and Steyn-Parvé[24] have reported that no activation of yeast acid phosphatase upon methanol addition was observed, and enzyme-phosphate covalently bound complex was not detected. The phosphate transfer rate from *p*NPP to methanol remained 9—21%, which is only as much as the intrinsic decomposition rate of *p*NPP under non-enzymatic conditions.

Activation types of EAPase were analyzed by means of Lineweaver–Burk plots, and noncompetitive-type activation was seen with unaffected K_m and increased V_{max} at low concentrations of alcohols. Above certain alcohol concentrations, activation plots having both K_m as well as V_{max} increased were seen; for example, 0.046 M in the case of pentanol (Fig. 3). Pyridine alcohols inhibited EAPase noncompetitively (not shown). Aliphatic alcohols affected PAPase only noncompetitively (Fig. 5). With both EAPase and PAPase, the activation types were thus not competitive but noncompetitive. If this activation were competitive, competition by *p*NPP and alcohol at the substrate binding site might be assumed, but since it is not, we consider that there is a phosphotransferase active site in addition to the phosphohydrolysis active site in the enzyme molecule.

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