STUDIES ON THE ENZYMATIC REACTION CONCERNING LIBERATION OF ETHANOLAMINE IN ASCITES HEPATOMA OF RAT

1. Substrate of the Enzymatic Reaction Concerning Liberation of Ethanolamine in Ascites Hepatoma of Rat
2. Properties of Acetone Insensitive Phosphatase from Ascites Hepatoma of Rat
3. Effect of Acetone on Alkaline Phosphatase Activity

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As reported in the previous paper (1), the apparent contents of ethanolamine of hepatoma cells and regenerating liver of rat were about eight to ten times greater than that of normal liver, and this difference was attributed to the effect of acetone, which was used in the determination process of ethanolamine. Namely, acetone inhibited the enzymatic reaction system concerning the liberation of ethanolamine in normal liver homogenate but not in others.

In the present work, isolation and identification of the substrate of this reaction in hepatoma cells were attempted. It was found that phosphorylethanolamine (PE)** is the direct precursor of ethanolamine in hepatoma cells, AH 130, and that the enzymatic hydrolysis of PE in the tumor cells was not

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* A part of this work was presented at the 34th Congress of the Japanese Biochemical Society held at Osaka in November, 1961.

** Follwing abbrevations are used throughout this paper: PE: phosphorylethanolamine, El: ethanolamine, GPE: glyceryl-phosphorylethanolamine, CDP-E: cytidine-5'-diphosphate ethanol-amine, Pd-E: phosphatidyl ethanolamine, P-Ser: phosphorylserine, TCA: trichloroacetic acid.

inhibited by acetone.

It has been shown by several investigators (2-4) that PE was present in a large amount in some neoplastic tissues, and the present data agree with their results in that the PE contents of AH 130 and regenerating liver of rat are much larger than that of normal liver.

MATERIALS AND METHODS

Male Sprague-Dawley strain rats weighing approximately 200 g. were used. Partial hepatectomy was operated by the method of Higgins and Anderson (5), by which about two thirds of the liver was removed. Animals were fed *ad libitum* before and after the operation. AH 130 cells were harvested eight to ten days after transplantation and washed with isotonic saline before use. The homogenates were made with Teflon-pestled Potter-Elvehjem homogenizer in 0.154 M KCl solution. The soluble fraction was obtained from above homogenates by centrifugation at 105,000 x g for 60 minutes. PE was synthesized by the method of Outhouse (6), and glycerylphosphorylethanolamine (GPE) was prepared from crude phosphatidylethanolamine (Pd-E) by mild alkaline hydrolysis according to the method of Dawson (7) and purified by cellulose powder column chromatography

(water saturated phenol: glacial acetic acid: water = 100 : 10 :12 (v/v)). Cytidine-5'-diphosphate ethanolamine (CDP-E) was purchased from Sigma Chemical Co., St Louis, U.S.A..

Quantitative determination of ethanolamine was made by dinitrophenylation, ether extraction and Amberlite CG-50 column chromatography as previously described (1). Inorganic phosphate was determined by the Fiske-SubbaRow's method (8).

Column Chromatography--- (1) Isolation of a precursor of ethanolamine from AH 130 cells: A 2.5 x 34 cm. column of Amberlite CG-120 (H^+ form, 200-300 mesh) was used. Aliquots of a concentrated dialysate of soluble fraction (15-25 ml.) were applied to the column, and eluted with deionised water. Effluent was collected in 10 ml. fractions. One ml. of samples was taken from each fraction for the analysis of ninhydrin positive substances by the method of Yemm and Cocking (9).

(2) Estimation of phosphorylethanolamine in tissues: Aliquots of aqueous homogenates of a tissue were treated with an equal volume of 10 per cent trichloroacetic acid (TCA) and the precipitate was washed twice with 5 per cent TCA. The supernatant solution and washings were combined and concentrated to 10 ml. in vacuo in a boiling water bath after removal of TCA

with diethyl ether. One ml. of the sample was applied to an 1 x 30 cm. column of Amberlite CG-120 (H^+ form) or Dowex 50 (H^+ form) and the column was eluted with deionized water. Every one ml. of effluent was collected and PE was quantitatively determined by the ninhydrin method (9). Ninhydrin colour yield of PE was determined by a synthetic sample in each experiment.

RESULTS

Ethanolamine libration in the homogenates of AH 130--- In the previous paper (1), it was found that ethanolamine was produced linearly for at least 5 hours when aqueous homogenates of both normal and regenerating liver were incubated at 37°C, and that the liberation of free ethanolamine was almost completely suppressed in normal liver homogenates, when acetone was added to the homogenates at the concentration of 50 per cent (v/v), while in homogenates of regenerating liver the suppression by acetone was only about 30 per cent. As shown in Fig. 1, ethanolamine was also liberated linearly for 5 hours when the homogenates of AH 130 cells were incubated at 37°C (Fig. 1 (A), solid line) and the reaction was not suppressed by 50 per cent of acetone as the case of regenerating liver (1) (Fig. 1 (A),

dotted line). The same phenomena were also observed in the case of the supernatant obtained by centrifugation of the homogenates of AH 130 at 105,000 x g for 60 minutes (soluble fraction) (Fig. 1 (B)). The recovery of the activity in sloouble fraction was approximately one third of the original homogenates.

Separation of substrate and enzyme---- Separation of a precursor of ethanolamine from the enzyme system concerning liberation of ethanolamine was attempted with soluble fraction of AH 130 cells, as follows: Soluble fraction was dialysed throughly against deionized water with use of a cellophane tube in a cold room, and the dialysate was concentrated to a small volume *in vacuo* in a boiling water bath. As shown in Table I, ethanolamine was liberated only when dialysed soluble fraction was incubated together with the concentrated dialysate at 37°C. This fact suggests that a substrate of the ethanolamine liberating reaction was separated in the dialysate from the enzyme system. The possibility that a compound separated in the dialysate was not a substrate but a cofactor for the reaction, was ruled out by the experiment described below.

Isolation and identification of substrate of the reaction--- The concentrated dialysate of soluble fraction was subjected to a column (2.5 x 34 cm.) of Amberlite CG-120 (H^+ form),

and elution was carried out with deionized water. Fig. 2 shows an example of the chromatogram. Authentic phosphoryl-serine and taurine were eluted at a column volume but authentic sample of PE was eluted just in the same position as the 2 nd peak in the chromatogram. The eluate corresponding to the 2 nd peak was concentrated and the concentrate was incubated with dialysed soluble fraction of AH 130 cells for 2 hours at 37°C. The liberated ethanolamine and inorganic phosphate were estimated by DNP-method (1) and the method of Fiske-SubbaRow (8). As shown in Table II, only when both concentrated solution of the 2nd peak and dialysed soluble fraction were incubated, liberation of ethanolamine and inorganic phosphate was observed. In this case, the ratio of liberated ethanolamine to liberated phosphate was found to be nearly 1 (Table II). Under the same condition, liberation of ethanolamine was not observed with use of substances in the first peak of the chromatogram in Fig. 2.

The substance of the 2nd peak was compared with an authentic sample of PE by means of paper-chromatography and paper-electrophoresis, as shown in Fig. 3. All maps showed that the pursued substance gave one ninhydrin- and molybdate reagent (7)- positive spot and the position of the spot coincided with the reference one. No other ninhydrin-positive spot was detected. Furthermore, the substance in the 2nd peak was analysed for contents of

amino group and phosphorous (Table III). It was found that the molar ratio of amine group to phosphorous was nearly 1.

In oder to examine whether there is another compound, beside PE, which liberates ethanolamine in AH 130 cells, following experiment was undertaken: The concentrated dialysate and its chromatographically purified fraction (2nd peak in Fig. 2) were incubated respectively with dialysed soluble fraction, until liberation of ethanolamine stopped. Assuming that amounts of precursors of ethanolamine were equal to amounts of ethanolamine liberated under the condition described above, amounts of the precursors in the dialysate and its purified fraction were compared. It was found that almost all amount of the precursor of ethanolamine found in the dialysate was recovered in the 2nd peak, PE fraction of chromatography on Amberlite CG-120. Therefore, it is thought that there is no other compound, beside PE, which participates in liberation of ethanolamine in soluble fraction of AH 130 cells. Namely, the direct precursor of ethanolamine in soluble fraction of AH 130 cells is considered to be phosphorylethanolamine.

Effect of acetone on the reaction--- With use of dialyzed soluble fraction of AH 130 cells and normal liver as enzyme sources, the rates of enzymatic hydrolysis of authentic PE

were compared in the absence and presence of acetone (Table IV). It can be seen in Table IV that the enzymatic activity of AH 130 is much greater than that of normal liver and that the activity is not suppressed by 50 per cent acetone for both AH 130 and normal liver.

When GPE, CDP-E and crude cephaline were added to the enzyme solution (dialysed soluble fraction of AH 130 cells), the rates of ethanolamine liberation from these compounds were small, compared with the case using PE as substrate, and the liberation of ethanolamine from these compounds were inhibited by 50 per cent acetone in a greater extent (Table IV).

Phosphorylethanolamine contents in AH 130 cells, regenerating and normal liver -----PE contents in AH 130 cells, regenerating and normal liver of rat were estimated as described in Materials and Methods. As shown in Table V, AH 130 cells contained PE about eight times and regenerating liver (48 hours after partial hepatectomy) about three times greater than normal liver.

DISCUSSION

In the previous paper (1), it was shown that an enzymatic reaction system concerning the liberation of ethanolamine is present in homogenates of normal and regenerating liver of rat.

The present work revealed that this system also exists in both homogenates and soluble fraction of an ascites hepatoma of rat (AH 130).

By means of dialysis, the precursor of ethanolamine was isolated from soluble fraction of AH 130 cells and proved to be phosphorylethanolamine. No other compounds which participate to the liberation of ethanolamine, beside PE, were found in the soluble fraction. Furthermore, the presence of a phosphatase hydrolysing PE was also proved in soluble fraction of both AH 130 cells and normal liver. Hydrolysis of PE by this enzyme was scarcely inhibited by 50 per cent of acetone in the case of normal liver as well as AH 130 cells (Table IV). Therefore, the fact that the ethanolamine liberation in normal liver homogenates was completely suppressed in 50 per cent acetone in contrast to homogenates of AH 130 cells (1), would be explained as mentioned below. For the ethanolamine liberation, two pathways are considered in mammalian tissues as follows;

(1) hydrolysis of PE by phosphatase (10)

(2) hydrolysis of GPE by phosphodiesterase (11), which is produced from Pd-E by phosphatidase A and lysophosphatidase (12). Reaction (1) was proved to be acetone insensitive (Table IV) but reaction (2) may be acetone sensitive because the hydrolysis of GPE by dialysed soluble fraction of AH 130 was inhibited by acetone (Table IV). Hence, ethanolamine liberated

in homogenates of normal liver and AH 130 would be due to both reaction (1) and (2) in the absence of acetone. When acetone is added to the homogenates, ethanolamine would be liberated only by reaction (1), since reaction (2) is suppressed by acetone. Thus, the difference in the ethanolamine liberation between normal liver and regenerating liver or AH 130 cells, observed when these homogenates were incubated with acetone, would be attributed to the quantitative difference of acetone-sensitive (reaction (2)) and acetone-insensitive (reaction (1)) enzyme systems. In fact, the amount of PE was much larger in AH 130 than in normal liver, and phosphatase is also present in a large amount in hepatoma, as reported by Greenstein (13).

As for the formation of PE in tissues, two pathways are known as follows;

(3) phosphorylation of ethanolamine by phosphokinase (14,15)

(4) hydrolysis of Pd-E by two-steps reactions by PE-glyceride transferase and PE-cytidyl transferase (16,17).

The reactions (4) are considered to play a synthetic role for Pd-E rather than a degradative role. The fact that PE content in hepatoma is much larger than normal liver, would be explained by two ways; stimulation of reaction (3) in hepatoma, or

suppression of the formation of Pd-E from PE by reactions (4), and the physiological significance of this elevated PE content of hepatoma is now under investigation.

The properties of the enzyme hydolysing PE will be reported in a forthcoming paper.

SUMMARY

1. The ethanolamine liberating enzymatic system was also present in AH 130 cells.

2. The main precursor of free ethanolamine in an ascites hepatoma of rat (AH 130) was identified as phosphorylethanolamine.

3. Phosphatase which hydrolyses phosphorylethanolamine was not inhibited at all even in the presence of 50 per cent of acetone in the case of normal liver as well as AH 130 cells.

4. Phosphorylethanolamine contents of ascites hepatoma and regenerating liver of rat were larger than that of normal rat liver.

5. The difference between normal liver and regenerating liver or tumour cells of rat in the reaction of formation of ethanolamine (1) was discussed from the results decribed above.

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Table I

Liberation of Ethanolamine from a Compound Isolated in the Dialysate of Soluble Fraction of AH 130 Cells.

Soluble fraction of AH 130 cells was dialysed against deionized water and the dialysate was concentrated to a small volume as described in the text. Dialysed soluble fraction was incubated with and without the concentrated dialysate at 37°C for 2 hours in 0.05 M veronal buffer (pH 8.5) and 1 mM $MgCl_2$. The reaction was stopped by heating in a boiling water bath for 5 minutes and ethanolamine was estimated by DNP-method (1).

	Ethanolamine (µmoles/flask)		
	1	2	3
Dialysed Soluble Fraction (without incubation)	0.00	0.00	----
Dialysate (without incubation)	0.878	0.190	0.190
Dialysed soluble Fraction (incubated)	0.00	0.00	0.009
Dialysed Soluble Fraction Plus Dialysate (incubated)	2.40	1.21	0.833

Table II

Enzymatic Digestion of the Compound Isolated in the 2nd Peak of the Chromatogram of Fig. 2.

	El** µmoles	Pi** µmoles	Pi/El
(1) Concentrated Fraction of the 2nd Peak in the Chromatogram of Fig. 2 (without incubation)	0.00	0.00	-----
(2) Dialysed Soluble Fraction of AH 130 (incubated*)	0.00	0.00	-----
(1) + (2) (incubated)	3.09	3.25	1.05

* Incubation was carried out at 37°C for 2 hours in 0.05M veronal-carbonate buffer (pH 9.5) and in 1 mM $MgCl_2$.

** Ethanolamine was determined by DNP-method (1), and inorganic phosphate by Fiske-SubbaRow's method (8).

Table III

Contents of Amino Group and Phosphorous in the Compound Isolated in the 2nd Peak of the Chromatogram

Exp. No.	Amino Group* (μmoles/ml.)	Phosphorous (μmoles/ml.)	P/Amino Group
1	2.40	2.60	1.08
2	3.17	3.31	1.04

* Contents of amino group were estimated as PE by the ninhydrin method .

Table IV

Effect of Acetone on Enzymatic Hydrolysis of Various Compounds

Dialysed soluble fractions of normal rat liver and AH 130 cells were used as enzyme sources. Incubation was carried out at 37°C for 1.5 hours in 0.05 M veronal-carbonate buffer (pH 9.5) and 1 mM $MgCl_2$ in the absence and presence of acetone (50 per cent (v/v)).

Enzyme Source	Substrate (final con.)	Specific Activity (El umoles/g-N/hr.)		Acetone Effect* (%)
		+Acetone	-Acetone	
Normal Rat Liver	PE (1.0mM)	32.6	23.5	72.0
	PE (1.5mM)	23.4	18.1	74.2
AH 130 Cells	PE (1.5mM)	1405.	1558.	109.
	PE (2.4mM)	2840.	3335.	117.
	GPE (0.69mM)	20.2	2.2	10.9
	CDP-E (0.75mM)	278.	41.6	15.0
	Cephalin (4.3mg/ml)	35.6	18.9	53.2

* Acetone effect = $\frac{\text{Specific Activity (+Acetone)}}{\text{Specific Activity (-Acetone)}} \times 100$

Table V Phosphorylethanolamine Contents in Various Tissues.

	Phosphorylethanolamine contents*	
	μmole/g wet wt.	μmole/g total-N
Normal rat liver	0.428 ± 0.023	16.1 ± 0.45
Regenerating rat liver (48 hrs after partial hepatectomy)	1.17 ± 0.19	44.8 ± 9.0
AH 130 cells	2.56 ± 0.68	131 ± 22

* Mean value and standard error of three experiments.

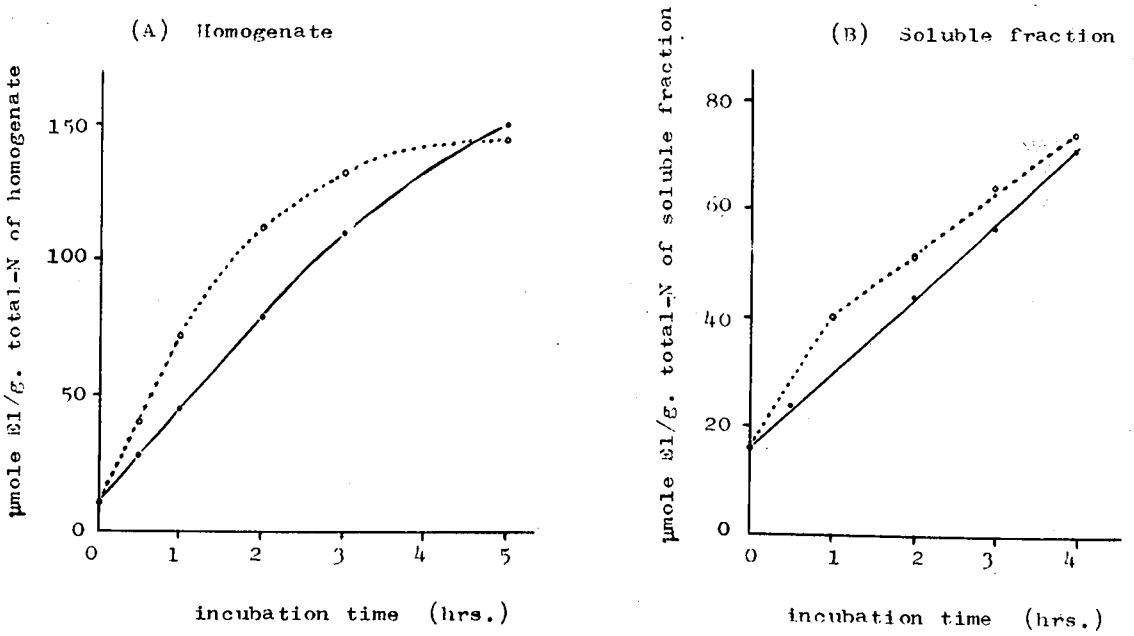

Fig. 1 Time curves of the reaction liberating free ethanolamine in homogenate(A) and soluble fraction(B) of rat ascites hepatoma cells. Incubation was carried out at 37°C, in the absence (solid line) and presence (dotted line) of 50 per cent acetone(v/v).

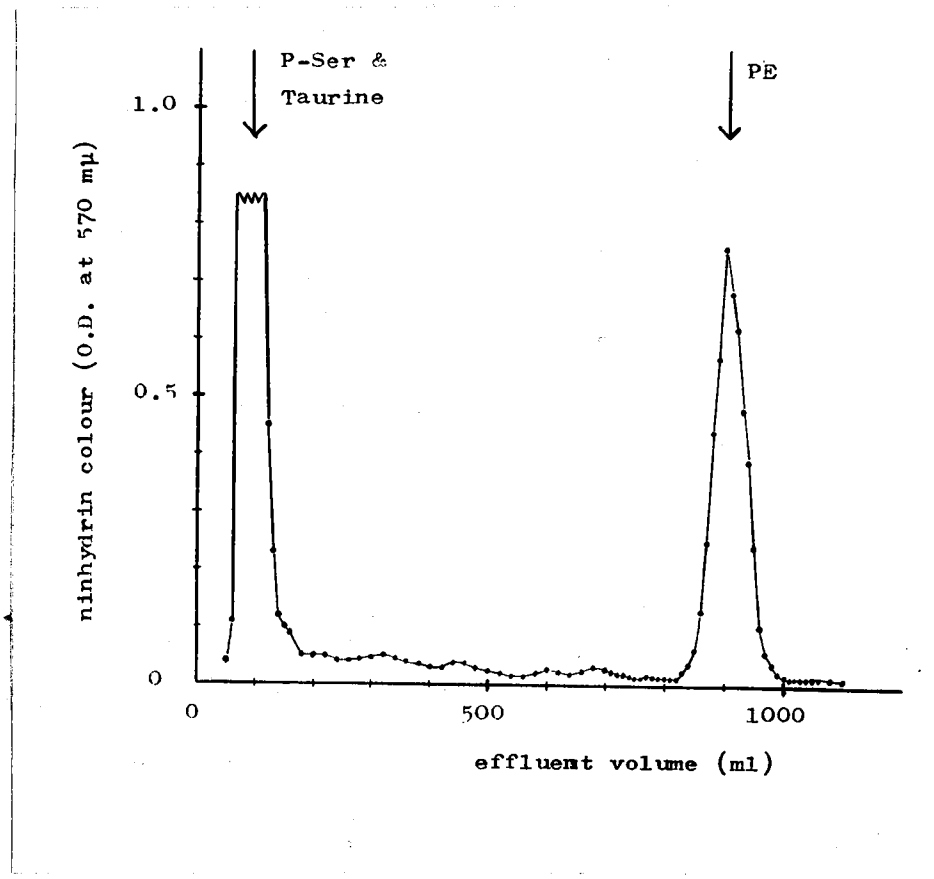

Fig. 2 Column chromatography of the concentrated dialysate of soluble fraction of AH 130 cells.

Fifteen ml. of the concentrated dialysate of soluble fraction (prepared from 45 ml. of packed cells of AH 130) was applied to a 2.5 x 34 cm. column of Amberlite CG-120 (H^+form) and elution was carried out with deionized water. Arrows indicate the elution positions of authentic samples.

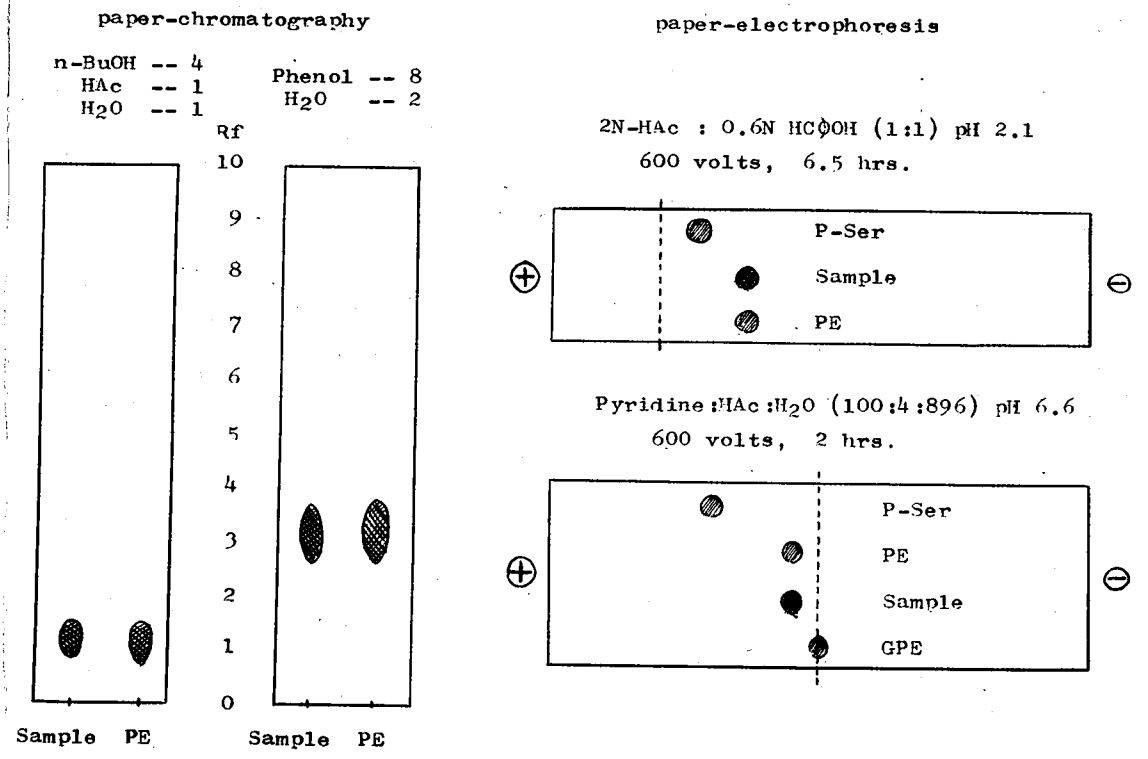

Fig. 3 Patterns of paper-chromatography and paper-electrophoresis of a compound isolated in the 2nd peak of the chromatogram of Fig. 2 .

PROPERTIES OF ACETONE INSENSITIVE PHOSPHATASE FROM ASCITES HEPATOMA OF RAT

MASAYORI INOUYE

PROPERTIES OF ACETONE INSENSITIVE PHOSPHATASE FROM ASCITES HEPATOMA OF RAT*

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Enzymatic reaction in which ethanolamine is produced from phosphoethanolamine occurs in homogenates of ascites hepatoma of rat and of normal rat liver (1,3). The activities in the two preparations differ in that the reaction is inhibited by the presence of organic solvents in 50 per cent concentration in normal liver homogenates but not in hepatoma homogenates (1-3).

In the present work the enzyme which hydrolyzes phosphoethanolamine has been partially purified from rat ascites hepatoma. Some properties of this enzyme and the effect of organic solvents on its activity have been studied. It was found that this enzyme is the same enzyme known as non-specific alkaline phosphatase. Whereas it was active toward phosphoethanolamine even in the presence of organic solvents, it was markedly supressed by organic solvents in its activity toward β-glycerophosphate, the usual substrate of alkaline phosphatase. Furthermore, the level of the enzymatic activity and the effect of acetone on the activity of preparations from hepatoma cells, regenerating rat liver, and normal rat liver were compared.

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* A part of this work was presented at the 9 th Kinki Local Meeting of the Japanese Biochemical Society held in Nara in June, 1962.

MATERIALS AND METHODS

Rats-----Male Sprague-Dawley strain rats weighing approximately 150 g. were used.

Hepatoma-----Ascites hepatoma cells, AH 130, were harvested by laparotomy 8 to 10 days after transplantation and washed with cold isotonic saline.

Regenerating Liver-----Rats were partially hepatectomized as described in the previous paper (1). Livers, 48 hours after the operation, were used.

Homogenization-----Normal liver and regenerating liver were perfused with cold isotonic saline and homogenized in 3 volumes of saline with a Teflon-pestled Potter homogenizer. Hepatoma cells were also homogenized as described above. Ten ml. of homogenates were dialyzed overnight against about 2 liters of deionized water at 3°C, befor enzyme assay.

Enzyme Assay-----Activity was estimated from the liberated inorganic phosphate by the method of Fiske-SubbaRow (4).

The usual assay system consisted of 0.3 ml. of the enzyme solution, 0.1 ml. of 0.1 M substrate, 0.1 ml. of 10^{-2} M $MgCl_2$ and 0.5 ml. of 0.1 M veronal-carbonate buffer, pH 9.45, in a total volume of 1.0 ml.. The reaction was started by adding the enzyme solution, and the mixture was incubated at 37°C. The reaction was stopped by adding 1 ml. of 10 per cent trichloroacetic acid, and the mixture was centrifuged. One ml. of the supernatant was taken for determination of inorganic phosphate. The amount of inorganic phosphate liberated was corrected for

the amount liberated from the enzyme preparation in the absence of substrate. When inhibitor was used, 0.1 Ml. of inhibitor solution was added instead of $MgCl_2$. In the experiments with organic solvents, 1 ml. of organic solvent or of organic solvent plus water was added to 1 ml. of the reaction mixture described above; for instance, 0.5 ml. of solvent and 0.5 ml. of water were added to 1 ml. of the reation mixture to make the final concentration of 25 per cent solvent. Since the color yield in phosphate estimation changed according to organic solvents involved in the reaction mixture, the amount of inorganic phosphate liberated was corrected for each case. Phosphoethanolamine and β-glycerophosphate were used as substrates. One unit was defined at the amount of enzyme which liberates 1 μmole of inorganic phosphate per hour under the conditions described above. The specific activity was expressed as units per mg. of total nitrogen or μg. of phosphate of DNA.*** Nitrogen contents were determined by the micro Kjeldahl method. DNA contents were estimated according to the method of Schneider (5).

Materials-----Phosphoethanolamine was synthesized by the method of Schmidt (6) (mp. 233-4°C, uncor.), and proved to be paper-chromatographically pure.

Partial Purification of the Enzyme from Ascites Hepatoma Cells ----The enzyme was extracted from the homogenate of tumour cells with use of n-butanol as described for alkaline phosphatase by Morton (7) as follows: Half volume of n-butanol

*** The following abbreviations are used: DNA; deoxyribonucleic acid, EDTA; ethylenediamine tetraacetic acid, PCMB; p-chloromercuribenzoate.

was added to the homogenate of tumour cells, and the mixture was incubated at 37°C for 1.5 hours with occasional stirring. After incubation, the mixture was centrifuged at 4,000 r. p. m. for 15 minutes, and the residue was extracted twice with water. The supernatants were collected, and the n-butanol layer was removed with a pipet (Fr. I in Table I). Cold acetone was added to the supernatant, and the precipitate formed between 25 to 50 per cent of acetone was collected and dissolved in a small amount of water (Fr. II in Table I). Before the enzymatic activity was assayed, the fractions were always dialyzed two times against about 50 volumes of deionized water at 3°C. About 13 fold purification was achieved. The procedure for purification is summarized in Table I.

(Table I)

RESULTS

pH-Activity Curves-----Fig. 1 shows pH-activity curves with use of the homogenate of tumour cells. Activities at each pH are expressed as per cent of the activity at pH 9.40 measured with β-glycerophosphate as substrate. As shown in Fig. 1, the pH optima are 9.4 for both phosphoethanolamine and β-glycerophosphate under the conditions used. Similar results were obtained for the enzyme partially purified from tumour cells. At acidic pH, 5.8 and 4.5, the activities, measured with phosphoethanolamine, were only 0.45 and 0.76 per cent, respectively, of the activity at pH 9.40. These facts suggest that the enzyme

hydrolyzing phosphoethanolamine in the homogenate of tumour cells (3) is what is known as non-specific alkaline phosphatase.

(Fig. 1)

Effect of Various Compounds on the Reaction-----With use of the partially purified enzyme from tumour cells (Fr. I in Table I), the effect of magnesium ion and various other compounds on the reaction were investigated. As shown in Fig. 2, magnesium ion acts as activator of alkaline phosphatase from tumour cells, and its presence at 10^{-3} M concentration results in about 45 per cent activation for both substrates. Ratios of the activity measured with β-glycerophosphate to that with phosphoethanolamine differ considerably according to the concentration of magnesium ion (Fig. 2-A). Similar phenomena were also observed in experiments with other inhibitors, as shown in Table II.

(Fig. 2)

(Table II)

It can be seen in Table II that EDTA and potassium cyanide markedly inhibit both activities of the enzyme. Zinc ion at

10^{-3} M inhibits about 90 per cent of the activity for both substrates. Cadmium ion is also inhibitory, whereas calcium ion is without effect, under the conditions used. PCMB does not inhibit the activities at 10^{-3} M. Ethanolamine and glycine stimulate the enzymatic activities. These results, mentioned above, are similar to those obtained for several alkaline phosphatases from different species (8-12).

Effect of Acetone on the Reaction-----The time curves of the reaction in the presence or absence of 50 per cent acetone are shown in Fig. 3, with use of the enzyme of Fr. I in Table I. It can be seen in Fig. 3-A that the activity measured with phosphoethanolamine is scarecely affected by 50 per cent acetone up to 40 minutes incubation, while the activity measured with β-glycerophosphate is markedly suppressed by 50 per cent acetone and is completely inhibited after 20 minutes of incubation. In order to determine wthether there are two kinds of alkaline phosphatases, acetone-sensitive and acetone-insensitive, the following experiment was undertaken: The enzyme solution was

(Fig. 3)

incubated at 37°C in the presence of 50 per cent acetone and 10^{-3} M of Mg^{++} without added substrate, and at intervals of 5 minutes 1 ml. of the solution was taken and added to 0.1 ml. of 0.1 M substrate. After 9 minutes incubation at 37°C, inorganic phosphate liberated was measured as mentioned in Methods.

(Fig. 4)

The results are shown in Fig. 4. In the case of phosphoethanolamine the activity gradually decrease just as in the case of β-glycerophosphate. By 20 minutes preincubation in the presence of 50 per cent acetone without added substrate, the activities were almost completely suppressed for both substrates. Furthermore, no inorganic phosphate was liberated from phosphoethanolamine, when it was added to the reaction mixture with use of β-glycerophosphate in 50 per cent acetone after 20 or 30 minutes incubation (broken line in Fig. 3-B). These facts suggest that phosphoethanolamine has a protective effect on the enzyme protein, not possessed by β-glycerophosphate.

The enzyme solution was incubated for 30 minutes at 37°C in 50 per cent acetone in the absence of substrate and then centrifuged. The precipitate was dissolved in a small amount of water, and this solution was assayed after overnight dialysis at 3°C against 50 volumes of deionized water. The recovery of the activities for phosphoethanolamine and β-glycerophosphate were 95.3 and 81.6 per cent of the original activities, respectively. Therefore, inactivation by acetone appears to be reversible.

Effect of Various Organic Solvents-----Fig. 5 shows the effects of various organic solvents on the reaction with use of phosphoethanolamine as substrate. It can be seen that dioxan, acetone and dimethylformamide increase the enzyme activity. Notably, about 80 per cent increase in activity was obtained with 30 per cent dioxan. In contrast to ribonuclease (13), which is activated by dimethylformamide and 2-chloroethanol, the enzyme of the present study was inhibited markedly by dimethylformamide at 50 per cent concentration and by 2-chloroethanol at much lower concentration. n-Propanol significantly increases the activity of the enzyme toward phosphoethanolamine and ethanol has a similar effect to a lesser extent in contrast to their inhibitory effect on ribonuclease (13). Methanol shows increase of inhibition with increase of concentration. The effect of chain length of alcohols on inhibition or activation was previously observed with ribonuclease (13) and the enzyme of the present study (2).

(Fig. 5)

Fig. 6 represents the effect of various organic solvents on the reaction with use of β-glycerophosphate as substrate. In contrast to the results obtained with phosphoethanolamine, all solvents used show increase of inhibition with increase of their concentration.

(Fig. 6)

Effect of Acetone on the Reaction in the Case of Regenerating Liver and Normal Liver of Rat-----Fig. 7 shows the effect of acetone on the activity of alkaline phosphatase from regenerating liver and normal liver of rat. The enzyme was partially purified by the same method described for alkaline phosphatase from tumour cells (see in "Methods"). About 13 and 4 folds purification was achieved for regenerating and normal liver, respectively.

(Fig. 7)

The similar results as for alkaline phosphatase from tumour cells (Fig. 3) were obtained for those from both regenerating and normal liver. Namely, 50 per cent acetone scarcely affected the hydrolysis of phosphoethanolamine but inhibited markedly the hydrolysis of β-glycerophosphate.

Alkaline Phosphatase Activity of Tumour Cells, Regenerating Liver and Normal Liver of Rat-----As shown in Table III,

(Table III)

the enzyme activity refered to DNA-P and total nitrogen was about 5 and 18 times, respectively, greater in tumour cells than in normal rat liver. The activity of regenerating liver was also larger than that of normal liver as already reported (14-16).

DISCUSSION

In previous papers (1-3), it was revealed that an enzymatically catalysed reaction for the liberation of ethanolamine occurs in homogenates of rat ascites hepatoma cells and normal rat liver, and that this reaction occurs in the homogenate of tumour cells even in the presence of 50 per cent acetone but not in the homogenate of normal liver. Furthermore, the substrate of the reaction was shown to be phosphoethanolamine (3). In the present paper, several properties of the reaction were investigated with use of the enzyme partially purified from tumour cells. From the fact that pH optimum of the reaction was 9.4, which was the same as that measured with β-phycerophosphate as substrate, and the results of experiments with various activators and inhibitors, it is concluded that the enzyme is the same as the enzyme known as non-specific alkaline phosphatase.

As shown in Figs. 3 and 7, 50 per cent acetone scarcely inhibited the hydrolysis of phosphoethanolamine by enzymes prepared from regenerating liver and normal liver as well as by the enzyme prepared from tumour cells. Therefore, the difference between tumour cells and normal liver, as mentioned

above, is not due to a difference in the acetone insensitive property of the enzyme, but may be due to differences in the contents of alkaline phosphatase and its substrate, phosphoethanolamine, in the two tissues. In fact, the content of phosphoethanolamine in ascites hepatoma, AH 130, was about 8 times as much as that of normal rat liver (3), and alkaline phosphatase activity of tumour cells was also 18 times as much as that of normal liver on the base of total nitrogen (Table III).

It was found in the present paper that alkaline phosphatase was quite active in several organic solvents, of even activated by these solvents, when phosphoethanolamine was used as substrate. But 2-chloroethanol and dimethylformamide, especially the former, inhibited the activity, in contrast to the marked activation of ribonuclease by these solvents as reported by Elödi (13). Therefore it is thought that the folding of the enzyme protein, caused by dimethylformamide and 2-chloroethanol as reported on ribonuclease by Yang et al. (17) and Webb et al. (18) is not suitable for the enzymatic action of alkaline phosphatase. Since organic solvents may affect physicochemical properties of the enzyme protein, the mechanism of the activation by organic solvents will be unknown until the changes of the enzyme protein caused by solvents are elucidated.

It is also interesting that alkaline phosphatase activity was markedly suppressed by all solvents examined, when β-glycerophosphate was used as substrate, in contrast with phosphoethanolamine. The causes of this difference are under investigation.

SUMMARY

1. The properties of the enzymatic hydrolysis of phosphoethanolamine were examined with use of the enzyme partially purified from AH 130 ascites hepatoma of rat.

2. From its pH optimum and experiments with various activators and inhibitors, it was concluded that the enzyme is the same as non-specific alkaline phosphatase.

3. The effect of various organic solvents on the activity was investigated. It was found that the hydrolysis of phosphoethanolamine was activated by several organic solvents, but that the hydrolysis of β-glycerophosphate was markedly suppressed by all solvents examined.

4. Partially purified alkaline phosphatases from regenerating liver and normal liver of rat behaved similarly to enzyme from hepatoma cells in the presence of acetone.

5. The difference between normal liver, and regenerating liver and tumour cells of rat in the reaction of formation of ethanolamine (1-3) was discussed from the results described above.

The authors wish to thank Prof. S. Akabori of the Institute for Protein Research, Osaka University, and Prof. Y. Sakamoto of this Institute and Drs. A. Oikawa and T. Matsushima of National Cancer Center Research Institute for their continuous encouragement and interest in this work. Thanks are also due to Miss T. Miyaji for her skilled technical assistance.

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Table I

Summary of the Purification Process of Alkaline Phosphatase from Tumour Cells

The enzyme assay was carried out at 37°C with use of β-glycerophosphate and phosphoethanolamine as substrates. Each fraction was dialyzed before use. In each experiment, a time curve was constructed and the activity was estimated from an initial velocity.

Fraction	Operation	Substrate						GP/PE
		β-Glycerophosphate(GP)			Phosphoethanolamine(PE)			
		Total Activity Units	Yield(%)	Specific Activity (Units/mg. N)	Total Activity Units	Yield(%)	Specific Activity (Units/mg. N)	
Homogenate		8.99×10^4	100	18.8	8.18×10^4	100	17.1	1.10
Fr. I	Extration with n-butanol	7.03×10^4	78.3	162.	6.39×10^4	78.2	147.	1.10
Fr. II	Fractionation with acetone	6.06×10^4	66.7	238.	6.00×10^4	73.3	238.	1.00

Table II

Effect of Various Compounds on the Reaction with Use of Phosphoethanolamine and β-Glycerophosphate as Substrates

The enzyme assay was carried out at pH 9.45 and 37°C under the conditions shown in the table. The enzyme partially purified from tumour cells (Fr. II in Table I) was dialyzed overnight against 50 volumes of deionised water at 3°C befor use. Incubation was carried out for 15 minutes with use of 3.1 units of the enzyme for β-glycerophosphate.

Compounds	Concentration(M)	Inhibition(%)		GP/PE
		GP[1]	PE[2]	
None	-	-	-	1.00
EDTA	10^{-2}	73.4	79.5	1.30
	10^{-3}	29.4	45.6	1.29
KCN	10^{-2}	100.	100.	-
	10^{-3}	72.2	39.5	0.46
$ZnCl_2$	10^{-3}	90.8	93.0	1.31
	10^{-4}	78.2	66.0	0.64
$CdCl_2$	10^{-3}	5.0	24.0	1.25
	10^{-4}	-0.2	16.0	1.22
$Ca(CH_3COO)_2$	10^{-3}	0	0	1.00
NaF	10^{-1}	27.6	44.0	1.29
	5×10^{-2}	13.2	21.0	1.10
PCMB	10^{-3}	0	0	1.00
Ethanolamine	10^{-2}	-22.0	-7.0	1.14
Glycine	10^{-2}	-10.5	-10.0	1.06

1) β-glycerophosphate
2) phosphoethanolamine

Table III

Alkaline Phosphatase Activity of Ascites Hepatoma, Regenerating Liver and Normal Liver of Rat

The enzyme assay was carried as described in the text. In each experiment, a time curve was constructed and the values indicated was estimated from its initial velocity.

Tissues	No. of Experiments	β-Glycerophosphate(GP)			Phosphoethanolamine(PE)			GP/PE
		Units/μg. DNA-P	Units/mg. Total-N.	Units/g. Tissue	Units/μg. DNA-P	Units/mg. Total-N.	Units/g. Tissue	
AH 130 Ascites Hepatoma	5	0.622 ±0.096[1)]	18.8 ±4.5[1)]	275 ±45[1)]	0.565 ±0.096[1)]	17.2 ±3.6[1)]	254 ±39.8[1)]	1.08 ±0.043[1)]
Regenerating Liver	3	0.550 ±0.048	4.63 ±0.48	112 ±7.4	0.483 ±0.051	4.07 ±0.42	98.0 ±6.7	1.14 ±0.014
Normal Liver	4	0.132 ±0.047	1.04 ±0.25	25.6 ±6.9	0.108 ±0.092	0.85 ±0.27	20.8 ±6.8	1.27 ±0.158

1) Standard deviation

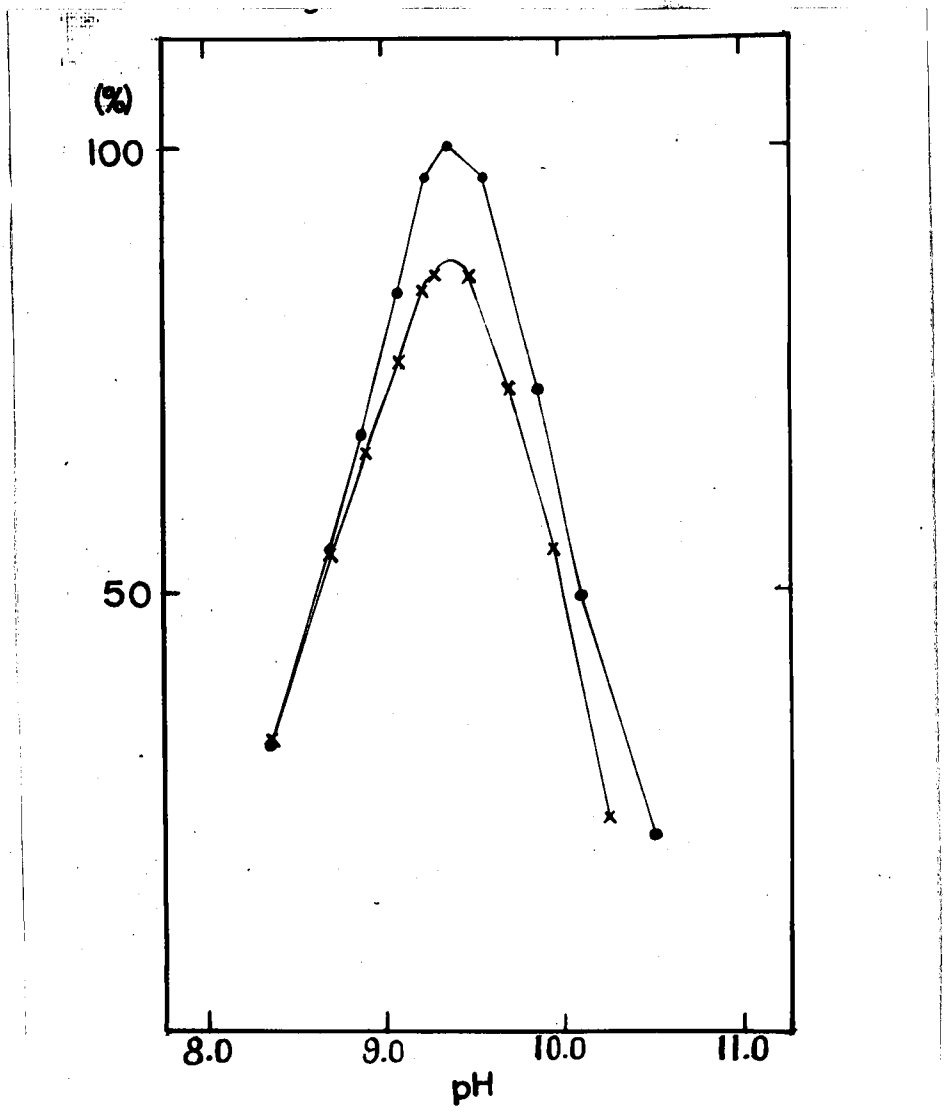

Fig. 1. pH-activity curves in the case of homogenate of tumour cells. The enzyme assay was carried out as described in the text. Activities indicated are expressed as per cent of the activity at pH 9.45, measured with β-glycerophosphate as substrate.

——o—— : Activities measured with β-glycerophosphate as substrate

——×—— : Activities measured with phosphoethanolamine as substrate

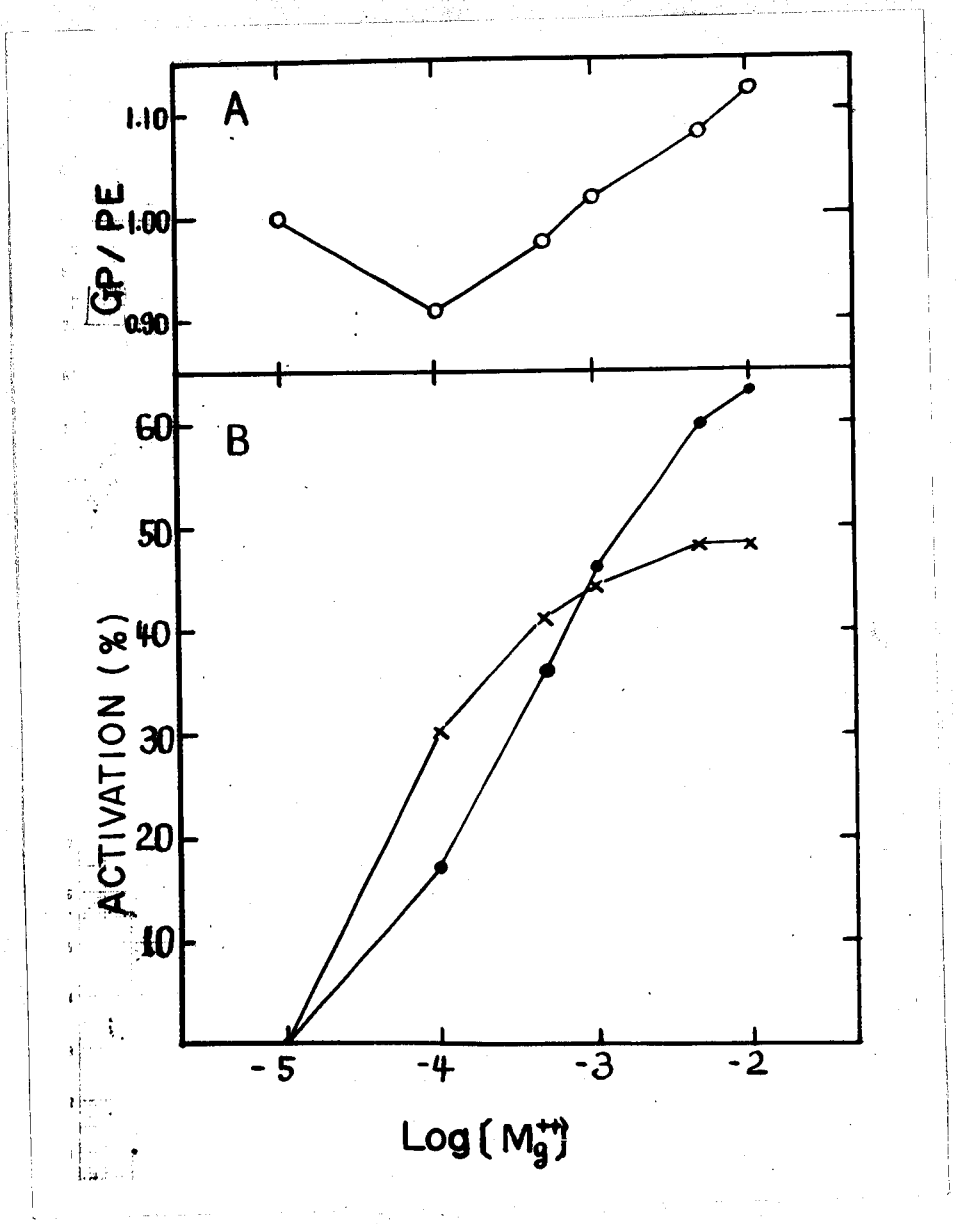

Fig. 2. Effect of magnesium ion on the reaction with phosphoethanolamine and β-glycerophosphate as substrates. The enzyme assay was carried out as described in the text.

——o—— : Activities measured with β-glycerophosphate (GP) as substrate

——•—— : Activities measured with phosphoethanolamine (PE) as substrate

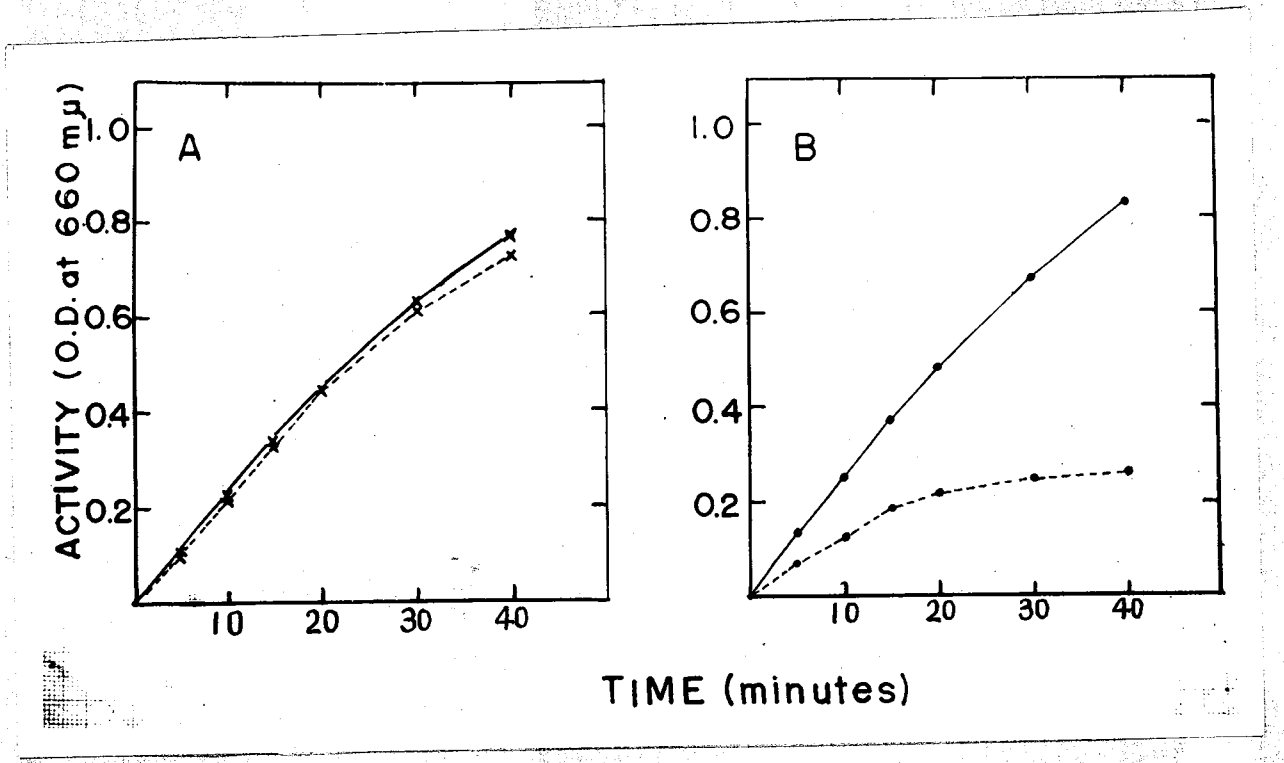

Fig. 3. Time curves in the absence and presence of 50 per cent acetone with use of phosphoethanolamine or β-glycerophosphate as substrate. The enzyme assay was carried out as described in the text. Activities were measured, A; with phosphoethanolamine, and B; with β-glycerophosphate, as substrates. Solid lines express the activities in the absence of acetone, and broken lines express the activities in the presence of 50 per cent of acetone.

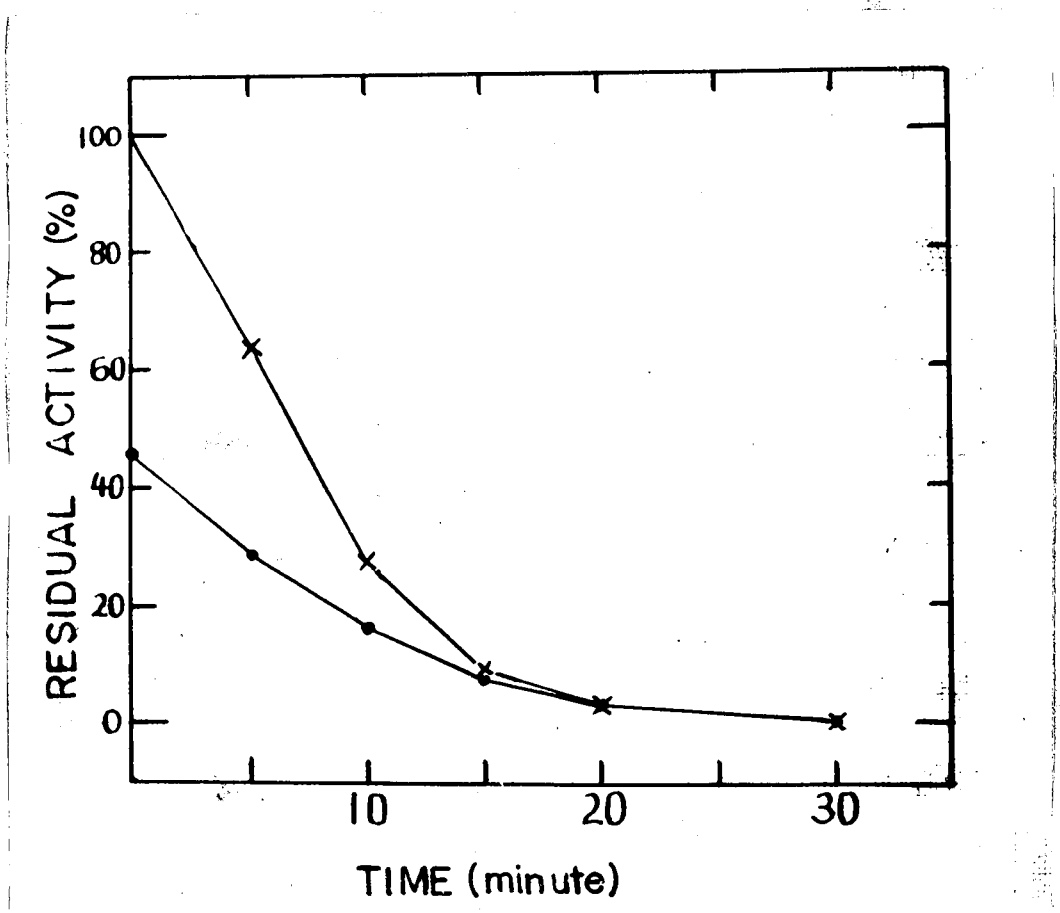

Fig. 4. Effect of 50 per cent acetone on the activities with use of phosphoethanolamine and β-glycerophosphate as substrates. The enzyme solution was incubated at 37°C in 50 per cent acetone without added substrate, and at each time indicated an aliquot was taken for the assay of residual activity as described in the text. Activities indicated are expressed as per cent of the activity at 0 time measured with phosphoethanolamine as substrate. Activity with β-glycerophosphate at 0-time preincubation corresponds to the initial velocity shown as the broken line in Fig. 3, B.

—o— : Residual activities with use of β-glycerophosphate

—•— , Residual activities with use of phosphoethanolamine

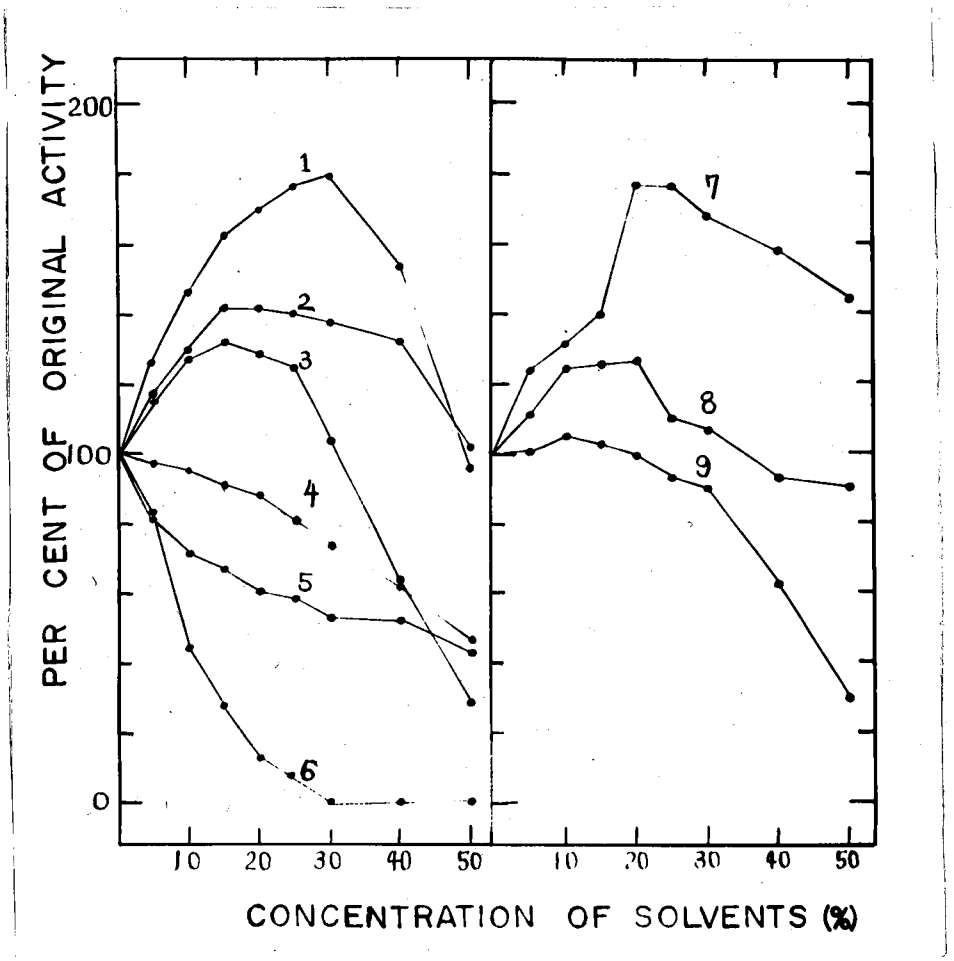

Fig. 5. Effect of various organic solvents on the reaction with use of phosphoethanolamine as substrate. The enzyme assay was carried out as described in the text. 1; activities in dioxan, 2; acetone, 3; dimethylformamide, 4; ethylene glycol, 5; glycerol, 6; 2-chloroethanol, 7; n-propanol, 8; ethanol, 9; methanol.

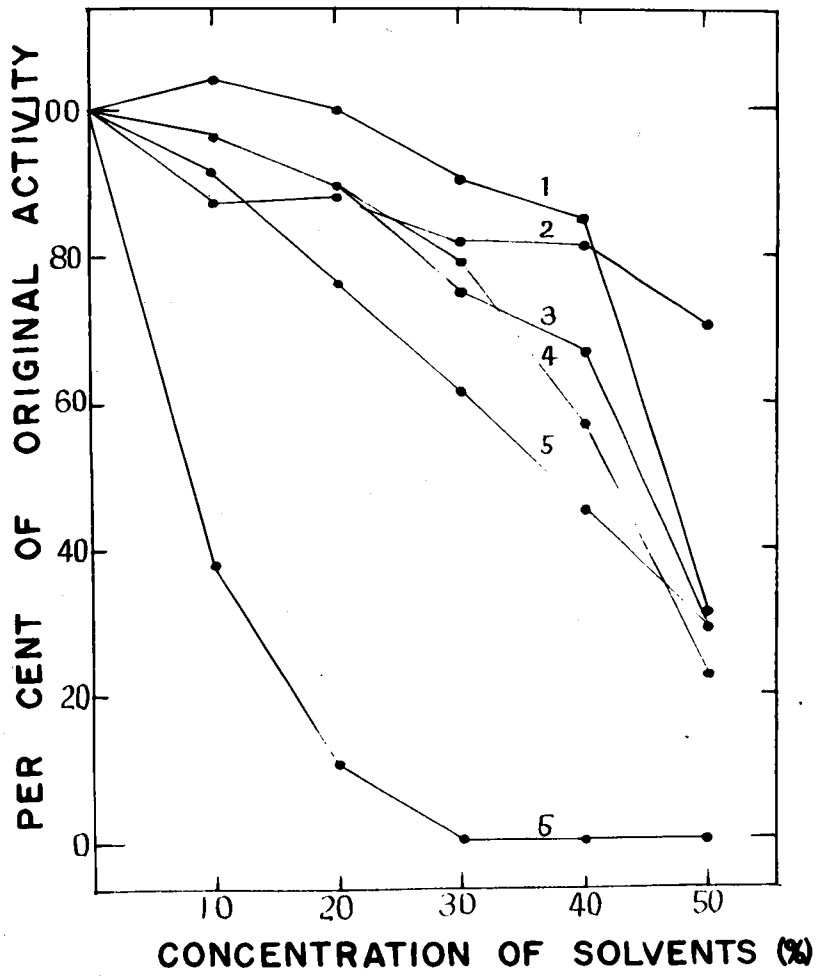

Fig. 6. Effect of various organic solvents on the reaction with use of β-glycerophosphate as substrate. The enzyme assay was carried out as described in the text. 1; Activities in dioxan, 2; n-propanol, 3; dimethylformamide, 4; acetone, 5; ethylene glycol, 6; 2-chloroethanol.

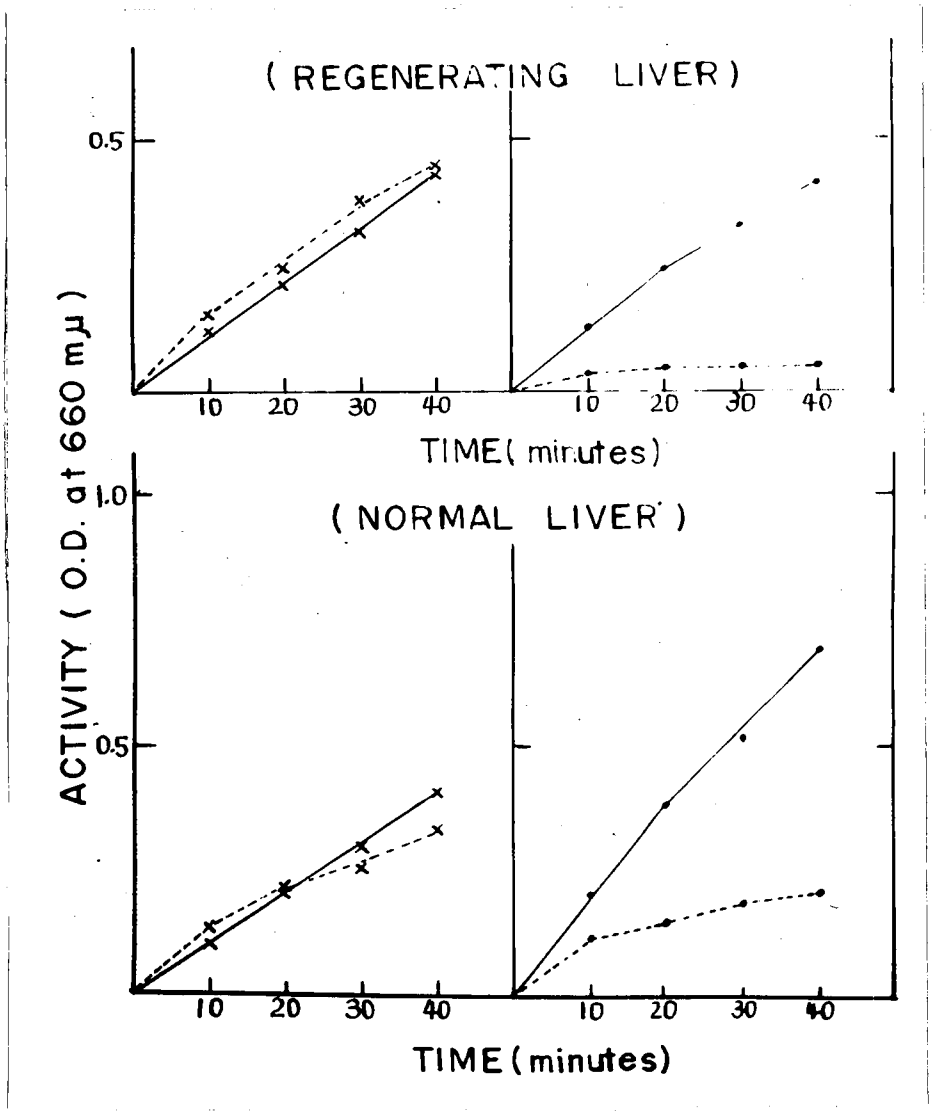

Fig. 7. Time curves in the absence and presence of 50 per cent acetone in the cases of regenerating liver and normal liver of rat. The enzyme assay was carried out as described in the text. Solid lines express the activities in the presence of 50 per cent acetone.

——o—— : Activities with use of phosphoethanolamine as substrate

——•—— : Activities with use of β-glycerophosphate as substrate

EFFECT OF ACETONE ON ALKALINE PHOSPHATASE ACTIVITY

MASAYORI INOUYE

EFFECT OF ACETONE ON ALKALINE PHOSPHATASE ACTIVITY

MASAYORI INOUYE

Alkaline phosphatase activity with phosphoethanolamine as substrate is increased by several organic solvents such as acetone, dioxan, dimethylformamide and n-propanol, while the activity with β-glycerophosphate is suppressed by these solvents (1).

In the present work, the effect of acetone on the activity with some other substrates was studied with partially purified enzyme from rat ascites hepatoma (AH 130). It was found that the activity with p-nitrophenyl phosphate was suppressed more strongly than with β-glycerophosphate not only by acetone but also by other solvents which caused activation with phosphoethanolamine (1). With phosphoethanolamine and p-nitrophenyl phosphate as substrates the kinetics of the reaction was analysed in the absence and presence of acetone and the difference in the effect of acetone on the activities with the two substrates is discussed.

MATERIALS AND METHODS

Enzyme Assay —— The activities with phosphoethanolamine

(PE*), β-glycerophosphate (GP*) and phospho-DL-serine (PS*) were estimated from the inorganic phosphate liberated during the reactions as described in the previous paper (1), except that the whole reaction mixture was used for the estimation of inorganic phosphate, since no precipitate was formed by adding 10 per cent trichloroacetic acid to purified enzyme in the present work. Activity with p-nitrophenyl phosphate (PNPP) was assayed from the p-nitrophenol liberated. This was estimated spectrophotometrically by measuring the change in optical density at 430 mμ in 0.25 N NaOH. The usual assay system consisted of 0.1 ml. of substrate solution, 0.1 ml. of 10^{-2} M $MgCl_2$, 0.5 ml. of 0.1 M veronal-carbonate buffer, pH 9.50, and 0.3 ml. of enzyme solution in a total volume of 1.0 ml.. In experiments with organic solvents, 1.0 ml. of organic solvent and/or water was added to the reaction mixture described above, in a total volume of 2.0 ml.. After incubation at 37°C, the reaction was stopped by adding 0.5 ml. of 10 per cent of trichloroacetic acid for the activities with PE, GP, and PS or 2.0 ml. of 0.5 N NaOH for the activity with PNPP. In experiments with low concentrations of PNPP, 3.0 ml. of reaction mixture, containing the same constituents as above in the same ratio

(Fig. 1)

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* The following abbrevations are used in the present paper: PE, phosphoethanolamine; GP, β-glycerophosphate; SP, phospho-DL-serine; PNPP, p-nitrophenyl phosphate.

and the reaction was stopped by adding 1.0 ml. of 1.0 N NaOH. The color yield was corrected in both inorganic phosphate and p-nitrophenol estimations in experiments with organic solvents, since the color changed with the amount and type of solvent, added to the reaction mixture.

In each experiment, a time curve was constructed and Fig. 1 shows an example obtained using PE and PNPP as substrates in the absence and presence of acetone. As shown in Fig. 1, linear results were obtained at least during the first 15 minutes of the incubation period even in the presence of acetone.

One unit of activity is definited at the amount of enzyme which liberates 1 μmole of inorganic phosphate or p-nitrophenol per hour, when 0.01 M of PE or 0.002 M of PNPP are used as substrate in 0.001 M $MgCl_2$ at 37°C and pH 9.50.

Enzyme ——Alkaline phosphatase was partially purified from rat ascites hepatoma (AH 130) as described in the previous paper (1). In the present work, partially purified preparations, (Fr. II in Table I of the previous paper (1)) were purified further with ribonuclease, bacterial protease digestion and gel filtration through Sephadex G-75 and G-200 (Pharmacia, Uppsala), as follows: A preparation extracted with butanol and fractionated with acetone (Fr. II in Table I) obtained from 365 g. of tumour cells, was incubated with 2 mg. of ribonuclease of bovine pancreas (Sigma Chemical Co.) at room temperature for 20 hours and the digest was fractionated with acetone. A fraction precipitating between 25 and 55 per cent acetone was collected by centrifugation and dissolved in water (Fr. III in Table I). Next,

the solution was digested with 4 mg. of Nagase (bacterial protease, Nagase Industrial Co.) at neutral pH at 37°C for 2 hours. After 2 hours incubation, 4 mg. more Nagase was added and the incubation was continued for 2 hours more. To the resulting solution, acetone was added to a concentration of 60 per cent and the precipitate formed was collected and dissolved in a small amount of water. This solution was subjected to gel filtration through a Sephadex G-75 column (3.5 x 23 cm.) in 0.1 M sodium acetate. The fractions eluted in the column volume were collected and concentrated by adding acetone (0 to 60 per cent) (Fr. IV in Table I). The procedure for purifica-

(Table I)

tion is summarized in Table I. As can be seen, about 140 fold purification was achieved. Further purification using ion exchangers such as DEAE- and CM- cellulose and Duolite A2 was not successful. However, by gel filtration of Fr. IV through Sephadex G-200, a further purified preparation was obtained, as shown in Fig. 2. Fraction Nos. 33 - 38 were collected. The recovery of protein (optical density at 280 mμ) and alkaline phosphatase activity were 19.9 and 65.5 per cent, respectively. Therefore the enzyme had been purified about 3.5 fold over Fr. IV in Table I.

(Fig. 2)

Substrate specificity and pH-activity curves with PE and PNPP

as substrates are shown in Table II and Fig. 3, respectively. In the present work, Fr. IV in Table I was used for all experiments.

(Table II)

(Fig. 3)

RESULTS

Effect of Acetone on Alkaline Phosphatase Activities with Various Substrates ——Fig. 4 shows the effect of acetone on the enzyme activities with PE, PNPP, GP and SP as substrates. It can be seen that acetone activates the enzyme with PE at concentrations of 10 to 30 per cent, but not with PNPP, as already reported in the previous paper (1). In the case of SP, a slight activation is observed at concentrations of 10 to 20 per cent acetone, while the activity with PNPP is markedly suppressed and the inhibitory effect increase with

(Fig 4)

increase in the acetone concentration. Table III shows the effects of some organic solvents at concentrations of 25 per cent on the enzyme activity with PE and PNPP. It can be seen that all the solvents tested inhibited the activity with PNPP in contrast to the acti-

vity with PE.

(Table III)

Identity of the Enzyme Which Hydrolyzes PE and PNPP ——
From the results mentioned above, the question arises as to whether the same enzyme participates in the hydrolysis of both PE and PNPP. Although there was a little difference between the two pH-activity curves (Fig. 3), the ratio of the activity with PE to the activity with PNPP was constant through the purification procedure, as shown in Table I and Fig. 2. This suggests that both activities are due to the same enzyme. Fig. 5 shows the heat inactivation curve. The enzyme solution was incubated at each of the temperatures indicated in Fig. 5, at pH 9.50 for 3 minutes, and after cooling in an ice-water bath the residual activities were measured with PE, PNPP, GP and PS as sub-

(Fig. 5)

strates. Inactivation of the activities toward all substrates began from 50°C and almost all the activities with all substrates were lost at 60°C.

The kinetics of the reactions was analyzed to examine this problem further. As shown in Fig. 6, PE acts as a competitive inhibitor of the activity with PNPP. Ki, the dissociation constant of the enzyme-inhibitor complex, was calculated from Fig. 6-B, since the slopes in the presence of PE are equal to $\frac{Km}{Vmax.}\left(1+\frac{I}{Ki}\right)$,

where Km, Vmax., and I are the Michaelis constant for PNPP (1.13×10^{-4} moles/liter, obtained from the line in the absence of PE in Fig. 6-B), the maximum velocity and the concentration of inhibitor (PE), respectively. The Ki's were found to be 1.90×10^{-3} and 2.08×10^{-3} moles/liter in the presence of 0.0050 M and 0.0025 M PE, respectively. These values are in quite good agreement with the Km for PE, 1.93×10^{-3} moles/liter, which was obtained from a Lineweaver-Burk plot (3) for PE (Fig. 6-A).

(Fig. 6)

From these results, it is evident that the same enzyme or the same active site reacts with both PE and PNPP.

Effect of Substrate Concentration on the Activation and Inhibition Caused by Acetone———Fig. 7 shows the effect of substrate concentration on the activation and inhibition caused by acetone. With PE as substrate, the activation caused by acetone increases with decrease in the concentration of PE. With 0.0010 M PE, more than 70 per cent activation was observed at a concentration of 40 per cent acetone. With PNPP as substrate, the inhibition caused by acetone

(Fig. 7)

increases with decrease in the concentration of PNPP. It should be noted that the suppression of the activity with PNPP is not due to the denaturation of the enzyme protein caused by acetone, since time

curves in the presence of acetone were linear for at least the first 15 minutes of the incubation period at 37°C, as shown in Fig. 1. Therefore it is thought that acetone acts as an activator for PE and as an inhibitor for PNPP.

Fig. 8 shows Lineweaver-Burk's plots for PE and PNPP in the absence and presence of acetone. As expected from Fig. 7, the slopes decrease with increase in the acetone concentration in the case of PE, and both lines cut the vertical axis very near the point

(Fig. 8)

given in the absence of acetone (Fig. 8-A). The apparent Michaelis constants were 1.42×10^{-3}, 1.11×10^{-3}, 0.81×10^{-3} and 0.45×10^{-3} moles/liter at 0, 5, 10 and 25 per cent acetone, respectively. With PNPP as substrate, on increase in the acetone concentration, the apparent Km values increased as follows: 0.97×10^{-4}, 1.37×10^{-4}, 1.61×10^{-4} and 1.66×10^{-4} moles/liter in 0,5, 10 and 25 per cent acetone, respectively. Furthermore, intercepts on the vertical axis increased with increase in acetone concentration, which means that the apparent k_2 values, the rate constants for dissociation of the enzyme-substrate complex to the enzyme and the product, decreased.

Effect of Acetone on the Activities with PE and PNPP in the Presence of PNPP and PE, Respectively——Table IV shows the effect of acetone on the hydrolysis of PE and PNPP by the enzyme in the presence of PNPP and PE, respectively. When both subst-

rates are present in the reaction mixture, they will be hydrolyzed at the same time by alkaline phosphatase, the one substrate competitively inhibiting the hydrolysis of the other substrate, and if the change of the enzyme protein caused by a certain concentration of acetone in the presence of PE is different from the change caused by the same concentration of acetone in the presence of PNPP, the increase in the activity with PE and the inhibition of the activity with PNPP caused by acetone will decrease in the presence of both substrates. However, as can be seen in Table IV, the activity with PE was increased by acetone even in the presence of PNPP, while the activity with PNPP was inhibited by acetone even in the presence of PE. Therefore, it is thought that the effects of acetone on the enzyme activities with PE and PNPP are independent.

(Table IV)

Activation Energy in the Absence and Presence of Acetone——

Activation energies were measured by Arrenius' plots in the absence and presence of acetone for both PE and PNPP as shown in Fig. 9. Activation energies calculated from the slopes are indicated in Fig. 9. It was found that there is little difference between them in the absence and the presence of acetone with either substrate.

(Fig. 9)

DISCUSSION

In the previous paper (1), it was shown that alkaline phosphatase activity with PE as substrate was activated by several organic solvents, while enzyme activity with GP as substrate was suppressed by these solvents. The present paper shows that beside GP, the activity with PNPP is inhibited more strongly than the activity with GP by several organic solvents. This paper shows that the same enzyme or the same active site participates in the hydrolysis of both PE and PNPP, and that the suppression of the activity with PNPP by acetone is not due to denaturation of the enzyme protein, since time curves for PNPP in the presence of acetone were linear, at least for the first 15 minutes of the incubation period at 37°C (Fig. 1). Therefore acetone acts as an activator with PE and as an inhibitor with PNPP.

Kinetical analysis (Fig. 8), showed that acetone decreased the apparent Km, Michaelis constant, for PE and increased it for PNPP. This fact suggests that in the presence of acetone the affinity of PE for the enzyme increased and that of PNPP for the enzyme decreased. It was also found that the rate constant for dissociation of the enzyme-substrate complex to the enzyme and product was scarcely changed by acetone in the case of PE, while it was decreased by acetone when PNPP was the substrate.

These differences between the activities with PE and PNPP in the effect of acetone appear to be due to the same change in the enzyme protein. This change is caused by acetone in the presence of either

PE or PNPP, since similar phenomena caused by acetone were observed even in the presence of both PE and PNPP (Table IV). Although PE had more of a protective effect than GP on the inactivation caused by acetone as shown in the previous paper (1), it is unknown whether this protective effect of PE has any relation with the phenomena described above. It would also be possible to explain the phenomena as due to changes in the substrates caused by acetone, rather than as due to a change in the enzyme protein caused by acetone. In fact, the activities with compounds having amino groups such as PE and PS, were increased by acetone, in contrast to those with other compounds (Fig. 4).

Effects of organic solvents on enzyme activities have been reported in various papers (4 - 10), and Brahms et al. (10) have shown a relationship between the enzyme activity of myosin A and its conformational changes caused by organic solvents. They mentioned that the activation by solvents would be due to a partial unfolding of myosin in the region of the active site. If the same is true for alkaline phosphatase, a change in the enzyme protein caused by acetone would be inhibitory with PNPP but activatory with PE. These problems will be elucidated by examining conformational changes in alkaline phosphatase caused by organic solvents, using more purified preparations.

SUMMARY

1. The effects of acetone on the activities of alkaline phospha-

tase with various substrates were investigated. It was found that the activity with p-nitrophenyl phosphate (PNPP) was markedly suppressed by several organic solvents, which increased the activity with phosphoethanolamine (PE).

2. It was shown that the same enzyme or the same active site participates in the hydrolysis of both PE and PNPP.

3. The kinetics of the reactions with PE and PNPP were analyzed in the absence and presence of acetone. It was found that the apparent Km, Michaelis constant, was decreased by acetone with PE but was increased with PNPP.

4. From these results, the effect of acetone on alkaline phosphatase activity are discussed.

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Table I

Summary of the Purification of Alkaline Phosphatase from Ascites Hepatoma of Rat

The enzyme assay was carried out as described in the text. Each fraction was dialyzed against deionized water before use.

Fraction	Volume ml.	Substrate							GP/PE	PNPP/PE
		PE (0.01 M)			GP (0.01 M)		PNPP(0.002 M)			
		Total Act. Units	Yield %	Specific Act. Units/Total mg.N	Total Act. Units	Yield %	Total Act. Units	Yield %		
Homogenate of 565 g. tumour	1,300	9.60×10^4	100.	17.1	10.6×10^4	100.	8.72×10^4	100.	1.10	0.91
Fr.I, extraction with n-butanol	440	7.00×10^4	73.0	97.5	8.40×10^4	79.2	6.55×10^4	75.0	1.20	0.94
Fr.II, fractionation with acetone	300	6.40×10^4	66.6	134.0	7.05×10^4	66.5	6.21×10^4	71.2	1.10	0.97
Fr.III, ribonuclease digestion	93	5.28×10^4	55.0	280.0	5.81×10^4	54.8	5.01×10^4	57.5	1.10	0.95
Fr.IV, Nagase digestion and gel-filtration	24	5.00×10^4	52.1	2400.	5.45×10^4	51.3	4.75×10^4	54.3	1.09	0.95

Table II

Substrate Specificity of Alkaline Phosphatase from Ascites Hepatoma of Rat

Activities are expressed as a percentage of the activity measured with PE as substrate at pH 9.50. Activities with ATP*, AMP** and G1P*** as substrates were assayed according to the method described for glycogen phosphorylase activity (2).

Substrate	Activity at pH 9.50
0.01 M PE	100.
0.01 M GP	110.
0.01 M G1P	123.
0.01 M PS	107.
0.002 M PNPP	95.0
0.005 M ATP	6.0
0.005 M AMP	87.0

* ATP: adenosine triphosphate

** AMP: adenosine monophosphate

*** G1P : glucose-1-phosphate

Table III

Effect of Various Organic Solvents on Alkaline Phosphatase Activities with Phosphoethanolamine (PE) and p-Nitrophenyl Phosphate (PNPP)

Activities were assayed in 25 per cent (v/v) concentrations of solvents and 0.005 M PE or 0.001 M PNPP under the following conditions; 0.0005 M $MgCl_2$, at 37°C and pH 9.50. Activities are expressed as a percentage of the activity with PE as substrate.

Solvent (25%)	Substrate	
	PE	PNPP
None	100.	100.
Acetone	116.	35.0
Dioxan	134.	32.8
Dimethylformamide	105.	24.7
n-Propanol	112.	22.6

Table IV

Effect of Acetone on the Activities with PE and PNPP in the presence of both PNPP and PE

Activities with PNPP in the presence of PE were measured from the liberated p-nitrophenol as described in the text. Activities with PE in the presence of PNPP were estimated as follows; inorganic phosphate (iP) liberated from PE) = (Total iP liberated from both PE and PNPP) - (iP liberated from PNPP, which was estimated from the p-nitrophenol liberated from PNPP). Enzyme assay was carried out at pH 9.50 in 0.0005 M $MgCl_2$ in the absence and presence of acetone. Activities are expressed as a percentage of the activity in the absence of acetone.

Acetone volume %	Substrate											
	PE						PNPP					
	0.0025 M			0.0050 M			0.0005 M			0.0010 M		
	None	PNPP 0.0005 M	PNPP 0.0010 M	None	PNPP 0.0005 M	PNPP 0.0010 M	None	PE 0.001 M	PE 0.005 M	None	PE 0.001 M	PE 0.005 M
0	100. (100.)[a]	100. (28.2)[a]	100. (13.3)[a]	100. (100.)[a]	100. (43.8)[a]	100. (23.0)[a]	100. (100.)[b]	100. (93.0)[b]	100. (66.0)[b]	100. (100.)[b]	100. (93.5)[b]	100. (72.0)[b]
5	110.	103.	170.	110.	104.	125.	78.0	73.2	74.5	79.0	75.5	71.0
10	127.	159.	185.	125.	131.	144.	56.5	55.8	49.5	57.5	55.8	51.0
25	152.	215.	315.	137.	156.	221.	37.7	34.1	25.7	39.2	39.1	25.1

a) per cent of the activity in the absence of PNPP b) per cent of the activity in the absence of PE

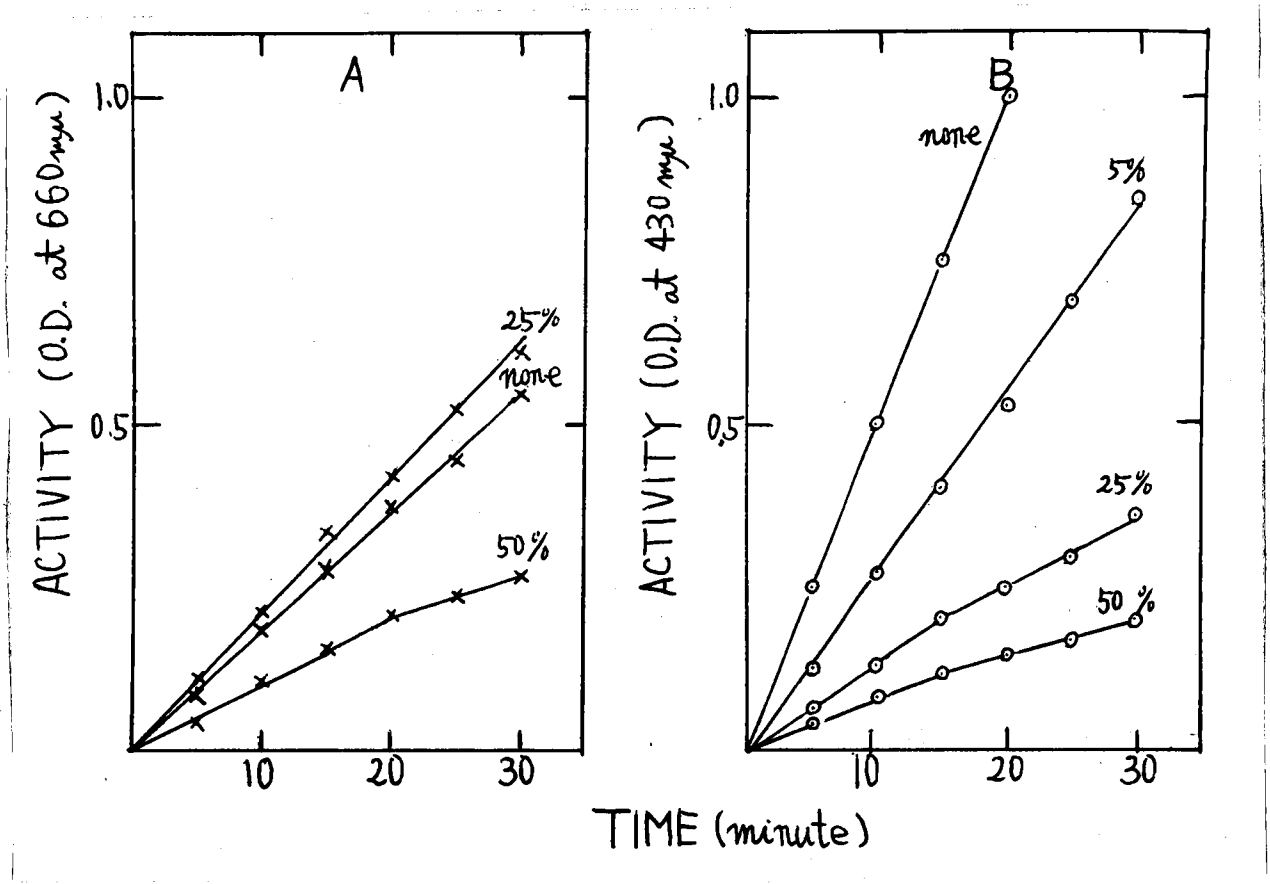

Fig. 1 Time curves of alkaline phosphatase activity in the absence and presence of acetone. Phosphatase activity was assayed as described in the text. A; PE (0.01 M) and B ; PNPP (0.002 M) were used as substrates in the absence and presence of acetone.

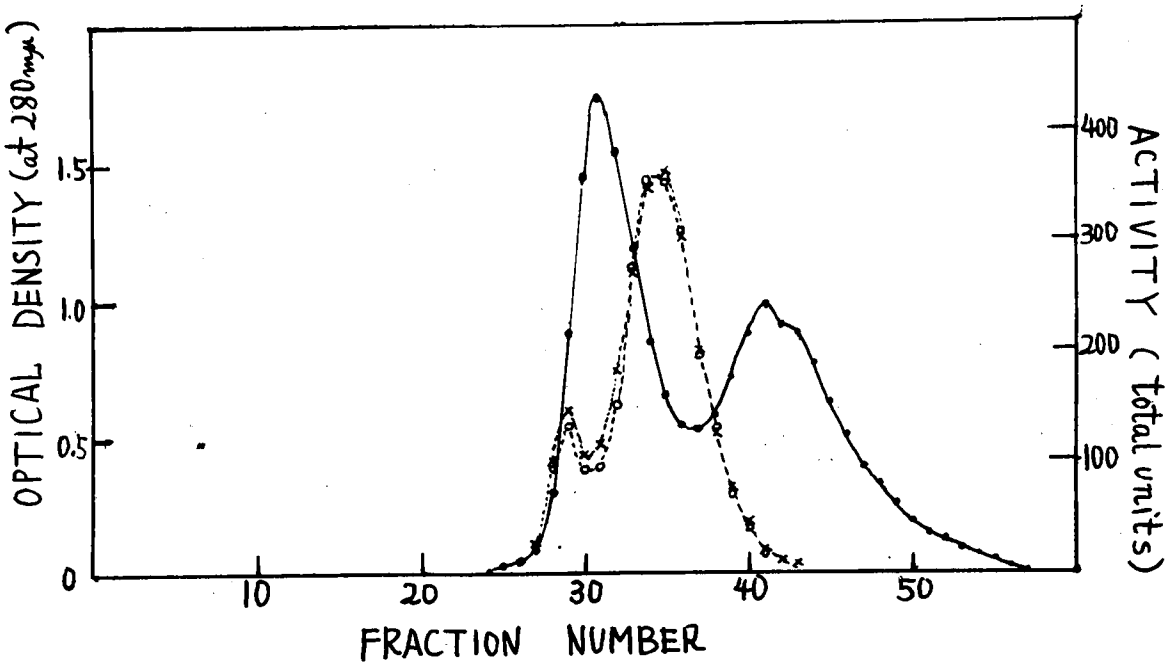

Fig. 2 Gel filtration of Fr. IV in Table I on Sephadex G-200. Two ml. of Fr. IV were applied to a column of Sephadex G-200 (3.5 x 23 cm.) in 0.1 M sodium acetate. The column was eluted with 0.1 M sodium acetate and 2 ml. fractions of effluent were collected.

•——• : absorbancy at 280 mμ

×——× : activity with PE (0.01 M)

○——○ : activity with PNPP (0.002 M)

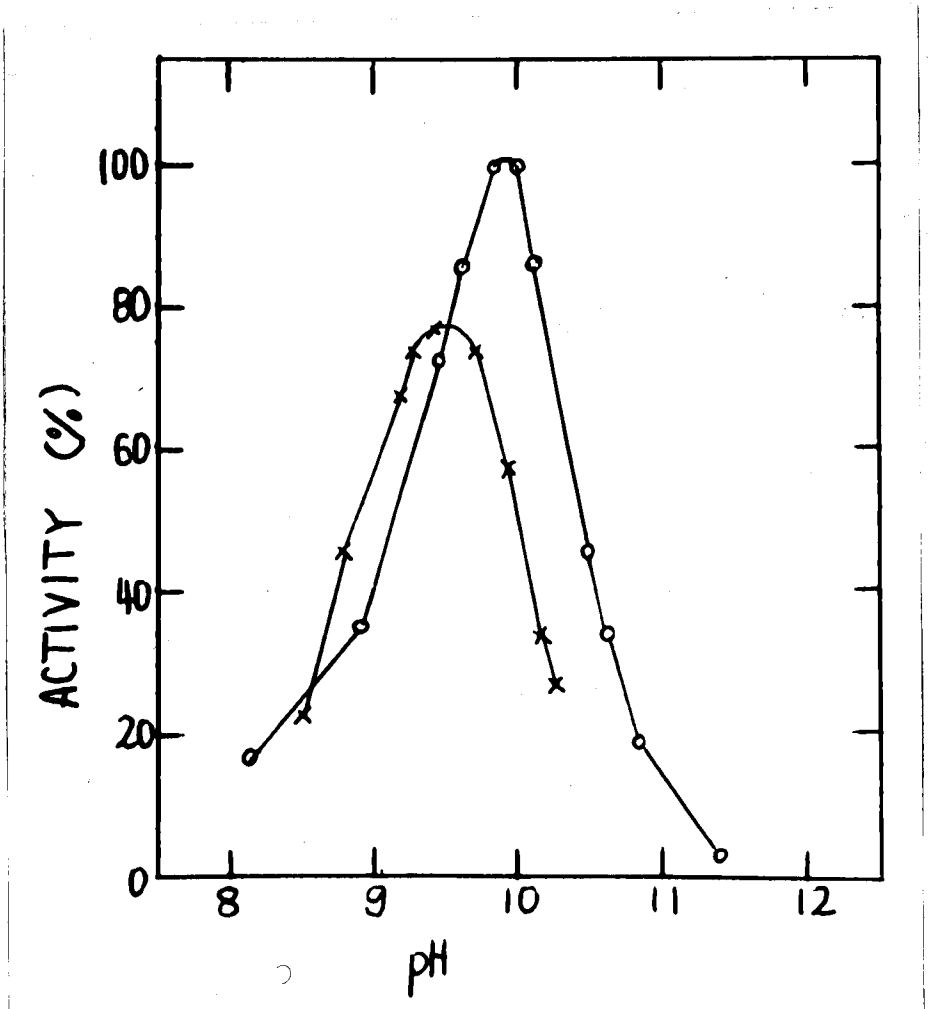

Fig. 3 pH-activity curves with PE and PNPP as substrates. Enzyme assay was carried out as described in the text. Activities indicated are expressed as a percentage of the activity at pH 10.0, measured with PNPP as substrate. ×—× : activities measured with PE (0.01 M) and o—o : with PNPP (0.002 M) as substrates.

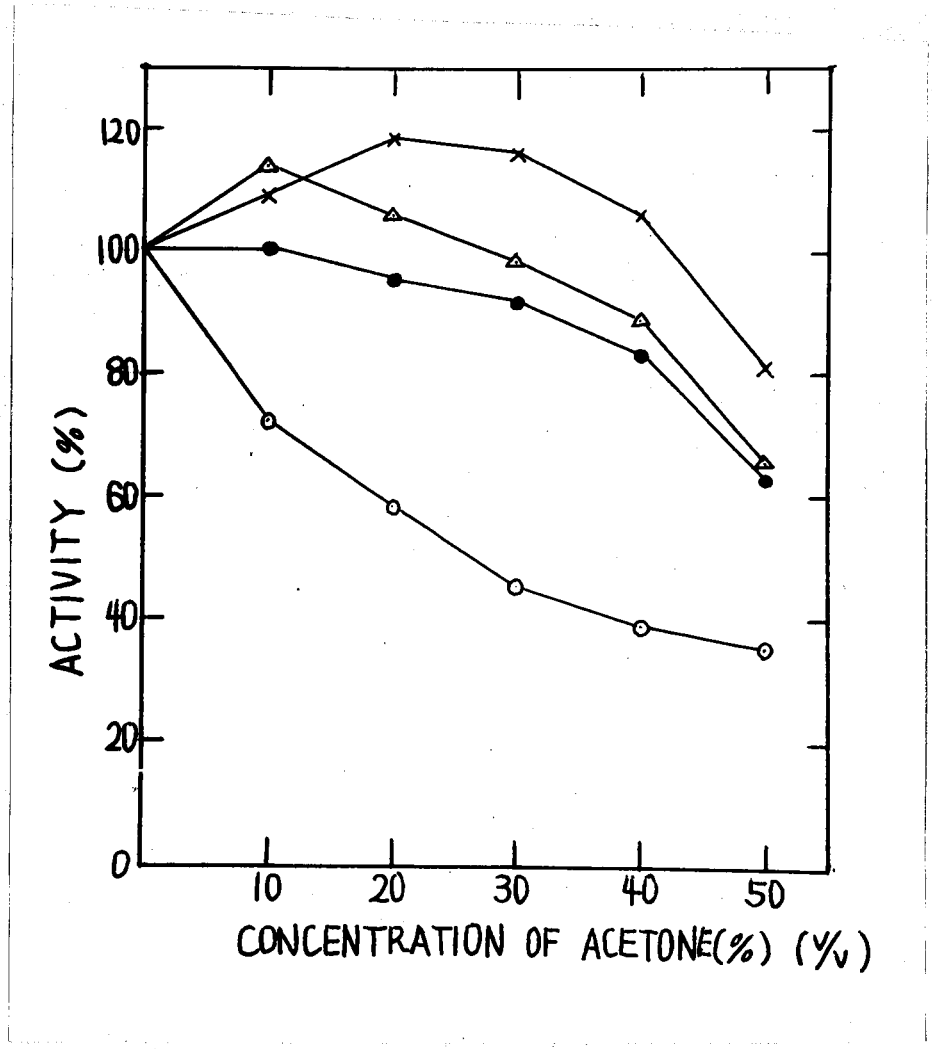

Fig. 4 Effect of acetone on alkaline phosphatase activities with various substrates. The enzyme assay was carried out as described in the text. Activities are expressed as a percentage of the activity in the absence of acetone. ×—× : PE (0.01 M), ○—○ : PNPP (0.01 M), △—△ : PS (0.01 M) and •—• : GP (0.01 M) were used as substrates.

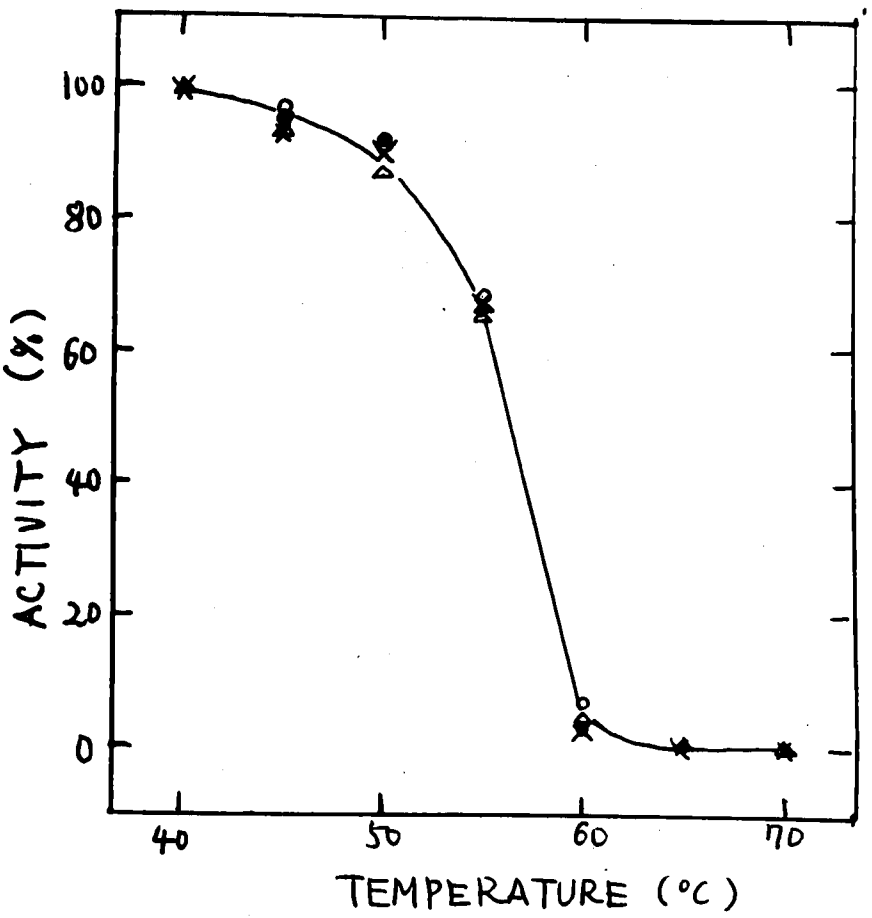

Fig. 5 Heat inactivation of alkaline phosphatase. The enzyme solution was incubated at the temperatures indicated at pH 9.50 for 3 minutes and after cooling in an ice-water bath the residual activities were assayed with ×—× PE (0.01 M), ○—○ ; PNPP (0.002 M), ●—● ; GP (0.01 M) △—△ ; and PS (0.01 M) as substrates.

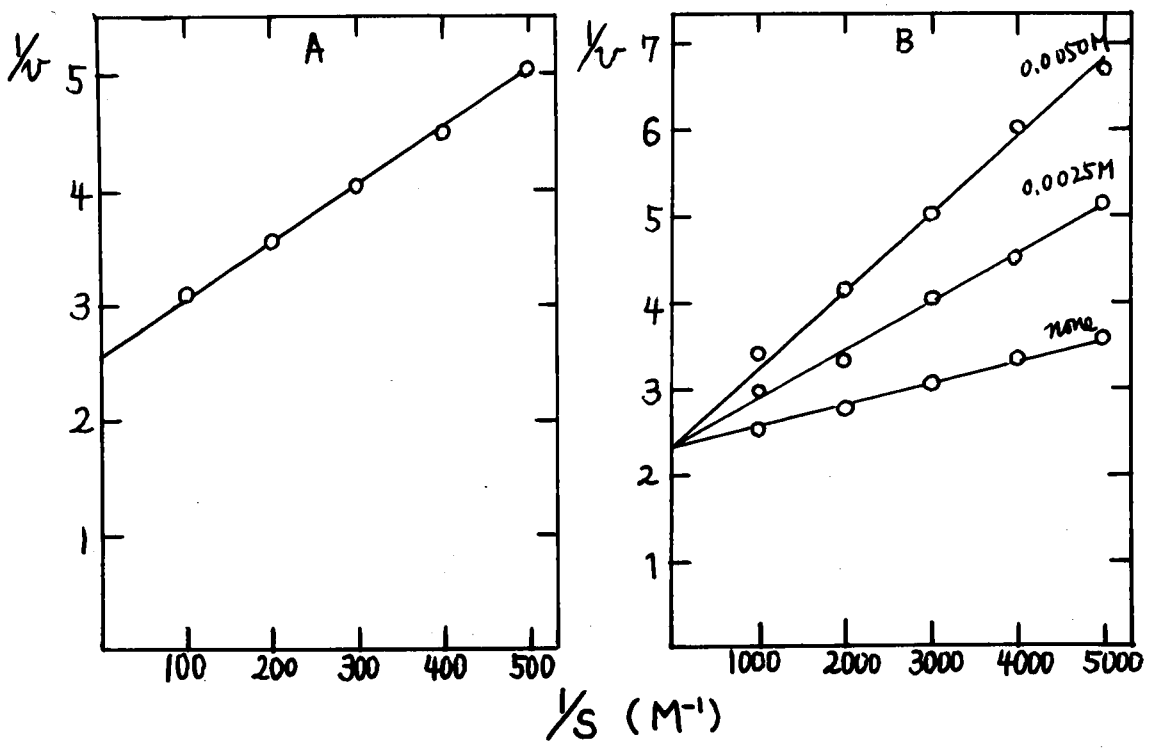

Fig. 6 Lineweaver-Burk plots with PE and PNPP in the absence and presence of PE.

A; Lineweaver-Burk plots with PE and B ; with PNPP in the absence and presence of 2.5 mM and 5.0 mM of PE at pH 9.50 in 1 mM $MgCl_2$.

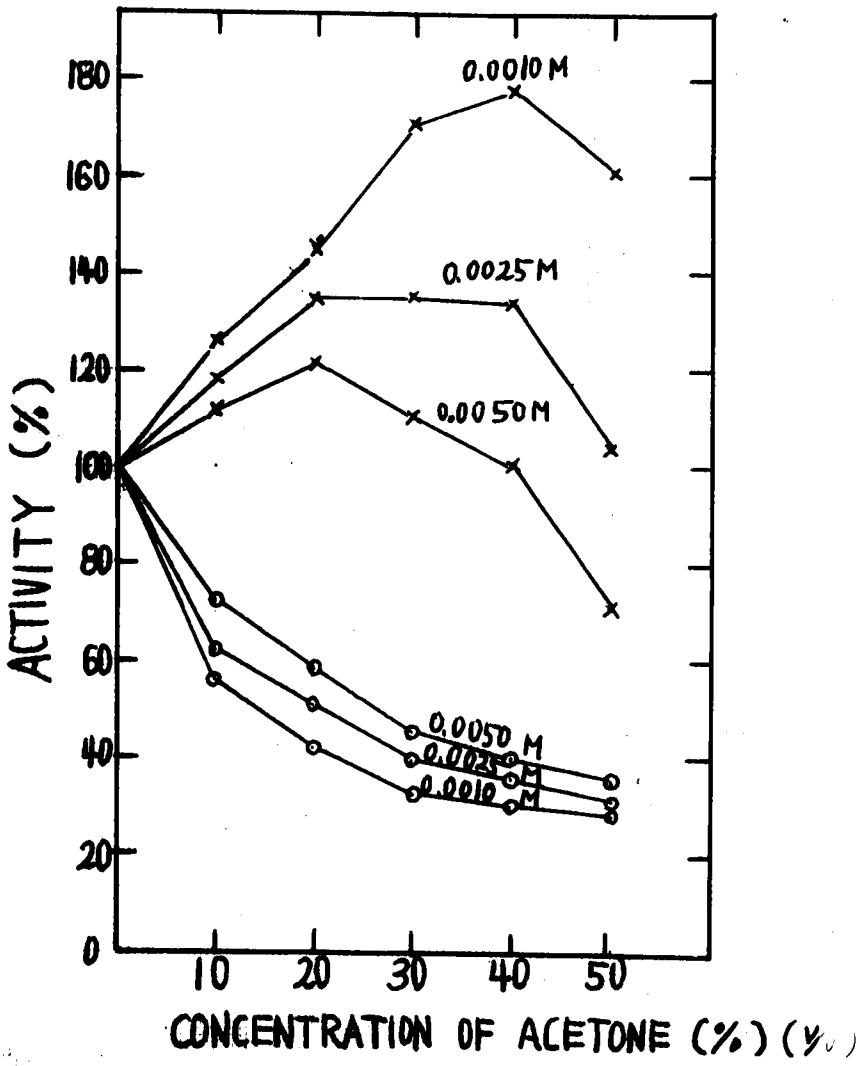

Fig. 7 Effect of substrate concentration on activation and inhibition by acetone. The enzyme assay was carried out as described in the text. Activities are expressed as a percentage of the activity in the absence of acetone. ×—× ; PE and ○—○ ; PNPP were used as substrates at the various concentrations indicated in the figure .

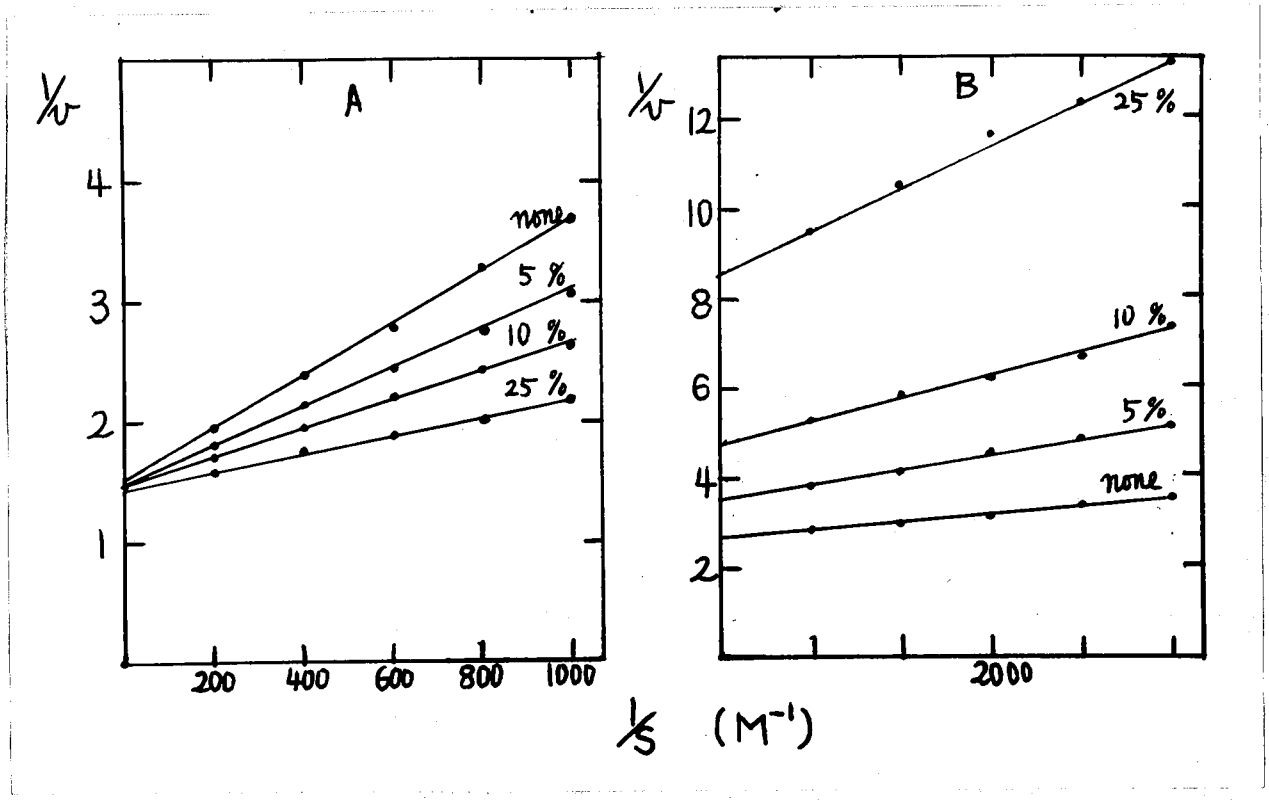

Fig. 8 Lineweaver-Burk plots with PE and PNPP in the absence and presence of acetone.

A; Lineweaver-Burk plots with PE and B; with PNPP in the absence and presence of acetone at pH 9.50 in 5 mM $MgCl_2$. The concentrations of acetone are indicated in the figure as volume per cent.

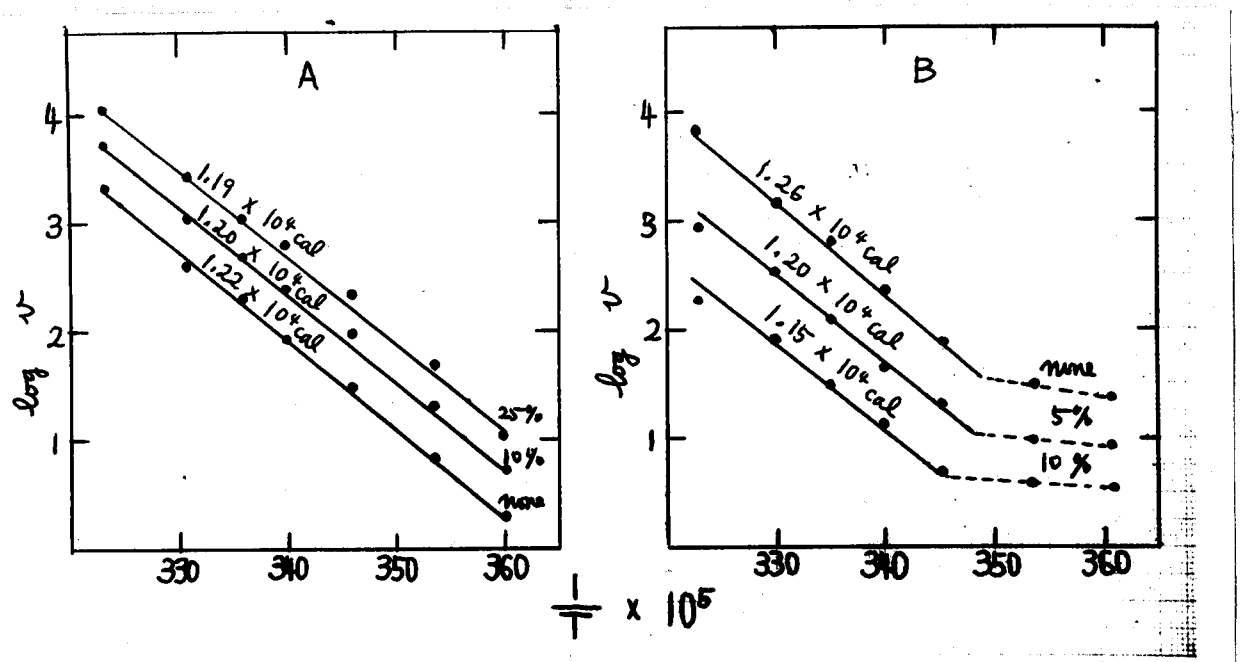

Fig. 9 Arrenius plots for alkaline phosphatase in the absence and presence of acetone.

Enzyme assay was carried out at pH 9.50 in 0.5 mM $MgCl_2$, A ; with PE (0.01 M) and B ; with PNPP (0.002 M) in the absence and presence of acetone. The concentrations of acetone are indicated in the figure as volume per cent.