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# SOLUBILIZATION AND PARTIAL PURIFICATION OF ALKALINE PHOSPHATASES OF SARCOMA 180/TG ASCITES CELLS

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## SUMMARY

A procedure for the solubilization of particulate-bound alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from Sarcoma 180/TG ascites tumor cells, and methods for their partial purification are described; the employment of these procedures resulted in the isolation of two distinct alkaline phosphatase activities. Both of the alkaline phosphatase enzymes are non-specific phosphomonoesterases which require $Mg^{2+}$ for maximal activity and exhibit optimum reaction velocities around pH 9.5. However, the two enzymes differ significantly in substrate specificity, degree of $Mg^{2+}$ activation, and electrophoretic mobility on polyacrylamide gels. The two enzymes are inhibited by $Be^{2+}$, several chelating agents, and $Zn^{2+}$ at relatively high concentrations, while L-phenylalanine is without major effect on either phosphatase.

## INTRODUCTION

The purine analogs, 6-thioguanine and 6-mercaptopurine, are effective agents in the treatment of acute leukemia of man. However, one of the factors which limits the usefulness of these drugs in these diseases is the acquisition of insensitivity. Despite more than a decade of extensive investigation, the biochemical mechanism(s) by which leukemic cells of man achieve resistance has not yet been fully delineated.

6-Thiopurines are known to require conversion to the nucleotide level for tumor inhibitory activity [1]. In both transplanted animal tumors and microorganisms, the most prevalent biochemical alteration responsible for resistance to these agents is loss or marked decrease in activity of guanine-hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) [1]; however, in human leukemic leukocytes this would appear to be an infrequent mechanism [2–4]. An alternate candidate mechanism was described in an experimental model system, a subline of the murine ascitic neoplasm Sarcoma 180 resistant to 6-thiopurines (Sarcoma 180/TG) [5]. In this system, evidence is available to support an increased rate of degradation of 6-thioguanine 5'-phosphate [6, 7]. The enzymatic basis for the increased rate of degradation of 6-thioguanine nucleotides in Sarcoma 180/TG was provided by the studies of Wolpert et al. [8] who found that cell-free extracts of this neoplasm possessed markedly increased levels of alkaline phosphatase (orthophosphoric mono-

ester phosphohydrolase, EC 3.1.3.1) activity relative to the parent sensitive line, and suggested that this enzyme was responsible for the increased catabolism of the nucleotide forms of the thiopurines, and the consequent insensitivity of the resistent subline to these antimetabolites. Wolpert et al. [8] further observed that more than 95% of the alkaline phosphatase activity of Sarcoma 180/TG was associated with insoluble cellular particulate matter.

The present investigation has been undertaken in an effort to obtain the phosphatase in purified form for more detailed studies on the role of this enzyme in the resistance of Sarcoma 180/TG to 6-thiopurines. A procedure for solubilization of the enzymes and preliminary results on the isolation and partial purification of two alkaline phosphatases from particulate preparations are described. Evidence that the two isolated alkaline phosphatase enzymes are distinct in several characteristics is also presented.

## MATERIALS AND METHODS

### *Materials*

Tris, *p*-nitrophenylphosphate, CMP, *β*-naphthyl acid phosphate, Fast Blue, 1,10-phenanthroline, and EDTA were purchased from Sigma Chemical Co. Other nucleotides were obtained from P-L Biochemicals, Inc. DEAE (Whatman DE-32) was purchased from W and R Balston Ltd. Other chemicals and organic solvents were of analytical grade.

### *Source and transplantation of cells*

Female CD-1 Swiss mice, 9–12 weeks of age, were obtained from Charles River Breeding Laboratories. Methods for the transplantation of Sarcoma 180 ascites cells in mice, and the development and maintenance of the resistant subline, Sarcoma 180/TG, were described previously [5].

### *Enzyme assays*

Unless otherwise indicated enzyme activity was measured at 25 °C by determining the initial rate of hydrolysis of *p*-nitrophenylphosphate using the change in absorbance at 410 nm with a Gilford thermostated recording spectrophotometer. The reaction mixture contained 3.0 ml of 1.0 M Tris–HCl (pH 9.2), except when otherwise specified, and $10^{-3}$ M substrate; the reaction was initiated by the addition of an appropriate amount of enzyme. 1 unit of activity is expressed as the hydrolysis of 1 nmole of substrate per min under the conditions employed, based on a molar absorbance of *p*-nitrophenol in the buffers used of $1.7 \cdot 10^4$ moles$\cdot l^{-1} \cdot cm^{-1}$. Specific activity refers to the nmoles of $P_i$/min per mg of protein. Protein was determined by the method of Lowry et al. [9].

### *Determination of inorganic phosphate*

Inorganic phosphate was determined according to the method of Fiske and SubbaRow [10].

### *Disc electrophoresis*

Electrophoresis was carried out according to the procedure of Davis [11].

Activity staining of the enzyme was based on the method described by Smith et al. [12] except that enzyme location buffer was substituted by 0.1 M $NaHCO_3$ buffer, pH 9.4, containing $5 \cdot 10^{-3}$ M $MgCl_2$.

## RESULTS

### *Solubilization and separation of alkaline phosphatases*

*Preparation of cell-free sonicated extracts.* Ascites tumor cells were collected through an incision in the peritoneal wall 7–9 days after implantation. Cells were rinsed with 0.9% cold NaCl into beakers chilled in ice. After filtration through cheese cloth, cells were collected by centrifugation (6000 × *g*, 5 min). Contaminating erythrocytes were lysed by suspending the tumor cells in cold 0.2% NaCl; an equal volume of cold 1.6% NaCl solution was then added to bring the concentration of NaCl in the medium to 0.9% before centrifugation (8000 × *g*, 5 min). This step was repeated three times until no erythrocytes could be detected visually. Neoplastic cells were then suspended in 4 vol. of distilled water and disrupted sonically with three 30-s bursts using a Branson sonifier. Concentrated Tris–HCl, pH 7.6, was added to bring the final concentration in the extract to 0.01 M.

*Solubilization of alkaline phosphatases.* It was reported earlier [8], and confirmed in the present investigation, that about 95% of cellular alkaline phosphatase activity of Sarcoma 180/TG ascites cells is associated with the cell-free particulate fraction sedimenting after centrifugation at 105 000 × *g* for 3 h. Enzyme activity was not solubilized by several techniques, such as treatment with sodium laurylsulfate, toluene, Triton X-100, Lubrol, sodium deoxycholate, and saponin. Alkaline phosphatase activity was also not released by treatment with proteolytic enzymes (i.e. trypsin, chymotrypsin, and protease) or by treatment with other enzymes (i.e. deoxyribonuclease, lysozyme and lipase). Furthermore, the enzyme could not be extracted with 1 M $Mg^{2-}$ [13, 14]. However, solubilization of activity was achieved by incubation of the sonicated suspension at 37 °C for 20 h with occasional stirring by hand. During this incubation period, enzyme activity was released into the supernatant fraction and the emulsion-like suspension separated into a clear supernatant fraction and a precipitate. The solubilized enzyme was then separated from the residue by centrifugation at 17 000 × *g* for 20 min. The residual particulate matter retained residual enzymatic activity. This residual activity could not be extracted by further incubation, by extraction with buffers, or by other treatments as described above. Solubilization of this fraction of enzyme activity was achieved, however, by butanol extraction. This was accomplished by resuspension of the pellet in the original volume of 0.01 M Tris–HCl, pH 7.6, and then 0.5 vol. of 1-butanol was added. The suspension was warmed to 37 °C and incubated at this temperature in a shaking water bath for 15 min. After centrifugation at 17 000 × *g* for 20 min the following phases were obtained: a lower aqueous phase, an interphase of cell debris and an upper organic phase. Enzymatic activity was present in the aqueous layer which was removed with a syringe. Contaminating butanol was removed by dialysis of the enzyme solution in running cold tap water for 6 h; a small precipitate formed during dialysis which was removed by centrifugation. Thus, two soluble alkaline phosphatase preparations were obtained: alkaline phosphatase A, which was solubilized by incubation at 37 °C and

pH 7.6 for 20 h, and alkaline phosphatase B, which was obtained by butanol extraction. The two enzymes were found to be significantly different in some of their characteristics.

*Some characteristics of the release of alkaline phosphatase A.* Incubation of the sonicated extract of Sarcoma 180/TG cells at 37 °C for 20 h released enzyme activity as described above. Such release resulted in a several-fold increase in enzymic activity; however, only about one-half of the total activity assayed was released into the supernatant fraction. The particulate fraction retained one-half of the enzymatic activity, which could not be solubilized either by further incubation or by other treatments previously described. As shown in Fig. 1, release of the enzyme from the particulate fraction was markedly inhibited by the addition of $5 \cdot 10^{-3}$ M iodoacetamide; whereas,

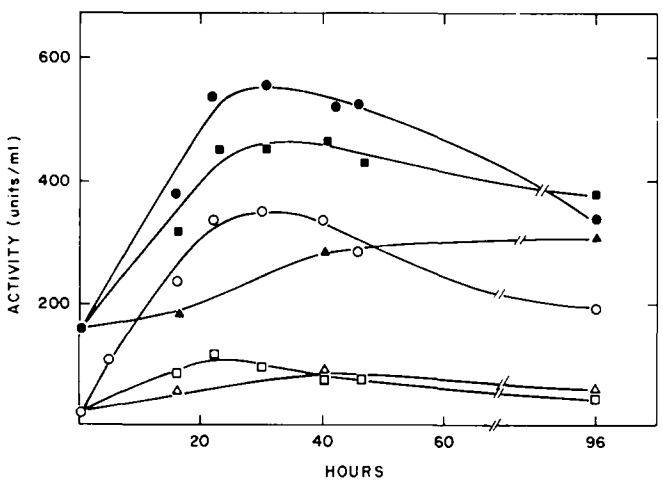


Fig. 1. Release of alkaline phosphatase A from the particulate fraction. About 250 ml each of sonicated suspension from Sarcoma 180/TG ascites cells in 500-ml Erlenmeyer flasks covered with parafilm were incubated in a 37 °C water-bath up to 96 h with occasional stirring by hand. Activity of sonicated suspension after incubation (●—●), and activity released into the supernatant fraction (○—○). Activity of sonicated suspension incubated in the presence of $5 \cdot 10^{-3}$ M iodoacetamide during incubation (■—■), and activity released into the supernatant fraction (□—□). Activity of sonicated suspension stored at 4 °C (▲—▲), and activity of its supernatant fraction (△—△).

residual alkaline phosphatase activity present in the particles was only minimally affected. Furthermore, alkaline phosphatase activity was not released into the supernatant fraction by incubation under the conditions described above when the sonicated extract was previously stored at 4 °C for 2 weeks, despite the finding that the total enzymatic activity of the extract was similarly increased when the previously stored extract was transferred to 37 °C for 20 h. Such storage at 4 °C also increased the activity of the extract slightly, but activity was not released into the supernatant fraction (Fig. 1). These observations suggest that some factor, possibly a sulfhydryl-dependent enzyme, may be involved in the release of the enzyme from the particulate fraction.

*Partial purification of enzymes*

*Alkaline phosphatase A*

*Fractionation with ethanol.* The enzyme, solubilized by incubation in 0.01 M Tris–HCl, pH 7.6, at 37 °C for 20 h, was cooled to −1 °C and 15 ml of −20 °C abs. ethanol per 100 ml of enzyme solution was added slowly with vigorous stirring. The mixture was maintained at −9 °C for 30 min, and the solution was centrifuged at 17 000 × *g* for 20 min at −9 °C. The precipitate was discarded, the supernatant fraction was cooled to −5 °C, and chilled ethanol (−20 °C) was added up to 35% of the original enzyme solution. The mixture was allowed to remain at −9 °C for 30 min, and after centrifugation at 17 000 × *g* for 20 min at −9 °C, the resulting precipitate was redissolved in 0.01 M Tris–HCl, pH 7.6.

*Chloroform treatment.* To the above cold enzyme solution, 1 vol. of chloroform was added in a separatory funnel, and shaken vigorously until the mixture became a milky emulsion. The emulsion was centrifuged at 17 000 × *g* for 30 min at 4 °C; the resulting white lipid phase remained at the interphase, and the upper aqueous enzyme solution was removed with a syringe.

*DEAE-cellulose chromatography.* A fraction of the enzyme from the chloroform treatment was concentrated by ultrafiltration to about one-tenth of the original volume. The enzyme was diluted with cold deionized water, with the conductivity of the solution being maintained below 0.5 mMHO. The pH of the solution was adjusted to 7.6, and the mixture was added to a DEAE-cellulose column previously equilibrated with 0.01 M Tris–HCl, pH 7.6. The enzyme was eluted with a linear gradient of NaCl from 0.05 to 0.7 M in the same buffer. The elution pattern is shown in Fig. 2.

*Filtration on Sephadex G-200.* A sample of concentrated effluent from DEAE-cellulose column chromatography was filtered through a Sephadex G-200 column

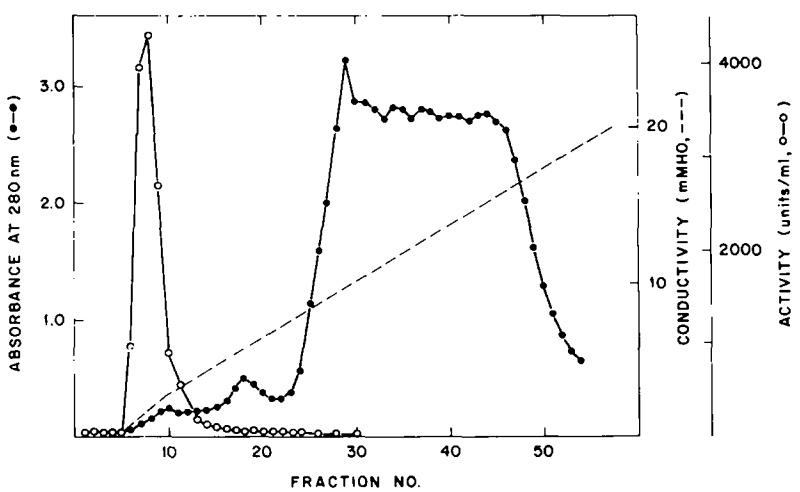


Fig. 2. Typical elution pattern of alkaline phosphatase A using DEAE-cellulose column chromatography. A column (1.6 cm × 30 cm) was packed with steadily increasing $N_2$ gas pressure from 2 to 10 psi and equilibrated with 0.01 M Tris–HCl, pH 7.6. Enzyme solution (210 ml, $A_{280\ nm}$ = 2.6) was applied to the column with the aid of a Technicon proportioning pump (Technicon Corporation, Tarrytown, N.Y.). The enzyme was eluted using a linear salt gradient of 100 ml each of 0.05 and 0.7 M NaCl in the same buffer. Fractions were collected in volumes of 3.8 ml.

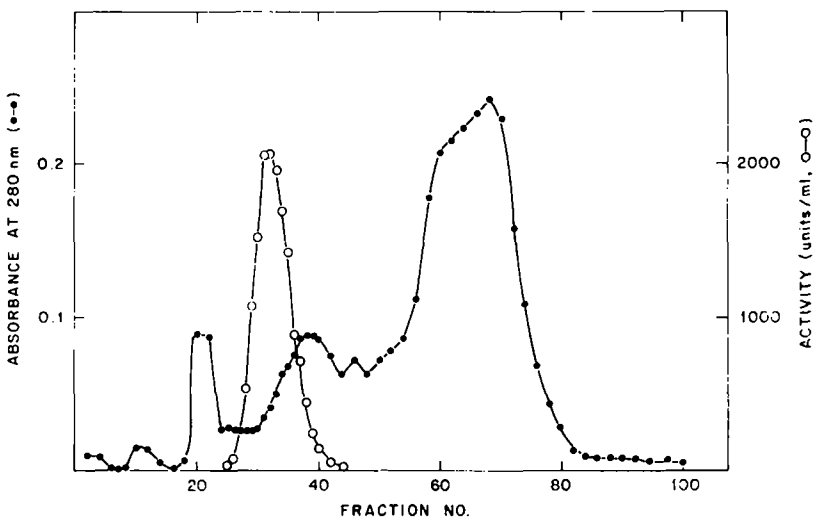


Fig. 3. Sephadex G-200 gel filtration of alkaline phosphatase A. An enzyme solution (186 ml, activity 427 units/ml, $A_{280\ nm}$ = 0.4) obtained from pooled fractions from DEAE-cellulose column chromatography was concentrated by ultrafiltration to 4.4 ml and applied to a Sephadex G-200 column (2.6 cm × 50 cm) previously equilibrated with 0.01 M Tris–HCl, pH 7.6. The enzyme was eluted with the same buffer and collected in 3.2-ml fractions; the flow rate was 0.21 ml/min.

previously equilibrated with 0.01 M Tris–HCl, pH 7.6. The elution profile is shown in Fig. 3. The entire partial purification scheme employed is summarized in Table I.

*Alkaline phosphatase B*

The steps involving fractionation with ethanol, chloroform treatment, and filtration on Sephadex G-200 (Fig. 4), employed for the purification of alkaline phosphatase B were essentially the same as described for alkaline phosphatase A, except that the former enzyme precipitated between 16 to 70% of ethanol added at the ethanol fractionation step. Alkaline phosphatase B was quite stable at this stage of the

TABLE I

SUMMARY OF THE PARTIAL PURIFICATION OF ALKALINE PHOSPHATASE A OF SARCOMA 180/TG

The starting material was isolated from the peritoneal cavities of 1000 mice bearing 7-day implants of Sarcoma 180/TG.

| Procedure | Volume (ml) | Total activity (units) | Total protein (mg) | Specific activity (units/mg) | Yield (%) | Fold purification |
|---|---|---|---|---|---|---|
| Sonicated extract | 5860 | 673 900 | 99 320 | 6.8 | 100 | 1 |
| Solubilization | 3491 | 712 160 | 17 450 | 41 | 118 | 6 |
| Ethanol | 788 | 747 810 | 1 820 | 411 | 124 | 61 |
| Chloroform | 795 | 778 300 | 1 700 | 455 | 129 | 67 |
| DEAE-chromatography | 339 | 144 750 | 41 | 3 558 | 24 | 525 |
| Sephadex G-200 | 29.5 | 90 270 | 0.4 | 54 710 | 15 | 8070 |

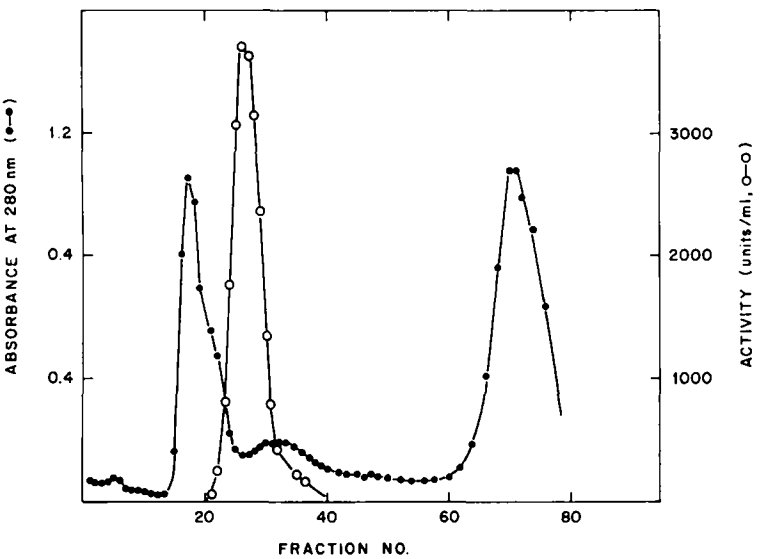


Fig. 4. Sephadex G-200 gel filtration of alkaline phosphatase B. 3 ml of concentrated enzyme (activity 20,470 units/ml, $A_{280\ nm}$ = 121) from chloroform treatment was applied onto the column (1.5 cm × 100 cm) and eluted as described in Fig. 3. Each 2.0-ml fraction was collected; flow rate 0.7 ml/min.

purification; however, when the enzyme was stored at 4 °C, part of the non-enzymatic protein denatured and precipitated, and an increase in enzymatic activity was obtained. The procedure employed in the partial purification of this enzyme is summarized in Table II.

TABLE II

SUMMARY OF THE PARTIAL PURIFICATION OF ALKALINE PHOSPHATASE B OF SARCOMA 180/TG

The source of the starting material was the same as in Table I.

| Procedure | Volume (ml) | Total activity (units) | Total protein (mg) | Specific activity (units/mg) | Yield (%) | Fold purification |
|---|---|---|---|---|---|---|
| Sonicated extract | 5860 | 673 900 | 99 320 | 6.8 | 100 | 1 |
| Butanol extract | 3150 | 548 100 | 3 150 | 174 | 74 | 26 |
| Ethanol | 1090 | 267 000 | 686 | 389 | 40 | 57 |
| Storage (3 months, 4 °C) | 1090 | 562 440 | 447 | 1 258 | 80 | 185 |
| Chloroform | 1070 | 571 380 | 407 | 1 405 | 84 | 207 |
| Sephadex G-200 | 87 | 236 200 | 7.8 | 30 160 | 35 | 4450 |

*Some properties of alkaline phosphatases A and B*

*Optimum reaction pH.* The effect of pH on the activity of the two enzymes is shown in Fig. 5; both enzymes had maximal activity at pH 9.5 with *p*-nitrophenyl-phosphate employed as the substrate.

*Substrate specificities.* As shown in Table III, both enzymes were capable of

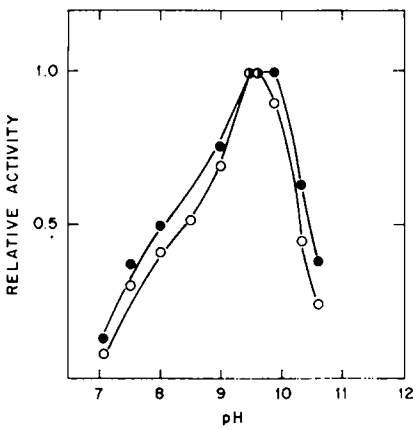


Fig. 5. Effect of pH on the activities of alkaline phosphatases A and B. Assays were performed in either 0.05 M sodium barbital, pH 7.0–9.0, or 0.05 M $NaHCO_3$, pH 9.0–11.0, with *p*-nitrophenylphosphate as the substrate. Activity of phosphatase A (○—○); activity of phosphatase B (●—●).

hydrolyzing a wide variety of phosphomonoesters. However, using *p*-nitrophenylphosphate as a standard, it was found that alkaline phosphatase B had much greater activity toward 5′-nucleoside monophosphates than toward other inorganic phosphomonoesters employed. While *p*-nitrophenylphosphate tended to be a better relative substrate for alkaline phosphate A, this enzyme was found to be more active with purine monophosphates than with pyrimidine monophosphates.

TABLE III

SUBSTRATE SPECIFICITIES OF ALKALINE PHOSPHATASES A AND B OF SARCOMA 180/TG

Enzyme activities were measured by release of orthophosphate as determined by the method of Fiske and SubbaRow [10]. The buffer was 3.0 ml of 0.1 M $NaHCO_3$, pH 9.4, containing $5 \cdot 10^{-3}$ M of each substrate, and an appropriate amount of enzyme. The reaction was carried out at 37 °C for a total of 30 min. At 0-, 5-, 10-, 20- and 30-min intervals, 0.5 ml each of reaction mixture was collected for determination of phosphate content.

| Substrate | Relative reaction velocity | |
|---|---|---|
| | Phosphatase A | Phosphatase B |
| *p*-Nitrophenylphosphate | 1.0 | 1.0 |
| IMP | 1.1 | 3.3 |
| AMP | 1.1 | 2.2 |
| CMP | 0.4 | 3.0 |
| UMP | 0.7 | |
| 3′-AMP | 0.8 | 0.5 |
| β-Glycerophosphate | 0.6 | 0.5 |

*Inhibition studies.* Alkaline phosphatases A and B were inhibited by chelating agents, such as EDTA and 1,10-phenanthroline. The 50% inhibitory concentrations for the two enzymes in 1.0 M Tris–HCl, pH 9.2, were very similar for the two chelating agents (Fig. 6). Both enzymes were inhibited by $Be^{2+}$ and by $Zn^{2+}$ at relatively high

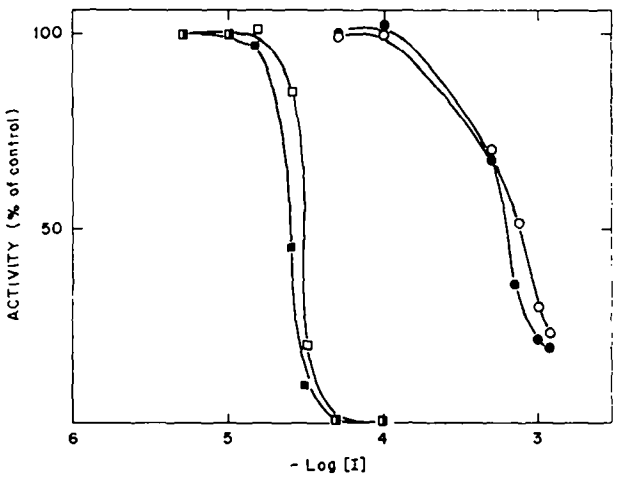


Fig. 6. Inactivation of alkaline phosphatases A and B by chelating agents. The assay buffer was 1.0 M Tris–HCl, pH 9.2. The enzymes were incubated with various concentrations of either 1,10-phenanthroline or EDTA at room temperature for 15 min prior to addition of *p*-nitrophenylphosphate. The activities were then determined spectrophotometrically as described in the Methods section. EDTA: phosphatase A (□—□); phosphatase B (■—■). 1,10-Phenanthroline: phosphatase A (○—○); phosphatase B (●—●).

concentrations, with phosphatase B being slightly less susceptible (Table IV). However, the stereospecific inhibitor L-phenylalanine was without much effect on both catalysts at concentrations up to $5 \cdot 10^{-3}$ M.

*Activation by* $Mg^{2+}$. Both enzymes required $Mg^{2+}$ for maximal activity; however, alkaline phosphatase B was much more sensitive to $Mg^{2+}$ activation. Thus, at a

TABLE IV

INHIBITION OF SARCOMA 180/TG ALKALINE PHOSPHATASES A AND B BY $Zn^{2+}$, $Be^{2+}$, AND L-PHENYLALANINE

The reaction was carried out in 0.1 M $NaHCO_3$, pH 9.4, using *p*-nitrophenylphosphate as the substrate, and enzymatic activity was determined spectrophotometrically as described in the Methods section.

| Inhibitor | Concentration (M) | % Inhibition | |
|---|---|---|---|
| | | Phosphatase A | Phosphatase B |
| $Be^{2+}$ | $1 \cdot 10^{-5}$ | 92 | 77 |
| $Zn^{2+}$ | $7.5 \cdot 10^{-4}$ | 94 | 60 |
| L-phenylalanine | $5 \cdot 10^{-3}$ | 4 | 25 |

concentration of $5 \cdot 10^{-3}$ M $Mg^{2+}$, where maximum effects occurred, in 0.1 M $NaHCO_3$, pH 9.4, the activity of phosphatase A increased 2–5 times with different enzyme preparations, while the activity of alkaline phosphatase B increased 10–22 times under the same conditions (Fig. 7).

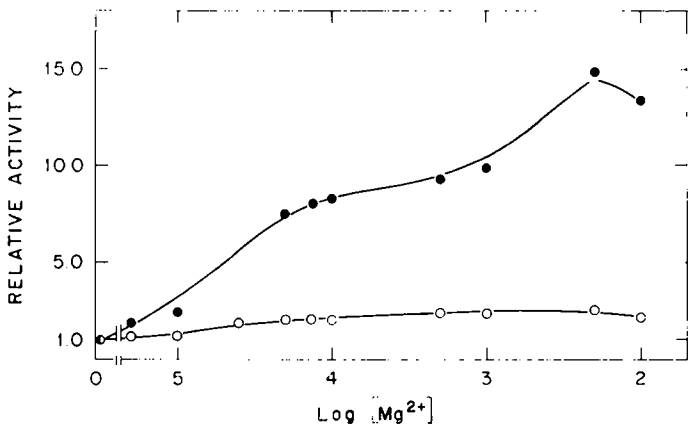


Fig. 7. Effect of $Mg^{2+}$ on the activities of alkaline phosphatases A and B. The enzymes were incubated with various concentrations of $Mg^{2+}$ at room temperature for 5 min in 2.7 ml of 0.1 M $NaHCO_3$ buffer, pH 9.4. At the end of the incubation, 0.3 ml of $10^{-2}$ M *p*-nitrophenylphosphate was added and mixed; the activity was determined spectrophotometrically as described in the Methods section. Activity of phosphatase A (○—○); activity of phosphatase B (●—●).

*Electrophoresis in polyacrylamide gels.* The two enzymes showed significant differences in mobility when subjected to electrophoresis on polyacrylamide gels. Alkaline phosphatase B had greater mobility under the conditions employed as shown in Fig. 8.

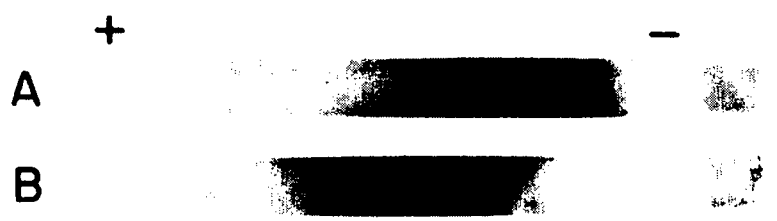


Fig. 8. Polyacrylamide gel electrophoresis of alkaline phosphatases A and B. Electrophoresis was carried out in 5% polyacrylamide gel at 4 °C for 3 h with 3 mA/tube using a Canalco disc electrophoretic apparatus. Activity was stained with β-naphthyl acid phosphate and Fast Blue as described in the Methods section. Gel A, migration of phosphatase A; Gel B, migration of phosphatase B.

## DISCUSSION

Like alkaline phosphatases from many other sources [13–16], the enzymes of Sarcoma 180/TG are particulate-bound, and presumably are for the most part insoluble under physiological conditions. The procedures employed in the present investigation for the solubilization of these proteins resulted in the isolation of two alkaline phosphatase activities which differed significantly in substrate specificity, $Mg^{2+}$ activation, and electrophoretic mobility on polyacrylamide gels. The butanol extraction technique used by Wolpert et al. [8], on freshly prepared cell-free sonicated suspensions of Sarcoma 180/TG, only resulted in partial extraction of activity. Furthermore, the

relatively crude enzyme obtained in this manner was not a fully solubilized form; thus the enzyme in this state could not be further purified by elution from columns of either DEAE-cellulose or Sephadex G-200. Total extraction of phosphatase activity by butanol was achieved only after incubation at 37 °C under the conditions described. During such incubation, alkaline phosphatase activity, designated as phosphatase A, was released into the supernatant fraction and was thereby separated from the residual butanol-extractable enzyme, designated alkaline phosphatase B.

The finding that storage of the crude sonicated extract at 4 °C for 2 weeks did not alter residual enzymatic activity, but did interfere with the release of alkaline phosphatase A activity upon incubation at 37 °C and at pH 7.6, suggested the presence of a labile releasing factor. That sulfhydryl groups might be important for the action of this factor was suggested by the sensitivity of the process to iodoacetamide. The presence and possible physiological role of such a releasing factor is presently under investigation in our laboratory.

Both alkaline phosphatase A and B hydrolyze a wide variety of phosphomonoesters, indicating that the enzymes are non-specific in nature. Phosphatase A is a more classical type of alkaline phosphatase because *p*-nitrophenylphosphate was among the best substrates tested; this characteristic is a feature shown by alkaline phosphatase enzymes from many other sources [14, 17–23]. The fact that alkaline phosphatase B has much greater activity towards 5′-nucleotides suggested that this enzyme might be a 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5); however, this possibility appeared to be ruled out, since the enzyme exhibited an optimum pH at 9.5 with either *p*-nitrophenylphosphate (Fig. 5) or IMP (Fig. 9) as substrates. No reaction maximum was seen in the pH range 6–8 for this enzyme when IMP was used as a substrate; the optimum pH for 5′-nucleotidase has been reported to be about pH 6.5 [24, 25], and between pH 7.0 to 7.5 in the absence of $Mg^{2+}$ [26]. The unusual reactivity of alkaline phosphatase B towards nucleotide monophosphates makes this enzyme the

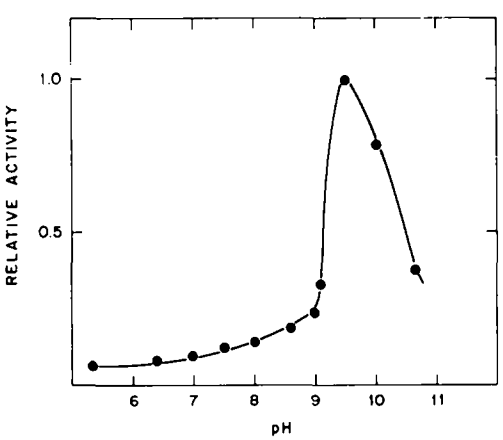


Fig. 9. Effect of pH on the activity of alkaline phosphatase B with IMP as the substrate. Enzyme activity was measured by release of orthophosphate as determined by the method of Fiske and SubbaRow [10]. The assay mixture contained 1.0 ml of various buffers, $5 \cdot 10^{-3}$ M IMP, and an appropriate amount of enzyme. The mixture was incubated at 37 °C for 30 min and the phosphate content was determined. The buffers employed were as follows: pH 5.2–6.4, 0.05 M sodium-maleate; pH 6.8–9.2, 0.05 M sodium-barbital; pH 9.6–11.0, 0.05 M sodium-bicarbonate.

prime candidate for the biocatalyst involved in the increased rate of degradation of the 6-thiopurine nucleotides, a phenomenon which confers resistance to 6-thiopurines in Sarcoma 180/TG ascites cells.

Both alkaline phosphatases of Sarcoma 180/TG are strongly activated by $Mg^{2+}$. In this respect, the enzymes behave like those of kidney [27, 28] and bone marrow [16] but are different from the intestinal [29] and the placental enzymes [21] which are much less susceptible to activation by this metal ion. Up to a 20-fold increase in catalytic activity was observed for alkaline phosphatase B, which was considerably more susceptible to $Mg^{2+}$ activation than was phosphatase A. This degree of activation (i.e. 20-fold) is not uncommon for alkaline phosphatase enzymes, since a 14-fold increase in activity was reported for bovine brain alkaline phosphatase [30].

The chelating agents, EDTA and 1,10-phenanthroline inhibit the two enzymes to comparable degrees. In addition, the inhibition caused by these chelating agents was partially reversed by $Zn^{2+}$ or other divalent metal ions (Lee, M. H. and Sartorelli, A. C., unpublished), suggesting that the alkaline phosphatases of Sarcoma 180/TG are also metalloenzymes [31]. Be and Zn salts at relatively high concentrations caused considerable inhibition of both enzymes, with the sensitivity of alkaline phosphatase A being slightly greater than that of B. The susceptibility of these enzymes to these inhibitors is similar to that of alkaline phosphatase from other sources [22, 32].

There are significant differences in the mobility of the two enzymes on polyacrylamide gels under the conditions employed. Alkaline phosphatase B migrates significantly faster than does phosphatase A. This characteristic is in agreement with the observation that at pH 7.6, in 0.01 M Tris–HCl buffer, alkaline phosphatase B is strongly bound to DEAE-cellulose; whereas, alkaline phosphatase A is eluted from DEAE-cellulose at very low ionic strength. Furthermore, at pH 5.8 in 0.01 M sodium–acetate buffer, phosphatase B is not absorbed by CM-cellulose, while alkaline phosphatase A, under the same conditions, can be eluted from CM-cellulose only at high ionic strength.

The results indicate that the alkaline phosphatase activities isolated and partially purified from Sarcoma 180/TG ascites tumor cells have many characteristics similar to those of the kidney and bone marrow type of alkaline phosphatase, rather than to an intestinal alkaline phosphatase. It is not clear whether the two alkaline phosphatase activities isolated from Sarcoma 180/TG actually represent two distinct enzymes in situ, since it is conceivable that the solubilization procedures employed may have altered a portion of the enzyme molecules, which accounts for the observed differences in enzymatic behavior. The limited amounts of enzymatic activity present in these neoplastic cells severely hampers the further purification and characterization of these enzyme activities; nevertheless, investigations are continuing to delineate their role in the acquisition of insensitivity by this clone to the cytotoxic action of the 6-thiopurines.

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