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Alkaline Phosphatase in the Healing Burn Wound of the Rat[1,2]

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Alkaline phosphatase has been extracted from the granulation tissue of healing burn wound of the rat, in quantity sufficient to allow testing for its response to heat and urea inactivation, neuraminidase treatment, pH, and a variety of amino acids and other compounds known to affect its activity. It is sensitive to chemical inhibition in a manner similar to isoenzymes from bone or liver, but its sensitivity to heat and urea inactivation falls between those of bone and liver isoenzymes. The possibility that it is composed of a mixture of bone- and liver-type isoenzymes cannot be ruled out by the means used in this study. Neuraminidase sensitivity indicates the presence of a terminal sialic acid residue on the polysaccharide portion of the enzyme. Optimum pH was determined to be 9.3 at the substrate concentration and in the buffer system employed. Levamisole was the most effective inhibitor tested. The inhibition by L-homoarginine was found to be pH dependent. The healing burn wound provides an excellent model for study of the nature and role of alkaline phosphatase in wound repair, due to the availability of relatively large amounts of enzyme.

INTRODUCTION

Alkaline phosphatase (EC 3.1.3.1) has been studied in developing, healing, normal, and neoplastic tissues from human and animal sources, generally demonstrating positive correlation with rate of healing, transport processes, and tumor growth. Among the tissues in which the enzyme has been studied are included rat sponge-induced subcutaneous granulation tissue [17] and skin palatal gingiva and tongues of rats during healing after experimental wounding [2]. This paper presents a study of alkaline phosphatase extracted from granulation tissue of rats which had been subjected to a standardized burn [18]. In an effort to characterize the enzyme from granulation tissue and, if possible, to distinguish it from alkaline phosphatase isoenzymes extracted from liver, bone, and intestine, a variety of inhibitors were employed, heat lability and neuraminidase sensitivity measured, and pH optimum and electrophoretic mobility on polyacrylamide gels determined. The following presents our findings.

METHODS

Adult male Sprague–Dawley (Holtzman strain) rats were anesthetized with pentobarbital administered intraperitoneally (1 mg/25 g body wt), sheared of hair over the back and belly, and placed in a device to limit and control burn size. The exposed dorsal and ventral areas were then immersed in boiling water for 10 sec on the dorsum and 2.5 sec on the abdomen, producing a full thickness burn over 50% of the body surface. On the 20th day after burning, the

[1] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. In conducting the research described in this report the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences National Research Council.

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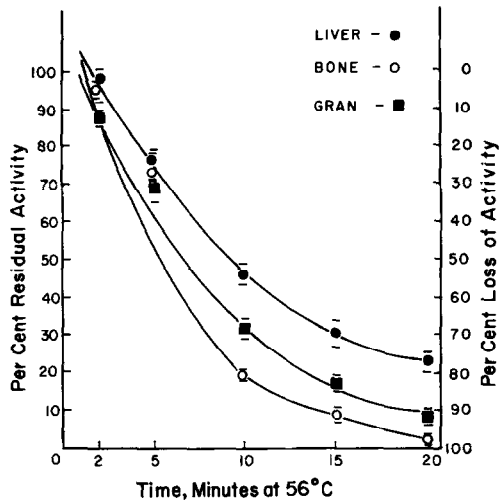

FIG. 1. Effect of heating on activity of RGTAP, bone, and liver alkaline phosphatase. Points represent the mean (±SD) of triplicate measurements of phosphomonoesterase activity of butanol extracts of homogenates of rat bone, liver, and granulation tissue after incubation in AMP buffer at 56°C for 0, 2, 5, 10, 15, or 20 min. The unheated enzymes represent 100% residual activity.

rats were killed by dry ice anesthesia followed by heart puncture and exsanguination. Livers were blanched by perfusion with normal saline. Pieces of rib bone were scraped clean of soft tissue. Sections of the small intestine in the region of the jejunum were excised and flushed of contents, and the kidneys were removed. The burn eschar was stripped from the granulation tissue. Tissue slices of normal skin were prepared by stripping the fascia and removing fibrous connective tissue and excising the deep dermal layer using a Stadie-Riggs tissue slicer. Each tissue was then placed in approximately 1 ml of water/100 mg of tissue and homogenized at 26,000 rpm in a Tissumizer (Tekmar Company, Cincinnati, Ohio). The homogenates were extracted with *n*-butanol as described previously [12].

Alkaline phosphatase was assayed by a modification [12] of the method of Bessey *et al.* [1]. *P*-Nitrophenyl phosphate at 3.8 m*M* was the substrate.

Inhibition studies were carried out by addition of aqueous solutions of the inhibitor to the assay mixture prior to the addition of substrate. Inhibitor concentrations were calculated with respect to final assay mixture. Duplicate assays were set up using the highest concentration of each inhibitor, and the pH of the final assay mixture was measured. In three experiments, alkaline phosphatase from rat bone, liver, and granulation tissue was preincubated with urea in concentrations ranging from 2 to 4 *M* for 9 min at 37°C, in 1 *M* diethanolamine buffer, pH 9.6. Urea was added to the reaction mixture to bring the final concentration of all to 4 *M* at the time of addition of substrate to start the reaction. Measurement of pH optimum was performed using 0.19 *M* AMP buffers prepared in the range of pH 8.4 to 10.4 at 37°C.

Heat lability was determined by heating the assay mixture containing the enzyme and buffer in an oil bath at 56°C. After the prescribed period of incubation, each sample was placed in an ice bath for a few minutes, allowed to return to room temperature, substrate was added, and the assay was carried out as usual. Neuraminidase sensitivity was measured by incubating the alkaline phosphatase with *Clostridium perfringens* neuraminidase, Type VIII (EC 3.2.1.18, Sigma N-5631) for 16 hours at pH 7.2, 37°C [11]. Electrophoretic mobility was determined by polyacrylamide gel electrophoresis on 7% gels (3). Alkaline phosphatase bands were visualized by incubating the gels in a histochemical stain composed of 100 ml of a 0.15 *M* Tris buffer, pH 10.3, containing 1.0 m*M* $MgCl_2$ and 200 mg each of α-naphthyl phosphate and Fast Blue BB diazonium dye (Dajac Laboratories, Haven Chemical Company, Philadelphia, Pa.). All reagents used were reagent grade. Amino acids were purchased from Pierce, Box 117, Rockford, Illinois, except for L-arginine (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) and D-cysteine (Sigma). Levamisole was procured from Scott-Pittman, Washington Crossing, N. J.

RESULTS

The phosphomonoesterase activity of the enzyme extract was determined to be linear

with respect to sample size and time of incubation. The rate of hydrolysis of *p*-nitrophenylphosphate at a final concentration of 3.8 m*M* in 0.19 *M* AMP buffer was found to be greatest at pH 9.33 at 37°C.

The time required for loss of RGTAP 50% activity was 7.5 min, in comparison with bone AP, 5 min, and liver, 9 min (Fig. 1). Treatment with neuraminidase caused a 40% decrease in mobility on polyacrylamide gel electrophoresis (PAGE). Several samples of RGTAP demonstrated three bands on PAGE, with electrophoretic mobilities similar to those observed in two kidney samples.

L-Histidine inhibited the isoenzymes from granulation tissue, bone, and liver to the same extent. L-Homoarginine was a much more effective inhibitor of bone and granulation tissue enzymes than of intestinal alkaline phosphatase (Fig. 2). For all three isoenzymes there was an increase in inhibition as the pH was increased from 9.6 to 10.3. Intestinal alkaline phosphatase was inhibited to a greater extent by L-phenylalanine than the isoenzymes from granulation tissue, liver, or bone. Inhibition of the granulation tissue, bone, and liver enzymes by urea is shown in Fig. 3. Levamisole was a very strong inhibitor at both pH 9.6 and 10.3 (Fig. 4). None of the inhibitors tested caused any measurable change in pH under these experimental conditions. The effects on RGTAP of 23 amino acids and

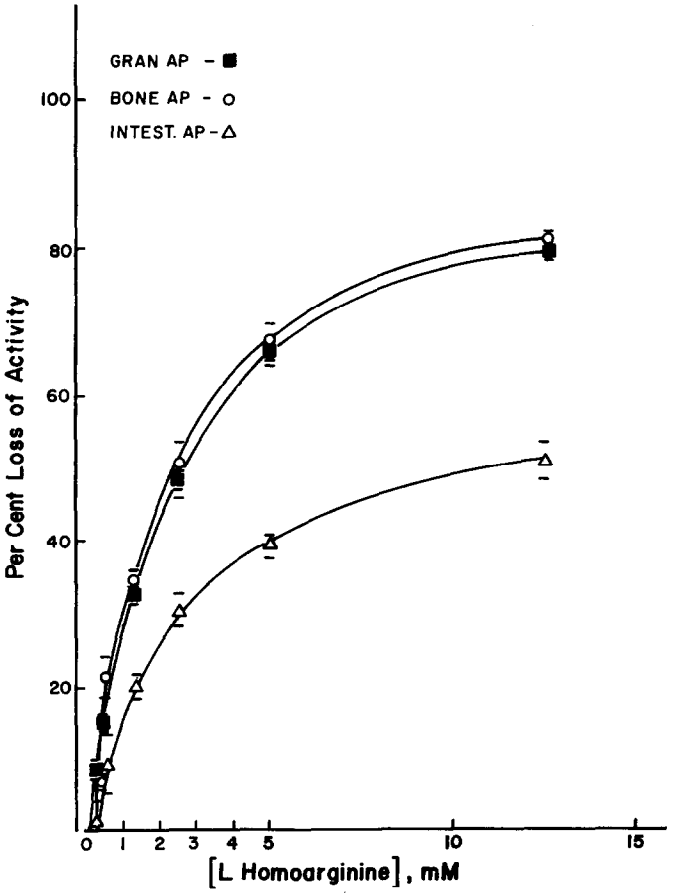

FIG. 2. Effect of L-homoarginine on activity of bone, intestine, and granulation tissue alkaline phosphatase at pH 9.6. Points represent the mean (±SD) of triplicate determinations of phosphomonoesterase activity of butanol extracts of homogenates of rat bone, intestine, and granulation tissue in the presence of L-homoarginine at pH 9.6.

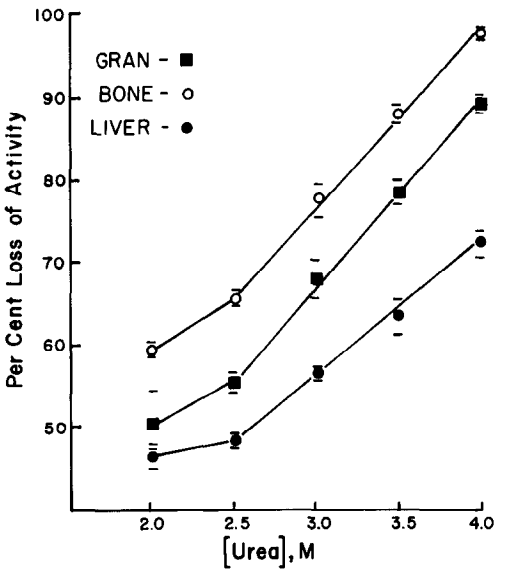

FIG. 3. Effect of incubation in urea on activity of rat bone, liver, and granulation tissue alkaline phosphatase. Points represent the mean (±SD) of triplicate measurements of residual percentage phosphomonoesterase activity of butanol extracts of homogenates of rat bone, liver, and granulation tissue after preincubation in urea. Extracts were incubated in diethanolamine buffer at 37°C for 9 min, substrate was added, and assay was carried out for 5 min.

four selected drugs, as well as zinc [11] and urea at single concentrations, are listed in Table 1. Ouabain, an inhibitor of some membrane-bound enzymes, was found to have no inhibitory effect at concentrations ranging from 10^{-6} to 10^{-3} mole/liter. The specific activity of alkaline phosphatase in dialyzed butanol extracts of granulation tissue and normal skin was found to be 2.13 ± 2.01 IU/mg protein and 1.06 ± 0.8 × 10^{-3} IU/mg protein, respectively.

DISCUSSION

Isoenzymes from bone or liver can be distinguished from those of intestine, placenta, or tumors by their thermolability [5]. The alkaline phosphatase from rat granulation tissue falls in the thermolabile group with bone and liver isoenzymes. The sensitivity of RGTAP to treatment with neuraminidase is similar to that observed in liver and bone [14] and distinguishes it from that of intestine which is not affected [11]; RGTAP responds to L-homoarginine in a manner similar to the isoenzymes of bone and liver, unlike that of intestine [9]. In contrast to Lin and Fishman's findings, we observed a greater inhibition at pH 10.3 than pH 9.6, possibly reflecting rat strain or tissue type differences. Treatment of these four isoenzymes with L-phenylalanine similarly groups RGTAP with bone and liver isoenzymes. The degree of inhibition of RGTAP by L-tryptophan is in close agreement with that reported for rat liver and bone [10] isoenzymes. Levamisole is a powerful uncompetitive inhibitor of alkaline phosphatase, unusual in that it inhibits

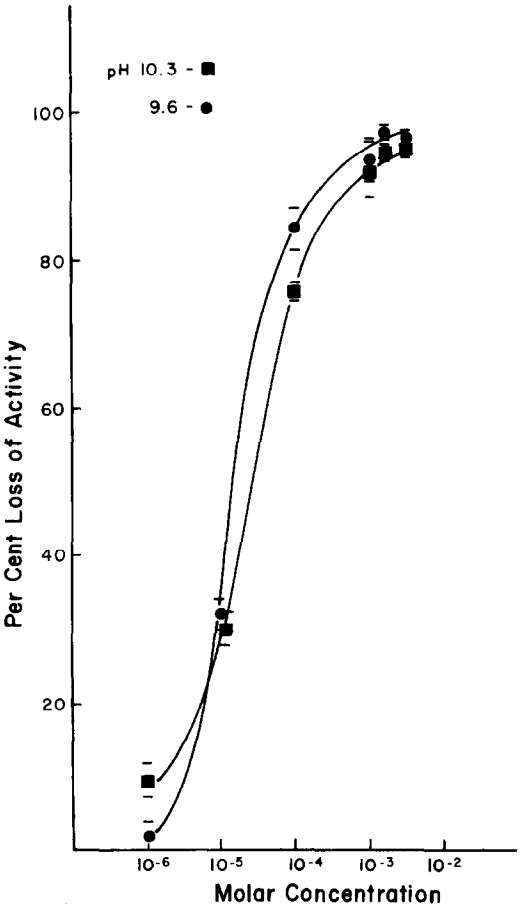

FIG. 4. Effect of levamisole on the activity of rat granulation tissue alkaline phosphatase at pH 9.6 and pH 10.3. Points represent the mean (±SD) of triplicate determinations of phosphomonoesterase activity of rat granulation tissue alkaline phosphatase in the presence of levamisole at pH 9.6 or pH 10.3, expressed as percentage loss of activity.

canine placental isoenzyme to the same extent as that from liver, bone, kidney, and tumor. The isoenzyme from intestine is relatively resistant [15]. However, the same author found that human placental and intestinal isoenzymes are much less sensitive to levamisole than those from bone, liver, kidney, and spleen [16]. Our RGTAP inhibition curve can be superimposed on his canine liver–bone group curve.

On the basis of data from these studies, it is apparent the alkaline phosphatase in rat burn wound granulation tissue most closely resembles that derived from bone or liver. Several authors have reported differential inhibition of bone and liver isoenzymes by urea treatment [6, 7]. These experiments do not suggest whether the RGTAP is a distinct isoenzyme or a mixture of bone and liver types. Inasmuch as several cell types, e.g., leukocytes, fibroblasts, and capillary wall epithelium demonstrate activity, the latter possibility should be considered.

Although alkaline phosphatase activity is very low in mature normal skin, it is relatively abundant in both developing normal skin and in regenerating skin following a wound. Such activity has been demonstrated by histochemical techniques to occur in the capillaries, subepidermal capillary plexus, and mast cells of embryonic normal skin at the 14th day with more intense activity in capillaries in the subdermal plexus at the 18th day and in dermal papillae and hair bulbs in the newborn rat [8]. Alkaline phosphatase has been reported to occur in polymorphonucleocytes, fibroblasts, and chondrocytes during the formation of hemopoietic ossicles induced by implantation of demineralized bone-matrix collagen in the rat [13]. The peak enzyme activity during the healing of experimental incised wounds and burns was concurrent with the maximum rate of collagen formation [4].

As expected, the granulation tissue formed in the healing burn wound of the rat about the third week postburn demonstrates a relative abundance of alkaline phosphatase activity. There remain questions about the physiologic role of the enzyme, its responses to other drugs and inhibitors, and its immunologic relationship to isoenzymes from other tissues.

TABLE 1

RAT GRANULATION TISSUE ALKALINE PHOSPHATASE RESPONSE TO AMINO ACIDS AND SELECTED DRUGS

Compound	Final concentration (m*M*) in assay	Percentage inhibition
L-Alanine	12.50	14.0
L-Arginine	12.50	49.9
L-β-Asparagine	12.50	10.1
L-Aspartic Acid	*[a]	4.4
D-Cysteine	12.50	92.5
L-Cystine	*	2.1
L-Glutamic Acid	*	21.6
L-Glutamine	12.50	14.6
Glycine	12.50	38.5
L-Histidine	12.50	83.0
L-Homoarginine	12.50	84.0
L-Hydroxyproline	12.50	13.2
L-Isoleucine	12.50	41.5
L-Leucine	12.50	44.8
L-Lysine	12.50	25.0
L-Methionine	12.50	38.3
L-Phenylalanine	12.50	48.2
L-Proline	12.50	2.1
L-Serine	12.50	18.3
L-Threonine	12.50	29.5
L-Tryptophan	12.50	59.0
L-Tyrosine	*	0.1
L-Valine	12.50	48.0
Epinephrine	*	5.7
Histamine	12.50	45.4
Imidazole	12.50	64.0
Levamisole	5.0	96.5
Urea	2000	83.8
Zinc (Zn^{2+})	1.0	91.0

[a] * Saturated at 22°C.

The burn wound offers a striking advantage over the incised wound as a model for the study of alkaline phosphatase and its role in healing. The greatly increased amount of tissue involved serves as a source of sufficient enzyme to make practicable isolation and purification of the enzyme, as well as kinetic inhibition studies.

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