The Possible Sources of Serum Acid Phosphatase in the Dog Following Hepatic Ischemia[1] (36261)

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Acid phosphatases are enzymes with the common property of catalyzing the hydrolysis of phosphate monoesters at acidic pH. Elevated levels of serum acid phosphatase have been observed following prolonged ischemia or hypoperfusion of organs. The elevation is considered to be a sign of lysosomal damage, since acid phosphatase is known to be present in the lysosomes and released following severe anoxia (1–3). However, acid phosphatases are found in circulating red cells, white cells, platelets, and in cell components other than lysosomes (4–6). The different acid phosphatases have different substrate specificities (7, 8). For example, all blood cells contain large amounts of acid phosphatase when *p*-nitrophenylphosphate (PNPP) is used as substrate but with α-naphthylphosphate (ANP) practically no acid phosphatase activity is found in red blood cells (9). White blood cells and platelets show moderate activity with ANP. It is for this reason more meaningful to characterize acid phosphatase activity in terms of its substrate.

Acid phosphatases also differ from each other in sensitivity to inhibitors. Human erythrocyte and thrombocyte acid phosphatases (substrate PNPP) are strongly inhibited by 0.5% formaldehyde or 2 m*M* copper sulfate, but not by 20 m*M* L-tartaric acid (4, 5). On the other hand, human prostatic and rat liver lysosomal acid phosphatase is inhibited by tartaric acid but not by formaldehyde or copper sulfate under the same conditions (4, 10).

In the following study the inhibitory characteristics and the substrate specificities of acid phosphatases from dog blood cells and from the subcellular elements of the canine liver were determined in order to investigate the origin of serum acid phosphatase following experimental liver ischemia.

Materials and Methods. Acid phosphatase activity was determined by incubating 0.2 ml plasma, serum, or cell lysate with PNPP or ANP at 37° for 30 min in a citrate buffer at pH 4.8 and 5.6, respectively. The concentration of free *p*-nitrophenol or α-naphthol was then measured colorimetrically. Substrates and color developing reagents were supplied by the Boehringer-Mannheim and the Warner-Chilcott Co. (Boehringer-Mannheim's Acid Phosphatase and Warner-Chilcott's Phosphastrate Acid Kit). When the acid phosphatase content of erythrocytes was measured the red color of the lysate interfered with the colorimetric determination of the products. In this case, at the end of the incubation period *p*-nitrophenol and α-naphthol were extracted from the medium by diethyl ether and were reextracted into water phase by alkali prior to color development.

Separation of blood cells. Erythrocytes were separated from other blood cells by repeated centrifugations and washings with physiological saline. Platelet-rich plasma, free of white and red blood cells, was prepared by the method of Abrahamsen (11). White blood cell-rich preparations were obtained by treating the blood after the removal of the platelet-rich plasma with 1% ammonium oxalate to lyse the red blood cells. The white blood cells were then sedimented by centrifugation. The pellet was repeatedly washed with 1% ammonium oxalate until no hemoglobin color was seen in the washing. The pellet was resuspended in cell free plasma and the ratio of white blood cells to platelets

[1] Supported by grants from the Medical Research Council of Canada and the John A. Hartford Foundation, Inc.

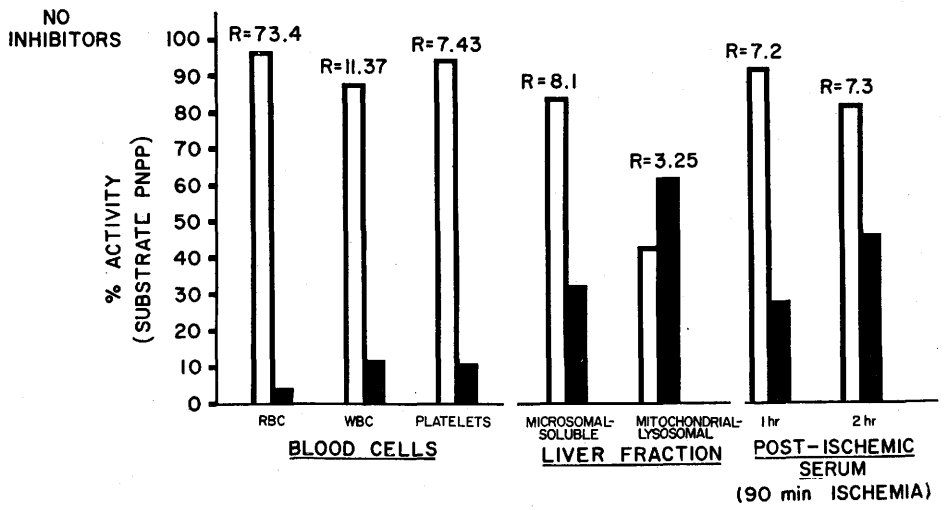

FIG. 1. Inhibitory characteristics and substrate specificities of acid phosphatases of various origin. The light bars represent activity in the presence of 20 m*M* L-tartaric acid, the dark bars stand for activity with 2 m*M* $CuSO_4$. R = activity against PNPP/activity against ANP in the acid phosphatase preparations of different origin.

determined under the microscope, using a Spencer Bright-Line Hemacytometer. The cell suspensions were diluted in a Unopette disposable pipetting system (Becton-Dickinson, Co.). The white cell count was determined under low magnification (× 100) with a precision of ± 15%. The platelets were counted at a × 450 magnification with a 25% precision. If the preparation contained many platelets the suspension was centrifuged repeatedly at 1000 rpm to sediment the white blood cells and leave the platelets in suspension. This procedure was repeated until the ratio of white blood cells to platelets could not be further improved. A ratio of one to one was acceptable in these studies.

Following the preparation of the erythrocyte, leukocyte, and thrombocyte suspensions, the cell count was determined and an aliquot was treated with 1% Triton X-100 (Packard) to lyse the cells completely. The lysates were then diluted with cell-free plasma to a suitable degree, corresponding to about 10^7 white blood cells and about 10^8 platelets or red blood cells per ml. A 0.2-ml aliquot was then tested for acid phosphatase activity as described earlier.

Dog liver lysosomal acid phosphatase was prepared by homogenizing 2–4 g of liver tissue in 10 vol of 0.25 *M* sucrose and 0.2 m*M* EDTA (ethylenediaminetetraacetic acid disodium salt) at 0°. The homogenate was centrifuged at 1800 rpm for 10 min to eliminate blood cells, cell debris, and nuclei, following which the mitochondrial–lysosomal fraction was sedimented by centrifugation of the supernatant at 15,000 rpm for 20 min at 0° in an International Refrigerated Centrifuge Model B-20. The mitochondrial–lysosomal pellet was washed once in sucrose–EDTA, centrifuged, resuspended and treated with Triton X-100 in 1% concentration to disrupt the subcellular membranes. The lysate was then diluted to a suitable degree (50–100 mg wet tissue wt/ml) and 0.2 ml tested for enzyme activity. The supernatant was also treated with Triton X-100, diluted to a concentration of 50 mg wet tissue wt/ml, and tested as above.

Serum acid phosphatase following liver ischemia was determined on 32 dogs undergoing complete hepatic inflow stasis at normothermia for periods of time ranging from 30–100 min. Details of the operative procedure have been described (12).

Results. Figure 1 shows the substrate specificities and inhibitory characteristics of acid phosphatases from dog red and white cells and platelets and from liver mitochondrial–ly-

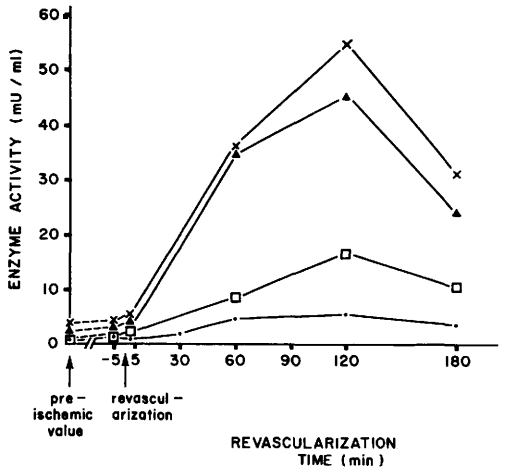

FIG. 2. Serum acid phosphatase levels following 90 min. of hepatic inflow stasis. Acid phosphatase activity, substrate PNPP: ×—× no inhibitor; ▲—▲ in the presence of 20 m*M* L-tartaric acid; □—□ in the presence of 2 m*M* $CuSO_4$. Acid phosphatase activity, substrate ANP: •—•, no inhibitor present.

sosomal and microsomal-soluble supernatant fractions compared to those of the postischemic serum. It can be seen that the acid phosphatase activity (substrate PNPP) of the blood cell and microsomal-soluble supernatant enzymes are strongly inhibited by 2 m*M* $CuSO_4$ and weakly by 20 m*M* L-tartaric acid, while the mitochondrial–lysosomal enzyme shows opposite behavior. The postischemic serum is weakly tartaric acid and strongly $CuSO_4$ inhibited. The relative enzyme activities against PNPP and ANP—expressed as a ratio of the former to the latter—show that all enzyme preparations tested hydrolyze PNPP better than ANP. The ratio is the highest for the erythrocyte and lowest for the mitochondrial–lysosomal enzyme preparation. The postischemic serum activity shows a ratio similar to the white blood cell, platelet, and microsomal-soluble supernatant enzyme.

Figure 2 illustrates the level of serum acid phosphatase activity (substrate PNPP) before, during, and following a 90 min period of complete hepatic inflow stasis in a dog. This period of liver ischemia was invariably fatal to dogs within 20 hr postoperative. There is no elevation of serum acid phosphatase during the ischemic period and the peak of activity is at 2 hr after the reestablishment of circulation. In some experiments activity was the highest one hour following revascularization and it was almost at normal levels by 3 hr even though the animals eventually died. The acid phosphatase activity with ANP followed the same pattern, although at a lower level.

The peak postischemic serum acid phosphatase activities (substrate PNPP) of 32 dogs which suffered 30–100 min of complete hepatic inflow stasis are summarized in Fig. 3. Eleven animals survived the procedure (ischemic time 30–80 min) and 21 died within 20 hr postoperative (ischemic time 70–100 min). It can be seen that when the duration of the inflow stasis was shorter than 60 min, acid phosphatase activity did not rise above normal levels. There was a strong correlation between high postischemic serum acid phosphatase levels and death of the experimental animal, the point biserial correlation coefficient being 0.782.

Table I shows the number of blood cells per milliliter or the weight of liver containing the amount of mitochondrial–lysosomal and microsomal-soluble fractions in the same volume which when completely lysed elevates the level of serum acid phosphatase activity by 50 mU/ml (substrate PNPP). A moderate lysis of the erythrocytes suffices to provide the high acid phosphatase activity

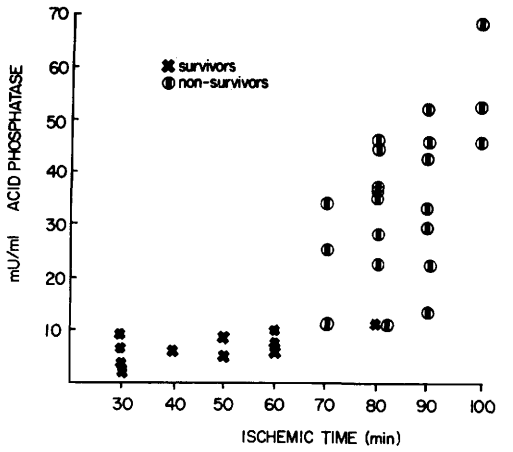

FIG. 3. Peak postischemic acid phosphatase levels following liver ischemia.

TABLE I. Acid Phosphatase Content of Dog Blood Cells and Liver Subcellular Fractions.

	Number of cells in millions/ml or liver tissue weight in mg/ml which, when completely lysed give 50 mU/ml acid phosphatase activity		
	red blood cells	white blood cells	platelets
Mean number of cells/ml	193.0	10.3	97.2
std. deviation	±55.9	±3.3	±31.9
	Mitochondrial–lysosomal		microsomal–soluble
Mean liver tissue weight in mg/ml	94.9		59.3
std. deviation	±36.5		±8.8

with PNPP but not with ANP seen in the postischemic period. The destruction of about one-third of the normally circulating platelets or almost all of the white blood cells could be the source of the postischemic acid phosphatase activity both with PNPP and ANP. The subcellular fractions examined show considerable acid phosphatase activity with both substrates and thus could also serve as a source of the postischemic serum acid phosphatase.

Discussion. The elevated serum acid phosphatase activity following tissue ischemia was presumed by many investigators to originate in the lysosomes. Sutherland *et al.* (2) found high serum acid phosphatase levels in the late stages of experimental hemorrhagic shock in the dogs. They calculated the amount of acid phosphatase coming from blood cells from plasma hemoglobin values and speculated that the source of the excess acid phosphatase was the lysosomes of the intestinal mucosa. Rangel and coworkers (3) observed delayed appearance and lower activity of circulating acid phosphatase following hepatic ischemia when the animals were pretreated with chlorpromazine or phenoxybenzamine. They considered this phenomenon as a sign of lysosomal membrane stabilization.

Although lysosomes and red blood cells were most frequently quoted as the source of elevated serum acid phosphatase following anoxic damage, Neil and Horner (6) and others (7, 8) reported the presence of large amounts of nonlysosomal acid phosphatase in livers of rats, mice and guinea pigs. They investigated the inhibitory characteristics and substrate specificities of various subcellular fractions. It was established that acid phosphatase from the lysosome-rich large granule fraction is strongly inhibited by tartaric acid, fluoride, and molybdate (the Group I inhibitors of Neil and Horner) while the microsomal-soluble supernatant activity is formaldehyde, alloxan and copper-sensitive (Group II inhibitors). In the dog the patterns of inhibition and substrate preference are the same as reported for the rodents, but the differences between the characteristics of the large granule and supernatant fractions seem to be less pronounced. However, even in the dog the mitochondrial–lysosomal acid phosphatase can be distinguished from the supernatant acid phosphatase by the former's higher sensitivity to tartaric acid than to copper sulfate and by its relatively high activity against ANP.

Whether the postischemic serum acid phosphatase originates from the white blood cells, platelets, or from the microsomal-soluble fraction of the liver—all of which release acid phosphatases with similar characteristics to the postischemic serum acid phosphatase —cannot be deduced from these experiments. To reach high serum acid phosphatase activity against PNPP, about 100,000 platelets or 10,000 white blood cells per microliter would have to undergo lysis and release of their total acid phosphatase content. Although there is a sharp decline in platelet count following liver ischemia due probably to sequestration of the platelets in the liver it

is not known whether this leads to the release of platelet acid phosphatase. It has been shown that platelets release their content selectively under different stimuli (13).

Severe liver ischemia is followed by the appearance of very high levels of glutamate–oxalacetate transaminase, lactic dehydrogenase, and other cytoplasmic enzymes (12). Since acid phosphatase is present in the cytoplasm, it could be released at the same time and by the same mechanism as the transaminases and dehydrogenases. The good correlation between death of the experimental animal and abnormal levels of serum acid phosphatase might be due to the fact that very extensive liver damage is needed for a definite increase in circulating acid phosphatase activity while the sensitivity of the transaminase and dehydrogenase determinations allow the diagnosis of moderately severe liver damage.

Summary. The inhibitory sensitivities and substrate preferences of dog erythrocyte, leukocyte, thrombocyte, and liver subcellular fraction acid phosphatases were determined and compared to those of the serum acid phosphatase appearing after long periods of hepatic inflow stasis. This postschemic acid phosphatase is inhibited moderately by tartaric acid and strongly by copper sulfate and the ratio of activities with PNPP to that of ANP shows values around seven. These characteristics are similar to those of platelet, white blood cell, and liver microsomal-soluble supernatant fraction acid phosphatases. Since there is extensive leakage of other cytoplasmic enzymes into circulation following liver ischemia it seems likely that cytoplasmic nonlysosomal acid phosphatase contributes considerably to the postischemic serum acid phosphatase activity.

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Received Oct. 1, 1971. P.S.E.B.M., 1972, Vol. 139.