Metal Requirements of Alkaline Phosphatases of Human and Rabbit Leukocytes. (23110)

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(Introduced by G. L. Hobby)
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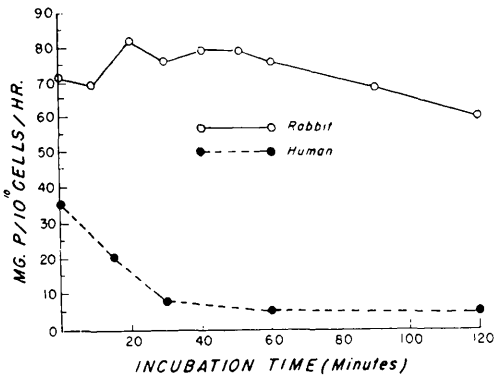
Histochemical and biochemical studies have clearly shown that leukocytes of both man and rabbit are rich in alkaline phosphomonoesterase. Haight and Rossiter(1) showed that the rabbit leukocyte was richer in alkaline phosphatase than the human leukocyte and that magnesium was apparently necessary for complete activation of the enzyme. Valentine and Beck(2) applying the technics of Haight and Rossiter studied alkaline phosphatase activity of the human leukocyte in health and disease. They were able to demonstrate large increases in enzyme content of the leukocyte in infection, polycythemia vera, and in leukemoid states, while sharp decreases were seen in leukemias. In preliminary incubation studies undertaken as part of an investigation into the mechanism of this disease-induced enzymatic change of the leukocyte, striking inhibition of alkaline phosphatase activity became manifest when di-Na ethylenediamine tetraacetate (EDTA) was used. The effects of this chelating agent on leukocyte alkaline phosphatase and results obtained on metal requirements of the enzyme in man and rabbit will be presented. The data indicate the presence of at least 2 alkaline phosphatases in the leukocyte.

Materials and methods. Leukocytes. Fresh human blood was obtained by venipuncture using siliconized syringes. 10 ml of whole blood was added to 1 ml of several anticoagulants. To each 10 ml of blood 5 ml of 2.4% (w/v) bovine fibrinogen in 5% (w/v) polyvinylpyrrolidone (PVP) was added and blood mixed by inversion. The leukocyte-rich supernatant was removed periodically as rapidly as sedimentation of red cells allowed. The leukocytes were transferred to siliconized tubes and were then centrifuged at 500 x G for 5 minutes and the clear supernatant discarded. Cells were washed 3 x with 0.9% saline and resuspended in a measured quantity of the same medium for WBC, RBC, and total polymorphonuclear cell counts. This procedure resulted in average leukocyte recovery of about 75% with a red cell contamination of 2:1. Fresh rabbit blood was obtained by cardiac puncture using siliconized syringes and treated in the same manner. Sedimentation was generally slower than for human blood and leukocyte yield lower. *Anticoagulants.* EDTA (1.5% in 0.7% saline), heparin (1 mg), or sodium citrate (3.8%) as applicable. *Incubation studies.* The packed cells were resuspended in either 1 ml of 0.9% saline or 1 ml of 0.3% EDTA in 0.9% saline and incubated for varying intervals at 37°C. After incubation, all cells were washed 3 x with 0.9% saline prior to assay for phosphatase activity. *Phosphatase determination.* Assays for alkaline phosphatase activity were carried out as described by Valentine(2); the only modifications being in amount and type of heavy metal employed. The general plan of study was to investigate (1) the effects of EDTA on alkaline phosphatase activity of both human and rabbit leukocytes, and (2) to determine the metal requirements of the EDTA-inhibited enzyme.

Results. Effects of incubation of isolated leukocytes with EDTA. Leukocytes of pooled human and of pooled rabbit blood containing EDTA as anticoagulant, were isolated and incubated with EDTA. The cells were then assayed at varying intervals for alkaline phosphatase activity with no metal added. Fig. 1 shows the difference in behavior between rabbit and human leukocytes. The initial activity of pooled normal rabbit leukocytes was higher than that of pooled normal human leukocytes. Further, the alkaline phosphatase activity of the rabbit leukocyte was not appreciably inhibited during a 2 hr incubation period, whereas the human cell showed a rapid decrease so that in 30 min. approxi-



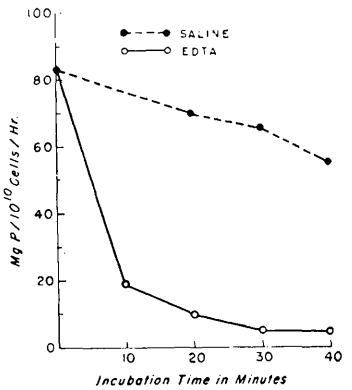


FIG. 1 (top). Effect of EDTA on alkaline phosphatase of isolated human and rabbit leukocytes (anticoagulant: EDTA).

FIG. 2 (bottom). Effect of EDTA on alkaline phosphatase of isolated human leukocytes (anticoagulant: heparin).

mately 80% of initial activity had been lost. After 60 min. no further inhibition was seen.

To determine whether the demonstrated inhibition of alkaline phosphatase of the human cell was due solely to the effects of EDTA. human leukocytes were isolated from heparinized blood. Aliquots were incubated in EDTA and in 0.9% saline and the phosphatase activity compared at intervals. Fig. 2 again demonstrates striking inhibitory action of EDTA. However, the control cells incubated in saline also showed a progressive, though considerably smaller, decrease in enzyme activity. There was no significant decrease in cell count throughout the period of study. In view of the chelating properties of EDTA, an attempt was made to reactivate alkaline phosphatase activity by addition of known metal activators. The addition of magnesium in final concentration of 2.5×10^{-3}M in the phosphatase assay had no effect on level of activity. Upon addition of zinc in final concentration of 2.5×10^{-4}M however, phosphatase activity was restored to original levels in both cases.

Effects of magnesium and zinc on leukocyte alkaline phosphatase activity. The results of a study on effect of varying concentrations of zinc and magnesium on EDTA exposed human and rabbit leukocytes are shown in Table I. The rabbit cell showed a high alkaline phosphatase activity despite 1 hr incubation in EDTA. Addition of magnesium in various concentrations up to 5×10^{-2}M produced some decrease in alkaline phosphatase activity. Similarly, final concentrations of zinc at 2.5×10^{-4}M produced a sharp drop in activity. These inhibitory effects have been attributed(3) to metal substrate complexes formed at high metal concentrations. The EDTA-inhibited human leukocyte, on the other hand, showed a slight rise with increasing concentration of magnesium. However, addition of 5×10^{-5}M zinc caused approximately a 50-fold increase in alkaline phosphatase activity. Here too a drop in activ-

TABLE I. Effect of Zinc and Magnesium on Human and Rabbit Leucocytes Incubated for 1 Hr at 37°C with EDTA.

Magnesium			Zinc		
	mg P/10^{10} cells/hr			mg P/10^{10} cells/hr	
Final molarity	Human*	Rabbit†	Final molarity	Human*	Rabbit†
0	2.4	70.4	0	2.4	70.4
2.5×10^{-4}	2.8	69.2	5×10^{-6}	2.8	64.0
$5 \times$ "	2.0	64.0	1.25×10^{-5}	71.2	69.2
2.5×10^{-3}	3.6	60.0	$2.5 \times$ "	100.0	77.2
$5 \times$ "	4.8	57.2	$5 \times$ "	121.0	81.2
2.5×10^{-2}	4.0	47.0	2.5×10^{-4}	39.0	15.6
$5 \times$ "	4.0	53.2			

* Pooled polycythemic + normal. † Pooled normal.

TABLE II. Effect of Magnesium and Zinc on the Alkaline Phosphatase Activity of Normal Human Leukocytes.

Anti-coagulant	No. of patients	Final molarity metal	Mean activity, mg P/10^{10} cells/hr	Range
EDTA	39	None	3.8 ± 2.1	0.8- 7.8
"	46	2.5×10^{-3} Mg	8.6 ± 5.4	0 -26.4
"	25	5.0×10^{-5} Zn	16.7 ±11.0	2.4-44.8
"	25	2.5×10^{-4} Zn	1.5 ± 1.6	0 - 6.0
Citrate	23	None	25.5 ±15.1	8.0-61.2
"	18	2.5×10^{-3} Mg	31.2 ±16.6	4.8-56.4
"	22	5.0×10^{-5} Zn	23.2 ±11.2	7.6-44.4

ity at high zinc concentration was seen. Loss in phosphatase activity of human leukocytes upon incubation with saline seen in Fig. 2 may possibly be attributed to leakage of zinc from the cell during incubation period, and in the wash periods after incubation.

The addition of EDTA to whole blood produced a progressive decline in alkaline phosphatase activity of leukocytes. After 90 min. at 25°C, there was a 65% decrease in activity of cells subsequently isolated. This activity could be restored by the addition of zinc in final concentration of 5 x 10^{-5}M.

Normal values. Normal values for leukocyte alkaline phosphatase have been determined in man using cells isolated from citrated blood and compared with those obtained with and without the subsequent addition of metals. Table II shows that in man the highest alkaline phosphatase activity can be obtained by using cells isolated from citrated blood and that addition of either magnesium or zinc to this system contributed little. On the other hand, cells isolated from EDTA treated blood required zinc for activation; magnesium produced little response. The maximum activation of EDTA treated cells with zinc or magnesium does not reach the level obtained with cells isolated from citrated blood and this may be due to the fact that we are working at sub-optimal zinc concentrations, or possibly sub-optimal levels of other activators; *e.g.* the alpha-amino acids.

Discussion. The phosphatases require an alpha-amino acid as well as a bivalent metallic ion for their activation(4). There have, however, been many contradictory reports concerning the role of metal ions in activation of alkaline phosphatases. In addition, the question as to number and identity of different phosphatases has still not been resolved. Cloeten(5-7) presented evidence that alkaline phosphatases are metal complexes and concluded from dialysis and inhibition studies that both magnesium and zinc were involved in these complexes. He argued that the alkaline phosphatases of different organs were not identical, probably being a mixture of 2 enzymes. Further, activity of the enzyme extracted from any given tissue was determined by concentrations of these 2 alkaline phosphatases which varied from organ to organ. More recently, Gryder, Friedenwald, and Carlson(3) working with rat kidney reported on the presence of 2 alkaline phosphatases which they called phosphatases A and B. Phosphatase A required both glycine and zinc for activation while phosphatase B required magnesium only and was inhibited by calcium and zinc. The data here presented suggest that there are also 2 alkaline phosphatases in the human leukocyte, one requiring magnesium and the other zinc for activation. In this regard, the rise in leukocyte alkaline phosphatase obtained by Wiltshaw and Moloney(8) following incubation of human leukocytes (from EDTA blood) with serum can probably be attributed to the zinc content of the serum (5 x 10^{-4}M(9)), rather than to some "factor in the leukocyte." Our own studies confirm the activating properties of serum on the EDTA inhibited leukocyte alkaline phosphatase.

Of interest is the fact that Vallee(9) has found a high zinc concentration in the human leukocyte and there seems to be a gross parallel between alkaline phosphatase activity and zinc content of the cell in disease states. The relationship, if any, existing between these 2 cellular constituents is not known. Preliminary results of studies now being carried out suggest that elevation of alkaline phosphatase activity seen in the leukemoid states and in infection in man is due primarily to elevation of the zinc activated phosphatase rather than the magnesium activated enzyme.

In the rabbit, the situation is grossly different inasmuch as EDTA shows no appreci-

able effect on leukocyte alkaline phosphatase activity and zinc or magnesium have no activating effect. Obviously, there is a species difference either in the leukocyte or in the kind of phosphatase present. By histochemical studies we have been able to show a marked increase in the leukocyte alkaline phosphatase of the rabbit following injection of vaccines, pyrogens, etc., but the enzyme type has not as yet been characterized.

Freiman(10) has carried out rather comprehensive histochemical studies of inhibition of alkaline phosphatase activity of various tissues by EDTA and concluded that a number of alkaline phosphatases exist. Our findings indicate that even a brief incubation of human leukocytes with EDTA results in a decrease in alkaline phosphatase activity. EDTA currently enjoys much popularity as an anticoagulant for blood and is widely used in tissue decalcification. Obviously the chelating properties of EDTA for ions other than calcium must be kept in mind in any investigation of alkaline phosphatase activity in blood or tissues.

Summary. EDTA inhibits alkaline phosphatase activity of the human leukocyte. The rabbit leukocyte is, however, not so affected. There seem to be 2 alkaline phosphatases in the human leukocytes: one requiring magnesium and the other zinc for its activation.

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Spleen DPNH Cytochrome *c* Reductase Activity In X-Irradiated Rats.* (23111)

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With either α-ketoglutarate or succinate as substrate, several laboratories(1-3) have reported a decrease in oxidative phosphorylation by mitochondria and homogenates prepared from spleens of rats and mice exposed to lethal doses of X-irradiation. Oxygen consumption was also found to be diminished in the presence of either α-ketoglutarate(2,3) or malate(4). Since phosphorylation is coupled to DPNH oxidation in the sequence DPNH → cytochrome *c*, it was considered of interest to determine whether interference with DPNH cytochrome *c* reductase activity in the spleens of X-rayed animals was involved in the lowered oxidation rates or linked in some way to the diminished P:O ratios. Another area on which some light might be shed by the study of DPNH cytochrome *c* reductase activity in irradiated animals is in the relation of cell structure to function. The presence of a high concentration of this enzyme in liver(5) and spleen† microsomes is somewhat of an oddity, since most of the known linked respiratory systems are associated with the mitochondria. Loss of microsomal DPNH cytochrome *c* reductase to the exclusion of the mitochondrial enzyme would appear to cast

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The following abbreviation is employed: DPNH = reduced diphosphopyridine nucleotide.

† Eichel, H. J., *J. Biophys. and Biochem. Cytology,* 1957, v3, no. 3, in press.