

## A Histochemical Procedure for Localizing and Evaluating Leukocyte Alkaline Phosphatase Activity in Smears of Blood and Marrow

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**T**HE RESULTS of previous studies<sup>1-8</sup> on the histochemical localization of alkaline phosphatase activity in cells of blood and bone marrow have been varied and contradictory. All such studies, except that of Vercauteren,<sup>8</sup> were based on the Gomori-Takamutsu procedure.<sup>9, 10</sup> Numerous investigators<sup>11-19</sup> have shown that this procedure may produce false staining of nuclei and other structures, particularly with long incubation times. Furthermore, the fixation methods used in these studies did not always adequately preserve cytologic detail.

The present communication describes a rapid and simple procedure for demonstrating alkaline phosphatase activity in cells of blood and marrow, in which a new fixative procedure and the azo dye staining method are used.<sup>20-23</sup>

A "scoring" method is described whereby intracellular alkaline phosphatase activity in blood smears from different individuals may be compared and evaluated. Brodell and Swisher<sup>7</sup> reported a technic for deriving "leukocyte alkaline phosphatase indices." They described their method as being "based upon microscopic grading from 0 to 4 of the depth of staining of 100 successive leukocytes." Cells were selected for grading on smears prepared from separated leukocytes obtained by differential centrifugation. They recommended the use of a phase microscope. In deriving their indices, these authors counted leukocytes of all types. Since alkaline phosphatase activity appears limited to neutrophils only, their figures probably did not accurately reflect the degree of activity of the phosphatase-containing leukocytes.

The application of the staining and scoring methods herein described offers a convenient and practical means of estimating leukocytic alkaline phosphatase activity in health and disease.

### MATERIALS AND METHODS

#### *A. Preliminary Studies*

Various fixatives were evaluated for their ability to preserve cell structure and enzyme activity. Using fresh frozen sections of rat kidney and staining for alkaline phosphatase activity by the azo dye technic, sections fixed in formalin stained much more rapidly and intensely than did duplicate sections fixed in absolute methanol under similar conditions. Formalin, however, was unsuitable for fixing blood smears as it lysed and distorted erythrocytes. This was observed with 10 per cent formalin, 10 per cent buffered formalin (pH 7.4),

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15 per cent formol-saline or 15 per cent formol-saline in 50 per cent methanol. Absolute acetone and formol vapor were also discarded as fixatives as they distorted and cracked the blood film. The inhibitory effect of methanol was overcome by combining it with formalin and fixing for short periods at low temperature. A combination of 10 per cent formalin in absolute methanol at  $0 \pm 5$  C. for 30 seconds was chosen as the fixative of choice and was used in all subsequent experiments. Preservation of cytologic detail was excellent and enzyme destruction was minimal as judged by comparing the intensity and rapidity of staining of fresh frozen sections of rat kidney, both fixed and unfixed.

As observed by previous investigators,<sup>14, 24</sup> propanediol buffer was found more effective than veronal buffer in maintaining the pH of the substrate mixture. Despite more rapid disintegration of the diazonium salt, staining was more intense and more rapid with 0.05 M propanediol buffer at pH 9.6 than with veronal-acetate buffer at pH 9.6.

### B. Reagents and Solutions

1. *Fixative solution:* Formalin (36–39 per cent formaldehyde).....10 ml.  
Absolute methanol.....90 ml.  
(Between use, store in freezing unit of refrigerator.)
2. *Stock 0.2 M propanediol solution:* Dissolve 10.5 Gm. of 2-amino-2-methyl-1,3-propanediol\* in 500 ml. of distilled water. (Store in refrigerator.)
3. *Working 0.05 M propanediol buffer, pH 9.75:* to 25 ml. of stock 0.2 M propanediol solution, add 5.0 ml. of 0.1 N HCl and dilute to 100 ml. with distilled water. (Store in refrigerator.)
4. *Substrate mixture:* (prepare immediately before use)  
Sodium alpha naphthyl acid phosphate†.....35 mg.  
Fast Blue RR‡ (diazonium salt of 4-benzoyl-2:5-methoxy-aniline).....35 mg.  
Working 0.05 M propanediol buffer, pH 9.75.....35 ml.  
(filter directly onto slides or into Coplin jars and use at once, the pH of the mixture is 9.5–9.6)
5. *Counterstain:* Mayer's aqueous hematoxylin.
6. *Mounting Medium:* Glycerin gelatin, adjusted to pH 7.5–8.0.

### C. Staining Schedule

1. Prepare blood smears on slides as for routine differential staining.
2. Immerse slides in fixative solution for 30 seconds at  $0 \pm 5$  C. Wash in running tap water for 10 seconds.
3. Incubate in substrate mixture for 10 minutes at room temperature. Wash in running tap water for ten seconds.
4. Counterstain with Mayer's hematoxylin for 3–4 minutes. Wash in running tap water for 10 seconds and air-dry.
5. Mount in glycerol gelatin and examine under high dry or oil immersion objective.

### D. Scoring Technic

Count 100 consecutive neutrophilic granulocytes, rating each from *zero* to *four* on the basis of intensity and appearance of the precipitated dye in the cytoplasm:

- Zero*.....colorless  
*One*.....diffuse pale brown, no granules  
*Two*.....brown with or without occasional clumps of brownish-black precipitate  
*Three*.....brownish-black, unevenly distributed granular precipitate  
*Four*.....uniform deep black granular precipitate  
 The sum of the ratings of 100 cells is considered the "score" for a particular smear.

\* Eastman Kodak Co., Rochester, N. Y.

† Dajac Laboratories, Leominster, Mass.

‡ General Dyestuff Corp., 255 Atlantic Ave., Boston, Mass.

For purposes of comparison, in scoring select areas of similar cell thickness, preferably where the erythrocytes just touch.

### RESULTS AND DISCUSSION

In every smear examined from 75 normal adults and 65 patients with a variety of pathologic disorders, alkaline phosphatase activity was found exclusively in the cytoplasm of some mature and stab-form neutrophilic granulocytes. All other leukocytes, erythrocytes and thrombocytes were unstained. (See figures 1 and 2.) Basophilic granulocytes could not be distinguished with the counterstain used.

A limited number of bone marrow smears were similarly treated and showed staining not only in neutrophilic granulocytes but also in the cytoplasm of reticuloendothelial cells. Staining was much more intense in these cells than in the neutrophils. (See figure 3.)

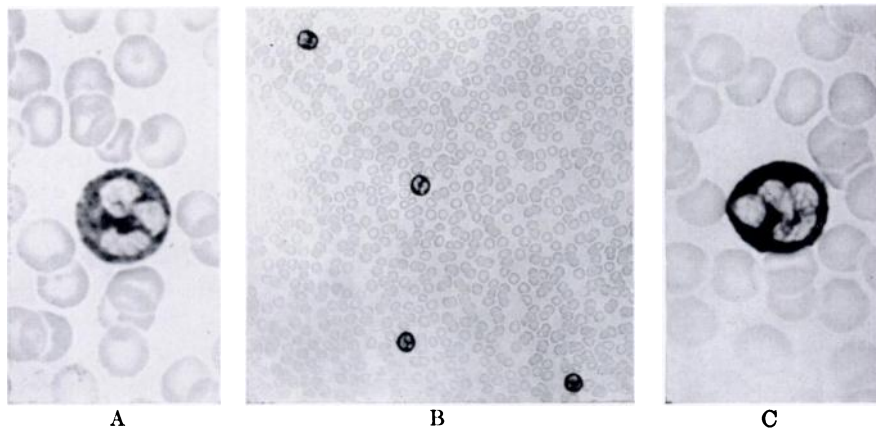


FIG. 1.—Peripheral blood smears stained for alkaline phosphatase activity, not counterstained, showing absence of nuclear staining. From patient with bacterial pneumonitis. A & C—( $\times 900$ ), B—( $\times 200$ )

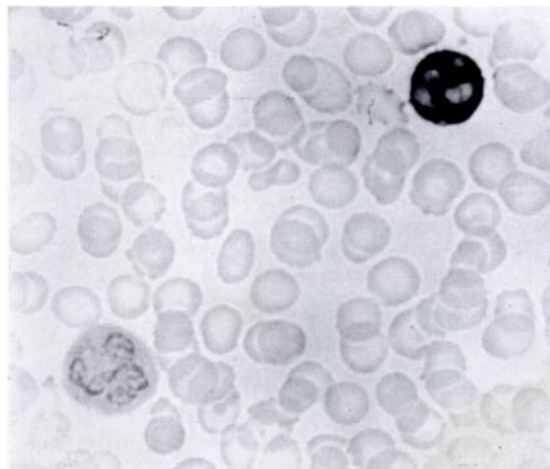


FIG. 2.—Blood smear stained for alkaline phosphatase, counterstained with Mayer's hematoxylin showing variation in staining intensity of two neutrophils. ( $\times 900$ )

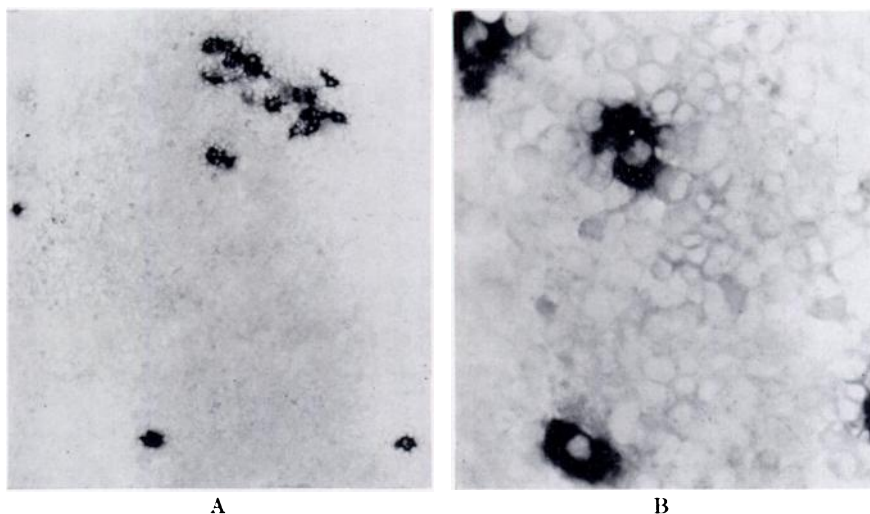


FIG. 3.—Bone-marrow smear stained for alkaline phosphatase, not counterstained, showing intense staining in cytoplasm of reticuloendothelial cells. A. 120X, B. 450X.

Previous investigators,<sup>1-8</sup> using the Gomori-Takamutsu technic, reported some nuclear staining of blood cells. The artifactual nature of nuclear staining in fixed tissues has been thoroughly reviewed by Pearse<sup>23</sup> and others. The absence of nuclear staining of blood cells as observed in this study and as reported by Vercauteren<sup>8</sup> indicates a similarity between the alkaline phosphatase of fixed tissues and that observed in peripheral blood cells. Vercauteren also reported activity in the cytoplasm of eosinophils. This was not confirmed in the present study. Alkaline phosphatase activity could not be demonstrated in any eosinophils in smears from a patient whose circulating eosinophil count was 34,000/cu.

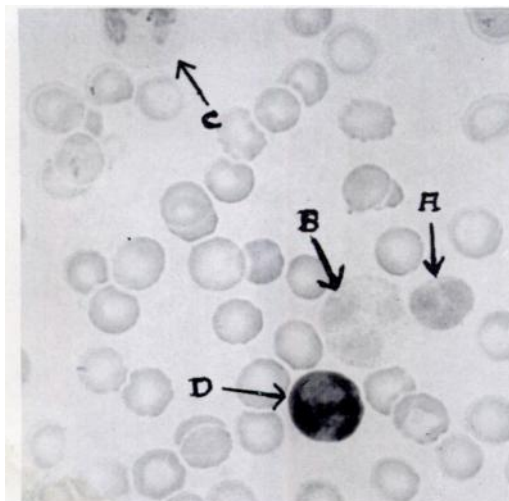


FIG. 4.—Peripheral blood smear stained for alkaline phosphatase activity, counterstained with Mayer's hematoxylin, from patient with erythroblastosis fetalis. A—Phosphatase negative lymphocyte, B—Phosphatase negative eosinophil, C—Phosphatase negative neutrophil, D—Phosphatase positive (3 plus) neutrophil. (X900)

TABLE 1.—*Comparison Between Serum Alkaline Phosphatase Levels and Leukocyte Alkaline Phosphatase Scores*

Patient	Serum Alkaline Phosphatase Levels (Bodansky units)	Leukocyte Alkaline Phosphatase "Scores"
1	36.5	129
2	29.1	128
3	22.7	27
4	16.4	82
5	14.9	10
6	14.2	37
7	7.7	147
8	4.6	25
9	2.8	86

mm. Unfortunately, Vercauteren did not report on any of the details of his method.

The neutrophils varied markedly in their degree of activity, particularly in patients whose smears exhibited high activity. (See figure 4.) This variation in the staining intensity of different neutrophilic granulocytes supports the speculation of Valentine et al.<sup>26</sup> who suggested that increased alkaline phosphatase activity may be initiated at the site of cell maturation, resulting in the appearance in the circulatory system of cells of two types: those with little or no activity originating prior to the increase, and those rich in alkaline phosphatase, originating after the increase.

No correlation was observed between serum alkaline phosphatase activities and the intensity of intracellular staining. Comparative figures are indicated in table 1.

Although staining was observed only in some mature or stab-form neutrophils, no correlation was observed between intensity of staining and total or differential leukocyte counts. Low scores and high scores were obtained on smears from patients with normal total counts and from patients exhibiting leukopenia or leukocytoses, although scores tended to be elevated in the latter. Similarly, both low and high scores were obtained from patients with normal differential leukocyte counts and those with shifts toward immaturity. These findings are consistent with the observations of Valentine and Beck<sup>25</sup> in their biochemical studies on separated leukocytes.

Using the scoring technic on smears from 68 healthy adults, values ranged from 2 to 76 with a mean of 22. Sixty-seven per cent of the smears had scores below 25 and only 11% had scores above 50. Most of the neutrophils on these smears were unstained, i.e., rated *zero*, and no cells rated above *two* were observed. Studies still in progress on smears from patients with a variety of conditions have indicated that neutrophilic alkaline phosphatase activity may be significantly increased in numerous pathologic conditions. (See table 2.) Wachstein<sup>1</sup> using histochemical methods reported increased intracellular alkaline phosphatase activity in infectious leukocytoses. Valentine and his group,<sup>26</sup> studying separated leukocytes biochemically, confirmed the observations of Wachstein and also reported increases in a variety of other "stress" conditions.

The staining and scoring methods described in this report are reproducible

TABLE 2.—Scoring Data on Blood Smears from Fourteen Bed-Ridden Patients

Patient	Diagnosis	Percentage of Neutrophils Rated					Total Score
		0	1	2	3	4	
1	Cerebrovascular accident	92	8	0	0	0	8
2	Hodgkin's disease	90	11	0	0	0	11
3	Lymphoma	88	12	0	0	0	12
4	Paget's disease	47	51	2	0	0	55
5	Diabetes (uncomplicated)	39	48	7	0	0	62
6	Megaloblastic anemia	40	48	12	0	0	72
7	Prostatic carcinoma	30	59	10	1	0	82
8	Diabetes (in acidosis)	39	38	18	5	0	89
9	Uremia	30	45	25	0	0	95
10	Craniotomy (24 hrs. post-op.)	21	47	32	0	0	111
11	Craniotomy (48 hrs. post-op.)	13	36	36	15	0	153
12	Poliomyelitis (acute)	6	38	30	21	5	181
13	Polycythemia vera	19	16	32	23	10	189
14	Pneumonia (bacterial)	4	4	27	25	40	293

and readily permit the detection of gross differences in neutrophilic alkaline phosphatase activity. The method requires a minimum of time and equipment and should facilitate studies associated with the present interest in leukocytic enzyme systems.

#### SUMMARY

A rapid and simple method is described for demonstrating the presence of alkaline phosphatase activity in cells of blood and bone marrow. Smears were fixed with 10 per cent formalin in methanol at  $0 \pm 5$  C. for 30 seconds and stained by the azo dye procedure, using propanediol buffer at a pH of 9.6.

A "scoring" technic is outlined, which permits the comparison of alkaline phosphatase activities of blood leukocytes of different individuals.

Alkaline phosphatase activity was found only in neutrophilic granulocytes and was localized exclusively in the cytoplasm of these cells. In normal adults, 43 to 95 per cent (mean of 78%) of all neutrophils were unstained. Staining was up to ten times more intense in smears from patients with a variety of pathologic disorders.

#### SUMMARIO IN INTERLINGUA

Es describe un simple e rapide methodo pro demonstrar le presentia de activitate de phosphatase alcalin in cellulas in frottis de sanguine e de medulla. Le frottis esseva fixate per medio de 10 pro cento de formalina in 90 pro cento de methanol durante 30 secundas a un temperatura de  $0 \pm 5$  C. Illos esseva colorate per le technica azoic con propanediol a un pH de 9,6 usate como tampon.

Es delineate un technica de tabulation que permette le comparation del activitate de phosphatase alcalin in leucocytos ab plure individuos.

Activitate de phosphatase alcalin esseva trovate solmente in granulocytos neutrophile e esseva localisate exclusivemente in le cytoplasma de iste cellulas. In adultos normal, inter 43 e 95 pro cento (i.e. 78 pro cento al mediano) de omne neutrophilos remaneva non-colorate. Le coloration esseva usque a 10 vices plus intense in frottis ab patientes con un varietate de disordines pathologic.

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