Histochemical Demonstration of Diaphorases and Dehydrogenases in Normal Human Leukocytes and Platelets

By Karoly Balogh, Jr., and Richard B. Cohen

The problems involved in the cytochemical demonstration of the activity of oxidative enzyme systems in the cells of bone marrow and peripheral blood relate mainly to methods of fixation and to the ultimate identification of the cells in which the enzyme reaction takes place. Approaches have been suggested for the demonstration of the activity of specific dehydrogenase systems in blood cells, some of which involve the use of deleterious physical or chemical agents and require the use of counterstains. In our experience counterstains which do not interfere with interpretation of the histochemical reaction are not adequate for proper identification of white blood cells.

It seemed desirable to develop a simple technic permitting the study of enzyme activity in minimally altered cells which can be easily identified. This has been achieved by using phase contrast microscopy along with histochemical methods.

Materials and Methods

For the purpose of describing the method we confined our study to the peripheral blood of healthy laboratory workers. The blood is obtained from the finger by the prick of a lancet and smeared in an even film on clean slides. The smears are dried for several minutes at room temperature and then a drop of substrate solution (see below) cooled to 4°C is pipetted on to it. A coverslip (Corning No. 1, 22 mm sq.) is placed on the drop which spreads as a thin film and serves as an incubating system for cytochemical study and a mounting medium for microscopy. The coverslip is immediately sealed with vaseline. The slides are then incubated for periods up to 1 hour at 37°C. Following incubation the slides are examined using a Zeiss standard GFL microscope equipped with an achromatic-aplanatic phase contrast condenser VZ. The positive image contrast obtained in the optical system is observed first under high dry magnification (x400), and then under oil immersion (x1000). By means of the phase microscope technic nuclei and cytoplasmic granules of white blood cells stand out clearly and may be identified according to the description of Bessis and Rind without counterstain. On identification of the cell by phase contrast microscopy the optical system may be switched without moving the slide to bright field illumination. Prior to incubation under bright field illumination the unstained white blood cells can be seen only with great difficulty and cytoplasmic granules appear as bright refringent bodies. Subsequent to incubation the

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The research was supported by Grant No. C-3424 of the National Cancer Institute, National Institutes of Health, Bethesda, Md. and by USPHS Research Training Grant in Pathology No. 2G-192.

Submitted Dec. 5, 1960; accepted for publication Jan. 5, 1961.
precipitated formazan stands out sharply against the unstained cell. The quantity and distribution of the indicator of enzyme activity may then be evaluated. Under some circumstances it may be advantageous to identify certain cells or cell groups by phase microscopy prior to incubation. This may be done easily, and the cell’s position noted using the micrometer and vernier scale attached to the mechanical stage of the microscope.

The oxidative enzyme systems studied are listed in table 1 which includes a reference to the method used for the histochemical demonstration of each enzyme. In all instances the basic technic involves the reduction of a tetrazolium salt, 2,2'-di-(p-nitrophenyl)-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride (Nitro-BT). This compound is colorless until it is reduced by H ions liberated from substrates via specific dehydrogenases or diaphorases. On reduction it forms a deep blue granular, fat and water insoluble substantive diformazan which serves as an indicator of the site of enzyme activity.

Control preparations to exclude non-specific reduction of Nitro-BT are treated in an identical manner, but incubated in solutions which lack specific substrate, but do include Nitro-BT, coenzymes, and the necessary inorganic ions.

RESULTS

The results are summarized and roughly quantitated on a 0 to 4 plus basis in table 1. Enzyme activity varied only slightly in a given cell group. It was in all instances confined to the cytoplasm of white blood cells and to thrombocytes. Definite enzyme activity in mature red blood cells was not demonstrated by this technic. The reason for this was not clear, however, it is probable that constituents of the incubating mixture may not penetrate into the red blood cells. Fine granules of formazan were noted on the surface of these cells, but on the basis of their distribution could well be artifacts. Fibrin filaments, or serum did not demonstrate enzyme activity by this method. The age and sex of subjects did not influence enzyme distribution. Controls were consistently negative.

Polymorphonuclear leukocytes showed varying degrees of moderate activity of all enzymes studied. The deposition of dye appeared to be independent of neutrophilic granules (figs. 1a and b). The distribution of enzyme activity in eosinophils and basophilic leukocytes was approximately the same, however, generally less intense. Occasionally glucose-6-phosphate dehydrogenase in eosinophils, and lactic dehydrogenase in basophils equaled the activity of these enzymes in neutrophils. The eosinophilic and basophilic granules were dissociated from the granules of the dye (figs. 2a and b, 3a and b).

Enzyme activity in the lymphocyte was localized at the site of mitochondrial concentration (figs. 4a and b). This was predominantly at one pole of the cell, where a small nuclear notch accentuated the frequent polarity of enzyme localization. Activity was not entirely confined to this region, some dye having been deposited in the thin cytoplasmic rim around the entire nucleus. Focal concentration of enzyme activity in the mitochondria of lymphocytes made quantitative comparisons with neutrophils difficult.

The pattern and relative intensity of dye deposition in monocytes were similar to those of lymphocytes. Monocytes also showed predominant localization of enzyme activity in the region of mitochondria (figs. 5a and b).

All these enzymes were very active in thrombocytes. Particularly striking in this regard was beta-hydroxybutyric dehydrogenase (figs. 6a and b). The enzyme activity in the platelets appeared to be located at the site of the granulomere.
### Table 1.—Results

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>POLYMORPHONUCLEAR LEUKOCYTES</th>
<th>LYMPHOCYTES</th>
<th>MONOCYTES</th>
<th>THROMBOCYTES</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NEUTROPHIL</td>
<td>EOSINOPHIL</td>
<td>BASOPHIL</td>
<td></td>
</tr>
<tr>
<td>DPNH DIAPHORASE 16</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>TPNH DIAPHORASE 16</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
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<tr>
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<td>+</td>
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</tr>
<tr>
<td>LACTIC DEHYDROGENASE 12 (DPN)</td>
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<td>+</td>
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<td>++</td>
</tr>
<tr>
<td>GLUTAMIC DEHYDROGENASE 12 (DPN)</td>
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<td>+</td>
<td>++</td>
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</tr>
<tr>
<td>β-HYDROXYBUTYRIC DEHYDROGENASE 12 (DPN)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>GLUCOSE-6-PHOSPHATE DEHYDROGENASE 13 (TPN)</td>
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<td>+</td>
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</tr>
<tr>
<td>6-PHOSPHOGLUCONIC DEHYDROGENASE 13 (TPN)</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>ISOCITRIC DEHYDROGENASE 12 (TPN)</td>
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<td>+</td>
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The figures after each enzyme indicate the reference for the histochemical method used. Abbreviations: Diphosphopyridine nucleotide (DPN); Reduced diphosphopyridine nucleotide (DPNH); Triphosphopyridine nucleotide (TPN); Reduced triphosphopyridine nucleotide (TPNH).

### DISCUSSION

The activity of oxidative enzyme systems in white blood cells has been investigated using techniques which destroy the morphologic integrity of the cell.2,4,7,10,17,18 These methods do not permit individual study of various cell types. The histochemical method affords an approach which to a considerable extent preserves the integrity of the cell, allows its identification and provides data on the distribution of enzyme activity.

A method for the cytochemical demonstration of dehydrogenases in white blood cells using Nitro-BT and specific substrates has been described.14 This method requires acetone fixation, and identification of the cells depends on counterstaining with nuclear fast red. Another method has been published recently, in which a drop of blood was incubated with a substrate solution containing sodium succinate and Nitro-BT for the purpose of demonstrating succinic dehydrogenase activity. Following incubation the mixture was inverted on a slide and examined under phase contrast and bright field illumination. The technic we propose is done on ordinary blood smears, and does not require fixation nor is counterstaining necessary since phase contrast microscopic examination provides the means of identification of the cell.
Fig. 1a.—Neutrophilic polymorphonuclear leukocytes as observed by phase contrast microscopy prior to incubation in substrate solution. X2140. b. The same cell as observed by bright field microscopy after incubation in the substrate designed to demonstrate lactic dehydrogenase activity. The neutrophilic granules are refringent and do not contain formazan deposits. X2140.

Fig. 2a.—An eosinophilic leukocyte as observed by phase contrast microscopy. X2140. b. The same cell as observed by bright field microscopy demonstrating DPNH diaphorase activity. The dye is absent from the eosinophilic granules. X2140.

Fig. 3a.—A phase contrast microphotograph of a basophilic leukocyte. X2140. b. The same cell in bright field showing the cytoplasmic distribution of DPNH diaphorase. The basophilic granules show no activity. X2140.

Fig. 4a.—A lymphocyte as observed by phase contrast microscopy. The dark granules observed in the upper portion of the cytoplasmic rim are at least in part mitochondria. X2140. b. The same cell in bright field showing malic dehydrogenase activity. Note the polarity of the formazan deposit in the cytoplasmic rim. The localization corresponds to the distribution of mitochondria in the lymphocytes. X2140.

Fig. 5a.—A monocyte as observed by phase contrast microscopy. X2140. b. The same cell in bright field showing the granular deposits of dye demonstrating the sites of glutamic dehydrogenase activity. X2140.

Fig. 6a.—Platelets observed by phase contrast microscopy. X2140. b. The same group in bright field showing strong lactic dehydrogenase activity. X2140.

The enzyme activities as noted in table 1 cannot be accurately compared to results obtained by quantitative technics which do not preserve cell structure. All white blood cells and thrombocytes demonstrate activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase which suggests a capacity to oxidize carbohydrate via the hexose-monophosphate pathway. This capacity has been indicated by other workers. Activity of lactic
dehydrogenase is generally high as determined by this and other methods.\textsuperscript{2,3} An interesting finding is the considerable oxidative enzyme activity of thrombocytes. This confirms the work of Koppel and Olwin,\textsuperscript{9} who observed active dehydrogenase systems in human thrombocytes using a quantitative method, and is consistent with the finding of an active oxidative metabolism in platelets as observed by others.\textsuperscript{18}

More detailed interpretation of enzyme activity in white blood cells and thrombocytes as determined by this histochemical approach will evolve from studies of normal and abnormal human peripheral blood and bone marrow, and from the study of blood cells under experimental conditions.

**Summary**

A method for the cytochemical demonstration of oxidative enzyme activity in the white cells and platelets of peripheral blood is described. The technic depends upon the reduction of a colorless ditetrazolium salt to a blue granular insoluble diformazan at the site of enzyme activity. Identification of the cells is accomplished by phase microscopy thus avoiding the deleterious effects of fixation and counterstaining. The distribution of oxidative enzyme activity in normal peripheral blood as observed by this method is discussed.

**Summario in Interlingua**

Es describite un methodo pro le demonstration cytochimic de activitate enzymatic oxydative in le leucocytos e plachettas de sanguine peripheric. Le technica depende del reduction de un sal incolor de ditetrazolium ad in un insoluble diformazano granular de color blau al sito del activitate enzymatic. Le identification del cellulas es accomplite per microscopia a phases, de manera que le effectos deletori del fixation e contratincturation pote esser evitate. Es discutite le distribution de activitate enzymatic oxydative in normal sanguine peripheric como illo es observate per medio de iste technica.

**References**


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