Brief Report

Simplified Myeloperoxidase Stain Using Benzidine Dihydrochloride

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THE DEMONSTRATION of leukocyte peroxidase activity has been of practical value to the hematologist since the beginning of the present century. Many technics have been described based on the use of benzidine base as an indicator. This compound when oxidized precipitates as brown or blue insoluble crystals or granules.

The problems encountered in staining for peroxidase activity are those common to most cytochemical enzyme staining methods, namely spurious staining, enzyme inhibition and cellular distortion. Furthermore, benzidine base is carcinogenic and its production has been limited because of manufacturing hazards. Benzidine dihydrochloride, although also carcinogenic, is more granular and less powdery than the base, and thus less dangerous to handle. It is commonly offered as a substitute by most laboratory suppliers. Methods using benzidine dihydrochloride to demonstrate leukocyte peroxidase have not been previously described. In our hands, attempts to substitute the salt in place of the base in older methods without modifications have been uniformly unsuccessful.

The present report describes a highly sensitive and rapid method for localizing intracellular peroxidase activity using benzidine dihydrochloride as the indicator compound in a single, stable, reusable staining solution. The method permits the use of fixed material resulting in excellent preservation of cell morphology.

METHOD

1. Use fresh smears of blood or bone marrow or organ imprints. Activity may be preserved for as long as 3 weeks if preparations are stored in the dark. Venous or peripheral blood is equally satisfactory. Heparin, oxalate and EDTA are not inhibitory.

2. Fix slides for 60 seconds at room temperature in 10 per cent formal-ethanol. (10 ml. of 37 per cent formaldehyde and 90 ml. of absolute ethyl alcohol). Wash for 15 to 30 seconds with gently running tap water. Shake off excess water.

3. Place wet slides in incubation mixture in a Coplin jar for 30 seconds at room temperature.

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This investigation was supported in part by Public Health Service Research Grants HE-07456 and HE-09344 from the National Heart Institute.

The earlier portions of this study were carried out in the Department of Pathology of the Medical College of Virginia, Richmond, Virginia.

Submitted Feb. 23, 1965; accepted for publication Apr. 25, 1965.

215

Blood, Vol. 26, No. 2 (August), 1965
Incubation mixture:

- 30 per cent ethyl alcohol ........................................ 100 ml.
- Benzidine dihydrochloride ....................................... 0.3 Gm.
- 0.132 M (3.8 per cent w/v) ZnSO₄ · 7 H₂O ...................... 1.0 ml.
- Sodium acetate (NaC₂H₃O₂ · 3 H₂O) ........................... 1.0 Gm.
- 3 per cent hydrogen peroxide ................................... 0.7 ml.
- 1.0 N sodium hydroxide .......................................... 1.5 ml.
- Safranin O .................................................. 0.2 Gm.

The reagents should be added in the order listed, mixing well with each addition. The benzidine salt may contain a small amount of inert residue which will not go into solution. A precipitate forms upon addition of the zinc sulfate. This dissolves upon addition of the remaining reagents. Omit the safranin if a nuclear counterstain is not required. The final pH is 6.00 ± .05. The solution should be filtered and stored in a capped Coplin jar or bottle at room temperature. The same solution can be used over and over again for as long as 6 months.

4. Wash briefly (5 to 10 seconds) in running tap water, dry and examine.

5. If greater nuclear detail is desired, the stained preparations may be recounterstained in 1 per cent aqueous cresyl violet acetate for 1 minute, or in freshly prepared Giemsa stain for 10 minutes. (Wright’s stain is less satisfactory as a counterstain.)

RESULTS

Peroxidase activity is represented by discrete blue granules in the cytoplasm of granulocytes and monocytes (fig. 1). Eosinophils are stained most intensely and are often tinged brown-black or green-black (fig. 1D). The cytoplasm of neutrophils is filled with blue dye. Rarely a neutrophil is observed which is weakly stained or unstained. Monocytes (fig. 1E) exhibit weak to moderate activity as compared to granulocytes. All other formed elements of blood and bone marrow, including basophils, are unstained (fig. 1F).

Satisfactory staining of tissue granulocytes was also obtained using fresh frozen sections.

Examination of blood smears stained for peroxidase from patients with a wide variety of disorders showed no deviation from normal.

No staining was observed if benzidine or hydrogen peroxide was omitted from the incubation mixture. Almost complete inhibition of peroxidase activity was observed when 10⁻⁵ M KCN was added to the incubation mixture.

DISCUSSION

In many instances, fixation is not possible with older peroxidase staining methods because of enzyme inhibition or the formation of long needle-like crystals. Undesirable erythrocyte staining may also be troublesome. The increasing difficulty in obtaining benzidine base and the hazards attendant to its use are additional items of concern. Quaglino and Flemans suggested using o-tolidine in place of benzidine base. This compound is noncarcinogenic and readily available. The final reaction product is yellow-brown. Attempts to substitute o-tolidine in place of benzidine dihydrochloride in the present method were unsuccessful.

Satisfactory staining was also obtained using benzidine sulfate. This reagent
Fig. 1.—Simplified myeloperoxidase stain using benzidine dihydrochloride. (A) Peripheral blood granulocytes. Safranin omitted from staining mixture. (B) Peripheral blood granulocytes. Safranin present in staining mixture. (C) Two polymorphonuclear neutrophils. Safranin present in staining mixture. (D) Eosinophil. Safranin present in staining mixture. (E) Monocyte. Safranin present in staining mixture. (F) Lymphocyte and platelets showing absence of peroxidase activity. Safranin present in staining mixture. (A, C, D, E, F—Mag. x 1500; B—Mag. x 250.)
is less soluble and less granular than the hydrochloride salt. It was considered almost as much of a hazard in handling as the base.

With the technic described, the pH optimum for leukocyte peroxidase was 5.8 to 6.5. At pH below 5.7 erythrocyte staining became evident. Best preparations were obtained using mixtures buffered with sodium acetate to pH 6.0.

The role of zinc sulfate, first used by Usami and Yamamoto\(^{1}\) is unclear. It appears to stabilize the blue form of oxidized benzidine and is essential for optimal staining. Other salts, particularly nickel ammonium sulfate, as originally recommended by Mitsui and Ikeda,\(^{6}\) and nickel sulfate are also satisfactory. For a more detailed review of the role of metallic salts and other aspects of leukocyte peroxidase staining, see the publications by Tsukamoto\(^{11}\) and Undritz.\(^{8}\)

**Summary**

A method is described for demonstrating leukocyte peroxidase activity in which benzidine dihydrochloride is used as the indicator compound instead of the more commonly used but potentially more hazardous benzidine base. The method is highly sensitive and rapid and permits the use of fixed blood smears and organ imprints. The incubation mixture, which incorporates safranin as a counterstain, may be used over and over again, for as long as 6 months. The method is also applicable to fresh frozen tissue sections.

**Summary in Interlingua**

Es describite un methodo pro le demonstration del activitate de peroxidase in leucocytos utilisante como composito indicatori dihydrochloruro de benzidina in loco del plus communmente usate sed potentially plus riscose base benzidinic. Le metodo es altemente sensibile e rapide e permette le uso de fixate frottis sanguinee e impressiones ab organos. Le mixtura de incubation, que contine safranina como contracolorante, pote esser re-usate de novo e de novo durante usque a 6 menses. Le metodo es etiam applicabile a sectiones de tissu post congelation in stato fresc.

**References**

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STAIN USING BENZIDINE DIHYDROCHLORIDE


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