The Demonstration of Dehydrogenases and Diaphorases in Cells of Peripheral Blood and Bone Marrow

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The presence of dehydrogenases and diaphorases in cells is demonstrated by placing blood smears or sections of tissues in a buffered incubating solution containing tetrazolium salts and specific substrates. These enzymes accept hydrogen from specific substrates and transfer it through diaphorase to tetrazolium salt. The latter, on accepting hydrogen ions, changes into insoluble formazan, which is precipitated in the form of small grains at the site of enzymatic activity. However, succinic dehydrogenase does not require intervention of a diaphorase in transferring electrons from succinate to tetrazolium salt.

The most commonly used tetrazolium salts are Nitro-BT, 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'(3,3'-dimethoxy-4,4'-biphenylene) di-tetrazolium chloride and MTT, 3-(4,5-dimethyl-thiazolyl-2)-2,5 diphenyl tetrazolium bromide.

De Souza and Kothara found succinic dehydrogenase in the cytoplasm of polymorphonuclear neutrophils after the addition of sodium succinate and Nitro-BT to whole blood. Ackerman, using vital preparations, demonstrated dehydrogenases activity in white cells from peripheral blood with buffered Nitro-BT-succinate incubating solution. Quaglino and Hayhoe showed succinic dehydrogenase activity in lymphocytes, monocytes and granulocyte precursors up to the stage of myelocytes and metamyelocytes, but polymorphonuclears were predominantly negative. They fixed the smears in acetone prior to incubating with Nitro-BT and sodium succinate as substrate. Using unfixed smears, Balogh and Cohen found activity of oxidative enzymes in leukocytes and platelets of peripheral blood. Hayhoe and Quaglino described the distribution of succinic, lactic and glucose-6-phosphate dehydrogenases in normal and leukemic cells in acetone-fixed and fresh unfixed preparations.

In the present work we employed a number of cytochemical methods to demonstrate specifically the presence amid the site of action of these enzymes in hematopoietic cells. We succeeded in proving the specific activity of succinic, lactic and glucose-6-phosphate dehydrogenases, dihydronicotinamide adenine dinucleotide diaphorase (NADH diaphorase, formerly called DPNH diaphorase) and dihydronicotinamide adenine dinucleotide phosphate diaphorase (NADPH diaphorase, formerly called TPNH diaphorase), using special methods for each enzyme. However, we failed to demonstrate a specific activity of glutamic, alcohol and β-hydroxybutyric dehydrogenases.

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MATERIALS AND METHODS

Fresh peripheral blood smears were taken from healthy subjects, including laboratory workers, doctors, nurses etc. The bone marrow smears were obtained from patients who suffered neither from hematologic nor from malignant diseases.

Incubating solutions were prepared specifically for every dehydrogenase.

I. Succinic Dehydrogenase (SDH)

Incubating solution includes equal amounts of sodium succinate, 0.2 M; Sörensen’s phosphate buffer M/15, pH 7.4; and Nitro-BT, 1 mg./1 ml. Two methods were used.

Method 1. A few drops of incubating solution are placed on peripheral blood and bone marrow smears which were dried in air. The smears may be covered with coverslips and incubated in a humid petri dish at 37 C. for 2 hours. Subsequently (it is possible to examine the slides now microscopically with transmitted light or with phase contrast) the coverslips are removed carefully and the smears are dried in air and fixed in formalin fumes at 25 C. for 7 minutes, washed under tap water for 2 minutes, and stained for 10 minutes with 0.1 per cent neutral red, prepared at least a week before.

Method 2. The smears are fixed in 60 per cent acetone for 1 minute, dried in air, incubated in the incubating solution, washed under tap water and subsequently stained with 0.1 per cent neutral red for 10 minutes.

II. Glucose-6-Phosphate Dehydrogenase (G-6-P DH)

The incubating solution contains equal amounts of D-glucose-6-phosphate disodium salt, 0.2 M; NADP, 1 mg./1 ml. of Sörensen’s phosphate buffer M/15 pH 7.4 and Nitro-BT 1 mg./1 ml. The procedure used was method 1 for SDH.

III. Lactic Dehydrogenase (LDH)

The incubating solution contains equal amounts of sodium DL-lactate, 0.2 M; NAD, 1 mg./1 ml. of Sörensen’s phosphate buffer M/15, pH 7; and Nitro-BT, 1 mg./1 ml.

The demonstration of the enzymatic activity is done on smears fixed in formalin fumes at 25 C. for 4½ minutes, washed under tap water for 3 minutes and dried in the air.

A few drops of incubating solution are placed on the slides and the smears are incubated in a humid petri dish at 37 C. for 2 hours. They are washed under tap water and counterstained with 0.1 per cent neutral red for 10 minutes.

Lactic dehydrogenase activity in the cells of peripheral blood and bone marrow was estimated in a semiquantitative way. The grading was done according to the number of stained granules present in the cytoplasm.

- No stained granules: +0
- 1–6 granules or diffuse very weak staining: +1
- 6–14 granules and diffuse weak staining: +2
- 14–30 granules and diffuse staining: +3
- More than 30 granules and/or diffuse strong staining: +4

Peripheral blood smears were taken from 20 healthy subjects; 100 mature neutrophils and 100 lymphocytes were examined. Bone marrow was investigated from 13 subjects. One hundred immature granulocytes, 100 neutrophilic polymorphonuclears, 100 pronormoblasts and basophilic normoblasts, and 100 polychromatophilic and acidophilic normoblasts were examined. The sum of the score of 100 granulocytes, or 100 lymphocytes, or other cells described above, constitutes the score as used in this study.

IV. Diaphorase

Two different incubating solutions were prepared for the demonstration of diaphorases.
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Incubating solution A (after Pearse)

Stock Solution: Working Solution:
- MTT 1 mg./1 ml. 1.25 ml. 0.5 ml. Stock solution
- CoCl₂ 0.5 M 1.25 ml.
- Tris buffer, pH 8 1.25 ml.
- H₂O dest. 2.35 ml.

Incubating solution B includes equal amounts of Nitro-BT, 1 mg./1 ml., and NADH or NADPH. 1 mg./1 ml. of Sorensen’s phosphate buffer M/15, pH 7.4. Fixation of the smears, their incubation with either of the two incubating solutions, and counterstaining is done in the same way as for LDH.

V. Alcohol, Glutamic and β-Hydroxybutyric Dehydrogenases

We prepared an incubating solution which consisted of the following substrates: ethyl alcohol or sodium L-glutamate or DL-β-hydroxybutyric acid sodium salt. The incubating solution was similar to the one for C-6-P DH, the substrate being different, and NAD was substituted for NADP. Fixed and unfixed smears were used.

SUPRAVITAL METHOD

For investigation of dehydrogenases, 1 ml. is drawn from a finger in a heparinized capillary tube. Incubation is done by mixing a drop of blood with the specific incubating solution for each dehydrogenase in a watch-glass. The watch-glass is placed in a moist petri dish and the mixture is incubated for 2 hours at 37 C. Subsequently smears are made from the mixture; they are fixed in formalin fumes for 7 minutes, washed under tap water for 2 minutes, and counterstained with 0.1 per cent neutral red for 10 minutes.

CONTROL

To demonstrate the specific activity of the enzymes under investigation, blood smears or capillary blood were incubated with the appropriate incubating solution but without the specific substrate. The control for succinic dehydrogenase activity was also done by the addition of the specific inhibitor sodium malonate in concentration of 0.02 M to 1.5 M. Iodo acetate, 0.5 M, was used to inhibit the activity of all dehydrogenases and diaphorases.

RESULTS

Using the methods described with Nitro-BT, the demonstration of the enzymatic activity of dehydrogenases depended on the formation of insoluble blue formazan dye, which was precipitated in the cytoplasm either in the form of small blue granules or in a diffuse way. Using MTT for diaphorase activity, the granules and the diffuse coloration were gray-black.

We consider enzymatic activity to be specific if it shows its action in the presence of the corresponding substrate and no activity in its absence.

In unfixed smears a nonspecific enzymatic activity was obtained for alcohol, glutamic and β-hydroxybutyric dehydrogenases when, in the presence of NAD, formazan was formed with or without a specific substrate; in fixed smears no reaction was observed. Using supravital methods and incubation solutions with or without a substrate, and on adding sodium malonate, we always obtained
reduction of tetrazolium salt to formazan in neutrophils of peripheral blood and bone marrow. The morphology of cells is well preserved after fixation with formalin vapors. Smears which were not fixed or fixed in acetone were poorly preserved.

A specific action of SDH, LDH, G-6-P DH, NADH and NADPH diaphorases was made evident in the cells of peripheral blood and bone marrow. The results are summarized below.

**Peripheral Blood Smears**

In lymphocytes the enzymatic activity was strong or moderate in the form of well-defined coarse and/or fine granules and diffused strong blue staining of the cytoplasm (Figs. 13 and 14).

In monocytes few to numerous fine granules and a strong diffused staining of the cytoplasm was obtained (Fig. 8).

**Neutrophilic Granulocytes.** The majority of neutrophils were destroyed in SDH (Fig. 14) and G-6-P DH assays (Fig. 13); those which did not disintegrate contained a few colored granules or parts of their cytoplasm were stained diffusely. In the LDH method, 70 per cent of neutrophils were positive; the number of stained granules present in the cytoplasm varied from 1 to 30. A positive reaction was seen in all the cells in the NADH diaphorase determination, and only about 50 per cent of the neutrophils showed positive staining for NADPH diaphorase.

**Eosinophilic Granulocytes.** The staining was positive for all the enzymes tested in the form of granules (Fig. 9).

**Thrombocytes.** Only occasional granules were seen when examined for SDH activity. Strong diffuse staining with fine granules was found in LDH, G-6-P DH, NADH and NADPH diaphorases activity determinations (Figs. 2, 8 and 13).

**Erythrocytes.** Erythrocytes showed enzymatic activity for G-6-P DH (Fig. 10).

**Bone Marrow Smears**

The positive reaction for LDH, G-6-P DH, NADH and NADPH diaphorases was similar. Blast cells showed a strong activity which decreased progressively with maturation of cells.

In pronormoblasts and basophilic normoblasts (Fig. 2), we found numerous fine or coarse granules and a diffuse staining of the cytoplasm more pronounced around the nucleus. In polychromatophilic and acidophilic normoblasts, the staining was diffuse and/or in the form of granules (Fig. 10).

All the cells of the myeloid series were positive. In younger cells the enzymatic activity was stronger (Figs. 3, 6, 7 and 11). Megakaryocytes showed a strong activity in the form of large and small granules and a diffuse blue cytoplasmic coloration (Figs. 5 and 12). Enzymatic activity was strong in plasma cells with greater intensity in the lighter perinuclear zone (Fig. 4).

The reaction of SDH was much weaker. Fine blue granules were observed
Fig. 1.—The score of LDH activity in cells of peripheral blood and bone marrow.

- X = young myeloid cells.
- O = polymorphonuclear neutrophils.
- ⭕ = pronormoblasts and basophilic normoblasts.
- △ = polychromatophilic and orthochromatic normoblasts.
- — = lymphocytes.

In the blast cells, young myeloid cells and normoblasts. In plasma cells cytoplasmic coarse and fine granules with a diffused cytoplasmic coloration were seen. Only rarely did megakaryocytes exhibit SDH activity.

The results of LDH activity are summarized in Figure 1.

NADPH diaphorase positive reaction was obtained only with the incubating solution B that contained Nitro-BT.
Figs. 2-5.—Bone marrow cells. Fig. 2: Normoblasts and thrombocytes with strong LDH activity. Fig. 3: LDH activity in myeloid cells. The enzymatic activity decreases with the maturation of the cells. Fig. 4: Very strong LDH activity in a plasma cell. Fig. 5: A mature megakaryocyte with very strong NADH diaphorase activity.

Figs. 6 and 7.—Bone marrow cells. Fig. 6: Myeloid cells with strong NADH-diaphorase activity. Fig. 7: NADPH-diaphorase activity in myeloid cells.

Figs. 8 and 9.—Peripheral blood. Fig. 8: A monocyte and thrombocytes with strong NADH-diaphorase activity. Fig. 9: An eosinophil with strong activity and neutrophils with slight NADH-diaphorase activity.
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Figs. 10–12.—Bone marrow cells. Fig. 10: Normoblasts with very strong G-6-P DH activity; the enzymatic activity is evident around the periphery of the erythrocytes. Fig. 11: Myelocytes and metamyelocytes with strong G-6-P DH activity. Fig. 12: A megakaryocyte with very strong G-6-P DH activity.

Fig. 13.—Peripheral blood. G-6-P DH activity in a lymphocyte and thrombocytes.

Fig 14.—Peripheral blood. A neutrophil granulocyte and a lymphocyte with SDH activity.

DISCUSSION

Using a supravital method, the addition of Nitro-BT without a substrate was sufficient for formazan formation. To explain this phenomenon we have assumed that the amount of substrates in the cytoplasm of cells is sufficient to activate their enzymatic reactions and to transfer the hydrogen ions to tetra-
Table 1.—Necessary Factors for Specific Activity of LDH and NADH Diaphorase

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The enzymatic activity in blood cells is graded:

+ + = granular and diffuse reaction.
+ = granular reaction.

zolium salt with the production of the insoluble formazan. Supravital methods, therefore, make the reducing activity of dehydrogenases and diaphorases evident in the living unfixed cells; however, it is impossible to distinguish between the activity of the different enzymes. Although malonate inhibits SDH activity in the living cells, other dehydrogenases present will reduce tetrazolium salts to formazan.

Using Balogh’s method⁴ to demonstrate enzymatic activity in unfixed smears, we could demonstrate specific activity of G-6-P DH, which is NADP-linked, and of NADH diaphorase and SDH, which are not dependent on the presence of NAD or NADP. Using the same method to demonstrate the activity of glutamic, β-hydroxybutyric and alcohol dehydrogenases, which are NAD-linked, the enzymatic reaction went through when only NAD was added to the incubation solutions; addition of the specific substrate did not increase the enzymatic activity. We could not therefore demonstrate the specific activity of those enzymes; the reaction taking place was due to the presence of NADH diaphorase.

In the fixed smears specific action of NADH and NADPH diaphorases could be shown only in the presence of NADH or NADPH, not NAD or NADP. The plausible explanation is that in the unfixed cells the various dehydrogenases continue to act on the intracellular substrates and can reduce NAD or NADP to NADH or NADPH; this action is missing in fixed smears (Table 1).

In demonstrating the activity of diaphorases with Nitro-BT, we found much stronger staining reaction for NADH diaphorase than for NADPH. With MTT tetrazolium, NADPH diaphorase gave negative results.

NADH diaphorase showed a stronger staining with Nitro-BT than with MTT due to a higher redox potential of the former. Another disadvantage of MTT is that its formazan dissolves in immersion oil.
Selective LDH activity in cells from peripheral blood and bone marrow (Table 1) was shown in the following way: On incubation of the smears with lactate but without NAD, with NAD but without lactate and without the two substrates, no reaction was obtained. Only when NAD and lactate were added to the incubation media was the tetrazolium salt reduced to formazan. When, however, NADH was used instead of NAD, the reaction took place in the absence of lactate due to the presence of NADH diaphorase. As shown in Figure 1, the highest score of LDH in bone marrow was found in pronormoblasts, basophilic normoblasts and young myeloid cells; in peripheral blood higher scores were observed in lymphocytes than in granulocytes. The maximal enzymatic activity takes place at 37°C. The enzymatic action starts after 10 minutes of incubation with the appearance of formazan; it increases in strength and reaches the peak after 1½ hours. When smears are left at 20°C, the enzymatic activity of lactic dehydrogenase decreases and disappears after 4 days; at −20°C the activity persists for 1 month.

Some investigators employed metallic ions as activators of dehydrogenases. We used aluminum and cobalt ions without any effect. On adding amital or sodium azide to the incubation media we did not find any increase of formazan production. Formalin vapors, which were used for fixation, inhibited the activity of all the tested dehydrogenases with the exception of LDH.

In order to prove the specific activity of NAD-linked dehydrogenases, it will have to be necessary to use fixing media other than those employed by us—namely, formalin-methanol, formalin vapors, acetone and methanol. Then, the specific enzymatic action will take place only in the presence of both the specific substrate and NAD. The dehydrogenases and the diaphorases which we investigated were found in leukocytes and thrombocytes in variable quantities. In bone marrow cells their activity was stronger in young cells and decreased progressively with their maturation.

The advantage of the cytochemical methods for the demonstration of dehydrogenases and diaphorases is that they enable us to detect the presence of these enzymes in the cell. Furthermore, they permit us to assess the changing amount of the enzymes during cell maturation.

**Summary**

Nicotinamide adenine dinucleotide-linked dehydrogenases, SDII, NADH and NADPH diaphorases were assayed with cytochemical methods in white blood cells in bone marrow cells using tetrazolium salts, Nitro-BT and MTT. In unfixed smears specific enzymatic activity was demonstrated for glucose-6-phosphate dehydrogenase, succinic dehydrogenase and NADH diaphorase. In fixed smears the activity of lactic dehydrogenase and succinic dehydrogenase was shown. The action of NADH and NADPH diaphorases was demonstrated in the fixed smears only in the presence of NADH and NADPH. Supravital methods are inadequate for demonstration of specific enzymatic activity.

Lactic dehydrogenase activity in cells was estimated in a semiquantitative way. The enzymatic action of the dehydrogenases and the diaphorases was stronger in younger cells and decreased progressively with their maturation.
SUMMARIO IN INTERLINGUA

Dehydrogenases ligate a nicotinamido-adenino-dinucleotida, dehydrogenase succinic, e diaphorases de dihydronicotinamido-adenino-dinucleotida e de phosphato de dihydronicotinamido-adenino-dinucleotida esseva essayate con methodos cytochiniic in leucocytos de sanguine e in cellulas de medulla ossee con le utilisation de sales de tetrazolium, chloruro de 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3' (3,3'-dimethoxy-4,4'-biphenylene)-ditetrazolium e bromuro de 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl-tetrazolium. In non-fixate frottis, specific activitate enzymatic esseva demontrate pro dehydrogenase de glucosa-6-phosphato, pro dehydrogenase succinic, e pro diaphorase de dihydronicotinamido-adenino-dinucleotida. In frottis fixate, le activitate de dehydrogenase lactic e de dehydrogenase succinic esseva demontrate. Le activitate del diaphorases de dihydronicotinamido-adenino-dinucleotida e de phosphato de dihydronicotinamido-adenino-dinucleotida esseva demontrate solo in le presentia de dihydronicotinamido-adenino-dinucleotida e de phosphato de dihydronicotinamido-adenino-dinucleotida. Methodos stupravital es inadequate pro le demonstration de un specific activitate enzymatic.

Activitate de dehydrogenase lactic in le cellulas esseva estimate de maniera semi-quantitative. Le activitate enzymatic del dehydrogenases e del diaphorases esseva plus marcate in juvene cellulas e declinava progressivamente con lor maturation.

REFERENCES

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