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FREEZE-PRESERVATION OF STAINING SOLUTIONS IN ENZYME HISTOCHEMISTRY

BY

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ABSTRACT

Staining solutions of acid phosphatase and esterase group (naphthol-AS-D-chloroacetate esterase, naphthol-AS-acetate esterase and alpha-naphthyl-acetate esterase) were stored for 6 months using the freezing facilities, and the staining results were compared with those of each set of controls.

The results showed that the staining solutions of all enzymes were stable for long-term preservation (at least 6 months) in the deep freezer (−80°C) when they were stored appropriately in separated forms. In such a freezing procedure, the staining results for each enzyme did not show any decrease of positive intensity nor any non-specific staining.

INTRODUCTION

Enzyme histochemical examination is frequently important for proper pathological diagnosis especially in the field of hematology. Usually the specimens, which require enzyme histochemical examination, are stored in the freezer at once and the accumulated specimens are stained together at one time, since it has been thought that the solutions should be freshly prepared and used for staining (Pearse [1], Lillie [2], Takeuchi et al. [3], Ogawa et al. [4]). But if the staining solutions could be preserved for a long time and easily employed as the occasion demands, enzyme histochemistry is being employed more frequently in routine examination and is useful for diagnosis in regular pathologic laboratories.

In the present study, the methods were investigated for the preservation of the staining solutions of acid phosphatase and esterase group (naphthol-AS-D-chloroacetate esterase, naphthol-AS-acetate esterase and alpha-naphthyl-acetate esterase). The results showed that the staining solutions were stable for long-term preservation in the deep freezer (−80°C) when they were stored appropriately in separated forms; i.e., one containing the substrate and the other containing the coupling agent.

MATERIALS AND METHODS

I. *Specimens*

Bone marrow stamps sampled from two autopsy cases were quickly air-dried and used for the present study. These autopsy cases (Table 1) were confirmed beforehand to be free from any hematopoietic dis-

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Table 1. Autopsy Cases Used for Specimens in the First and Second Experiments

Experiment	Clinical diagnosis	Age	Sex	P.M.
First	Liver cirrhosis with hepatoma	74 y.	m.	3 hr 46 ms
Second	Brain hemorrhage	77 y.	m.	3 hr 20 ms

P.M.: Duration until autopsy after death

Table 2. Methods of Enzyme Histochemistry

Enzymes	Substrate	Coupling agent	pH	Incubation
Acid phosphatase	Naphthol-AS-BI-phosphate	Hexazonium pararosaniline	5.0–5.1	1 hour
Naphthol-AS-D-chloroacetate esterase	Naphthol-AS-D-chloroacetate	Hexazonium pararosaniline	6.3	30 minutes
Naphthol-AS-acetate esterase	Naphthol-AS-acetate	Fast blue BB salt	6.8	30 minutes, two times
Alpha-naphthyl-acetate esterase	Alpha-naphthyl-acetate	Hexazonium pararosaniline	7.0	30 minutes

orders.

II. *Preparation of staining solutions*

Solution containing the substrate (hereafter referred to as solution "A") and the solution containing the coupling agent (hereafter referred to as solution "B") were prepared respectively and used for this study (Table 2).

A) Acid phosphatase (Leder [5])

Solution "A": Thirty drops of 4% pararosaniline in 2N-HCl and an equal volume of 4% natrium nitrite in distilled water were mixed, added with 150 ml of 0.1 M veronal acetate buffer (pH 6.2) after 60 seconds, and then the pH of the solution was adjusted to 5.0–5.1 with 2N-HCl. Solution "B": Fifty mg of naphthol-AS-BI-phosphate were dissolved in 7.5 ml of dimethylformamide.

B) Naphthol-AS-D-chloroacetate esterase (Leder [5])

Solution "A": Four drops of 4% pararosaniline in 2N-HCl and an equal volume of 4% natrium nitrite were mixed, added with 120 ml of 0.1 M veronal acetate buffer (pH 7.62) after 60 seconds, and then the pH of the solution was adjusted to 6.2 with 2N-HCl. Solution "B": Fifty mg of naphthol-AS-D-chloroacetate was dissolved in 7.5 ml of dimethylformamide.

C) Naphthol-AS-acetate esterase (Stutte [6])

Solution "A": Four hundred and eighty mg of fast blue BB salt were dissolved in 0.1 M phosphate buffer (pH 6.8) with 2.4 ml of propylenglycol. Solution "B": Seventy mg of naphthol-AS-acetate were dissolved in 10.5 ml of dimethylformamide.

D) Alpha-naphthyl-acetate esterase (Leder [5])

Solution "A": Five drops of 4% pararosaniline in 2N-HCl and an equal volume of 4% natrium nitrite were mixed, 60 seconds later added with 125 ml of 0.1 M phosphate buffer (pH 7.5). Solution "B": Forty mg of alpha-naphthyl-acetate were dissolved in 6 ml of acetone.

III. *Basic process of staining* (Fig. 1)

Each solution "A" was pipetted into the polystylene tube (CORNING 25310), 10 ml

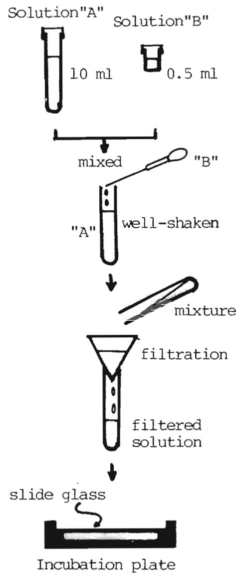


Fig. 1. Basic staining process used in this study.

in amount, and each solution "B" was pipetted into the glass vial (WHEATON, 224881), 0.5 ml in amount. Solutions "A" and "B" were mixed and well shaken, then the mixture was filtered through the filter paper (No. 2 TOYO ROSHI CO., LTD., Japan). The specimens on the sildes were incubated with the filtered solution on the incubation plate (8×20 cm, Lucite) each time at room temperature. After the incubation, the slides with the specimens were washed in water and counterstained by hematoxylin in the case of acid phosphatase, naphthol-AS-D-chloroacetate esterase and alpha-naphthyl-acetate esterase, and by kernechtrot in the case of naphthol-AS-acetate esterase. After washing slightly in water, the specimens were air-dried and mounted with glycerin-gelatin.

IV. *Treatment*

A) First experiment: This experiment was performed using freshly made solutions.

1. (A+B)R: Freshly made solutions "A" and "B" were mixed, filtered and used.

2. (AT80+BT80)R: Freshly made solutions "A" and "B" were frozen separately in the deep freezer (−80°C) for one hour and then thawed, mixed, filtered and used.

3. (A+B)R.T80: Freshly made solutions "A" and "B" were mixed, filtered and frozen in the deep freezer for one hour and then thawed and used.

B) Second experiment: This experiment was performed after the storage of the staining solutions for 6 months in each facility; i.e., refrigerator (4°C), freezer (−20°C) and deep freezer (−80°C).

1. (A+B)R.S4: Solutions "A" and "B" were mixed and filtered. The filtrate was stored in the refrigerator (4°C) for 6 months, and then used.

2. (AS4+BS4)R: Solutions "A" and "B" were stored separately in the refrigerator (4°C) for 6 months. Then both solutions were mixed, filtered and used.

3. (AS80+BS80)R: Each solution was stored respectively in the deep freezer (−80°C) for 6 months. After thawing, both solutions were mixed, filtered and used.

4. (AS20+BS20)R: Each solution was stored in the freezer (−20°C) for 6 months. After thawing, both solutions were mixed, filtered and used.

5. (A+B)R.S80: Solutions "A" and "B" were mixed and filtered. The filtrate was stored in the deep freezer (−80°) for 6 month. After thawing, the solution was used.

Results

1) Short-time freezing of each "A" and "B" solution did not disturb the staining results in all cases of enzymes.

2) Short-time freezing of the mixture of "A" and "B" solutions resulted in no

Table 3. Positive Intensity in Each Preparation of Staining Solutions

Treatment	Acid phosphatate	Naphthol-AS-D-chloroacetate esterate	Naphthol-AS-acetate esterase	Alpha-naphthyl-acetate esterase
(A+B)R	4+	4+	4+	4+
(AT80+BT80)R	4+	4+	4+	4+
(A+B)R.T80	4+	3+	1+	3+
(A+B)R.S4	—	—	—	—
(AS4+BS4)R	—	—	—	—
(AS80+BS80)R	4+	4+	4+	4+
(AS20+BS20)R	4+	—	—	—
(A+B)R.S80	4+	3+	—	3+

The symbols indicate: —, negative staining: +, unequivocal positive staining, graded 1 to 4: Each treatment is symbolized as mentioned under materials and methods.

change in the case of acid phosphatase, slight decrease of positive reaction in naphthol-AS-D-chloroacetate esterase and alpha-naphthyl-acetate esterase and marked decrease in naphthol-AS-acetate esterase.

3) When the mixture of "A" and "B" solutions was stored in the refrigerator (4°C) for 6 months, even when the "A" and "B" solutions were stored separately, no staining was observed in all cases of enzymes.

4) When each "A" and "B" solution was stored respectively in the deep freezer (−80°C), the staining of all enzymes was as strong as when fresh solutions were used.

5) When the mixture of "A" and "B" solutions was stored in the deep freezer (−80°C), the staining results were approximately the same as those in the case of short-time freezing of the mixture.

6) When each "A" and "B" solution was stored in the freezer (−20°C), the staining resulted in no change compared with that by deep freezing (−80°C) in the case of acid phosphatase, but completely negative in the esterase group.

7) In all the treatments, the staining was specific and was the same as that when the fresh solution was used (Figures 2–5), in spite of some decrease in the intensity according to the treatment.

These results mentioned above are summarized in Table 3.

Discussion

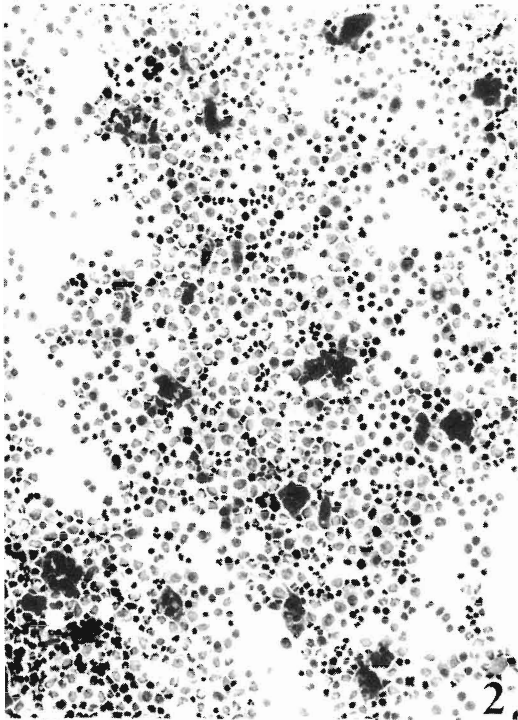
That the staining solutions should be freshly prepared and used for staining (Pearse [1], Lillie [2], Takeuchi et al. [3], Ogawa et al. [4]) has been a serious obstacle to the frequent and routine employ-

Fig. 2. Acid phosphatase, densely and homogeneously positive in the reticulum cells. ×170. Each "A" and "B" solution stored for 6 months in deep freezer, after thawing mixed, filtered and used for staining.

Fig. 3. Naphthol-AS-D-chloroacetate esterase, homogenously positive in immature myeloid cells. ×520. Preparation of staining solution same as that in Fig. 2.

Fig. 4. Naphthol-AS-acetate esterase, fine granularly positive in reticulum cells and monocytes. ×130. Preparation of staining solutions same as that in Fig. 2.

Fig. 5. Alpha-naphthyl-acetate esterase, hemogenously positive in reticulum cells. ×260. Preparation of staining solution same as that in Fig. 2.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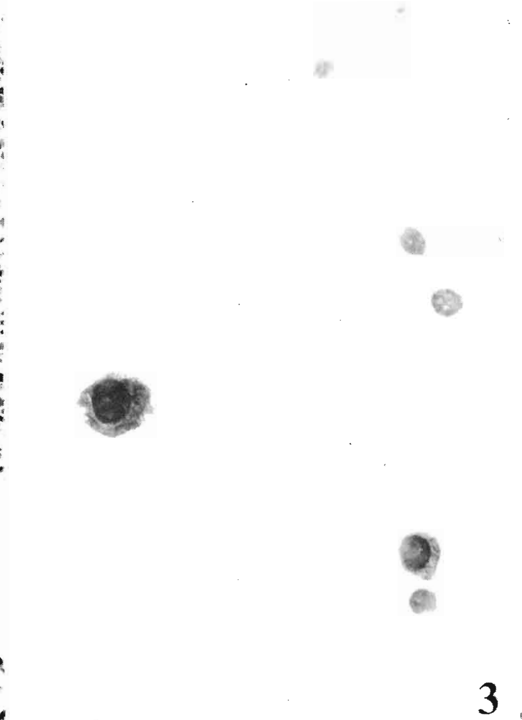
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ment of enzyme histochemistry in the regular pathologic laboratories.

The present study showed that the staining solutions of acid phosphatase and the esterase group (naphthol-AS-D-chloroacetate esterase, naphthol-AS-acetate esterase and alpha-naphthyl-acetate esterase) were all stable for long-term preservation when the solutions "A" and "B" were stored respectively in the deep freezer (−80°C) and mixed just before use. When the mixture of these two solutions was stored in the deep freezer for one hour or 6 months, there was a decrease in the positive reaction of various degrees regardless of the length of freezing. A long-term preservation of the staining solutions in the freezer (−20°C) showed successful results in the case of acid phosphatase but not in the esterase group. When the substrate solution of acid phosphatase was frozen for a short time, the pH of the solution did not differ before and after freezing (data not presented).. Therefore, deep freezing would induce neither decrease of the activities of the chemicals nor the change in the pH of the solution. Many chemicals used in enzyme histochemistry appeared to be stable when frozen and the staining solutions for many enzymes might be stable for long-term preservation in the deep freezer if they were stored appropriately in separated forms. Further examination of the other enzymes is now in progress.

In the use of these freeze preservation of staining solutions, histochemical examination of the enzymes will be easily applicable in the pathologic laboratories for routine examination and will be actually useful for diagnosis; i.e., the examination will be performed quickly as the occasion demands and the results will be obtained more quickly. Moreover, the staining solutions can be made on one occasion in a large amount and kept in the numberous small containers in the deep freezer, and these could be distributed from the main laboratory to the peripheral laboratories where enzyme histochemistry has not been employed.

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