Influence of Fixatives Upon the Peroxidase Staining

The 8th Report of Histochemical Study of Peroxidase

By

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The most important step in the preparation of sections of tissues for histological examination is proper and immediate fixation. We use generally three different fixatives in routine work; formalin, alcohol and Zenker's solution.

On the other hand, we use chiefly methanol as fixative for hematological examination, e. g. Giemsa staining as well as Wright staining. Of course, methanol being contained in Wright's stain itself, the previous fixation of blood smear before Wright staining is not necessary (1000 cc. of methanol contains 3 gm. of Wright's stain powder). However, when staining the blood smear with peroxidase stain, the fixatives often inhibit the reaction and the staining ends in failure. Shōji (1928) has already stated in the 10th Report of the peroxidase Reaction of Sato's School, that Sato's peroxidase reaction of blood cells never occurred when the smear had been fixed with methanol for over five minutes, and that methanol was therefore an inhibitor.

Mitsui and Ikeda (1951) reported also that a special fixative was not used in the NAS-Benzidine reaction, because the fixatives often acted as inhibitors of peroxidase, especially methylalcohol indicated a high inhibition. Therefore methylalcohol should not be used before and after staining.

In this paper, I am attempting to demonstrate which fixative is the most unsatisfactory for the peroxidase staining, and which is the most useful for routine work. Ethanol, methanol, formalin and acetone were tested, using the NAS-Benzidine solution as peroxidase stain of blood cells. Of course, the myeloid series were peroxidase positive, while the lymphoid were negative, and the eosinophil leukocyte showed

the most intense reaction. Human blood smears were used for all the experiments.

Results obtained

Experiment 1. On staining blood smears for one minute with NAS-Benzidine solution, the influence of fixatives upon the peroxidase staining was varied as shown in table 1. The technique of NAS-Benzidine reaction is as follows:

1. NAS-Benzidine solution contains the following components.

0.5 per cent aqueous solution of benzidine (saturated)..... 95 cc.

Hydrogen peroxide (3 per cent) 10 gtt (Sahli).

1 per cent aqueous solution of nickel ammonium sulfate (NAS) 5 cc.

2. Stain the fixed blood smear with NAS-Benzidine solution after washing out the fixatives with distilled water.

3. Wash the smear with distilled water gently again.

4. Counterstain, then wash, dry and examine.

It is clear from table 1 that methanol and formalin were inhibiting, while ethanol and acetone were valid. 10 per cent formalin in the table corresponds to about 4 per cent aqueous solution of formaldehyde.

Table 1. Peroxidase reaction of leukocytes stained with NAS-Benzidine solution for one minute. Fixative and its acting time are shown.

fixatives \ acting time	3 min.	5 min.	10 min.	1 h.	2 h.	3 h.	6 h.
ethanol	+	+	+	+	+	+	-
acetone	+	+	+	+	+	-	-
10% formalin	-	-	-	-	-	-	-
methanol	-	-	-	-	-	-	-

(min.=minute, h.=hour, d.=day)

Experiment 2. In this experiment the staining time with NAS-Benzidine solution was prolonged up to two minutes. By this procedure, the peroxidase reacting substance in the leukocyte could be more distinctly demonstrated. The results are shown in table 2. Within ten minutes of the acting time of fixatives, only methanol was inhibiting for the peroxidase staining. Other fixatives did not inhibit the staining so much.

Table 2. Peroxidase reaction of leukocytes stained with NAS-Benzidine solution for two minutes. Fixative and its acting time are shown.

acting time fixatives	3 min.	5 min.	10 min	1 h.	2 h.	3 h.	6 h.
ethanol	+	+	+	+	+	+	-
acetone	+	+	+	+	+	-	-
10% formalin	+	+	+	-	-	-	-
methanol	-	-	-	-	-	-	-

Experiment 3. The staining time with NAS-Benzidine solution was prolonged up to five minutes. In this case the peroxidase reacting substance in leukocytes became very sensitive to the reaction, so that a very slight amount of the substance could be demonstrated. If only air-dried, unfixed blood smear is stained for as long as five minutes with this solution, the myeloid leukocytes change into black clots on the smear because of too intense peroxidase reaction (so-called "After-darkening" after Mitsui). On the contrary, the reaction of the fixed blood cells were generally weak as indicated in table 3.

It is clear from this table that the myeloid leukocytes fixed with methanol did not become peroxidase positive even when stained with NAS-Benzidine solution for as long as five minutes, and that formalin as well as acetone were of value in practical use if the staining time is prolonged to some extent.

Table 3. Peroxidase reaction of leukocytes stained with NAS-Benzidine solution for five minutes. Fixative and its acting time are shown.

acting time fixatives	3 min.	10 min.	1 h.	12 h.	1 d.	3 d.	4 d.	36 d.	40 d.
10% formalin	+	+	+	+	+	+	+	+	-
acetone	+	+	+	+	+	+	-	-	-
ethanol	+	+	+	+	-	-	-	-	-
methanol	-	-	-	-	-	-	-	-	-

On the other hand, the peroxidase negative leukocytes, fixed with formalin for over forty days, began to degenerate, producing vacuoles in the cytoplasm and its cell body gradually shrunk. Finally its nucleus became no more stainable with fuschin solution.

Experiment 4. In this experiment the influence of fixatives upon the peroxidase staining and Wright staining was compared. It should be of prominent interest that the eosinophil as well as neutrophil granules in leukocytes appeared distinctly by Wright staining, though the blood smear had been fixed with methanol or ethanol for six hours, in which case the peroxidase reaction never occurred or only weakly occurred in all myeloid series (see table 4). Furthermore it was made clear by this experiment that there is a remarkable difference of peroxidase reacting substances in the leukocytes and the erythrocytes as the table shows.

Table 4. Effect of fixative upon the neutrophil and eosinophil granules in leukocytes. Peroxidase staining was done with NAS-Benzidine solution for two minutes.

fixatives	peroxidase reaction			Wright staining	
	neutrophil leukocyte	eosinophil leukocyte	erythrocyte	neutrophil granules	eosinophil granules
methanol (6 hours)	negative	negative	stronger than unfixed one	stained	stained
ethanol (6 hours)	very weak	very weak	stronger than unfixed one	stained	stained

Summary

There is no doubt that fixatives are necessary for histo- and cytological study, and fixation by methanol is most generally applied to the hematological routine work using blood smears. However, it should be noted that this methanol inhibited the peroxidase staining of leukocytes more intensely as compared with other fixatives examined. The peroxidase staining of leukocytes ended in failure by fixation with methanol for only three minutes. Strictly speaking, the fixatives inhibited the peroxidase reaction of leukocytes in various degrees, but of these, ethanol exerted the slightest inhibiting influence upon the reaction, and formalin as well as acetone were useful when the staining time of peroxidase reaction was adequately prolonged.

References

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