NAS-Benzidine Reaction of Blood Cells—New Simple Peroxidase Staining Method Using Nickel-Ammonium Sulfate

By

Tadao Mitsui and Sadao Ikeda

From the Anatomical Department, Dental School, Nihon University, Tokyo.

Special Reagents:

- 0.5 per cent aqueous solution of benzidine ------- 95 cc.
- Hydrogen peroxide (3 per cent) ------------------ 10 gtt. (Sahli)
- 1 per cent aqueous solution of nickel-ammonium sulfate (NAS) ----------------------------- 5 cc.

0.5 Gm. of benzidine (Benzidin puriss, E. Merck) is dissolved in 100 cc. of distilled water, and it is necessary to rub benzidine in a mortar unless fine powder. The preparation is filtered and ten drops (Sahli) of hydrogen peroxide are added, finally to its 95 cc. are added 5 cc. of 1 per cent aqueous solution of Nickel-Ammonium Sulfate (NAS is the capitals of this chemical). The NAS-Benzidine solution is thus prepared, which should have a pH of 5.8–6.0 approximately. Namely the mixture is necessary which includes 95 parts of benzidine solution, 5 parts of NAS-solution, and 10 drops of hydrogen peroxide per 100 cc. of the mixture. If this NAS-Benzidine solution is kept in the dark when not in use, it will keep half a year without significant loss of potency.

Procedure:

1. Stain fresh dried blood smear with the NAS-Benzidine solution for 45 to 60 seconds.
2. Wash the stained smear with water gently.
3. Counterstain with Pfeiffer's fuchsins solution for one minute. The latter solution is prepared by diluting Ziehl's Carbol fuchsin 5 or 10 times with distilled water.
4. Wash, dry and examine.
Results:
1. Neutrophils show fine blue or dark blue granules diffusely in the cytoplasm.
2. Monocytes show fine bluish granules coarsely in the cytoplasm.
3. Eosinophils show both brown and blue large granules which correspond to eosinophilic granules. Sometimes they show only blue large granules in the cytoplasm.
4. Basophils show only brown granules which vary in size.
5. Lymphocytes never show positive granules.

The Remarks and Meaning of this Staining Method.

1) In order to stain the smears with the NAS-Benzidine solution, we may use a pipette as usual, but the specially-made tube (Fig. 1) is recommended. At first, place the solution in the latter tube and put the smears into this solution, thus the smears stain homogenously and about 25 cc. of the solution can stain as many as fifty smears simultaneously. But the reagent in the tube becomes unavailable within a day after many smears are stained in it continuously.

Furthermore, the temperature of the stain can be kept constantly by means of surrounding hot water (Fig. 2). Again, the effect of

Fig. 1. Specially-made tube. Two smears can be stained in this simultaneously. 
(S = Slide glass, N = NAS-Benzidine solution)

Fig. 2. The temperature of the stain is kept constantly by surrounding hot water. 
(H = Hot water)
locality on blood smears can be eliminated by homogenous staining when the above tube is used. These are additional advantages of this method.

2) Special fixing solution is not used here, because the latter becomes often an inhibitor of peroxidase, especially methylalcohol indicates high inhibition number for it. Therefore, methylalcohol should not be used before and after staining. However, the ether-alcohol as fixing solution is preferable when the observation of erythrocytes is necessary; the solution contains the equal volume of ether and ethylalcohol. If the fixing solution is used, the smear should be stained a little longer than one minute.

3) As above described, the staining time of the NAS-Benzidine solution is 45 to 60 seconds in normal man. Of course, too prolonged staining is improper, because the peroxidase positive granules become intensely overstained, and appear much larger than usual. The staining time, however, should be longer when the peroxidase of blood cells is less active under various conditions. For instance, as many as two minutes are necessary for some patients, and more than three minutes for the leukocytes in saliva. In other words, the more the time is necessary for the staining, the less the activity of peroxidase is. This fact is observed in other peroxidase staining methods, too.

4) Counterstain. Fuchsin solution stains the nuclei of leukocytes simply as well as distinctly, in which case the peroxidase positive granules, excepting those of basophils, are blue, while the nuclei are red, therefore the color contrast between the two is blue and red, which is very clear. However, old fuchsin solution should not be used, because the brown granules may often occur in the cytoplasm of leukocyte by only this. Giemsa stain is recommended for counterstain by many investigators, because the latter stains the nuclei more finely and distinctly than the fuchsin solution. But, if Giemsa stain is used here, the color contrast between nuclei and peroxidase granules becomes less distinct, therefore the Illuminol red is preferable when we want to stain the nuclei more distinctly than fuchsin. The 0.005 per cent alcoholic solution of Illuminol red stains the nuclei red, erythrocytes yellow, in which case the color contrast between nuclei and peroxidase granules is as clear as fuchsin stain, and yet the fine structure of nuclei becomes as distinct as Giemsa stain.

5) The meaning of NAS-Benzidine solution. According to Sato’s copper method, at first the smear is covered with copper sulfate solution, next the benzidine solution is added to it. However, it should
especially be noticed that only the mixture of the two solutions is effective, i.e., if the copper sulfate solution on smear is washed off with water before adding the benzidine solution, the peroxidase staining ends always in failure, furthermore, if both the copper sulfate and benzidine-hydrogen-peroxide solution are mixed in a tube before staining, the mixture becomes soon useless due to the rapid chemical changes. Therefore the above two solutions should be added to smears separately in all cases. Besides, the mixing rate of the two is not always constant, because the two solutions are roughly mixed on the slide glass of smear, and this is the reason why the perfect results of Sato’s method are sometimes not obtained even when the technic described is rigorously followed. Consequently, if we can prepare the mixture which never loses the effect for a long time and yet can stain the peroxidase granules at a time as well as the method of Osgood or Sato, the staining method will become much more simple and rapid. For the latter purpose, many sorts of salts were examined by us and the following results were obtained. Most of salts, including copper sulfate, nitroprussidnatrium, ten sorts of alum, uranyl nitrate, and nickel sulfate, were unavailable, while nickel-ammonium sulfate (NAS) and zinc sulfate were only suited for the purpose. As already mentioned, the mixture of NAS and benzidine-hydrogen-peroxide solution keeps for six months without significant loss of potency.

The mixture including benzidine-hydrogen-peroxide, nitroprussidnatrium, and fuchsin, was devised by Goodpasture (1919), but this stain becomes useless two or three days after mixing; the fuchsin itself also accelerates the chemical change of the mixture. For this reason Osgood improved Goodpasture’s method, and prepared better staining solutions (1938).

Again, nickel sulfate and ammonium sulfate are each available for color change of peroxidase granules into blue, so their double salt, namely, nickel ammonium sulfate (NAS) will be more effective on the staining.

6) We should not use a thick smear because of the fact that the reagent does not act on the granules of leucocytes and most, if not all, of them show the transformed nuclei.

7) We should not wash the smear with the service-water before the benzidine solution is added, because the latter water itself may have sometimes an ability to change the brown granules into blue, though in slight degree.

8) Applications of NAS-Benzidine solution for other purpose.
i) Fresh blood cells. (Washed with 0.85% NaCl solution).
First, place 10 cc. of the NAS-Benzidine solution in a tube, then add one drop of fresh blood to it. After one or two minutes, the solution becomes a little bluish, and some bluish fine clots, which are visible to the naked eye, appear little by little, descending to the base of the tube. The deposits become dark blue and increase its volume with the lapse of time. This is due to the fact that the solution can stain leucocytes in liquid blood as intensively as in dry smear. But many drops of blood should not be added to the solution, because too much blood may interrupt the staining. Furthermore, if one drop of acetic acid is added to the above solution including blood, all the solution becomes immediately dark blue. This corresponds to the examination of occult blood which is usually carried out by clinician. In this case, however, nickel-ammonium sulfate is not always necessary.

ii) Staining of sections.
This is of value when we want examine the peroxidase granules in tissue. Frozen sections are usually stained, and the NAS-Benzidine solution is also effective here. By this, the peroxidase positive granules rapidly stain blue at a time, but the appropriate treatment before and after staining is of course necessary. The wandering leucocytes in connective tissue can be demonstrated very finely.

Summary

We have devised a simple peroxidase staining method using nickel-ammonium sulfate, and named this NAS-Benzidine reaction. By means of this we can stain the peroxidase positive granules at a time and more rapidly than the existing methods which use separate solutions to be mixed freshly each time. The NAS-Benzidine solution including benzidine, hydrogen peroxide and nickel-ammonium sulfate (NAS), keeps for six months without significant loss of potency. An additional advantage of this stain is the fact that it may be used not only for dry smears but also for the staining of sections and fresh blood.

This paper has already been read before the 56th annual Japanese Anatomical Society, April, 1951, and the study was carried out with the grant in aid for fundamental scientific research.
T. Mitsui and S. Ikeda.

References