A NEW METHOD FOR STAINING LEPROSY BACILLI

by

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To a paper on a new method for staining tubercle bacilli with Nachtblau which the writer published in 1941, Reenstierna added a short note (1), together with a plate, on its use in staining leprosy bacilli in nasal smears, films of blood, and sections of lepromata. As was the case with tubercle bacilli, the bacilli of leprosy were found to take a dark-blue color, to appear larger and more distinct*, and consequently, when occurring sparsely in the material, to be easier to discover than in corresponding films stained according to Ziehl-Neelsen.

Moreover, it was mentioned in this note that by the use of the Nachtblau method, it had been possible, in 1939, to detect in thick films of venous blood from a lepromatous patient in a moderately acute stage of the disease, besides sporadic Hansen's bacilli, acid-fast, oval and pear-shaped bodies resembling the cells of a fungus which had been isolated in 1912 by Reenstierna (3,4) from venous blood of a lepromatous patient in a very acute stage. This organism according to Reenstierna is the maternal fungus of Hansen's bacillus. Thus he considers the spindle-shaped conglomerations of leprosy bacilli as nothing but the differentiated contents of oval fungus cells (2) which, before bursting, generally swell. From the blood of the patient of 1939, a mycotic micro-organism was isolated, which was composed of ovoid elements like those found in the direct blood films. Only parts of it showed, both by Ziehl-Neelsen and Nachtblau staining, a certain degree of acid-fastness. It was further noticed that similar fungus-cells occurred in leprous nasal smears from lepromatous patients in different countries, and that the same were partly acid-fast.

Finally, there was reported the behaviour of a peculiar micro-organism which Reenstierna in 1924, isolated from the cerebro-spinal fluid of a third patient suffering from the nodular type in a fairly acute stage. In films from the culture, stained with Ziehl-

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* These facts did not at all become manifest from the microphotographs of the plate mentioned, which had been taken by use of the German "Agfacolor Neu" film with which experience was somewhat limited. But in a more recent paper by Reenstierna (2) is a picture (section of leproma) where full justice is done to the method.

Neelsen in that year and stained in 1941 with Nachtblau, there appeared, when hydrochloric 96 per cent alcohol was used as a decolorizing solution, instead of the usual 70 per cent, a number of dark-blue corpuscles between the counter-stained specimens of micro-organism.

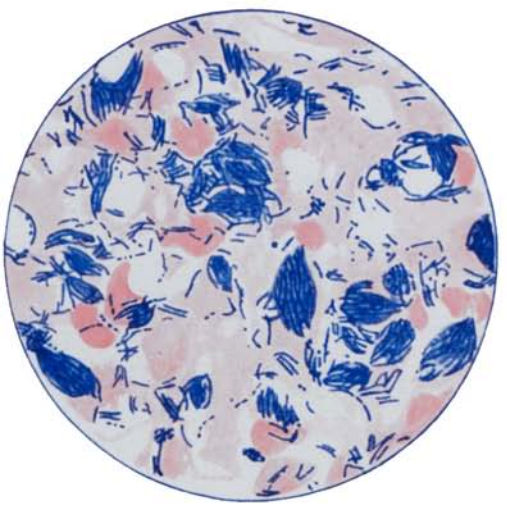
Roulet (5) who tried the Nachtblau stain on different acid-fast bacilli says with regard to its use on Hansen's bacillus: "Also the discovery of the leprosy bacilli was easy, agreeing with the statement of J. Reenstierna, even in preparations which had been kept for years in preserving solutions."

Since the appearance of the note by Reenstierna the writer has made a closer study of the value of the Nachtblau method for staining the leprosy micro-organism in films from cultures, nasal smears, thick blood films, and sections from lepromata. Not only has the successive procedure as described in 1941 been used but also a simultaneous previously unpublished procedure. The staining procedures and the solutions used are given in the appendix.

Two cultures, kept at the Upsala Institute, were examined: One of the micro-organism which Kedrowski (4,6), isolated in 1900 from a leproma of a Russian patient, the other of Reenstierna's micro-organism of 1912. Both grow on the surface of glycerin bouillon where they form greyish and greyish-yellow pellicles composed respectively of rods and filaments, which behave partly as acid-fast, partly as non-acid fast when stained by Ziehl-Neelsen. Thus it was several years ago (4) and thus it is now. On examining such films as were stained afterwards with Nachtblau the impression was gained that acid-fast bacterial elements were more numerous.

Regarding leprosy bacilli in thin nasal smears, it may be questioned whether the Nachtblau method is superior to the Ziehl-Neelsen, apart from their larger and more distinct appearance. In thicker films, on the other hand, they appear definitely in greater number when the former method is used, due to the fact that the methylene-blue of the latter method covers a good many of the red-stained bacilli. Also the reddish tint which this dye-stuff shows at times exercises a disturbing influence in the detection of the micro-organism in question.

In 1942 the writer examined several thick films made from the circulating blood of a fourth leprosy patient suffering from the nodular form in a relatively inactive stage. In one it was possible to detect, by the use of the Nachtblau staining (successive procedure), a spindle-shaped acid-fast fungus-cell closely resembling those previously found in the blood by Reenstierna, Walker (7), and probably also by Bosma (8). Its culture was not successful.



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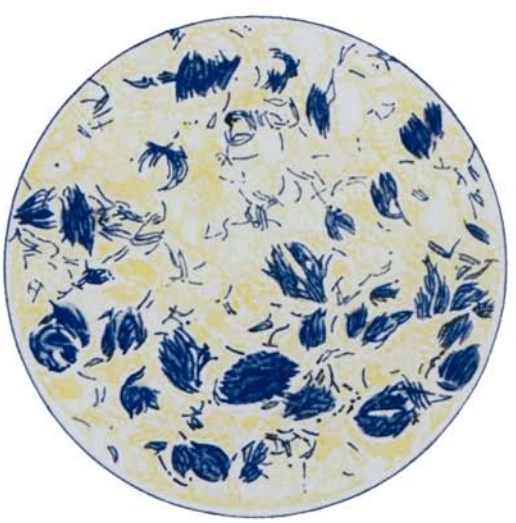

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With regard to sections of lepromata it is a well-known fact that their staining with Ziehl-Neelsen gives very capricious results. Either the preparations are underdecolorized so that the tissue also appears red in a disturbing manner, or they are overdecolorized with the result that a large number of the leprosy bacilli, which lose fuchsin more readily than tubercle bacilli, remain faintly stained or even unstained.

Illustrating the latter case is the following experiment carried out in 1945. A very skillful technician, experienced in the method concerned, stained and re-stained, according to Ziehl-Neelsen, a good many sections from lepromata originating from different Swedish patients. The first group consisted of sections freshly cut from nodules which for fully twenty years had remained embodied in paraffin, while the second consisted of sections which, stained with Ziehl-Neelsen twenty years ago, had remained mounted in Canada balsam.

In some of the sections belonging to the first group the bacilli were distinctly stained; in all of the second group, on the other hand, the bacillus-staining failed more or less completely. It is well known that such re-staining is difficult. In the unsuccessful preparations of both groups it was, however, possible to detect balls of bacilli, some faintly rose-colored, others quite unstained and refractive.

The sections were afterwards stained according to the Nachtblau method, successive as well as simultaneous procedure, with the following results: All the preparations of the first group showed bacilli clearly, stained very distinctly dark-blue, as previously unstained ones do (Figs. 1 and 2); in those of the second group the bacilli likewise took the Nachtblau stain nicely with the modification, however, that in some odd spots of a few sections the color of the micro-organisms appeared somewhat faded and the outlines of the bacterial bodies less sharp.

Thus, with regard to staining sections of lepromata, the Nachtblau method must be considered as superior to that of Ziehl-Neelsen.

APPENDIX

SUCCESSIVE METHOD

Preparation of stock- and staining solutions, decolorizing solutions, and counter-stains

I. a. *Saturated stock solution.*

1) Nachtblau (Grübler & Co., Leipzig)[1]	5 Gm.
2) 95 per cent alcohol	100 c.c.

The flask is shaken repeatedly during the day and left, at least overnight, for sedimentation of any possibly undissolved stain.

I. b. *Staining solution.*

1) Distilled water	90 c.c.
2) 10 per cent potassium hydrate[2]	max. 0.2 c.c.
3) Phenolum liquefactum	2.5 c.c.
4) Saturated stock solution I a	10 c.c.

— The saturated Nachtblau solution is taken by pipette from the upper layer, not from the bottom. It is added after the potassium hydrate and the phenol have been dissolved in the water.

II. a. *Saturated stock solution.*

1) Nachtblau	5 Gm.
2) Phenolum liquefactum	25 c.c.
3) 95 per cent alcohol	75 c.c.

—The Nachtblau is added after the phenol has been dissolved. Then the flask is shaken.

II. b. *Staining solution.*

1) Distilled water	90 c.c.
2) Saturated stock, solution II a	10 c.c.

III. *Decolorizing solution.*

1) 25 percent hydrochloric acid	3 c.c.
2) 70 (sic!) per cent alcohol	100 c.c.

—Hydrochloric 70 per cent alcohol decolorizes more strongly than hydrochloric 95 per cent alcohol. In decolorizing with the latter some other bacterial form also retain their blue color.

IV. *Counter stains.*

A. Pyronin

1) Distilled water	100 c.c.
2) Pyronin	0.25 c.c.
3) Phenolum crystallisatum	0.5 Gm.

—The pyronin is first dissolved in the water after which the phenol is added.

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[1] This factory has not been able during the war to produce a dye-stuff giving the originally stable solutions.

[2] Not absolutely necessary. If potassium hydrate is added, the bacilli are stained more intensely.

B. *Neutral red.*

1) Distilled water	100 c.c.
2) 1 per cent acetic acid	0.2 c.c.
3) Neutral red	0.1 Gm.

C. *Carbolfuchsin.*

1) Distilled water	95 c.c.
2) Concentrated carbolfuchsin	5 c.c.

D. *Vesuvin or Bismarckbraun.*

1) Distilled water	100 c.c.
2) Of these two dyes—respectively	2 c.c

STAINING PROCEDURE

Films

Thin films, well air-dried, and fixed in the flame are treated as follows:

1) Pour plenty of Nachtblau staining solution (Ib or IIb) on the slide. Heat carefully over the flame until boiling. Allow the stain to act for about 5 minutes during which period the slide will have time to cool.

2) Pour off stain.

3) Decolorize with solution III until no more blue clouds are given off.

4) Wash in distilled water.

5) Counter-stain with any of the five above solutions: for 5-10 seconds if neutral red, carbolfuchsin, vesuvin or Bismarckbraun are used; pyronin requires a somewhat longer time (15 seconds or even more).

6) Wash in water, blot and dry.

Sections

1) Place the slide on which the section has been well fixed for about 5 minutes in the usual manner in a cuvette containing xylol in order to remove the paraffin.

2) Wash consecutively in 95, 80, and 65 per cent alcohol.

3) Wash in distilled water.

4) Blot, slightly, with filter-paper.

5) Pour on Nachtblau staining solution (Ib or IIb).

6) Heat three times until vaporization sets in, and allow the stain to act for about 5 minutes after the last heating.

7) When the slide has cooled, decolorize the section with solution III and wash in distilled water. Counter-stain with pyronin for 2 minutes.

8) Wash in distilled water, blot with filter-paper and dry completely over the flame.

9) Mount in cedar oil.

SIMULTANEOUS METHOD

Preparation of stock-, staining-, and decolorizing solutions.

I. *Saturated stock solution.*

1) Sodium propionate (or potassium acetate)	1 Gm.
2) 90 per cent alcohol	100 c.c.
3) Nachtblau	5 Gm.
4) Bismarckbraun	0.4 Gm.

—The sodium propionate (or potassium acetate) is first dissolved in 100 c.c. alcohol. After that Nachtblau and Bismarckbraun are added. The flask is shaken repeatedly during the day and left, at least overnight, for sedimentation of any possibly undissolved stain.

II. *Staining solution.*

1) Distilled water	90 c.c.
2) Stock solution I	10 c.c.

III. *Decolorizing solutions.*

A. 1) 25 per cent (approximately) nitric acid	4 c.c.
2) 70 per cent alcohol	100 c.c.

or

B. 8 per cent (approximately) nitric acid.
C. 70 per cent alcohol.

STAINING PROCEDURE

Films

Thin films, well air-dried, and fixed in the flame are treated as follows:

1) Pour plenty of Nachtblau staining solution (II) on the slide. Heat carefully over the flame until boiling. Allow the stain to act for about 5 minutes during which period the slide will have time to cool.
2) Pour off stain.
3) Wash in distilled water.
4) Decolorize with nitric acid alcohol (III A) until no more blue clouds are given off.
5) Blot well with filter-paper and dry over the flame.

or

4) Dip the slide for 15 seconds in solution III B.
5) Decolorize directly (without previous washing in water) with solution III C until no more blue clouds are given off.
6) Blot well with filter-paper and dry over the flame.

Sections

1) Place the slide on which the section has been well fixed for about 5 minutes in the usual manner in a cuvette containing xylol in order to remove the paraffin.
2) Wash consecutively in 95, 80, and 65 per cent alcohol.
3) Wash in distilled water.
4) Blot, slightly with filter-paper.
5) Pour on plenty of Nachtblau staining solution (II).
6) Heat three times until vaporization sets in and allow the stain to act for about 5 minutes after the last heating.
7) When the slide has cooled, pour off the stain and wash in distilled water.
8) Decolorize with nitric acid alcohol (III A) until no more blue clouds are given off.
9) Blot well with filter-paper and dry over the flame.
10) Mount in cedar oil.

or

8) Dip the slide for 15 seconds in solution III B.

9) Decolorize directly (without previous washing in water) with solution III C until no more blue clouds are given off.

10) Blot well with filter-paper and dry over the flame.

11) Mount in cedar oil.

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Explanation of Plate

Figure 1. Section of a leproma. Stain: Nachtblau-pyronin (successive procedure). Magnification, 1,000 diameters.

Figure 2. Section of the same leproma. Stain: Sodium propionate-Nachtblau-Bismarckbraun (simultaneous procedure). Magnification, 1,000 diameters.