NOTES
Method for Staining Both Acid-Fast and Chromophobic Tubercle Bacilli with Carbolfuchsin

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It is well known that lesions which are unquestionably tuberculous may remain negative for acid-fast bacilli if they are fixed routinely and stained with carbolfuchsin according to the Ziehl-Neelsen technique. This is due to the property of the tubercle bacillus of being at times acid-fast and at others chromophobic. Numerous variants of the original technique have been devised in an effort to demonstrate the causative agent in negative lesions, assumed to be there but brought out neither by the acid-fast dye nor the counterstain. Carbolfuchsin was, therefore, abandoned by the present writer, and a new staining method, consisting of impregnating with silver tissues that were strongly oxidized with periodic acid, was devised (W. Nyka, Am. Rev. Respirat. Diseases 88:670, 1963). This method reveals large numbers of bacilli where carbolfuchsin shows few or none. Further studies of the influence of oxygen on the staining affinities of Mycobacterium tuberculosis showed that additional oxidation of sections of tuberculous material fixed in an oxidizing mixture makes the chromophobic bacilli acid-fast, and therefore liable to staining with carbolfuchsin. Chromophobic bacilli in smear preparations respond in the same way when oxidized as described below.

Seventy-eight surgical specimens taken from the lungs of tuberculous patients, 8 tuberculous lymph nodes, 12 cases of thickened pleura from tuberculous empyema, 21 cultures (1-month-old) of weakly acid-fast bacilli of human type, and 3 old cultures, aged 6, 9, and 12 months and all grown on American Trudeau Society medium, were used. The fixing procedure has already been published, but, since it is essential for the staining of tubercle bacilli with carbolfuchsin according to the new method, it is necessary briefly to mention it here. The specimens were fixed in one of the following solutions. Solution 1: saturated solution of mercuric chloride, 70 ml; absolute alcohol, 10 ml; Formol (40%), 10 ml; hydrogen peroxide (33%), 10 ml. Solution 2: saturated solution of mercuric chloride, 90 ml; Formol (40%), 10 ml; periodic acid, 5 g. Neither of the mixtures was stable, and both had to be prepared fresh each time. Both gave equally good results as far as the staining of the bacilli was concerned, but their effect on the tissues differed. The hydrogen peroxide solution left a pale background after destaining of carbolfuchsin, and was particularly indicated for processing solid material. When used on softened lesions, however, it tended to produce bubbles of oxygen which displaced the bacillary masses and disrupted the natural relationship of the organisms. Softened caseous material should, therefore, always be fixed in the periodic acid solution, which has far less tendency to develop free gas. The only drawback with this fluid, which has good penetrating power and good fixing properties, was that after its use the sections tended to remain red after decolorization of carbolfuchsin, and the bacilli were, therefore, less sharply defined. This red color of the sections could be toned down by washing the blocks with tap water overnight after fixation. Large specimens, such as lobes of lungs or whole lungs, had to be injected with the fluid via the bronchial tree. At 1 or 2 hr after injection, they were cut into slices about 5 mm thick and cut into blocks; the blocks were fixed for 24 to 48 hr in the same fixing fluid which was used for injection. Smaller specimens, such as lymph nodes, thickened pleura, etc., were cut into slices about 3 mm thick and fixed for 24 to 48 hr, according to the thickness of the blocks. The blocks were washed in tap water, dehydrated with alcohol, and embedded in paraffin. To secure good permeation of thick blocks of lung tissue with paraffin, a vacuum oven was used. Sections 5 μ thick were cut.

Paraffin was removed from the tissue sections;
they were then rehydrated, cleared of sublimate crystals, and washed in tap water to remove the hyposulfate. They were transferred to troughs filled with a 10% aqueous solution of periodic acid and were left for 4 hr, washed for a few minutes in tap water, and rinsed in distilled water. Longer exposure to periodic acid, although desirable, required caution, because after 4 hr the sections became loosened and had a tendency to come off the slides.

At the time of use, the sections were placed in troughs filled with carbolfuchsin prepared as follows: basic fuchsin, 1 g; absolute alcohol, 10 ml; distilled water, 100 ml; phenol, 5.6 ml. The troughs were put in the slide dryer for 0.5 hr at about 70 C. Then the fuchsin was discarded; the sections were next rinsed in tap water, and were decolorized in three changes of acid alcohol prepared as follows: alcohol (70%), 100 ml; lactic acid, 2 ml. Finally, they were washed in tap water, counterstained lightly with hematoxylin, washed, blued in lithium carbonate, washed, dehydrated with acetone, and mounted in Permount.

In the resulting preparations, nuclei were bluish, cytoplasm and caseous material were pink, and bacilli were red.

Smear preparations. The air-dried and flamed films were left overnight in a paraffin oven at 60 C to secure good adhesion of the organisms. The slides were then oxidized in a 10% solution of periodic acid for 4 to 24 hr, according to the intensity of staining desired. They were rinsed, first in tap water and then in distilled water, stained with carbolfuchsin and decolorized in the same way as tissue sections, rinsed in water, dried, and examined without counterstaining. After this treatment, bacilli appeared homogeneously dark red or deep purple.

**Fig. 1.** (A) Section through a cavity fixed in solution 2 and stained with the Ziehl-Neelsen procedure. (B) Taken from a companion section stained in a similar way after 4 hr of additional oxidation in 10% periodic acid. (A) shows one or two faintly stained, granular bacilli lying on an amorphous mass, which in (B) is shown to be a clump of tubercle bacilli. $\times 1,200$.

**Fig. 2.** One-month-old culture of bacilli of human type grown on American Trudeau Society medium. (A) Stained with the Ziehl-Neelsen procedure; shows numerous weakly acid-fast, slightly granular bacilli. (B) The same field in the same slide after decolorization with absolute alcohol at 60 C, oxidation for 14 hr in 10% periodic acid, and restaining with fuchsin as before. The bacilli are intensely stained, normal in shape and size, and more numerous through the addition of many which remained unstained in (A). $\times 1,200$. 
There is no doubt that in oxidized tissue sections bacilli which were completely decolorized when stained routinely with carbolfuchsin (Fig. 1A) became fully acid-fast after additional oxidation (Fig. 1B), a result which could not be obtained with Formol-fixed material. Likewise, originally weakly acid-fast bacilli grown on artificial media (Fig. 2A) were made strongly acid-fast by oxidation (Fig. 2B). In aged cultures, the well-known granular bacilli stained homogeneously and regained their regular, rodlike shape; the chromophobic ones recovered their acid-fastness and retained the stain.

The new technique for acid-fast staining regularly brought out both single and agglomerated bacilli in as great numbers as impregnation with silver, although it did not provide pictures with as much contrast. However, when properly applied, it brought them out in both tissue sections and smear preparations as sharply as is illustrated in Fig. 1B and 2B. Since it is technically much simpler than the rather involved silver method, it is recommended as a routine procedure for staining tuberculous material. In this laboratory, it is being used routinely parallel with the silver stain.