ON THE DIFFERENTIATION OF DEAD FROM LIVING MYCOBACTERIUM LEPRÆ

On the availability of a technique to differentiate the dead from living bacilli in a lesion depends the scientific evaluation of the therapeutic value of even Hydnocarpus preparations. Hansen discovered the bacillus in 1874 but even in 1939 we find Cowdry[1] stating: "What proportion, if any, of the bacilli in a given lesion are dead is at present purely a matter of speculation." The new technique devised by one of us (Subramaniam[2]) suggested a possible mode of attack of this problem. Serial sections of formol fixed skin clippings from an advancing lepromatous case when stained with Ziehl-Neelsen showed innumerable bacilli. These identical slides when destained with potassium permanganate and oxalic acid and stained with Heidenhain's hæmatoxylin presented a curious picture. The bacilli were red, blue or of mixed hues. The proportions of these variously stained bacilli differed from slide to slide but what was more important the number of bacilli seen in the sections was far less. What is the cause for the disappearance of a considerable number of bacilli seen in the same section after the preliminary Ziehl-Neelsen staining? The question was whether the bacilli which disappear get washed off during the potassium permanganate and oxalic acid treatment or whether they are the dead ones which cannot be stained with iron hæmatoxylin after the above treatment.

Since dead bacilli have been known to persist in tissues for over an year (Lowe[3]) any smear or section ought to contain living and dead bacilli. Attention was, therefore, directed to the application of the technique to smears. The usual method of fixation of smears is by heat. In slides fixed in the above manner one can possibly expect only *dead* and *killed* bacilli. For, even those bacilli which were alive at the time of smearing are killed by heat. If, on the other hand, a wet smear is fixed in 25 per cent. formalin for 30 minutes one ought to expect *dead* and *fixed or preserved* bacilli. The behaviour of the bacilli in the differently treated smears were different.

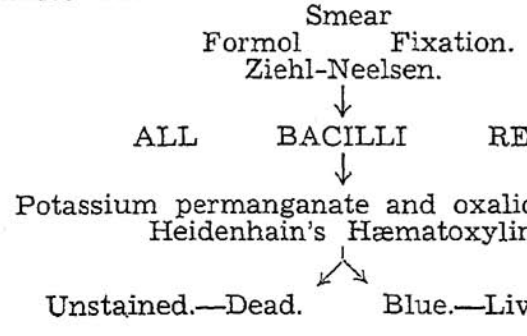
When formol and heat fixed smears are stained in carbol-fuchsin overnight, differentiated in 1 per cent. hydrochloric acid alcohol and counterstained with Loffler's methylene blue, then in each field could be seen hundred or more bacilli. If instead of methylene blue the slides are treated for two hours with Heidenhain's iron alum, an hour with hæmatoxylin and differentiated in the usual way, then also the same picture is seen. The bacilli appear red on a yellow background and the nuclei appear light or dark blue. Treatment of smears for even 24 hours with 1 per cent. acid alcohol does not destain any of the bacilli. But when the differently fixed smears are destained in stages with 0·25 per cent. potassium permanganate and 5 pere cent. oxalic acid there is a distinct difference. The bacilli in the heat fixed smears resist decolourization. While repetition of permanganate and oxalic acid treatment would destain most of the bacilli in formol fixed smears in about an hour, heat fixed bacilli require often two hours of alternating treatment with the above reagents. If especially the smear is very thin as if made with a bacterial emulsion, then a considerably longer time was found necessary. The destaining should be carried out very carefully and in stages, as otherwise the bacilli get loosened from the smear. In such cases the clear spaces originally occupied by the bacilli could be definitely distinguished in finished preparations. Our procedure was to keep the slides

in 0·25 per cent. potassium permanganate till the smear goes brown, and after washing it, to transfer it to 5 per cent. oxalic acid. The slide is then examined under the high power and if the bacilli are still red the process is repeated till no red bacilli could be made out. Many workers have commented on the remarkable acid-resistant nature of *M. leprœ* in smears as compared with that in sections (Lowe[3]). This becomes perfectly intelligible from the foregoing observations. Dead and killed bacilli are highly acid-fast while living bacilli fixed in formol are less so.

When heat and formalin fixed Ziehl-Neelsen stained smears destained in the above manner are stained with Heidenhain's hæmatoxylin two different pictures are obtained. The alterations in time of treatment with the mordant and stain make, if at all, very little difference. Our usual procedure was to keep the slides overnight in 4 per cent. iron alum, wash in running water for 10 minutes and keep it in the stain for two or three hours. Even if the above timings are reversed we found very little difference. Destaining correctly is an exacting procedure and unless great care is exercised the result will be a thorough failure. But when carefully done, the formalin fixed smears would show bacilli stained red, blue or of mixed hues. In heat fixed smears, a few red ones alone could be made out. The blue ones are conspicuous by their absence. Not only that. The blue bacilli in formalin fixed smears form only a varying percentage of those seen originally in the same smear after the preliminary Ziehl-Neelsen staining. In smears they constitute 10 and 15 per cent. and in sections of two lepromatous cases 4 and 40 per cent. respectively.

It appears from the above observations that the bacilli coloured blue or of a mixed hue alone are living while the red ones seen along with the above are dead. We have preparations showing blue bacilli alone in sections. Cowdry's[1] observations that individual globi differ in their acid-fastness, becomes intelligible in the light of the above results.

These blue staining bacilli could be demonstrated in both smears and sections fixed in formol, by staining ordinarily with Heidenhain's hæmatoxylin. On comparison with routine Ziehl-Neelsen stained smears and sections, the debris of the others tinted light blue could just be made out. When heat fixed smears are stained with Heidenhain's hæmatoxylin a few bacilli with indistinct outlines could be located. Since they roughly correspond in number with the blue bacilli seen in formol smears stained according to the new technique, it appears as if there is even a possibility of distinguishing between dead and killed bacilli.



Our results suggest that in the hands of experienced workers the above technique offers a method to differentiate between dead and living bacilli. We believe, the technique offers the leprologist a method to judge the progress of the disease and the scientist a method to evaluate the therapeutic value of the various drugs administered.

M. K. SUBRAMANIAM.
A. SHAMA RAO.
Department of Biochemistry,
Osmania Medical College,
Hyderabad (Dn.),
October 10, 1944.

1. Cowdry, E. V., *Puerto Rico J. Publ. Health and Trop. Med.*, Dec. 1939, pp 95–117. 2. Subramaniam, M. K., "Variations in the Acid-fastness of Mycobacterium Lepræ" (In press). 3. Lowe, J., *Internat. J Leprosy* 5, 1937 463–87.

* One of us (M. K. S.) would like to thank the Vice-Chancellor of the Osmania University for permission to work in Dr. S. Mahdihassan's Laboratory and as Cytologist to Messrs. The Biochemical and Synthetic Products Ltd. for their encouragement.