STAINING NODULES OF THE LEPROSY BACILLUS.

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During the course of a study of the lepra bacillus I have elaborated two staining methods: Method I for staining structures which in this paper are called " bacilli nodules," or " nodules " for short, and which I believe to be identical with those seen by Albert Neisser as early as 1881 and represented in pen drawings in his " Weitere Beiträge zur Aetiologie der Lepra "; Method II which stains the well known " Coccothrix granules " fully reported by Adolpho Lutz in " Zur Morphologie des Mikroorganismus der Lepra " published in 1886.

I have obtained very good results with both. Method I has been submitted to the test of routine work with success, Method II is still under test and I hope to publish it in an early number of this *Leprosy Review*.

METHOD I.

By utilising the property of salts of precipitating the dye of a carbol-fuchsin solution, this method is connected with F. B. Cooper's modification of Ziehl-Neelsen, but it is different in other respects.

For the sake of clarity I shall begin by stating the chief points of Cooper's modification in his own words:

" Ammonium chloride, ammonium sulphate, barium chloride, calcium chloride, magnesium chloride, ferric chloride, lead acetate, mercuric chloride, sodium chloride, sodium dichromate and secondary sodium phosphate all cause the precipitation of the dye material from carbol-fuchsin when added in proper amounts. The

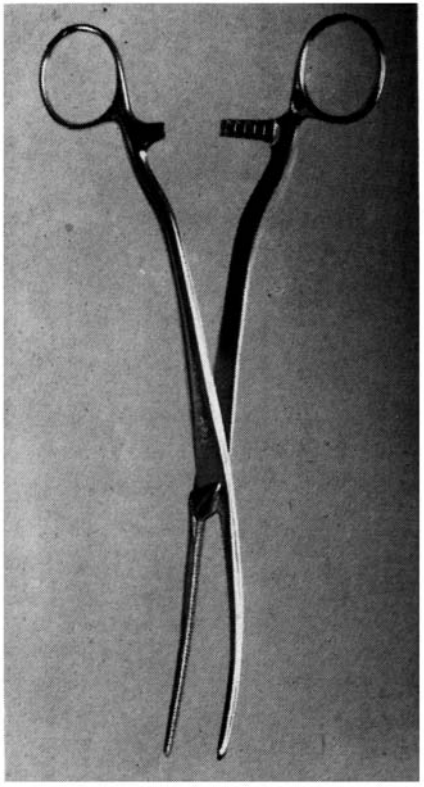

***Fig. 1.—Clamp** used for obtaining lymph*

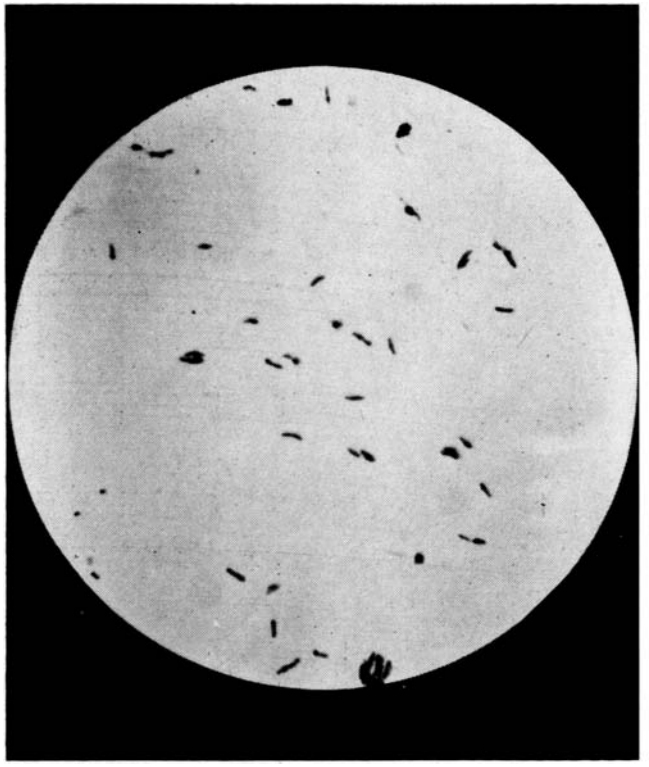

Fig. 2.—Bacilli stained by method of Dr. de Oliveira Castro

precipitate comes down at room temperature, but dissolves when warmed to 20-30 C., and remains in solution if that temperature is maintained." . . . . " Since the precipitant used makes no appreciable difference, sodium chloride was selected for the development of the modified stain. The amount necessary to give maximum staining was found to be 3 c.c. of a 10 per cent. solution per hundred cubic centimetres of carbol-fuchsin. Although this modified carbol-fuchsin precipitates at room temperature, it is stable if kept at incubator temperature, and keeps at least two months. ' . . . . " The technique for the modified stain, using the rapid method, is as follows: The slide is flooded with stain, steamed for four minutes and allowed to cool until the precipitate forms. This takes place in about two minutes, and may be hastened by gently blowing on the slide. The precipitate must be allowed to form, for on it depends the success of the method. It is then washed with tap water, decolorized from one to ten minutes in acid alcohol (5 c.c. of nitric acid, specific gravity 1.42, to 95 c.c. of 95 per cent ethyl alcohol); washed in water, then two minutes in 95 per cent ethyl alcohol; washed again, counterstained with Loeffler's methylene-blue for one minute, washed, dried and examined." . . . . ". . . . it was suggested that the methylene-blue, being an intense stain, might mask a number of bacilli by super-imposition and also by rendering the heavier portions of the smear non transparent. Various counterstains were tried and brilliant green 1 per cent. in 1:10,000 sodium hydroxide was selected, since such a solution failed to stain tubercle bacilli in ten minutes, gave transparent smears and stained secondary organisms pus and epithelial cells sufficiently to give nicely balanced fields in one to ten minutes."

I tried the salts with similar results to those of Cooper. It must be pointed out however that basic and acid salts, e.g. secondary sodium phosphate (Na_2HPO_4, pH8.7—9.5) and primary potassium phosphate (KH_2PO_4, pH 4.4—9.5), as well as neutral ones, cause immediate clouding of the dye irrespective of the different values of pH.

Potassium hydroxide, sodium hydroxide and ammonium hydroxide precipitate the dye, but hydrochloric acid, nitric acid and sulphuric acid do not.

As the element common to salts and bases which is not present in acids is the metallic cation, the precipitating action must be credited to this.

The precipitate dissolves when warmed, comes down if cooled again, and this reversible process may be repeated a number of times, after which it becomes more and more difficult to dissolve the precipitated dye.

Acids, in the concentrations used in decolorizing, ethyl alcohol and acetone easily dissolve the precipitated dye.

The precipitate, when washed from the salt solution, dissolves slowly in distilled water and in very diluted acids.

The precipitation of the dye before the acid-alcohol treatment is of the utmost importance. It may occur in Ziehl-Neelsen's method when, after heating the stain on the slide, sufficient time is allowed for it to form, which may take from a few minutes to more than an hour. When this happens the best results are obtained. On the other hand, if slides in which carbol-fuchsin remains perfectly clear are treated with acid-alcohol a great number of bacilli do not take the stain.

Having emphasised this basic point I shall summarise the experimental data which led me to a different process of treatment as compared with Cooper's staining technique.

The bacillary nodules always remain unstained when decolorising is done with nitric acid, in aqueous as well as in alcoholic solution; previously examined slides showed the bacilli with well stained nodules, using 95 per cent. ethyl alcohol as a second decolorising agent as proposed by Cooper, and treatment with alkalinised solutions to wash the stain from the nodules.

The study of stained smears obtained from excised lepromata, in which the very great number of bacilli and globi enables one to form an easy appreciation of the different results of various decolorising agents and precipitating salts used, led me to the following staining technique.

STAINING TECHNIQUE.

Add to the Ziehl-Neelsen's set of reagents a solution of an acid salt. I selected a 10 per cent. solution of primary potassium phosphate, KH_2PO_4, (with a small addition of calomel to avoid moulds).

Just before starting to stain, add 3 c.c. of the salt solution per hundred cubic centimeters of carbol-fuchsin, or in routine work 5 drops per 10 c.c. respectively.

Flood the slide with stain, steam for five minutes, allow to cool until clouding and the coming down of a precipitate.

Pour the precipitated solution from the slide, without troubling about the metallic green coating which is formed.

Without any washing in water decolorise in hydrochloric acid-ethyl alcohol (3 c.c. of hydrochloric acid, 97 c.c. of 96 per cent. ethyl alcohol), until the dye ceases to flow off and the slide is cleaned.

Counterstain in a diluted solution of methylene-blue (0.1 gm. of methylene-blue in 1,000 c.c. of water).

Wash again and dry.

The counterstaining must not be too prolonged, otherwise it interferes with the staining of the nodules. Half to one minute is sufficient, as well as for decolorising and washing. Smears with no counterstain are best for the study of bacillary nodules.

I use air dried smears as well as fixed and dehaemoglobinised ones in acetic-alcohol (acetic acid 25 c.c., ethyl alcohol 75 c.c.)

Results—The acid fast bacilli are selectively stained red, and show the nodules very dark red or brown; the cells and non acid fast bacteria take the blue colour. The nodules appear as more or less spherical structures of larger size than the diameter of the bacilli. They occur in small numbers, 1 to 3, rarely 4 or 5, in each bacillus. When 1 or 2 they are frequently at the ends of the bacillus, giving them a pin or a dumb-bell-like appearance. When in the middle portions, they appear as outstanding nodules or may be asymetrically placed on one side of the bacillus like drops hanging from a wire. These nodules are present in practically all bacilli.

I obtained the same results staining the tuberculosis bacillus from sputum smears.

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