A METHOD OF DIFFERENTIATING IN SECTIONS OF TISSUE BACTERIA DECOLORIZED BY GRAM'S STAIN.

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A number of methods are given in the text books on histological technique for staining in the tissues bacteria which are decolorized by Gram's method, but owing to the fact that the demonstration of the bacteria is accomplished at the expense of the tissue differentiation they have all proved unsatisfactory.

Good results are only occasionally obtained by the ordinary routine eosin-methylene-blue method (recommended in Mallory and Wright's text book on Pathological Technique) and are too inconstant to be of practical value in staining bacteria in sections. The failure of the method to give constant results does not seem to be the fault of any one step in the technique, therefore attempts to correct or modify this method have been unsuccessful.

In the course of a study of the histological lesions produced by certain bacteria which do not retain the Gram stain, the attempt was made to demonstrate the organisms and at the same time to obtain a clear differentiation of the tissues. The well differentiated cell-picture obtained with the various modifications of Leischman's blood stain (especially that devised by Wright), suggested to me this method, or some modification of it, as a possible means of overcoming the difficulty heretofore encountered. The experiment succeeded beyond all expectation.

The staining solution used is a modification of the Leischman-Wright solution; the essential difference being in the amount of eosin used. The ordinary Leischman stain and the various modifications will not give color differentiation to the tissues. They all stain the tissues uniformly blue with little if any nuclear differentiation so that the bacteria if stained are not distinguishable.

Since the method of preparing this special stain differs somewhat from that described for the various polychrome-staining mixtures, the technique will be given here in detail.

Thoroughly dissolve 0.5 gram of sodium carbonate in 100 c.c. of hot distilled water, and while hot add 1 gram of Grübler's methylene-blue. The mixture is now steamed in an Erlenmeyer flask over a water bath until there is formed on the surface a distinct metallic luster, which usually appears within from twenty to thirty minutes. The solution is now cooled and 900 c.c. of 0.25 per cent. aqueous solution of Grübler's eosin is added slowly, the flask being thoroughly shaken after each addition. Finally a precipitate forms which is collected on a filter paper and while in the thick, moist state is turned out into a dish and dried in the incubator at 55° C. The drying is accomplished in one to two hours. Dissolve one gram of the dry precipitate in 100 c.c. of a 0.5 saturated alcoholic (methyl) solution of eosin. The stain should be made fresh before using. The dried precipitate can be kept as a stock from which the fresh stain is prepared as needed.

Method of Application.—The tissues are cut in sections from 2 to 4 micro-millimeters in thickness and fixed in eighty per cent. methyl alcohol for twenty-four hours and embedded in paraffin. Zenker's fixation also gives good results though it is not as satisfactory as with methyl alcohol. Paraffin sections are cut as thin as possible and fixed on slides in the usual way by means of an albumen solution. The paraffin, Zenker's fluid, and iodine are removed from the tissues by the ordinary method of procedure. The sections are now placed in ninety-five per cent. methyl alcohol until ready to stain.

The slide containing the section is removed from the methyl alcohol and placed in a level position over a small tumbler or slide support and covered evenly with the staining solution, care being used not to run the stain over the edges of the slide. The stain should be added before the methyl alcohol has time to evaporate in order to avoid the drying of the section. Distilled water is now slowly added by means of a dropper, until a metallic sheen forms upon the surface of the stain. This usually occurs when the phenomenon of ebullition ceases. The section is left covered in the diluted stain thirty minutes.

The next step is the differentiation of the tissue which is the most important one in the whole procedure. The excess of stain is first poured off and the slide placed in a dish of distilled water in which it is constantly moved to and fro for from ten to fifteen minutes. The water should be changed two or three times in the course of the process and the section examined from time to time under the low power of the microscope to determine the progress of the differentiation.

The stained section when completely differentiated appears to the naked eye of a distinct purple red color, whereas in the beginning it was of a deep purple blue, varying in degree with the tissue under examination. The microscopic examination shows the nuclei a distinct blue and the intercellular tissues a deep pink. The differentiation in water, particularly when the water is constantly agitated, intensifies the eosin stain of the tissue and cell protoplasm and to some extent tones down the blue stain of the nuclei.

The most satisfactory method of dehydrating and clearing is to first quickly blot off the excess of water by means of absorbent tissue paper (using every care to avoid a drying of the section), then to add a few drops of ninety-five per cent. methyl alcohol followed immediately with xylol. The secret of the whole procedure, after the section is stained and differentiated, is in properly dehydrating without decolorizing the bacteria or taking the eosin stain from the cell protoplasm and intercellular tissues.

Sections dehydrated in alcohol for more than fifteen seconds may be completely decolorized of their eosin. By first removing the excess of water by means of blotting paper a few seconds are sufficient to dehydrate the section.

The summary of the steps is as follows:

1 Fix tissues in methyl alcohol (or Zenker's fluid) and embed in paraffin.

2. Treat sections in the usual way to remove the paraffin (or Zenker's fluid and iodine) and place in 95 per cent. methyl alcohol.

3. Remove slide from methyl alcohol and cover evenly with the staining solution, using care not to run the stain over the edges.

4. Add to the stain-covered slide an equal quantity of distilled water (avoid running the stain over the edges) and allow it to stand in the admixture from 15 to 30 minutes.

5. Pour off excess of stain and differentiate in distilled water for 15 to 30 minutes, constantly keeping the water in motion.

6. Blot off excess of water with tissue paper (do not allow to dry) and dehydrate for a few seconds in 95 per cent. methyl alcohol, or dehydrate and clear in analin oil, first blotting.
7. Clear in xylol.
8. Mount in balsam.

This method not only renders possible the demonstration of bacteria in tissue sections but gives a perfectly satisfactory tissue stain in which the various elements are clearly differentiated. The microscopic picture is even more delicate and distinct in color contrast than that obtained with the eosin-methylene-blue method after Zenker's fixation. The color contrast is especially well marked in sections of the spleen and lymph nodes where the protoplasm of the individual cells is well preserved. Here the various elements can be as readily differentiated by their nuclear and protoplasmic staining reaction as the cells of a blood film. The phagocytic cells with their inclusions are especially striking in color contrast. Blood platelets and fibrin are well stained. The connective and muscle tissues are stained a deep pink color. Sections of the cornea and skin are especially well differentiated by this method of staining. The epithelial cells are colored a distinct robin's-egg blue in contrast to the clear pink staining of the underlying connective tissue. Bacteria take a deep blue-black stain and are readily detected either in the cells or the intercellular substance.

The perfect staining of the various tissues with the differentiation of the chromatin and cell protoplasm renders the method most useful in the demonstration of protozoa, such as the amoeba-coli, in tissues and offers a more certain method of recognizing protozoan-like bodies in the cells and intercellular spaces of the skin in certain contagious diseases.