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SOME

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FOR

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# Some New Methods of Using the Aniline Dyes for Staining Bacteria.

By

**E. Hanbury Hankin.**

I HAVE lately been staining bacteria in tissues for Dr. Klein by some new methods that seem to offer certain advantages over those already in use.

Dr. Klein has kindly supplied me with tissues containing various bacteria. But most of my results have been obtained with Bacillus anthracis.

In the methods about to be described the hardening of the tissue that generally precedes section cutting must be carefully attended to. It is always necessary to use Müller's fluid. The tissues must be cut into very small pieces, and the liquid frequently changed. If this is not done the nuclei will be found to show more affinity for the dye than the bacteria, and only those organisms that are placed near the margin of each section will be visible.

Nearly equally good results are obtained with perfectly fresh material.

Method A. Materials required:

(1) A strong watery solution of methyl blue or Weigert's aniline oil solution of the same dye.

(2) A saturated alcoholic solution of eosin. This should be made simply by shaking up the dye with the alcohol and filtering. By heating the alcohol or letting the solid eosin remain for some days in contact with the liquid a stronger solution is obtained, which is undesirable for the present purpose.

(3) A pipette to hold the above. A test-tube fitted up as a wash bottle is very convenient for holding the eosin.

(4) Absolute alcohol, which must be used in a capsule provided with a cover, to prevent the access of watery vapour from the air. Every precaution should be taken to keep the alcohol as free from water as possible.

(5) Benzine and clove-oil mixture, made by mixing equal volumes of benzine and oil of cloves, and adding sufficient absolute alcohol to dissolve the turbidity that appears on shaking together the above reagents.

(6) Oil of cloves, which must be fresh and nearly colourless. By exposure for a few hours to light and air oil of cloves loses a great deal of its power of dissolving the aniline colours.

(7) Benzine. This may be replaced by xylol or cedar oil.

Modus Operandi.—The sections are taken from spirit and placed in the methyl blue solution. Immediately the eosin is dripped in from the pipette. About equal parts of the eosin and methyl blue solutions are employed. If too much eosin is used the background of the section will have a dull purplish tinge, contrasting badly with the blue-tinted bacteria; while if too little eosin has been dropped in its red shade is scarcely visible.

By adding eosin to the solution its power of dissolving methyl blue is diminished. Part of the excess is precipitated in the form of a granular deposit; part of it combines with the tissue in the form of stain. The precipitate thus rapidly formed is readily dissolved by the alcohol used in dehydrating, and never spoils the appearance of the specimen. As soon as the eosin has been added the sections are removed one by one to absolute alcohol, shaken about in it for a few seconds, and then placed in the benzine and clove-oil mixture.

The sections are washed in this till the effects of the eosin begin to be apparent, then washed rapidly in another capsule of the same mixture, spread out on a section lifter, placed in benzine, and mounted. If, however, they are not sufficiently decolorised they should be removed to oil of cloves, which will readily dissolve the excess of methyl blue. If the red tint of

the background is not sufficiently pronounced they may be treated for a minute or less with eosin dissolved in clove oil, afterwards washed in benzine and clove-oil mixture, lastly in benzine, and then mounted.

The whole process does not take much more than a minute, of which about twenty seconds are occupied in adding the eosin solution to the methyl blue. The dehydrating in alcohol should be accomplished as rapidly as possible.

Sections stained in this manner show the bacteria stained blue; the nuclei are similarly coloured, but of a lighter shade, the eosin has stained the red blood-corpuscles orange, and the background of the tissue is of a rose-red tint.

Ganglion cells are stained purple if the section has not had too much of the colour removed. Besides the blood-corpuscles eosin can stain several other things of a yellow or orange tint, and if these structures have also an affinity for methyl blue the two dyes combine, giving a bright green shade. Hæmoglobin crystals and sometimes red blood-corpuscles are thus stained.

In sections through the lung of a sheep which had suffered from foot-and-mouth disease this eosin and methyl blue method showed large amorphous caseous deposits of a bright emerald-green colour, while other methods of staining failed to differentiate them.

Sections of the lung of another sheep, which contained encysted thread-worms, showed within their rose-red epidermis the protoplasm of the nematodes stained green, and their nuclei of a purple tint.

Sections through a congested spleen showed orange-red blood-vessels on a background of blue and green nuclei almost as well as an injected specimen.

While oil of cloves is generally used to help the alcohol in taking the excess of colour out of a section, I, on the contrary, use it for keeping the colour in, so far as the bacteria are concerned; for bacteria stained as here described would rapidly become invisible if left for long in the more powerful solvent, namely, the alcohol; and sections may often be kept indefinitely in oil of cloves without the bacteria losing their stain,

although this solvent is quite capable of washing out all the colour from nuclei, connective tissue, &c.

Not only clove oil, but also other clearing reagents, such as benzine, xylol, creosote, aniline, and cedar oil have a greater or less power of fixing the colour in the bacteria, though several of them can dissolve the aniline dyes freely.

Sections stained by any of these methods, quickly dehydrated and cleared, can then be washed for a longer time in alcohol, without the dye leaving the bacteria, than can sections which have been taken direct from the dye into spirit. I even found that clearing the tissue before staining, and then washing in alcohol, would slightly improve the result in some cases.

Concerning this method of staining it may be remarked that, firstly, a mixture of methyl blue and eosin has scarcely any staining power. If the sections are put into the dye after the addition of the eosin no result is obtained, and consequently it is immaterial how long the sections are left in the mixed staining solution. Secondly, the eosin solution is much stronger than that in general use. If a section is stained in methyl blue, and then placed in a saturated alcoholic solution of eosin, as here used, in a very few seconds all the blue colour is turned out, and the section stained of a uniform red tint; but in this method the presence of the methyl blue in solution seems to protect the tissue from the eosin. Thirdly, when eosin and methyl blue solutions are mixed some of the methyl blue is precipitated, and after some time will be found sticking to the sides of the containing vessel. Fourthly, if a greater quantity of the eosin solution than that above mentioned is employed, instead of a preponderance of red in the resulting stain, it will be found that the methyl blue has commenced to stain the background besides the nuclei, and the whole section will have a purplish colour. Fifthly, if the conditions of solution of the dyes are reversed, and alcoholic methyl blue is added to watery solution of eosin, no staining effect is produced, unless the saturated eosin is diluted with about five times its bulk of water. By this method the bacteria are stained slightly better than in the manner above described

the only objection to its use being that the dilute eosin employed (though stronger than the eosin solutions in general use) is incapable of staining the background of the tissue strongly. I have sections stained in this way showing dark blue bacilli on a nearly colourless background.

Moreover, the eosin may be replaced by certain other reagents, which have the property of precipitating methyl blue; for instance, by dilute alcoholic solution of picric acid and by a dilute alcoholic solution of tetrabromofluorescein. This last reagent is made from a strong watery solution of eosin by adding hydrochloric acid, filtering off, and washing the precipitate, and then dissolving it in alcohol.[1]

In the former case I noticed that if the picric acid solution is too strong all the methyl blue is precipitated in the solution, and no staining effect is produced. By diluting the picric acid the bacteria were stained, and by still further dilution of the picric acid no more effect was produced than in the first experiment. These facts seem to show that the precipitation of the methyl blue plays some part in producing the stain.

Most of the aniline dyes in common use are more soluble in alcohol than in water. It occurred to me that if a saturated alcoholic solution was added to a strong watery solution of the same dye a precipitate would occur, and that possibly bacteria present would be stained. I first tried with Weigert's aniline water solution of Spiller's purple, and obtained very poor

[1] See 'The Chemistry of the Coal Tar Colours,' by Benedict and Knecht, published by Bell and Son. Since writing the above I have been looking over some of the first specimens that I made by the eosin and methyl blue method about six months ago. I find that some of them are faded, but in others the bacteria are still dark blue on a pink background. In the latter case, instead of using the eosin solution above described, I had used a mixture of three parts eosin saturated in alcohol, and one part of tetrabromofluorescein saturated in alcohol. When first made the bacteria were stained nearly black.

The bacteria in question were small bacilli, which Dr. Klein found associated with some as yet undescribed disease of a sheep. They were not very easy to stain by this method. Scarcely any anthrax specimens stained in this way show any sign of fading.

results. On adding the alcoholic Spiller's purple a whitish tinge was produced, as if milk had been added to the solution. This I found to be due to minute globules of aniline. I also found that these globules have a great affinity for the dye, for if aniline or any other oily liquid that can dissolve Spiller's purple is shaken up in a test-tube with a watery solution of this dye nearly all the colouring matter is removed from the solution and dissolved in the oily liquid.[1]

I then tried with watery solutions of Spiller's purple, and obtained successful results, in the following manner:

Method B. The materials required are:

(1) Saturated watery solution of Spiller's purple.

(2) Saturated alcoholic solution of the same dye.

It is very important that both these solutions should be saturated. The best way to effect this result is to boil the dye with the solvent used in each case, allow the mixture to cool, and then filter.

(3) Absolute alcohol, benzine and clove-oil mixture, and benzine as in Method A.

(4) Eosin dissolved in oil of cloves made by mixing about as much eosin as can be lifted on the point of a penknife with a watch-glass full of oil of cloves. The mixture should be used fresh, as after standing a quantity of the eosin is precipitated in crystals, especially if any acid fumes or traces of picric and other acids are about.

Modus operandi.—The sections are removed from spirit and placed in the watery Spiller's purple.

At once an equal bulk of alcoholic Spiller's purple is dropped in from a pipette. The sections are then dehydrated in absolute alcohol as quickly as possible and removed to the benzine and clove-oil mixture.

Sometimes it may be necessary to dehydrate, not in alcohol, but in alcohol saturated with Spiller's purple. By this means

[1] All dyes that stain nuclei also stain the globules of an oil emulsion. Hence when staining—at any rate by these methods—care must be taken that no trace of oil of cloves or any other oil is introduced (on needles, section-lifters, &c.) into the staining solution. Mere traces will often spoil the result

the exit of the dye from the bacteria can be effectually prevented.

When cleared the sections are removed from the benzine clove-oil mixture to oil of cloves containing eosin. The eosin stains the background red and at the same time turns out the excess of Spiller's purple; sometimes a few seconds, sometimes a few minutes, are required to do this. The sections are then washed in oil of cloves, passed through the benzine and clove-oil mixture to benzine and mounted.

The removal of the excess of colour can be greatly hastened by moving the sections backwards and forwards between the oil of cloves and benzine and clove-oil mixture.[1]

I attempted also to stain in this manner with fuchsin and gentian violet, but found that although the sections were left in the dyeing solution only for a few seconds they were hopelessly overstained, and it was impossible to remove the excess of colour by means of oil of cloves.

After some trouble I succeeded in finding out how to avoid this difficulty. The method is as follows:

The sections are subjected to double treatment with fuchsin or gentian violet in the same way as above described for Spiller's purple, then rapidly dehydrated in alcohol and placed in the benzine and clove-oil mixture. They are then removed to oil of cloves in which picric acid has been dissolved. This quickly turns out the excess of fuchsin or gentian violet and at the same time stains the background. The sections are then washed in pure oil of cloves, benzine and clove-oil mixture, placed in benzine and mounted. Or if a green background is preferred to the yellow tint of picric acid the sections may be contrast stained with a mixture of methyl blue snd picric acid dissolved in oil of cloves or aniline. They must then be washed rapidly in benzine clove-oil mixture and placed in benzine.

The only objection to the use of gentian violet by this method

[1] This is really the reiteration of a "tip" kindly shown me by Mr. Lingard at the Brown Institution. He showed me that in staining by Gram's method it is possible to turn out the excess of colour by moving the sections from alcohol to oil of cloves, and back again repeatedly.

is that the bacteria, if at all easy to stain, are dyed black, which result, for obvious reasons, should generally be avoided.

In all these methods of staining, advantage is taken of the well-known fact that benzine does not dissolve, and therefore fixes the aniline dyes. Sections when stained so feebly that prolonged washing in alcohol would render them quite colourless, are placed in benzine after dehydration, and the loss of colour being thus checked, they are mounted as permanent sections. If, however, they were removed directly from the alcohol to the benzine, a precipitate of dye would probably be formed on the surface of the section, which would spoil the result. Hence the necessity for the benzine and oil of cloves mixture, which, refusing on the one hand to dissolve much more of the dye, and on the other to precipitate any, forms a link between the alcohol and the benzine. Sections should generally be placed successively in two or three watch-glasses or capsules full of this mixture before removal to benzine; and in the last of the series they should only remain while they are being spread out on a lifter. By taking this precaution the last capsule will contain little dissolved dye, and all granular precipitate on the section is prevented.

Another advantage in placing sections in benzine before mounting is that any residue of clove oil is removed. By this means the section is far more likely to be permanent than if the excess of clove oil is merely drained off with a piece of blotting paper, as is usually the case.

In using these methods it is necessary to remember that fuchsin and other dyes which show such an affinity for nuclei when dissolved in water or alcohol, act quite differently when dissolved in oil of cloves. Under these conditions they are almost incapable of staining bacteria or nuclei, but the whole of the tissue becomes dyed of a uniform tint. This influence of the solvent in modifying the action of the dye is not of a chemical but rather of a physical nature, for the oil of cloves may be replaced by aniline, creosote, or any clearing agent, with scarcely any alteration in the result.

If a section is placed in water and some alcoholic fuchsin

dropped in, the nuclei and bacteria will be found to have taken the dye. But if the section is placed in an alcoholic solution of fuchsin, and if the dye is precipitated by the addition of a considerable quantity of benzine, the whole of the tissue will be found to be strongly stained a uniform crimson tint. The dye has acted as a background stain. From these considerations it is evident that firstly, after the nuclei or bacteria in a section have been stained with Spiller's purple, fuchsin, or gentian violet, if the excess of colour is being removed by means of oil of cloves, care must be taken to move the sections into a fresh quantity of the reagent as soon as it has become strongly tinted by the dye. And, secondly, after staining bacteria by double treatment with Spiller's purple or methyl blue, they may be contrast stained with fuchsin dissolved in an oily medium. Aniline is the best for the purpose. After leaving in this reagent for about a minute they should be removed to picric acid and clove oil for a few seconds, then washed and mounted. If methyl blue was the dye previously used, the bacteria will be found to be stained green on a brick-red background.

Another method of staining, which, though a little more complicated seems to offer certain advantages, is as follows:

Sections are subjected to double treatment with fuchsin as above described, then washed for about five minutes in picric acid dissolved in oil of cloves; this last reagent is removed by washing in benzine and clove-oil mixture. They are then treated with methyl blue dissolved in aniline oil for about ten minutes. The effect of this is that every structure that was previously stained red with fuchsin is turned black, blue black, or dark purple. This, then, is the colour of the bacteria, and the background tint is green or blue. This can be changed to red by placing the section in oil of cloves and eosin for a minute. The sections are then washed and mounted as in the other methods. Not only do sections stained in this way promise to be remarkably permanent, but the contrast between the dark blue of the micro-organism and the pale pink of the background is as strong as can be desired.

The question arises whether sections stained by these methods

are likely to be permanent. It is well known that, cæteris paribus, bacteria that have been stained for six hours are more likely to retain their colour than those that have been in the staining solution for only a few minutes. Is it then likely that these methods will be permanent, where the whole process takes only a few minutes, and the bacteria are only exposed to the action of the dye for a few seconds? Though the only way to settle this question is to observe how long sections thus stained will last, I thought that some indication of their permanency or otherwise might be obtained by exposing them to the action of sunlight. To do this I fixed up several sections in a window with a southern aspect on sunshiny days and observed the effect of the light at intervals. The results obtained were very different in different cases, but seemed to show that the permanency under these conditions depended rather on the amount of colour that had been washed out of the section after staining, than on the time that the section had been left in the staining solution.

A section that had been stained in gentian violet for twenty-four hours in the ordinary manner was completely bleached by sunlight in three hours. A section stained by the eosin and methyl blue method, and in which all blue colour had been washed out of everything except the bacteria, faded in the same time, while other sections stained by the same method, but in which a trace of colour had been left in the nuclei, stood a whole day's sunlight without much change, so far as the micro-organisms were concerned. Another section which had been stained in this method for histological purposes when exposed to six hours' sunlight lost the colour previously adhering to the nuclei, but left the bacteria present still strongly dyed. It is thus clear that the fastness to light is due to the quantity of dye present in any stained part of the section; and I employed this fact in differentiating out certain bacteria by means of light as follows. I had some sections of the liver of a mouse that had died of some form of septicæmia investigated by Dr. Klein. The sections when stained with Spiller's purple and eosin showed here and there dark blue apparently homo-

geneous masses plugging the smaller blood-vessels and expanding the capillaries. There was absolutely nothing to show whether or not these were masses of bacteria. But after I had exposed the mounted sections to sunlight for a day, the Spiller's purple present in the interspaces between the bacilli was completely destroyed, and the masses that before seemed to be amorphous were now seen to consist of clusters of minute bacilli marked out with perfect distinctness.

Some sections stained by the method last described were exposed to ten days' sunlight during the hottest part of last July, without appreciable change, and they still show everywhere the bacilli of a dark blue black tint, though the background of the tissue has become so bleached as to be nearly invisible. This permanency of the stain produced by methyl blue and fuchsin in combination is remarkable, when it is remembered that manufacturers regard a dye as "fast to light" when a tissue stained by it is unchanged by three hours' exposure.

These methods of staining are generally inapplicable to coverslip specimens. But preparations of anthrax blood or pneumonia sputum, very excellent for demonstration purposes, can be made as follows:

The films are stained with methyl blue or Spiller's purple, washed and dried as usual, and then a drop of eosin dissolved in oil of cloves is placed on them for a few minutes; this is then washed off with clove oil and benzine mixture. This again is removed with benzine and the coverslips are then mounted. Bacteria are seen to be stained blue, red blood-corpuscles are red, pus-cells or leucocytes generally have purple nuclei, and the background has a pinkish tint.

The method of staining by means of eosin and methyl blue gives very good results from a histological point of view, but will only succeed with bacteria that are easy to stain.

The methods of double treatment with fuchsin and Spiller's purple are successful with nearly all bacteria.

Tubercle bacilli, however, cannot be satisfactorily stained by any of these methods.