NOTE

ON

A SIMPLE AND RAPID METHOD OF PRODUCING
ROMANOWSKY STAINING IN MALARIAL
AND OTHER BLOOD FILMS.

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SINCE the appearance of a note by me in the BRITISH MEDICAL JOURNAL of March 16th, 1901, on Romanowsky staining, with special reference to its action on the parasite of malaria, I have endeavoured still further to simplify the method by isolating the active staining ingredient of the compound dye and using it as a single stain. The results have exceeded my expectations, and the method which is described below gives not only the characteristic appearances of Romanowsky staining, but is so easy in its application, and necessitates so little time and skill in its manipulation that I venture to think it may with advantage be used for ordinary blood staining, in preference to many of the methods in general use. Beautiful results have also been obtained in the examination of "smear" preparations from bone marrow, from glands such as the spleen and liver and from cancer juice. The presence of bacteria in blood films and smear preparations is also demonstrated by this method with the greatest clearness and precision, while as a selective tissue stain for sections it appears to present many advantages. As a blood stain, from the point of view of time saving, it is also no small gain that in this method no separate process of fixation of the film is necessary, fixing and staining being performed at one and the same time by the staining fluid.

The isolation of the active staining ingredient from the mixed solutions of methylene blue and eosin presented no great difficulty, but the selection of a suitable solvent and the practical application of the stain so as to simplify and shorten the technique was not so easy. It was found that the new dye when dissolved and applied to blood films only gave Romanowsky staining feebly and after being allowed to act for some hours, and it thus appeared to present little advantage over the method previously described by me. But, after further experiment, it was found that the simple addition of distilled water in a particular manner produced brilliant Romanowsky staining in from two to five minutes according to the thinness and evenness of the blood film.

The method which I will now describe as briefly as possible has been used by myself and others in this laboratory for the last five months, and the results have proved extremely regular and satisfactory. I have also recently received a good report of its efficacy in the staining of malaria from Lieutenant McKendrick of the Indian Medical Service, who is at present attached on behalf of the Indian Government to Major Ross's Malaria Expedition to the West Coast of Africa.

Preparation of the Stain.

Solutions of alkaline medicinal methylene blue and eosin were prepared in the manner described in my previous note. *Solution A:* Medicinal methylene blue (Grübler). A 1 per cent. solution of this was made in distilled water, and then rendered alkaline by the addition of 0.5 per cent. of sodium carbonate. The solution was then heated to 65° C. in a paraffin oven for twelve hours, and afterwards allowed to stand at room temperature for ten days before use. *Solution B:* Eosin, extra B. A. (Grübler). A 1 in 1,000 solution in distilled water. Equal volumes of these two solutions, A and B, were then mixed in a large open vessel and allowed to stand for from six to twelve hours, being stirred from time to time with a glass rod. The abundant flocculent precipitate which resulted was then collected on a filter, thoroughly washed with distilled water until the washings were colourless or had only a pale blue tinge, and the insoluble residue carefully collected, dried, and powdered. The resulting powder, which has a greenish, metallic lustre, constitutes, or at least contains, the active staining ingredient in Romanowsky's method. Full direc-

tions for the manufacture of this dye have been furnished by me to the firm of Dr. G. Grübler and Co., Leipzig.

Solvent.

The best solvent which I have found for this new dye is pure methyl alcohol (Merck's "for analysis"), and, by taking advantage of the powerful fixative properties of this alcohol—as in the case of the well-known compound blood stain of Louis Jenner—I have been able to dispense with any separate process of film fixation.

The dye, prepared as above, should be dissolved in methyl alcohol in the proportion of 0.15 per cent., and the resulting solution kept in stoppered glass bottles until required for use. The solution does not deteriorate by keeping; some of the dissolved dye which I have had in use for over five months has lost none of its fixing and staining properties. The solution is of a clear dark-blue colour, and shows a greenish iridescence when illuminated by reflected light.

Method of Application.

Films of blood or smear preparations from bone marrow, spleen, cancer juice, etc., are made in the usual way upon perfectly clean polished coverglasses and allowed to dry in the air. In the case of blood films the thinner and more even the layer the better will be the results, especially when staining for malaria. A coverglass, with the film uppermost, is picked up with a pair of Cornet's forceps, and three or four drops of the stain are allowed to fall upon the film. The forceps are gently rotated from side to side, so as to ensure the stain being evenly distributed over the whole surface of the glass. No attempt is to be made to check evaporation. After about half a minute, double the quantity of distilled water—that is, 6 to 8 drops—is added and allowed to mix with the alcoholic solution of the dye. Intimate mixture is hastened by rotating the forceps as above.

The film is now allowed to stain for five minutes. This time, in my hands, has given the best results, but in the case of a thick blood film, or of smears from cellular structures, ten minutes may be necessary.

The stain is now gently washed off with distilled water, and a few drops of the water are allowed to rest on the film for one minute, at the end of which time the specimen is ready for examination, either directly in water under a $\frac{1}{4}$ or $\frac{1}{8}$ inch objective, or, after drying (without heat) and mounting in xylol balsam, under an oil-immersion lens.

The whole operation, from the withdrawal of the blood from the finger to the mounting of the stained film for microscopical examination, can be completed within seven or eight minutes, and no reagents are needed beyond a few drops of the stain and a little distilled water. Where the latter is unavailable, it may be replaced by rain water or soft tap water without greatly affecting the results.

The part of the procedure in which the stained film is allowed to soak in distilled water for a minute after staining and washing has a triple importance. It intensifies the Romanowsky staining, it removes the remains of the deposit, and it alters the tint of the red blood corpuscles from a greenish blue to a transparent pink. The same effects are produced by absolute alcohol almost instantaneously, but it is apt to decolorise the specimen unless its action is very carefully controlled.

Appearances of Blood Films stained by the above Method.

Red Blood Corpuscles.—Pale pink or greenish in tint, semi-transparent.

Polynuclear Leucocytes.—Nuclear network—stained a deep ruby-red colour with sharply-defined margins. Extra-nuclear protoplasm, colourless. Fine eosinophile granules red.

Mononuclears.—Nuclei—ruby-red with extremely sharp clear outlines. Extra-nuclear protoplasm—pale eau-de-Nil or blue, occasionally showing a few red granules.

Lymphocytes.—The same as mononuclears, except that the nuclei are, as a rule, more deeply stained.

Coarse-grained Eosinophiles.—Nucleus ruby-red but not so densely stained. Granules pale pink.

Basophiles.—Granules very densely stained, of a deep purplish-black tint. Nucleus red, but usually more or less masked by granules overlying it.

Nucleated Red Cells.—Nucleus almost black, with sharp outline; extranuclear portion, grey.

Blood Plates.—Deep ruby-red with spiky margins, frequently showing a pale-blue peripheral zone surrounding the red centre.

Bacilli and Micrococci.—Speaking generally, these stain evenly blue, but, by prolonging the period of staining, and subsequently decolorising with absolute alcohol, many interesting differences may be noted with different organisms by which structural details are brought out, not generally observed by other staining methods.

Malaria Parasites.—The body of the parasite stains blue and its chromatin ruby-red; in the case of the tertian parasite Schüffner's dots are well marked in the containing red blood corpuscle. The coloured plate accompanying the article on Romanowsky staining, previously referred to, might have been drawn from specimens stained by this method, the appearances being identical.

For most clinical work in connection with blood examination for malaria parasites, census of leucocytes, etc., where a permanent preparation is not wanted, it is quite sufficient to examine the films in water with a $\frac{1}{4}$ or $\frac{1}{8}$ -inch objective. The staining is so sharp and clear that it is unnecessary to use an oil-immersion lens except to determine the presence of bacteria or the detailed structure of parasites or cells.

Postscript.

While this note was in preparation for publication articles on the same subject, by Dr. Karl Reuter, appeared in the *Munch. med. Woch.* of July 30th, 1901, and in the *Centralbl. f. Bakt.*, vol. xxx, 6, of August 26th, 1901. While it is evident that Dr. Reuter has been working on similar lines, and has also succeeded in isolating the active staining ingredient in Romanowsky's method and applying it as a single stain, I may briefly indicate a few material points in which his method differs from mine. (1) He employs as a solvent absolute alcohol. (2) A separate process of film fixation occupying "at least one hour" is necessary before staining. (3) The staining process in itself "occupies two to three hours." (4) More elaborate precautions are necessary to avoid precipitation on the film.

NOTE.

I have found that by treating Louis Jenner's blood stain with distilled water, in a similar manner to that described for the stain in question in this note, the results are greatly improved, the usual pale-blue nuclear staining being intensified to a deep Oxford blue, while the granular staining is as well marked.

THE VALUE OF NEISSER'S STAIN IN THE DIAGNOSIS OF DIPHTHERIA.

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(Preliminary Communication.)

SINCE Neisser published the account of his differential stain for the bacillus of diphtheria many bacteriologists have used it, but they appear to differ in opinion as to its value in the diagnosis of this organism. There seems to be no doubt that the great majority of the bacilli obtained from a young and pure culture exhibit the differential staining described by Neisser, but a doubt exists whether the stain is altogether satisfactory when used for mixed cultures such as are obtained from the throats of patients suffering from diphtheria.

We determined therefore to make a series of observations under the conditions which so frequently holds nowadays, namely, when the bacteriologist is not also the physician, in order to test the value of the stain in the diagnosis of diphtheria. Each of the cases was diagnosed independently at the South-Western Hospital by Mr. A. W. Daniel and confirmed by one of us (F. F. C.) from a consideration of the whole of the clinical evidence, and by the others of us at Guy's simply by the presence in the cultures of bacilli having the characteristic staining reactions described by Neisser, and the clinical and bacteriological evidence was subsequently compared.

Swabbings were taken with every necessary precaution from the throats of all cases certified as diphtheria which were admitted into the South-Western Fever Hospital; tubes of Loeffler's blood serum were inoculated, and both swabs and culture tubes were sent to the laboratory at Guy's Hospital without having been previously incubated.

When the cultures and swabs reached the laboratory, the swab was examined at once, and the culture was placed either in the incubator or the ice chest and subsequently in the incubator; the reason for this latter proceeding was that it was desirable to examine the cultures after eighteen hours incubation at 37° C. At the end of this time preparations of the growth were made and stained.

The method of preparation adopted was the usual one, the films being fixed by heat. After fixation, the films were stained for two minutes in the methylene-blue solution, washed well in water, stained for two minutes in the watery vesuvin, dried and mounted in canada balsam. It will be noticed that in our experiments we have not adhered to the method of Neisser as regards the time necessary for staining in each of the fluids. The reason for this alteration was that we found that, even when dealing with pure cultures of the Klebs-Loeffler bacillus, the bacilli did not always show the characteristic appearance after being stained for a few seconds as recommended by Neisser. When the films were stained for two minutes, however, we found that the bacilli invariably exhibited the characteristic appearances.

All the specimens were examined by two of us separately (R.M.B. and W.C.C.P.), and the bacteriological diagnosis arrived at independently of each other. If, upon comparing notes, we agreed, the diagnosis was made accordingly. When, as happened on three or four occasions, there was any discrepancy in the verdict, the films were again examined by each of us, and fresh specimens were made and examined. The bacteriological diagnosis of each case was, therefore, that arrived at by both of us.

On several occasions we found that blue granules were present in bacteria which appeared to us to be definitely cocci. We had to satisfy ourselves, therefore, in order to arrive at a positive diagnosis, that the bacteria in which were the blue granules were definitely bacilli.

In several cases we have found Klebs-Loeffler bacilli present in only small numbers in comparison with those of other bacteria. We therefore made series of subcultures in order to determine whether the bacilli retained the property of staining in this particular manner when they were in an obvious numerical inferiority, and therefore under a disadvantage as regards ease of growth.

The cultures which we chose for these experiments were those containing only Klebs-Loeffler bacilli and cocci, generally staphylococci. These were subcultivated daily and examined. Each subculture, when the staphylococcus aureus was present, was found to contain fewer Klebs-Loeffler bacilli than the culture from which it was made, and finally this bacillus disappeared altogether. The bacilli became increasingly difficult to find, but when they were found they showed the granules as well marked as in the case of a pure culture. We conclude, therefore, that under the conditions of our experiment the Klebs-Loeffler bacillus will show the blue granules, even when it is growing with difficulty owing to the synchronous growth of other bacteria.

Our observations are concerning 100 cases, with the following results:

Clinical and bacteriological diagnosis both positive	53
Clinical and bacteriological diagnosis both negative	17
Clinical diagnosis positive, bacteriological diagnosis negative	25
Clinical diagnosis negative, bacteriological diagnosis positive	5

The clinical and bacteriological diagnoses, therefore, were in accord in 71 cases and differed in 30. Of the cases, 5 in number, in which the clinical diagnosis was negative and the bacteriological positive, one had the appearance of a follicular tonsillitis, another was a case of scarlet fever with yellow spots upon the tonsil, another was suffering from measles and pronounced laryngeal stridor; another, admitted on the fifth day of illness, showed an ulceration of the tonsils which suggested the recent separation of membrane. So that in 4 out of these 5 cases the condition of the throat was compatible with the presence of diphtheria, and in one instance only in this series of 100 cases did the Neisser test warrant a diagnosis of diphtheria when the clinical evidence was entirely