AN IRON-HAEMATEIN STAIN.
WITH REMARKS ON THE GIEMSA STAIN.

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Plate V.

FOR general purposes, especially in haematological work, the Giemsa method, based on the Romanowsky principle, is certainly the most satisfactory stain for Protozoa. For certain structures, however, and in general as a control of the Giemsa stain, it is always necessary to have at one's disposal another method which will give particularly clear pictures of the nuclear elements (see *e.g.* Minchin, 1909). Of the different methods which are employed for this object the iron-haematoxylin stain is probably the most valuable and the most frequently used. There are, however, several circumstances which are unfavourable to its use as a universal stain. Its principal disadvantage is, that a differentiation is essential: the preparation is first deeply over-stained and the colour is afterwards extracted to the degree which is deemed convenient in each particular case. This extraction is always somewhat irregular, so that the observation, especially of intra-corpuscular parasites, is sometimes made very difficult, and it is not often possible to obtain in a single specimen a satisfactory stain of all the different elements. This difficulty is enhanced by the fact, that the red blood-corpuscles generally retain a deep grey, or almost black, colour. Further, a rather serious drawback is that in sections where greater masses of blood, and particularly blood-clots, are present, it is almost impossible to distinguish either intra- or extra-corpuscular parasites; to make such masses of blood

transparent it is necessary to differentiate so much, that the nuclei are also decolourized. A certain inconvenience is also experienced because of the long duration of the staining process, at least 30 hours when the original Heidenhain's method (1896) is adhered to; and although the more recent modifications, especially that of Rosenbusch (1908), have to a great extent overcome this difficulty, they still suffer from the two other disadvantages referred to.

I have therefore endeavoured to develop a quick and reliable stain of a similar nature, which does not require a differentiation. Beginning with the fact that haematein, as a rule, stains less diffusely than hæmatoxylin, I tried systematically mixtures of alcoholic haematein solutions and watery solutions of lithium carbonate, and obtained an excellent colouration of the tissue-nuclei, but did not find it satisfactory for Protozoa. The following formula gave the best results, staining 5 minutes:

Haematein sol. (1 % in 96 % alc.)	...	...	1 c.c.
Distilled water	...	...	4 c.c.
Saturated aqueous solution of lithium carbonate			5 drops.

Afterwards different combinations of iron-alum and haematein were tried, but always gave diffuse stains, so that a differentiation could not be dispensed with. Then I proceeded to experiment with alcoholic haematein and watery iron-perchloride solutions, after the manner of Weigert's iron-hæmatoxylin method (1904), which is known as an excellent nuclear stain for histological purposes, but as a rule does not stain Protozoa well. The following combinations were tried, A being a 1 per cent. solution of haematein and B the iron-perchloride solution of Weigert:

- 4 A + 1 B diffuse stain
- 3 A + 2 B good, both for sections and films
- $2\frac{1}{2}$ A + $2\frac{1}{2}$ B good for sections, but too weak for films
- 2 A + 3 B faint
- 1 A + 4 B very faint.

I shall now describe the necessary details concerning the process. It is evidently only a slight modification of Weigert's method, but I believe that the difference will be found to make the present method useful in a certain number of cases, where Weigert's cannot be used.

Fixation. Sublimate-mixtures give the best results. I prefer Schaudinn's liquid: two parts of a saturated aqueous solution of mercury

bichloride and one of absolute alcohol. When this fixation is used, the pieces of tissue must be very thin, not more than 2–3 mm., but squares of about one cm. may be allowed. The tissues should be left in the fixative for 12 hours or more—two hours are sufficient for coverslip-films—but both may remain in it for over 24 hours without any bad effect. The fragments are then passed into 60% alcohol, and subsequently into 70% alcohol containing a small quantity of iodine, sufficient to give it a pale yellow colour; thereafter to 90% alcohol and absolute alcohol, allowing one day in each of the four liquids. Clear thoroughly in xylol, pass to xylol-paraffin then paraffin, and finally embed quickly in pure paraffin. The sections should be cut as thin as is compatible with the particular purpose in view. For trypanosomes it is generally not advisable to use sections of less than five μ in thickness. They should be fixed on clean slides after simply floating them on warm distilled water (about 40° C.) and without using any fixative such as albumen glycerin. Films are treated in the same way, only they are not brought further forward than 90% alcohol. I have also stained formalin-fixed sections with fairly good results. Osmic vapour fixation of films has so far not proved successful, but I have not had the opportunity of trying to bleach these preparations with hydrogen peroxide previously to the staining, which might well modify the results. On the other hand, the bleaching of sections of tissue fixed in Flemming's liquid has not made it possible to obtain a good stain.

The staining process. After dissolving the paraffin in xylol the sections are passed through alcohols of diminishing strength to tap water (or distilled water). This is important, as they do not stain well when taken directly from alcohol into the stain, in spite of the latter being an alcoholic solution. The staining liquid is prepared about 15 minutes before being used, as it does not stain well immediately after mixing; it may be kept for about two hours, but will not stain after a longer period. It is prepared by thoroughly mixing three parts of solution A and two of B. The solution A is prepared by adding one gram of pure haematein (Grübler) to 100 c.c.s. of 96% alcohol, and shaking repeatedly without heating. The whole quantity is not dissolved, but the solution is preserved with the residuum and, if not completely transparent, filtered before use. The solution B is composed as follows, after Weigert: perchloride of iron 4, hydrochloric acid 1, distilled water 100. The slides are left for about five minutes in the mixture, with the sections downwards, and for this purpose there

are in use in Prof. Minchin's laboratory some very convenient glass-dishes of the exact size of a slide, so that a comparatively small quantity of the staining solution is necessary. A longer time than five minutes is only very rarely necessary, but the staining may be controlled at any moment by examining the preparation with a low power after washing, and the slide may then, if necessary, be returned to the mixture. For showing the structure of certain nuclei, for instance of lymphocytes, an even shorter time in rare cases may be preferable. The sections are washed in tap water for several minutes; a longer washing, even of several hours, does not harm them, and may sometimes be useful in making the colour blacker, if it is too blue. They are subsequently taken through alcohols of increasing strengths to absolute and then xylol, and are mounted in Canada-balsam, or Damarlack.

Results. The stain has been tried on the different materials containing Protozoa, which have been available at the present time, especially on smears of rat-blood containing *Trypanosoma lewisi*, also on the contents of the digestive tube of leeches containing trypanosomes from fresh-water fishes; most of the latter forms were very small, and many of them extremely slender and very difficult to stain by any method. The stain was further applied to many sections, *e.g.* of leeches containing the above mentioned trypanosomes, and of human spleen infected with *Leishmania donovani*; also some sections without Protozoa were treated with the stain in order to try its value as an ordinary histological method. The tests, to which the methods have been subjected, have therefore been varied, if not very numerous, and several of them have been rather severe. As a rule it may be said that the stain has given the same results as the iron-haematoxylin method, but in much less time. In some cases it has proved distinctly superior, especially in some of the sections of leeches, which contained compact masses of half digested blood; an excellent result was obtained by the present method, whilst the preparations stained after Heidenhain or Rosenbusch were quite useless, as either the whole mass presented a nearly uniform black colour, or else, when the decolouration was carried far enough to make out details, the trypanosome nuclei were decolourized also.

The karyosome and chromatin-structures stain black, the kinetoneucleus being especially dark; the flagellum is grey and shows very sharply whilst the protoplasm takes a pale grey colour. As mentioned above, instead of the proportion of 3 to 2, equal parts of the two solutions may be used in sections when a particularly strong contrast

is desirable. The protoplasm then remains nearly unstained, the structure of the tissue-nuclei is shown beautifully, and it deserves especial mention, that the limits between the cells in the several different types of epithelia are remarkably well defined.

For histological purposes a combination of this stain with the acid fuchsin—picric acid—acetic acid solution of Hansen (1898) gives far clearer pictures than the so-called van Gieson's stain as ordinarily employed. It may of course also be combined with any other counter stain, just as the ordinary haematoxylin-method, but generally with better results, as the protoplasm comes out more sharply in the colour of the counter stain, because it absorbs haematein to a much less degree than it does haematoxylin.

I consequently believe, that the staining process will not only be a useful substitute for Heidenhain's method in protozoological work, but also that it may take the place of the different haematoxylin-combinations in most cases, especially as it is both rapid and easy to carry out. However, as to its principal advantages, I may emphasize the fact that it gives a good stain in certain cases where no other haematoxylin-method can be used, and that it does away with all differentiation, a circumstance which adds greatly to the reliability of any stain. The rapid execution will be of value especially for work in tropical countries, where it is always inconvenient to keep preparations for a long time in watery solutions.

This method certainly does not compete with the Romanowsky stains, especially the Giemsa and Leishman methods, which have a field of their own in the rapid diagnosis of malaria and other blood diseases, and which have the great advantage of not producing any confusion between chromatin and pigment, between which it is sometimes difficult to distinguish in sections stained by iron-haematein, or haematoxylin. In more detailed work the use of both the Giemsa and the iron-haematein-stains will generally be necessary.

In Pl. V some figures are given, which illustrate the results obtained. The method may be summarized as follows:

I. *Sections.*

1. Fixation in sublimate-alcohol with ordinary subsequent operations, embedding in paraffin.
2. Sections $5\ \mu$ in thickness, or less. Sections to be fixed on slides without any medium.

3. Passage through xylol, and alcohols of diminishing strengths to distilled, or tap water.
4. Staining for 5-10 minutes in a mixture of 3 pts. Solution A, and 2 pts. Solution B, which has been prepared not less than 15 minutes and not more than 2 hours. Sometimes equal parts of A and B may be used.
5. Washing in tap water for 5 minutes or more.
6. Passage through alcohols of increasing strengths to absolute and then xylol.
7. Canada-balsam or Damarlack.

II. *Films.*

The only differences are, that these are not brought up to xylol after hardening, but passed from 90% alcohol to water, and that the mixture of 3 A + 2 B is the only one that gives good results.

After the staining they are treated exactly like sections, never being allowed to dry.

REMARKS ON THE GIEMSA STAIN.

The Romanowsky-stain has always been very difficult to apply to sections, in all its different modifications. Leishman's method gives fairly good results, but it is a great drawback that, for obtaining them, it is always necessary to be in possession of fresh serum (1904). Also Giemsa's method was originally intended only for smears which had been dried and fixed. But fortunately it now gives, when the principles laid down by Giemsa in his later publications (1910, 1 and 2) are strictly adhered to, excellent results also after wet fixation of films, and when applied to sections. In details some slight modifications may be made, whereby still better results may, in my experience, be obtained. As the method, which should certainly prove extremely useful, strangely enough does not seem to have come into general use, I shall describe the technique as I have now been using it on different materials for a considerable length of time. In fact, I had employed the acetone-differentiation a long time before the appearance of Giemsa's papers on that subject, but I only obtained the very best results after having become acquainted with the elaborate details, with which the author himself describes the method. Therefore the reader also must be

referred to the original publications, more especially to the first of the two referred to.

Fixation. Tissues must be fixed in sublimate-alcohol, as recommended above. No other method gives, as far as I have tried, useful results, and it must be especially mentioned, that the addition of acetic acid to the sublimate-alcohol detracts from the value of the stain. When films are used, a preliminary fixation may take place in osmic acid vapours, as recommended by Minchin (1909). The following procedure is the same as that described for the iron-haematein method. It is important not to use glycerin albumen for fixing the sections on the slides.

Staining. After taking the sections through the different alcohols I have found it preferable, instead of passing them first to distilled water, to leave them in tap water until transferred to the staining solution. Sometimes it is not necessary to use the somewhat complicated process of dissolving any traces of sublimate that may remain, which Giemsa recommends. If very thin fragments of tissue are fixed the sublimate is completely taken away by the iodine-alcohol which is employed during the hardening procedures; but if only few sections are at one's disposal it may be the wiser course not to omit this precaution. A prolonged staining gives the best results. I generally use a dilution of 1 to 20 (*i.e.*, 1 drop of Giemsa's solution to each c.c. of water) for 1 hour, and then change to a dilution of 1 to 40 (1 drop to each 2 c.c.'s), in which the sections are left for about 20 hours, but sometimes I have left them for 2 or 3 days in the weaker solution and still obtained very good results. The water employed for the dilution must be distilled and have been exactly neutralized, or slightly alkalized, after titration, with a haematoxylin solution as indicator, as Giemsa describes it. (The water must turn a faint blue in the course of from one to five minutes after the addition of one to two drops of an alcoholic haematoxylin solution to ten c.c.'s, or potassium carbonate solution must be added till that point is reached.) The water, which I have been using, has generally needed the addition to each ten c.c.'s of about two drops of a cold saturated potassium carbonate solution, but the actual proportion undoubtedly varies very much, so that a frequent titration is inevitable. An omission of this step in the technique invariably spoils the results. The slides are preferably stained with the sections downwards, using for that purpose the glass dishes mentioned above. It is also important, that both the stronger and the weaker staining solutions should be prepared immediately before being used; when left

even for a short time they lose in staining power. From the staining solution the preparations are passed to tap water, not to distilled water, and then into pure acetone, or this liquid is continually dropped on the slide until the desired differentiation is obtained. When the preparations have been strongly over-stained the decolouration may take a long time, very often half an hour or more, and then one seems to get the best results. The progress of the differentiation should be frequently controlled under the microscope. From pure acetone, which I prefer to the first mixture of Giemsa of 95 acetone and 5 xylol, the section passes into the second mixture of 70 acetone and 30 xylol, and after 5 minutes into a third mixture of 30 acetone and 70 xylol. It is decidedly better to use these three steps, instead of only two as Giemsa recommended. It sometimes happens, however, that the last named mixture of acetone and xylol becomes cloudy, but it seems to keep clear when both the xylol and the acetone are very pure; if it becomes cloudy at least equal parts of the two substances may be used. It is certainly not convenient to pass the specimens directly from 70 acetone and 30 xylol into pure xylol. After five minutes in each of the two mixtures pass into pure xylol, which should be changed once so that no trace of acetone may be left. The sections may be mounted in Canada-balsam, but I have for some time been using Damarlack, in which they should keep much longer without fading, if we can draw any conclusions from experience with other stains, which would seem to last much longer in Damarlack, as I have learned from Prof. C. J. Salomonsen in Copenhagen.

Results. This method has been tried during the last few months on the same material as the iron-haematein stain and at an earlier date on a good deal more. The different elements stain in the same way as by the ordinary Giemsa method and the results have been uniformly good on material which had been fixed in sublimate-alcohol, whilst after formalin and osmic acid they have been as constantly negative. If there is any difference, the chromatin stains still better than in dry preparations, and I can quite confirm Giemsa's assertion, that in blood smears and other films better results can be obtained after wet fixation than by the dry method.

It may be convenient to give a brief summary of this technique also, though the differences from the one given in Giemsa's paper are very small :

1. Fixation, hardening, and embedding as above.
2. Sections of a uniform thickness of 5μ , or less.
3. Xylol, alcohols of diminishing strengths, tap water.

4. Water, to which Lugol's iodine-solution has been added in the proportion of 2 to 100, 5–10 minutes.
5. Water.
6. 0·5 % solution of sodium thiosulphate, 5–10 minutes.
7. Tap water, 5 minutes or more.
8. Diluted Giemsa-solution 1:20 in distilled water, which has been neutralised (haematoxylin-indicator) with potassium carbonate solution, 1 hour.
9. The same 1:40, 20–24 hours.
10. Wash in tap water.
11. Differentiate in pure acetone. (Control under microscope.)
12. Acetone 70, xylol 30, 5 minutes.
13. Acetone 30, xylol 70, 5 minutes.
14. xylol, 5 minutes.
15. Fresh xylol.
16. Damarlack.

I wish to express my thanks to Prof. E. A. Minchin for his kind permission to work in his laboratory, and to Dr H. M. Woodcock and Miss Muriel Robertson for material and for having tried the stain. I am also indebted to Dr J. W. W. Stephens for some material.

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Illustrating the paper by Dr H. Seidelin.
(Description: see p. 103.)

EXPLANATION OF PLATE V.

All the figures have been drawn from iron-haematein preparations with Zeiss's prism, using Zeiss's apochr. obj. 3 mm. or 2 mm. and compens. oc. 18. Figs. 1-5 $\times 2000$, Fig. 6 $\times 3000$. My thanks are due to Miss M. Rhodes, of the Lister Institute, for executing the drawings.

- Fig. 1. *Trypanosoma lewisi* in film, using the wet method. (Fixation in Schaudinn's liquid.)
- Figs. 2, 3. Fish trypanosomes from digestive tube of leech (*Hemiclepsis marginata*); wet method. (Schaudinn's liquid.)
- Fig. 4. The same in the interior of the half digested mass of blood, in section of leech. (Schaudinn's liquid, paraffin section.)
- Fig. 5. Part of digestive tube of the same leech. To the right are the epithelial cells; to the left the partially digested blood, and between both the trypanosomes, of which only a part of the body is to be seen, most of them having been cut through in an irregular manner.
- Fig. 6. *Leishmania donovani* in section of spleen. The outline of the nucleus of the host cell has come out too darkly in the reproduction. (Formalin, paraffin.)