XVI. ON A GREATLY IMPROVED HAEMIN TEST FOR BLOOD, WITH NOTES ON SOME RECENTLY PROPOSED METHODS.

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Since Teichmann in 1853 brought forward his test for blood by the production of haemin crystals, a host of modifications have been suggested by as many writers, and a fresh crop of the same appears each year. This in itself is sufficient indication that the method in its original and modified forms is far from satisfactory. Indeed, even an experienced and skilful worker may, apparently most unaccountably, fail to obtain a positive result, though working with fresh and unaltered blood specimens. With those encountered in ordinary laboratory practice, often minute in quantity and altered by age, the action of heat or sunlight, or the presence of rust or earth, or consisting of stains partly washed out by soap and water, the difficulties and uncertainty are increased to such an extent that in many laboratories the method has fallen more or less into disrepute. A number of sorting tests, presumptive only, have taken its place, the final decision resting upon the detection of corpuscles by the microscope, some form of spectroscopic examination—often exceedingly difficult with the limited amount of material at hand—or, in certain cases, the use of “precipitin” reagents.

None of the above-mentioned sorting tests, e.g. those employing guaiacum, benzdine, leuco-base of malachite green, phenolphthalin, etc., can satisfactorily replace the haemin test, since the latter alone affords absolute and unmistakable proof of the presence of blood.

In a hot dry climate like that of the Sudan, blood stains alter more rapidly than in more temperate zones, and the difficulties experienced with the haemin test are therefore usually greater. The need for some more reliable method than any of those yet advanced was actually felt, and in

the investigation which was consequently undertaken, the following considerations were kept in mind.

(a) The amount of blood present being usually small, it is essential that the whole of the portion taken be brought into solution. To insure this the acid must remain in contact with the suspected material for a long time—some hours at least.

(b) For the reason already stated above, the operation must be carried out so that no loss may occur.

(c) In many cases, especially when the blood is mixed with rust, earth, etc., it is highly desirable that some means be provided for filtering the liquid.

(d) If crystals of good size are to be obtained from so small a bulk of liquid—a few drops at most—special precautions should be taken to insure slow evaporation. Slow evaporation has often been recommended but the means prescribed for securing it have been totally inadequate, the operation being completed in, at most, a few minutes. Our endeavour has been to extend this to hours or even, if necessary, to days.

All the above essentials have been happily provided for in the following simple procedure the success of which, in all classes of cases, has exceeded our most sanguine anticipations.

A small quantity of the suspected material is placed at the bottom of a flat arsenic sublimation tube about 6 by 3 mm. and 35 mm. long. A thin round ignition tube will answer if no flat tube is procurable, but for examination under the microscope the flat form is obviously better. A few drops of acetic acid containing from 0.01 to 0.1 per cent. sodium chloride are added and a very fine cotton thread adjusted so that its upper end is near the top of the tube and the lower end reaches to the bottom of the liquid. The thread should be everywhere in contact with the tube, to which it adheres readily by being moistened with the liquid (Fig. 1). The adjustment is readily made by means of a glass rod one end of which is drawn out for the purpose. The tube is now placed in a rack, or supported by forcing its base into a small blob of plasticine, and allowed to remain until crystallisation has taken place.

Before placing the thread into position, the solution of the blood may be aided, if desired, by cautiously heating the tube over a small flame, loss of the liquid by explosive boiling being prevented by holding the finger tightly over the mouth of the tube.

While such heating appears rarely to be essential, the long contact with

the acid in the cold sufficing for solution in practically all cases, it is nevertheless advisable in that it gives at once an indication of the strength of blood solution obtained. For the best results this should be quite weak.

The clear liquid, filtered by its passage through and along the thread, is slowly drawn up, by capillary action, to the mouth of the tube. Under these conditions evaporation takes place so slowly that, even when the temperature of the atmosphere is above 38°C ., it is complete (if the tube is held in an upright position) only after 12 to 24 hours or more. The rate

Fig. 1. Actual size.

[Fig. 2. $\times 25$.

of evaporation is readily controlled, within wide limits, by altering the dimensions of the tube and the angle at which it is supported.

The crystals usually begin to appear, on or near the upper half of the thread, and of sufficient size to be distinguished with a power of 75 diameters, in about an hour; and they ultimately become so large that, in place of requiring a magnification of 250 to 300 diameters, as usually recommended, they may readily be seen with one of 25 diameters or even, in many cases, with a good hand lens (Fig. 2). The crystals are best viewed against a dull white background. A piece of white paper is suitable.

The extraordinarily large size of the crystals is due, in great part, to the slow evaporation of the liquid; but it is also a result of the removal, by the thread, of the interfering action of the blood albumin on crystallisation. Examination of the thread at the end of the experiment shows that the soluble, non-crystallisable, constituents of the blood are carried to its upper end, and that the maximum growth of haemin crystals is found lower down.

If the solution is sufficiently weak the first crystals form at the point of greatest concentration—the upper end of the thread; but as the albuminous matter accumulates these cease to grow and the largest and most numerous crystals are found, later, about midway down the tube.

For the best results the following precautions should be observed.

The tube should be thoroughly clean and dry. It is best cleaned by swabbing out successively with acetic acid, water, and alcoholic soda, followed by thorough rinsing with distilled water and drying on a hot plate.

The thread should be clean and as fine as can be obtained. A good plan is to take No. 80, "six cord sewing machine cotton" and to untwist it, using one of the "cords" for the test.

The solution of the blood in acetic acid should not be concentrated, but very weak. The liquid should only be faintly pink. This insures large crystals and clean fields.

If the method is to be applied experimentally to fresh blood, the latter must be completely dried before the addition of the acid, and especial care should be taken to work with very dilute solutions.

The test has been applied as described to:

- (1) Human blood stains—fresh.
- (2) Human blood mixed with earth—a forensic case.
- (3) Human blood stains—10 years old.
- (4) Sheep's blood stains—fresh.
- (5) Sheep's blood stains—18 months old.
- (6) Sheep's blood stains, heated to 110° C. for 1 hour.
- (7) Sheep's blood stains on a rusty knife, two months old and also exposed to the air and sun for several days.
- (8) Sheep's blood stains which had been, in great part, removed by washing with soap and water.
- (9) Cow's blood stains—12 years old.
- (10) Gamoose's blood stains—12 years old.
- (11) Dog's blood stains—two months old.

Upwards of 300 trials have been made, using the method as described or slight modifications of it, and in no single case did the crystals fail to appear.

Many trials were also made with modifications employing other acids in place of acetic and other forms of halide. These experiments need not be recorded in detail, but the following are some of the conclusions arrived at.

Acetic acid without any saline addition will suffice for stains, fresh or old, which have not been treated with water, the salts present in the blood being sufficient for the test. Stains thoroughly washed with water will not respond unless some salt, chloride or bromide, is added.

As halides, the following were tried. To make the comparisons conclusive the blood stains were previously thoroughly extracted with water. Unwashed stains were also employed.

- (a) Hydrochloric acid and hydrobromic acid.
- (b) Sodium fluoride.
- (c) Ammonium, sodium, potassium, calcium and magnesium chlorides.
- (d) Ammonium, sodium and potassium bromides.
- (e) Sodium and potassium iodides.

Hydrochloric and hydrobromic acids gave invariably negative results.

Sodium fluoride gave positive results in some cases, but inferior to those with chlorides or bromides.

All the chlorides and bromides tried gave good results. With magnesium chloride and calcium chloride (one tenth per cent. solutions) there is a marked tendency to the formation of a greater number of crystals, of smaller size but long and very slender.

The iodides, with washed blood stains, usually gave negative results. In some cases a very few small crystals were formed but these appeared to be due to the incomplete removal of chloride from the blood, or its presence as impurity in the reagent. With unwashed stains iodides gave results inferior to those with chlorides or bromides. Similarly a reagent composed of a mixture of the three halides gave results in some cases negative and, in all, inferior to those with chloride or bromide alone. From these results the conclusion appears obvious that the presence of iodide in the reagent is distinctly contra-indicated.

As substitutes for acetic acid, formic and propionic acids were tried. Lactic acid, mentioned by Teichmann and recommended recently by Symons (*vide infra*), is obviously not suitable for this form of the test as it is not sufficiently volatile.

The results with formic acid, alone and with the addition of the various halides mentioned above, were invariably negative.

Propionic acid gave results, in some cases negative, and in all cases inferior to those with acetic acid.

In short, of the solvents experimented with, acetic acid was found to be by far the best and is in fact, for this form of the Teichmann test, the only reliable one. Further, no appreciable advantage was found to result from the substitution of other forms of halide for sodium chloride.

Fig. 3. $\times 50$.

It was noted that in the case of very old blood specimens there is a marked tendency to the formation of short, broad haemin crystals and also of hemp seed forms (Fig. 3).

NOTES ON SOME RECENTLY PROPOSED METHODS.

Nippe [1912] recommends the following reagent as affording a "ready and certain" means for the identification of blood stains: potassium bromide 0.1 g.; potassium iodide 0.1 g.; potassium chloride 0.1 g.; glacial acetic acid 100 g. A drop of the solution of the stain in the reagent, on a

microscope slide, is covered with a cover glass and warmed until bubbles just form. "As it cools the growth of the haemin crystals can be watched under the microscope."

It is not easy to see any scientific basis for this modification of the reagent. In our hands it has been found to offer no advantages whatever and the method as a whole suffers from all the disabilities of the original procedure. In the light of the experiments already detailed it would seem likely that any positive indications were obtained, not as a result of the presence of iodide, but in spite of it.

W. F. Whitney [1912] published a note on a method of performing the haemin test which appeared to have advantages. It was noted that formic acid of sp. gr. 1.2 when placed on a glass slide and allowed to evaporate, disappeared gradually from the periphery to the centre. A drop of acetic acid, on the other hand, did not hold together but spread over the slide and evaporated instantly. Formic acid appeared therefore to be a better solvent for the blood, as it permitted of concentration of the solution and the deposition of the crystals in a much more confined area. The method recommended was as follows. A minute fragment of the suspected substance—a scraping from a stain or a bit of stained fibre—is placed on a slide and a small drop of formic acid applied with a glass rod. Over the drop is inverted a small watch glass which should not touch it nor project beyond the edge of the slide. Gentle heat is applied beneath the drop and the moment condensation is seen inside the watch glass, the heat is discontinued, the watch glass removed, and the remainder of the liquid allowed to evaporate in the air. When dry the spot is examined under the microscope. The crystals are said to be small, requiring a high power objective to see them well, but so numerous that they cannot be overlooked.

The remarkable statement is added that "the fact they are so small shows that only a minute quantity of blood is necessary for their formation."

We have made many trials of the above method with most disappointing results. Haemin crystals were obtained in one case using quite fresh blood of which a larger quantity was taken than is usually available for test. Aside from this all trials were fruitless, although the field was searched most carefully under a 1/7 inch objective. Even when precautions were taken to insure much slower evaporation than is possible with the method as detailed, the issue was still wholly unsatisfactory.

These results bear out those obtained by the method detailed in the body of the paper which indicate that formic acid cannot safely be substituted

for acetic acid for the haëmin test. This point was very thoroughly investigated by the use not only of formic acid alone but of formic acid containing hydrobromic acid, sodium fluoride, sodium chloride, potassium bromide and potassium iodide. In no case were haëmin crystals formed either with fresh or with old blood stains.

An improvement on the procedure as recommended by Whitney was effected by substituting for formic acid a mixture of equal parts of formic acid and acetic acid, the test being applied as described except that the slide is best slightly heated before the drop of liquid is placed upon it. The results were, however, at best, unsatisfactory and the method failed entirely in the case of old stains or those which had been partially washed out.

A far better method than either of the above, but taking advantage of the same principle of prevention of spreading of the liquid, is the following. The solution of the suspected material is effected in a very small test tube, such as an ignition tube, the reagent being a few drops of acetic acid containing 0.01 per cent. of sodium chloride. The tube should be heated cautiously over a small flame, or for ten or fifteen minutes in a steam bath or oven, in order to insure complete solution. The liquid is then transferred by means of a small pipette to a glass slide coated with a very thin layer of paraffin wax, and set aside in a place protected from dust, for evaporation. The amount of wax on the slide should be so small as to be only just visible. The slide may be prepared by melting on it a small quantity of wax, the excess of which is then removed by means of a dry cloth.

The rate of evaporation of the liquid will depend upon the amount of paraffin wax on the slide. It has been found that the wax dissolves in the acetic acid, and after the first rapid concentration has taken place the liquid becomes coated by a continuous film of wax which checks evaporation to such an extent that it is in some cases not complete even after two or three days. Under these conditions the crystals formed are usually few in number but very large. With less paraffin wax present, so that evaporation is complete in about an hour, crystals are formed of sufficient size to enable them to be distinguished by a magnifying power of about 50 diameters.

The method has been found to be applicable even to very old blood stains, which points clearly to the fact that the difficulties experienced with the ordinary haëmin test, when so applied, were not due, as has been frequently claimed, to decreased solubility of the blood, but rather to too rapid evaporation of the solvent.

The method is, as stated, a marked improvement on the ordinary one; but it is not intended to recommend it in preference to that described above, which is far superior to it on the score both of reliability and delicacy.

C. T. Symons [1913] recommends the following modification of Teichmann's test as especially suited to old stains which have been exposed for a long time to air and sun in the tropics: "Solid sodium iodide, freshly prepared, is dissolved in lactic acid (sp. gr. 1.2) to about one per cent. The solution becomes brown on keeping but this change does not appear to impair its action, at any rate for some months. The fragment of stain is covered with the solution, the cover glass is placed in position and the whole slowly warmed to such a temperature that the solution is just about to boil under the cover glass. In most cases five minutes' treatment is sufficient to produce crystals. If they have not then appeared the heating must be continued; more solution must be allowed to run under the cover glass if necessary, but this is not often the case as the lactic acid does not evaporate as rapidly as acetic acid. The resulting crystals differ very much in size in different cases, being usually rather small, but dark and perfectly characteristic."

In our hands this method gave, at first, very unsatisfactory results. It failed entirely in the case of old stains and even with quite fresh specimens positive indications were by no means the rule. Later, using another sample of lactic acid, much better results were obtained. It was found however that success was to a great extent dependent upon the control of the heating. A small water oven containing a saturated salt solution (boiling at about 107° C.) is probably the safest means, but the temperature of an ordinary water oven is usually sufficient. The best results were obtained not, as recommended, by long heating but by heating for about five minutes only. In some cases, when heating was prolonged, what appeared to be crystals too small for positive identification were formed, but disappeared entirely on further heating. Success in ordinary cases appears to depend upon the entanglement of a supersaturated liquid in the meshes of the fabric. In other words if the blood material is allowed to become diffused throughout the entire mass of the liquid under the cover glass, no crystals may be formed.

"Freshly prepared" sodium iodide is prescribed for the reagent. In our experience this has not been found to be necessary, the salt which had been kept a long time answering equally well.

SUMMARY.

(a) Of the reagents which have been recommended for use in Teichmann's test for blood, acetic acid is by far the best and is the only one on which entire reliance may be placed if the method by evaporation is employed.

(b) Acetic acid, without salt of any kind, suffices for the test with blood stains, fresh or old, provided the stains have not been extracted with water. As a precautionary measure it is best however to use a reagent containing a minute proportion—about 0.01 per cent.—of sodium chloride.

(c) No advantage results from the substitution of other salts for sodium chloride in the acetic acid reagent.

(d) Iodides may not be used to substitute chlorides or bromides in the above. This does not apply to the reagent made with lactic acid.

(e) Where positive results have been obtained from the use of an acetic acid reagent containing iodide, the effect is apparently due to the chloride naturally present in the blood, or as impurity in the reagents.

(f) The difficulties experienced with Teichmann's test when applied to stains, both fresh and old, are due chiefly to the too rapid evaporation of the solvent and, to a less extent, to interference of the albuminous matter of the blood with the crystallisation. Evaporation should be extremely slow and when carried out in the manner detailed, which also eliminates the harmful effect of albumin, crystals are obtained with the greatest certainty and of remarkably large size, even though only a minute amount of blood be present.

(g) The test as described was found to be equally applicable to blood stains fresh or old (12 years), stains partially removed by soap and water, or heated to 110° C., or mixed with earth, or to old stains on rusty iron which had been exposed to strong sunlight and atmospheric conditions for several days.

REFERENCES.

- Nippe (1912). *Deutsch. med. Woch.*, 2, 222 (abstracted in *Pharm. J.*, 89, 811).
Symons, C. T. (1913). *Biochem. J.*, 7, 596.
Whitney, W. F. (1912). *Lancet*, 1, 1484 (quoting the *Boston Medical and Surgical J.*, April 11, 1912).