Miscellaneous Contributions to Microtechnique

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SUMMARY

1. The glycerine in Mayer's glycerine and albumen serves no useful purpose.

2. If about *one-half* of the haematoxylin in such solutions as those of Heidenhain and Ehrlich be oxidized by sodium iodate, the dyes are ready for use the moment they have been made up and also keep well.

3. In using crystal violet for staining chromosomes, it is best to fix the dye by an aqueous (instead of the usual alcoholic) solution of iodine.

DURING the course of our teaching and research, we have worked out what we believe to be better methods of performing certain familiar techniques. We think that others might be glad to have particulars of them. The three techniques that will be discussed in the present paper are the attachment of sections to slides, the preparation of solutions of haematoxylin, and the staining of chromosomes with crystal violet and iodine.

Adhesive albumen for the attachment of sections to slides

For the past 70 years Mayer's glycerine and albumen has been the standard adhesive used in attaching sections to slides (Mayer, 1883). The inventor of this method mixed glycerine with an equal volume of filtered egg-white, dipped a brush into this fluid, and spread a thin layer of it over a slide. He then placed a paraffin section on it and put the slide on a water-bath. Thus the section was flattened on undiluted glycerine and albumen. The sole purpose of the glycerine, as Mayer stated clearly, was to keep the adhesive wet during this process. Finally, the albumen was precipitated by warmth and drying, and the section adhered to the glass.

Although Mayer's solution is used to this day, almost to the exclusion of other adhesives, yet it is improbable that anyone nowadays flattens sections as Mayer did. It is usual either to put 5 drops of his solution in about 10 c.c. of water and to flatten the sections on this, or else to flatten them on warm water in a basin and then transfer them to Mayer's solution smeared on a slide. In neither case does the glycerine perform the function intended by Mayer.

The apparent uselessness of the glycerine suggested a trial of albumen alone. The results were from the first perfectly satisfactory, so far as flattening and adhesion were concerned, but the solution did not keep well. Mayer himself at first used phenol (1883) and then changed to sodium salicylate at 1 per cent. (1887). The latter acts well in the presence of glycerine, but does not preserve simple albumen solutions indefinitely. We have found sodium

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p-hydroxybenzoate an admirable medium for this purpose. (If there should be any difficulty in obtaining the pure substance, 'Moldex' may be used instead. It is obtainable from Messrs. R. Campbell and Co., 7 Idol Lane, Eastcheap, London, E.C. 3.)

Our solution is prepared as follows:

To 100 c.c. of 1 per cent. (or 0·9 %) aqueous sodium chloride solution, add 0·2 gm. of sodium *p*-hydroxybenzoate. Mix this solution with an equal volume of egg-white. Stir well. Centrifuge until the supernatant fluid is clear. The supernatant fluid is the adhesive.

We call this product 'adhesive albumen' (AA). For paraffin sections we dilute the AA in the usual proportions (5 drops to about 10 c.c. of distilled water), flood the slide with this, float the sections on it, and flatten them by warming. When the excess of fluid has been drained off and the preparation dried on the hot plate, the sections adhere very firmly. Workers who attach sections after first flattening them in a basin of warm water should use AA exactly as though it were Mayer's fluid.

AA is perfectly suitable for the attachment of celloidin sections by Richardson's technique (1952).

Half-oxidized haematoxylin

It was pointed out by Unna long ago (1891) that the ordinary solutions of haematoxylin used for staining contain three constituents—unripe, ripe, and over-ripe portions of the dye, the latter forming a precipitate. In other words, the solutions contain haematoxylin, haematein, and further oxidation-products. Only the haematein is a dye. The haematoxylin keeps up the strength of the solution by gradual conversion to haematein. The further oxidation-products are useless. If haematein is used instead of haematoxylin in making up the solution, there is continual reduction in the strength of the stain with the passage of time.

Unna added sulphur to partially ripe haematoxylin solutions, to check oxidation at an early stage (presumably by the production of small quantities of sulphuretted hydrogen). He sometimes used this 'half-ripe' haematoxylin as a dye, sometimes kept it as a stock solution, fully oxidizing the required quantity from time to time with hydrogen peroxide.

Our purpose has been to produce haematoxylin solutions that would be usable the moment they had been prepared, but would nevertheless be stable. To do this we have used sodium iodate as oxidizing agent, but have allowed only about one-half of the amount that would oxidize the whole of the haematoxylin. The rest of the haematoxylin remains in the solution and is gradually oxidized to haematein by atmospheric oxygen, thus maintaining or increasing the strength of the solution.

Sodium iodate was used to oxidize haematoxylin by Mayer (1903), who allowed 0·2 gm. of the salt to 1 gm. of haematoxylin, in the belief that this amount was a little less than what was necessary to oxidize the haematoxylin

fully. Actually 0·187 gm. of sodium iodate suffices to oxidize 1 gm. of haematoxylin to haematein (Hansen, 1905). In our solutions we allow 0·1 gm. of sodium iodate to 1 gm. of haematoxylin, and thus oxidize a little more than one-half of the latter.

We boil the haematoxylin with the iodate, and the roughly half-oxidized solutions thus produced can be used directly they have been made up. In a stoppered bottle they gradually increase slightly in staining power. Heidenhain's, Regaud's, Ehrlich's, and Mayer's haematoxylins, made up in half-oxidized form, were stored for 6 months and then compared with the same solutions made up on the day of the test. In all cases the fresh dye acted well, but the stored dye was slightly stronger.

We make up the *half-oxidized versions* of the various dyes as follows:

Heidenhain's haematoxylin (1896)

Haematoxylin .	5 gm.	Distilled water .	950 c.c.
Sodium iodate .	0·5 gm.	Alcohol (96 %) .	50 c.c.

Put the haematoxylin and the sodium iodate into the water. Heat till the water just boils. Cool. Add the alcohol.

We find that the alcohol may be omitted, 1,000 c.c. of water being used instead of 950 c.c. Heidenhain used the alcohol to dissolve the haematoxylin quickly, but this is not necessary when heat is used.

Regaud's haematoxylin (1910)

Haematoxylin .	10 gm.	Glycerine	100 c.c.
Sodium iodate .	1 gm.	Absolute (or 96 %) alcohol .	100 c.c.
Distilled water .	800 c.c.		

Put the haematoxylin and sodium iodate into the water. Heat till the water just boils. Cool. Add the glycerine and alcohol. (Distilled water might presumably be substituted for the alcohol.)

Ehrlich's haematoxylin (1886)

Haematoxylin .	6·7 gm.	Glycerol	333 c.c.
Sodium iodate .	0·67 gm.	Absolute (or 96 %) alcohol .	333 c.c.
Distilled water .	333 c.c.	Acetic acid (glacial) . .	33 c.c.
Potassium alum .	18 gm.		

Put the haematoxylin and sodium iodate into the water. Heat till the water just boils; then remove from the flame and add the alum. Stir from time to time. Mix together the glycerol, alcohol, and acetic acid, and stir them into the haematoxylin solution when the latter has cooled to 50° C.

The amount of alum is just sufficient to leave a small precipitate.

Mayer's acid haemalum (1903)

Haematoxylin .	1 gm.	Potassium alum .	50 gm.
Sodium iodate .	0·1 gm.	Chloral hydrate .	50 gm.
Distilled water .	1,000 c.c.	Citric acid . .	1 gm.

Put the haematoxylin and sodium iodate into the water. Heat till the water

just boils; then remove from the flame and add the alum. Stir from time to time. When the solution is cool, add the chloral hydrate and citric acid.

Dobell's haematein (1914)

Haematoxylin . . .	10 gm.
Sodium iodate . . .	1 gm.
Alcohol, 70 % . . .	1,000 c.c.

Put the constituents in a flask provided with a reflux condenser. Heat till the fluid boils. Cool. Do not detach the reflux condenser until the fluid is cool.

Hansen's 'Trioxyhaematein'

Haematoxylin .	7·1 gm.	Iron alum . .	44·4 gm.
Sodium iodate .	0·71 gm.	Ammonium sulphate .	6·2 gm.
Distilled water .	1,000 c.c.		

Put the haematoxylin and sodium iodate into the water. Heat till the water just boils; then remove from the flame, add the iron alum and ammonium sulphate, and allow to cool, stirring from time to time.

Hansen himself believed that his 'Trioxyhaematein' represented a particular stage in the oxidation of haematoxylin, beyond haematein, and he gave careful instructions for preparing it. A complicated routine, based on Hansen's instructions, is usually followed. However, our simple method of preparation gives a stain that appears to act in the same way as Hansen's. Whether the solution is made up according to Hansen's instructions or ours, it is not stable under the ordinary conditions of laboratory use, for a precipitate forms and the ability to stain falls off rather quickly. However, if a bottle is filled to the top with our solution and well stoppered, the stain will still be in good condition 6 months later, though it will require filtering.

Crystal violet and iodine for staining chromosomes

This is an invaluable technique, especially for the study of meiotic prophases. The chromosomes are precisely stained, while the cytoplasm is of glassy transparency. Very thick sections can be used.

The method was introduced by Newton (1926), who gave no detailed instructions. He stained with gentian violet (a mixture of crystal violet in unstated proportions with other dyes), and secured it in the chromosomes by adding 1 per cent. of iodine (and 1 per cent. of potassium iodide) to the dehydrating alcohols. The principle is the same as that of Gram's bacterial stain. More precise details of technique were given by Huskins (1927) and La Cour (1931). Their techniques give excellent results, but are nevertheless open to the following criticisms.

After staining with gentian violet, both Huskins and La Cour treated the sections (or smears) with iodine/potassium iodide/80 per cent. alcohol. Since iodine is soluble in 80 per cent. alcohol, the function of the potassium iodide is unexplained. In the alcoholic iodine two antagonistic processes are at work. The alcohol tends to remove the dye from the chromosomes, while the iodine

tends to fix it in them. If the final result is unsatisfactory, it is difficult to judge whether to increase or decrease the period in this solution. Very little resistance to extraction by alcohol is given, for, as Huskins says, the slides must be taken 'almost as rapidly as possible' through absolute alcohol. Since dehydration is incomplete, the sections must pass through the expensive clove oil before entering xylene; and to remove the clove oil, prolonged soaking (15 min.) in three lots of xylene is advised. The first xylene becomes badly contaminated with clove oil, and is thus spoilt for use in other methods. As La Cour says of this method, 'Staining is an art which requires practice to reach perfection'.

It seemed rational to fix the crystal violet in the chromosomes by means of iodine dissolved in a medium (aqueous potassium iodide solution) that does not extract the stain. If this is done, there is no need to rush the dehydration of the sections, and the use of clove oil is unnecessary. We believe that our method is not only more rational, but also much simpler and easier than the one usually employed. We prefer pure crystal violet to the redder mixture of dyes (gentian violet).

Our technique is as follows. We usually fix in Sanfelice's fluid (1918), but sometimes in Zenker's; the latter, though generally used only as a routine fixative in histology and micro-anatomy, is quite a good fixative for some chromosomes. We embed in soft paraffin and usually cut sections at 14 μ.

Two solutions are required for staining:

Crystal violet, 1 per cent. aqueous.
Iodine, 1 per cent. in 2 per cent. aqueous potassium iodide solution.

The technique is as follows:

(1) Bring sections to water.
(2) Crystal violet, 15 minutes.
(3) Rinse in distilled water.
(4) Iodine solution, 1 minute.
(5) Rinse in distilled water.
(6) 70 per cent., 90 per cent., and first absolute alcohol, about 10 seconds in each.
(7) Second absolute alcohol, about 1 minute.
(8) Xylene, two lots.
(9) Mount in D.P.X. (Canada balsam is also suitable, but the stain sometimes fades in this medium.)

If a section is understained, this can be corrected with the next slide by lengthening stage (2) or shortening stage (7). If it is overstained, the reverse applies.

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