Although several neurological silver impregnation methods have been used in the past to demonstrate argyrophile enterochromaffin cells most workers now exclusively employ the Bodian method or its modifications (see Chapter 2) for this purpose. With suitable modifications the Bodian method gives an excellent display of these cells. This method, however, required the use of special brands of silver proteinate that are not always obtainable. Another disadvantage is the considerable time (24 hours or more) required for the staining procedure. Attempts have, therefore, been made to develop a method that is rapid and does not require the use of silver proteinate. Numerous trials show that the method described below fulfils these desirata.

TECHNIQUE

Special solutions required: (Modified from Holmes, 1943)

(1) Buffer solutions:
   (a) Boric acid (A.R.) 12.4 g
   Distilled water 1000 ml
(b) Borax(A.R.) 19 g
Distilled water 1000 ml

(2) Impregnating solution:

To 70 ml of the boric acid buffer solution add 30 ml of silver nitrate(A.R) and 5 ml of 10% the borax buffer solution. Add 1 ml of 1% aqueous solution of pure pyridine. Make up to 500 ml with distilled water. Mix thoroughly.

(3) Developer:

Hydroquinone 1 g
Sodium sulphite crystals 10 g
Distilled water 100 ml

Fix tissues in 10% formalin, formol-saline or Bouin's fluid. Dehydrate the tissues in ascending series of ethyl alcohol and embed in paraffin. Cut sections at 10μ and mount on slides.

Staining procedure:

1. De-paraffinise slides and take down to 95% alcohol.
2. Transfer to 1% solution of iodine in 95% alcohol for 5 minutes.
3. Take sections down through 90% and 70% alcohol to water.
4. Treat with 5% aqueous sodium thiosulphate till the sections are decolourised (1 to 3 minutes).
5. Wash in running tap water for 5 minutes.
6. Rinse well in distilled water.
7. Place the slides in the impregnating solution and impregnate for one hour at 60°C in a clean covered container, using about 25 ml of solution for each slide.
8. Take out slides, shake off superfluous solution and place them in the developer till no further browning of the sections occurs (about 2 minutes).

9. Rinse well in distilled water.

10. Return the slides to the impregnating solution and impregnate at 60°C for a further 1⁄2 to 1 hour.

11. Repeat development in the developer.

12. Examine the slides under a microscope at adequate magnification. Intensely black argyrophile granules should stand out against very lightly stained background of nuclei and other tissues. If the argyrophile granules are not adequately blackened, but are brown, repeat the impregnation for another 15 minutes to 3⁄4 hour followed by another development. This procedure can be repeated as many times as is necessary, but as a rule only two impregnations of one hour and 1⁄2 hour, (vide steps 7 and 10 respectively) are necessary.

13. Transfer the slides to 5% sodium thiosulphate for 2 minutes.

14. Wash in running tap water for 5 minutes.

15. Counterstain, if desired, with neutral red or safranin.

16. Dehydrate and mount.

Result:

The argyrophile granules in enterochromaffin cells are stained black (Fig. 2). When a counterstain is not used the nuclei and other tissue elements are stained varying shades of brown.

Argyrophile granules in normal enterochromaffin cells are stained
more easily than those in carcinoids. The latter can be satisfactorily impregnated (Fig. 3) by doubling either the impregnating time or the silver nitrate content of the impregnating solution.

COMMENT:

(a) Role of fixatives:

Proper fixation of tissues is essential for satisfactory demonstration of enterochromaffin cells by any technique and this holds true for the present method also. The choice of fixative is equally important as shown by trials with the following: 10% formalin; 10% formol-saline; Bouin's fluid; Carnoy's fluid; Alcoholic formalin; Acetic alcohol formalin; 10% formol-saline for \( \frac{1}{2} \) hour followed by formol-dichromate-chromate mixture (10 volumes of 5% potassium dichromate, 1 volume of 5% potassium chromate, 2 volumes of formalin and 7 volumes of distilled water); Formol-dichromate-chromate mixture; Dichromate-chromate mixture (10 volumes of 5% potassium dichromate, 1 volume of 5% potassium chromate) for \( \frac{1}{4} \) hour followed by formol-dichromate-chromate mixture; Heidenhain's Susa fluid; immersion in boiling normal saline for 2 minutes.

Satisfactory results are obtained by fixation in the first three of these fixatives. Prolongation of fixation time in formalin reduces the staining of background considerably without affecting impregnation of the argyrophile cells. Results with tissues fixed in Heidenhain's Susa fluid are almost as satisfactory as with tissues fixed in formol-
saline though somewhat longer impregnation times are required. All fixatives containing alcohol yield negative results. Fixation by heat fails to preserve the argyrophile granules. Contrary to what is often stated argyrophile cells can be demonstrated after the use of fixatives containing potassium dichromate provided formalin is also present. Of the three such procedures used best results are obtained when tissues are fixed in formol-saline for $\frac{1}{2}$ hour before being transferred to a formol-dichromate-chromate mixture. Results are less satisfactory when the tissues are directly placed in a formol-dichromate-chromate mixture, and are poor when the tissues are fixed first in dichromate-chromate solution and then transferred to formol-dichromate-chromate solution. These results indicate that it is the formalin that is responsible for such staining as is obtained after fixation in these mixtures.

(b) The iodine-sodium thiosulphate treatment:

The treatment in the iodine-sodium thiosulphate sequence is an important part of the technique. Omission of this step does not prevent impregnation of the argyrophile granules, but results in heavier staining of the background thereby reducing contrast. The definition of individual granules is also somewhat less distinct. Longer impregnation times are required especially for the second impregnation. With tissues fixed in Susa this step is essential to avoid mercuric precipitates.
(c) The silver impregnation:

The impregnating solution prepared as described above consists of a 0.002% solution of silver nitrate containing 0.1% pyridine, buffered to a pH of 8.0. This solution keeps well. It can be used only once. It is better not to include sections cut from different blocks in the same staining jar. Some tissues take up the silver sooner than others and if such slides are mixed in the same jar it is sometimes found that the more rapidly staining slides take up all the silver leaving little for the more slowly staining sections.

The repetition of the impregnation and of the development is an essential feature of the technique. A single prolonged impregnation followed by development leads to a generalised browning of the section. The argyrophile granules either do not show up at all or are brown. It is obvious that the first development produces some change in the argyrophile granules enabling the second impregnation to selectively blacken them.

The pH of the impregnating solution is not critical. Trials at pH varying from 7 to 8.4 show that the impregnation of argyrophile cells is satisfactory throughout this range. Slight differences in intensity of impregnation and definition of granules are, however, observable and a pH of 8.0 gives the best and most consistent results. Although the impregnation of the argyrophile granules is not affected, variation in pH does have a significant influence on the staining of other tissue elements and thus indirectly influences the clarity and contrast of the preparation. Raising the
pH to 8.4 intensifies background staining while lowering the pH reduces it.

The influence of pyridine is to retard impregnation of both the argyrophile cells and of the background, but not to the same degree. When pyridine is omitted the browning of nuclei is enhanced and the argyrophile cells stand out less distinctly. Doubling the pyridine content (0.2%) greatly reduces background staining; argyrophile cells stand out well, but their impregnation is somewhat less intense. Further increase in pyridine content (0.4 to 0.8%) completely abolishes background staining, but longer impregnation time is required to adequately blacken the argyrophile granules.

Doubling the concentration of silver nitrate (0.004%) causes much heavier deposition of silver, but if the impregnation times are reduced to half (i.e., ½ hour initially followed by development and further impregnation of 15 minutes to ½ hour) the results are indistinguishable from those obtained with the method described. Critical control of the degree of impregnation is, however, more difficult and there is a greater tendency for extraneous deposits to form. Further increase in the concentration of silver nitrate is undesirable as extraneous deposits become numerous.

The temperature at which impregnation is carried out is important. A variation of 5°C can cause considerable alteration in impregnation time required. The temperature should not exceed 60°C and should not be below 55°C.

The various factors on which the intensity of impregnation depends
may be summarised as follows:

1. Length of time of fixation of the tissue;
2. The pH of the impregnating solution;
3. The pyridine content of the impregnating solution;
4. The silver nitrate content of the impregnating solution;
5. The impregnation time; and
6. The temperature at which the impregnation is carried out.

With most tissues the staining procedure described gives excellent results. The impregnation time alone needs to be controlled. A heavier staining of background is achieved by prolongation of impregnation by a few minutes. With some tissues background staining may be too heavy by the time the argyrophile granules are satisfactorily and uniformly blackened. This is specially apt to occur in tissues fixed for short periods. In such cases an increase in pyridine content may be desirable.

(d) The development:

The time required for development varies with the room temperature. At room temperatures over 30°C one dip in the developer with slight stirring is adequate; two minutes should be ample under most conditions. Slides should be left in the developer till no further browning of sections occurs. Leaving them longer does no harm.

As stated earlier (pages 14, 15) it is possible to stain sections initially by an argentaffin or equivalent method (e.g., Gomori-hexamine-silver; Masson-Hemperl; Schmorl; Diazonium), to bleach them if necessary and then to restain them by the Bodian method.
allowing a successive study of cells on one and the same section by both an argentaffin and argyrophile method. The present method has been successfully substituted for the Bodian method in this procedure, and has been found to be distinctly preferable to the latter for such studies.

Results obtained by the method described are at least as good, if not better than, the best results of the Bodian method. The results are more consistent and reproducible and more easily controlled. Beginning with mounted paraffin sections the preparations are ready for examination in two hours or less. The technique dispenses with the use of silver proteinate and of gold chloride and is thus much cheaper to use.