SOME PRACTICAL OBSERVATIONS ON THE BIELSCHOWSKY – GROS IMPREGNATION METHOD

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During research into the peripheral innervation of organs we have succeeded in introducing a number of modifications into the Bielschowsky-Gros silver method, which we mainly used. This paper is devoted to a description of these modifications, which enable some of the inherent faults of this method to be corrected.

1). Obtaining, fixing and preservation of the material. For impregnation it is best to use fresh material obtained soon after death, but if human tissue is to be examined this can be done during the first 48 hours provided that proper conditions of preservation are provided. Specimens of organs (or tissues) taken for examination must be well rinsed free of blood and from water-soluble proteins. This is best achieved by washing in a 5% solution of magnesium sulfate for 5-40 minutes, depending on the thickness and firmness of the specimen. After being washed in this solution, the material is fixed in 15-20% neutral formalin. Fixation continues for not less than 7 days. The material may be preserved in neutral formalin for months without losing its ability to undergo impregnation, especially if 10-25 g of chalk is placed in the bottom of the vessel containing the neutral formalin.

If after prolonged keeping the quality of impregnation of the specimens deteriorates, they may be stained again, but this time a 20% solution of magnesium sulfate must be used, and the specimens must be placed in freshly prepared 12% neutral formalin.

The superficial layers of the fixed material may impregnate poorly, and so it is best to cut them away before impregnation. When the superficial layers are themselves of interest to the investigation and may not therefore be removed, the material is fixed in two stages: at first for 2-3 days in a 6% solution of neutral formalin, and for the remainder of the time in 15% formalin. The neutral formalin is prepared by standing on purified chalk, on magnesium carbonate or calcined magnesia, or by titration with caustic alkali.

2). Impregnation. Immediately before sections are cut, the pieces of fixed material are washed (for a sample 1 cm^3) in tap water for 3-5 minutes, after which they are transferred to a Petri dish with distilled water, and quickly cut on a freezing microtome. The sections are collected in the same dish, without changing the distilled water in it, so that traces of formalin remain in the sections. This is one of the essential conditions for successful impregnation.

In case of unsuccessful impregnation, the sections or pieces of tissue which have been washed excessively free from formalin may be replaced for 1-3 hours or overnight in 5% neutral formalin. Before impregnation such material is washed for 2-5 minutes in the same sample of distilled water.

The washed sections are placed in a 10% solution of silver nitrate for 5-30 minutes until they acquire a yellowish color. When they are taken out of the distilled water they are lightly dried by wrapping in filter paper.

After their first treatment with silver nitrate solution, the sections are passed through the same sample of 20% acid formalin, which it is advisable to heat to 25-30°C. This operation is carried out as quickly as possible (not longer than 5 seconds). The sections are placed on a glass rod and immersed in formalin (in one dish) to wash each section on all sides with the solution, and they are then just as quickly and carefully dried free from formalin between layers of filter paper. Light expression of the formalin by pressure on the filter paper may bring about removal of formalin from the sections. If the nature of the material does not permit the use of this method of removal of formalin from the sections, they may rapidly be taken through distilled water. Freeing the impregnated sections from formalin by one of the methods indicated is essential, otherwise they will momentarily blacken in the ammoniacal silver solution, which is one of the main causes of failure of impregnation.

After the sections have been freed from formalin they are placed in the ammoniacal silver, prepared according to Gros's formula. The sections may be kept in the ammoniacal silver solution without harm to the subsequent treatment for 5 minutes to 1-2 hours. After immersion in the solution, the sections rapidly acquire a brownish hue. In the first place test sections must be stained; if these become brown in patches, then 2-5 drops of ammonia must be added to the ammoniacal silver solution.

The metallic silver in the nerve cells is readily reduced in boiled and cooled water (15-25 ml) with the addition of 1-2 drops of 20% acid formalin. It is best to take these 1-2 drops of formalin not from pure (unused) formalin, but from the dish in which the sections were immersed after the first silver bath. Impregnation proceeds evenly in this weak formalin solution; the sections acquire a brown tone with an orange hue. If impregnation proceeds too quickly or too slowly it is necessary to vary the number of drops of formalin added to the water; where impregnation is extremely rapid, 1 drop of formalin per Petri dish (15-25 ml) of boiled water is sufficient, and when it is slow, drops of formalin may be added up to a volume of 0.1-1 ml. As a control, isolated sections are taken out of the dish at different times, and placed on a glass slide for examination under the microscope. On the appearance of a clear pattern of nerve cells (which takes place quite quickly), the reduction must be brought to an end without delay and the sections are transferred to cooled, boiled water. Should the sections continue to darken, glacial acetic acid is added to the water at the rate of 1 drop per 25 ml. After washing (for 1-2 hours) in acidified water, the sections are rinsed in distilled water and taken up through alcohol for clearing and mounting in balsam.

If the sections have been kept too long in the reducing formalin solution, so that they have acquired a dark brown tone, they may be placed in a 1% solution of ammonia and allowed to remain there until lighter tones have been produced (but not for longer then 1 second), after which they are quickly washed in distilled water and transferred to alcohol.

If impregnation is unsuccessful on account of an inadequate length of stay of the sections in the silver solutions, and they appear pale yellow in tone with no clear pattern of the nerve cells, these sections may be placed in distilled water for a few minutes and then transferred to the first (10%) solution of silver nitrate, after which the procedure is repeated as described above.

When the sections are to be counterstained with aniline dyes, gold impregnation is required as a preliminary step. The bath for impregnation with gold is prepared as follows. To 10-20 ml of distilled water is added 5-10 drops of 1% gold chloride, and at the bottom of the dish is added 2-3 g of purified chalk; this is stirred with a glass rod, the particles of chalk allowed to settle, and the purified gold chloride solution is then poured into a clean dish. The sections are placed in this dish after they have been washed for a few seconds in distilled water until they turn grey, and they are then quickly transferred to 3% hyposulfite (chemically pure) for 5-10 minutes. After the sections have been thoroughly rinsed free from hyposulfite they are counterstained with aniline dyes, dehydrated with alcohol, cleared and mounted in balsam. If no chalk is used, the gold chloride causes severe decolorization of the sections, so that the pattern of the neurofibrils may completely disappear.

Our modification of the Bielschowsky -Gros method is simpler and more convenient. It is possible to stain a large number of sections (hundreds) simultaneously, thereby resulting in considerable economy of time and of expensive chemicals. Furthermore serial treatment of the material is possible, for all the sections without exception are impregnated to the same desired color — from dark yellow to dark brown; under these circumstances the neurofibrillar apparatus of the nerve formations acquires a black tone.

SUMMARY

The author suggests a simplified modification of the method of impregnation for staining the peripheral nervous system and the nerve endings. The tecnique of impregnation is as follows: the specimen is washed in magnesium sulphate solution for 5 to 40 minutes; it is fixed and kept in 15% neutral formalin; then it is washed and sections are prepared; next the sections are impregnated by 10% $AgNO_3$ and are transferred into 20% acid formalin and then into distilled water (second) and are thouroughly dried between pieces of filter paper (important), then it is passed into 10% $AgNO_3$ where it is kept for 5-10 minutes; metallic silver is reduced (under microscopic control) in cups with boiled and cooled water with an addition of 1 to 5 or more drops of 20% acid formalin (a test is to be made); then it is rinsed in distilled water and dehydrated in alcohol of increasing concentration after which the sections are embedded in the fir tree balsam. Sections may be stained with gold (before dehydration in alcohols). 1% gold chloride should be mixed with pure calcium chloride (3:1) and 1-4 drops of this solution per cup of distilled water should be taken for gold impregnation of the preparation, then it may be additionally stained by 5% hyposulphite (not necessarily).