SILVER IMPREGNATION OF RETICULUM IN PARAFFIN SECTIONS *

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Since Maresch first used the Bielschowsky silver stain for the demonstration of connective tissue fibrils, and especially since its results in paraffin sections proved to be at least as reliable if not better than those obtainable with frozen material, the Bielschowsky-Maresch silver impregnation has become one of the most widely used special staining methods. Although many connective tissue stains of different types have been devised since, silver impregnation still ranks first because of its sharp delineation of the finest fibrils. Like all important methods it too has many modifications, each claiming some superiority to the original technique. As I was unable to find data in the literature concerning the relative merits and reliability of the various modifications, and, secondly, as the rôle and importance of the individual steps of the impregnation process are almost unknown, I decided to investigate this problem systematically. For the time being I used formalin-fixed material only, embedded in paraffin, or in celloidin-paraffin according to the rapid method of Erös. It is my plan also, however, to extend further my investigations concerning the action of the fixatives. My results to date are as follows: First, I was impressed by the occasional high degree of similarity, almost identity, of the results obtained by methods seemingly most dissimilar. On the other hand, there is no known method, the results of which would be as reliable and constant as those of, for instance, the hematoxylin nuclear stain, all silver methods being liable to yield more or less variable pictures. Even unexplainable complete failures are by no means rare. There is, however, a great difference in the reliability index of the various methods, some of them being notoriously prone to complete failure, and even in the event of success yielding pictures of variable quality; whereas with other methods failure is most exceptional and the results are remarkably uniform. A second essential difference between the various methods lies in the different amount of reticular meshwork demon-

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strated. Some methods almost invariably reveal a decidedly smaller number of fibrils than are demonstrated by other methods. If one happens to be accustomed to use routinely one of the methods demonstrating a relatively sparsely woven reticulum, he may be convinced, by lack of comparison, of the effectiveness of his method and quite unaware of the fact that other methods demonstrate a much richer fibrillar structure than the method he adopted. He probably would be surprised if informed that he never had seen a really complete fibrillar picture.

The successive steps of the silver impregnation method are discussed below and it is hoped that the information given will be of value to the various workers interested in the demonstration and study of reticulum.

1. *Oxidation-Reduction:* Oxidation of the sections with potassium permanganate, followed by reduction with oxalic or hydrobromic acid, or, according to my experience, even better with acid potassium sulphite (so-called metabisulphite), is an essential part of the procedure, greatly reducing the number of failures and ensuring more uniform results. Wilder suggests the use of a 10 per cent phosphomolybdic acid solution instead. I, too, obtained many beautiful preparations with this latter method, but its reliability being decidedly inferior to that of the permanganate treatment, the routine use of the latter is more recommendable. The undesirable action of the permanganate treatment, consisting of loosening of the sections on the slide and often their actual floating away in the further course of impregnation, can easily be obviated by a suitable technique to be described later. According to Foot, the permanganate is liable to impair nuclear staining; therefore he suggests the use of a pyridine-glycerin mixture instead. I have never observed impairment of nuclear staining caused by permanganate treatment. Moreover, I found the effect of the pyridine-glycerin mixture to be much inferior to that of permanganate oxidation and by no means suitable to replace it. The mechanism of action of the permanganate solution is not well understood. That it is not due to mere oxidation has been proved by Foot. He was unable to obtain the same effect with any other oxidizing agent. I repeated his experiments and tested a few additional substances, such as sodium perborate, ammonium persulphate, chromic acid and a compound solution of iodine. The results were

identical. I also concur in Foot's finding that both manganese salts and oxalic acid in themselves are inert.

2. *Sensitization:* I wish to apply this term to any treatment of the section with metal salts or other substances preceding the use of the ammoniacal silver solution. Suitable sensitization also is an important step, causing the fibrillar structure to become more completely visualized. Tannic acid and certain metal salts are sensitizing agents. The tannic acid treatment seems to be inferior to that of the metal salts as it is less reliable and the ground substance is likely to become dark brown, greatly impairing the clarity of the picture. Hence, metal salts are to be preferred. I assayed the salts of the following metals: aluminum, silver (used in the original method of Bielschowsky), gold, cadmium, chromium, cobalt, copper, iron (ferrous and ferric), mercury, magnesium, manganese, nickel, lead, stibium, tin, uranium (suggested by Wilder) and zinc. Marked and uniform sensitization was obtained with silver, gold, cadmium, ferric, lead, tin and uranium salts. The richest reticulum is obtained with iron sensitization. The sensitizing action of the other metals is either nil, slight, inconstant, or not selective. Gold, lead and tin salts often cause undesirable precipitates, though otherwise the reticular structure is excellent. There remain silver, cadmium, ferric and uranium compounds. As mentioned, silver sensitization is a part of the original Bielschowsky method and of certain of its modifications. Originally the use of a 2 per cent solution of silver nitrate for 24 to 48 hours was recommended. I found that exactly the same effect can be obtained within 2 minutes if a 10 per cent solution is used. In this way time can be saved. The original method warns against a more than superficial rinsing of the sections in distilled water after sensitization, as longer rinsing is liable to weaken the stain. This I cannot confirm. On the contrary, I strongly advise thorough washing of the sections in several changes of distilled water. This slight modification will result in distinctly clearer pictures, better nuclear staining, and complete absence of precipitates. The same technique applies to all other metal sensitizations (cadmium, uranium, and especially iron). I used the nitrates of cadmium and uranium in 1 to 2 per cent solutions. The duration of sensitization is about 1 minute; longer exposure does not enhance the effect nor does the combined use of several metal salts. The ferric com-

pound I used was iron ammonium sulphate, the same substance used in Heidenhain's iron hematoxylin stain. I employed freshly prepared 1 to 2 per cent solutions. The time of exposure should be 1 minute. The peculiarity of iron sensitization is the brilliant metachromasia obtained on gold toning.

3. *Silver Impregnation:* Most modifications concern the preparation of the ammoniacal silver solution. In general, three different types of solutions are used:

1. From silver nitrate silver hydroxide is precipitated with sodium or potassium hydroxide and the precipitate is dissolved in ammonia.

2. From silver nitrate silver carbonate is precipitated by some soluble carbonate and the precipitate is dissolved in ammonia.

3. To the silver nitrate solution ammonia is added drop by drop, until the precipitate which forms on addition of the first few drops is again dissolved.

There are many formulas for the preparation of the solutions, some of which are characterized by almost extravagant accuracy — for instance, the formulas of Kubie and Davidson. I started my experiments with solutions of the Type 1 (silver-ammonia hydroxide). I varied the amount of added alkali from one-half to three volumes. The only difference I noticed was the proportionately quicker action of the more alkaline solutions. However, the final results obtained with the different solutions were extremely similar, indeed identical to such extent that I would have been unable to distinguish the sections stained with the different solutions had I not marked them beforehand. Therefore, in my opinion, too great accuracy in preparing the ammoniacal silver solution is entirely superfluous. Of course, it is better not to use too strongly alkaline solutions as they are liable to damage the sections. Solutions prepared with one-half to three-fourths equivalent amount of alkali are the most suitable, *i.e.* to one volume of 10 per cent silver nitrate solution one-sixth to one-fourth volume of a 10 per cent solution of potassium hydroxide is added. The same applies to carbonate solutions. The amount of ammonia seems to be more important. According to most formulas even a slight excess of ammonia is likely to produce inferior results, especially if the hydroxide type of solution is used. According to my experience there is a certain optimal amount of ammonia; both more or less will produce unsatisfactory results. If there is an excess

of ammonia the picture will be very sharp and distinct, but a part of the fibers will escape impregnation; whereas if too little ammonia is used the ground substance will be dark and the picture blurred. There are different methods for securing the right amount of ammonia, the simplest of which are the following: to the precipitate ammonia is added drop by drop, while the container is continuously shaken, until the last grains are just dissolved and then either (1) silver nitrate is again added cautiously until it is easily dissolved on stirring the solution, or (2) the vessel containing the solution is placed in hot water until black silver precipitate begins to form on its surface. Solutions prepared in either way can be used for 2 or 3 days if kept in stoppered bottles. The silver precipitate that collects on the bottom of the bottle does not interfere with the staining capacity of the solution. Solutions of the carbonate type and those prepared with ammonia only, keep well for at least 5 to 6 days. Solutions of the hydroxide type are to be diluted with distilled water to twice their volume and used at room temperature. The time of exposure is about 1 to 3 minutes. If cadmium, iron or uranium sensitization is used, 1 minute will suffice and the sections will show almost no change in color; whereas if silver sensitization is used it is better to prolong impregnation to 3 minutes, until the sections become pale tobacco brown. Solutions of Types 1 and 2 stain only at higher temperatures (37 to 50° C.) and should be diluted to 4 to 5 times their volume before use. When comparing the different solutions I found that those of the hydroxide type are the most reliable and yield the most uniform results; whereas with the carbonate solution and with the solution prepared with ammonia only, failures are not uncommon. However, in the case of success the silver carbonate stain excels in producing absolutely even, delicately shaded pictures, free of precipitate. All solutions stain the cells also. At times excellent nuclear staining is obtained, on other occasions the cytoplasm will be stained. Very often different parts of the same section show different cellular staining. The cause of this phenomenon is unknown. In general, with cadmium and uranium sensitization the chromatin pattern is more distinct than if iron or silver is used. After impregnation the sections are washed in distilled water for 5 to 10 seconds. Longer exposure to distilled water weakens the stain.

4. *Reduction:* Formalin is used for this purpose, the concentra-

tion of which within wide limits does not appreciably influence the result. In contrast with the findings of Foot I found the reaction of formalin unimportant. Simple commercial formalin, neutralized, slightly alkalinized or acidulated solutions, gave identical results. The duration of reduction should be at least 3 minutes. After reduction the sections are washed in running water.

5. *Gold Toning:* Successful toning produces beautiful shades. The reticulum is dark black, collagen fibers are rose to brick red, nuclei rusty brown to deep red. Unfortunately, it is not always possible to produce this range of shades. The cause of occasional failures is unknown. However, there are several factors decidedly enhancing metachromasia. These are iron sensitization, prolongation of gold toning to at least 10 minutes, and finally the reduction of the toning with oxalic acid (according to Laidlaw), or even better with potassium metabisulphite. The action of the latter compound is instantaneous. By employing this combination failures can be prevented with almost absolute certainty.

6. *Fixation:* Fix in a 1 to 2 per cent solution of sodium thiosulphate (hyposulphite) for 1 minute. Longer fixation will impair the distinctness of the finest fibers. After fixation the sections are thoroughly washed in running water, then treated with 2 changes of alcohol, cleared with xylol and mounted in balsam. Foot suggested counterstaining of the sections with hematoxylin and picro-fuchsin. In my opinion this counterstaining is unnecessary; moreover, the fact that after the van Gieson stain it is often impossible to determine whether certain fibers are stained by fuchsin or by gold, outweighs its possible advantages.

In summarizing my results, I may say that all methods omitting either permanganate oxidation or sensitization, or both, are decidedly unreliable and the reticulum picture they yield is, even in the case of success, incomplete. Far the best sensitizing agent I have tried is iron ammonium sulphate.

I wish now to describe my own modification of the Bielschowsky-Maresch reticulum impregnation which gave complete satisfaction in a series of several hundreds of sections. The only material I had failures with is bone marrow, especially the fatty type, whereas highly cellular marrow, as seen in leukemia and in some cases of pernicious anemia, gave beautiful pictures. The poor impregnability of bone marrow is well known to all who have

tried to study its reticulum by means of silver impregnation, and it is mentioned also by Orsós. After having tried all methods described I am convinced that no method is certain of reliability in this respect.

My modification is as follows:

Run paraffin sections through xylol, then 2 changes of alcohol and wash under the tap.

1. Oxidize with a 0.5 to 1 per cent solution of potassium permanganate for 1 to 2 minutes. Rinse in tap water.

2. Decolorize with a 1 to 3 per cent solution of potassium metabisulphite for 1 minute. Wash under the tap for several minutes.

3. Sensitize in a 2 per cent solution of iron ammonium sulphate (violet crystals) in distilled water for 1 minute. Wash under the tap for a few minutes, then run through 2 changes of distilled water.

4. Impregnate with the following solution for 1 minute:

To a 10 per cent silver nitrate solution add one-sixth to one-fourth its volume of a 10 per cent solution of potassium hydroxide. Add strong ammonia water drop by drop, while shaking the container continuously, until the precipitate is completely dissolved. Add again, cautiously, silver nitrate solution drop by drop until the resulting precipitate easily disappears on shaking the solution. Make up the solution with distilled water to twice its volume. It can be kept in a stoppered bottle for 2 days.

5. Rinse quickly in distilled water for 5 to 10 seconds.

6. Reduce for 3 minutes in commercial formalin diluted with tap water to 5 to 10 times its volume. Wash under the tap for a few minutes.

7. Tone in a 0.1 to 0.2 per cent solution of gold chloride for 10 minutes. Rinse in distilled water.

8. Reduce toning in a 1 to 3 per cent solution of potassium metabisulphite for 1 minute.

9. Fix in a 1 to 2 per cent solution of sodium thiosulphate (hyposulphite) for 1 minute.

Wash under the tap. Run through alcohol of increasing percentages. Clear in xylol and mount in balsam.

As mentioned before, paraffin sections occasionally will float away during impregnation with the strongly alkaline silver solu-

tion. This annoyance can be easily prevented by affixing the sections to the slide with gelatin instead of egg albumin-glycerin. The gelatin must be subsequently hardened by formalin fumes. The method is as follows: Dilute the glycerin-gelatin mixture commonly used for fluid preservation of sections with water or glycerin until it remains fluid at room temperature. Spread a thin layer of this solution on the slide and affix sections. Dry the slides in the incubator at 37° C. in formalin fumes for at least 10 hours. (Pour commercial concentrated formalin into an open Petri dish and place it in the incubator.) The formalin has to be removed from the sections as even traces of it will inhibit impregnation. This is easily accomplished by exposing the slides in a similar manner to the action of ammonia vapor for several hours.

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DESCRIPTION OF PLATES

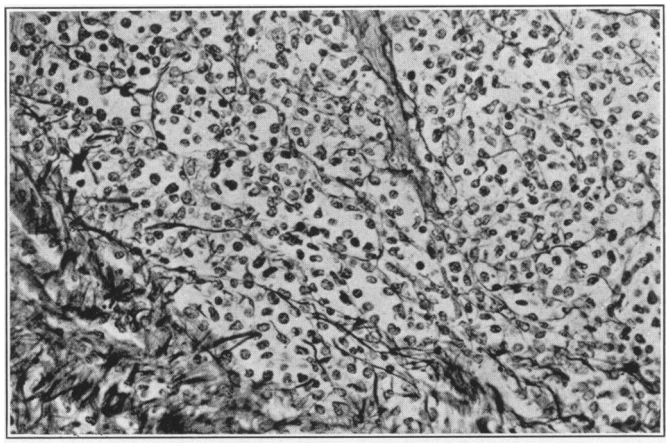
Microphotographs of Figs. 1 to 5 show corresponding fields of serial sections of a block from a case of subcutaneous round cell sarcoma. Figures 6 to 9 show corresponding fields of serial sections from a leukemic spleen. (The distention of the vascular spaces is artificial and was produced by the injection of formalin solution into the splenic vessels.) All microphotographs have been made under strictly identical optical conditions.

PLATE 146

FIG. 1. Foot's stain, Variant II.

FIG. 2. Silver sensitization (author's modification).

FIG. 3. Uranium sensitization (method of Wilder).



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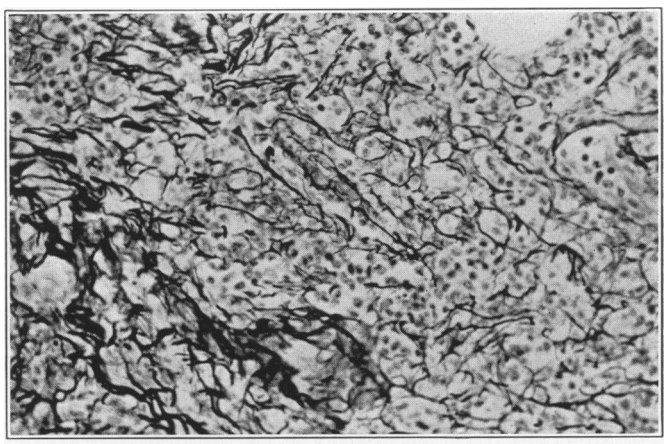

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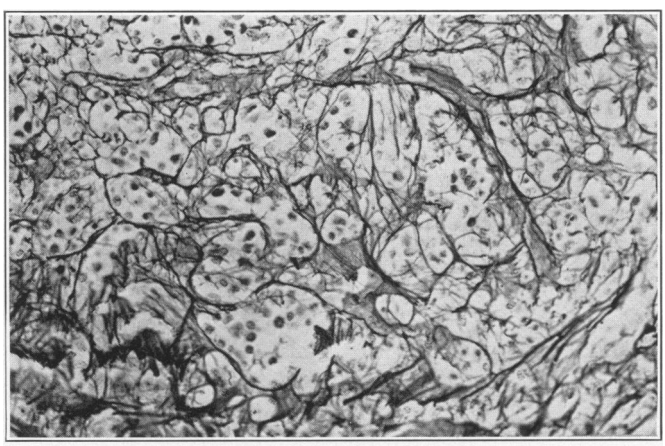

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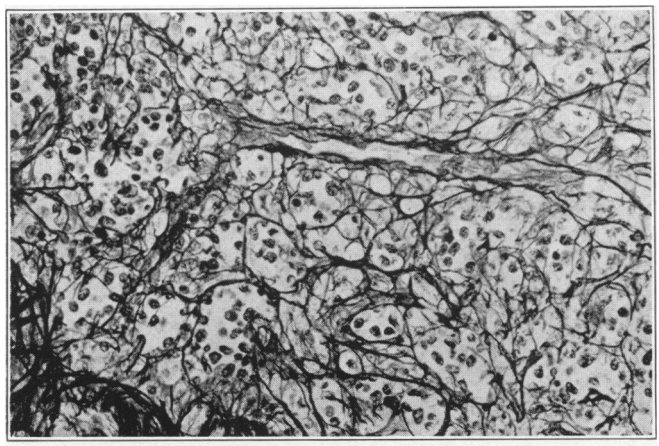
Gömöri Silver Impregnation of Reticulum

PLATE 147

FIG. 4. Cadmium sensitization.

FIG. 5. Iron sensitization.

FIG. 6. Foot's stain, Variant II.



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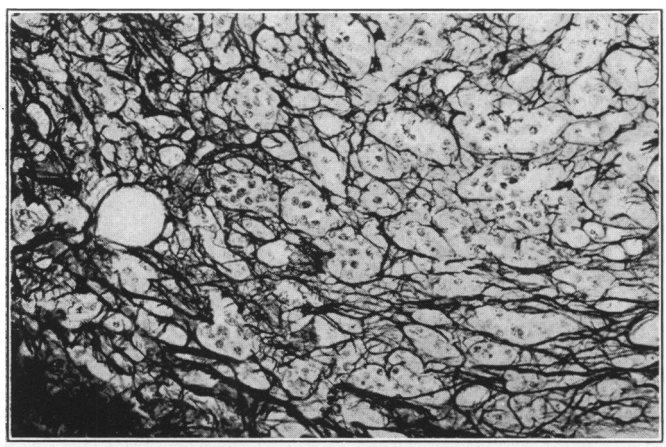

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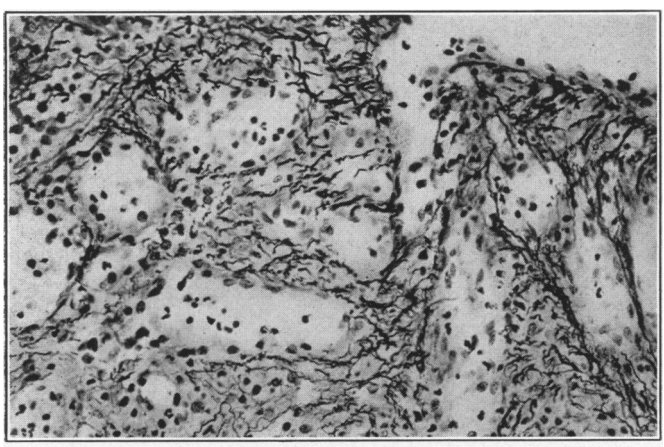


6

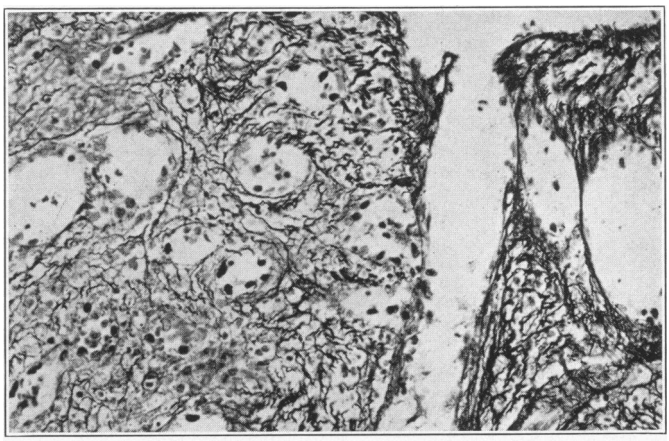
Gömöri Silver Impregnation of Reticulum

PLATE 148

FIG. 7. Uranium sensitization (method of Wilder).

FIG. 8. Cadmium sensitization.

FIG. 9. Iron sensitization.



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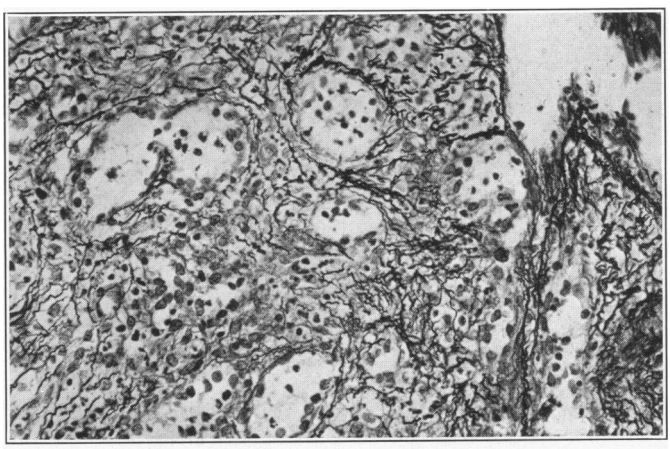

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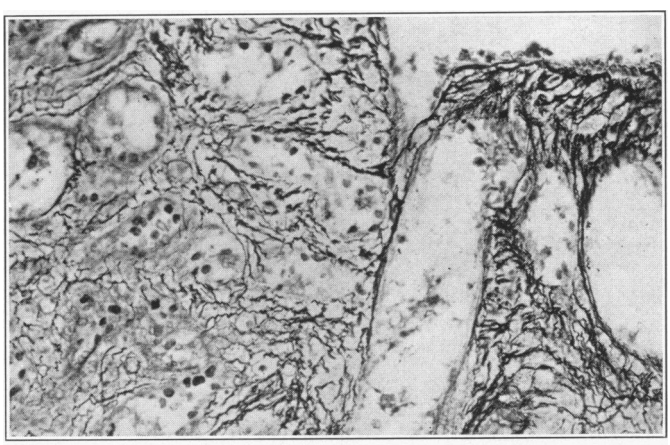

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Gömöri Silver Impregnation of Reticulum