SILVER STAINING OF THE SKIN AND OF ITS TUMORS *

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Long known as a capricious staining substance, silver does not always deserve this reputation. Provided that certain conditions are fulfilled, silver staining becomes as simple and as certain as the staining of nuclei with hematoxylin; at least, this is true of the peripheral nervous system, of the skin, and of the argyrophil † reticula generally.

The conditions are: (1) suitable fixation; (2) suitable mordanting; (3) the use of silver solutions ten to twenty times stronger than those in vogue.

The fixative determines the result of the stain. When the silver worker has grasped this principle, half of his difficulties disappear. Differential silver staining might well be called differential fixation, for a given silver stain will produce quite different effects according to the fixative employed. This paper will deal with the application of these principles to the skin and to tumors of the skin, the fixatives selected being Bouin's fluid and neutral formol. The resulting preparations will be described separately, for these fixatives are not interchangeable.

BOUIN FIXATION

Figure 1 illustrates a paraffin section of normal skin that has been (1) fixed in Bouin's fluid; (2) mordanted with the Mallory bleach; (3) stained with del Río-Hortega's lithium silver made ten times stronger than the original formula.

The epithelial cells hold the stain, not only the surface epithelia but also those of the hair follicles, the sebaceous glands and the sweat glands. The staining of the epithelia is peculiar in that only the cytoplasm is colored; the nuclei are colorless. This is a constant feature of Bouin-fixed tissue, both normal and pathological.

There is a curious exception to the positive staining of the epithelia. The basal layer does not stain; it looks as if the epithelium had

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† Schiefferdecker's popular word *argentophil* is a hybrid. Professor Schaffer points out that the correct form is *argyrophil*, which he attributes to Merk, 1899.

separated from the corium, but counterstains show that the basal cells are in place. Unna noted the same thing in his permanganate stain for reduction points.

On examination of the corium beneath the epithelia, in the actual section the most striking feature is the reddish purple collagen with its fine graining, indicating the ultimate fibrillae. Here and there, traced in black, are the delicate reticula of the arrector pili muscles, of the walls of the vessels, of the sheaths of the sweat glands and of the nerve fibers. Only the high magnification of an immersion lens brings out the full beauty of these silver collagen stains, presenting the fine lines and sharp contrasts of an engraving.

Our first conclusion is that we have here a good collagen-reticulum stain. In truth, it is one of the shortest, simplest and most constant; but it is more than this. In contrast to the prominent epithelia, note that every cell native to the corium has vanished; fibroblasts, mast cells, endothelia of the vessels, smooth muscle cells of the vessels and of the arrector pili muscles, all are gone. When we recall that the epithelia are ectodermic and that the vanished cells of the corium are of mesodermic origin, we begin to perceive the selective possibilities of this technique.

Tumors

It is interesting to observe this differential staining of ectodermic and mesodermic cells continued in their progeny as they form tumors in the skin. The daughter cells are true to type. In tumors of mesodermic origin, fibromata, angiomata, lipomata and myomata, all cells disappear, leaving the reticula clearly outlined in black. In ectodermic tumors, adenoma, papilloma and epithelioma, the epithelial cytoplasm holds the stain, while the mesodermic cells of the interstitial tissue disappear; that is, the descendants of the fibroblasts disappear, lymphocytes remain, as will be related under sarcoma.

The sarcomas present a more complex problem, which will be discussed in a future paper. Still, workers wishing to experiment with this technique may be interested in our tentative conclusions in this none too well illuminated subject. Sarcoma, of course, is a mesodermic tumor, and one would say that if this technique is truly selective the sarcoma cells should be silver-negative. In the embryo, however, the mesodermic cells differentiate in two directions. One

line forms the framework of the body; these cells descend as fibroblasts, osteoblasts, vessel endothelia and, eventually, fibroblastic sarcoma and osteogenic sarcoma. With Bouin fixation, this lineage, from the simple fibroblast to the fibroblastic and osteogenic sarcoma, is silver-negative. In the other line, the mesodermic cells differentiate into blood- and lymph-forming tissues, red cells, bone marrow, spleen, lymphatic tissues, lymphocytes and leucocytes, continuing under favoring conditions as lymphosarcoma and sarcoma of bone marrow. In Bouin-fixed tissue, this lineage, from beginning to end, is silver-positive.

Holding rigorously to the principle that function determines structure, Menetrier long ago divided the sarcomas into these two groups. It is curious to see how this selective silver technique duplicates his functional grouping in every detail.

Of the Rous chicken sarcomas thus far examined, the cells are silver-positive, agreeing with Carrel's observation that they spring from macrophages and not from fibroblasts.

In sarcomas of every kind, giant cells are positive, as they are in tuberculosis, in nevi and wherever found.

Nerves and Nerve Tumors

The nerves of the skin present a special problem. With this technique, the axis cylinders, the neurokeratin of the myelin sheath, and the Schwann cells are constantly negative. This picture does not harmonize with Harrison's well known experiment which seems to prove that the Schwann cells are derived from the ectodermic neural crest. We may recall, on the one hand, that the basal cells of the epidermis are silver-negative and, on the other hand, that more than one keen observer has asserted the mesodermic origin of the Schwann cells (quoted by Cajal, page 203). The question cannot yet be regarded as settled beyond dispute.

On the peripheral nerves there occurs a puzzling tumor, known in this country as perineurial fibroblastoma, sometimes as neurosarcoma, and held to be derived from the fibroblasts of the nerve sheaths. Most European pathologists call it Schwannoma or peripheral glioma and derive it from the Schwann cells, the "peripheral neuroglia." The cells of this tumor are silver-negative, not throwing much light on their disputed origin; for both the Schwann cells and the fibroblasts are silver-negative also.

Formol Fixation

Figure 2 shows a paraffin section of the same skin fixed in 10 per cent neutral formol. Bearing in mind our maxim that the fixative determines the result of the stain, we should expect this fixative to produce a different result from Bouin; and so it does. As with Bouin fixation, the epithelia take the stain; but, in formol fixation, it is the nuclei that are black, while the cytoplasm is colorless. This epithelial nuclear selection is constant in formol-fixed tissue, both normal and pathological, from all parts of the body.

On examination of the corium, we seem at first sight to have the same picture as with Bouin, the mesodermic cells having vanished. On closer examination, we see that formol fixation makes a further differentiation in the corium. The endothelia of many of the vessels, the smooth muscle cells of the vessels and of the arrector pili muscles hold the stain, or, at least, their nuclei do, following the rule of formol fixation. This curious differentiation in the corium is frequent but not constant.

Tumors fixed in formol follow the general lines of Bouin fixation in differentiating between epithelia and mesoblastic cells and between structural and blood-forming mesoderm; but the results are not so constant as with Bouin fixation. The question merits further study.

Discussion

Of all the sharp eyes that have studied silver preparations, those of the Spanish master, del Río-Hortega, seem to have been the only ones to detect the differential staining of the epithelia and the connective tissue cells. In his paper on various methods for staining the connective tissue reticulum, using formol fixation, occurs this phrase:

"Los protoplasmas de las células epiteliales (especialmente epidérmicas), del hígado, suprarrenal, riñon, células cianófilas, etc., muéstranse difusamente teñidos, contrastando su tinción con la incolorabilidad de los elementos conjuntivos normales." The illustrious scientist of Madrid does not seem to have pursued the matter further.

The silver negativity of the basal epithelia and of the Schwann cells and the division of the mesenchyme into silver-positive and silver-negative lines show that the principle of embryonic origins

cannot be applied too strictly. Function rather than embryological origin may determine the reaction; in fact, if we exclude collagen, a feeble reducer, the reaction of the cells to silver parallels the reduction staining so thoroughly studied by Unna. Whatever the explanation, it seems that when, in the course of its development, a cell has acquired a positive or negative silver relation, its retention of that quality through many pathological vicissitudes enables us to recognize that cell when found far from its natural habitat and in activities that seem foreign to its nature.

Technique

1. Fix in Bouin for 3 days or in 10 per cent neutral formol for 3 days. Old formol material may be improved by immersion in fresh neutral formol for 3 days. Formol-fixed tissue immersed in Bouin for 3 days will give nearly perfect but not quite perfect Bouin pictures; a positive endothelial or smooth muscle nucleus here and there betrays the original formol fixation.

2. Embed in paraffin or celloidin; or make frozen sections.

3. Stick paraffin sections on the slide with Masson's gelatin glue and harden in hot formol fumes; sections so treated seldom float off.

4. After removal of the paraffin, wash Bouin sections in running water for 20 minutes to remove the picric acid; wash formol sections for 5 minutes.

5. Mordant with the Mallory bleach. Tissue recently fixed in formol (2 to 10 days) often gives the reaction without mordanting, but not constantly.

(*a*) 1 per cent tincture of iodin, 3 minutes; rinse in tap water.

(*b*) 5 per cent hypo (sodium thiosulphate), 3 minutes; rinse in tap water.

(*c*) ¼ per cent potassium permanganate, 3 minutes; rinse in tap water.

(*d*) 5 per cent oxalic acid, 3 minutes; wash well in running water for 10 minutes.

6. Distilled water; change 3 times within 5 or 10 minutes to ensure clean slides entering the silver solution.

7. Río-Hortega's lithium silver augmented to 10 per cent. Heat the stock solution in the oven to 50° C and stain in the oven

for 5 minutes. We filter the used solution into a glass-stoppered bottle and use it a dozen times or more.

8. Rinse the slides by pouring distilled water over both sides.

9. Formol, 1 per cent in tap water; flood the sections frequently for 3 minutes.

10. Rinse both sides of the slide with distilled water.

11. Yellow gold chlorid, 1 to 500 of distilled water in a Coplin jar; immerse the slides at room temperature for 10 minutes. The gold solution may be used many times.

12. Rinse both sides of the slide with distilled water.

13. Oxalic acid 5 per cent; pour on slide and leave for 10 minutes.

14. Rinse with distilled water.

15. Hypo, 5 per cent; pour on slide. Change as often as it becomes turbid for 10 minutes.

16. Wash well in running water. Counterstain if desired and mount in balsam.

The times advised are those that have given us the clearest pictures in the shortest time, but this is not a finical technique; the time of the different steps may vary widely without affecting the result. The Mallory bleach solutions and the formol may be left on the slides for an hour or more without harm. Even the silver solution may vary in strength from 2 per cent to 15 per cent, the time in silver from 2 to 10 minutes and the temperature of the silver solution from room temperature (20 minutes) to 60° C; though the longer times and the higher temperatures are likely to give precipitates and dirty slides. After the formol, the slides may be left overnight in gold or in oxalic acid; and at any point in the technique the slides may be left overnight or over Sunday in distilled water or in tap water. In fact, some of us believe that counterstaining is better if the slides are left in tap water overnight between the hypo and the counterstain.

Historical Note

The use of potassium permanganate followed by a reducer entered histological practice in 1885, when Lustgarten borrowed it from the dyeing industry. In the next year Pal adopted it in his modification of Weigert's myelin stain, changing the reducer from sulphurous acid to a mixture of oxalic acid and potassium sulphite.

In both instances the reaction was used after staining to differentiate overstained tissue.

The use of permanganate and oxalic acid as a mordant before staining begins with Weigert's neuroglia and fibrin techniques in 1895. Against much opposition, Weigert remained convinced of the necessity of "oxidation and reduction" as a preparation for staining neuroglia and all other tissue difficult to stain. In 1898, Rosenthal applied the new Weigert neuroglia technique to a Zenker-fixed tumor and necessarily preceded the bleach with iodin-alcohol extraction of the mercury. In 1900, Mallory included the permanganate-oxalic acid mordant in his phosphotungstic acid neuroglia stain. In 1908, he changed the fixative to Zenker and established the bleach as it has been used ever since in American laboratories to prepare Zenker sections for staining.

All silver stains for reticulum go back to del Río-Hortega, Maresch and Bielschowsky; but mordanting with permanganate and oxalic acid was not part of the original technique. It begins with Perdreau, in 1921, who mordanted formol sections with permanganate and Pal's decolorizer. In 1924, Foot advised the full Mallory bleach for paraffin sections of Zenker material. In 1925, del Río-Hortega published his Variant C for reticulum, mordanting frozen sections of formol material with permanganate and oxalic acid; he mentions Biondi as using it in his adaptation of Cajal's gold technique to connective tissue.

The writer's contributions are the use of the Mallory bleach as a mordant for Bouin-fixed tissue, the increase of lithium silver to 10 per cent (indispensable for the reticulum on nerve fibers), and the intercalation of oxalic acid between the gold and the hypo.

Formulas

Bouin's Fluid (Masson's Formula): To 300 cc. tap water add 100 cc. commercial formol and 20 cc. glacial acetic acid. Add an excess of picric acid. Shake frequently and keep an excess of picric acid in the fluid. Ready for use in 3 days and keeps indefinitely.

Masson's Gelatin Glue: Dissolve 0.05 gm. gelatin (in practice, a bit of ordinary sheet gelatin 5 millimeters square) in 20 cc. distilled water, warming it over the flame. Place a row of slides on the warm plate. Filter a large drop of gelatin solution on each slide and float

the paraffin section on it. As soon as the section spreads, stand the slide upright to drain, holding the section for a moment in the desired place with a brush or needle. At this stage do not permit the section to dry; when the excess gelatin has drained off, blot the section and place immediately in the oven at 45 to 50° C in formol vapor, secured by leaving in the oven an open dish of formol. For staining with hematoxylin and anilin dyes, 20 minutes in the hot formol vapor is sufficient; for silver staining, leave the slides for several hours or, better, overnight.

Ten Per Cent Lithium Silver (Modified del Río-Hortega): To make 120 cc. In a 250 cc. glass-stoppered graduate, dissolve 12 gm. silver nitrate C. P. in 20 cc. distilled water.

Add 230 cc. saturated solution lithium carbonate C. P. in distilled water; shake well; let settle to about 70 cc. of precipitate; wash well with distilled water 3 or 4 times.

After settling to about 70 cc. of precipitate, decant the wash-water; add aqua ammonia fortior, shaking constantly, until the fluid is almost clear.

Add distilled water to a total of 120 cc.; shake and filter into stock bottle. The solution keeps for many months. It is so strong that a slight precipitate is negligible.

Ordinary filter paper is apt to turn brown and discolor the solution while filtering. Use Whatman filter paper No. 42 or No. 44 or Schleicher and Schüll No. 589.

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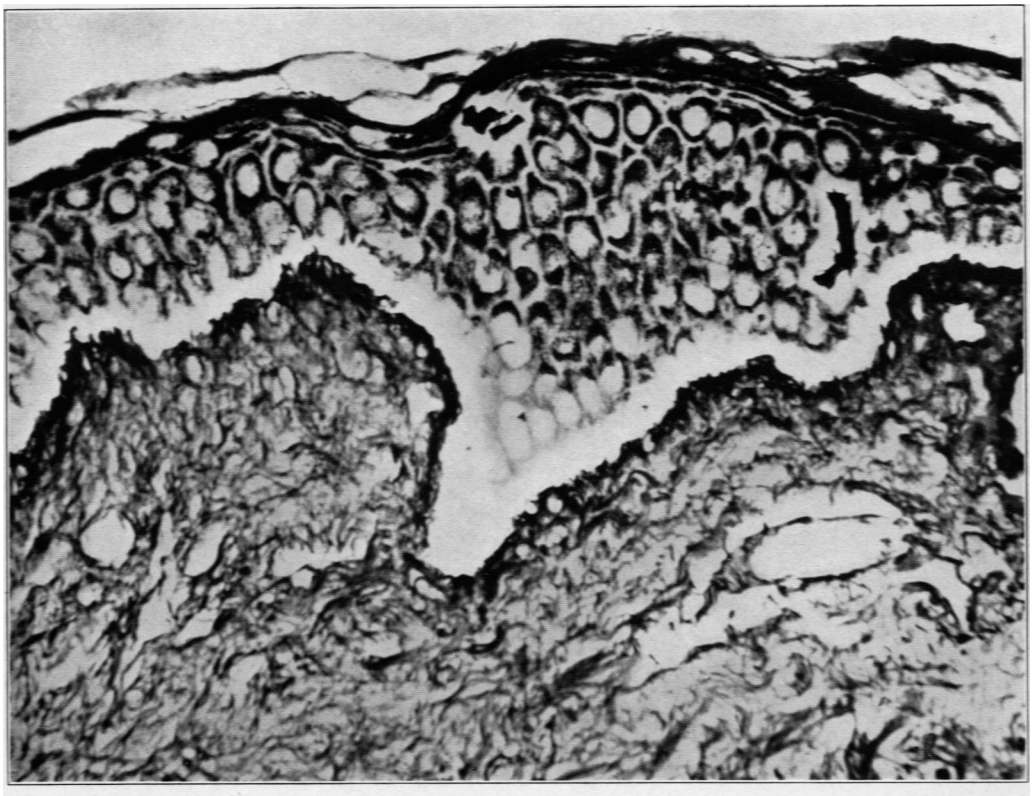
DESCRIPTION OF PLATE

PLATE 51

FIG. 1. Normal skin fixed in Bouin. Epithelial nuclei and basal cells colorless; mesodermic cells of the corium colorless. × 500.

FIG. 2. Normal skin fixed in neutral formol. Epithelial nuclei positive, cytoplasm colorless; mesodermic cells of corium colorless. × 500.



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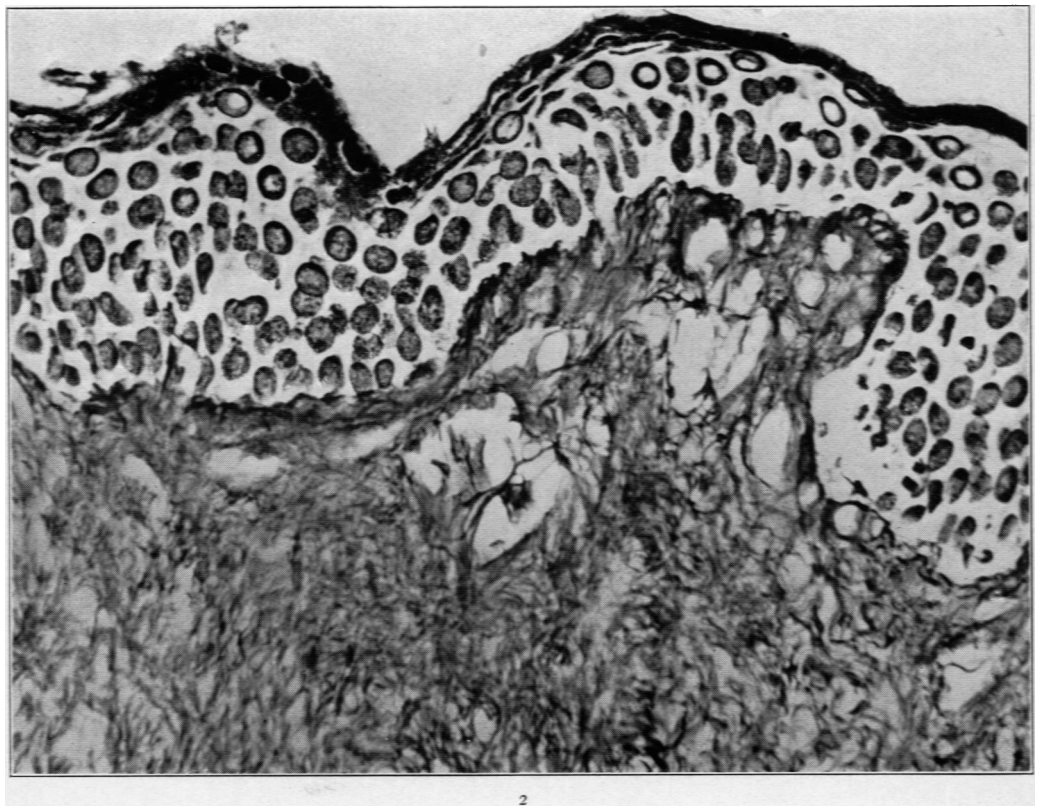

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