A METHOD FOR SUPRAVITAL STAINING OF ANIMALS WITH NEUTRAL RED AND ITS PRESERVATION IN PARAFFIN SECTIONS

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The only method which satisfactorily preserves neutral red in sections of tissue supravitally stained is that reported by Cash (1)—or certain modifications of it—for the study of monocytes and epithelioid cells in the lungs of animals experimentally infected with tuberculosis.

Cash slowly injected into the ear vein 15 cc. of a 1 per cent solution of neutral red, dissolved in normal salt solution. In this manner he secured a true supravital staining of the connective tissue cells of the lungs. Any damage to the cells brings about diffuse staining of cytoplasm and nuclei and obscures those structures which are significant in the so-called supravital reaction. It is an essential part of the technique, therefore, to secure this reaction first. 10 minutes after the injection the rabbit was quickly killed. Thin slices of the lungs were fixed in Zenker-formol solution for from 12 to 24 hours. These blocks of tissue were quickly dehydrated, without washing, in acetone, cleared in benzene or xylol, and rapidly embedded in paraffin. Thin sections were then stained with various dyes, avoiding alcohols and water as much as possible. This method gave uniformly good results with lung tissue.

Prior to the experiments of Cash, McJunkin (2) reported a method for staining tissue supravitally and embedding it in paraffin. He exsanguinated animals from the carotids under light ether anesthesia and then injected saline solution, saturated with neutral red, directly into the tissues. The tissues were fixed in a Zenker formol solution. McJunkin states that the contact with the dye is irregular and that no observations can be made within the areas in which the cells are injured. Indeed, the weakness of the interstitial method of introducing the neutral red is the unevenness and uncertainty of obtaining a supravital reaction of the cells.

Gardner (3) states that the technique of McJunkin proved to be inapplicable

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for use on lung tissue and he therefore modified the methods of McJunkin and Cash. He injected the dye solution either intratracheally or intravenously. The fixation was carried out in the same fluid as that used by McJunkin, except that the formaldehyde was carefully adjusted to a pH of 7.6 with sodium hydroxide. Gardner claims that the sodium has a specific mordanting effect upon staining.

It is well-known that the supravital technique of staining monocytes in the blood or tissues is the most reliable method we have for the accurate differentiation of these cells from other cytological elements. Good descriptions of it can be found in the papers of Simpson (4) and Sabin (5). Forkner (6) has recently described in some detail the methods, as used in Dr. Sabin's laboratory, of applying the technique to tissue cells. This description includes some minor modifications in the methods as originally reported by the above authors. Forkner (7) has shown that lymph nodes from certain areas of the body contain an abundance of monocytes, but that the exact relations of these cells in the tissues could not, by the methods then available, be demonstrated. The technique here recorded was elaborated for the study of this point. It has been found to be equally applicable for the study of all supravitally stainable cells throughout the body. The process of staining and rapid fixation was developed by the writer. The method of dehydration and embedding is a modification of that recorded by Cash (1), to whom I am indebted for suggestions in the development of this technique.

Method

Apparatus.—An instrument which has been of great value in this work is a modified, self-retaining cannula. It is made by inserting a fish-hook (Fig. 1, *a*), the curve of which has been eliminated by heating and straightening (Fig. 1, *b*), into the lumen of a No. 18 or 20 gauge transfusion needle in such manner that the barb of the hook protrudes over the edge of the needle, as shown in the accompanying figures (Fig. 1, *c*). About 1.5 or 2 cm. of the shaft of the hook lies within the lumen of the needle and if a slight bend is made in this shaft, it will retain its position within the lumen but can be removed for cleaning. When such a needle is inserted into the heart, or into a large vessel, the barb catches and prevents its coming out. In this way the labor, time, and trauma caused by the insertion and ligation of a cannula are avoided.

A large pressure flask (about 5 liters) is tightly stoppered and connected by means of rubber tubing with three outlets (Fig. 2) to (1) a blood pressure pump, (2) an Erlenmeyer flask containing 500 to 1000 cc. of 0.6 per cent neutral red (Grübler,

vital nach Ehrlich) in 0.9 per cent sodium chloride solution, and (3) Erlenmeyer flask containing 500 cc. of Zenker's fixing fluid containing 15 per cent formalin and no acetic acid (Fig. 2). The flasks containing neutral red solution and the fixing fluid each has a glass tube extending to its bottom, in order that all the fluid can be pumped out when an increased pressure in the flask obtains. These glass tubes

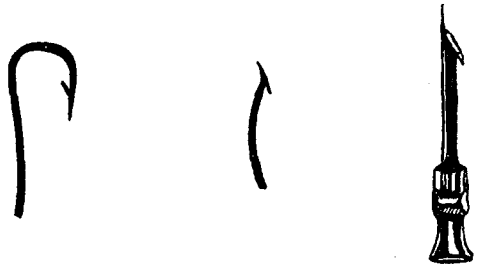

FIG. 1. Diagram illustrating construction of special cannula for perfusion of small animals through the heart.

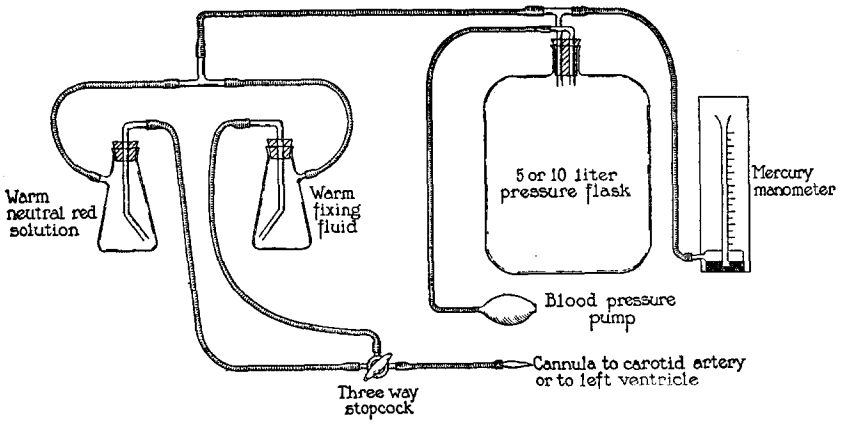

FIG. 2. Apparatus used for staining of tissues and fixation of supravital dyes in cells of the entire body.

are connected by about 1.5 meters of small rubber tubing to two of the outlets of a three way stopcock. The remaining outlet of the stopcock is connected by a short rubber tube to the barbed cannula, as above described.

Staining of Tissues with Neutral Red.—An animal (rabbit, rat, guinea pig) is anesthetized with ether and fastened, ventral side up, with cords to an animal board. A jugular vein is exposed and ligated at the end proximal to the heart. An ordinary cannula is inserted into the distal segment. The animal should now

be under deep anesthesia. Two routes may be used for injection of the neutral red. One can either insert a cannula into a common carotid artery, ligating the distal segment of the artery, and inject the neutral red through the carotid artery into the aorta, or a window about 2 cm. square can be quickly made in the lower left chest near the sternum, care being exercised not to injure the lungs. With a pair of blunt forceps, the parietal pericardium is torn away, the apex of the heart held, and the special self-retaining cannula quickly inserted into the left ventricle and the neutral red solution allowed to pass into the heart, under a pressure of from 100 to 150 mm. of mercury. It is understood, of course, that the injection system must be free of air. At the moment of injection, the cannula in the jugular vein is opened and the blood and neutral red solution are allowed to escape. The cords binding the legs are immediately released to promote a ready circulation through the entire vascular system. The perfusion proceeds for from 15 to 25 minutes, at the end of which time the entire animal is deep red in color. The perfusion fluid should be warmed to from 38° to 40°C. and the rubber tubing carrying the fluid immersed in a vessel of warm water at this temperature. If the heart becomes greatly distended, an incompetency of the mitral valve results with a back flow of neutral red into the lungs through the pulmonary veins and considerable fluid may thus escape through the trachea into the mouth. This may be avoided by clamping the trachea with a hemostat or by clamping off the left auricle. Likewise, intercostal or internal mammary arteries may be clamped off if excessive fluid is being lost.

The amounts of fluid described above are for rabbits. For animals of smaller or larger size, the amounts can be more or less proportionate. Considerably less neutral red solution is required if one injects by way of the carotid artery instead of through the left ventricle. In the rabbit or larger animals, the injection through the carotid is the method of choice. With rats, mice, and all small animals, the route through the left ventricle is much more feasible and it is for these smaller animals that the special barbed cannula is especially adapted.

Fixation of Neutral Red in Tissues.—After all of the neutral red has been passed through the circulation, the stopcock is adjusted so that the fixing fluid warmed to body temperature is injected immediately following the neutral red and through the same channels. About 500 cc. of fixing fluid perfused over a period of from 10 to 20 minutes will reach every organ. The cannulae and all instruments are then immersed in running water to prevent tarnishing with mercury. Instruments made of nickel-steel are not as readily injured by the fixing fluid. Organs are now removed and small blocks placed in a vessel or vessels containing the fixing fluid, where they are allowed to remain for from 12 to 24 hours at room temperature.

Dehydration and Embedding.—It is necessary that blocks be cut not over 2 mm. in thickness, in order that rapid dehydration can take place. Such blocks are dipped in tap water and blotted to remove the excess of fixing fluid. They are then carried through the following solutions, blotting gently after each step up to No. 7.

Solution 1.	Absolute ethyl alcohol—rinse for	1	minute
" 2.	" " "	5	minutes
" 3.	Pure acetone	20	"
" 4.	" "	20	"
" 5.	" "	20	"
" 6.	Xylol	20	"
" 7.	"	20	"
" 8.	56°C. Paraffin	1–2	hours
" 9.	56°C. "	1–2	"

The blocks are then embedded in 56°C. paraffin.

Staining.—The sections are cut from 4 to 10 microns in thickness, mounted on slides previously prepared with albumin fixative, and allowed to dry in a 37° incubator for 4 hours, or preferably over night. The sections on the slides can be excellently treated by the drop bottle method as follows: (1) Xylol to remove paraffin 10 to 20 seconds; (2) Absolute alcohol 5 seconds; (3) 95 per cent alcohol 3 seconds; (4) 95 per cent alcohol containing 1 per cent iodine 15 seconds; (5) 95 per cent alcohol several washings; (6) Absolute alcohol 5 seconds; (7) Clear in xylol; (8) Mount in balsam.

With the above method the macrophages, monocytes, and other vitally stained cells will stand out beautifully with the identical appearance of these cells, as seen in supravitally stained films of living cells.

An excellent counterstain can be employed after step (5) as follows:

(6 a) Distilled water for just long enough to overcome surface tension (3 to 5 seconds); (7 a) Goodpasture's acid polychrome methylene blue, full strength, for from 2 to 7 seconds; (8 a) Rinse quickly with water; (9 a) 95 per cent alcohol 5 seconds; (10 a) Absolute alcohol 5 seconds; (11 a) Xylol to clear; (12 a) Mount in balsam. Other methylene blue solutions may be used, but Goodpasture's permits of rapid staining, does not obscure the neutral red, and causes no troublesome precipitates.

Another method which is probably better than any other for demonstrating and preserving the cytoplasmic structures in supravitally stained macrophages and monocytes, and at the same time giving a satisfactory nuclear stain and a beautiful staining of the reticulum-fibers is a modification of Foot and Mènard's (8) method for staining reticulum by silver impregnation. The method is carried out in exactly the same manner as described by Foot, except that the sections are not bleached. When stained in this manner, the neutral red is of course washed out or obscured, but all the cytoplasmic structures which are stained with neutral red become intensely black. The

monocytes, pre-monocytes, and macrophages show the typical characteristics, except that the cytoplasmic bodies are black. Detailed descriptions of these cells stained in the tissues by the above methods can be found in an accompanying paper (9).

SUMMARY

A simple, rapid method for staining all the supravitally stainable cells in the body, as in supravital preparations with neutral red, is described, together with a method for faithful preservation of the dye in paraffin sections. The essential points of the technique are, first, to secure the reaction of cells to neutral red which corresponds to the so-called supravital technique, involving the reaction of only those substances which respond to the dye while the cell is living; and second, to preserve the stain through the processes of fixation, embedding, and counterstaining.

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