MELANOMA STUDIES *

II. A SIMPLE TECHNIQUE FOR THE DOPA REACTION

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Bloch's dopa reaction is a specific stain for melanoblasts and for myelogenous leucocytes. These cells are believed to contain an organized ferment which oxidizes dioxyphenylalanin (dopa) to melanin. The dopa-melanin colors the reacting cell black. This blackening of the cell is the dopa reaction.[1]

In the published descriptions success with the dopa reaction is attributed to a somewhat meticulous observance of certain chemical details. Blackberg proved that many of these precautions can be dispensed with. In fact, we have come to treat the dopa reaction with so little ceremony that when a specimen comes into the laboratory late in the day we put frozen sections in a small vial of buffered dopa in an inside pocket until the solution turns sepia brown, wash the sections in tap water and mount them the next day. However, we prefer to work with a uniform temperature and to control the reaction with the microscope. The following simple method gives constant and accurate results. The reagents required are a stock solution of dopa and the Sorensen buffers.

The Stock Dopa Solution

This is a 1:1000 solution of 3, 4-dioxyphenylalanin (abbreviated to dopa) † in distilled water. Dopa is a phenol extracted from *Vicia faba*, a common vetch or sow bean. The levorotatory preparation should be used, since Bloch and Schaaf[2] and Peck and coworkers[3] have shown that melanoblasts have little or no oxidizing power over

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† When ordering, specify "for Bloch's dopa reaction." Supplied by the American branch of Hoffmann-LaRoche, Nutley, New Jersey, at 95 cents per gram. With the minute quantity used, the cost of staining a dozen or more sections is less than 2 cents.

the dextrorotatory form. In powder, as purchased, dopa keeps indefinitely at room temperature.

Dissolve 0.3 gm. of dopa powder in 300 cc. of cold distilled water. Keep well corked in the refrigerator, where it will remain good for many weeks. The solution is usable as long as it is colorless or only slightly tinged with red. Darker red solutions should be rejected; they oxidize too quickly and overstain the sections.

Correcting an Error

Dr. Peck calls our attention to a printer's error that has dogged the steps of the dopa reaction. Bloch uses dopa in a solution of 0.1 of 1 per cent and he has never used anything else. Unfortunately in the literature, even in Bloch's and Peck's own papers, in Romeis' popular Taschenbuch (p. 295), and in Krause's Enzyklopädie der mikroskopischen Technik (Vol. 3, p. 1785), the concentration has been printed incorrectly as from 1 to 2 per cent. Let it be understood then that the correct proportion is 1:1000 and that any statement to the contrary is a printer's error.

The Buffers

Dissolve 11 gm. of disodium hydrogen phosphate (Na_2HPO_4+ 2 H_2O) in 1000 cc. of distilled water.

Dissolve 9 gm. of potassium dihydrogen phosphate (KH_2PO_4) in 1000 cc. of distilled water. Both of these buffers are kept in the refrigerator.

Just before cutting the sections, buffer to 7.4 by adding 2 cc. of the potassium phosphate and 6 cc. of sodium phosphate buffer to 25 cc. of the stock dopa solution. For a small batch of a dozen sections we use 15 cc. of the buffered solution, but we prepare double the quantity immediately required in order to have enough to renew the solution in half an hour. Return the stock dopa solution and the surplus of the buffered solution to the refrigerator immediately; at room temperature the stock solution soon oxidizes and turns red, the buffered solution tends to turn brown.

At a given temperature, the speed of the reaction is determined by the pH. At 7.4 the reaction will be finished in 4 or 5 hours at 37° C. When in a hurry, we hasten the reaction by using only 1 cc. of the potassium buffer, giving a pH of 7.7; or we may omit the potassium

buffer, obtaining a pH of 8.2. Such solutions react quickly, in about 60 minutes at 37° C. They should be inspected every 20 minutes to forestall overstaining. These hurried reactions are apt to be overstained; the slow reactions give much more delicate pictures.

A trace of acid inhibits the reaction. A trace of alkali hastens it. All glassware therefore should be scrupulously clean.

Fresh Tissue Required

Melanoblasts: For a dopa reaction of melanoblasts the tissue must be fresh. After death or excision of the tissue from the living body, the intracellular ferment soon diffuses into the surrounding tissue and it is quickly destroyed by most fixatives and preservatives. The ideal material is a frozen section of fresh tissue made immediately after excision from the living body. However, it is difficult to cut fresh tissue neatly. In practice we follow Bloch's present custom of hardening thin slices of freshly removed tissue in 5 per cent formalin for 2 to 3 hours. After this short fixation the tissue cuts better and such experienced observers as Bloch, Miescher, Becker and Peck testify that the reaction is in no way impaired by this short stay in 5 per cent formalin. Neither the gross specimen nor the sections should be permitted to lie in water for more than a few seconds. Water and dilute alcohol extract the ferment rapidly.

In our experience, Walthard goes too far in permitting a stay of 3 days in formalin. We have made innumerable efforts to preserve tissue overnight for a dopa reaction the next day. We have tested commercial formalin (plain and neutralized with sodium hydrate), and Merck's neutral formalin, both plain and further neutralized with chalk (Cajal's practice), from 1 per cent to 100 per cent. Melanoblasts last best in 5 per cent formalin, whether neutralized or not. However, the result is always a gamble and depends on the quantity of ferment originally present in the cells. If in the fresh tissue the dopa-positive cells are black and numerous, they may still be found after 3 weeks or more in 5 per cent formalin. If in the fresh tissue the dopa-positive cells are pale, indicating little or feeble ferment, they will disappear in any formalin within 10 to 12 hours. All workers agree that the best concentration of formalin is 5 per cent. Weaker solutions extract the ferment; stronger formalins abolish the reaction quickly.

Refrigeration: We have tried with little success to preserve melanoblasts in the refrigerator. As with formalin, strongly positive melanoblasts survive for several days; faintly reacting cells disappear overnight.

Leucocytes: The ferment of the myelogenous leucocyte endures much longer than that of the melanoblast. In fact, fresh leucocytes react all the better for a few days in strong formalin and after 2 or 3 months they may still react well. Even for leucocytes there is a time limit, 3 to 4 months, beyond which most of them no longer blacken in dopa solutions.

Cutting the Sections

Frozen sections are obligatory since the chemicals of celloidin and paraffin embedding would destroy the ferment. It is important to remember that water extracts the ferment quickly. Neither the block nor the sections should lie in water longer than the few seconds of a quick rinse. Before cutting the sections, the dopa solution should be buffered and poured into dishes ready to receive the sections without delay.

In order to exhibit the long dendrites of melanoblasts some of the sections should be very thick, 75 to 100 microns; others may be from 20 to 30 microns for better detail. Sections of fresh tissue are dropped from the knife directly into dopa. If the tissue has been in formalin, the sections are rinsed for a few seconds in distilled water and placed promptly in the dopa solution. Since the reaction is an oxidation the dish is left uncovered for free access of air.

Temperature and Time

The dish of dopa containing the sections is put in the incubator at 37° C for about half an hour. Then the fluid is replaced by fresh solution, which in the meantime has been kept cold in the refrigerator. For this renewal of the solution there is a reason. Some tissues are sufficiently acid to lower the pH below 7.0, in which event the fluid remains red and the cells do not oxidize dopa to melanin. They remain colorless. On the other hand, tissue that has been in formalin, especially neutralized formalin, hastens the oxidation, darkens the fluid prematurely and easily overstains. Since it is impossible to foresee the presence of these disturbing factors, and since water

cannot be used to wash them out, we make it a routine practice to change the dopa. The first dopa washes out any objectionable substances and the reaction proceeds unhindered in the fresh solution. At times we have found sections of rectum and colon so acid that two changes of dopa were required before the red of the acid solution changed to the sepia brown of a correct reaction. Under these circumstances a liberal quantity of dopa solution should be used.

Having replaced the first dopa with fresh solution, the reaction is inspected every half hour. In 2 or 3 hours the fluid turns reddish, then sepia brown. The appearance of the sepia tint signals the end of the reaction. At this point a section is rinsed and examined under the microscope. In the perfect reaction the bodies of the dopa-positive cells (melanoblasts and leucocytes) are gray or black, melanin retains its natural yellowish brown color, and collagen is colorless or the palest shade of gray. If a darker stain of melanoblasts is desired, the section is returned to the dopa solution for another half hour or so. The beginner will do well to mount a section every half hour from the beginning to the end of the reaction, continuing until the solution has become black. Such a series of sections is an instructive panorama of the progress of the reaction. It will show him clearly that much of the criticism of the dopa reaction is based on overstained sections.

The time in the incubator will vary with different specimens. Of two tissues prepared alike, cut and dropped into separate dishes of dopa at the same time, one may darken more quickly than the other. The color of the section is a fair guide to the progress of the stain. A well stained section is colorless or pale gray; a pronounced smoke gray indicates overstaining. However, it is much better practice to control the reaction with the microscope.

Bloch, Walthard and European writers generally prefer a slow reaction at a lower temperature, leaving the sections in dopa for from 12 to 24 hours "at room temperature," which they state to be 18° C. In an American laboratory it is difficult to secure a constant temperature of 18° C (64° F). Our own laboratory is 23° C (73° F) in winter and from 26 to 28° C in summer. Sections left in dopa overnight are invariably found to be overstained in the morning. For this reason we use 37° C as more easily controlled.

The reaction proceeds much more quickly in the paraffin oven at 56° C. At this temperature the sections should be inspected more

frequently to prevent overstaining. The objection to rapid reactions at high temperatures is that the fluid soon darkens from spontaneous oxidation of dopa to melanin. The dopa-melanin stains the whole section an even dark brown. In these rapid reactions it is not easy to seize the exact point where the reaction should be checked to prevent overstaining. Bloch is certainly right in insisting that the slower reactions give the more delicate pictures.

Counterstaining and Mounting

The reaction finished, wash the sections in water, dehydrate, clear and mount in balsam, or counterstain in any way desired. The dopa stain is a fast black which resists all the usual reagents except hydrogen dioxide and similar oxidizing bleaches. The paradox of melanin being produced by the oxidation of dopa, and disappearing with further oxidation, is explained by its being the one colored stage in a series of oxidations, the stages before and after it being quite colorless.

The browns, blacks and grays of a correct dopa reaction form an extremely delicate picture. We dislike to obscure it with a counterstain. A dopa section, a silver stained section and a wholly unstained section mounted side by side constitute a very instructive series. Some sections of the batch may be counterstained for general topography or for special features, such as mast cells, plasma cells, or elastic fibers. As a counterstain Bloch and dermatologists generally use methyl green-pyronin. We prefer cresyl violet well differentiated with alcohol, as giving a paler ground. All counterstains take better if the dopa sections are first dehydrated, cleared, and brought back through alcohol to water.

Surgical Preparation of the Skin

Through all dopa literature runs the warning that surgical preparation of the skin with chemicals, especially with iodin, inhibits the dopa reaction. If true, this would be unfortunate; for most of the skin coming into a surgical laboratory has been painted with iodin. However, the statement cannot be strictly true because the greater part of our collection, including some of our finest specimens of dopa-positive dendritic cells, consists of skin that was painted with iodin and washed with alcohol in the usual surgical way.

In order to test this point, in the excision of a series of scars and pigmented moles Dr. Jerome Webster kindly offered to use no skin preparation other than washing with soap and water followed by alcohol. These specimens were sectioned and placed in dopa within an hour of their excision, without contact with formalin or even with water, the sections being dropped from the knife directly into dopa solution. The results were very fine but we cannot say that they were uniformly better than in many similar specimens that had been painted with iodin and immersed in dopa with equal promptitude. As already noted in the efforts to preserve melanoblasts in formalin and with refrigeration, it is possible that strongly positive cells survive the iodin, while weakly positive cells disappear. Be that as it may, our experience indicates that the prospective investigator of the dopa reaction need not be deterred from using surgical material, even though it has been treated with iodin. We have even had good reactions from skin and mucous membranes that had been painted with picric acid, as used in the Squier Urological Clinic, to which we are indebted for some very fine specimens.

Leucocytes

Myelogenous leucocytes stain more quickly than melanoblasts. To secure a delicate picture of leucocytic granules, the reaction must be checked before the melanoblasts are fully stained. As in the Schultze-Winkler reaction, leucocytes stain more uniformly in a strongly alkaline solution. Taking advantage of this principle, Bloch and Peck [4] have recently recommended a special dopa technique for myelogenous leucocytes in blood films; it is useful for sections also.

The films are fixed in hot formol fumes for 20 minutes and immersed in 1:1000 dopa prepared with physiological salt solution. Then 0.2 cc. of 0.1 normal sodium hydrate is added for each 10 cc. of the stock dopa. The mixture turns yellow, then brown. It is left at room temperature for from 1 to 2 hours. A slide is examined microscopically every half hour. When the leucocytic granules are stained uniformly, usually in 1 to 1½ hours, the slides are washed in running water and treated like ordinary films.

Bloch and Peck have devised an ingenious method of accentuating the sharpness of the granule staining by washing the film in distilled water and immersing it in 2 per cent silver nitrate for 2 hours. Wash

again in distilled water; leave 10 minutes in saturated solution of hypo, wash in distilled water, stain the nuclei with hematoxylin and mount in balsam.

Summary

A simplified technique for the dopa reaction is described and discussed in detail.

Note: For the interpretation of the dopa reaction with illustrations and complete literature, the reader is referred to the previous paper.[5]

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