

## ENZYME LOCALIZATION DURING MELANOGENESIS

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### ABSTRACT

The DOPA-reaction was used to identify tyrosinase in the nucleus and cytoplasm of the neural crest melanoblast of *Taricha torosa*, the California newt. In this urodele there is a nuclear DOPA-positive response during the normal embryonic development from the late blastula stage to the nucleus of the early melanocyte. During the gastrula stages, all nuclei of this newt are DOPA-positive. This positive nuclear response fades away after the formation of the neural crest, save in the melanoblasts. The only cells that give a positive DOPA marking in the cytoplasm are the melanoblasts. This cytoplasmic reaction appears while the melanoblast nucleus still gives a DOPA-positive reaction. Tyrosinase activity, as marked by unlabeled DOPA, has ceased in the fully mature melanocyte. The red nuclei, seen in some of the animals in the maturing melanocyte and adjacent tissues, may be in the hallachrome stage of melanin formation. There is a diffuse distribution of DOPA reactivity in the resting nucleus, and an adherence of the DOPA-marking in the region of the dividing chromosomes in the mitosis of DOPA-positive nuclei of the melanoblast. These observations suggest that tyrosinase may be among the chromosomally bound enzymes of the chromatin space.

The melanin-forming cell of the vertebrate is the melanocyte. Its precursor, the melanoblast, is present in the transitory neural crest. From the neural crest, the large, round, amoeboid melanoblast migrates to the dermis and epidermis. Here, enzymatic action in the cytoplasm of the melanocyte forms the melanin pigment that visibly identifies this cell.

Histochemistry and histochemical staining (3, 13-15, 7, 10, 18, 25, 27), density-gradient centrifugation (25), electrophoresis (25), chromatography (10), tissue culture (10, 18, 25), electron microscopy (4, 7, 10, 15, 18, 25) have all contributed to a clear picture of the localization of the melanin-forming enzyme, tyrosinase, during melanogenesis, in the melanocyte cytoplasm of fishes, amphibians, birds and mammals, in both normal and pathologic tissues. These authors have used variations of Bloch's technique for the DOPA reaction to identify tyrosinase in vertebrate melanin-forming cells.

The DOPA reaction is a brown, black or grey staining in the area where the copper enzyme (tyrosinase) and the substrate (DOPA) react in the presence of molecular oxygen. This staining indicates the presence of tyrosinase in the melanoblast (3, 13, 19).

Very recently, tritiated DOPA has been used elegantly in an autographic study of tyrosinase distribution in populations of cultured neural crest cells of the Mexican axolotl, *Ambystoma mexicanum* (19). The authors have presented a properly cautious interpretation of possible intranuclear labeling, pointing to the probability that perinuclear cytoplasmic label was responsible for the exposures revealed in their illustrations.

In the present study of the California newt, *Taricha torosa*, the DOPA reaction reveals tyrosinase in a diffuse state in both nucleus and cytoplasm of the neural crest melanoblast.

In still earlier stages of development of the embryo, exogenous DOPA gives a blackening in

the nucleus only, in three species of urodeles: *T. torosa*, *T. granulosa* and *Ambystoma gracile*, the Northwestern salamander.

The question arose: Did tyrosinase appear de novo in both the nucleus and the cytoplasm of the melanoblast when the cell was identifiable in the neural crest by the DOPA reaction (19), or did the enzyme have an earlier history in the embryo?

The findings on *Taricha torosa* are recorded in this paper: In the California newt, DOPA reactive nuclei appear at random throughout the late blastula. In the gastrula, all nuclei are DOPA-positive. Subsequently, the cytoplasm becomes reactive, but only in those cells associated with actual melanin production in normal embryology. Thereafter, as melanin is produced, nuclei gradually lose reactivity, and, ultimately, so does the cytoplasm.

#### TERMINOLOGY

The terminology of Fitzpatrick et al (8), is followed in describing melanogenesis: "The *melanoblast* is the precursor of the melanocyte. The *melanocyte* synthesizes a specialized melanin-containing organelle, the melanosome. The *melanosome* is the fully developed, melanin-containing organelle in which melanization is complete; *tyrosinase activity is not usually demonstrable*. *Premelanosome* covers all distinctive particulate stages in the maturation of melanosomes; *the premelanosome possesses an active tyrosinase system after the onset of melanin synthesis*."

Here, also, might be mentioned the features that distinguish the *egg pigment* from the *melanin granule*. Already present in the periphery of the gamete and the fertilized egg, the *egg pigment* is composed of small, discrete, brown spheres that enter into the cytoplasm of every cell. As division proceeds, the egg pigment is found, in diminishing amount, among the yolk platelets. The *melanin granule* (mature melanosome), developed by the embryo, is formed and contained only in the cells designed for melanogenesis, and does not appear until the melanoblasts have left the neural crest. The melanin granule appears black, in the larval *T. torosa*.

#### MATERIALS AND METHODS

Clusters of *Taricha torosa* eggs were collected and shipped periodically from Berkeley and Stanford, California during five spawning seasons for the paraffin serial section material. Tissue cultures were made from several shipments during one season.

Embryos and tissue cultures, made by Algard's

method (1), were DOPA-treated according to Laidlaw's technique (14). It must be emphasized that Laidlaw's "delicate browns, blacks and greys of a correct DOPA reaction" depend on meticulous attention to technique. *T. torosa* cells lend themselves to such discriminatory staining.

Both DL and L DOPA gave the DOPA reaction in *T. torosa*. pH ranges of 6.4 to 6.8 gave selective staining of nuclei in the early stages of embryonic development. The nuclei of the ectoderm stained light grey, other nuclei stained red. Both colours were pale and unsuitable for photography. In later stages, the reaction could be photographed. Such nuclei are depicted in Figures 9, 10, 11, and 13.

At pH 7.4, specified by Bloch and Laidlaw for identifying the melanin-producing enzyme in the pigment cell, the ectodermal nuclei at these early stages in *T. torosa* stained a deeper grey or a black and the other nuclei stained a grey of various intensities during the gastrula and early neurulation stages. The recognizable melanoblast stained brown to black in both nucleus and cytoplasm. In *T. torosa*, the reaction was clear-cut in both nucleus and cytoplasm. There was no brown diffusion of the stain in the tissues. For this reason, no enzyme inhibitor (i.e. cyanide, azide, or sulfide) (16) was used in the incubation with DOPA. These trials resulted in the following satisfactory procedure for the paraffin serial sections:

Normal embryos at Twitty and Bodenstein's stages 1 to 35 (24), were fixed in 5% formalin at room temperature for 3 hr. The animals were then rapidly rinsed in distilled water. Some were placed in buffered solution alone. Some were placed in freshly made and buffered, colorless L-DOPA solution (25 ml of 0.1% DOPA solution, plus 8 ml of Sorenson's buffer, pH 7.4). The DOPA solution was freshly made each time, to avoid any autoxidation before use.

Further treatment of all embryos was identical: solutions were changed at  $\frac{1}{2}$ ,  $1\frac{1}{2}$  and 3 hr. Both DOPA-treated and nontreated animals were maintained in open dishes at room temperature (20-21°C) during the DOPA treatment. When the sepia color of the DOPA solution signalled the end of the DOPA-reaction, all animals were placed in 50% ethanol alcohol for 2 hr, shifted to 70% ethanol for 24 hr, and stored in 80% ethanol for sectioning. Serial sections were cut at 8-10 $\mu$ ; some were lightly counterstained with 1% Orange G.

Tissue cultures made and maintained from stage 15 (medullary plate) through stage 22 (closure of the medullary tube) were fixed and stained in a similar manner, and whole-mounted in Adams' "Histoclad."

#### RESULTS

DOPA-treated animals from stage 1 to stage 9, the middle blastula, show no DOPA staining: Sections

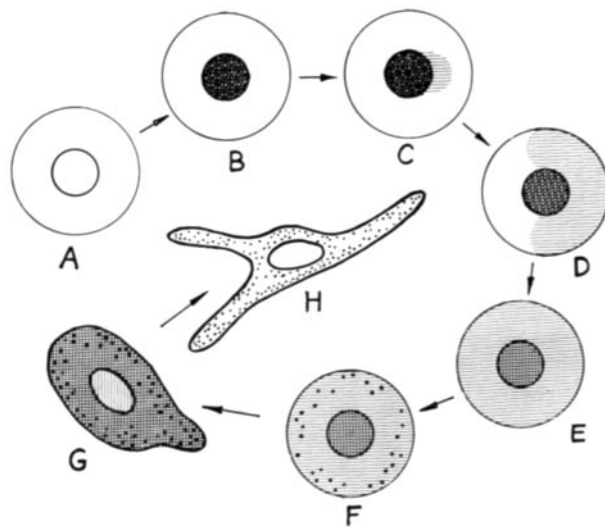


DIAGRAM 1 Diagrammatic representation of the sequence of tyrosinase-DOPA reactivity in developing melanocytes of *Taricha torosa*. Shading represents a positive DOPA reaction; black stippling represents melanosomes.

with an Orange G counterstain show yolk platelets, cytoplasm and nucleus with an even, yellow colour (Fig. 1); DOPA-treated sections without counterstain, show no stain.

In stages 9, 10, and 11 (late blastula) the picture changes. In DOPA-treated animals, the bulk of the nuclei still stain yellow with Orange G (Fig. 1), but in 20 to 40 nuclei scattered throughout the embryo, the colour is grey or black, not yellow (Figs. 2-4). The nuclei are large, between 20 to 30  $\mu$  in diameter, for each nucleus occupies three sequent sections.

As gastrulation proceeds, the number of marked nuclei rapidly increases, until every cell in the gastrula (stages 14 to 15) has a DOPA-positive nucleus (Fig. 6). The nontreated animal has no such marking (Fig. 5). There is no concentration of lighter or darker nuclei in any one region of the DOPA-treated animal. Each nucleus seems to mark individually. In stage 17, there is a crowding of the nuclei (and, therefore, of the cells) into the anlage of the medullary fold (Fig. 6).

During neurulation, (stages 16 to 21), every nucleus of the DOPA-treated animals still has a DOPA-marking, in contrast, again, to the nontreated animals (Figs. 7 and 8). After the neural tube is fully formed (stage 22), the general nuclear marking gradually fades, leaving the relatively few propigment cells in the neural crest region as the only ones with black nuclei (Figs. 8 and 10). There, mitosis is very active (Figs. 8 and 9). It is to be noted that in the mitosis of an early propigment cell (Fig. 9) only the cytoplasm immediately

around the chromosomes contains the DOPA marking.

The propigment cell, now a melanoblast, retains the heavy, nuclear marking, but now for the first time, and only in the melanoblasts, does the cytoplasm give a DOPA-reaction (Fig. 10). There, from a small area proximal to the nucleus, a black or brown marking gradually diffuses, until the whole cytoplasm shows DOPA-reactivity (Figs. 11-14). As the marking spreads and intensifies in the cytoplasm, it fades in the nucleus (Figs. 13 and 14). Now, the melanosomes appear in the cytoplasm, sparsely at first, then in ever-increasing numbers. Nuclei tend to show reactivity no longer (Figs. 13 and 15); some cytoplasmic reactivity is detected in some cells (Figs. 13 and 15). As the melanosome population becomes denser, the reaction can no longer be detected in the melanocyte, using unlabeled DOPA (Fig. 15).

This is summarized in Diagram 1.

At the comparable stages in the tissue cultures the DOPA marking appears first in the nuclei of all cells then later, as in the serial sections, in the cytoplasm of the melanoblasts fading from the melanoblast nuclei as it does in the serial sections when the melanosomes are formed (Figs. 12 and 14).

In Figs. 13 and 15, the red colour of the nuclei in the DOPA-treated animals obviously is not a DOPA-reaction, by definition. Yet no stain but DOPA was used. This red nuclear staining appears in some animals incubated in DOPA at pH 6.4-6.8 and throughout such an animal. In

DOPA-treated animals of the same stage incubated in DOPA at pH 7.4, the nuclei will be colourless, similar to the nuclei of the nontreated animals.

## DISCUSSION

The DOPA-reaction has a well established usage to reveal the presence of tyrosinase in the melanocyte. This reaction recognizably localizes the melanin-forming enzyme in the cytoplasm and the nucleus of the melanoblast in *Taricha torosa*. That certain white blood cells containing polyphenol-oxidases and embryonic red blood cells react also with DOPA (13, 23, 28), should be noted; such cells are not present during early amphibian development. In the California newt, a DOPA-positive protoplasmic marking from the late blastula stage to the melanoblast, is located specifically in the nucleus.

If the positive DOPA marking in this study reveals tyrosinase it follows that in *Taricha torosa*, (1) the enzyme appears in the nucleus before it appears in the cytoplasm; (2) there is a widespread, intra-nuclear localization of the enzyme in embryos prior to the formation and eventual dispersion of the neural crest tissue; (3) the melanoblasts are the only cells that develop reactive cytoplasm, and (4) when the full complement of melanin has been formed the enzyme cannot be revealed by unlabeled DOPA in either nucleus or cytoplasm.

To interpret (1), there are two obvious possibilities: (A) quantities of tyrosinase, too minute to be detected by this histochemical staining method, may be synthesized in the cytoplasm and then stored in the nucleus, or (B) the enzyme may be synthesized in the nucleus and exported subsequently to the cytoplasm.

(A) Since the method used here has not detected tyrosinase in the cytoplasm before the enzyme appears in the nucleus of the melanoblast, (A) will have to rest as a possibility without further comment.

(B) The second possibility is that the enzyme may be synthesized in the nucleus and exported subsequently to the cytoplasm. It is evident that the DOPA reaction reveals tyrosinase first in the nucleus of the developing melanoblast. (B) may be examined, however, in the light of present-day knowledge of enzyme synthesis in the nucleus:

One enzyme, DPN pyrophosphorylase, is of exclusive nuclear localization (12). This has been

confirmed by many other investigators since that time. Further, a coenzyme that is known to be formed in the nucleus is DPN. Exported to the cytoplasm, DPN seeks the mitochondria and is incorporated there (26).

DPN pyrophosphorylase (which responds in the nucleus to experimental stimuli) and DPN are firmly bound to chromatin-like material in the chromatin space (26). Siebert and Humphrey (26) define the chromatin space as a "nuclear region where enzymes are tightly bound to nucleoproteins, presumably to chromosome material. It is expected that in vivo enzymes belonging to the chromatin space also act in their bound state . . . Only the substrates of these enzymes can be expected to move around and give rise to an impact which may lead eventually to the formation of an enzyme-substrate complex; the enzyme itself will not move. Furthermore, since the chromatin-bound enzymes are not soluble, there will be a lowered substrate concentration only in the close vicinity of the enzymes and not in more remote regions."

In the melanoblast nucleus of the resting stage (Figs. 8 and 12) the DOPA-reaction is diffuse, and so is the chromatin. Reasonably then, the enzyme could, be bound to the chromatin. In mitotic figures of melanoblasts DOPA-reactivity closely adheres to the region immediately around the chromosomes (Fig. 9). This would be the picture if the enzyme were bound chromosomally.

DPN seeks the mitochondria in the cytoplasm. Tyrosinase may seek the ribosomes of the endoplasmic reticulum. Seiji et al (25) have already documented this cytoplasmic association.

Under (B) also should be mentioned nuclear involvement in melanin formation described by von Szily (29), Meirovsky (17), and Radaeli (21), all of whom reported a nuclear origin of melanin granules, the granules being extruded from the nucleus in normal development. Radaeli used a modified DOPA technique with acid medium under anaerobic conditions, to blacken isolated nuclei and reveal an enzyme "DOPA quinonase" which, he states, is to be distinguished from tyrosinase.

The second conclusion about *Taricha torosa* was: "(2) There is a widespread intranuclear localization of the enzyme in embryos prior to the formation and eventual dispersion of the embryonic neural crest tissue."

Serial sections, cutting through the large (20 to

PLATE 1 DOPA-reaction in the early stages of development of *Taricha torosa*.

FIGURE 1 Early blastula. Experimental animal. 2 yellow nuclei in large cells. Yolk platelets of various sizes are in the cytoplasm.  $\times 170$ .

FIGURE 2 Late blastula. Experimental animal. 1 grey nucleus in lower right corner. All other nuclei are yellow. Low power.  $\times 42$ .

FIGURE 3 Late blastula. Same field as Fig. 2. 1 grey, 1 yellow nucleus. Small dark dots are egg pigment spheres. Higher power.  $\times 170$ .

FIGURE 4 Late blastula. Experimental animal. 2 grey nuclei, showing nucleoli. The upper grey nucleus may be in the telophase.  $\times 170$ .

FIGURE 5 Gastrulation. Control. Medullary fold forming, to curve up and over medullary plate, to form the neural tube. Egg pigment spheres aggregate in the ectodermal cytoplasm toward the outer edge of the cells. There are a few aggregates of egg pigment spheres within the body of the embryo.  $\times 42$ .

FIGURE 6 Gastrulation. Experimental animal. Nuclei in forming neural fold and medullary plate are grey-black. There is a concentration of nuclei in the fold. Egg pigment spheres congregate toward the outer edge of the ectoderm, on the right.  $\times 42$ .

FIGURE 7 Stage 22. Control. Neural tube completely formed. Between the ectoderm, on the right, and the dorsum of the neural tube is the neural crest. Brown aggregates of pigment spheres are in the body of the animal and around the periphery of the neural tube.  $\times 95$ .

FIGURE 8. Stage 22. Experimental animal. DOPA-positive. Nuclei in the neural crest region are actively dividing, and are dark brown or black. The DOPA-reaction is fading from the nuclei on either side of the neural tube. Egg pigment spheres appear in the cytoplasm of the neural tube cells lining the cavity. Nucleoli are visible. Ectoderm is on the right.  $\times 95$ .

FIGURE 9. Stage 24. DOPA-positive mitosis of a melanoblast in the neural crest, before the reaction appears in the cytoplasm of the resting stage. The DOPA-reaction adheres to the area of the chromosomes.  $\times 170$ .

FIGURE 10. Stage 24. Experimental animal. DOPA-positive nucleus in a melanoblast of the neural crest. There is a diffuse DOPA-reaction in the cytoplasm adjacent to the nucleus, in a small area on the right.  $\times 170$ .

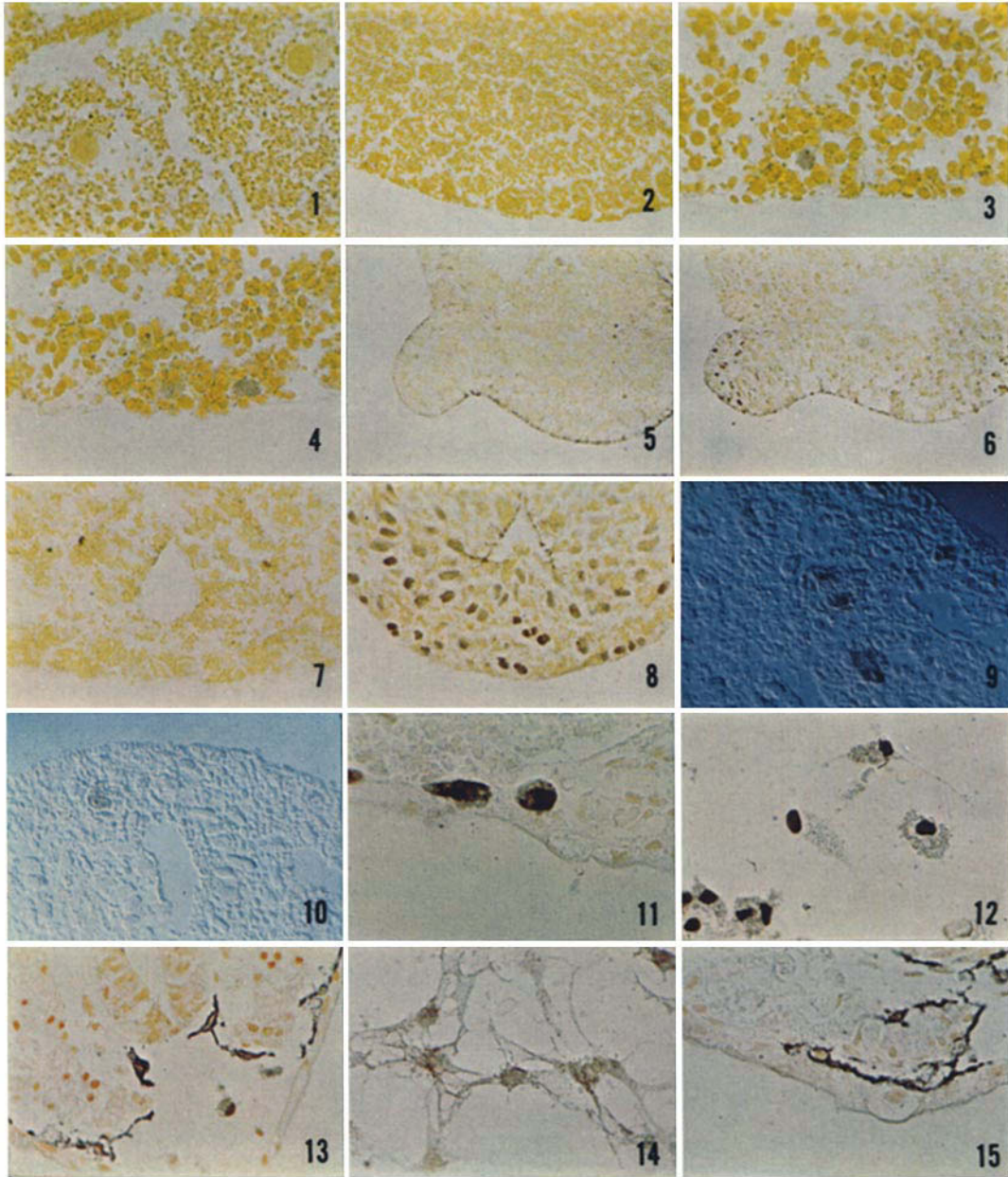
FIGURE 11. Stage 28. DOPA-positive melanoblasts migrating to their position in the dermis to form the dorsal stripe, typical of this species. Both pigment cells have DOPA-positive nuclei and cytoplasm. Yolk platelets are still profusely present. Egg pigment spheres are visible in the epidermal cells, to the right.  $\times 170$ .

FIGURE 12. Tissues culture melanoblasts. Stage 28, as in Fig. 11. Nuclei are still heavily DOPA-positive. Cytoplasm shows reaction around the yolk platelets in the cytoplasm.  $\times 170$ .

FIGURE 13. Stage 35. Between the neural tube and the dorsal finfold (on the right) are various stages in the development of the melanoblast into melanocyte: The round, nucleus-and-cytoplasm marking melanoblast; evolving melanocytes, with 2, 3, or more dendrites and with nuclei in which the DOPA-reaction is fading. In the mature melanocyte (lower right) and all of the non-melanocyte cells, the nuclei are red.  $\times 95$ .

FIGURE 14. Tissue culture. Same stage as early melanocytes of Figs. 13 and 15. Cytoplasm is granular (melanosomes), with some greying, while the nuclei retain a fading, but definite DOPA-marking. The cells are dendritic, as in Figs. 13 and 15.  $\times 170$ .

FIGURE 15. Mature melanocytes along the dermis, forming the dorsal stripe. The epidermis is on the right. The red stain is fading from the nuclei of all cells. The cytoplasm of the melanocytes is filled with melanosomes, and the cell is fully dendritic. The DOPA-reaction can no longer be demonstrated by unlabeled DOPA.  $\times 95$ . Figs. 9 and 10 are made with an interference contrast microscope.



30  $\mu$ ) nucleus, give certainty that the DOPA reactivity occurred throughout the nucleus, not just on the nuclear membrane. In tissue cultures, the DOPA marking might be only on the surface of the nuclear membrane.

The red nuclei (Figs. 13 and 15) of some of the DOPA-stained sections, obviously do not show a DOPA reaction following Bloch's definition (3). Raper (22) recognizes hallachrome (red) as the first visible sign of the oxidation of DOPA to melanin. Hallachrome can be reduced to the colourless leuco compound, and the reaction is readily reversible (5, 9). The red nuclei may be a hint that tyrosinase is still in the nucleus but can react with DOPA only to the hallachrome stage.

No other stain, nuclear or cytoplasmic, was used on these sections. There is no red staining of the cytoplasm at any time.

Figge's work (6) may throw some light on this red colour. He regulated tyrosinase-melanin formation by oxidation-reduction systems, and found melanin to be a reversibly oxidizable substance. Sodium hydrosulfite or ascorbic acid can act on jet black melanin to produce light tan, reversing the action of tyrosinase on tyrosine. The tan-coloured melanin produced with sodium hydrosulfite can be changed to black again if potassium ferricyanide be added to the reaction mixture. Lerner and Fitzpatrick (15) add: "However, vigorous oxidation of the black melanin apparently produces a substance which is first red and then colorless". This may be the course of tyrosinase reaction in the nucleus as the DOPA reactivity fades.

The third conclusion was: "(3) The melanoblasts are the only cells that develop reactive cytoplasm."

Their position in the neural crest, their size, shape, and final location in the dermis are all typical of melanoblasts, and further identification comes with the DOPA-reaction in the cytoplasm of these cells alone.

If the histochemical staining reveal the accurate picture of the course of tyrosinase, the enzyme would leave the nucleus, presumably through the nuclear pores and enter the endoplasmic reticulum which is continuous with the outer layer of the nuclear membrane. Ribosomes are associated with the endoplasmic reticulum, and it is proximal to the ribosomes that tyrosinase has been documented first in the cytoplasm. Because of this, Seiji et al (25) hypothesized that tyrosinase is synthesized in

the ribosomes. Near the enzyme associated with the ribosomes is the site of premelanosome formation (20, 25, 2). Tyrosinase is incorporated in this premelanosome. Here, tyrosinase oxidizes either substrate (3,4-dihydroxyphenylalanine, or its immediate precursor, tyrosine) to melanin in the presence of molecular oxygen (11). The pigment is deposited in and on this premelanosome. This is then the visible melanin granule. When this organelle has received its full complement of melanin, the enzyme activity is turned off in the granule.

Diffuse marking of tyrosinase in cytoplasm is mentioned in the recent literature: Clark (4) reported an opacity in the cytoplasm of DOPA-treated melanocytes, in the early stages of a DOPA-reaction, that was clear-cut and specific for this reaction in this cell. This specific opacity was revealed at both low and high powers of the electron microscope. Seiji et al (25) found that the diffused small-granule fraction from density-gradient centrifugation gave a high yield of tyrosinase, and stated that there was sufficient evidence for the precursor-end-product between the small granule fraction associated with RNP particles and the large-granule fraction, composed predominantly of melanosomes. Greenberg (10) stated that "one autography type (using DOPA-C<sup>14</sup>) in fish melanomas, indicated a generalized label, evenly distributed throughout the tissue, with a moderate pigment synthesis."

In some nonmelanizing premelanosomes, tyrosinase has been shown to be present, but no melanin is formed in vivo. This is in either melanoblasts of albinos, or melanoblasts of amelanotic melanomas (18). In vitro, however, this same nonmelanizing premelanosome, if it contains tyrosinase, will convert L-tyrosine to melanin (18).

Tyrosinase has been shown to be present in a diffuse form in both nucleus and cytoplasm before the large, easily seen melanosomes are present, in *Taricha torosa*. It follows that, in this species, tyrosinase can be in the melanoblasts in both an active and an inactive state in the cytoplasm, and, so far as melanogenesis is concerned, in an inactive state in the nucleus, in normal development.

The last conclusion was: "(4) When a full complement of melanin is formed, tyrosinase cannot be revealed by unlabeled DOPA in either nucleus or cytoplasm."

Laidlaw (13) notes that "not all cells capable of producing melanin are at all times DOPA-positive."

Labeled tyrosine, DOPA-<sup>3</sup>H, and DOPA-C<sup>14</sup> (10, 15, 19) can reveal tyrosinase in the premelanosome of the maturing melanocyte, where the full complement of melanin has not been deposited in that premelanosome. Labeled substrate does not reveal tyrosinase activity in the fully mature melanocyte. Activity is then turned off in all melanosomes.

The histochemical staining localization of tyrosinase as appearing first in the nucleus of the melanoblast, and the DOPA reactivity of the nucleus from the late blastula stages to the differentiated melanin-forming pigment cell, are offered as

circumstantial evidence for the synthesis of tyrosinase in the nucleus.

It is a pleasure to acknowledge the photographic aid of William Wowchuk and Jack Dietrich, the tissue cultures made by Dr. F. T. Algard, the diagram drawn by Annette Algard, the opportunity and assistance to do this work, given by Dr. W. Gordon Fields, Head of the Department of Biology.

This work was supported by the Faculty Research Fund of the University of Victoria, administered by Dr. W. Gordon Fields.

Received for publication 28 March 1969, and in revised form 27 June 1969.

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