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Histochemical Evidence that Peroxidase Does Not Affect Melanin Formation in Feather Melanocytes[1]

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Animal peroxidases are iron-containing proteins that catalyze the oxidation of a variety of substances by hydrogen peroxide. Histochemically, the myeloperoxidase of granulocytes is the most easily detected (1). Tyrosinase (dopa oxidase) is a copper-containing enzyme complex capable of converting both tyrosine to dopa (slowly) and dopa to dopa quinone (rapidly) in the melanin synthetic pathway (2). Histochemically, dopa-oxidase activity is easily demonstrated.

Okun and his collaborators, using various histochemical tests, have shown that peroxidases are present in mast cells, granulocytes, neurons, some melanomas, and possibly some mammalian melanocytes (3–7). These peroxidases are capable of converting both tyrosine and dopa to melanin. These workers have concluded that peroxidase, rather than tyrosinase, is primarily responsible for converting tyrosine to dopa in melanocytes and that tyrosinase actually possesses only dopa-oxidase activity.

Since these peroxidase studies were limited to mammalian systems, it was imperative that we make similar investigation of the melanocyte system of the fowl so that results involving enzyme activity in this species could be correctly interpreted. Histochemical tests were conducted using the melanocytes and granulocytes of three pigment mutations of the fowl. The results indicate that a copper-containing tyrosinase does exist in the fowl which is capable of oxidizing both tyrosine and dopa.

MATERIALS AND METHODS

There were two main groups of experiments. In the first group, the tyrosinase, dopa oxidase, and peroxidase activities of feather melanocytes of the three selected

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genotypes were determined. Results were quantitated with the electron microscope. In the second group, the dopa-oxidase and peroxidase activities of peripheral blood smears of the three genotypes were ascertained using the light microscope.

Melanocytes from the regenerating breast feathers of adult standard, pink-eye, and albino-like males were examined. Standard males ($+^{Pk}/+^{Pk}$ $+^{c}/+^{c}$) have black breast feathers, pink-eye males (*pk/pk* $+^{c}/+^{c}$) have gray feathers, and albino-like males ($+^{Pk}/+^{Pk}$ *c/c*) have white feathers. Stocks are maintained in the Department of Zoology of the University of Nebraska–Lincoln. The standard stock is of junglefowl origin (8). The pink-eye stock has previously been described (9) and the albino-like effect is due to the presence of the recessive white (*c*) mutation (10).

Breast feather tracts were plucked and allowed to regenerate for 2 weeks prior to sampling. Only the central follicles of corresponding feather tracts were plucked as samples. Each feather cylinder was then split longitudinally, one piece serving as control and the other piece as experimental. Whenever possible sections from the same feather were compared. In no case, however, were comparisons made between feathers that were more than one follicle away in that feather-tract row.

Melanocytes differentiate in linear fashion as the feather grows due to the mitoses of the basal collar cells (11). The collar becomes organized into several longitudinally-oriented barb ridges as it develops. Melanoblasts differentiate rapidly in this barb-ridge forming region. To ensure that the same stage of differentiation was being compared, only melanocytes occupying the most basal portion of the barb ridges were examined.

The ability of melanocytes to use tyrosine was determined using the quasi-organ culture technique of Brumbaugh *et al.* (12). Unfixed tissues were incubated for 20 hr at 38°C in medium containing either 0.22 m*M* (control) or 4.0 m*M* (experimental) L-tyrosine. After incubation the tissues were fixed in 3% glutaraldehyde. Dopa-oxidase activity was determined by incubating tissues, which had previously been fixed, in 5 m*M* L-dihydroxyphenylalanine (L-dopa) at pH 6.9 for 3 hr at 38°C (13). Diaminobenzidine (DAB) was used as the substrate to visualize peroxidase activity. Tissues were fixed for 2 hr in 3% glutaraldehyde, rinsed for 3 hr in cold buffer, and incubated for 2 hr in Graham and Karnovsky's medium (14) containing DAB and H_2O_2 at 38°C. This method is similar to that used by Okun *et al.* (5).

Some tissues were incubated in 10^{-2} *M* diethyldithiocarbamate (DDC) for 2 hr at 38°C to block the copper-containing tyrosinase complex. Before being placed into their respective histochemical solutions, the DDC was washed out (5) in six changes of buffer over a 30-min period. To inhibit peroxidase activity by the removal of H_2O_2, some tissues were incubated in DAB without H_2O_2 and in the presence of 0.1–1.0 mg/ml of beef-liver catalase (Sigma) (5).

After treatment, feather tissues were osmicated for 1 hr (2% OsO_4), dehydrated, and embedded in epon. Ultrathin sections were mounted on Formvar coated grids and viewed with an RCA EMU 3-B (modified) electron microscope after being stained with uranyl acetate and lead citrate (9).

Several fields of each genotype and treatment, each containing at least one melanocyte, were photographed. The individual melanogenic organelles on each negative were classified according to maturity in order to determine the amount of pigment produced by a given treatment. They were classified as stage II premelanosomes (filament matrix only), stage III premelanosomes (some melanin on

matrix), or stage IV melanosomes (no internal matrix discernible) (15). The total number of each type of melanogenic organelle was determined for each genotype and treatment and the percentage of organelles at each stage calculated (13).

Granulocytes were studied from peripheral blood obtained from the wing vein. Smears were air dried and fixed in 10% buffered formalin. They were subjected to Laidlaw's dopa reaction at pH 7.2 (16, 17), and to the peroxidase test of De-Robertis and Grasso using benzidine as the substrate (16, 17). DDC and catalase were used in combination with each genotype and treatment using the procedures already described for electron microscopic histochemistry. Results were observed with the light microscope.

Some simple mushroom tyrosinase experiments were performed to test substrate responses. Mushroom tyrosinase (Sigma) was added to test tubes containing buffered solutions (pH 6.9) of 5 m*M* L-dopa, 5 m*M* L-dopa plus 10^{-2}*M* DDC, 1.4 m*M* DAB, and 1.4 m*M* DAB plus 10^{-2}*M* DDC, to produce a final concentration of 450 units/ml. Any resulting reactions were noted and recorded.

RESULTS

When standard tissues were incubated in tyrosine, the percentage of stage IV melanosomes increased from 32 to 47, indicating increased melanin deposition (Table 1). The decrease in stage III premelanosomes, from 63 to 33%, might also be indicative of maturation. The increase in stage II premelanosomes from 5 to 20% is perplexing, however. This is probably due to a sampling discrepancy rather than to tyrosine treatment. When standard tissues were used the results were sometimes equivocal because the melanocytes in the areas sampled were already quite melanized.

Pink-eye melanocytes responded more decisively to tyrosine treatment than standard melanocytes. The percentage of stage IV melanosomes and stage III premelanosomes increased from 0 to 5% and from 47 to 76%, respectively (Table 1). Accordingly, tyrosine incubation decreased the percentage of stage II premelanosomes from 53 to 19. The inherent immaturity of pink-eye melanogenic organelles during the developmental stages examined enables clearer reaction product visualization.

Albino-like melanocytes are characterized by a paucity of melanogenic organelles. No stage IV melanosomes or stage III premelanosomes were seen in con-

TABLE 1

GENOTYPIC RESPONSES OF MELANOCYTES TO TYROSINE TREATMENT

	Melanogenic organelles at stage					
	II		III		IV	
Genotype	#	%	#	%	#	%
Standard						
Control	15	5	184	63	91	32
Experimental	20	20	32	33	45	47
Pink-eye						
Control	63	53	56	47	0	0
Experimental	76	19	288	76	18	5
Albino-like						
Control	Rare		0	0	0	0
Experimental	Occasional		Rare		0	0

trol cells and only rarely were stage II premelanosomes encountered (Table 1). The cytoplasm, however did contain an overabundance of Golgi material (12). No stage IV melanosomes developed during tyrosine treatment but the number of premelanosomes increased although stage III premelanosomes were only rarely seen (Table 1). Concomitantly, the Golgi compartment of the cytoplasm decreased (12). Thus all three genotypes used tyrosine, but the response was most clearly discernible in pink-eye melanocytes. It should be noted that no catalytic amount of dopa or other co-factor was present to speed up the reactions other than that already present in the melanocytes at the time of incubation.

Standard melanocytes were strongly dopa positive. Many Golgi-related cisternae and vesicles as well as premelanosomes became electron opaque due to the deposition of dopa melanin within their membrane boundaries during treatment. Figure 1 shows three such reacted areas (arrows) associated with three Golgi stacks.

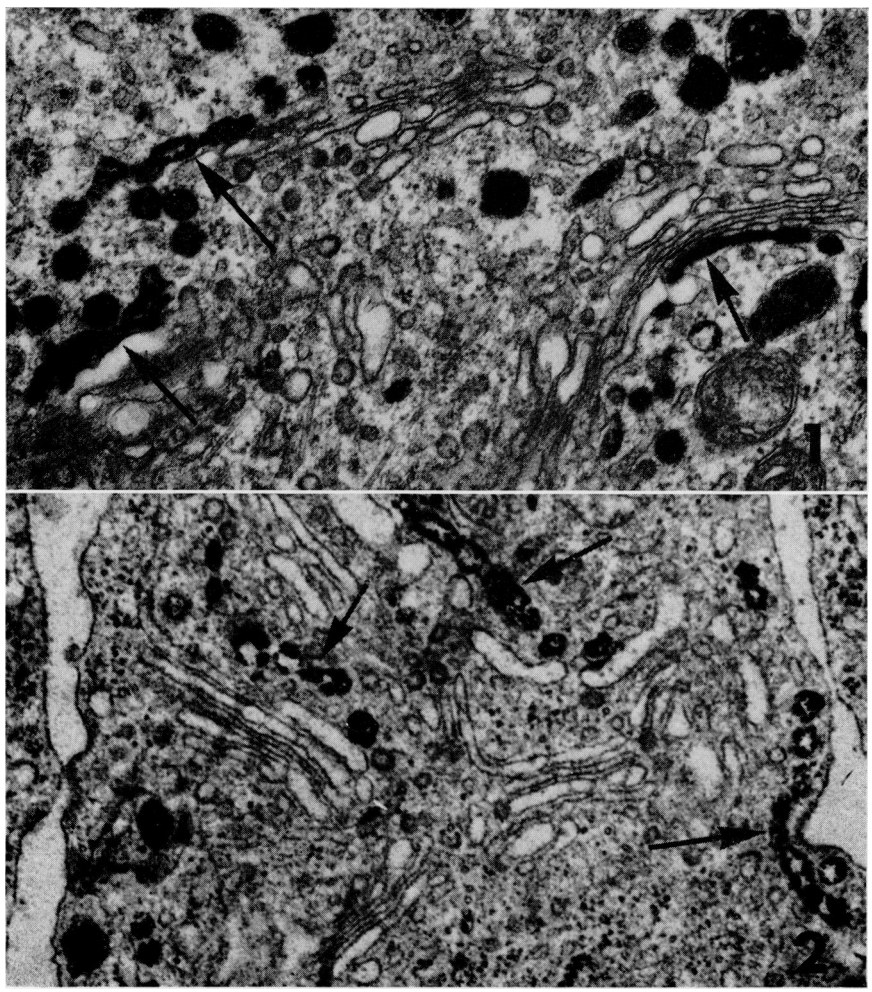

FIG. 1. Portion of dopa-treated standard melanocyte showing three (arrows) dopa-positive Golgi-related cisternae. ×40,000.

FIG. 2. Portion of dopa-treated pink-eye melanocyte showing three (arrows) dopa-positive Golgi-related cisternae. ×40,000.

Pink-eye melanocytes also responded to dopa treatment but not as vigorously as standard melanocytes. As shown in the three reacted areas (arrows) of Fig. 2, pink-eye cisternae and vesicles were not as heavily packed with dopa melanin as those in standard melanocytes. The proportion of Golgi stacks which contained reaction product was also smaller. When pink-eye tissues were treated in DDC prior to dopa incubation, the reaction was blocked. The pieces of tissue did not darken during incubation and no Golgi-related reaction product was observed. Figure 4 shows a tangential view of a Golgi cluster (G) and associated vesicles from a DDC-treated, dopa-reacted pink-eye melanocyte. No visible reaction product is present, either in the Golgi region or surrounding premelanosomes (arrows).

In spite of their hypertrophied Golgi system, albino-like melanocytes were dopa negative. Figure 3 shows three Golgi stacks and their associated vesicular and tubular system. No reaction product is apparent, not even in the very immature premelanosome (arrow).

The granulocytes of all three genotypes responded the same to all the tests involving benzidine and dopa as summarized in Table 2. They were peroxidase positive as visualized with benzidine and H_2O_2. When only benzidine was used the cells were negative as they were when catalase was added, indicating that the reaction was definitely due to peroxidatic activity. Pretreatment with DDC did not interfere with the reaction. When dopa was used as the substrate the granulocytic responses were similar. In this case dopa treatment both with and without H_2O_2 was positive, since dopa produces H_2O_2 on autoxidation (18). The reaction was, however, catalase sensitive and DDC insensitive indicating that the dopa melanin was formed by peroxidatic action and not by a copper-containing dopa oxidase.

The melanogenic organelles of standard melanocytes darkened when incubated in DAB and H_2O_2. The percentage of stage IV melanosomes increased from 55.0 to 68.4, while the percentage of stage III and stage II premelanosomes decreased from 25.9 to 23.4 and from 19.1 to 8.2%, respectively (Table 3). Unlike the dopa test, no reaction product was detected in Golgi-related cisternae or vesicles in any of the genotypes. Okun *et al.* (19), however, found peroxidase-mediated, tyrosine reaction product in the Golgi-associated regions of melanoma cells.

Albino-like melanocytes did not react with DAB. There was no reaction product visible in any of the immature premelanosomes or Golgi-related cisternae and vesicles present in the cytoplasm (Fig. 5).

As with the tyrosine test, pink-eye melanocytes changed more dramatically than standard melanocytes when incubated in DAB. Again, this is due to the abundance of immature melanogenic organelles in this genotype. The percentage of stage IV melanosomes increased slightly from 0.0 to 0.5 (Table 3). The number of stage III premelanosomes increased from 25.4 to 41.5%, while the number of stage II premelanosomes decreased from 74.6 to 58.0%. The darkened premelanosomes typical of the DAB-positive response of pink-eye melanocytes is shown in Fig. 6.

Pink-eye melanocytes were selected for the tests which distinguish peroxidases from copper-containing dopa oxidases and tyrosinases because they gave more obvious responses to both tyrosine and DAB than standard melanocytes and yet paralleled them in being tyrosine, dopa, and DAB positive. Pink-eye melanocytes which were incubated in DAB and catalase varied little from melanocytes incubated in DAB without catalase but with H_2O_2. Their melanogenic organelles were distributed as follows: stage IV, 1.5%, stage III, 44.5%, and stage II, 54.0% (Table 3). Pretreatment with DDC, however, blocked the ability of premelanosomes to

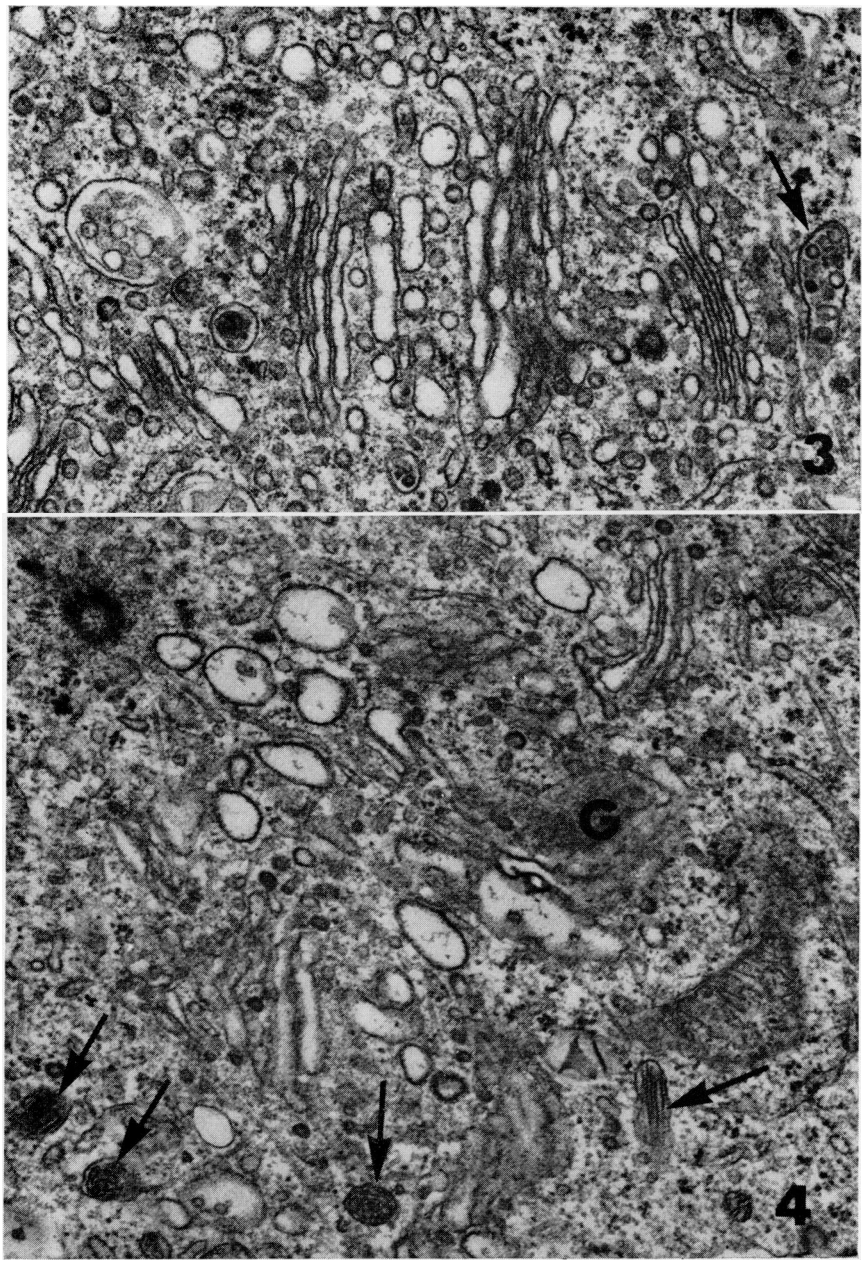

FIG. 3. Portion of dopa-treated albino-like melanocyte showing absence of reaction product in the Golgi system and immature premelanosome (arrow). ×40,000.

FIG. 4. Portion of dopa-treated, DDC-blocked, pink-eye melanocyte showing absence of reaction product in the tangentially sectioned Golgi system (G) and accompanying premelanosomes (arrows). ×35,000.

darken in the presence of DAB and H_2O_2. There were no stage IV melanosomes, only 11.5% stage III premelanosomes and 88.5% stage II premelanosomes in the DDC and DAB-treated melanocytes (Table 3). This is clearly shown by comparing the darkened premelanosomes of the DAB-treated melanocyte in Fig. 6 with the

TABLE 2

GENOTYPIC RESPONSES OF GRANULOCYTES TO BENZIDINE AND DOPA TREATMENTS

Treatment	Standard	Pink-eye	Albino-like
Peroxidase test (benzidine and H_2O_2)	+	+	+
Benzidine only	−	−	−
Peroxidase block (benzidine and catalase)	−	−	−
Dopa oxidase block (benzidine fol. DDC)	+	+	+
Dopa and H_2O_2	+	+	+
Dopa only	+	+	+
Peroxidase block (dopa and catalase)	−	−	−
Dopa oxidase block (dopa fol. DDC)	+	+	+

TABLE 3

GENOTYPIC RESPONSES OF MELANOCYTES TO DIAMINOBENZIDINE (DAB) TREATMENT

	Melanogenic organelles at stage					
	II		III		IV	
Genotype and treatment	#	%	#	%	#	%
Standard						
Control (no DAB)	31	19.1	42	25.5	89	55.0
Experimental (DAB and H_2O_2)	42	8.2	120	23.4	350	68.4
Pink-eye						
Control (no DAB)	262	74.6	89	25.4	0	0.0
Experimental (DAB and H_2O_2)	123	58.0	88	41.5	1	0.5
Peroxidase block (DAB and catalase)	105	54.0	87	44.5	3	1.5
Dopa oxidase block (DAB and H_2O_2 fol. DDC)	144	88.5	19	11.5	0	0.0
Albino-like						
Control (no DAB)	No reaction; no difference between controls and experimentals					
Experimental (DAB and H_2O_2)						

lighter premelanosomes of the DDC and DAB-treated melanocyte shown in Fig. 7. Thus, it is possible to conclude that the DAB reaction in pink-eye melanocytes is catalase insensitive and DDC sensitive.

If the DAB reaction in fowl melanocytes is catalase insensitive and DDC sensitive, then tyrosinases and dopa oxidases must be able to use DAB as a substrate. When dopa was subjected to mushroom tyrosinase, a dark-colored precipitate immediately formed. When the reaction mixture contained DDC as well as dopa and tyrosinase, the reaction was delayed and produced a fine white precipitate. When DAB was substituted for dopa a dark-colored precipitate also formed immediately, but again the reaction was delayed and a white precipitate formed if DDC was present.

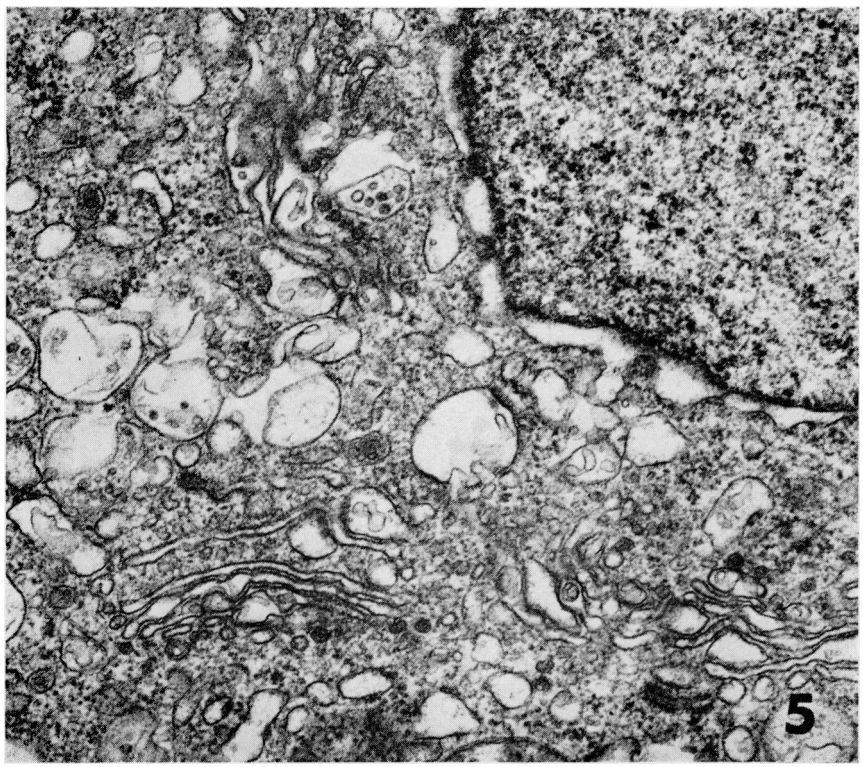

FIG. 5. Portion of DAB-treated albino-like melanocyte showing absence of reaction product. ×35,000.

DISCUSSION

The pink-eye mutation served as a "tool" and allowed the differences between treatments to be definitely quantitated at the ultrastructural level. Pink-eye melanocytes were valid substitutes for standard melanocytes because they responded qualitatively in the same way to each histochemical test.

The results indicated that granulocytes contain peroxidases capable of using dopa as a substrate. Okun *et al.* (3–5) have shown that peroxidases are also capable of oxidizing tyrosine. Although tyrosine was not used as a substrate in this study, it is logical to assume that fowl peroxidases are also capable of oxidizing both substrates.

The ability of pink-eye melanocytes to use DAB in the absence of H_2O_2 and in the presence of catalase, showed that they contain some enzyme, not peroxidase, which is capable of using DAB. The enzyme is obviously a copper containing tyrosinase and/or dopa oxidase because the reaction is DDC sensitive. The *in vitro* production of a dark precipitate from DAB by mushroom tyrosinase showed that tyrosinases and/or dopa oxidases are capable of converting peroxidatic substrates. This ability of phenol oxidases to use benzidine and related compounds was shown by Keilin and Mann (20), and takes place readily in the presence of a small concentration of catechol. Knight (21) found that a phenol oxidase present in the perisarc of the hydroid *Laomedea flexuosa* was capable of a benzidine reaction even in catalase-containing medium.

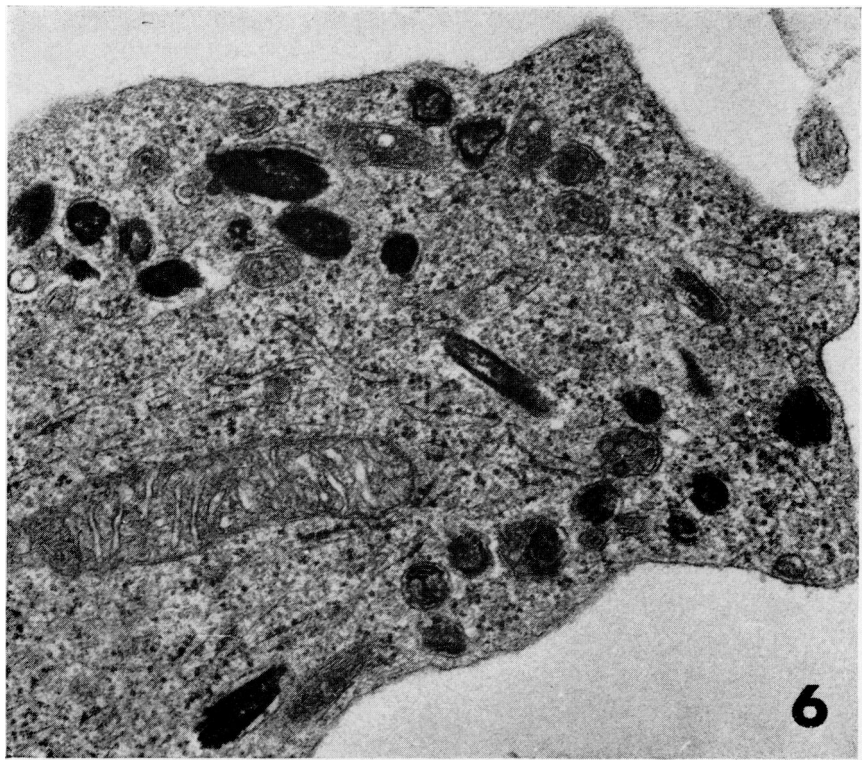

FIG. 6. Portion of DAB-treated pink-eye melanocyte showing darkened premelanosomes due to reaction product. ×35,000.

Okun *et al.* (3, 7) have affirmed that the conversion of tyrosine to dopa in mammalian melanocytes is nearly always mediated by peroxidase. This is especially true for melanoma cells. If peroxidase is present in pink-eye melanocytes, then at least part of the DAB reaction product should have been catalase sensitive and DDC resistant, since these cells were definitely tyrosine positive. Since no DDC-resistant reaction product was noted, it seems logical to conclude that a true tyrosinase is present in fowl melanocytes. It should also be noted that even though albino-like melanocytes were slightly tyrosine positive, they were completely DAB negative.

The comparison of the gene-specific responses of albino-like melanocytes also indicate that peroxidase does not mediate melanin synthesis in fowl melanocytes. To produce albinism using the peroxidase theory, peroxidase activity, but not necessarily dopa-oxidase activity, must be extinguished. It is interesting to note that albino-like melanocytes are dopa negative while granulocytes are definitely peroxidase positive. Body-wide peroxidase negativity does not exist. If the melanocytic and granulocytic peroxidases were products of different structural loci, then melanocytes could be peroxidase negative while granulocytes were peroxidase positive. The hypothetical melanocytic peroxidase of normally functioning melanocytes, however, would still have to be catalase sensitive and DDC resistant.

The results of this investigation when compared to the results of Okun *et al.* (3–5) suggest that fowl melanocytes are distinctly different from mammalian

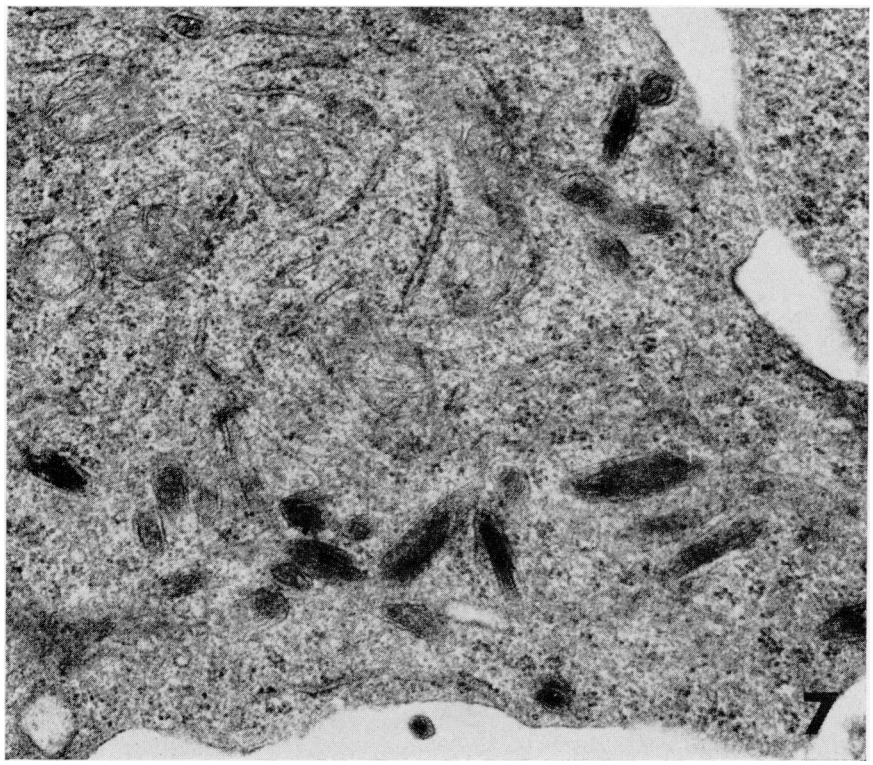

FIG. 7. Portion of DAB-treated, DDC-blocked, pink-eye melanocyte showing absence of premelanosomal reaction product. ×35,000.

melanocytes. Dopa-oxidase activity was blocked by DDC in basal melanocytes as reported by Okun *et al.* (3–5), and yet they were not tyrosine positive. How is the dopa which is oxidized to melanin in these cells made available to them? It is unlikely that enough dopa is present in body fluids to supply their requirements. Perhaps these cells were capable of oxidizing tyrosine with tyrosinase at a prior stage of development. Okun *et al.* (3–5) seem to have examined only rather mature normal melanocytes which may have had only residual tyrosinase activity remaining. This study examined melanocytes that were definitely differentiating. It would be advantageous to study normal differentiating mammalian melanocytes and then compare them to melanoma cells. Perhaps the peroxidase-mediated tyrosine reaction is a modification associated with malignancy rather than a distinctive difference between normal mammalian melanocytes and those of the fowl.

SUMMARY

Standard melanocytes were tyrosine, dihydroxyphenylalanine (dopa), and diaminobenzidine (DAB) positive, but ultrastructural quantitation was difficult because a majority of melanogenic organelles were already melanosomes. Albino-like melanocytes were slightly tyrosine positive, but dopa and DAB negative. Pink-eye melanocytes are characterized by reduced melanin deposition. When treated with tyrosine the percentage of stage II premelanosomes decreased while the percentage at stage III and IV increased. Pink-eye melanocytes were dopa positive, containing

reaction product in Golgi-related cisternae and vesicles like the standard. The dopa reaction was sensitive to pretreatment with diethyldithiocarbamate (DDC).

The melanogenic organelles of pink-eye melanocytes also darkened significantly when incubated in DAB even in the presence of catalase. The same reaction was sensitive to DDC which suggests that dopa oxidase is capable of using DAB as a substrate. This was confirmed *in vitro* by the production of a DAB reaction product by mushroom tyrosinase.

Peroxidase does not seem to be functioning in feather melanocytes since no DDC-resistant, catalase-labile reaction product was detected. This is substantiated by the fact that albino-like individuals have melanocytes, which are both dopa and DAB negative and granulocytes which are peroxidase positive. This mutation affects only the copper-containing phenol oxidase system which apparently functions as both tyrosinase and dopa oxidase in the fowl.

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