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SUBCELLULAR DEMONSTRATION OF PEROXIDATIC OXIDATION OF TYROSINE TO MELANIN USING DIHYDROXYFUMARATE AS COFACTOR IN MOUSE MELANOMA CELLS*

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ABSTRACT

Peroxidatic oxidation of tyrosine was directly demonstrated in tumor cells of Harding–Passey mouse melanoma using dihydroxyfumarate (DHF) as cofactor; there was no evidence of oxidation of tyrosine by aerobic dopa oxidase ("tyrosinase") in these cells. Peroxidative oxidation of tyrosine was correlated with a positive benzidine reaction and peroxidase-dependent oxidation of dopa to melanin. Melanoma cells with peroxidase activity were designated as type A cells.

Many of the type A cells also demonstrated oxidation of dopa to melanin which was mediated by aerobic dopa oxidase ("tyrosinase"). In cells with both enzymes, peroxidase activity was present in melanosomes,whereas aerobic dopa oxidase activity was present in both melanosomes and in the Golgi-associated cisternae of endoplasmic reticulum (GERL).

Without inhibitors, cells containing both enzymes showed reaction product in melanosomes and in the GERL after incubation in tyrosine, DHF, and hydrogen peroxide. After preincubation with sodium diethyldithiocarbamate (DDC), new melanin was still formed in melanosomes, but was eliminated in the GERL; when catalase was present in the incubation medium, reaction product was completely eliminated in all areas. These results indicate that melanin in the GERL induced after incubation with tyrosine, DHF, and hydrogen peroxide was due to the action of dopa oxidase on dopa synthesized by peroxidase.

Some larger, more heavily melanized tumor cells (type B cells) were present which had aerobic dopa oxidase activity, but no demonstrable peroxidase activity. These may represent end-stage cells in which aerobic dopa oxidase acts on dopa formed by previous peroxidase catalysis.

Synergism of peroxidase and dopa oxidase may be a mechanism of melanogenesis in vivo.

Experiments in our laboratory [1–4] have shown that peroxidase activity is present in normal and neoplastic melanocytes and that this activity can be correlated with peroxidase-dependent oxidation of dopa or tyrosine (in the presence of dopa cofactor) to melanin. The ability of peroxidases to oxidize tyrosine (as well as dopa) to melanin was confirmed by in vitro studies with purified peroxidases, using a variety [5, ‡, §] of assay methods. In these biochemical studies, as well as in the study of Mason et al [6], dihydroxyfumarate (DHF) was an effective cofactor for peroxidase-mediated hydroxylation of tyrosine. Since DHF, unlike dopa, is not itself melanogenic, its use as cofactor has allowed the direct visualization of peroxidase-mediated oxidation of tyrosine to melanin [7] and the subcellular localization of peroxidase-mediated oxidation of tyrosine to melanin in granulocytes [8]. The studies reported in this paper used this method to delineate the subcellular localization of peroxidase-mediated oxidation of tyrosine to melanin in mouse melanoma cells.

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MATERIALS AND METHODS

The Harding–Passey melanoma studied was the moderately melanotic line (obtained from Arthur D. Little, Inc., Cambridge, Mass.) used in our previous histochemical studies.

Light microscopy. Light microscopic studies were performed on 6-μ cryostat sections prefixed in 10% buffered formalin (pH 7.4) for 15 min. Sections were incubated in substrate solution containing 20 mg% L-tyrosine, 32 mg% DHF, and 10^{-4} M hydrogen peroxide in 0.1 phosphate buffer (pH 7.4) at 37° C for 3 hr. Tyrosine was dissolved by boiling for 5 min; the solution was then cooled and DHF was dissolved by stirring at 40° C for 2 hr. Hydrogen peroxide was added last. Controls included: (a) incubation in buffer alone; (b) exposure to 100° C wet heat for 15–30 min before incubation in substrate solu-

TABLE

Results of histochemical reactions

	Tyrosine-DHF-H_2O_2	Dopa Reaction	Benzidine/Diaminobenzidine Reaction
	Light microscopic Groups of positive melanoma cells (type A)	*Light microscopic* Groups of positive type A and type B melanoma cells	*Benzidine* Groups of positive melanoma cells (type A)
	EM Reaction product in melanosomes and GERL	*EM* Reaction product in melanosomes and GERL	*Diaminobenzidine* Reaction product in melanosomes; none in GERL
Effect of Catalase	*Light microscopic and EM* Complete suppression of reaction product	*Light microscopic* Reaction completely or partially suppressed in type A melanoma cells; reaction unaffected in type B melanoma cells *EM* Reaction in GERL of melanoma cells unaffected; number of reacting melanosomes reduced in some cells	*Benzidine and Diaminobenzidine* Reaction suppressed
Effect of Preincubation with DDC	*Light microscopic* Partial suppression of reaction in some type A melanoma cells *EM* Reaction in GERL eliminated; reaction in melanosomes reduced	*Light microscopic* Reaction slightly reduced in type A melanoma cells; reaction eliminated in type B melanoma cells *EM* Reaction in GERL eliminated; number of reacting melanosomes reduced in some cells, reacting melanosomes eliminated in others	*Benzidine and Diaminobenzidine* Reaction unaffected
Effect of Boiling	Reaction suppressed	Reaction suppressed	*Benzidine and Diaminobenzidine* Reaction suppressed

tion; (c) omission of tyrosine, DHF, or hydrogen peroxide; (d) preincubation of sections in 10^{-4} M sodium diethyldithiocarbamate (DDC) at 37° C for 2 hr; this was thoroughly removed by washing before incubation of sections in substrate solution (thiols in the incubation medium produce a nonspecific inhibition of melanogenic oxidase activity [1–4, 9–12, ‖]; (e) addition of 0.2 mg/ml beef liver catalase (Sigma # C-100) to the incubation medium.

For comparison, sections were subjected to the benzidine reaction for peroxidase (method of De Robertis and Grasso [13]) and to the dopa reaction of Laidlaw [14], with controls as described above.

Electron microscopy. 40-μ sections of melanoma were prefixed in Karnovsky's fixative and, after washing with multiple changes of phosphate buffer, were incubated in tyrosine–DHF–hydrogen peroxide substrate solution (as described above) overnight at 4° C. Substrate solution was then changed and incubation was allowed to proceed for an additional 3 hr at 37° C. Controls were performed as described under *Light microscopy*.

For comparison, 40-μ sections were subjected to the EM dopa reaction, following the method of Novikoff et al [15] and the diaminobenzidine (DAB) reaction of Graham and Karnovsky [16], as described in our previous reports [2, 4]. Controls were performed as described under *Light microscopy*.

After osmication, all tissues were washed, dehydrated, and embedded in Araldite. Thin sections were obtained from appropriate areas as judged by observation of 1-μ sections with light microscopy. The thin sections were lightly stained (Venable and Coggeshall lead citrate method, for 2 min) to facilitate observation of reaction product. Sections were viewed with an RCA EMU-3G

‖ Okun M, Patel R, Donnellan B, Edelstein L: Histochemical and biochemical studies of inhibition of peroxidase by thiols (In preparation)

electron microscope or with a JEM 100B electron microscope.

RESULTS (Table)

Light Microscopy

Tyrosine-DHF-hydrogen peroxide reaction. After incubation in substrate solution, foci containing numerous, relatively small tumor cells (designated as type A tumor cells) with grayish-black induced melanin were observed (Fig. 1A). This induced melanin was easily distinguished from yellowish-brown preformed melanin. These reacting cells appeared to be most numerous in areas of tissue damage (e.g., edges of sections, tears). Omission of tyrosine or DHF eliminated the grayish-black reaction product. Reaction product was also absent in boiled sections and sections incubated in buffer alone. Preincubation with DDC resulted in partial suppression of reaction product in some type A cells (Fig. 1B); the presence of catalase in the incubation medium resulted in total suppression of reaction product (Fig. 1C). Omission of hydrogen peroxide without addition of catalase resulted in partial suppression of reaction product; the persistent reaction product under these conditions was due either to preformed hydrogen peroxide or to hydrogen peroxide generated by autooxidation of DHF.

Benzidine reaction. Cells with a positive benzidine reaction for peroxidase corresponded to those with a positive tyrosine–DHF–hydrogen peroxide reaction (type A cells). Reaction was eliminated by boiling or by the absence of hydrogen peroxide. Preincubation with DDC had no inhibitory effect.

Dopa reaction. There were positive cells which corresponded to those observed in the tyrosine–DHF–hydrogen peroxide reaction (type A cells) (Fig. 1D). There were also larger positive cells (Fig. 2) which had no analogues in the tyrosine–DHF–hydrogen peroxide reaction or benzidine reaction. These were designated as type B tumor cells. The type B cells generally contained more preformed melanin than the type A cells. However, grayish-

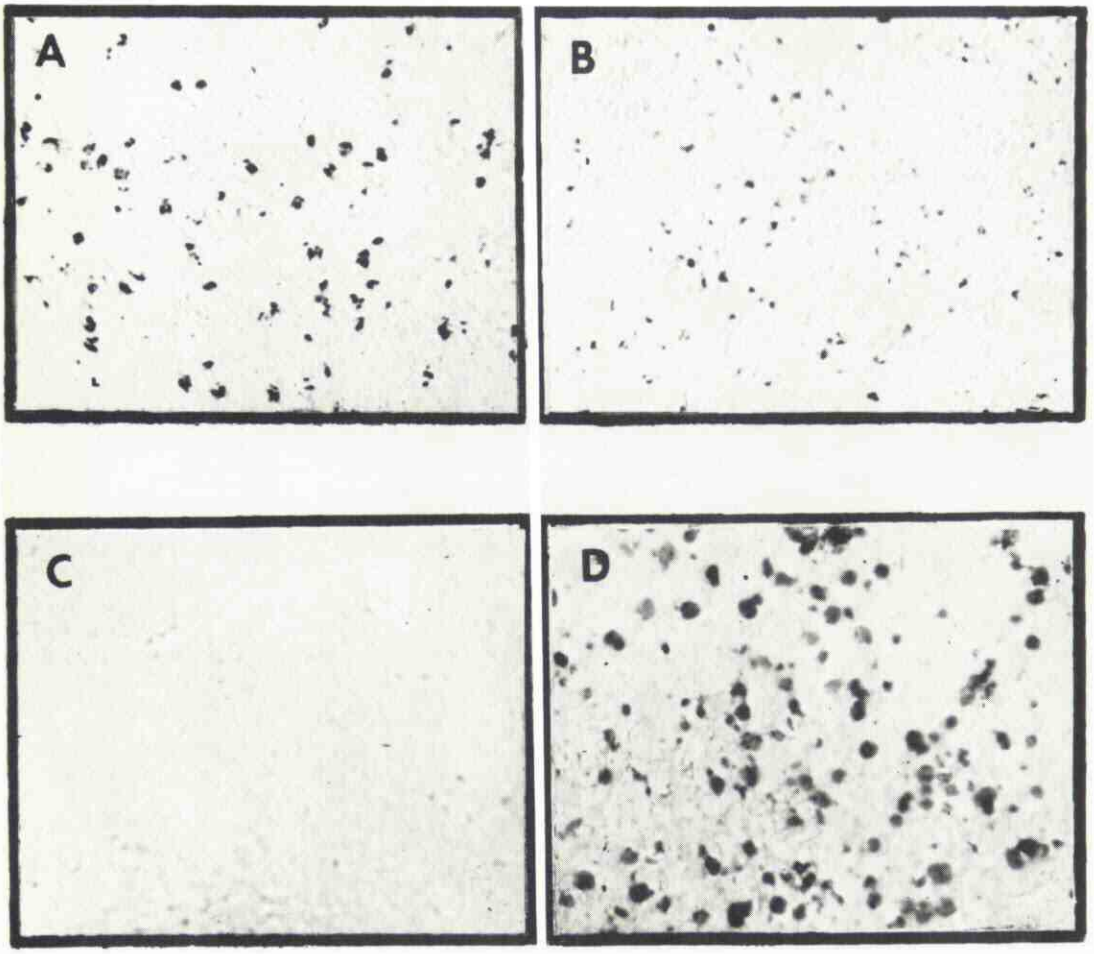

FIG. 1. Corresponding fields of serial cryostat sections of Harding-Passey melanoma illustrate tyrosine-DHF-hydrogen peroxide reaction in tumor cells, the response of the reaction to inhibitors, and comparison with the dopa reaction. A: Without inhibitors. B: After preincubation with DDC. C: Catalase present. D: Dopa reaction. No counterstain. × 190.

black dopa melanin was always easily distinguished from yellowish-brown preformed melanin (Fig. $2a_2$ and $2b_2$).

The dopa reaction was eliminated by omission of substrate (Fig. $2a_1$ and $2b_1$) and was suppressed by boiling sections before incubation.

Preincubation with DDC eliminated the dopa reaction in type B cells (Fig. 2), but had only a partial suppressive effect on type A cells (Fig. $2a_3$ and $2b_3$).

Conversely, incubation in dopa solution containing catalase produced no suppression of the dopa reaction in type B cells, but a high degree of suppression of the dopa reaction in type A cells (Fig. $2a_4$ and $2b_4$).

Electron Microscopy

Tyrosine–DHF–hydrogen peroxide reaction. Induced melanin resulting from incubation in substrate solution was present as particulate or homogeneous areas of electron density (Figs. 3, 4). Reaction product was observed in melanosomes and in the Golgi-associated cisternae of smooth-surfaced endoplasmic reticulum (Fig. 3). In melanosomes, induced melanin was easily differentiated from filamentous preformed melanin (Fig. 4) observed in this moderately pigmented melanoma line.

In preparations preincubated with DDC, reaction product persisted in melanosomes (Fig. 4), but was completely absent from the GERL. The presence of catalase in the incubation medium eliminated reaction product in all areas. The results of other controls paralleled those observed by light microscopy.

Diaminobenzidine reaction. Reaction product of the DAB reaction for peroxidase activity was observed in melanosomes, but not in the GERL, as in our previous study [4]. The results of other controls paralleled those observed by light microscopy.

EM dopa reaction. Results paralleled those of our previous study [4]. Induced melanin was present in both melanosomes and GERL. After preincubation with DDC, induced melanin was present in melanosomes (in reduced numbers), but not in the GERL. The presence of catalase in the incubation medium similarly reduced the number of reacting melanosomes, but had no effect on the reaction in the GERL. Other controls paralleled those observed by light microscopy.

DISCUSSION

The results of this study: (a) add to the evidence that peroxidase can oxidize tyrosine to melanin in the presence of cofactor; (b) confirm the effectiveness of DHF as cofactor for the peroxidatic oxidation of tyrosine and its effectiveness for the light and electron microscopic demonstration of peroxidatic conversion of tyrosine to melanin; (c) confirm the presence of peroxidase activity in melanoma cells; (d) add to the evidence that mam-

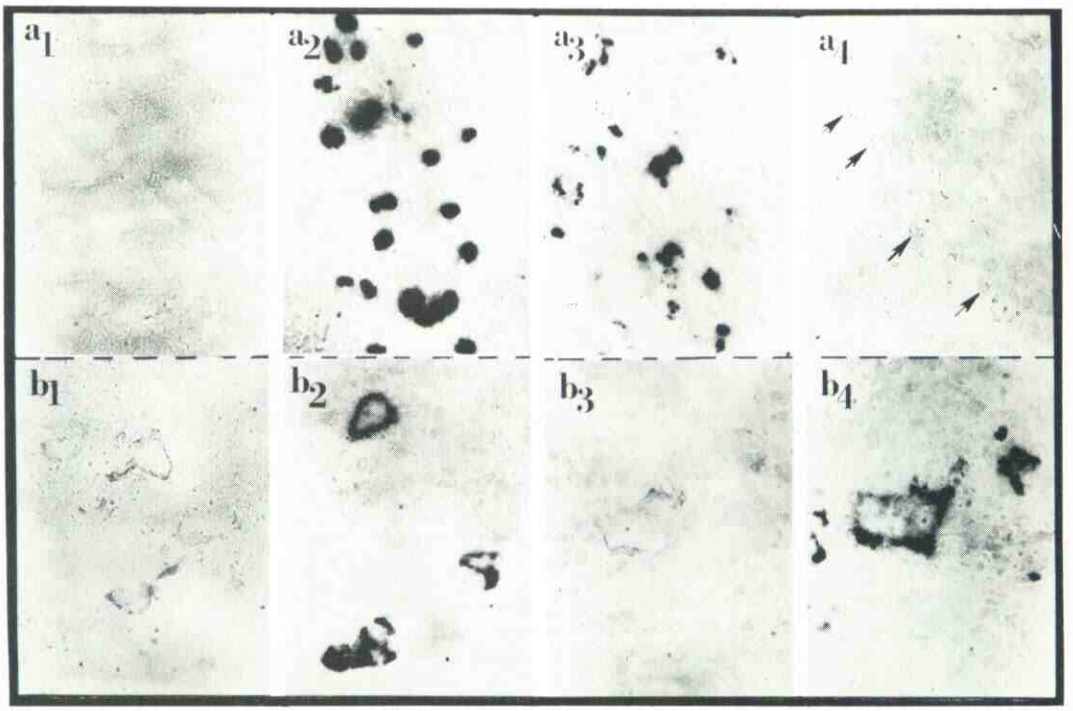

FIG. 2. Corresponding fields of serial cryostat sections of Harding–Passey melanoma illustrate the dopa reaction in type A and type B tumor cells and the response of the reaction to inhibitors. Fields separated by the broken line are from the same section. a_1 and b_1: Incubated in buffer alone. a_2 and b_2: Dopa without inhibitors. a_3 and b_3: DDC preincubation. a_4 and b_4: Catalase. No counterstain. × 380.

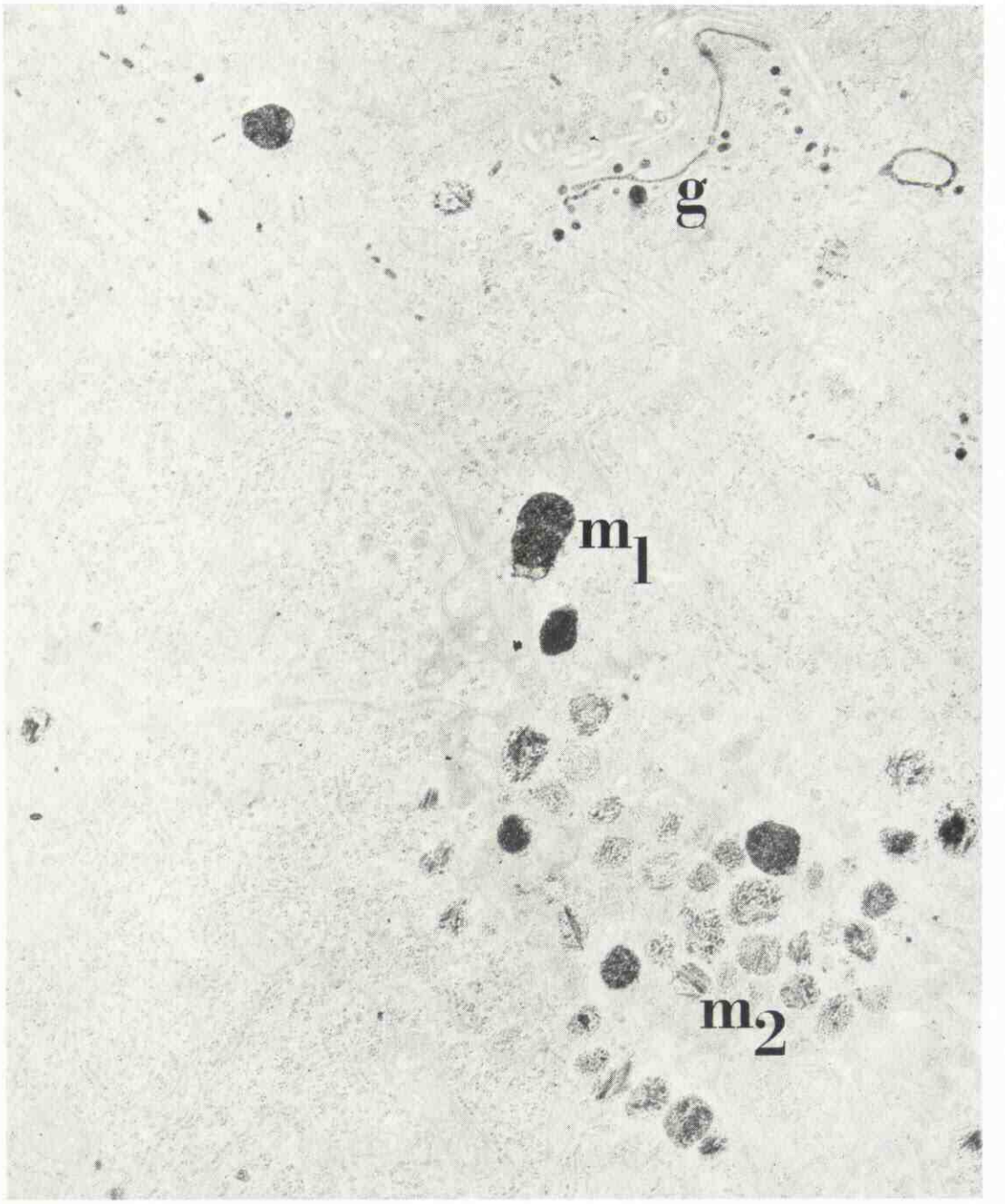

FIG. 3. Electron micrograph of type A melanoma cell subjected to the tyrosine-DHF-hydrogen peroxide reaction without inhibitors shows particulate induced melanin in the GERL (g) and in melanosomes (m_1). Other melanosomes (m_2) show filamentous preformed melanin, but little or no induced melanin. 2-min lead citrate stain. × 25,000.

malian "tyrosinase" is a dopa oxidase with no ability to oxidize tyrosine (evidence paralleled by current biochemical studies of melanoma "tyrosinase" [17]); (e) further delineate the distribution of peroxidase and aerobic dopa oxidase ("tyrosinase") in tumor cells of Harding–Passey melanoma; (f) provide evidence of synergism between peroxidase and aerobic dopa oxidase.

The oxidation of tyrosine to melanin was in all instances entirely peroxidase-dependent as indicated by the complete suppression by catalase; it was also cofactor-dependent. In our previous autoradiographic–histochemical study [3], there was minimal peroxidatic oxidation of tyrosine in peroxidase-containing cells in the absence of added cofactor. This suggests that endogenous cofactor (dopa) may be sufficient to produce enough end-product for detection by the sensitive autoradio-

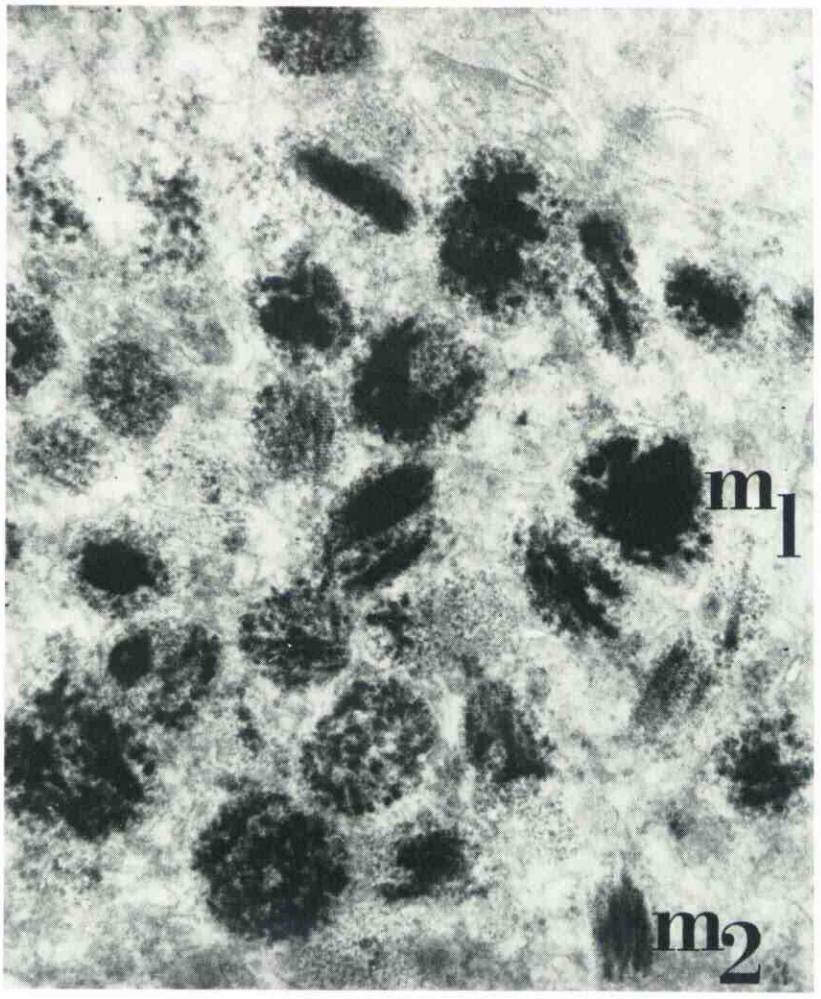

FIG. 4. Electron micrograph of a type A melanoma cell subjected to the tyrosine-DHF-hydrogen peroxide reaction after preincubation in DDC shows particulate and homogenous induced melanin in melanosomes (m_1). Some melanosomes (m_2) show only filamentous preformed melanin. Controls incubated in buffer alone showed only melanosomes with preformed melanin, corresponding to m_2. Induced melanin was therefore easily distinguishable from preformed melanin in this lightly melanized melanoma line. 2-min lead citrate stain. × 55,000.

graphic–histochemical method, but not enough for visualization by histochemical or high resolution histochemical methods. Histochemical studies [18, 19] attributing oxidation of tyrosine to melanin by mammalian "tyrosinase" lacked peroxidase controls and utilized prolonged incubation times which would facilitate the nonenzymatic oxidation of tyrosine.

As our previous studies indicated, the Harding–Passey melanoma line had two principal cell types on the basis of peroxidase and aerobic dopa oxidase ("tyrosinase") activity: the peroxidase-active type A cell and the peroxidase-negative, aerobic dopa oxidase-active type B cell. However, the present study indicates that type A cells have some degree of aerobic dopa oxidase activity.

The results of the inhibitor studies suggest that reaction product in the GERL observed after incubation in tyrosine–DHF–hydrogen peroxide solution was the result of aerobic dopa oxidase acting on dopa synthesized by peroxidase. The reaction product could not have been due to the action of aerobic dopa oxidase ("tyrosinase") on tyrosine, since it was completely catalase-labile. It could not have been due to peroxidase with increased vulnerability to DDC, since the diaminobenzidine reaction showed that peroxidase was not present in the GERL of melanoma cells.

This apparent synergistic action of peroxidase and aerobic dopa oxidase in melanoma cells may be a mechanism of melanogenesis in vivo, and further knowledge of this interaction may be important in understanding this biosynthetic pathway.

The phase of high peroxidase activity may precede the phase of high aerobic dopa oxidase activity in the melanoma cell, with an intermediate stage showing both types of activity. The phase of high peroxidase activity may accumulate dopa for subsequent oxidation by aerobic dopa oxidase;

this would presuppose a system for retarding oxidation of dopa by peroxidase, since peroxidase can convert dopa to melanin. Some melanin formation in vivo may be mediated entirely by peroxidase.

It cannot be entirely excluded that the apparent peroxidase negativity of type B cells is related to relative inaccessibility of peroxidase, with greater membrane damage required for its demonstration (latency) than for that of type A cells. Preliminary histochemical experiments with a heavily melanized Harding–Passey melanoma line revealed benzidine reactivity widely distributed throughout the tumor. Peroxidase activity in normal melanocytes also exhibits latency. Benzidine reactivity of basal melanocytes is much more readily demonstrated in whole mounts of epidermis (particularly when cut into small pieces) than in vertical sections of skin.

The ability to oxidize tyrosine to melanin in the presence of cofactor appears to be a generic property of heme protein peroxidases. It remains to be determined whether other heme proteins, such as catalase, which under certain circumstances can act as a peroxidase (20), can also oxidize tyrosine to melanin. There are no reliable histochemical methods for differentiating the peroxidatic action of catalase from that of peroxidase (21). However, it is unlikely that catalase is solely responsible for peroxidase activity in melanoma cells, since electrophoretic studies in our laboratory (22) have demonstrated peroxidase bands from Harding–Passey melanoma and B16 melanoma which were distinct from the catalase band.

The isolation and characterization of melanoma peroxidase is in progress in our laboratory.

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