Peroxidase, "Protyrosinase," and the Multiple Forms of Tyrosinase in Mice

THOMAS J. HOLSTEIN, CHRISTOPHER P. STOWELL, WALTER C. QUEVEDO, JR., ROBERT M. ZARCARO, AND THERESA C. BIENIEKI

Department of Biology, Roger Williams College, Bristol, Rhode Island 02809, Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912, and Department of Biology, Providence College, Providence, Rhode Island 02918

Mammalian tyrosinase as currently defined has two catalytic functions: 1) the oxidation of tyrosine to L-3,4-dihydroxyphenylalanine (dopa) and 2) the oxidation of dopa to dopa quinone (1-9). Although an imposing body of evidence has been advanced in support of this interpretation, Okun and associates have recently challenged the prevailing concept of the action of tyrosinase (10, 11). They have proposed that within mammalian melanocytes, the oxidation of tyrosine to dopa is mediated by peroxidase whereas the oxidation of dopa to dopa quinone is regulated by dopa oxidase. In their view it is the coupling of peroxidase and dopa oxidase which accounts for "tyrosinase" activity in mammals; i.e., a tyrosinase with catalytic action directed toward both tyrosine and dopa does not exist.

Our previous studies have demonstrated that polyacrylamide gel electrophorograms of supernatant fractions from homogenates of mammalian pigmented hair bulbs reveal the presence of multiple molecular forms of tyrosinase (12, 13). Extensive examination of hair bulbs from various coat-color mutants of the mouse indicate that the multiple molecular forms of tyrosinase are under genetic control (12, 13). Depending on genic constitution, a maximum of three electrophoretically separable forms of tyrosinase (T₁, T₂-T₃) are demonstrable.² In keeping with general practice (14, 15), dopa has been the melanogenic substrate used in our laboratory to demonstrate tyrosinase within polyacrylamide gels. Accordingly, there have been two basic assumptions: first, that tyrosinase revealed by dopa also is able

¹ This investigation was supported by PHS Research Grant CA-06097 from the National Cancer Institute.

² T₁ and T₂ initially are observed as two closely migrating discrete bands of activity but rapidly merge as melanin is deposited in polyacrylamide gels incubated in melanogenic substrates. Since only a single T₁ band is observed in the soluble fraction of mouse melanoma extracts (14, 15), it is possible that a distinction between T₁ and T₃ may not be warranted; hence, in referring to T₁ and T₃, they are joined by a hyphen to reflect this unresolved problem.
to oxidize tyrosine; second, that peroxidase is not involved in the deposition of dopa-melanin under the conditions established for demonstrating tyrosinase activity within polyacrylamide gels.

This paper presents evidence that the multiple forms of tyrosinase from mouse melanoma and hair bulbs possess the ability to utilize both tyrosine and dopa in the initial steps of melanin synthesis. The evidence suggests that, although peroxidase is present in hypodermal ("hair bulb") and melanoma extracts, it can be distinguished from multiple forms of tyrosinase defined by their ability to oxidize both tyrosine and dopa. Specifically, it will be shown that 1) all forms of tyrosinase (T1 and T2-T3) retain the ability to convert tyrosine and dopa to melanin when catalase is present in quantities sufficient to block peroxidase activity within polyacrylamide gel electropherograms, 2) peroxidase overlaps T3-T5 in its electrophoretic mobility but is clearly distinct from T1 in polyacrylamide gels, and 3) T1, selectively isolated from polyacrylamide gels, hydroxylates tyrosine in the presence of catalase as demonstrated by the sensitive Pomerantz radioassay (9).

The enhancement of tyrosinase activity by trypsin treatment of hair bulb and melanoma supernatant fractions observed during this study provides preliminary evidence for the existence of a mammalian protyrosinase. The concept of a vertebrate protyrosinase activated by an appropriate endopeptidase has received strong support from research on amphibian epidermis by McGuire and associates (16, 17). In the present study the term "protyrosinase" is used guardedly since neither its precise nature nor its position in the biosynthetic pathway leading to active mammalian tyrosinase is known.

MATERIALS AND METHODS

The following strains of mice were used: C57BL/6J (a/a), Y/Wi (A0/A0), C3HB/St (mibw/mibw), BUB/Wi (a/a, c/c), C57BL/Ha-e (a/a, e/e). The genes designated indicate departures from the agouti or "wild-type" genotype (13).

Hair bulbs, together with the adipose tissue in which they were embedded, were collected 10 days postplucking (18). Each hair bulb preparation was homogenized in a Potter–Elvehjem homogenizer in chilled sucrose (wet weight/volume = 0.3 g/ml). Homogenates of Harding-Passey melanoma maintained by serial transplantation in C3HB mice were treated in a similar fashion with the exception that the wet weight/volume was 0.1 g/ml. The homogenates were centrifuged at 35,000 g for 30 min at 0°C. In a few cases, the 35,000 g supernatant fractions were re centrifuged at 100,000 g for 1 hr at 0°C. When TPCK–trypsin (Worthington 230 U/mg) was used, it was added to portions of the supernatant fractions (0.3 mg trypsin/ml supernatant) 5–10 min prior to electrophoresis. Aliquots (0.2 ml) of trypsin-treated and untreated supernatant fractions were subjected to polyacrylamide gel electrophoresis according to the method of Davis (19) as modified by Burnett, Seiler, and Brown (14) and Holstein, Burnett, and Quevedo (12).

Demonstration of Tyrosinase within Polyacrylamide Gels

After electrophoresis, the gels were neutralized in 1.0 M phosphate buffer (pH 6.8) for 30 min. The multiple forms of tyrosinase were routinely visualized by incubation of the gels for several hours in a solution of 0.1 M phosphate buffer (pH 6.8) containing either 0.15% L-3,4-dihydroxyphenylalanine (dopa) or 0.2 mM L-tyrosine and 0.2 mM dopa. In one series of experiments involving C57BL hair bulb supernatant fractions, dopa was maintained constant at 0.2 mM and tyro-
sine varied from 0.2 to 8 mM, a variation in tyrosine/dopa concentration of 1:1 to 40:1. The ability of the multiple forms of tyrosinase to convert tyrosine and dopa to melanin in the presence of catalase (Worthington 3100 U/mg) was demonstrated by adding catalase to the gel incubation medium in a concentration of 0.1 mg/ml. The quantity of catalase added was sufficient to block the activity of peroxidase. To preserve the tyrosinase patterns, all gels were stored in 7.5% acetic acid.

The effect of trypsin on isolated tyrosinase was determined as follows: After polyacrylamide gel electrophoresis and neutralization, one of 6-to-12 gels was placed in dopa buffer while the others were maintained in 0.1 M phosphate buffer. The $R_s$ values of the $T_1$ and $T_2$-$T_3$ bands in the dopa-treated gel were determined. Based on these $R_s$ values, the approximate $T_1$ and $T_2$-$T_3$ regions in the unstained gels were cut out, diced, and placed in a beaker containing 3–5 ml of 0.25 M sucrose. Elution of the tyrosinase from the diced gels was accomplished by continuous stirring for 15–20 hr at 4°C. Subsequently, the sucrose solution containing eluted enzyme was separated from the diced gel by low-speed centrifugation. The isolated $T_1$ and $T_2$-$T_3$ tyrosinases from non-trypsin-treated supernatant fractions from hair bulbs of C57BL mice were treated with trypsin, resubjected to electrophoresis, and tested for activity toward dopa.

**Demonstration of Peroxidase within Polyacrylamide Gels**

Peroxidase activity was determined by placing the electropherograms of hair bulb and melanoma supernatant fractions in benzidine reagent following the procedure reported by van Duijn (11, 20). No peroxidase activity was observed when peroxide was omitted from the benzidine reagent. The addition of catalase to the standard benzidine reagent in a concentration of 0.1 mg/ml completely inhibited peroxidase activity within the electropherograms of hair bulb and melanoma supernatant fractions.

**Tyrosine Hydroxylating Capacity of Isolated $T_1$**

The ability of $T_1$ isolated from trypsin-treated and untreated supernatant fractions of Harding–Passey melanoma to hydroxylate tyrosine to dopa was measured according to the method of Pomerantz (9) with certain modifications. The reaction mixture contained 0.15 µmole L-tyrosine, 0.0025 mCi 3, 5-3H-L-tyrosine (ICN, sp act 14 Ci/µmole) and 0.15 µmole dopa in each 1.25-ml volume of 0.1 M phosphate buffer (pH 6.9). To 10 ml of this reaction mixture 1 ml of 0.25 M sucrose containing eluted $T_1$ was added. The background included the counts from a control to which no enzyme had been added. The $T_1$ was eluted from the polyacrylamide gels following the method described above. An additional method employed to ensure precise localization of $T_1$ was to incubate all gels in dopa reagent until a light deposit of melanin was evident at the tyrosinase positions. The $T_1$ region was then cut out, diced, and the enzyme eluted for the radioassay.

**RESULTS**

**Enhancement of Tyrosinase Activity by Trypsinization of Supernatant Fractions**

Trypsin treatment of 35,000 g and 100,000 g supernatant fractions of hair bulb extracts from C57BL (black) mice resulted in a marked increase in melanin deposition at the $T_1$ position when the polyacrylamide gel electropherograms were incu-
bated in 0.15% dopa (Fig. 1). No definite enhancement of activity was found at the T2-T3 position. Also, based on visual inspection of the electropherograms, there was no marked difference in the response of the 35,000 g and 100,000 g supernatant fractions to trypsinization. When T1 and T2-T3 from untreated black hair bulb extracts were isolated from polyacrylamide gels, treated with trypsin, and resubjected to electrophoresis, no enhancement of dopa-melanin deposition was found at either the T1 or T2-T3 positions (Fig. 2). In addition, trypsinization did not appear to convert T1 into T2-T3 or vice versa.

The 35,000 g supernatant fractions of hair bulb extracts from lethal yellow (A'/A) and recessive yellow (e/e) mice revealed enhanced deposition of dopa-melanin at the T1 position when treated with trypsin prior to electrophoresis (Fig. 3). T2-T3 activity normally absent in hair bulbs of lethal yellow and recessive yellow mice was not “induced” by trypsinization of the supernatant fractions. Occasionally, a slight darkening was noted at the T2-T3 position in gels incubated for prolonged periods in dopa. This appeared to be associated with the presence of hemoglobin which approximated T2-T3 in its electrophoretic mobility. Trypsinization of supernatant fractions of hair bulb extracts from albino mice did not result in the deposition of dopa-melanin within the electropherograms. Treatment of 35,000 g supernatant fractions from Harding-Passey melanoma extracts with trypsin resulted in markedly enhanced dopa-melanin deposition at the T1, but not the T2, position in electropherograms (Fig. 4).

Tyrosine Utilization by Tyrosinase from Trypsinized and Nontrypsinized Supernatants

Tyrosine utilization by T1 and T2-T3 in melanin formation was demonstrated in several ways. Increased melanin deposition was found in polyacrylamide gel elec-
trophopherograms of nontrypsinized C57BL hair bulb extracts when tyrosine and dopa were both present in the incubation medium at a concentration of 0.2 mM. The melanin bands were significantly darker than in gels incubated in 0.2 mM dopa alone (Fig. 5). Similar findings were obtained with extracts of Harding–Passey melanoma (Fig. 6). In both cases tyrosine utilization was particularly evident at the T₁ position when the supernatant fractions were treated with trypsin prior to electrophoresis; the elevated melanogenic activity presumably was the result of an increased amount of tyrosinase at the T₁ position. Examination of trypsinized hair bulb supernatant fractions from C57BL mice revealed that the molar ratio of tyrosine to dopa was critical in demonstrating tyrosinase activity. When dopa was maintained at 0.2 mM in the gel incubation medium and tyrosine presented in concentrations of 1:1 and 2:1 relative to dopa, melanin deposition was enhanced compared to that in gels incubated in dopa alone. At tyrosine/dopa ratios from 10:1 to 40:1, melanogenesis was clearly inhibited within the electropherograms (Fig. 7).

Peroxidase overlapped T₂-T₃ in electrophoretic mobility and was clearly distinct from T₁ (Figs. 4, 5, 8, 9). As revealed by the benzidine test, peroxidase from hair bulb preparations usually took the form of two major bands of activity, but, on occasion, additional minor bands were noted. Peroxidase from Harding–Passey melanoma routinely exhibited several prominent bands. In both hair bulb and melanoma preparations tyrosine utilization by T₁ clearly occurred in the absence of comigratory peroxidase.
Fig. 3. Activity patterns of tyrosinase from follicular melanocytes of yellow mice: (A) untreated supernatant fraction from recessive yellow; (B) trypsin-treated supernatant fraction from recessive yellow; (C) untreated supernatant fraction from lethal yellow; (D) trypsin-treated supernatant fraction from lethal yellow. (All gels incubated in 0.15% dopa.)

Fig. 4. Activity patterns of tyrosinase and peroxidase from Harding-Passey melanoma: (A) tyrosinase in untreated supernatant (substrate = 0.15% dopa); (B) tyrosinase in trypsin-treated supernatant fraction (substrate = 0.15% dopa); (C) peroxidase in trypsin-treated supernatant fraction (benzidine reagent).
In all cases, addition of catalase in a concentration of 0.1 mg/ml to the benzidine reagent completely inhibited peroxidase activity within the electropherograms (Fig. 9). In striking contrast, catalase did not inhibit the utilization of tyrosine and/or dopa by the multiple forms of tyrosinase from hair bulbs and melanoma (Fig. 9). The intensity of melanin deposition at all tyrosinase positions within electropherograms was unaffected by the addition of catalase to the incubation medium.

The radioassay method demonstrated tyrosine hydroxylation by melanoma $T_1$ eluted from electropherogram “$T_1$-fragments” which were dissected so as to exclude contamination by peroxidase. $T_1$ catalyzed the formation of $^3$H-OH from 3, 5-$^3$H-L-tyrosine as a byproduct in the conversion of tyrosine into dopa. $T_1$ from melanoma supernatant fractions trypsinized prior to electrophoresis showed a 2- to 4-fold greater tyrosine hydroxylating capacity than that from nontrypsinized supernatants. Addition of catalase (0.1 mg/ml of reaction mixture) did not prevent the conversion of tyrosine into dopa (Table 1).

DISCUSSION

This study clearly indicates that murine tyrosinase derived from pigmented hair bulbs and Harding–Passey melanoma is capable of catalyzing both the oxidation of tyrosine to dopa and of dopa to higher melanogenic intermediates. In this respect, it joins numerous other papers which have supported this interpretation for
**Fig. 6.** Activity patterns of tyrosinase from Harding–Passey melanoma. Electropherograms of untreated supernatant fractions incubated in (A) 0.07 mM dopa, and (B) 0.07 mM dopa and 0.83 mM L-tyrosine; electropherograms of trypsin-treated supernatant fractions incubated in (C) 0.07 mM dopa, and (D) 0.07 mM dopa and 0.83 mM L-tyrosine.

**Fig. 7.** Activity patterns of tyrosinase in electropherograms of trypsin-treated supernatant fractions of C57BL hair bulb extracts: gels incubated in (A) 0.2 mM dopa, (B) 0.2 mM dopa and 0.2 mM L-tyrosine, (C) 0.2 mM dopa and 0.4 mM L-tyrosine, (D) 0.2 mM dopa and 2 mM L-tyrosine, (E) 0.2 mM dopa and 4 mM L-tyrosine, and (F) 0.2 mM dopa and 8 mM L-tyrosine.
the role of mammalian tyrosinase in the initial steps of the biosynthetic pathway leading to melanin (1–9). It does not support the view that peroxidase acting either alone or coupled with a dopa oxidase accounts for the enzymatic activities currently attributed to mammalian tyrosinase (10, 11). Although the present study does not rule out the possibility that peroxidase and tyrosinase (dopa oxidase) activities are in some way coupled or synergistic in vivo, it does demonstrate that peroxidase activity is not required to support the action of a mammalian tyrosinase defined by its ability to convert tyrosine and dopa to melanin in vitro. Three lines of evidence support this conclusion. First, all multiple forms of tyrosinase deposit tyrosine- and dopa-melanin when polyacrylamide gel electropherograms bearing these enzymes are incubated in the presence of catalase in concentrations adequate to block peroxidase activity. Second, tyrosine- and dopa-melanin are deposited at the T1 position in polyacrylamide gels in the absence of comigratory peroxidase. Third, melanoma T1 carefully isolated from polyacrylamide gels so as to exclude the peroxidase comigratory with T2 carries out the hydroxylation of tyrosine to dopa in the presence and absence of catalase.

An appropriate molar ratio of tyrosine to dopa appears to be critical for demonstrating the utilization of tyrosine by hair bulb tyrosinase within polyacrylamide gels. This is most likely the result of substrate inhibition of the type reported by Pomerantz (9). Since melanoma tyrosinase converts tyrosine to melanin at tyrosine/dopa molar ratios inhibitory to hair bulb tyrosinase, there is probably less tyrosinase in the hair bulb extracts and hence a greater opportunity for substrate inhibition to occur. This interpretation is consistent with the relatively fewer melan-
nin-forming cells in hair bulb preparations compared to those of melanomas and the significant differences in the ease with which the two tyrosinase sources are homogenized.

Based on evidence available to date, the enhanced T₁ activity observed after trypsinization of hair bulbs and melanoma supernatant fractions might result from

**TABLE 1**

**Tyrosine Hydroxylating Activity of Isolated T₁ from Harding-Passey Melanoma as Determined by the Method of Pomerantz (9)**

| Activity of T₁ isolated from electropherograms of untreated and trypsin-treated supernatant fractions | cpm (²H-OH)⁶ Experiment |
|---------------------------------------------------------------|-------------------------|
|                                                              | 1⁶                       |
| Without trypsin treatment                                     | 517                      |
| With trypsin treatment                                        | 923                      |
| With trypsin treatment + catalase added to the radioassay reaction mixture (0.1 mg/ml) | -                        |

*Background subtracted in all experiments (background = counts from a control sample to which no T₁ was added).*

*Dopa treatment of one gel used to detect T₁ position in remaining gels (T₁ isolated from unstained gels).*

*T₁ isolated after short incubation of all the gels in dopa.*

*T₁ isolated as in b; incubation time 1.5 hr as compared to 1 hr in Expts. 1 and 2.*
1) the conversion of prototyrosinase to tyrosinase by enzymatic removal of a polypeptide forming part of its primary structure, or 2) the removal of an inhibitor (21, 22) forming part of an inhibitor-tyrosinase complex possibly involving covalent linkages, or 3) the release of tyrosinase bound to cytomembranes (23). The latter explanation would seem unlikely based on the observation of significant tyrosinase activation in trypsinized supernatant fractions obtained at 100,000 g. At present it is not possible to distinguish between the first and second possibilities. Indications of a “true” prototyrosinase in vertebrates comes from research on the frog in which McGuire and associates have advanced considerable evidence for a proenzyme activated by an appropriate endopeptidase (16, 17). They have tentatively identified the fragment detached from the apoenzyme by the endopeptidase. This finding suggests at least for lower vertebrates the existence of an additional site for posttranslational control of tyrosinase activity and hence of melanogenesis. The results of the present study, in a tentative way, raise the same possibility for mammalian melanogenesis. A variety of proteases and protease inhibitors have been demonstrated in mammalian skin (24, 25). Possibly represented in this assemblage is a protease devoted to the activation of prototyrosinase. Lee and Lee (26) have proposed that the MSH-induced enhancement of melanogenesis within frog skin may involve the hormone-induced release of a protease which in turn activates prototyrosinase. In view of the observed enhancement of mammalian melanogenesis by MSH (22, 27), it is possible that a similar mechanism may be involved. Lee, Lee, and Lu (22), however, proposed that MSH enhances mammalian tyrosinase activity by the removal of an inhibitor from tyrosinase molecules.

The observation that additional T₁ is formed by trypsinization of supernatant fractions lends further support to a view advanced earlier that T₁ stands close to the primary action of the c locus (13). More direct evidence for the existence of mammalian prototyrosinase is currently being sought.

SUMMARY

The multiple forms of tyrosinase isolated from pigmented hair bulbs and Harding–Passey melanoma of mice have been demonstrated to utilize tyrosine and dopa in the synthesis of melanin within polyacrylamide gel electropherograms. Although peroxidase activity can be demonstrated in electropherograms of extracts of these tissues, there appears to be no requirement for peroxidase support of tyrosinase in the utilization of tyrosine or dopa as a melanogenic substrate. Hair bulb and melanoma tyrosinases utilize tyrosine and dopa in the presence of catalase sufficient to block peroxidase activity within polyacrylamide gels. The sensitive Pomerantz radioassay reveals that tyrosinase, isolated from electropherograms without associated peroxidase, catalyzes the hydroxylation of tyrosine both in the presence and absence of catalase. Although alternative theories are not excluded, the observed enhancement of tyrosinase (T₁) activity on treatment of hair bulb and melanoma supernatant fractions with trypsin might indicate the existence of a mammalian “protyrosinase” akin to that demonstrated in amphibians by other investigators.

REFERENCES

1. Duchon, J., Fitzpatrick, T. B., and Seiji, M. Melanin 1968: Some definitions and problems. In "1967–68 Year Book of Dermatology," (A. W. Kopf and R. Andrade, Eds.), p. 6. Year Book Medical Publishers, 1968.

2. Pomerantz, S. H., and Warner, M. C. 3,4-Dihydroxy-L-phenylalanine as the tyrosinase cofactor. J. Biol. Chem. 242, 5308 (1967).
3. Pomerantz, S. H. Separation, purification, and properties of two tyrosinases from hamster melanoma. *J. Biol. Chem.* 238, 2351 (1963).

4. Pomerantz, S. H., and Li, J. P-C. Tyrosinases (hamster melanoma). In "Methods in Enzymology," (S. P. Colowick and N. O. Kaplan, Eds.), Vol. 17 (part A), p. 620. Academic Press, New York, 1970.

5. Brown, F. C., and Ward, D. N. Studies on mammalian tyrosinase. I. Chromatography on cellulose ion exchange agents. *J. Biol. Chem.* 233, 77 (1958).

6. Fitzpatrick, T. B., Miyamoto, M., and Ishikawa, K. The evolution of concepts of melanin biology. *Arch. Dermatol.* 96, 305 (1967).

7. Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and summerson, W. H. Mammalian tyrosinase: preparation and properties. *J. Biol. Chem.* 178, 185 (1949).

8. Lerner, A. B. Metabolism of phenylalanine and tyrosine. *Adv. Enzymol.* 14, 73 (1953).

9. Pomerantz, S. H. The tyrosine hydroxylase activity of mammalian tyrosinase. *J. Biol. Chem.* 241, 161 (1966).

10. Okun, M. R., and Donnellan, B. Ultrastructural relationship of melanocytes to mast cells and "melanophages" in a lesion of alopecia mucinosa. *J. Invest. Dermatol.* 59, 211 (1972).

11. Okun, M. R., Edelstein, L. M., Or, N., Hamada, G., Donnellan, B., and Lever, W. F. Histochemical differentiation of peroxidase-mediated from tyrosinase-mediated melanin formation in mammalian tissues. *Histochemie* 23, 295 (1970).

12. Holstein, T. J., Burnett, J. B., and Quevedo, W. C., Jr. Genetic regulation of multiple forms of tyrosinase in mice: action of a and b loci. *Proc. Soc. Exp. Biol. Med.* 126, 415 (1967).

13. Holstein, T. J., Quevedo, W. C., Jr., and Burnett, J. B. Multiple forms of tyrosinase in rodents and lagomorphs with special reference to their genetic control in mice. *J. Exp. Zool.* 177, 173 (1971).

14. Burnett, J. B., Seiler, H., and Brown, J. V. Separation and characterization of multiple forms of tyrosinase from mouse melanoma. *Cancer Res.* 27, 880 (1967).

15. Burnett, J. B. The tyrosinases of mouse melanoma. Isolation and molecular properties. *J. Biol. Chem.* 246, 3079 (1971).

16. McGuire, J. S. Activation of epidermal tyrosinase. *Biochem. Biophys. Res. Commun.* 40, 1084 (1970).

17. Barisas, B. G., Newman, D., and McGuire, J. S. A proteolytically activated tyrosinase from frog epidermis. In "Abstracts of Yale University Conference on Pigment Cell Biology, *Yale J. Biol. Med.*, 46, 472-482 1973.

18. Burnett, J. B., Holstein, T. J., and Quevedo, W. C., Jr. Electrophoretic variations of tyrosinase in follicular melanocytes during the hair growth cycle in mice. *J. Exp. Zool.* 171, 369 (1969).

19. Davis, B. J. Disc electrophoresis. *Ann. N. Y. Acad. Sci.* 121, 404 (1964).

20. Pearse, A. G. E. "Histochemistry," 2nd ed., p. 903. Little, Brown, Boston, 1960.

21. Hamada, T., and Mishima, Y. Intracellular localization of tyrosinase inhibitor in amelanotic and melanotic malignant melanoma. *Brit. J. Dermatol.* 86, 385 (1972).

22. Lee, T. H., Lee, M. S., and Lu, M-Y. Effects of α-MSH on melanogenesis and tyrosinase of B16 melanoma. *Endocrinology* 91, 1180 (1972).

23. Seiji, M., Itakura, H., and Irimajiri, T. Tyrosinase in the membrane system of mouse melanoma. In "Pigmentation: Its Genesis and Biologic Control" (V. Riley, Ed.), p. 525. Appleton-Century-Crofts, New York, 1972.

24. Fräki, J. E., and Hopsu-Havu, V. K. Human skin proteases. Partial purification and characterization of a protease inhibitor. *Arch. Dermatol. Forsch.* 243, 153 (1972).

25. Troll, W., Klassen, A., and Janoff, A. Tumorigenesis in mouse skin: inhibition by synthetic inhibitors of proteases. *Science* 169, 1211 (1970).

26. Lee, T. H., and Lee, M. S. Studies on MSH-induced melanogenesis: effect of long-term administration of MSH on the melanin content and tyrosinase activity. *Endocrinology* 88, 155 (1971).

27. Pomerantz, S. H., and Chuang, L. Effects of β-MSH, cortisol and ACTH on tyrosinase in the skin of newborn hamsters and mice. *Endocrinology* 87, 302 (1970).