Tyrosinase Inhibition: Its Role in Suntanning and in Albinism

Abstract. *Tyrosinase inhibitor (molecular weight less than 5000; extracted from various melanomas) fully inhibits soluble tyrosinase but only partially inhibits tyrosinase "aggregated" into melanosomes; the inhibitor can be inactivated by ultraviolet light. S91 Albinotyrosinase Type B apparently cannot "aggregate" into melanosomes because its protein carrier is genetically altered. Therefore, albinotyrosinase remains vulnerable to its inhibitor and cannot produce melanin, even though the enzyme has a functioning active center.*

Tyrosinase inhibition was postulated as an important factor in melanogenesis many years ago (*1*). We obtained evidence of a tyrosinase inhibitor and its site of action in vivo during investigations with a nonpigmented albino strain of the S 91 mouse melanoma. We deduced the presence of a tyrosinase inhibitor because, in one of the two albino mutants of this tumor, we found extractable tyrosinase that, although very active after purification, was inactive in the tumor in vivo and in homogenates of whole tumors in vitro. The postulated tyrosinase inhibitor was purified from the S 91 albino mutant by the method outlined below. We used the same methods with the original wild, S 91 pigmented strain, the Harding-Passey and B 16 mouse melanomas, and the Fortner melanoma in the hamster (*2*).

After having been harvested, the tumors were stored at −20°C and thawed just before use. They were homogenized with distilled water (one part by weight to one part by volume) in a Waring blendor, and thereafter ground in a Potter-Elvehjem vessel. We centrifuged the whole homogenate at 10,000*g* to remove nuclei, mitochondria, debris, and all heavily melanized melanosomes. The remaining supernatant was boiled for ½ hour, after which all proteins coagulated by heat were removed by centrifugation at 40,000*g* for 20 minutes. The clear supernatant containing the heat-stable inhibitor could be used against tyrosinase preparations. We further purified the inhibitor by passing the clear supernatant over a Sephadex-G 25 column. The inhibitor was retained in the gel and could easily be eluted with distilled water.

Tyrosinase exists in melanoma cells in a form bound to ribonucleoprotein (RNP) particles on which the enzyme is synthesized (Ty I). It is then released into the cytoplasm where it exists in a freely extractable, soluble form (Ty II). Apparently Ty II then "aggregates" into the quarternary structure of the melanosomes (Ty III) where the enzyme oxidizes tyrosine to melanin by way of dihydroxyphenylalanine (dopa).

These three tyrosinase fractions were prepared as follows: whole homogenates of melanomas (one part by weight of tumor to ten parts by volume of water) were treated with one-tenth volume of saturated ammonium sulfate and with an amount of acetone equal to that of homogenate plus ammonium sulfate according to the method of Brown and Ward (*3*).

A heavily colored precipitate formed which contained a mixture of pigmented compounds as well as the RNP-bound tyrosinase (Ty I). The supernatant, after treatment with another volume of acetone, yielded a fine, almost white precipitate that contained freely extractable tyrosinase, soluble in vivo (Ty II). This tyrosinase was further purified by column chromatography in 0.03*M* barbital buffer at *p*H 8.6 on a diethylaminoethyl (DEAE) Sephadex column. The enzyme was retained and could be eluted with 0.08*M* phosphate buffer at *p*H 7.2. For special purposes the enzyme could be further purified with curtain electrophoresis in barbital buffer at *p*H 8.6 (*4*). Tyrosinase "aggregated" into melanosomes was prepared from melanosomes (Ty III) obtained in density-gradient fractions free of other cell constituents (*5*).

Table 1. Percentage of inhibition of dopa oxidation in presence of RNP-bound tyrosinase (Ty I), soluble tyrosinase (crude Ty II or purified Ty II), or the melanosome fraction (Ty III) by the inhibitor treated with ultraviolet light (UV) or dialyzed (D).

Treatment of inhibitor	Ty I	Crude Ty II	Purified Ty II	Ty III
S 91 Pigmented mouse melanoma				
None	100	100	100	25
D	0	0		0
UV	0	0		0
S 91 Albino mouse melanoma				
None	100	100	100	25
D	0	0		0
UV	0	0		0
Harding Passey mouse melanoma				
None	0	15	100	0
D	0	0		0
UV	0	0		0
B 16 Mouse melanoma				
None	0	15	100	0
D	0	0		0
UV	0	0		0
Fortner hamster melanoma				
None	0	20	100	0
D	0	0		0
UV	0	0		0

The inhibitor was tested for suppression of dopa oxidation by the three types of tyrosinase prepared as outlined above. Tyrosinase activity or its inhibition was followed spectrophotometrically (*6*). The concentrations of enzymes and of the inhibitor were adjusted so that their ratios in the test tube reflected their assumed original concentrations in vivo. In the S 91 melanomas the inhibitor completely suppressed dopa oxidation when tested against both crude (before DEAE-Sephadex chromatography) and purified (after DEAE-Sephadex chromatography) preparations of soluble tyrosinase. The inhibitor was also effective against RNP-bound tyrosinase. But, when it was tested against melanosome fractions, the inhibitor effected only a 25 percent suppression of dopa oxidation (Table 1).

In the Harding-Passey and B 16 mouse melanomas and in the Fortner hamster melanoma (*7*), only purified soluble tyrosinase fractions could be inhibited.

The inhibitor is dialyzable. Ultraviolet irradiation of solutions containing the purified inhibitor inactivated it; this was accomplished by either of these methods: (i) 3 ml of a solution containing purified inhibitor was irradiated for 1 hour in a petri dish (9 cm in diameter) with a Model B-100A Blak-Ray ultraviolet lamp emitting ultraviolet light (3400 to 3800 Å), or (ii) 2 ml of a solution containing the purified inhibitor was exposed to monochromatic irradiation of 2800 Å in a quartz tube for 30 minutes (2.5×10^8 erg/cm^2 per second) (*8*).

Since the inhibitor is retained in G-25 Sephadex gel, its molecular weight can be assumed to be less than 10,000. Its kinetics are those of competitive inhibition (*9*).

The inactivation of the inhibitor by ultraviolet light may account for the induction of suntanning after ultraviolet exposure (*10*). It is conceivable that the tyrosinase inhibitor not only inhibits the fully functioning enzyme but represses the gene which regulates the synthesis of tyrosinase. When the repressor is inactivated or destroyed,

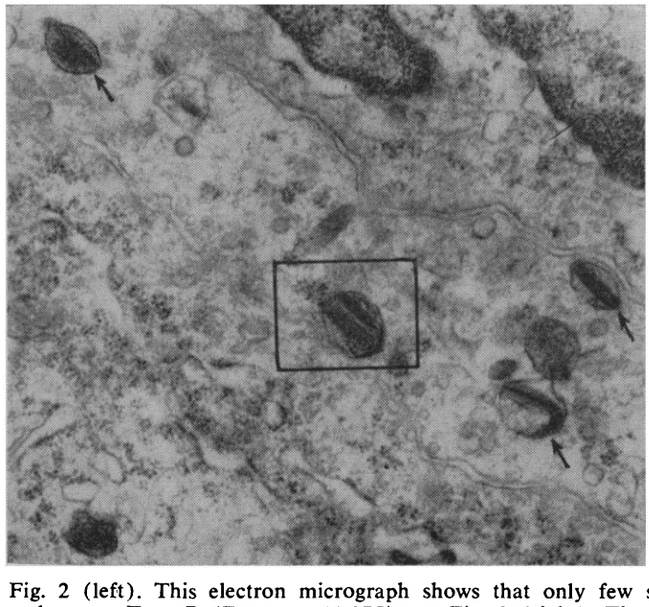
Fig. 1. A melanosome. Note the regular arrangement of fine "ripples" in each longitudinal strand of this melanosome in the early stages of melanization (Epon, × 53,250).

the gene is derepressed. This, in turn, would lead to an increased synthesis of tyrosinase, followed by an increased number of melanosomes into which the tyrosinase has aggregated.

The inhibition of the RNP-bound tyrosinase and of freely soluble tyrosinase would seem to explain why these two enzyme fractions do not oxidize tyrosine in vivo. Our data suggest that tyrosinase begins to oxidize tyrosine to melanin only after the enzyme has "aggregated" into the stable, protected quaternary structure of the melanosome where it can no longer be inhibited (Table 1). The lack of inhibition of crude preparations of tyrosinase from the Harding-Passey, B 16, and Fortner melanomas suggests that in these tumors there is a substance which either destroys the inhibitor or which interferes with its action. Purified tyrosinase obtained from these tumors is readily inhibited (Table 1).

Two mutations that consisted of distinctly white areas amidst pigmented tissue were observed in the pigmented S 91 mouse melanoma. Careful selection of these nonpigmented areas in transplantation produced two types of albino melanomas. Type A had no trace of tyrosinase activity when extractions were carried out as described above. With the electron microscope no melanosomes or structures resembling them could be detected in the Type A albino mutant. It was possible, however, to extract the inhibitor which suppressed tyrosinase from the albino as well as from the wild strain. Therefore, we concluded that the loss of color in the Type A mutation was not due to a change in the inhibitor, but rather to the assumed deletion or complete lack of activity of the enzyme tyrosinase.

In the Type-B mutation, melanosomes that differed in structure from those of the wild pigmented strain and that had a low incidence could be detected with the electron microscope (Fig. 1–3). Whole homogenates of Type B, S 91 albino melanomas did not give a positive dopa reaction, whereas whole homogenates of the wild pigmented strain did. Upon fractionation in ammonium sulfate and acetone, however, Type B albino melanomas were found to have activity of RNP-bound tyrosinase (Ty I) and of soluble tyrosinase (Ty II). Freely



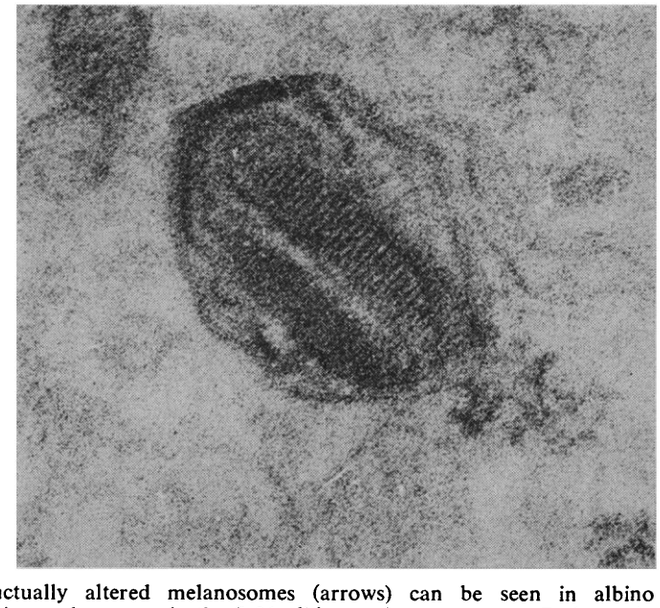


Fig. 2 (left). This electron micrograph shows that only few structually altered melanosomes (arrows) can be seen in albino melanomas Type B (Epon, × 11,875). Fig. 3 (right). The albino melanosome in the S 91 albino melanoma mutant B shown in the rectangular area of Fig. 2 is enlarged. It can be seen that one of the two strands in the melanosome shows "ripples" similar to the ones observed in melanosomes of the wild pigmented strain (Fig. 1), but the other strand shows a complete blurring out of structure. Apparently in this cell small traces of normal tyrosinase had been manufactured together with larger amounts of the mutant albino tyrosinase (Epon, × 54,000).

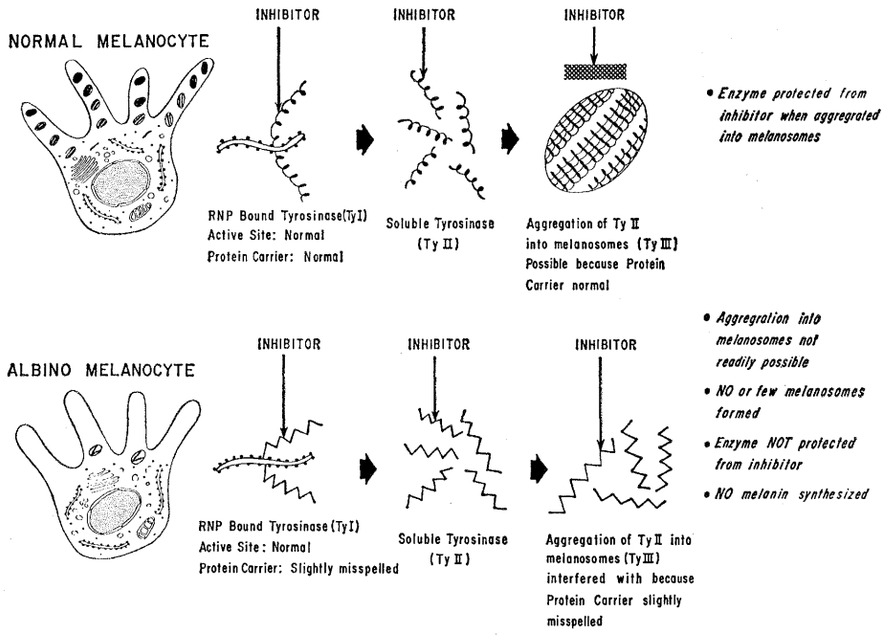


Fig. 4. Concept of the molecular cause of albinism.

extractable, soluble albino tyrosinase (Ty II) was further purified by DEAE-Sephadex chromatography and curtain electrophoresis as described above. The electrophoresis on acrylamide gel (*11*) of highly purified tyrosinase from the wild pigmented melanoma was compared with that of the albino mutant enzyme. The albino tyrosinase consistently had a slightly lower anodic electrophoretic mobility which suggested a small change in the overall charge of the protein carrier. Michaelis constants of the enzymes from the wild pigmented and the albino mutant melanomas were about equal (2.2 and 2.4 $\times$ $10^{-4}M$ dopa) which suggested that little or no difference existed in the active centers of the two enzymes. The specific activity of the enzyme from the albino mutant was slightly higher than that of the enzyme from the wild pigmented strain (110 $\pm$ 7 and 98 $\pm$ 6, respectively).

When albino tyrosinase was tested against its own albino inhibitor, prepared as described above, the RNP-bound (Ty I) and the freely soluble forms of tyrosinase (Ty II) were fully inhibited. Tyrosinase "aggregated" into melanosomes (Ty III), however, was inhibited only 25 percent (Table 1). The melanosome fraction of the Type B melanoma had only weak activity to begin with, because, as shown by electron microscopy, only a few structurally altered melanosomes were present. Therefore only small amounts of albino tyrosinase apparently had "aggregated" into the stable form of the melanosome where it was protected from its inhibitor.

Thus, we conclude that in the Type A mutation of the S 91 mouse melanoma the enzyme tyrosinase seems to be completely deleted, because tyrosinase is not extractable and melanosomes cannot be detected with electron microscopy. A fully functioning inhibitor is present. In the Type B mutation of the S 91 mouse melanoma, an albino tyrosinase is extractable and a few melanosomes are detectable with electron microscopy. The ultrastructure of these few albino melanosomes is different from that of the abundant melanosomes in the wild pigmented strain. Tyrosinase activity can be demonstrated in vitro after the inhibitor is removed by cell fractionation. Both the RNP-bound and the freely soluble forms of this albino enzyme are susceptible in vitro to the normally present inhibitor. We also believe that through mutation a small change took place in the protein carrier of the albino enzyme, as detected by refined electrophoresis. The active center of the mutant enzyme does not seem to be altered since the Michaelis constant is almost identical to that of the wild enzyme. In the wild pigmented strain, tyrosinase apparently "aggregates" itself into melanosomes where the enzyme is protected from its inhibitor. Therefore, despite the presence of an inhibitor, tyrosinase activity can be shown in the whole tumor as well as in whole homogenates. In the Type B albino mutant the small change in the protein carrier of the enzyme does not allow the "aggregation" of albino tyrosinase into melanosomes. As a result, the albino tyrosinase does not reach its stable form in the melanosome where it would be protected from its inhibitor. Thus, melanosome formation and melanin production cannot take place in vivo, although albino tyrosinase with a fully functioning active center is present (Fig. 4).

The application of these findings in mouse and hamster tumors to cutaneous albinism in man is not easy, but there are indications that in human albino hair bulbs a few malformed melanosomes are present and that traces of tyrosinase are demonstrable with histochemistry under favorable substrate conditions (*12–14*). It seems likely that the tyrosinase inhibitor is washed out during histochemical procedures. Since RNP-bound tyrosinase and tyrosinase "aggregated" into a few melanosomes remain in the histologic tissue section, a positive dopa reaction may be obtained under these circumstances. Thus it appears that the Type B mutation in the S 91 albino melanoma may be an experimental model of the conditions as they prevail in albinism in man.

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References and Notes

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7. Independently of our own investigations, Satoh and Mishima, at the Federation Meetings, Atlantic City, April 1966, reported the presence of an inhibitor in the Fortner hamster melanoma.
8. We thank Dr. Madhukar Pathak for help and collaboration in carrying out the ultraviolet irradiation at 2800 Å.
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