Regulatory Mechanisms of Tyrosinase Activity in Melanocyte. I.

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The activity of tyrosinase (EC 1.10.3.1 o-diphenol:O₂ oxidoreductase) was found to be in melanosomes, smooth-surface membranes, rough-surface membranes, and ribosomes when they are isolated from Harding-Passey mouse melanoma. In vivo incorporation studies of ¹⁴C-dopa into these cell particles were carried out in order to clarify the site of melanin formation in melanocytes. ¹⁴C-dopa incorporation occurred only in melanosomes. If the site of the dopa incorporation could be accepted as the site of melanin formation, it was concluded that melanin was synthesized only in melanosomes (1). Although, in mammals, tyrosinase is generally believed to occur only in the presence of melanin, and vice versa, tried out in order to clarify the site of melanin formation in melanocytes. ¹⁴C-dopa despite the presence of tyrosinase activity. It is considered, therefore, that the presence of tyrosinase activity is not necessarily correlated to the formation of melanin in melanocyte. How does tyrosinase exist in smooth-surface membranes, rough-surface membranes or ribosomes? Is it in a form of protyrosinase or is it inhibited in some way?

The presence of naturally occurring inhibitors of tyrosinase has been reported in the serum (2) and melanoma tissues (3–5). Satoh et al. (4) found the presence of tyrosinase-inhibitory materials in the hamster melanomas, and they were shown to be heat stable, dialyzable and did not exhibit sulfhydryl properties. Chian et al. (5) have isolated the tyrosinase inhibitor from mouse melanomas, and it was found to be heat stable, dialyzable and could be inactivated by ultraviolet irradiation. The inhibitor isolated fully inhibited soluble tyrosinase but only partially inhibited melanosome tyrosinase.

In the present studies, the regulatory mechanisms of tyrosinase activity in the melanocytes have been studied in vivo and in vitro. The tyrosinase-inhibitory material was found to be present in the soluble fraction of melanoma tissue. The inhibitory effect was examined in vitro on soluble tyrosinase and on tyrosinase associated with smooth-surface membranes and melanosomes obtained from melanomas, and also on melanogenesis in the melanocyte in vivo.

MATERIALS AND METHODS

1. Preparation of soluble tyrosinase. Harding-Passey mouse melanomas used in the experiments reported here were serially transplanted into a strain of Swiss mice.
The entire, actively growing tumors were excised when their diameter reached 1.0–1.5 cm, and were promptly homogenized in 5 vol ice-cold distilled water at about 0°C. The homogenate was centrifuged at 700 g for 10 min and the supernatant (1 mg protein/ml) was incubated with 200 μg of trypsin in \( \frac{1}{15} M \) phosphate buffer, pH 7.2 at 6°C for 18 hr. After the digestion, the mixture was centrifuged at 105,000 g for 60 min. The supernatant obtained was condensed and subjected to gel filtration through a Sephadex G-100 column (2.5 × 100 cm), equilibrated with a 0.01 M potassium phosphate buffer, pH 7.0; the elution was conducted with the same buffer. Fractions of 5 ml were collected and analyzed for protein and tyrosinase activity. Tyrosinase activity was recovered in fractions 28–54. The tyrosinase-rich fractions of the Sephadex eluate (fractions 44–54) were combined, lyophilized, and kept in a deep freezer at −20°C until use.

2. Preparation of melanosomes and smooth-surface membranes. The entire, actively growing melanoma was excised and homogenized in 0.25 M sucrose at about 0°C. The cell fractionation method was essentially that of Seiji (1). The nuclear fraction was prepared by centrifuging the homogenate for 10 min at 700 g. The resulting supernatant, when centrifuged at 11,000 g for 10 min, yielded a sediment which was resuspended in 0.25 M sucrose and recentrifuged at 15,000 g for 10 min. The sediment was again suspended in 0.25 M sucrose and designated as the large-granule fraction. The small-granule fraction was prepared by centrifuging the combined washings and supernatant from the large-granule fraction at 105,000 g for 60 min by an ultracentrifuge. Melanosomal fraction was prepared from the large-granule fraction by density gradient centrifugation (6). The small-granule fraction was further separated into smooth-surface membranes and rough-surface membranes (7) with a slight modification.

3. Determination of tyrosinase activity. Tyrosinase activity was estimated manometrically by measuring the oxygen consumption, using L-dopa as substrate in M/10 phosphate buffer, pH 6.8, and was determined colorimetrically by the initial rate of increase in optical density (expressed as \( \Delta E/10 \) min), with 1 mg of L-dopa in 5 ml of M/10 phosphate buffer, pH 6.8 (8). The optical density was determined with Klett-Summerson photoelectric colorimeter (\( \Delta E = \) scale reading).

4. Determination of tyrosinase activity in the melanocyte. The melanotic cell line C54, isolated from a culture cell line of mouse melanoma B-16 established by Furuse et al. (9), was used in this series of experiments. Tyrosinase activity in cultured melanocytes was assayed as described by Oikawa (10). The method involved measurement of the amount of tritium released into the medium as water from L-[3,5-\( ^3 \)H]tyrosine (11), since this was proportional to the amount of melanin synthesized in the culture. \( 2 \times 10^5 \) dispersed cells in 2 ml of culture medium were incubated at 37°C in a plastic dish with a surface area of 5 cm². The medium consisted of 2 ml of Eagle’s minimum essential medium supplemented with 10% calf serum, 120 μg of Kanamycin and L-[3,5-\( ^3 \)H]tyrosine (2 μCi/ml), and was changed every 24 hr. Used media were analyzed for tritium released as water from the labeled tyrosine. The rate of melanin synthesis is expressed as cpm of radioactive hydrogen released as water from positions 3 and 5 of L-tyrosine. The cell number was counted with a hemocytometer after trypsinization. Aliquots of the filtrate were counted in a liquid scintillation counter (Packard model 3203 spectrometer).
5. **Chemicals.** L-[3,5-3H2]tyrosine (51.7 Ci/mmole) came from The Radiochemical Center, Amersham, England. Culture medium and calf serum came from Igaku-Seibutsugaku-Kenkyusho, Kawasaki, Japan.

6. **Chemical analysis.** The protein content was determined according to Lowry et al. (12).

**RESULTS**

1. **Preparation of tyrosinase inhibitor.** The melanomas were excised and promptly homogenized in 5 vol of ice-cold distilled water at about 0°C. The homogenate was centrifuged at 105,000 g for 60 min by a Hitachi ultracentrifuge. The supernatant thus obtained was condensed. Ten milliliters of condensed supernatant was subjected to gel filtration through a Sephadex G-100 Column (2.6 X 100 cm), equilibrated with 0.01 M phosphate buffer, pH 7.0; the elution was conducted with the same buffer. Fractions of 5 ml were collected, and the elution rate was about 15 ml/hr, at 4°C. The absorbances at 280 nm were measured. Roughly four peaks (I–IV) were obtained, as shown in Fig. 1, and the III and IV peaks showed the inhibitory activities; the fractions between No. 88 and No. 120, which correspond to the III peak and IV peak, were collected and again condensed. The condensed material was chromatographed on a 1.5 X 30 cm column of diethylaminoethyl cellulose-Sephadex A-25, equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. Continuous gradient elution was then performed: A linear gradient elution from 0 to 0.2 M NaCl in 0.01 M potassium phosphate buffer, pH 7.0, was used. Fractions of 5 ml were collected, and the absorbancies at 280 nm were measured. Figure 2 shows the elution pattern of the inhibitory materials. Four peaks: TyI1, TyI2, TyI3, and TyI4 were obtained.

2. **Inhibitory effects of TyI subfractions on soluble-tyrosinase activity.** The inhibitory activities of the four fractions thus obtained were examined manometrically on the soluble-tyrosinase activity. The results obtained are listed in Table 1. The inhibitory activities of Fractions III and IV were reasonably high, but those of Fractions I and II were very low.

![Fig. 1. The gel filtration pattern of the melanoma extract on a Sephadex G-100 column. Melanoma extract (118 mg) was applied on the top of the 2.6 X 100 cm column, equilibrated with 0.01 M phosphate buffer, pH 7.0. Fractions of 5 ml were collected, and the elution rate was about 15 ml/hr at 4°C. The absorbances at 280 nm were measured. Four major peaks were obtained.](image-url)
Fig. 2. The gradient elution pattern of TyI on a DEAE Sephadex A-25 column. Fifty-six milligrams of TyI in 5 ml of 0.01 M phosphate buffer, pH 7.0, in the mixing chamber and 100 ml of 0.5 M NaCl in the same buffer in the reservoir. Fractions of 5 ml were collected, and the elution rate was about 15 ml/hr at 4°C. The absorbances at 280 nm were measured. Four peaks were obtained.

### TABLE 1
**Inhibitory Effect of TyI on Soluble Tyrosinase**

| Fract. | TyI (μg protein) | O₂(μl/30 min) | Inhibition (%) |
|--------|------------------|---------------|----------------|
|        |                  |               |                |
| Soluble tyrosinase<sup>a</sup> | — | 51.6 | — |
| + Fr. I | 30 | 49.3 | 4.4 |
| + Fr. II | 33 | 47.0 | 8.8 |
| + Fr. III | 27 | 36.9 | 28.4 |
| + Fr. IV | 50 | 30.8 | 41.3 |

<sup>a</sup> Soluble tyrosinase: D.U. = 3.7.

### TABLE 2
**Inhibitory Effect of Tyrosinase Inhibitors on Melanosome Tyrosinase**

| Fract. | TyI (μg protein) | O₂(μl/30 min) | Inhibition (%) |
|--------|------------------|---------------|----------------|
|        |                  |               |                |
| Melanosome<sup>a</sup> | — | 61.4 | — |
| + Fr. I | 30 | 62.8 | —1.8 |
| + Fr. II | 33 | 60.4 | 1.6 |
| + Fr. III | 55 | 60.7 | 1.1 |
| + Fr. IV | 50 | 59.8 | 2.6 |

<sup>a</sup> Melanosome: D.U. = 3.75.

3. *Inhibitory effect of TyI subfractions on melanosomal tyrosinase.* The inhibitory activities on these four fractions were examined manometrically on the melanosomal tyrosinase. As shown in Table 2, no inhibitory activity was found in all four fractions.

4. *Inhibitory effect of the TyI<sub>3</sub> on melanosomes, smooth surface membranes, and soluble tyrosinase.* Then the inhibitory effect of the TyI<sub>3</sub> was examined on the particle-bound tyrosinases (melanosomal and smooth-surface-membrane tyro-
TABLE 3
INHIBITORY EFFECT OF TyI₃ ON THE MELANOSOMES, SMOOTH SURFACE MEMBRANES, AND SOLUBLE TYROSINASE

| Enzyme          | Fr. III (µg protein) | Inhibition (%) |
|-----------------|----------------------|----------------|
| Melanosome      | 58.5                 | 1.5            |
| Smooth membrane | 58.5                 | 33.8           |
| Soluble tyrosinase | 58.5              | 23.8           |

* Enzyme activity (ΔE/10 min). Melanosome, 66; smooth membrane, 61; soluble tyrosinase, 65.

TABLE 4
INHIBITORY EFFECT OF HEAT-TREATED TyI₃ ON SMOOTH SURFACE MEMBRANE TYROSINASE

|           | Fr. III (µg protein) | ΔE/10 min | Inhibition (%) |
|-----------|----------------------|-----------|----------------|
| Smooth membrane | —                  | 90        | —              |
| Nontreated | 39.3                 | 70.5      | 21.7           |
| Heat treated | 39.3                | 88.5      | 1.7            |

* TyI₃ was heat treated at 100°C for 10 min.

sinase) and on soluble tyrosinase. The results obtained are listed on Table 3. Although the protein contents and tyrosinase activities of the three tyrosinase preparations used in this particular experiment are almost equal, the inhibitory effect of TyI₃ was found to be present on smooth-surface-membrane tyrosinase and on soluble tyrosinase but not on melanosomal tyrosinase.

5. The effect of heat treatment of TyI₃ on smooth-surface-membrane tyrosinase. The protein nature of the active principle was investigated by observing the effects of heat on the biological activity. The TyI₃ suspension in M/10 phosphate buffer, pH 6.8, was treated at 100°C for 10 min. The experimental result of the heat treatment on the inhibitory effect is listed in Table 4. The inhibitory effect of TyI₃ on smooth-surface membrane tyrosinase was almost completely abolished.

6. Dose response of TyI₃ on soluble tyrosinase. The relationship between the inhibitory effects on soluble tyrosinase and the amount of TyI₃ assayed was studied. As shown in Fig. 3, the more TyI₃ used, the more effective the inhibition on soluble

![Fig. 3](image-url)

Fig. 3. Dose response curve between the inhibitory effects on soluble tyrosinase and the amounts of TyI₃ assayed. There was a limit in the inhibitory effect.
tyrosinase. The inhibitory effect, however, became limited when about 55% inhibition had occurred.

7. Inhibitory effect of TyI3 on the tyrosinase activity in melanocytes. Inhibitory effect of TyI3 on melanogenesis in the melanocytes was investigated by adding various amounts of TyI3 to the culture medium. As shown in Table 5, there does not seem to be present any significant inhibitory effect on the melanogenesis in cultured melanocytes.

**DISCUSSION**

In mammals, tyrosinase, the enzyme responsible for melanin formation, is believed to be synthesized in the ribosomes and transferred via the Golgi area, where tyrosinase is separated into small units, each of which becomes surrounded by a membranous envelope (smooth surface membrane). Within each envelope, the tyrosinase molecules become aligned in an ordered pattern, after which melanin biosynthesis begins, and the particle is known as a melanosome (1). Tyrosinase activity was present in isolated ribosomes, where tyrosinase is synthesized; also in rough endoplasmic reticulum and smooth-surface membranes, where tyrosinase is located before deposition in the melanosome. On the other hand, tracer studies have indicated that the specific site of melanin formation in the melanocyte is on the melanosome and that the presence of tyrosinase activity is not necessarily correlated to the formation of melanin in situ. If this is so, there must be present in the melanocyte some sort of regulatory mechanism to control tyrosinase activity.

Pro-tyrosinase, a precursor form of the enzyme found in body fluids of insects, is activated (by the activators) to tyrosinase during metamorphosis (13). In frog skin it was shown that tyrosinase could be activated significantly by trypsin digestion (14). Mammalian tyrosinase, however, has not been known to possess the pro-tyrosinase stage. The tyrosinase inhibitors are known to be present in the various mouse melanomas (3–5). The inhibitor was extracted from various mouse melanomas as a heat-stable, dialyzable substance. It fully inhibited soluble tyrosinase, but only partially inhibited melanosomal tyrosinase (3), and could be inactivated by ultraviolet light. Tyrosinase isolated from a nonpigmented strain of the S91 mouse melanoma apparently could not aggregate into melanosomes, because its protein carrier was genetically altered. Therefore, albino-tyrosinase remained associated with its inhibitor and could not produce melanin, even though the enzyme had a functioning active center.

The tyrosinase inhibitor described above is of interest with respect to the regulatory mechanisms of tyrosinase activity in the melanocyte. The naturally occurring tyrosinase inhibitor was investigated in Harding-Passey mouse melanomas. Tyrosinase inhibitor (TyI) was isolated from the soluble fraction of melanoma homogenate. TyI was found to consist of four fractions: TyI1, TyI2, TyI3, and TyI4.

**TABLE 5**

| TyI3 | Inhibition (%) |
|------|----------------|
| Control | 0 |
| 0.1 γ  | −12 |
| 1.0 γ  | −3  |
| 10.0 γ | 5   |
The inhibitory effect of TyI was shown on the soluble tyrosinase isolated from the postnuclear supernatant by trypsin digestion. As there is a sigma-shaped dose response curve between the values of percent inhibition and the amount of TyI, it may not be so meaningful to compare their biological activities in terms of percent inhibition per mg protein. The high inhibitory effect was shown in TyI₃ and TyI₄. The inhibitory effects shown in TyI₃ and TyI₄ were not shown on melanosome tyrosinase. The smooth-surface membranes were isolated in order to examine whether the inhibitory effect of TyI is active on the other particle bound tyrosinase. The smooth-surface membranes were chosen since the tyrosinase molecule is known to be a constitutional element of the membranes (15). Tyrosinase activity of the smooth-surface membranes was partly inhibited by TyI₃, as was that of the soluble tyrosinase. It is assumed, therefore, that the structural situation of tyrosinase molecule in the smooth-surface membranes is not the same as that in the melanosomes. Dose response of TyI₃ was investigated on the soluble tyrosinase. As shown in Fig. 3, the dose response curve showed that there was a limit in the inhibitory effect. When TyI₃ was heat treated, the inhibitory activity on the smooth-surface membrane tyrosinase was abolished.

The melanin-synthesizing activity has been determined in a culture of melanocytes (10), and it is now possible to investigate the biological effect of any substances on the melanogenesis in the living melanocytes. Various amounts of TyI₃ were added to the culture media, and the inhibitory effect was examined on the melanogenesis in the melanocytes. The results obtained are listed in Table 5. No inhibitory effect was shown. TyI₃ did not show any inhibitory effect on the cell proliferation in the dosage used. As described above, melanogenesis is assumed to occur only on the melanosomes in the melanocyte, and the TyI₃ did not possess any inhibitory effect on melanosomal tyrosinase. Therefore, the results obtained are thought to be reasonable since it is expected that TyI₃ might not affect melanogenesis in the melanocyte in vivo. Other factors, however, could account for such negative results: (1) TyI₃, the molecular weight of which would be about 10,000, may not be able to penetrate into the cytoplasm of the melanocyte, (2) TyI₃ may be denatured and lose its biological activity during the incubation at 37°C for 24 hr, or (3) TyI₃ may not act on the cultural B-16 mouse melanoma melanocyte because of cell specificities. The preliminary experiment did not show that the inhibitory effect of TyI on the tyrosinase activity is explained by the sulfhydryl group contained.

Further experimental studies are needed to clarify the nature of TyI and the inhibitory mechanism of the TyI on the various tyrosinases.

SUMMARY

The regulatory mechanisms of tyrosinase activity in melanocytes have been studied. The inhibitory factors of tyrosinase were investigated in the soluble fraction of Harding-Passey mouse melanoma homogenate. The protein fractions obtained after the soluble-tyrosinase profile in chromatography through a Sephadex G-100 column showed the inhibitory effect on soluble tyrosinase. Further purification was carried out by continuous gradient elution through a DEAE Sephadex A-25 column. Four protein profiles were obtained. Two fractions of these possessed the inhibitory effect on soluble tyrosinase. Soluble tyrosinase and tyrosinase in smooth membranes were inhibited, but that in melanosomes was not. The inhibi-
tory effect was completely abolished by boiling. *In vivo* melanin formation does not seem to be disturbed by these inhibitors in melanocytes in tissue culture.

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