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Action of Trypsin and Detergents on Tyrosinase of Normal and Malignant Melanocytes[1] (39116)

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Polyacrylamide gel electropherograms of supernatant fractions from homogenates of hair follicle-bearing hypodermal ("hair-bulb") preparations from pigmented mice reveal soluble or readily solubilized multiple molecular forms of tyrosinase (1–4). Depending on genic constitution, a maximum of three soluble tyrosinases (T_1, T_2 and T_3) are demonstrable in mouse "hair bulb" extracts subjected to routine electrophoresis. (In hair bulb preparations, T_2 and T_3 are observed as two closely migrating discrete bands of activity, but they rapidly merge as melanin is deposited in polyacrylamide gels incubated in melanogenic substrates. Since only a single T_2 band is observed in the soluble fraction of mouse melanoma extracts (5, 6), it is possible that a distinction between T_2 and T_3 might not be warranted; hence, in the present study, T_2 refers to the more slowly migrating double bands of hair bulb extracts and the single band of melanoma.) Soluble T_1 and T_2 are present in homogenates of eumelanic hair bulbs of nonagouti black mice, but only T_1 is evident in those of pheomelanic (yellow) mice (1–3). Both soluble and particulate T_1 and T_2 as well as an additional particle-bound tyrosinase have been reported to occur in mouse melanoma cells (5–9). The relationship of the various tyrosinases to each other and their significance in melanogenesis remains to be established. Recently it was shown that trypsin treatment of supernatant fractions from homogenates of black hair bulbs leads to enhanced T_1 activity in electropherograms (3). It remained to be settled whether the mechanism involves the trypsin-induced 1) conversion of a soluble "protyrosinase" to tyrosinase, 2) removal of an inhibitor joined to soluble tyrosinase, or 3) solubilization of particulate tyrosinase. In supporting the third interpretation, this paper provides evidence that the T_1 of normal mouse melanocytes is present in both particulate and soluble (or readily solubilized) forms. It further demonstrates that trypsin solubilizes particulate T_1 in supernatant fractions of pigmented hair bulbs and of Harding–Passey melanoma.

Materials and methods. Hair bulbs together with the adipose tissue in which they were imbedded were collected from 8-week-old C57BL/6J mice 10 days postplucking (4). Each hypodermal (hair bulb) preparation was homogenized gently in a Potter Elvehjem (teflon pestle–glass vessel) homogenizer in chilled 0.25 *M* sucrose (wet wt/vol = 0.3 g/ml). Homogenates made from Harding–Passey melanoma maintained by serial transplantation in C3HB/St mice were treated in a similar fashion with the exception that the wet wt/vol was 0.1 g/ml. All homogenates were centrifuged at 35,000*g* for 30 min at 0°C and the 35,000*g* supernatants were used for further analysis.

Triton X-100 (Sigma) or the sodium salt of deoxycholic acid (DOC) (Matheson, Coleman, and Bell) was added to portions of the 35,000*g* supernatants in a final concentration of 0.1% in 0.25 *M* sucrose. Subsequently, both treated and untreated supernatants were centrifuged twice at 100,000*g* for 1 hr at 0°C. In one case, Triton was added to the previously untreated 100,000*g* supernatant. In another, the 100,000*g* pellet obtained from an untreated 35,000*g* supernatant was rinsed in 0.25 *M* sucrose and centrifuged again at 100,000*g* for 1 hr. The resulting supernatant was discarded and the pellet resuspended in 0.1% Triton-in-0.25 *M* sucrose. Except as noted, when TPCK-trypsin (Worthington 230 U/mg) was used, it was added in a concentration of 0.3

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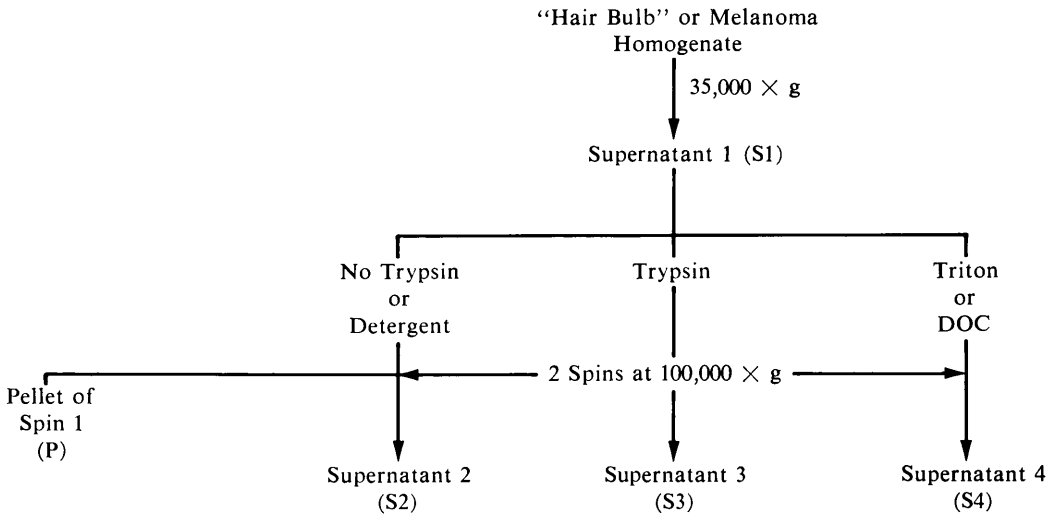

FIG. 1. Cell fractionation of "hair bulb" and Harding-Passey melanoma preparations. With the exception noted, all pellets were discarded. The 35,000*g* supernatants were treated as indicated, and either used directly or centrifuged twice to produce the 100,000*g* supernatants.

mg/ml of sample 10–15 min prior to electrophoresis. A slow-moving "tyrosinase" associated with detergent treatment of 35,000*g* supernatants was eluted from electropherograms of 100,000*g* supernatants, and after either no treatment or trypsin treatment, it was resubjected to electrophoresis following methods described elsewhere (3).

Aliquots (0.2 ml) of all treated and untreated supernatant fractions were subjected to polyacrylamide gel electrophoresis according to the method of Davis (10) with the following modifications: distilled water was substituted for solution F, the sample gel was omitted, the enzyme preparation was layered directly on top of the spacer gel, and the bath buffer was one-fifth the stock concentration. The running time for the electrophoresis was 20–30 min at 3–5 mA/tube. 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 30–60 min in a solution of 0.1 *M* phosphate buffer (pH 6.8) containing 0.15% L-3,4-dihydroxyphenylalanine (dopa). Previous studies have established that the mouse tyrosinases are capable of utilizing both tyrosine and dopa as substrates in polyacrylamide gel electropherograms (3, 11). To preserve the tyrosinase patterns, all gels were stored in 7.5% acetic acid.

Results. The relationship and composition of the various supernatants (S1, S2, S3, and S4) examined are summarized in Fig. 1. Gentle homogenization of fresh eumelanic

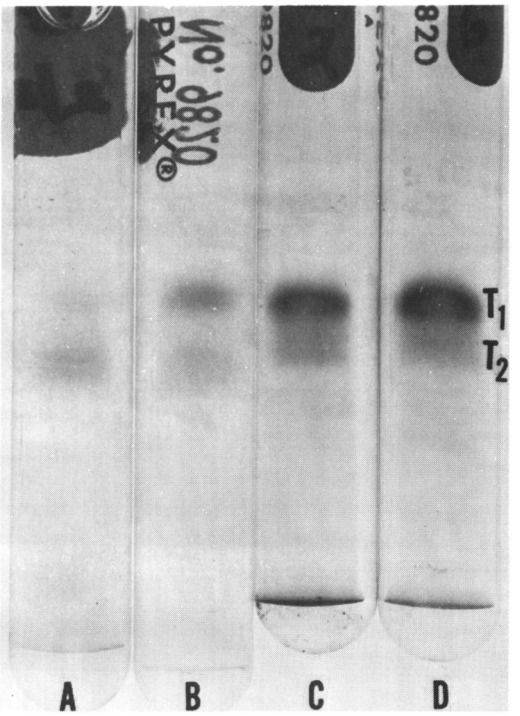

FIG. 2. Tyrosinase activity patterns of 100,000*g* trypsin-treated and nontreated supernatants: (A) hair bulbs, nontreated; (B) hair bulbs, trypsin; (C) melanoma, nontreated; (D) melanoma, trypsin. (All gels incubated in 0.15% dopa.)

hair bulb and melanoma preparations consistently resulted in the appearance of T_1 and T_2 in the electropherograms of 100,000*g* (S2) supernatants. Addition of trypsin to the 100,000*g* (S2) supernatants produced a slight increase in T_1 activity but not of T_2 as judged by the amount of dopa-melanin deposited within the electropherograms (Fig. 2). However, when trypsin was added to 35,000*g* (S1) supernatants and the 100,000*g* (S3) supernatants derived from them were examined for tyrosinase activity, the T_1 activity of the trypsinized supernatants was much greater compared to nontrypsinized control (S2) supernatants (Fig. 3). Identical results were obtained when 35,000*g* (S1) supernatants were incubated in trypsin for 15 min prior to electrophoresis. This corresponded precisely to the time of trypsin incubation of the 100,000*g* (S2) supernatants.

The addition of Triton or DOC to 35,000*g* (S1) supernatants, on centrifugation, yielded

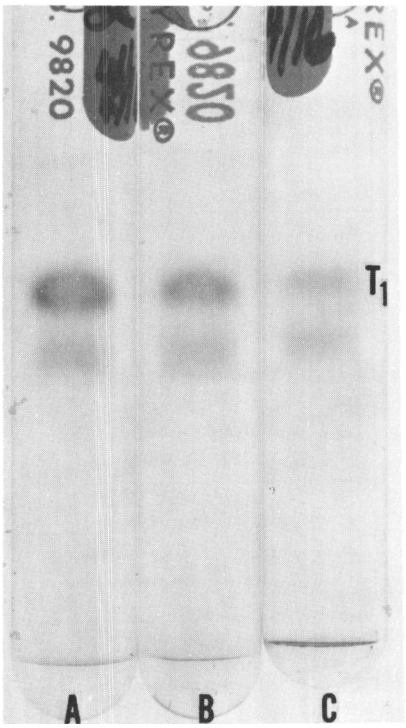

FIG. 3. Tyrosinase activity patterns of 100,000*g* melanoma supernatants: (A) trypsin treatment of 35,000*g* supernatant prior to centrifugation at 100,000*g*; (B) trypsin treatment after 100,000*g* centrifugation; (C) nontreated. (All gels incubated in 0.15% dopa.)

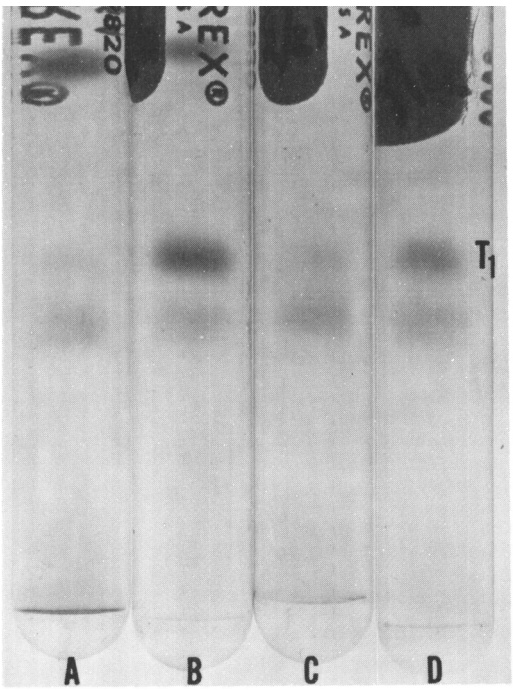

FIG. 4. Tyrosinase activity patterns of 100,000*g* trypsin-treated and nontreated hair bulb supernatants derived from DOC-treated and nontreated 35,000*g* supernatants: (A) DOC only; (B) DOC and trypsin; (C) no treatment; (D) trypsin only. (All gels incubated in 0.15% dopa.)

100,000*g* (S4) supernatants in which tyrosinase activity was markedly increased by trypsin treatment (Figs. 4, 5). In the absence of trypsin, T_1 and T_2 activity in the S4 supernatants was the same as in nontreated control (S2) supernatants (Figs. 4, 5). Uniquely, the electropherograms of detergent-treated (S4) supernatants were characterized by a slow moving band of dopa-reactive ("T_s") material (Fig. 5A). The T_s band was not found in electropherograms of nontreated (S2) control supernatants (Fig. 5C) or in those of trypsin treated (S4) supernatants (Fig. 5B). The activity of T_s varied in different preparations. In some it was sharply localized, in others it was diffuse. When T_s was eluted from the polyacrylamide gels and subjected to electrophoresis, a T_1 band appeared in the electropherograms (Fig. 6). Trypsin treatment of the T_s eluate prior to electrophoresis increased activity at the T_1 position within electropherograms.

When microsomal (P) pellets (Fig. 1) obtained from centrifugation of 35,000*g* supernatants at 100,000*g* were resuspended in 0.1% Triton in sucrose and treated with trypsin, T_1 was found within the electropherograms, but T_2 was absent (Fig. 7). When trypsin treatment of the detergent-treated resuspended (P) pellets was omitted, T_s was found in the absence of T_1 and T_2.

Discussion. This study indicates that T_1 and T_2 exist in soluble or readily solubilized forms both in the hair bulbs of C57BL mice and in Harding–Passey melanoma (9). In addition, it demonstrates that trypsinization of the microsomal ("small granule") fraction (12) releases a tyrosinase with mobility equivalent to T_1. Seiji and associates have reported that detergent-treatment of a smooth membrane fraction of melanoma forms a suspension that is not sedimented by centrifugation at 100,000*g* (12, 13). They found that tyrosinase was solubilized on trypsin treatment of the suspended membranes. The tyrosinase released migrated as a single band in acrylamide gel electrophoresis with mobility (R_x = 0.37) equivalent to one of the isooctylphenoxypolyoxyethylene alcohol ethanol (IPPE) solubilized particulate T_3 tyrosinases obtained by Burnett *et al.* (5). Under the conditions of the present experiments, the tyrosinase released from the microsomal (small granule) fraction in which smooth membranes were included had an R_x of 0.62–0.65 which most closely approximates the T_1 (R_x = 0.61–0.62) of Burnett *et al.* (5). Detergent treatment of the microsomal (small granule) fraction resulted in a slow-moving band T_s of variable width which was estimated to have had an average R_x of 0.15. Trypsin treatment of the detergent treated 100,000*g* (S4) supernatants led to a loss of the slow-moving band coupled with increased activity at the T_1

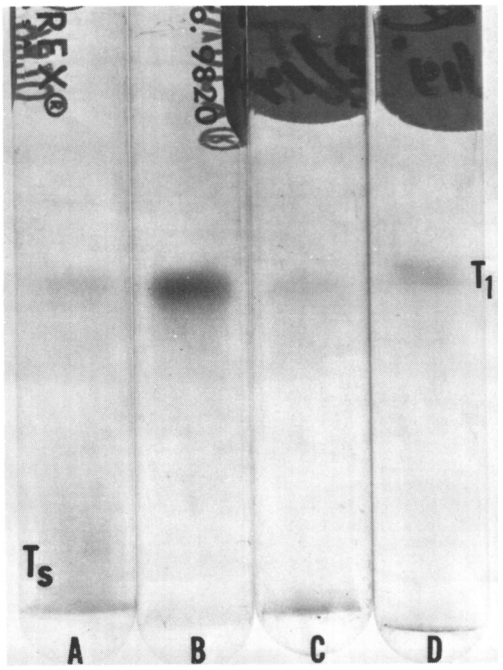

FIG. 5. Tyrosinase activity patterns of trypsin-treated and nontreated 100,000*g* supernatants derived from Triton-treated and nontreated 35,000*g* melanoma supernatants: (A) Triton only; (B) Triton and trypsin; (C) no treatment; (D) trypsin only. (All gels incubated in 0.15% dopa.)

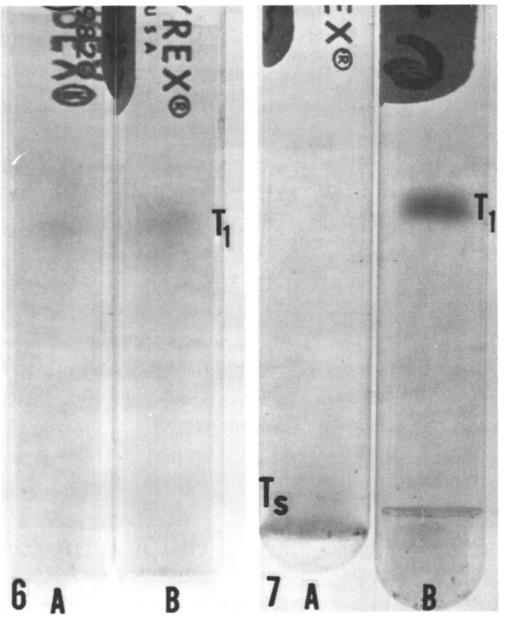

FIG. 6. Reelectrophoresis of low mobility "tyrosinase" eluted from electropherograms of 100,000*g* melanoma supernatant (with or without subsequent addition of trypsin) obtained from 35,000*g* supernatant treated with Triton: (A) nontreated; (B) trypsin. (Gels incubated in 0.15% dopa.)

FIG. 7. Tyrosinase activity patterns of 100,000*g* pellet (from 35,000*g* hair bulb supernatants) resuspended in Triton-sucrose with and without added trypsin: (A) trypsin omitted; (B) trypsin added. (Gels incubated in 0.15% dopa.)

position. The appearance of T_1 activity following elution and reelectrophoresis of the slow-moving tyrosinase either with or without prior trypsin treatment suggests that it consists at least in part of T_1 units in an association that is disrupted by resolubilization and to a greater extent by resolubilization combined with trypsin treatment (14).

Lipase treatment of particulate fractions of B-16 mouse melanoma has been reported to release three isozymes of tyrosinase, two of which correspond to T_1 and T_2 (7–9). If T_2 is present in particulate form in Harding–Passey melanoma and hair bulb melanocytes, it is either not responsive or accessible to trypsin under conditions favorable to the solubilization of T_1.

The present results on the gentle homogenization of fresh tissues are most consistent with the conclusion that increased T_1 activity in trypsin treated 100,000*g* supernatants of relatively vigorous (glass on glass) homogenates of pigmented hair bulb preparations is best explained by release of particulate tyrosinase (3). In all likelihood, disruption of cytomembranes caused by mechanical shear produced fragments that remained suspended after 100,000*g*, giving results that parallel those found with detergent- or trypsin-treated 35,000*g* supernatants. Whether this fully explains the slight enhancement of T_1 activity in the "gentle" 100,000*g* supernatants treated with trypsin remains to be determined. If the foregoing interpretation should prove correct, the present study lends no direct support to the view that in mammals an inactive soluble protyrosinase gives rise to active tyrosinase by the proteolytic activity of appropriate enzymes. Hearing (15, 16) has reached a similar conclusion based on an examination of tyrosinase activation in homogenates of eyes from pigmented mice. A protyrosinase which can be activated by a variety of endopeptidases has been identified in the pigmented and unpigmented skin of the frog *Rana pipiens* (17, 18).

Summary. The T_1 variety of tyrosinase is present in both particulate and soluble or readily solubilized forms in the pigmented hypodermis (hair bulbs) of C57BL mice and Harding–Passey mouse melanoma. Trypsin treatment of 35,000*g* supernatants containing the microsomal (small granule) fraction of gentle homogenates of hair bulbs and melanoma results in significantly increased T_1 activity within polyacrylamide gels. Similar treatment of 100,000*g* supernatants results in a slight increase in T_1 activity. Addition of Triton-X or DOC to 35,000*g* supernatants of hair bulb and melanoma homogenates followed by centrifugation at 100,000*g* results in a marked enhancement of T_1 when the latter supernatants are treated with trypsin. In the absence of trypsin treatment, T_1 activity is comparable to nondetergent-treated controls. A slow-moving dopa-reactive band (T_s) is found in electropherograms of the nontrypsinized 100,000*g* supernatants of detergent-treated 35,000*g* supernatants. It is absent in those treated with trypsin. The slow-moving enzyme appears to give rise to T_1 molecules when eluted from acrylamide gels and even to a greater extent when elution is combined with trypsin treatment prior to reelectrophoresis. In mammals, tyrosinase apparently is not derived by a proteolytic activation of protyrosinase.

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