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# Electrophoretic Pattern of Human Hairbulb Tyrosinase

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**A small gel electrophoretic system has been developed for the study of tryosinase from human hairbulb melanocytes. Enzyme is released from the hairbulb with Triton X-100. Electrophoresis is run in 60 mm microcapillary pipettes with an internal diameter of 1.5 mm. With this technique, tyrosinase from normally pigmented brown, black, blond, and red hairbulbs gives a single band when the gel is stained with L-dopa for enzyme activity. Tryosinase from tryosinase-positive oculocutaneous albino and Hermansky-Pudlak syndrome hairbulbs gives a single L-dopa band. Neuraminidase pretreatment of the tyrosinase from normal and albino hairbulbs changes the migration of the tyrosinase band to a less anodal position. Trypsin pretreatment has no effect. We conclude that human hairbulb tyrosinase is a glycoprotein and that tyrosinase-positive albino tyrosinase has no major electrophoretic difference from normal enzyme.**

Tyrosinase initiates melanin synthesis by catalyzing the hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone [1,2]. Mammalian tyrosinase has been characterized as a copper-containing glycoprotein that exists in soluble and in insoluble bound forms within the melanocyte [3–12]. Electrophoretic studies have revealed multiple forms for tyrosinase from mouse melanoma [8,9,13–17], hamster melanoma [11,18], human melanoma [6,13,19–24], mammalian eye [25, 26], mouse hairbulb [27–31], and human serum [32,33]. Biochemical studies suggest that the multiple forms are the result of posttranslational modification of the basic enzyme polypeptide [15,16,21,23]. Electrophoretic analysis of normal human melanocytic tyrosinase has not been reported, most likely because of the difficulty in obtaining an adequate tissue sample for analysis. In this report, we describe a small gel electrophoretic system that can be used with human hairbulb samples and give the results from studies with normal and tyrosinase-positive oculocutaneous albino (TPA) hairbulb tyrosinase.

## METHODS

### *Preparation of Enzyme*

Freshly plucked anagen (growing) scalp hairbulbs were used as a source of enzyme. After plucking, the hairbulbs were separated from the hairshaft, immediately placed in 0.1 M phosphate buffer, pH 6.8, with 0.5% Triton X-100, and incubated at 20°C for 60 min for enzyme release. For most experiments, 4 hairbulbs were used for each gel, at a concentration of 4 hairbulbs per 20 μl of Triton buffer. The hairbulbs were removed from the buffer and 0.1% bromophenol blue was added as a marker dye.

The effect of trypsin on the electrophoretic mobility of tyrosinase was determined as follows: Trypsin (hog pancreas, Type IX, 14,190 BAEE units per mg protein, Sigma) at a concentration of 0.1–0.4 mg per 0.1 ml of enzyme sample was added and the mixture was incubated at 37°C for 60 min. The trypsin-treated enzyme was then placed directly on the gel for electrophoresis. The effect of neuroaminidase was determined in a similar manner. Neuraminidase (Cl. perfrigens, Type VI neuraminidase, 5.4 units per mg protein, Sigma) was used at a concentration of 15–60 μg per 0.1 ml of sample.

### *Electrophoresis*

*Preparation of gels:* Electrophoresis in 7.5% polyacrylamide gel was carried out in capillary tubes with an internal diameter of 1.5 mm, and was based on the method by Davis [34] and the microgel method of Condeelis [35]. Disposable micropipettes (200 μl) were cut to a length of 60 mm, cleaned by boiling in distilled water for 30 min, rinsed, and oven dried at 100°C. The dried tubes were coated with a 1:10 dilution of Photo-Flow 200 (Kodak) in water, oven dried for 30 min, and stored in a sealed beaker.

The capillary tubes were filled by dipping the tubes in the acrylamide solution and holding them in a horizontal position until the acrylamide reached the desired level. The bottom was then capped with dental wax, and the tubes were placed in a vertical rack, water layered, and allowed to polymerize for at least 1 hr.

After polymerization the bottom 5 mm of the tube was cut away and the water layer removed. The tubes were placed in a Buchler gel electrophoresis apparatus and held in place by rubber stoppers. The sample was layered onto the gel and the electrophoresis performed in a routine manner. A 20 μl enzyme sample was placed on each gel.

*Preparation of solutions:* The stock acrylamide solution contained 30% acrylamide and 0.8% bis-acrylamide. The gel solution contained 2.5 ml of the acrylamide stock solution; 1.0 ml of 0.25 M Tris-glycine buffer, pH 8.2; 0.01 ml of TEMED (N,N,N′,n′-tetramethylethylene diamine); 0.15 ml of 10% ammonium persulfate solution, 0.7 ml of 1% Triton X-100 in 0.1 M phosphate buffer, pH 6.8; and 5.64 ml water. The gel solution was mixed and degassed in a 50 ml flask. The reservoir buffer contained 90 ml of 0.25 M Tris-glycine, pH 8.2; 10 ml of 0.5% Triton X-100 in 0.1 M phosphate buffer, pH 6.8; and 800 ml water.

*Electrophoresis and staining:* Electrophoresis was carried out at 4°C at a current of 0.2 mA per tube, and was stopped when the marker dye reached the bottom of the tube (approximately 100 min). The gels were removed from the capillary tubes with pressure from a 2 ml syringe filled with water and attached to the tube by rubber tubing. The gels were stained for protein with 0.12% Coomassie Blue in 50% methanol and 7% acetic acid. Destaining was with a 5% methanol, 7% acetic acid solution. For tyrosinase activity staining the gels were neutralized in 0.5 M phosphate buffer, pH 6.8–7.0, for 30 min, then placed in a solution of 0.1 M phosphate buffer containing 150 mg% L-3,4-dihydroxyphenylalanine (dopa). The gels were stored in 7% acetic acid.

## RESULTS

Tyrosinase released from human hairbulb melanocytes with Triton X-100 gave a characteristic banding pattern with the dopa stain. Enzyme obtained from individuals with brown, black, blond, and red hair gave a single dopa band (Fig 1). Determination of mean $R_x$ values for normal tyrosinase for different hair colors is given in Table I. The difference between the mean value for the red hairbulb enzyme and the mean value for enzyme from the other hair colors is significant (Student $t$-test, $p<0.01$), but there is no significant difference between the values for the brown, black, and blond enzyme ($p<0.1$). There were 13 major protein bands present with normally pigmented and TPA hairbulb samples. No single protein band corresponded to the tyrosinase dopa band. White hairbulb samples had a protein pattern similar to that of the pigmented and TPA samples but no dopa band was present.

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Abbreviations:
TEMED: N,N,N′-tetramethylethylene diamine
TPA: tyrosinase-positive oculocutaneous albino

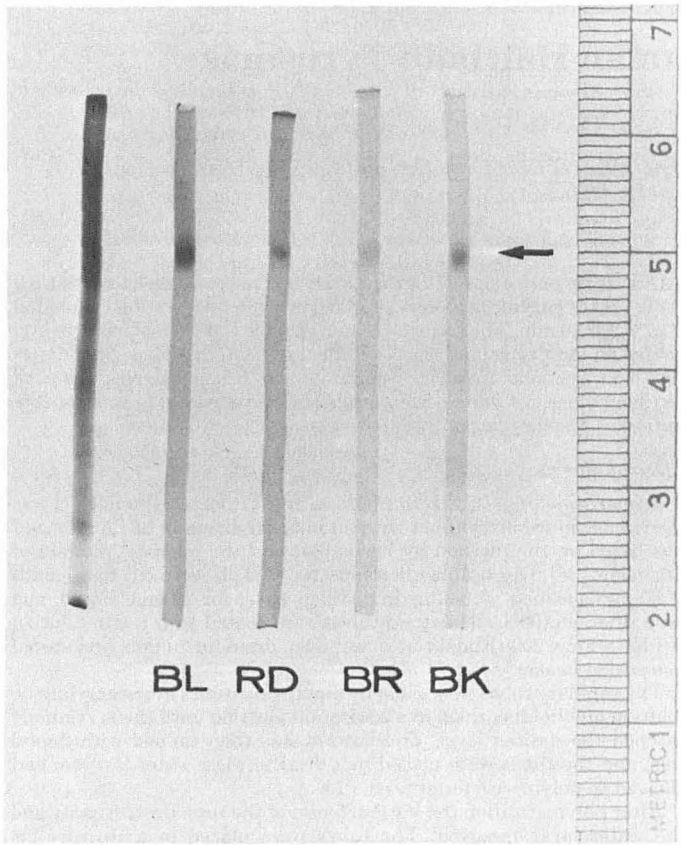


FIG 1. Electrophoretic pattern for tyrosinase from normally pigmented hairbulbs. The protein gel is stained with Coomassie Blue. The gels for the different hair colors are stained with L-dopa for tyrosinase activity. *BL*=blond, *RD*=red, *BR*=brown, *BK*=black.

TABLE I. *Mobility of soluble hairbulb tyrosinase*

| Hair Color | No. of Samples[a] | $R_x$[b] | Range[c] |
|---|---|---|---|
| Brown | 16 | 0.276 ± 0.029 | 0.198–0.317 |
| Black | 6 | 0.282 ± 0.017 | 0.250–0.297 |
| Blond | 9 | 0.284 ± 0.032 | 0.253–0.341 |
| Red | 11 | 0.309 ± 0.038 | 0.258–0.364 |
| TPA[d] | 6 | 0.331 ± 0.036 | 0.287–0.376 |
| HPS[e] | 1 | 0.297 | |

[a] Approximately 8 gels were run per each sample.
[b] Mean ± SD.
[c] Range of values for each hair color.
[d] Tyrosinase-positive oculocutaneous albino.
[e] Hermansky-Pudlak syndrome.

TABLE II. *Effect of trypsin pretreatment*

| Subject | Hair color | $R_x$[a] Trypsin concentration—mg/0.1 ml sample | | | |
|---|---|---|---|---|---|
| | | 0 | 0.1 | 0.2 | 0.4 |
| 1 | Brown | 0.270 | 0.253 | 0.250 | 0.244 |
| 2 | Brown | 0.198 | 0.196 | 0.209 | 0.203 |
| 3 | Black | 0.250 | 0.213 | 0.223 | 0.218 |
| 4 | Blond | 0.265 | 0.264 | 0.264 | 0.259 |
| 5 | Red | 0.286 | 0.270 | 0.272 | 0.265 |
| 6 | TPA | 0.287 | 0.288 | 0.280 | 0.275 |

[a] Usually 2 gels were run for each trypsin concentration.

Hairbulb tyrosinase from one male with tyrosinase positive oculocutaneous albinism (TPA) gave a single dopa band with a mean $R_x$ value of 0.331 (Table I). The difference between the mean TPA and normal $R_x$ values is significant (Student *t*-test, $p<0.01$). Hairbulb tyrosinase from one female with Hermansky-Pudlak syndrome gave a single dopa band with an $R_x$ of 0.297 (Table I).

Pretreatment of the hairbulb tyrosinase with trypsin had only minor effects on the mobility of the dopa band (Table II). The migration of the band was slightly shifted to a less anodal position for subject 1 (brown), subject 3 (black), and subject 5 (red), with little change in the others. There was no obvious change in the intensity of the staining of the dopa band after trypsin treatment. All major bands became less intense and several new faint bands appeared closer to the anode with protein staining after trypsin treatment (Fig 2).

Pretreatment of the hairbulb tyrosinase with neuraminidase produced a significant shift in the dopa band to a less anodal position (Table III, Fig 3). In all cases, the single band was altered by the neuraminidase, with a shift of 20–34% from the untreated value. The effect on the TPA enzyme was less (20%) than on most of the normal enzymes (28–32% for subjects 1–3, 5) but the shift was only 23% for one blond enzyme (subject 4).

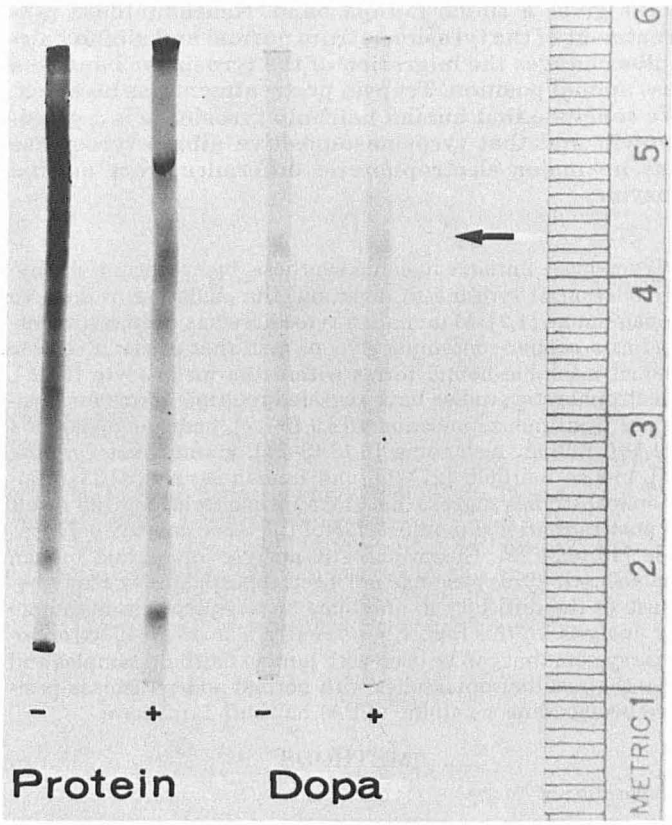


FIG 2. Electrophoretic pattern for trypsin treated tyrosinase from brown hairbulbs. The 2 gels on the left are stained for protein with Coomassie Blue. The 2 gels on the right are stained for tyrosinase activity with L-dopa, (+)=trypsin pretreatment, (−)=no trypsin pretreatment.

TABLE III. *Effect of neuraminidase pretreatment*

| Subject | Hair color | $R_x$[a] Neuraminidase concentration—μg/0.1 ml sample | | | |
|---|---|---|---|---|---|
| | | 0 | 15 | 30 | 60 |
| 1 | Brown | 0.254 | 0.185 | 0.177 | 0.173 |
| 2 | Black | 0.290 | 0.203 | 0.203 | 0.196 |
| 3 | Blond | 0.278 | 0.205 | 0.207 | 0.200 |
| 4 | Blond | 0.253 | 0.189 | 0.174 | 0.196 |
| 5 | Red | 0.323 | 0.228 | 0.236 | 0.228 |
| 6 | TPA | 0.303 | 0.242 | 0.237 | 0.244 |

[a] Usually 2 gels were run at each neuraminidase concentration.

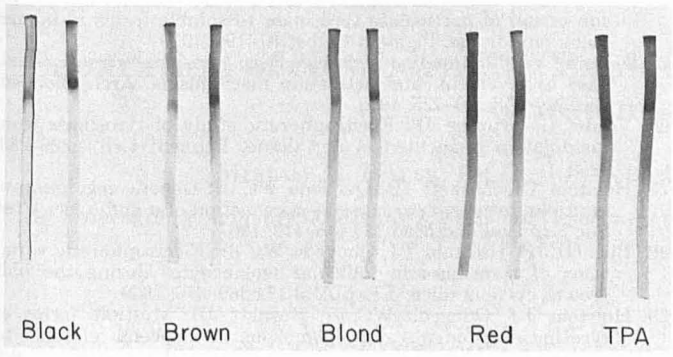


FIG 3. Electrophoretic pattern for neuraminidase treated tyrosinase from normally pigmented and tyrosinase-positive oculocutaneous albino (TPA) hairbulbs. For each hair color and for TPA, the left gel is without neuraminidase treatment and the right gel is after neuraminidase treatment. Gels stained with L-dopa.

All 3 concentrations of neuraminidase had similar effects. There was no change in the intensity of the dopa band after neuraminidase treatment.

## DISCUSSION

The electrophoretic study of normal human tyrosinase has proved difficult because of the lack of an easily available source of the enzyme. Previous work in our laboratory has shown that Triton X-100 will rapidly release soluble tyrosinase from human hairbulb melanocytes for determination of activity [36–38]. We now report the development of a small gel method for electrophoretic analysis of hairbulb tryosinase. With this method, the banding pattern of soluble tyrosinase from a sample as small as 4 fresh hairbulbs can be visualized. For enzyme from normally pigmented brown, black, blond, and red hairbulbs, there is a single dopa band. There is no evidence for nonmigrating enzyme as there is no dopa stain at the top of the gel. All tyrosinase released from the hairbulbs with Triton migrates to a single position.

Trypsin treatment of tyrosinase preparations has been shown to enhance the enzyme activity [12,30,31] or to change the electrophoretic pattern [15,20,21,23] of specific bands. This has been interpreted as the solubilization of membrane bound tyrosinase with conversion to a soluble phase of the enzyme [15,29]. Trypsin does not alter the electrophoretic position or activity of our Triton released hairbulb tyrosinase, indicating that the enzyme released with Triton is in a soluble phase and is not bound to small melanosomal fragments that can be further dispersed with trypsin [37]. Trypsin appears to have no direct effect on the tyrosinase itself under the incubation conditions used in these experiments.

Tyrosinase from a variety of mammalian sources has been shown to be a glycoprotein containing sialic acid and other carbohydrate components [4–6,17,21,23], and the electrophoretic mobility of the predominant band of soluble tyrosinase can be converted to a slower migrating band with neuraminidase [4,5,17,21,23,24]. Our studies demonstrate similar findings with human hairbulb tyrosinase. Neuraminidase treatment of hairbulb tyrosinase converts the dopa band to a less anodal position and the effect on enzyme from all hair colors and from TPA is similar.

The electrophoretic pattern of human hairbulb tyrosinase is different from that reported for tyrosinase from many other sources. Mouse melanoma [8,12–17], hamster melanoma [18], human melanoma [13,19,22,23], and mouse hairbulbs from normally pigmented animals [27–31] contain 2 or 3 distinct soluble tyrosinase bands in the supernatant obtained from centrifugation of a homogenate of the tissue. There is usually one major soluble enzyme band ($T_1$) and a single ($T_2$) or a complex ($T_2$-$T_3$) minor band. Hearing has suggested that part of this double minor band is an artifact of electrophoresis and that there are only two soluble bands [17]. Human melanoma also contains an additional distinct slowly migrating soluble tyrosinase band ($T_4$) [22]. The particulate fraction contains bound tyrosinase which can be released with trypsin, lipase, and detergents, yielding a band that is equivalent to the major soluble band ($T_1$).

Hairbulbs from lethal yellow (AY/A) and recessive yellow (e/e) mice contain a single form of soluble tyrosinase [27,29, 30]. The position of the band is similar to the $T_1$ found in hairbulbs from normally pigmented mice, but the activity of the enzyme is greatly reduced. Ocular tissue (choroid, ciliary process, retina) from a variety of mammals also contains a single soluble form of tyrosinase with an electrophoretic position similar to $T_1$ from other tissues [26].

The single form of tyrosinase in human hairbulbs is most likely equivalent to the major soluble ($T_1$) form of tyrosinase present in other tissues. With the present experimental system, it may be impossible to demonstrate the minor forms of the enzyme because of the reduced concentration of enzyme in our samples. Alternatively, it may be that there is only one form of soluble tyrosinase in plucked hairbulbs. Burnett, Holstein, and Quevedo showed that the $T_2$-$T_3$ minor band disappeared in a maturing mouse hairbulb as melanogenesis was reduced at the end of anagen and $T_1$ disappeared at a later anagen-catagen phase [28]. Voulet and Ortonne suggest that the lack of active melanogenesis in ocular tissue accounts for the lack of the second minor form of tyrosinase in this tissue [26]. Although it is possible that all plucked human hairbulbs are late anagen-catagen in phase and contain only residual $T_1$ activity, it seems unlikely because of the high enzyme activity and the microscopic appearance of the hairbulbs [36]. The most likely explanation is low enzyme concentration.

The electrophoretic position of the dopa band from red hairbulbs was statistically different than that from blond, brown, and black hairbulbs, and the position of the dopa band from the TPA hairbulbs differed from all normal hair colors. The differences in electrophoretic position were not great, however, and there was overlap of values between groups. Previous kinetic studies of hairbulb tyrosinase showed that the response to temperature and pH, and the $K_m$ for tyrosine as substrate and for dopa as cofactor was similar for normal and TPA enzyme [38]; however, there were minor differences in the contours of the pH optima and temperature activity curves for red, blond, and TPA enzyme as compared to brown and black enzyme. The biological significance of the differences that we have demonstrated in hairbulb tyrosinase cannot be determined with the present studies. It is possible that the electrophoretic and kinetic changes of TPA enzyme are related to or account for the lack of in vivo enzyme activity in this condition. A better explanation is that the changes are related to normal individual variation in enzyme as seen with that from pigmented hairbulbs, or to experimental variation. Characterization of purified enzyme is required to define these issues.

## REFERENCES

1. Lerner AB, Fitzpatrick TB, Calkins E, Summerson WH: Mammalian tyrosinase: Preparation and properties. J Biol Chem 178: 185–195, 1949
2. Lerner AB, Fitzpatrick TB: Biochemistry of melanin formation. Physiol Review 30:91–126, 1950
3. Lerner AB, Fitzpatrick TB, Calkins E, Summerson WH: Mammalian tyrosinase: the relationship of copper to enzymatic activity. J Biol Chem 187:793–802, 1950
4. Miyazaki K, Ohtaki N: Tyrosinase as glycoprotein. Arch Dermatol Forsch 252:211–216, 1975
5. Miyazaki, Ohtaki N: Tyrosinase as glycoprotein. Pigment Cell 3: 113–120, 1976
6. Hermann WP, Uhlenbruck G: Serological studies on the carbohydrate moiety of human tryosinase. Arch Dermatol Res 254:275–280, 1975
7. Brown FC, Ward DN: Studies on mammalian tyrosinase. I. Chromatography on cellulose ion exchange agents. J Biol Chem 233: 77–80, 1958

8. Burnett JB, Seiler H, Brown IV: Separation and characterization of multiple forms of tyrosinase from mouse melanoma. Cancer Res 27:880–889, 1967
9. Burnett JB: The tyrosinase of mouse melanoma. Isolation and molecular properties. J Biol Chem 246:3079–3091, 1971
10. Shimao K: Partial purification and kinetic studies of mammalian tyrosinase. Biochim Biophys Acta 62:205–215, 1962
11. Pomerantz SH: Separation, purification, and properties of two tyrosinases from hamster melanoma. J Biol Chem 238:2351–2357, 1963
12. Miyazaki K, Seiji M: Tyrosinase isolated from mouse melanoma melanosome. J Invest Dermatol 57:81–86, 1971
13. Chen YM, Huo A: Biochemical characterization of tyrosinase in vertebrates. Pigment Cell 1:82–89, 1973
14. White R, Hu F: Characteristics of tyrosinase in B16 melanoma. J Invest Dermatol 68:272–276, 1977
15. Iwata K, Takeuchi T: Granule-bound tyrosinase: solubilization and its relation to the soluble form of tyrosinase. J Invest Dermatol 68:88–92, 1977
16. Hearing VJ Jr, Ekel TM, Montague PM, Hearing ED, Nicholson JM: Mammalian tyrosinase: isolation by a simple new procedure and characterization of its steric requirements for cofactor activity. Arch Biochem Biophys 185:407–418, 1978
17. Hearing VJ Jr, Nicholson JM, Montague PM, Ekel TM, Tomecki KJ: Mammalian tyrosinase. Structural and functional interrelationships of isozymes. Biochim Biophys Acta 522:327–339, 1978
18. Pomerantz SH, Li JP-C: Purification and properties of tryosinase isoenzymes from hamster melanoma. Yale J Biol Med 46:541–552, 1973
19. Burnett JB, Seiler H: Multiple forms of tyrosinase from human melanoma. J Invest Dermatol 52:199–203, 1969
20. Nishioka K, Romsdahl MM: Particulate and soluble tryosinases of human malignant melanoma. Pigment Cell 3:121–126, 1976
21. Nishioka K: Conversion of particulate tyrosinase to soluble form and to desialylated tyrosinase in malignant melanoma. FEBS Letters 80:225–228, 1977
22. Nishioka K, Romsdahl MM, McMurtrey MJ: Comparative studies of tyrosinases of malignant melanoma and correlation to serum tyrosinase in patients with malignant melanoma. Cancer Biochem Biophys 2:145–150, 1978
23. Nishioka K: Particulate tyrosinase of human malignant melanoma. Solubilization, purification following trypsin treatment, and characterization. Eur J Biochem 85:137–146, 1978
24. Nishioka K, Romsdahl MM, McJurtrey MJ: Purification of particulate tyrosinase following tryptic cleavage and possible artificial con ersion of particulate tyrosinase to soluble forms in human melanoma tissue. Pigment Cell 4:191–196, 1979
25. Hearing VJ: Mammalian melanogenesis: tyrosinase versus peroxidase involvement, and activation mechanisms. Arch Biochem Biophys 158:720–725, 1973
26. Voulot C, Ortonne JP: Electrophoretic study of tyrosinase from mammalian pigmented ocular tissue. Pigment Cell 4:226–233, 1979
27. Holstein TJ, Burnett JB, Quevedo WC Jr: Genetic regulation of multiple forms of tyrosinase in mice: action of *a* and *b* loci. Proc Soc Exp Biol and Med 126:415–418, 1967
28. Burnett JB, Holstein TJ, Quevedo WC Jr: Electrophoretic variations of tyrosinase in follicular melancoytes during the hair growth cycle in mice. J Exp Zool 171:369–376, 1969
29. Holstein TJ, Quevedo WC Jr, Burnett JB: Multiple forms of tyrosinase in rodents and lagomorphs with special reference to their genetic control in mice. J Exp Zool 177:173–184, 1971
30. Holstein TJ, Stowell CP, Quevedo WC Jr, Zarcaro RM, Bienieki TC: Peroxidase, "protyrosinase," and the multiple forms of tyrosinase in mice. Yale J Biol and Med 46:560–571, 1973
31. Quevedo WC Jr, Holstein TJ, Bienieki TC: Action of trypsin and detergents on tyrosinase of normal and malignant melanocytes. Proc Soc Exp Biol Med 150:735–740, 1975
32. Chen YM, Chavin W: Serum tyrosinase in malignant disease. Oncology 31:147–152, 1975
33. Chen YM, Lim BT, Chavin W: Serum tyrosinase in malignant disease, its activity, and the electrophoretic patterns of the enzyme as carried by immunoglobulins. Cancer Res 39:3485–3490, 1979
34. Davis BJ: Disc electrophoresis. II. Method and application to human serum proteins. Ann NY Acad Sci 121:404–427, 1964
35. Condeelis JS: A sodium dodecyl sulfate micro-gel-electrophoresis technique suitable for routine laboratory analysis. Analyt Biochem 77:195–207, 1977
36. King RA, Witkop CJ Jr: Hairbulb tyrosinase activity in oculocutaneous albinism. Nature 263:69–71, 1976
37. King RA, Witkop CJ Jr: Detection of heterozygotes for tyrosinase-negative oculocutaneous albinism by hairbulb tyrosinase assay. Am J Hum Genet 29:164–168, 1977
28. King RA, Olds DP, Witkop CJ Jr: Characterization of human hairbulb tyrosinase: properties of normal and albino enzyme. J Invest Dermatol 71:136–139, 1978
39. Prota G, Thompson RH: Melanin pigmentation in mammals. Endeavour 35:32–38, 1976