Purification and Properties of Tyrosinase Isoenzymes from Hamster Melanoma

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Tyrosinase, which catalyzes the formation of melanin from tyrosine (Eq. 1) has only recently been purified to homogeneity from mammalian sources (1, 2). This paper reports a rapid and improved method of purification of one of the several tyrosinase isoenzymes from hamster melanoma and the determination of its amino acid composition. The molecular weights of three of the isoenzymes have been determined.

L-tyrosine → L-dopa →→ dopachrome →→ 5,6-dihydroxyindole →→ melanin (1)

EXPERIMENTAL METHODS

Enzyme source. The melanomas were propagated in random bred Syrian golden hamsters (Lakeview Hamster Colony, Newfield, NJ) by subcutaneous implantation of a small amount of tissue. This melanoma occurred spontaneously and was obtained originally from Dr. A.B. Lerner, Yale University. The animals were killed 6–7 wk after tumors were implanted and the tumors were frozen at −20°C for future use. Frozen tumors retained full enzyme activity for at least 3 mo.

Enzymatic activity. During purification of the enzyme, activity was determined spectrophotometrically by measuring the rate of formation of dopachrome from L-dopa2 as described earlier (3). A unit of enzyme is the amount catalyzing the formation of 1 μmole of dopachrome per minute. For the tyrosine Km and lag experiments, activity was measured by the release of 3H2O from 3,5-3H-L-tyrosine (4). Protein concentrations were determined by the method of Lowry et al. (5).

Analytical polyacrylamide gel electrophoresis. The 12-port polyacrylamide gel system (Canal Industrial Corp.) was modified to take gel tubes 100 × 9 mm (i.d.). Gel concentrations of 3.5, 5, and 7% were employed at electrode buffer pH values of 8.5 (0.025 M Tris–0.15 M glycine) (6) or 7.0 (0.0083 M Tris–0.03 M diethylbarbituric acid) (7). Electrophoresis was performed at 5°C for 1–1.5 hr

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2 Abbreviations used are dopa, 3,4-dihydroxy-L-phenylalanine; SDS, sodium dodecyl sulfate.
with a current of 2.5 mamp for each gel column. Samples (0.1–0.25 ml) of enzyme preparation containing 10–20% sucrose were layered directly onto the top of the stacking gel, and bromophenol blue was used as a tracking dye. Gels were stained for enzyme activity by incubation of the gel at 37°C with 2.5 mM dopa—0.05 M phosphate, pH 6.8 for 10 min. The reaction was stopped by dipping the gel in 7% acetic acid. Gels were stained for protein with 0.05% Coomassie Blue in 12.5% trichloroacetic acid for 1–2 hr and then destained overnight with 10% trichloroacetic acid.

**Molecular weight determinations.** The molecular weights of tyrosinase isoenzymes were determined by electrophoresis in SDS-polyacrylamide gel (8) and by chromatography on Bio-Gel P100. The electrophoresis gel contained 5% acrylamide and all buffers contained 1% sodium dodecyl sulfate and 0.1 M sodium phosphate, pH 7.0. Cytochrome c was used as mobility reference in place of the tracking dye which produced a diffuse band in this gel. The concentrations of standards, reference protein, and tyrosinase were between 0.2 and 0.6 mg per ml when incubated individually at 37°C for 2 hr in buffer containing 1% β-mercaptoethanol and 8 M urea. Then 0.08 ml of each protein solution and 0.05 ml of 40% sucrose were mixed and applied on the gels. Electrophoresis was performed for 4 hr at a current of 3 mamp per gel with a tap water cooling system keeping the temperature near 25°C. After electrophoresis the gels were stained with 0.05% Coomassie Blue for 2 hr and then destained with several changes of 10% trichloroacetic acid until the background of each gel was clear. The gels were stored in 7% acetic acid solution until the measurement of migration was made. The mobility of each protein was calculated from the distance of protein migration and the distance of migration of cytochrome c.

The Bio-Gel P100 column had dimensions of 1.9 × 100 cm and the exclusion volume for blue dextran was 109 ml. The column was calibrated using trypsin, pepsin, ovalbumin, and albumin.

**Materials.** L-Dopa was purchased from Sigma or Calbiochem; the chemicals for acrylamide gel electrophoresis from Eastman or Canalco; Bio-Gel P100, calcium phosphate gel (A grade), and DEAE-cellulose (standard grade) from Calbiochem and 3,5-3H-L-tyrosine from New England Nuclear and Amersham Searle. The proteins used for determining molecular weights were obtained as follows: from Sigma, L-glutamate dehydrogenase (bovine pancreas, Type II), cytochrome c (horse heart) and trypsin (bovine pancreas, Type III); from Worthington, catalase (bovine liver), ovalbumin (egg white), and aldolase (rabbit muscle); from Calbiochem, pepsin (porcine stomach mucosa), pyruvate kinase (rabbit muscle), and albumin (bovine). Sodium dodecyl sulfate (Matheson) was recrystallized from ethyl alcohol. Coomassie Brilliant Blue R 250 was obtained from Colab and the Diaflo ultrafiltration apparatus and PM-10 membranes from Amicon.

**RESULTS**

**Purification of tyrosinases.** The following is representative of a large number of purification experiments. Hamster melanoma (265 g) was homogenized in a blender for 1 min in 0.003 M sodium phosphate, pH 6.8 (1060 ml). After centrifugation for 1 hr at 30,000 rpm, the dark red supernatant was stirred in the cold with calcium phosphate gel (1.75 g gel per g of protein) for 2 hr. Certain batches of commercial gel remove only small amounts of pigment and protein; pilot runs should be made on small volumes of crude enzyme until a good prepara-
tyrosinase from hamster melanoma

The supernatant was centrifuged at 30,000 g for 0.5 hr. The supernatant was now generally a pale straw color.

The supernatant was passed onto a column of DEAE-cellulose (0.65–0.70 meq per g; 2.0 × 40 cm) which had been washed with 0.003 M sodium phosphate, pH 6.8 before use. After loading, the column was washed with 0.005 M sodium phosphate, pH 6.8 until a red pigment band was removed (1000–1500 ml), followed by 200 ml of 0.01 M sodium phosphate pH 6.8. None of these fractions contained more than traces of activity. The resin was then eluted with a linear gradient formed by adding 0.06 M sodium phosphate pH 6.8 (1000 ml) to 0.01 M sodium phosphate, pH 6.8; elution proceeded at the rate of about 0.7 ml per min, with about 7–8 ml collected per tube. Tubes were assayed qualitatively for enzyme activity by adding 0.05 ml from every fourth tube to a solution of 0.5 μmole dopa in 0.4 ml of 0.05 M sodium phosphate, pH 6.8. After the enzymes were located, quantitative measurements of activity were made.

The gradient was maintained until the second peak of activity was eluted. Another buffer, 0.05 M sodium phosphate, pH 6.8–0.15 M NaCl was used to elute the next peak until yellow color was washed from the column. The pattern of elution of enzyme fractions from the column is depicted in Fig. 1. It shows three peaks of activity: the first, eluted from about 0.022 to 0.031 M phosphate, is a major peak; the second, eluted from about 0.031 to 0.041 M phosphate and the third, eluted by 0.05 M phosphate, pH 6.8–0.15 M NaCl. The last fraction was dialyzed against 0.003 M phosphate, pH 6.8 immediately after elution; otherwise it rapidly lost activity. All fractions were stored at −90°C.

Identifying multiple forms of tyrosinase. Acrylamide gel electrophoresis at pH 8.5 of samples from tubes labeled B, E, and F (Fig. 1) indicated that there were at least three tyrosinases, designated as E₁, E₂, and E₃ in order of elution from DEAE-cellulose. This is also the order of appearance on acrylamide gel electro-

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**Fig. 1.** Elution diagram of hamster tyrosinases by DEAE-cellulose chromatography. The supernatant from the first calcium phosphate gel step was applied to a column of DEAE-cellulose, 2.0 × 40 cm. The resin was eluted as described in the text. The gradient was substituted by 0.15 M NaCl–0.05 M sodium phosphate, pH 6.8, beginning at tube 210 (arrow). The letters refer to tubes from which samples were drawn for analytical gel electrophoresis.
Fig. 2. Schematic diagram of acrylamide gel electrophoresis patterns of fractions from DEAE-cellulose chromatography. B, E, F, A0, C, and D correspond to the particular fractions shown in Fig. 1; the others are combinations of respective fractions. The gels were 3.5% acrylamide. Each gel was loaded with 20–30 m units of tyrosinase activity. After electrophoresis at pH 8.5, activity bands were developed by soaking the gels in dopa as described in the text.

Electrophoresis, with E1 migrating slowest and E3 migrating fastest toward the anode, as shown in Fig. 2.

Frequently the elution diagram had a pronounced shoulder on the trailing edge of the first peak. Electrophoresis of samples from tubes C and D in the area of the shoulder showed that these tubes contained two activity bands (Fig. 2). Electrophoresis of a mixture of equal amounts of enzyme from B and D showed that the slower migrating band of the two bands in D became much darker than the corresponding band in D alone, so the slower band in D was probably E1. When equal amounts of enzyme from tubes D and E (E2) were mixed and subjected to electrophoresis (not shown in Fig. 2) three closely spaced bands were seen, indicating that the faster moving band in tube D was probably not E2. The width of the shoulder was variable. Since the shoulder was more pronounced if there was a delay in the operation of the DEAE-cellulose column, it is likely that the extra band seen in tubes C and D was a degradation product of E1.

An early tube in the first peak (A0) also contained two activity bands (Fig. 2), but tube A had only one activity band (not shown). Electrophoresis of a mixture of equal amounts of activity from tubes A0 and B showed that the faster migrating band in A0 was probably E1.

Purification of E1. Tubes from point A to point C (Fig. 1) were combined to form the fraction rich in E1. It was concentrated by Diaflo ultrafiltration to a protein concentration of 1.5 mg/ml while the buffer was replaced with 0.003 M sodium phosphate, pH 6.8, using a PM-10 membrane. The enzyme solution was then stirred for 0.5 hr with an amount of calcium phosphate gel equal to 20 times the weight of protein. The mixture was centrifuged for 1 hr at 40,000 rpm and the supernatant saved. The gel pellet was washed with 3 ml of 0.003 M sodium phosphate, pH 6.8, and centrifuged once more. The supernatants were combined and concentrated to a protein concentration of 1–2 mg/ml by Diaflo ultrafiltration.

Preparative acrylamide gels were prepared in 1.1 cm i.d. tubes. The separating gel (5%) was 12 cm, and the stacking gel, 1.5 cm long. These gel columns were joined with polyethylene tubing connectors to the regular Canalco analytical acrylamide gel electrophoresis equipment. The concentrated E1 fraction was introduced to each column in the proportion of 1.0–1.5 ml of enzyme solution to 0.30–0.50 ml of 40% sucrose. Electrophoresis was performed at pH 8.5 for 3.5 hr at 5 mamp for each gel column.

After removing the gel from the tube the enzyme was located by placing it on a strip of filter paper previously soaked with 0.5 m M dopa, 0.05 M sodium phos-
phate, pH 6.8 and incubating at 37°C for 3–5 min until a red color appeared in the strip. The section of gel containing the enzyme was then sliced into four or five discs, each was homogenized with 2 ml of 0.02 M sodium phosphate, pH 6.8, and then stirred in the cold for 2 hr to elute the enzyme. The gels were allowed to settle and the activity of each sample was measured. The high activity fractions were combined, mixed with calcium phosphate gel (50 mg), and then filtered with suction through filter paper. The calcium phosphate gel removed the pigment which formed during the process of locating the activity zone and also helped sediment the acrylamide gel particles. Most of the activity was present in the supernatant; additional enzyme was obtained by washing the gel pellet with 1 ml of the same buffer. The supernatants were combined, concentrated, and dialyzed against 0.003 M sodium phosphate, pH 6.8, saturated with calcium phosphate until the activity was at least 13 units/ml. The enzyme was stored in liquid N₂. A summary of steps in a typical purification procedure is shown in Table 1. The final dialysis buffer is saturated with calcium phosphate in order to prevent loss of activity.

**Purification of E₂.** E₂ was purified by procedures similar to the E₁ purification. Two crude E₃ preparations were combined (68 units, 200 mg) and stirred for 0.5 hr with an amount of calcium phosphate gel equal to 20 times the weight of protein. The mixture was centrifuged for 1 hr at 40,000 rpm and the supernatant, containing 14% of the activity, was saved. The gel was extracted first with 0.005 M sodium phosphate, pH 6.8 as above and then with 0.01 M sodium phosphate, pH 6.8. All of the supernatant fractions were then combined, concentrated to 10 ml by Diaflo ultrafiltration, dialyzed vs 0.01 M sodium phosphate, pH 6.8, and stirred once again for 0.5 hr with an amount of calcium phosphate gel equal to 10 times the weight of protein. The mixture was centrifuged for 1 hr at 40,000 rpm and the supernatant saved. The pellet was washed with 3 ml of 0.01 M sodium phosphate, pH 6.8, and the supernatants were combined and concentrated to 4 ml.

Preparative acrylamide gel electrophoresis was performed as before with about 2 mg of protein for each gel column and at current of 5 mamp per gel for 3.5 hr. The enzyme was located on each gel in the same manner described for E₁. The area containing the activity was sliced into five 1 mm pieces. Each slice was homogenized with 0.01 M sodium phosphate, pH 6.8 (2 ml), centrifuged, and the supernatant activity measured. In the example shown here the middle three

| Fraction                          | Volume (ml) | Protein (mg/ml) | Enzyme | Specific activity (units/mg) |
|-----------------------------------|-------------|-----------------|--------|-----------------------------|
| 1. High speed supernatant         | 1200        | 12.0            | 175    | 100                         | 0.012 |
| 2. Ca phosphate gel               | 1200        | 0.86            | 155    | 89                          | 0.15  |
| 3. DEAE-cellulose                 |             |                 |        |                             |       |
| a. First peak (E₁)               | 145         | 0.10            | 59     | 34                          | 4.07  |
| b. Second peak (E₂)              | 300         | 0.044           | 36     | 21                          | 2.70  |
| c. Third peak (E₃)               | 75          | 1.85            | 32     | 18                          | 0.23  |
| 4. E₁ Ca phosphate gel eluate    | 12.8        | 0.27            | 49     | 28                          | 14.0  |
| 5. E₁ Prep. acrylamide gel electrophoresis | 1.15 | 0.42          | 25     | 14                          | 52.0  |
TABLE 2

Purification of E₄ Tyrosinase

| Fraction                             | Volume (ml) | Protein (mg/ml) | Protein (units) | Specific activity (units/mg) |
|--------------------------------------|-------------|-----------------|-----------------|-------------------------------|
| 1. E₄ from DEAE-cellulose             | 68          | 2.94            | 68              | 100                           |
| 2. First calcium phosphate gel eluates| 130         | 0.102           | 38              | 56                            |
| 3. Second calcium phosphate gel eluates| 12.6        | 0.440           | 33.6            | 49                            |
| 4. Prep. acrylamide gel electrophoresis| 6.1         | 0.050           | 12              | 17.7                          |
| E₃ₐ                                  | 2.6         | 0.031           | 2               | 2.9                           |
| E₃ₜ                                 |             |                 |                 |                               |

slices from each large gel were combined (E₃ₐ) as were the upper and lower two slices (E₃ₜ). The acrylamide gel particles were removed as before and the enzyme fractions were concentrated by ultrafiltration. A summary of these steps is shown in Table 2.

*Absolute purity of enzyme fractions.* E₄ tyrosinase routinely had a specific activity in the range of 50–65 units per mg of protein after step 5 (Table 1) of the purification procedure. In Fig. 3 are shown the results of an analytical gel electrophoresis of purified E₄. Samples of E₁ from step 5 (0.3 μg of protein on the left gel and 30 μg on the right gel) were layered on 3.5% acrylamide gels and the electrophoresis was run at pH 8.5. The left gel was stained for enzymatic activity with dopa and the right gel was stained for protein with Coomassie blue.

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**Fig. 3.** Acrylamide gel electrophoresis of purified E₄. Samples of E₁ from step 5 (0.3 μg of protein on the left gel and 30 μg on the right gel) were layered on 3.5% acrylamide gels and the electrophoresis was run at pH 8.5. The left gel was stained for enzymatic activity with dopa and the right gel was stained for protein with Coomassie blue.
Fig. 4. Acrylamide gel electrophoresis of purified E₃a and E₃b. Samples of E₃a and E₃b (22 μg each) were loaded on 7% gels and electrophoresis was performed at pH 8.5. Each gel was sliced lengthwise; one piece was stained for enzymatic activity with dopa and the other, for protein. Left tube: E₃b (left slice, activity; right slice, protein). Right tube E₃a (left slice, activity; right slice, protein).

Electrophoresis of a preparation of high purity E₁. The single protein band obtained from 30 μg of protein coincided with the activity band from 0.3 μg of protein. When 100 μg of this E₁ preparation was applied to acrylamide gel, the resulting gel electrophoresis showed a wider E₁ protein band and a very weak contaminating protein band which migrated more slowly than E₁. This contaminant was estimated to be weaker in intensity than 5 μg of protein. Accordingly this particular preparation of E₁ was judged to be about 95% pure. Electrophoresis at pH 7 did not reveal additional protein bands.

Electrophoresis of E₃a and E₃b (Fig. 4) revealed that the E₃ fraction from the DEAE-cellulose resin was probably a mixture of two tyrosinases: E₃a appeared to be one protein, although the band was broad, while E₃b was composed of two bands, one of which migrated near the one band in E₃a. Additional electrophoresis of mixtures of E₁, E₃a, and E₃b at pH 8.5 (Fig. 5) and at pH 7 (not shown) did not lead to further resolution of E₃.

Since it was difficult to obtain the pure E₃ tyrosinases in quantity, the enzymes in this fraction were usually purified together and the component isoenzymes were not separated. The tyrosinase in E₃ has not been purified routinely beyond the stage of DEAE-cellulose chromatography.

Molecular weight determinations. The molecular weight of E₁ was estimated by SDS-acrylamide gel electrophoresis (Fig. 6) to be 53,500; the estimate for the
Fig. 5. Acrylamide gel electrophoresis of $E_1$, $E_{3a}$, $E_{3b}$, and mixtures of the purified fractions. About 25 m units of activity were applied to each gel (7%). Electrophoresis was performed at pH 8.5. The gels were stained for enzymatic activity with dopa. Left to right: $E_1$, $E_{3a}$, $E_{3b}$, $E_1 + E_{3a}$ (equal amounts), $E_1 + E_{3b}$ (equal amounts), $E_{3a} + E_{3b}$ (equal amounts).

![Enzyme bands on SDS-acrylamide gel](image)

Fig. 6. Determination of the molecular weights of $E_1$ and $E_3$ by SDS-acrylamide gel electrophoresis. The six marker proteins used were catalase, pyruvic kinase, l-glutamate dehydrogenase, ovalbumin, aldonase, and pepsin. All proteins were run in duplicate. The mobilities refer to cytochrome c as 1.00. The arrow indicates the mobility of tyrosinase $E_1$ (0.66). The molecular weights of the marker proteins were taken from Weber and Osborn (8).

$E_3$ enzymes by the same method was 70,000. The two enzyme bands in $E_3$ also migrate as one protein band in SDS-acrylamide gel. Efforts to estimate molecular weights by chromatography on Bio-Gel P100 were not as satisfactory because repeated runs with the standards continued to show ovalbumin falling off the line constructed with the other three standards (Fig. 7). The values obtained using serum albumin, pepsin, and trypsin as standards were 54,000 for $E_1$ and 66,500
Fig. 7. Determination of the molecular weights of $E_1$ and $E_3$ by Bio-Gel P100 chromatography. The four marker proteins used were serum albumin, ovalbumin, pepsin, and trypsin. The mobilities are referred to blue dextran as 1.00.

for $E_3$, in accord with the values obtained with SDS-acrylamide gel electrophoresis.

_Amino acid composition of $E_1._$ The approximate amino acid composition of $E_1$, obtained on a Beckman Amino Acid Analyzer, is shown in Table 3; trypophan was not determined. The composition is quite close to that of $T^2$ tyrosinase from mouse melanoma (2).

_Kinetic data for $E_1$ tyrosinase._ The kinetics of both tyrosine hydroxylation and of dopa oxidation were restudied with $E_1$ enzyme of high specific activity (62 units/mg). The $K_m$ values for tyrosine ($2.6 \times 10^{-4} \text{ M}$) and for dopa as sole substrate ($7.6 \times 10^{-4} \text{ M}$) were essentially the same as determined earlier (3) with crude enzyme. Using a molecular weight of 53,500 the turnover number for tyrosine was calculated to be 1000, while that for dopa was 5350.

_Lag period for tyrosine hydroxylation._ Earlier work (9) had demonstrated that lag periods in tyrosine hydroxylation, using enzyme which had a specific activity of about 1.0, could be shortened and then eliminated by adding increasing concentrations of dopa. Data was also obtained (9) which showed that endogenous dopa was the compound responsible for the ability of boiled melanoma extracts to shorten the lag in tyrosine hydroxylation. In principle, no tyrosine hydroxylation should occur in this system in the absence of dopa. We felt that homogeneous enzyme might be free of endogenous dopa and thus we might be able to demonstrate very lengthy or indefinite lag periods. In order to exclude dopa from the reaction, solutions of both tyrosine and 3,5-$^3$H-tyrosine were prepared fresh, the reaction tubes were scrupulously cleaned, and the incubations were protected from light and airborne particles. However, the longest lag period achieved was 195 min.

$^3$ Unpublished work of S. H. Pomerantz.
TABLE 3
AMINO ACID COMPOSITION

| Amino acid | Ratiosa | Nearest integer for minimum Residues/molecule based on mol. wt. of 54,000 |
|------------|---------|-------------------------------------------------|
| Lysine     | 1.98    | 4                                               | 28 |
| Histidine  | 1.09    | 2                                               | 14 |
| Arginine   | 1.74    | 3                                               | 21 |
| Cysteic acidb | 0.47    | 1                                               | 7  |
| Aspartic acid | 3.80    | 8                                               | 56 |
| Threonine  | 2.00    | 4                                               | 28 |
| Serine     | 3.11    | 6                                               | 42 |
| Glutamic acid | 3.31    | 7                                               | 49 |
| Proline    | 2.68    | 5                                               | 35 |
| Glycine    | 3.46    | 7                                               | 49 |
| Alanine    | 2.51    | 5                                               | 35 |
| Valine     | 2.51    | 5                                               | 35 |
| Methionine | 0.416   | 1                                               | 7  |
| Isoleucine | 1.00    | 2                                               | 14 |
| Leucine    | 3.44    | 7                                               | 49 |
| Tyrosine   | 1.41    | 3                                               | 21 |
| Phenylalanine | 1.45    | 3                                               | 21 |
| Residue weight | 7908    |                                                  | 55,356 |

a Based on isoleucine = 1.00.
b Determined in a separate run after performic acid oxidation (15).

It appeared quite likely that very small amounts of dopa or other reducing agents were being contributed by the tyrosine reactants (10) or by the enzyme.

DISCUSSION

Tyrosinases in hamster melanoma are largely soluble, or easily solubilized during homogenization, in contrast to the particulate tyrosinases of mouse melanoma (2). This makes for a somewhat easier purification procedure which is carried out routinely in 9 working days from homogenization of the tumor to storage of homogeneous E₁. Hamster tumor, however, contains tyrosinases at only about 15% of the concentration in mouse melanoma.

All of the tyrosinases studied in mammals and all of the polyphenol oxidases of plants occur as isoenzymes. At present there is no understanding of this phenomenon. In this case we considered the possibility that at least some of the isoenzymes were formed by proteolytic action during the homogenization or later in the procedure. However, in one instance in which phenylmethyl sulfonil fluoride, an inhibitor of some proteases (11) was added to the homogenization fluid, the same isoenzyme pattern was observed as in its absence. The one positive clue that at least a small conversion of one isoenzyme into another was taking place came during DEAE-cellulose chromatography. There was formation or broadening of a shoulder on the trailing edge of the first peak when chromatography was delayed a few days.

The molecular weight of E₁ (53,500) is near that of T² from mouse melanoma (56,000–62,000) as shown by Burnett (2), while the molecular weight of the en-
zymes in the $E_3$ fraction (66,500–70,000) is close to that of $T^1$ from mouse melanoma (65,000–76,000) (2). All of these tyrosinases are composed of single polypeptide chains, as judged by SDS-acrylamide gel electrophoresis. By comparison, the molecular weight of mushroom tyrosinase, a tetramer, is about 124,000 (12); that of bacterial tyrosinase from Vibrio tyrosinaticus, a monomer, about 36,000$^8$ and that from Neurospora crassa, also a monomer, 33,000 (13). In amino acid composition, $E_3$ also closely resembles $T^2$. Frog skin tyrosinase is a tetramer, with each monomer unit having a molecular weight of 50,000 (14).

Although we have not been successful in observing a lag of more than 195 min in the hydroxylation of tyrosine in the absence of exogenous dopa, we think it likely that very small amounts of dopa might be introduced as a contaminant of the tyrosine solutions or perhaps some tyrosine is converted to dopa nonenzymatically in the early reaction period as a result of the action of ultraviolet light. From the work of Arnow (10) we have calculated that in tyrosine solutions exposed to ultraviolet light, about 0.13% of the tyrosine was destroyed per hour and about 0.05% of the tyrosine accumulated as dopa each hour. The reaction was monomolecular for several hours and the rate of dopa accumulation was constant for 4 hr. Arnow stated that even more dopa was probably formed but that it, too, was decomposed by ultraviolet light. Although we cannot apply this data on irradiated solutions directly to the present case, it is clear that small amounts of dopa might accumulate during 3 hr of incubation, even though dopa was entirely absent at the start. Since the $K_m$ for dopa as a cosubstrate in the tyrosine hydroxylation reaction is about $2 \times 10^{-6} M$ (9), it is quite possible that sufficient dopa would accumulate from both non enzymatic and tyrosinase catalyzed reactions to produce a full scale enzymatic hydroxylation after about 3 hr.

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