in an active form in the crude tissue extracts. There are, however, examples of tyrosinase preparations in which enzymatic activity appears only after treatment of homogenates with detergents or proteolytic enzymes (9–10). This behavior has been generally ascribed either to solubilization of particulate tyrosinase or to dissociation of a tightly bound inhibitor. We have reported (11, 12) that the epidermis of the Northern grass frog Rana pipiens pipiens contains large amounts of a soluble protein which is, as extracted, incapable of catalyzing the oxidation of DOPA or tyrosine, but which can be rapidly activated by a wide variety of endoproteases. Lee and Lee have confirmed the activation of frog tyrosinase by trypsin (13).

We have purified this inactive tyrosinase and compared it physicochemically with the active enzyme produced from it by chymotryptic proteolysis. The inactive enzyme is a true zymogen and the activation process consists in cleavage of a low molecular weight peptide, probably from the N-terminal portion of the molecule. Our studies of the enzymic properties of the active frog epidermal tyrosinase show it to be similar to purified tyrosinases from other sources.

**EXPERIMENTAL PROCEDURE**

**Materials**

Northern and Rio Grande Grass Frogs (R. pipiens pipiens and Rana pipiens berlandierii) were supplied by Mogul-Ed, Oshkosh, Wis.

L-DOPA and L-tyrosine were obtained from the J. T. Baker Chemical Co. and Mann Research Laboratories, respectively.

L-[3,3,5-3H]Tyrosine (36 Ci per mmole) was supplied by Amersham-Searle.

Carboxymethylcellulose CM52 and Sepharose 6B were products of H. Reeve Angel and Pharmacia, respectively. α-Chymotrypsin (bovine pancreatic) and trypsin inhibitor (soybean) were obtained from Sigma. Other enzymes and proteins were purchased, according to availability, from Sigma, Worthington, or Calbiochem. Other chemicals were of highest available grade and were, except as noted, used without further purification. Glass-distilled water was used throughout.

**Methods**

**Assays and Activation—**Assays of DOPA oxidase and tyrosine hydroxylase activity were performed at 25°C. All solutions were equilibrated with air before use, and, in both assays, quantities

1 The abbreviations used are: DOPA, 3,4-dihydroxyphenylalanine; bis-tris, 2,2'-bis(hydroxymethyl)-2,2'-2'-4-nitroloctanol.
of enzymes were used such that measured activities were linearly dependent upon the amount of enzyme present.

Tyrosinase activity was routinely determined at 25°C using L-DOPA as substrate as suggested by Fling et al. (2). The reaction mixture consisted of 1 ml of 5 mM L-DOPA in 0.1 M sodium phosphate, pH 6.8. $v_{	ext{max}}$ for the conversion of DOPA to dopachrome was taken as 3600 cm$^{-1}$ (14), and the unit of enzymic activity was micromoles of dopachrome produced per min. All measurements of enzymic activity refer, unless specified, to this DOPA oxidase assay.

Tyrosine hydroxylase was measured at 25°C by the technique of Pomerantz (15). Tyrosine and DOPA concentrations were, respectively, $5 \times 10^{-4}$ M and $2.5 \times 10^{-5}$ M in a 1 ml reaction volume of 0.1 M sodium phosphate buffer, pH 6.8. The unit of tyrosine hydroxylase activity is micromoles of L-tyrosine oxidized per min.

Aliquots of enzyme were generally assayed both with and without the addition of chymotrypsin, thus permitting the determination of both active tyrosinase (directly) and proenzyme (by difference). Total activity was developed by adding 50 $\mu$g (10 $\mu$l) of chymotrypsin to the enzyme sample contained in the assay cuvette, incubating 3 min at 25°C, and stopping the reaction by addition of 400 $\mu$g (20 $\mu$l) of soybean trypsin inhibitor. This material was also added to samples not being proteolyzed, because we found that occasionally small amounts of chymotrypsin adsorbed to the cuvettes gave rise to opurious activity in otherwise pure samples of proenzyme. Active enzyme was prepared in quantity by adding to purified proenzyme 2% chymotrypsin by weight and incubating at 37°C until maximum activity developed (about 30 min). Proteolysis was stopped, if necessary, by addition of an 8-fold excess by weight of soybean trypsin inhibitor.

Amino Acid Analysis—Amino acid analyses were performed by Dr. William Konigsberg (Yale University) on 0.2-ml samples of pure proenzyme hydrolyzed by 6 $N$ HCl for 24 and 48 hours in evacuated tubes. No correction for loss of cysteine was applied. Tryptophan content was evaluated spectrophotometrically by two methods.

Protein Concentrations Determination—Routine determinations of pure proenzyme protein were performed spectrophotometrically using an extinction coefficient of 1197 cm$^{-1}$ for tyrosine and 5559 cm$^{-1}$ for tryptophan. Because of turbidity, the method of Lowry et al. (18) was used at all steps of purification before CM-cellulose chromatography.

Polyacrylamide Gel Electrophoresis—Acrylamide and N,N',N''-trimethylethylenediamine were used for crosslinking. Polyacrylamide molecular weights were determined by the sodium dodecyl sulfate gel procedure of Weber and Osborn (19). The proenzyme and active enzyme were studied on 10% acrylamide gels containing 0.17% bisacrylamide; while for the peptide proenzyme, 4% acrylamide gel was used with 0.77% bisacrylamide.

Analytical polyacrylamide gel electrophoresis of the intact proenzyme and active enzyme were performed in a continuous 5% acrylamide gel system containing 0.25% bisacrylamide. The running buffer was 0.025 M bis-tris-acetate, pH 6.0. Samples were applied in a 20-fold dilution of this medium. Under these conditions the enzyme migrates toward the cathode and crystal violet is a suitable tracking dye. Enzymic activity was visualized on the gels by treatment with 10 mM DOPA in 0.1 M sodium phosphate, pH 6.8, containing, for proenzyme samples, 0.01 mg per ml of chymotrypsin. Protein was stained with Coomassie blue 6B.

Analytical Gel Filtration—The molecular weights of proenzyme and active enzyme were estimated by gel filtration through a column (100 x 1.2 cm) of Sepharose 6B. Total and void volumes were determined using water calibration and blue dextran, respectively. Standard proteins emerging from the column were human y-globulin (153,000), bovine liver catalase (248,000), horse spleen apoferritin (467,000), and Escherichia coli $\beta$-galactosidase.

Copper Analysis—Copper was determined by atomic absorption analysis by Dr. J. E. Coleman (Yale University). A Jarrell-Ash spectrometer was employed at 324.7 nm using the "spike-height" method of absorbance determination (20).

Sucrose Density Gradient Centrifugation—Sedimentation coefficients for the enzymes were evaluated in a Beckman model L ultracentrifuge by the sucrose density technique of Martin and Ames (21). Bovine catalase was used as an internal standard with an assumed $s_{20,w} = 11.3 S$.

Isoelectric Focusing—Enzyme samples were subjected to isoelectric focusing at 4°C and 600 volts on a pH 7 to 10 Ampholine gradient for 90 hours. A 110-ml capacity apparatus from LKB Instruments, Rockville, Md. was employed.

RESULTS

Purification of Enzyme

The simple and highly effective purification procedure used in this study depends upon the basicity of this tyrosinase. Both the proenzyme and the activated protein can consequently be adsorbed on CM-cellulose under conditions where few other proteins bind the resin. The procedure detailed below is summarized in Table I. Separation of frog skins and chromatography over CM-cellulose were performed at room temperature; all other steps were carried out at 4°C. Buffer was, unless otherwise stated, an equimolar mixture of mono- and disodium phosphates, pH 6.8, diluted to the appropriate total phosphate concentration.

Extraction of Tyrosinase—Thirty to forty medium sized frogs (2.5 to 3.5 inch bodies) were decapitated, pithed, and skinned. The skins were soaked for 1 hour in 2 M KBr. The epidermis was manually removed from dermis and placed in 0.1 M Tris-HCl, pH 7.5, measured at 4°C. The epidermal tissue was homogenized in a motor driven Potter-Elvehjem apparatus with 2.5 ml of buffer for each frog epidermis. The extract was sedimented for 20 min at 40,000 x g in a Sorvall RC2-B refrigerated centrifuge; 50 ml volume of 1 M MnSO$_4$ was then added to the supernatant fraction to precipitate nucleic acids (22). Centrifugation for 1 hour, 100,000 x g in a Beckman model L ultracentrifuge removed manganese precipitate together with particulate tyrosinase and debris.

This solution was stable at -70°C, so that when desired, extracts of several batches of frogs could be pooled for purification.

<table>
<thead>
<tr>
<th>Purification</th>
<th>Total Specific</th>
<th>Total Specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>mg/mg units</td>
<td>mg/mg units</td>
</tr>
<tr>
<td>Fraction</td>
<td>vol mg</td>
<td>units units</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg/mg %</td>
</tr>
<tr>
<td>Epidermal homogenate</td>
<td>89.0 56.0 43.6 0.077 240 0.52</td>
<td></td>
</tr>
<tr>
<td>MnSO$_4$ supernatant</td>
<td>92.0 56.0 42.2 0.072 285 0.49</td>
<td>97.0</td>
</tr>
<tr>
<td>30 to 65% (NH$_4$)$_2$SO$_4$ precipitate...</td>
<td>11.2 23.4 43.8 0.191 265 1.18</td>
<td>90.0</td>
</tr>
<tr>
<td>Redissolved precipitate after dialysis</td>
<td>19.0 184.0 26.4 0.142 236 1.28</td>
<td>80.0</td>
</tr>
<tr>
<td>CM-cellulose eluate</td>
<td>6.1 1.89 10.9 5.77 67.0 35.4</td>
<td>23.0</td>
</tr>
</tbody>
</table>
Ammonium Sulfate Fractionation—The tyrosinase was isolated from the material precipitating between 30 and 65% saturated (NH₄)₂SO₄. To the supernatant fraction obtained after sedimentation at 100,000 × g, 0.43 volume of saturated (NH₄)₂SO₄ was added and the mixture sedimented for 20 min at 12,000 × g. Saturated (NH₄)₂SO₄ (1 volume) was then added to the supernatant fraction and the mixture resedimented at 12,000 × g. The pellet was dissolved in a minimal volume (10 ml) of 0.01 M sodium phosphate, pH 6.8. The solution was then twice dialyzed against 4 liters of this buffer, 8 hours per change. The conductance of the protein solution was then checked to ensure its being within 10% of the conductance of the dialysis buffer. There is some loss of enzymic activity in this step and perhaps a better yield would be obtained by the use of hollow fiber dialysis to remove (NH₄)₂SO₄.

CM-cellulose Chromatography—From microgranular CM-cellulose (Whatman CM52), equilibrated with 0.01 M sodium phosphate, pH 6.8, a column was prepared with a volume about equal to that of the dialyzed enzyme and with a length to diameter ratio close to 5. The absorbance of column effluent was continuously monitored at 280 nm and the pH and conductivity of the effluent were checked to ensure complete equilibration. This column preparation was done in advance to minimize the time the enzyme spent in 0.01 M buffer. The dialyzed enzyme was then sedimented for 20 min at 12,000 × g and the supernatant fraction applied to the column at a flow rate of 10 ml cm⁻² hr⁻¹. The column was washed with 0.01 M buffer at the maximum feasible flow rate until the A₂₈₀ of the effluent reached zero. A linear buffer gradient spanning 0.01 M to 0.1 M in 4 column volumes was then applied at a flow rate of 10 ml cm⁻² hr⁻¹. The enzyme emerged in a sharp, symmetrical peak centered at 0.03 M. The fractions of highest specific activity were stored at -70°C. Although the enzyme is stable for days at room temperature in unpurified epidermal extracts, dilute solutions of the purified enzyme are rather labile, even at 4°C.

Polyacrylamide Gel Electrophoresis Verification of Purity

The proenzyme fraction of highest specific activity appears homogeneous at pH values of 5, 6, and 7. We chose pH 6 for routine analysis of our samples since, at pH 5, resolution of the various proteins is poor while, at pH 7, the mobility of the enzyme is inconveniently small. Fig. 1 (left) shows the electrophoresis on two identical pH 6 gels of proenzyme from the highest activity tube of the eluted protein peak. The gel on the left was stained for protein, whereas the gel on the right was stained for DOPA oxidase activity with DOPA and chymotrypsin.

Proteolytic Activation of Tyrosinase

There is no measurable tyrosinase activity in crude frog epidermal extracts or in the purified proenzyme. Only upon proteolysis does enzymic activity appear. We have activated frog prototyrosinase with a variety of endopeptidases. Such activation was tested by adding protease to prototyrosinase (1:1 by weight) in 0.1 M buffer, incubating at 37°C for 30 min, and assaying. Chymotrypsin, trypsin, papain, and pronase all activate the enzyme, chymotrypsin being the most efficient. Carboxypeptidase A does not activate prototyrosinase. Prolonged incubation at 37°C of purified prototyrosinase with carboxypeptidase A (1:1 by weight) in 0.025 M Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl does not yield activity in the DOPA oxidase assay. These conditions are optimal for carboxypeptidase-catalyzed hydrolysis of hippuryl-L-phenylalanine and the prototyrosine can be activated in this buffer by the addition of chymotrypsin.

Some activation is achieved by leucine aminopeptidase upon prolonged incubation at 37°C of equal weights of prototyrosinase and diisopropylfluorophosphate-treated leucine aminopeptidase in 0.02 M Tris-HCl, pH 8.5, containing 5 mM MgCl₂. Results have been somewhat variable; but we have achieved, on the average, tyrosinase activities 15% of those obtained with chymotrypsin.

Fig. 2 illustrates the effect of adding varying amounts of chymotrypsin for a fixed duration of incubation. The time course of activation of proenzyme with a fixed amount of chymotrypsin follows simple first order kinetics; at 25°C, activation with 2% chymotrypsin has a half-time of 7 min. The kinetics thus suggest very rapid proteolysis of a peptide bond. This conclusion is borne by the observation that sodium dodecyl sulfate gel electrophoresis reveals in enzyme solutions carefully activated by chymotrypsin a single peptide not corresponding to any trace
contaminant of the proenzyme. From its mobility on such gels we estimate the molecular weight of this material to be 5700 ± 700 (19). Fig. 1 (right) shows the gels of the proenzyme and the active enzyme. The lowest band on the active enzyme gel is the peptide. The location of this peptide following proteolysis is not known; it may remain bound to the active enzyme or be released into solution.

Early in our studies of the enzyme we considered the possibility that tyrosinase, as extracted from the frog skin, was inactivated by a noncovalently bound inhibitor which was released by proteolysis. Consequently we attempted to activate the enzyme by removing this hypothetical inhibitor. Prolonged dialysis of the enzyme produced no activation, nor did dialysis against anisate, the most effective competitive inhibitor of DOPA oxidation. Urea and sodium dodecyl sulfate were similarly ineffective in evoking activity.

The tyrosinase from R. pipiens berlandieri exhibits qualitatively the same phenomenon of proteolytic activation as does that of R. pipiens pipiens. The isoelectric points of Tyrosinase

The isoelectric points of the proenzyme and active enzyme are not perfectly coincident, as shown in Fig. 3 which superimposes the isoelectric focusing patterns for these two materials. We estimate the isoelectric point of the proenzyme to be 9.35 and that of the chymotrypsin-activated material to be 9.25. By comparison, R. pipiens berlandieri yield an enzyme which is not bound by CM-cellulose at pH 6.8 and which focuses off of the linear portion of a pH 7 to 10 Ampholine gradient at about pH 6.

Structural Characterization of Enzyme

The molecular weights of both the active and proenzyme were estimated by gel filtration to be 200,000 ± 12,000. This agrees with a sedimentation coefficient $s_{20, w}$ of 10.0 S for both proteins obtained through sucrose density gradient centrifugation.

Both the proenzyme and the active enzyme are probably composed of four polypeptide chains, the molecular weights of which are 54,100 ± 700 and 50,000 ± 700, respectively. Fig. 1 (center) shows the mobility difference in sodium dodecyl sulfate gel electrophoresis between the two polypeptides. If the enzyme is indeed composed of identical subunits, then there are almost certainly an even number of such protomers. The molecular weights for the enzyme and for its constituent polypeptide chain are clearly incompatible with a dimeric or hexameric structure. Moreover, the copper content of the proenzyme is 0.15%, which corresponds to 4.6 moles of copper per 200,000 molecular weight, approximately 1 mole of copper per monomeric unit.

The amino acid analysis of the proenzyme is shown in Table II. No correction has been made for loss of cysteine, but it is at least clear that this amino acid is present in the protein in

![Fig. 2. Effect of varying amounts of chymotrypsin on activation of frog epidermal protyrosinase. Proenzyme (2.1 μg) was incubated for 3 min at 25° with the specified amount of chymotrypsin in a total volume of 0.5 ml. Proteolysis was stopped by adding 0.4 mg of soybean trypsin inhibitor and the DOPA oxidase activity of the sample determined.

![Fig. 3. Isoelectric focusing patterns for frog epidermal protyrosinase (—) and chymotrypsin-activated enzyme (---). Both the proenzyme (1.8 mg) and the active enzyme (0.3 mg) were mixed and simultaneously focused for 90 hours at 600 volts in the same pH 7 to 10 Ampholine gradient which was then divided into 2-ml fractions. For each fraction, DOPA oxidase activities corresponding to both proteins were determined as described in the text.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Grams/100 g of protein</th>
<th>Moles/200,000 molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>12.66</td>
<td>190.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.52</td>
<td>129.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.28</td>
<td>113.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.35</td>
<td>75.3</td>
</tr>
<tr>
<td>Valine</td>
<td>4.17</td>
<td>71.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.49</td>
<td>129.4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.64</td>
<td>70.8</td>
</tr>
<tr>
<td>Proline</td>
<td>5.21</td>
<td>90.5</td>
</tr>
<tr>
<td>Serine</td>
<td>4.05</td>
<td>77.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.45</td>
<td>74.8</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>1.43(3.37)</td>
<td>23.7</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.77</td>
<td>37.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.03</td>
<td>97.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.52</td>
<td>94.1</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.45</td>
<td>33.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.40</td>
<td>69.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.97</td>
<td>150.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>13.77</td>
<td>158.1</td>
</tr>
</tbody>
</table>

* Cystine is not recovered quantitatively after the hydrolysis.
* Tryptophan analyses were performed spectrophotometrically (16).
substantial quantities. This is not the case with tyrosinase from Neurospora (2). The high ratio of basic to acidic residues reflects the high isoelectric point observed for the protein. In particular, to calculate (23) the isoelectric point of 9.35 observed for the proenzyme, 60% of the acidic residues found on hydrolysis must be assumed to be present as amides in the native protein. Our samples were too small to permit analysis for amide nitrogen; however, 60% is within the range of a number of well characterized proteins (24).

One interesting point of comparison between the active tyrosinase and the proenzyme is the thermostability of the two proteins. While the active enzyme has a thermal inactivation halftime of 120 min at 60°C, one-half of the latent activity of the proenzyme is lost in only 20 min at this temperature. We conclude that proteolysis permits the active enzyme to assume a more compact and thermostable structure than that available to the proenzyme.

Kinetic Results

The homogeneous enzyme, after activation, functions as a DOPA oxidase and a tyrosine hydroxylase, but is devoid of peroxidase activity (25) using guaiacol as a substrate. Although guaiacol is slowly oxidized by the enzyme, neither the rate of this process nor that of DOPA oxidation is affected either by addition of peroxide or by prolonged incubation of the enzyme with catalase. Crude enzyme preparation contained tyrosine oxidase, DOPA oxidase, catalase, and guaiacol oxidase activities. The most highly purified enzyme retained the ability to oxidize DOPA, tyrosine, and guaiacol in the same ratio found in the crude preparation. Catalase activity was lost in the first purification steps.

The $K_m$ values of both tyrosine and DOPA are $5 \times 10^{-4}$ M. The $k_{cat}$ values for both substrates are also equal at 800 min$^{-1}$. There is, however, a marked difference between the $K_m$ for DOPA for this enzyme and that from R. pipiens berlandieri, the Rio Grande frogs having a 10-fold lower $K_m$ for DOPA ($5 \times 10^{-4}$ M) than the R. pipiens pipiens variety.

The pH dependence of DOPA oxidase activity was investigated in a buffer 0.05 M each in citric acid, phosphoric acid, glycine, and glycine/lysine. The buffer was noninhibitory to the enzyme and appropriate additions of NaOH permitted a smooth variation of pH from 3 to 11. Fig. 4 shows the pH dependence of the enzyme activity after appropriate correction for autoxidation of DOPA at alkaline pH. Maximum activity is at approximately pH 7.0.

A variety of substituted benzoic acids inhibits DOPA oxidase activity competitively with respect to DOPA. Table III shows the inhibition constants for the compounds examined. The copper complexing agents, cyanide and diethyldithiocarbamate, also inhibit the enzyme but do not compete with DOPA. The reversibility of inhibition by these compounds is shown by the fact that an enzyme sample inhibited by either material may be restored to activity by addition of excess copper. Diethyldithiocarbamate effects 50% inhibition at a concentration of $2.5 \times 10^{-4}$ M and cyanide at a concentration of $1 \times 10^{-4}$ M. The question of whether these compounds inhibit competitively with respect to oxygen was not explored, although it has been shown that cyanide competes with oxygen in binding to mushroom tyrosinase (26).

Copper can be removed from the proenzyme or active enzyme by dialysis against 10 mM cyanide or diethyldithiocarbamate. Following removal of the complexing agent by dialysis, prolonged incubation with 1 mM Cu$^{2+}$ restores up to 40% of the activity of the original protein to the apoenzyme, or to the chymotrypsin-treated apoprotein. Alternatively, the copper may be replaced in the apoprotein before proteolysis with the same result. Other divalent cations used at 1 mM concentration in an attempt to restore activity to the apoproteins, Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Fe$^{2+}$, were without effect.

The enzyme is quite stable toward sodium dodecyl sulfate. At the highest detergent concentrations used (0.2%), the activity of the enzyme is indistinguishable from that observed when none of this material is present.

**Discussion**

We have isolated frog epidermal prototyrosinase in high purity and yield. The effectiveness of the technique depends upon the extremely high isoelectric point of the R. pipiens pipiens enzyme and thus cannot be applied to the prototyrosinase from the related subspecies R. pipiens berlandieri which also exhibits proteolytic activation.

Of interest to us is the phenomenon of proteolytic activation. This example of post-translational control regulates the frog's pool of active tyrosinase by the presence or absence in the appropriate epidermal structures of only a small amount of proteolytic enzyme(s). Whether such a control mechanism for active tyrosinase levels exists in higher organisms is an important

**Fig. 4.** DOPA oxidase activity of frog epidermal tyrosinase as a function of pH. Ten microliters of a suitable enzyme solution was added to 0.5 ml of a buffer 0.05 M each in citric acid, glycine/lysine, glycine, and phosphoric acid previously adjusted to the desired pH with sodium hydroxide. An equal volume of 10 mM DOPA in distilled water was added, the enzymic reaction was allowed to proceed for 10 min at 25°C, and 100 microliters of the reaction mixture was transferred to a 1 cm cell, and the optical density was measured at 500 m.$

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Competitive with DOPA</td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>$1.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>$1.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>Anisic acid</td>
<td>$4.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Veratric acid</td>
<td>$6.1 \times 10^{-5}$</td>
</tr>
<tr>
<td>Noncompetitive with DOPA</td>
<td></td>
</tr>
<tr>
<td>Cyanide</td>
<td>$1.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>$2.5 \times 10^{-5}$</td>
</tr>
</tbody>
</table>
question. The best characterized mammalian tyrosinases are from mouse and hamster melanoma (5, 7) and both are fully active in the crude extracts. It is possible that tyrosinase is synthesized as a proenzyme in a variety of higher species, but that disruption of the tyrosinase-bearing tissue releases proteases which activate the proenzyme and prevent its isolation. Treating such intact tissue, particularly epidermis, with a spectrum of irreversible protease inhibitors might permit identification of tyrosinase proenzymes if they exist.

The turnover rates of both DOPA and tyrosine are about 800 min⁻¹ for the frog epidermal enzyme. If, as in the Neurospora (2) and hamster melanoma (3) enzymes, 2 moles of DOPA are oxidized per mole of dopachrome formed, the oxidation of DOPA is then twice as fast as that of tyrosine. Compared with the frog enzyme, hamster melanoma tyrosinase exhibits a similar turnover number for tyrosine of 1000 min⁻¹ but a much higher one for DOPA of 5400 min⁻¹. Both the Neurospora and mushroom tyrosinases also oxidize DOPA at a rate considerably greater than the frog enzyme; the importance of this fact is not known. It is possible that activation in vivo by some unknown protease produces in the frog a much more active product than that afforded by chymotryptic digestion in vitro.

The NH₂-terminal residues of the proenzyme and the active tyrosinase from frogs have not yet been determined. These data, when obtained, should resolve the important question of whether or not the activation peptide remains bound to the active enzyme.

Acknowledgments—The authors are grateful to Miss Dawn Newman for her invaluable assistance during this study and to Drs. William Konigsberg and Joseph Coleman for their courtesy and interest in performing the amino acid and copper analyses, respectively, of the proenzyme.

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