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MAMMALIN TYROSINASE
STOICHIOMETRY AND MEASUREMENT OF REACTION PRODUCTS

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Summary

The substrates and intermediates involved in the conversion of tyrosine or 3,4-dihydroxyphenylalanine into melanin by autooxidation, or tyrosinases (monophenol, dihydroxyphenylalanine:oxygen oxidoreductases, EC 1.14.18.1) of mushroom or mammalian melanocyte origin, were studied by a variety of enzymic assays, and by amino acid analysis. It was found that the classic pathway of melanin formation was followed, and that the proposed alternate pathway involving formation of the intermediate 3,4,6-trihydroxyphenylalanine was not a functional route, since nascent trihydroxyphenylalanine was not detectable. The ability of isolated mammalian tyrosinases to convert tyrosine into dihydroxyphenylalanine was unequivocably demonstrated. The polymerization of monomers into melanin was followed by the use of specifically labelled precursors, and the data indicate that uncyclized and carboxylated derivatives are not incorporated into the polymer in vitro. It was found that although in most respects the melanin produced from tyrosine by mushroom and mammalian tyrosinases are similar, the control mechanisms involved in the expression of melanin formation in these organisms must differ greatly.

Introduction

The biochemical characterization of the reactions involved in the synthesis of melanin from tyrosine by an enzyme called tyrosinase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) has not yet

been completed [1—6]. In part, this has been due: (a) to the inherent difficulty in the analysis of melanin, a black insoluble polymer; (b) to the differences in the enzyme preparations from the various sources that have been studied, and (c) to the general complexity of the reaction, some steps of which are enzymically catalyzed and some of which are not enzyme dependent. Recently, there has been a challenge [7—9] to the generally accepted scheme that the initial reaction involves the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine by tyrosinase; in addition, this challenge has questioned the validity of some of the methodologies employed in previous studies. Lastly, some recent evidence has suggested that new intermediates might be formed as the result of the oxidation of dihydroxyphenylalanine [10—12] by tyrosinase, resulting in the production of 3,4,6-trihydroxyphenylalanine, instead of the previously expected intermediate of dopaquinone.

This study was undertaken to identify some of the substrates and intermediates involved in the tyrosinase reactions, using new methodology. Additionally, our studies were carried out not only with enzyme of mushroom origin (used almost exclusively by other laboratories to date for identification of reaction products), but by way of comparison, we also used purified preparations of mammalian tyrosinases from both normal and malignant melanocytes.

Materials and Methods

Sources of Materials. Phenylalanine, tyrosine, dihydroxyphenylalanine, trihydroxyphenylalanine, tryptophan, 5-hydroxytryptophan, 5-hydroxyindole, 5-hydroxyindole-3-acetic acid, 5-hydroxyindole-2-carboxylic acid and mushroom tyrosinase were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals for the enzymic assays were obtained from the commercial suppliers listed in an earlier paper [13]. Tyrosinases from normal (C57Bl mouse epidermis) and malignant (B-16 murine malignant melanoma) melanocytes were purified as detailed in a previous report [14]. Only T-1 isozymes [15] which had been purified to apparent homogeneity were used. Isotopically labelled substrates were purchased from the following suppliers (specific activities were adjusted to those noted with unlabelled amino acids): L-[2,5,6-^{3}H]dihydroxyphenylalanine (1.07 Ci/mmol, 131 μCi/ml), L-[α,β-^{3}H]-dihydroxyphenylalanine (1.24 Ci/mmol, 142 μCi/ml), L-[U-^{14}C]dihydroxyphenylalanine (17.8 Ci/mol, 2.13 μCi/ml), L-[*carboxyl*-^{14}C]dihydroxyphenylalanine (8.26 Ci/mol, 0.94 μCi/ml), L-[β-^{14}C]dihydroxyphenylalanine (8.05 Ci/mol, 0.94 μCi/ml), L-[3,5-^{3}H]tyrosine (0.86 Ci/mmol, 96.6 μCi/ml), L-[α,β-^{3}H]-tyrosine (0.97 Ci/mmol, 119 μCi/ml), L-[*carboxyl*-^{14}C]tyrosine (56.2 Ci/mol, 7.87 μCi/ml), L-[U-^{14}C]tyrosine (106 Ci/mol, 123 μCi/ml) were from Amersham-Searle (Arlington Heights, IL); L-[3,5-^{3}H]tyrosine, L-[2,6-^{3}H]-tyrosine (1.21 Ci/mmol, 144 μCi/ml), L-[2,3,5,6-^{3}H]tyrosine (1.16 Ci/mmol, 148 μCi/ml), L-[U-^{14}C]tyrosine, L-[*carboxyl*-^{14}C]tyrosine, and DL-[β-^{14}C]-tyrosine (16.5 Ci/mol, 4.55 μCi/ml) were from New England Nuclear (Boston, MA). Purity of the isotope preparations was determined by amino acid analysis (see below), and when necessary, further purification was carried out as detailed in an earlier communication [13].

Tyrosinase assays. Two basic radiometric assays were used and are described in another paper [13]. Briefly, each assay was set up at least in duplicate and contained in a final volume of 25 μl, the following components; 0.1 M phosphate buffer (pH 7.4), chloramphenicol and cycloheximide (0.2 mg/ml), 200 units/ml penicillin G, 20 μg/ml bovine serum albumin, 50 μM labelled substrate, plus any cofactors or other agents as listed in the text. Routinely, 50 μM tyrosine was used as the labelled substrate with 5 μM unlabelled dihydroxyphenylalanine added as a cofactor and the unit (U) of enzyme activity reported was at those concentrations. Controls used to establish backgrounds included blanks from which the substrate and/or enzyme was omitted, boiled enzyme preparations, and enzyme inhibited with diethyldithiocarbamate [16]. The assays were incubated at 37° C for 60 min, or as detailed in the figure and table legends; they were then placed on ice, and 20-μl aliquots removed for processing as follows:

When the formation of acid-insoluble products (melanin) was to be measured, the aliquots were placed on 25-mm discs of Whatman 3 MM filter paper, allowed to air dry, and washed in the following sequence: one 15 min wash in 0.1 N HCl (with 50 mM unlabelled tyrosine), two 15-min washes in 0.1 N HCl, two 15-min washes in aqueous 99% C_2H_5OH, and one 15-min wash in acetone. The filters were again air-dried, then placed in scintillation vials with 0.4% (w/v) 2,5-diphenyloxazole in toluene and the radioactivity measured with a Packard 3375 liquid scintillation spectrometer. The efficiency of counting the ^{14}C and ^{3}H labels was 70 and 3.5%, respectively.

The other assay methodology measures the formation of $^{3}H_2O$ as a byproduct of the reactions of the enzyme with tritiated substrates [17,18]. When $^{3}H_2O$ was to be measured, the 20-μl aliquots of the reaction mixture were each placed in 0.98 ml 0.1 N HCl containing approx. 100 mg Celite 545 and 100 mg Norit A. The tubes were shaken at room temperature for approx. 90 min, centrifuged at $1000 \times g$ for 10 min, then 200-μl aliquots of the supernatant were removed and counted for radioactivity in Aquasol (at 30% efficiency). Appropriate corrections were made to facilitate direct comparisons with the melanin filter assays detailed above.

Amino acid analysis. In experiments where the concentration of tyrosine, dihydroxyphenylalanine and other intermediates were to be measured, the assays were set up with a final volume of 200 μl, but with all concentrations identical to those as listed above. After the specified time of incubation, the assays were placed on ice and 20 μl 10 N HCl were added to stop the reaction and stabilize the products. The samples were then separated on a Beckman 119 CL amino acid analyzer, equipped with a Beckman 126 data analyzer. The resin column was operated as described by Beckman for a simple hydrolysis run, completing the entire sequence in 98 min. After the sample had passed through the ninhydrin reaction vessel and had been quantitated in the spectrophotometer, the eluant was directed to a fraction collector, where samples were collected at 1 min intervals. 200-μl aliquots of these fractions were then placed in scintillation vials with Aquasol and the radioactivity of each fraction was measured (^{3}H at 25% efficiency, ^{14}C at 95% efficiency). The elution times of the various intermediates were determined by analysis of the known reagents listed in the materials section (see also legend to Fig. 1).

Statistics. All statistical analyses were carried out using Student's *t*-test.

Results and Discussion

The classical concept [19,20] of melanin formation is summarized in Scheme I. Tyrosinase has been considered to be a bifunctional enzyme, and obligatory for the initial step, that is, the hydroxylation of tyrosine (I) to dihydroxyphenylalanine (II). The oxidation of dihydroxyphenylalanine to dopaquinone (IV), although not strictly dependent on tyrosinase, is catalyzed at a much faster rate by the enzyme. The remaining steps of the reaction are thought to be enzyme independent, although it has not been demonstrated whether tyrosinase will affect their rates or not. Dopaquinone is cyclized and reduced to form an hydroxylated indole ring (leukodopachrome, V); this indole is then oxidized to dopachrome (VI). This latter compound is decarboxylated, yielding 5,6-dihydroxyindole (VII), which in turn is oxidized to an indole-quinone (VIII). Although there is still a great deal of disagreement as to the exact makeup of melanin, it is pretty well agreed that it is an irregularly linked heteropolymer, made up of several different melanogenic precursors, primarily V—VIII. There is some evidence that non-cyclized precursors (I, II and IV) are also incorporated into the melanin structure [2,6,21].

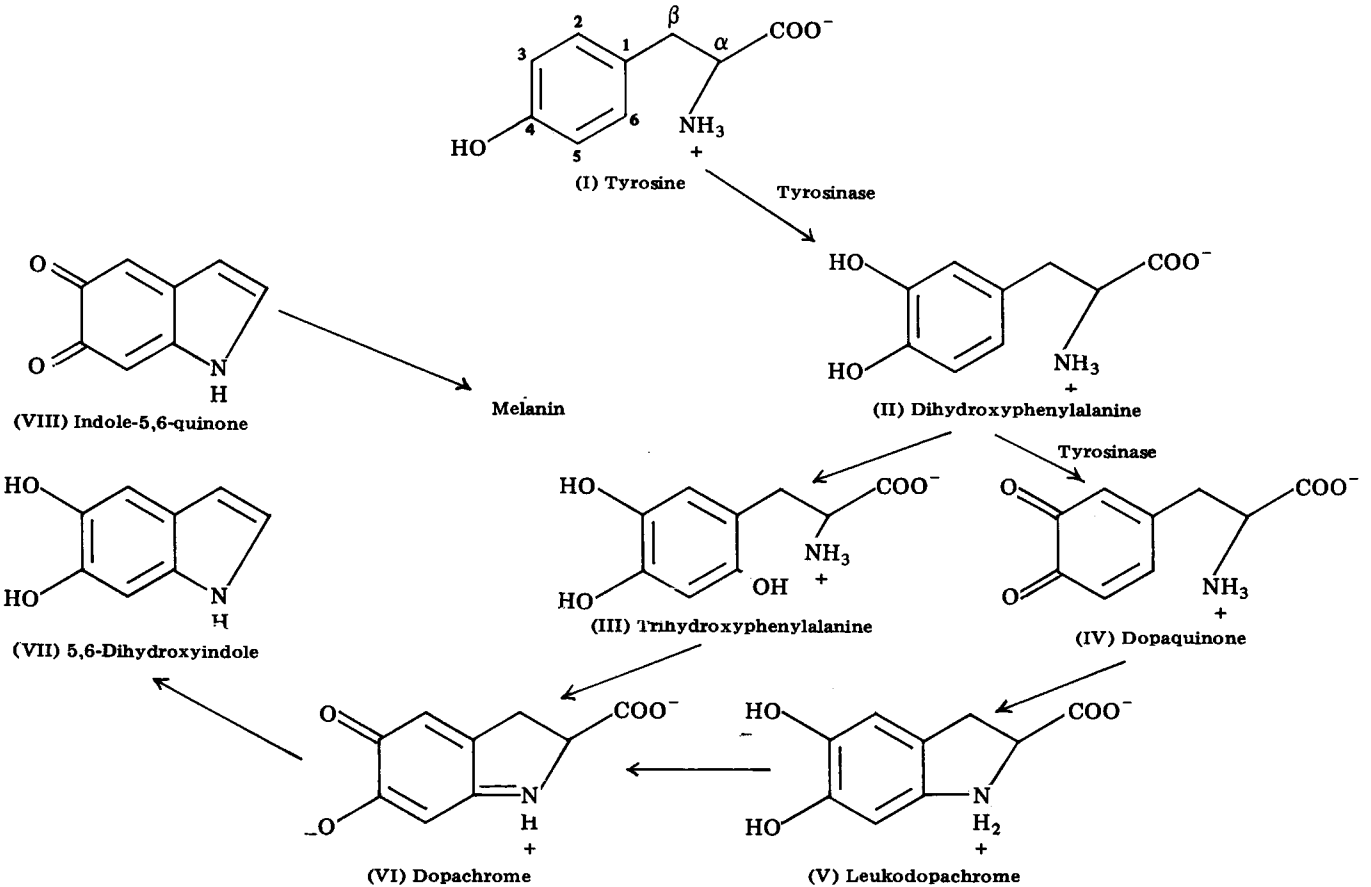
Recently, it has been suggested [10,11] that the reaction may also proceed via an alternative pathway, namely, that dihydroxyphenylalanine (instead of being oxidized by tyrosinase to dopaquinone) is further hydroxylated by tyrosinase to 3,4,6-trihydroxyphenylalanine (III), which would be subsequently oxidized and cyclized to dopachrome (VI). It has been shown that autooxidation [12] or periodate oxidation [10] of dihydroxyphenylalanine results in the formation of trihydroxyphenylalanine; this reaction can also be carried out by a tyrosinase found in one type of bacteria [11]. In each of these cases, dopachrome was formed as the result of cyclization of the nascent trihydroxyphenylalanine. Our initial experiments were aimed at determining if trihydroxyphenylalanine could be identified as an intermediate in the synthesis of melanin by mammalian enzyme.

It should be mentioned that dihydroxyphenylalanine, the first product of the reaction sequence, is also a necessary cofactor for optimal tyrosinase activity. Although slow rates of tyrosine hydroxylation do occur in the absence of added dihydroxyphenylalanine, the kinetics of the reaction are maximized when low levels of dihydroxyphenylalanine are present. This relationship is more fully discussed in a previous paper [13].

The elution profiles of reaction mixtures utilizing B-16 tyrosinase with either L-[^{14}C]tyrosine or L-[3,5-^{3}H]tyrosine at various reaction times are presented in Figs. 1 and 2, respectively. There are four peaks shown which affected the baseline but contained no radioactivity and remained constant throughout the reaction, and should be ignored (No. 21, glutamic acid; No. 25, glycine; No. 31, alanine; No. 43, buffer change). In Fig. 1, one can see that at time zero, virtually all of the ^{14}C radioactivity was in the tyrosine fraction (No. 51), and as the reaction proceeded, gradually that peak diminished, as more radioactivity was found in the dihydroxyphenylalanine fraction (No. 47), and in the indole fractions (Nos. 61 and 88).

Similarly, in Fig. 2, most of the ^{3}H radioactivity is found in the tyrosine

SCHEME I. Schematic pathway of alternate routes of melanin synthesis from tyrosine by tyrosinase.



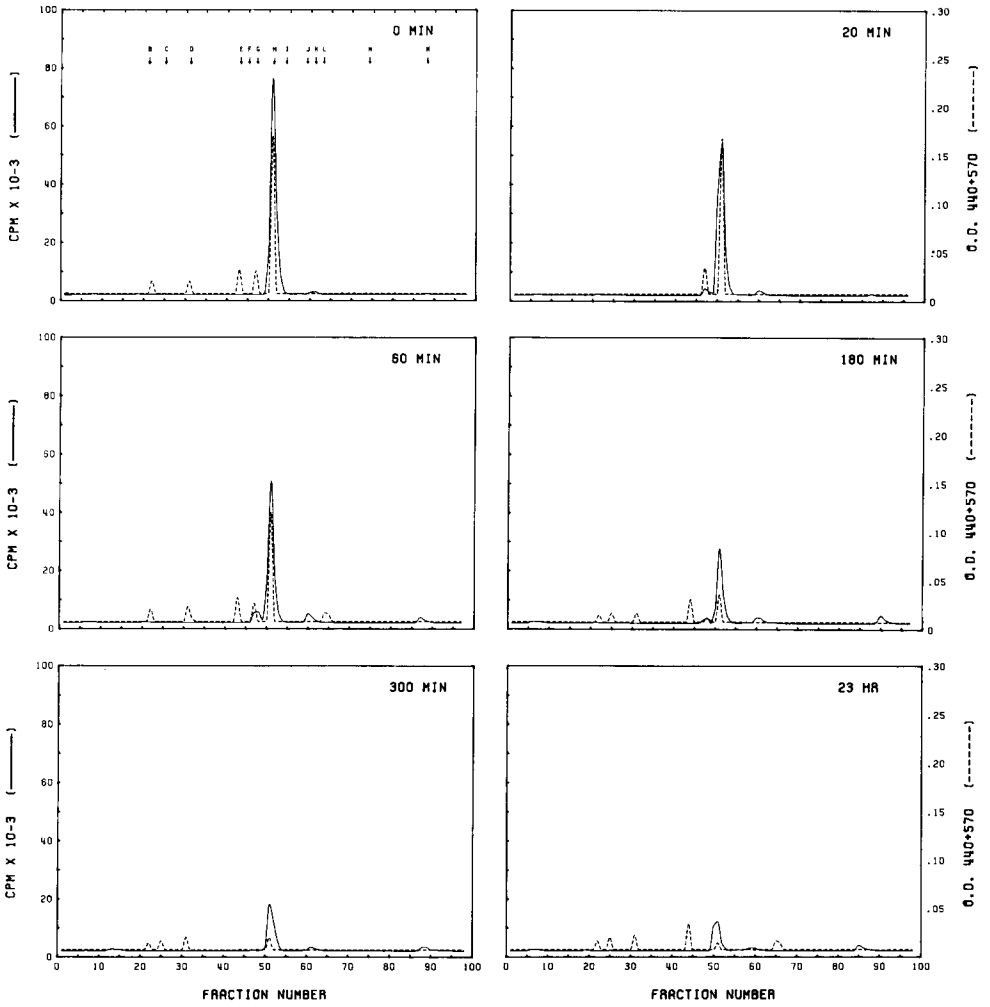


Fig. 1. Amino acid analysis of products of tyrosinase action on L-[U-^{14}C]tyrosine. (A) 0 min; (B) 20 min; (C) 60 min; (D) 180 min; (E) 300 min; (F) 23 h. 200-μl assays were set up as detailed in Materials and Methods; the reaction products at the noted times were then separated on an amino acid analyzer. Fractions were collected at 1 min intervals, and counted in a liquid scintillation spectrometer (the baseline was below 200 cpm but was raised in the figures for the sake of visibility). The products resolved showed elution characteristics as follows: A, H_2O; B, glutamic acid; C, glycine; D, alanine; E, buffer change; F, trihydroxyphenylalanine; G, dihydroxyphenylalanine; H, tyrosine; I, phenylalanine; J, 5-hydroxyindole-2-carboxylic acid; K, 5-hydroxyindole-3-acetic acid; L, 5-hydroxytryptophan; M, tryptophan; N, 5-hydroxyindole.

peak, with about 2% of the total found the ^3H_2O fraction (No. 10). As the reaction proceeded, again the amount of label in the tyrosine fraction dropped, while that in the dihydroxyphenylalanine fraction increased initially then decreased, and that in the indole fractions and the ^3H_2O fraction increased and remained stable. Virtually identical reaction products were obtained from normal (C57Bl) tyrosinase (data not shown).

The data from Figs. 1 and 2 are consistent with the classic theory of melanin

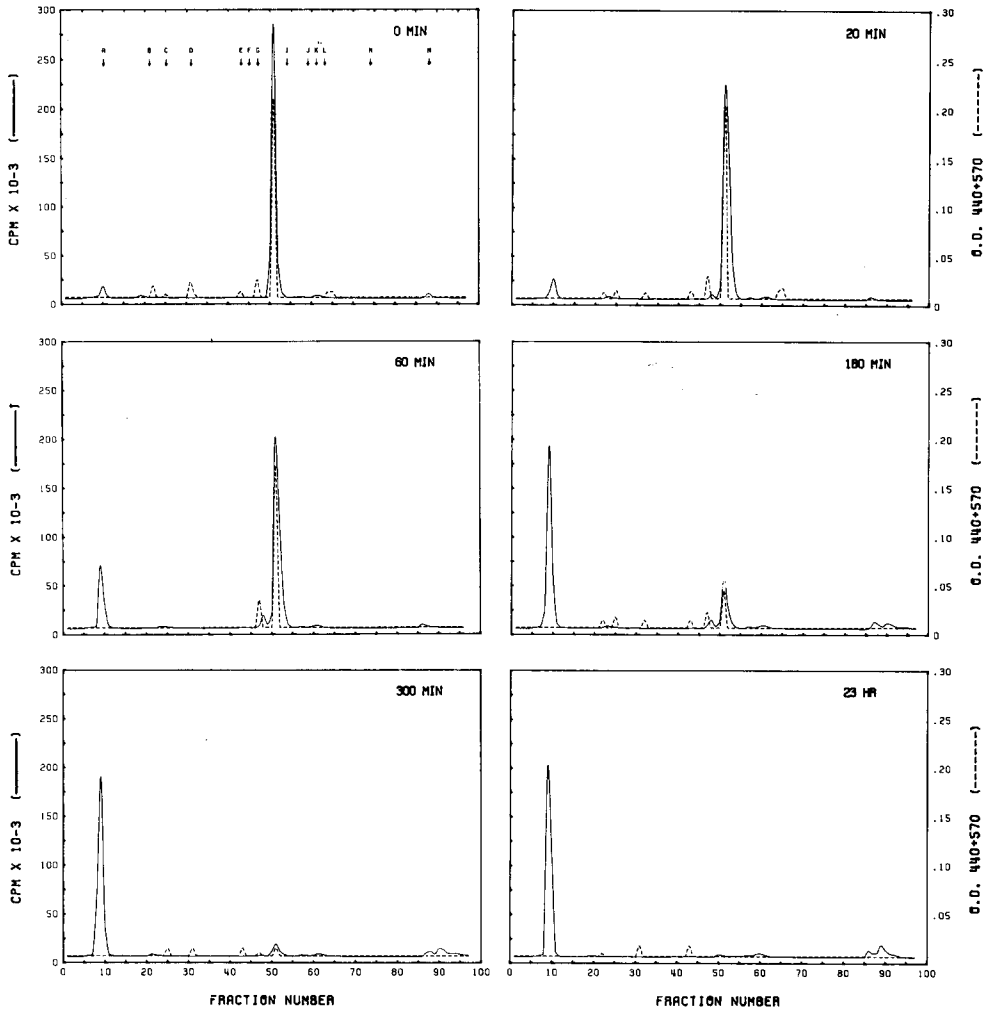


Fig. 2. Amino acid analysis of products of tyrosinase action on L-[3,5-^{3}H]tyrosine. Details as in Fig. 1.

formation, that is, formation of dopachrome through the dopaquinone intermediate. In the early stages of the reaction, there is an increase in the radioactivity in the dihydroxyphenylalanine fraction, but as the reaction proceeds the dihydroxyphenylalanine is eventually used as a substrate and depleted. There was no evidence, in any of the experiments carried out, for the formation of trihydroxyphenylalanine; this amino acid is relatively stable in acid solution (half-life of 12 h at 20° C), yet we were never able to resolve any peaks in the trihydroxyphenylalanine fraction (No. 45). When trihydroxyphenylalanine was added to the reaction mixture, it could be readily seen in fraction 45 using the amino acid analysis technique. Calculating from the resolving capability of the spectrophotometer system in the analyzer, if any trihydroxyphenylalanine is being formed it must be less than 1.2% of the total reaction pathway; similarly, by isotope measurement, we calculate that trihydroxy-

phenylalanine formation must be less than 0.5% of the total pathway. This is in good agreement with the data of Graham and Jeffs [10], whose results indicate that although the hydroxylation of dihydroxyphenylalanine to trihydroxyphenylalanine, and eventually oxidation to dopachrome, is feasible using mushroom tyrosinase, it appeared that the major pathway for dopachrome formation was through the oxidation of dihydroxyphenylalanine to dopaquinone, and thence to leukodopachrome.

These data have added significance in one other respect: Okun and coworkers [7—9] have questioned the capacity of mammalian tyrosinase to catalyze the initial reaction of the hydroxylation of tyrosine to dihydroxyphenylalanine. They feel that peroxidase is the enzyme responsible for this initial catalytic step, and that tyrosinase functions only in the oxidation of dihydroxyphenylalanine (which is not really an essential function since dihydroxyphenylalanine can autooxidize slowly to melanin, and the rate-limiting step is the initial one). While many laboratories have shown that peroxidase activity is not functional in the varous melanogenic systems under study [16,22—26], this is the first demonstration to date of a fully isolated, well-characterized [14,15] mammalian tyrosinase (other than from hamster melanoma [17,18]) catalyzing the conversion of labelled tyrosine into labelled dihydroxyphenylalanine. These data substantiate the essential correctness of the initial description of tyrosinase as bifunctional in nature by Lerner et al. [27]. In addition, this shifts the burden of responsibility to Okun's group to isolate their peroxidase and determine in this type of a definitive manner its capacity to convert tyrosine to dihydroxyphenylalanine; even if this could be shown, it will then become necessary to demonstrate the presence and functional significance of peroxidase in mammalian melanogenic systems. We have been unable as yet, using the methodology described herein for mushroom and mammalian tyrosinases, to demonstrate the production of dihydroxyphenylalanine from tyrosine by horseradish peroxidase. Our data, including further unpublished results using variously labelled tyrosine substrates, indicates that Smith and Swan were probably correct [28] in an earlier paper when they suggested that peroxidase cannot catalyze the formation of dihydroxyphenylalanine from tyrosine, but rather causes the condensation of tyrosines at the 3- and 5-positions into dimers, etc. This would explain the 'false positives' given by peroxidase when assayed by either of the two melanogenic assays as previously reported [16].

We next directed our attention to the analysis of the various fractions that were resolved, and correlation of these fractions with the assays currently used to measure them. Table I shows the results from one such experiment carried out with L-[U-^{14}C]tyrosine as the substrate; this isotope is routinely used to measure melanin formation. One can see that when comparing this parameter, as measured by the acid-insoluble filter assay and by the radiolabelled material not eluted from the resin column, there is generally good agreement after initially slight differences early in the reaction. The dihydroxyphenylalanine concentration remained level for 1 h then declined to zero; this fraction increases in isotopic labelling from 0% to approximately 75% of the dihydroxyphenylalanine at 1 h, then back again to 0% after 23 h. We have not been able to demonstrate a significant increase in dihydroxyphenylalanine con-

TABLE I

RECOVERY OF SUBSTRATES AND PRODUCTS PRODUCED BY TYROSINASE ACTION ON L-[U-^{14}C]TYROSINE

Assays were set up with 5 μM dihydroxyphenylalanine cofactor as per Materials and Methods; at the noted times 200-μl aliquots were removed and 10 μl of 10 N HCl were added; the samples were placed on an amino acid analyzer, and fractions were collected and counted. The recoveries of various substrates and products were determined by either ninhydrin assay, or by isotope recovery and are reported as the percent of the original tyrosine present at time zero. The data presented are those from one experiment; two others were carried out with similar results. 12 mU of B-16 tyrosinase were used in each assay.

Fraction assay	Time					
	0 min	20 min	60 min	180 min	300 min	23 h
Tyrosine						
ninhydrin	100.0	95.1	67.7	19.2	8.6	6.0
isotope	100.0	93.0	71.7	39.5	29.5	20.0
Dihydroxy-phenylalanine						
ninhydrin	10.7	11.3	9.9	2.0	0.0	0.0
isotope	0.0	4.2	7.3	3.4	1.2	0.0
Melanin						
isotope [a]	0.0	2.5	13.0	45.7	60.1	74.3
isotope [b]	0.5	0.7	6.6	47.0	62.6	68.1
Miscellaneous						
isotope [c]	4.0	7.8	9.0	9.7	6.0	6.2
Total						
isotope	104.5	105.7	94.6	99.6	99.3	94.3

[a] Determined as isotope not eluting from the column.
[b] Determined by 3MM filter assay, described in Materials and Methods.
[c] Total isotope present in peaks at fractions 61 and 86.

centration in any of our experiments using a 1 : 10 substrate/cofactor ratio, supporting our earlier contention [13] that dihydroxyphenylalanine will not accumulate at this cofactor ratio. This occurs even though there is measureable leakage of the nascent dihydroxyphenylalanine from the initial catalytic site on the enzyme before the further oxidation of dihydroxyphenylalanine to dopaquinone.

The levels of tyrosine in the reaction mixture revealed an interesting phenomenon: although the reaction is essentially completed at 23 h, approximately 6% of the tyrosine (probably in the D-form) remains in the medium and is never used, while 20% of the label remains in this fraction. It can also be seen that the specific activity of the tyrosine increases with the progress of the reaction; this is not a significant effect until more than 30% of the substrate has been depleted. All the kinetic data we have reported for mammalian tyrosinase [13] was measured at substrate depletion levels far below this figure, but the effect should be kept in mind. The recovery of isotope in the fractions labelled 'miscellaneous' are from two types of products: fraction 61, which contains primarily the carboxylated indole derivatives (V, and VI), and fraction 86, which contains the decarboxylated indole derivatives (VII and VIII). It can be noticed in Figs. 1 and 2 that the radioactivity at fraction 61 increases initially, then decreases, while the amount of decarboxylated indoles (fraction 86) is

TABLE II

RECOVERY OF SUBSTRATES AND PRODUCTS PRODUCED BY TYROSINASE ACTION ON L-[3,5-^{3}H]TYROSINE

Assays were set up with 5 μM dihydroxyphenylalanine cofactor as per Materials and Methods; at the noted times 200-μl aliquots were removed and 10 μl of 10 N HCl were added; the samples were placed on an amino acid analyzer, and fractions were collected and counted. The recoveries of various substrates and products were determined by either ninhydrin assay, or by isotope recovery and are reported as the percent of the original tyrosine present at time zero. The data presented are those from one experiment; two others were carried out with similar results. 12 mU of B-16 tyrosinase were used in each assay.

Fraction assay	Time					
	0 min	20 min	60 min	180 min	300 min	23 h
Tyrosine						
ninhydrin	100.0	93.1	76.5	17.8	3.6	0.0
isotope	100.0	90.0	74.7	20.5	6.2	2.0
Dihydroxy-phenylalanine						
ninhydrin	10.1	10.2	8.4	2.0	0.0	0.0
isotope	0.0	1.8	4.7	3.8	1.7	0.0
Melanin						
isotope [a]	0.0	0.5	7.6	9.5	20.0	22.3
isotope [b]	0.4	0.1	0.8	16.2	23.5	31.6
Miscellaneous						
isotope [c]	3.6	3.5	3.9	10.3	13.1	8.2
^3H_2O						
isotope [d]	0.0	7.2	23.7	46.6	56.1	62.0
isotope [e]	1.2	5.2	13.2	41.4	57.6	61.9
Total						
isotope	105.2	100.6	97.3	92.2	102.1	103.7

[a] Determined as isotope not eluting from the column.
[b] Determined by 3MM filter assay, described in Materials and Methods.
[c] Total isotope present in peaks at fractions 61 and 86.
[d] Determined as isotope eluting from the column at fraction 10.
[e] Determined by ^3H_2O assay, described in Materials and Methods.

relatively stable with time. The recovery of isotope from all these fractions is excellent, ranging from 94 to 106%.

Data from identical experiments carried out with L-[3,5-^{3}H]tyrosine are reported in Table II. This isotope is characteristically used to measure the production of ^3H_2O during the reaction, and comparison of ^3H_2O formation as determined by the Pomerantz method and the elution from the resin column (fraction 10) reveals an excellent correspondence, again with slight differences at early reaction times (20 and 60 min). Similar to the data presented for [^{14}C]tyrosine, the dihydroxphenylalanine content remains stable initially then drops to zero, while the isotopic content of this fraction rises initially, then decreases to zero at 23 h. The tyrosine concentration also decreases from time zero parallel to that in the [^{14}C]tyrosine experiment, except that at 23 h, all the ninhydrin-detectable tyrosine has been used, and only about 2% of the isotopic content of this fraction remains; this indicates that again there may be an isotope-enrichment effect, but to a lesser degree than with the [^{14}C]-tyrosine. The presence of the indoles in fractions 61 and 86 (labelled miscellaneous) again increases to approximately 10% of the total, then decreases to

TABLE III

SPECIFIC RADIOACTIVITIES OF TYROSINE AND DIHYDROXYPHENYLALANINE DURING TYROSINASE REACTION

Assays were set up with 5 μM dihydroxyphenylalanine cofactor as per Materials and Methods; at the noted times 200-μl aliquots were removed and 10 μl of 10 N HCl were added; the samples were placed on an amino acid analyzer, and fractions were collected and counted. The recoveries of tyrosine and Dihydroxyphenylalanine were determined by either ninhydrin assay, or by isotope recovery. The data are presented as nmol of substrate present, along with the amount of radioactivity and the calculated specific activity of the substrate. The data reported are those from one experiment; two others were carried out with similar results. 12 mU of B-16 tyrosinase were used in each assay.

Time	Tyrosine			Dihydroxyphenylalanine		
	nmol	dpm ($\times 10^{-3}$)	Spec. act. (dpm/pmol)	nmol	dpm ($\times 10^{-3}$)	Spec. act. (dpm/pmol)
L-[U-^{14}C]Tyrosine						
0 min	8.39	1 822	217.2 [a]	1.07	0	0.0
20 min	7.98	1 694	212.3	1.13	76	67.1
60 min	5.68	1 306	270.5	0.99	133	134.2
180 min	1.61	719	446.6	0.20	62	308.6
300 min	0.72	537	745.6	0.00	22	—
23 h	0.51	392	768.7	0.00	0	—
L-[3,5-^{3}H]Tyrosine						
0 min	11.00	23 544	2140.4 [b]	1.01	0	0.0
20 min	10.24	21 172	2067.6	1.25	431	344.9
60 min	8.41	17 579	2090.3	1.43	1103	771.5
180 min	1.96	4 825	2462.2	0.99	889	1010.0
300 min	0.40	1 462	3655.8	0.00	186	—
23 h	0.00	333	—	0.00	0	—

[a] Theoretical dpm/pmol for L-[U-^{14}C]tyrosine is 235.
[b] Theoretical dpm/pmol for L-[3,5-^{3}H]tyrosine is 1915.

a final figure of about 8% after 23 h. Again, when comparing melanin formation, [^{3}H]melanin as determined by the acid-insoluble filter assay agrees fairly well with the melanin content as determined by isotope not eluting from the column, after a slight lag, even though the final figures from the filter assay are routinely significantly higher. Again, the isotope recovery from these fractions is excellent, ranging from 92 to 105%.

The formation of radioactive dihydroxyphenylalanine from both [^{14}C] and [^{3}H]tyrosines by mammalian tyrosinase is summarized in Table III. It can be seen that the specific activity of the dihydroxyphenylalanine from both tyrosine isotopes increases quickly from time zero and approaches the specific activity of the tyrosine fraction at each time examined (the [^{3}H]dihydroxyphenylalanine is only one-half that of tyrosine since one label position is lost). The specific activity of the tyrosine fraction in both isotopic forms increases with time; however, as stated above, this increase is more dramatic with the [^{14}C]tyrosine.

Having corroborated the facts that: (1) the acid-insoluble fraction we were measuring was an accurate reflection of the melanin formed: (2) the $^{3}H_2O$ assay was measuring the enzymic formation of $^{3}H_2O$, and (3) dihydroxyphenylalanine and not trihydroxyphenylalanine are intermediates in the tyrosinase reactions, we turned our attention to the study of the fate of a number of different specifically labelled melanogenic precursors, in an attempt to deter-

TABLE IV

DISTRIBUTION OF RADIOACTIVITY AFTER TYROSINASE ACTIVITY ON DIFFERENTLY LABELLED SUBSTRATES

Assays were set up in quadruplicate with the amounts of dihydroxyphenylalanine cofactor listed and with the labelled substrates as detailed in Materials and Methods; after 60 min incubation at 37°C, the assays were removed and aliquots taken to determine the formation of labelled melanin and labelled water from the various labelling positions. The results are presented as the mean cpm ± S.E.; the data reported are those from one experiment, three others were carried out with similar results. 0.90 mU B-16 tyrosinase were used per assay. DOPA, dihydroxyphenylalanine.

Labelled substrate	50 μM cofactor [a]		5 μM cofactor	
	cpm	%	cpm	%
Melanin				
[*carboxyl*-^{14}C]DOPA	38 ± 20	0.3	58 ± 23	0.5
[β-^{14}C]DOPA	4 765 ± 238	39.0	5 148 ± 283	42.2
[U-^{14}C]DOPA	8 144 ± 407	29.2	8 656 ± 476	31.0
[α,β-^{3}H]DOPA	59 783 ± 4484	42.7	60 240 ± 4819	43.0
[2,5,6-^{3}H]DOPA	44 594 ± 1115	36.5	42 932 ± 2147	35.0
[*carboxyl*-^{14}C]tyrosine	bkg ± 15	0.0	11 ± 24	0.1
[β-^{14}C]tyrosine	3 737 ± 374	12.4	1 516 ± 182	5.0
[U-^{14}C]tyrosine	13 116 ± 1377	8.2	5 686 ± 853	3.5
[α,β-^{3}H]tyrosine	8 645 ± 865	7.2	2 532 ± 342	2.1
[3,5-^{3}H]tyrosine	13 453 ± 538	14.6	3 772 ± 207	4.1
[2,6-^{3}H]tyrosine	11 899 ± 178	8.6	3 098 ± 170	2.2
[2,3,5,6-^{3}H]tyrosine	15 340 ± 1151	10.1	3 956 ± 336	2.6
$^{3}H_2O$				
[α,β-^{3}H]DOPA	192 135 ± 7685	19.6	201 885 ± 8075	20.6
[2,5,6-^{3}H]DOPA	260 395 ± 3905	26.6	279 840 ± 4200	28.6
[α,β-^{3}H]tyrosine	52 260 ± 2350	5.7	30 675 ± 1225	3.4
[3,5-^{3}H]tyrosine	129 460 ± 1940	18.7	52 120 ± 520	7.5
[2,6-^{3}H]tyrosine	98 300 ± 490	9.5	45 390 ± 1135	4.4
[2,3,5,6-^{3}H]tyrosine	150 545 ± 755	12.1	56 035 ± 840	4.5

[a] Assays with labelled tyrosine contained the listed quantity of dihydroxyphenylalanine as cofactor; assays with labelled dihydroxyphenylalanine contained the listed quantity as a competitive inhibitor.

mine the number of carbon or hydrogen atoms lost or retained at each of the positions as tyrosine is converted to melanin. The data from one such experiment are presented in Table IV; B-16 tyrosinase is shown at two cofactor concentrations, and although the data are not shown, the results of similar studies with C57 tyrosinase and mushroom tyrosinase were completely analogous. In each case we will examine the effect of the dihydroxyphenylalanine substrates first, since they are the more simple to follow in the synthetic pathway, undergoing only one enzymic step, then we will go back one further reaction and look at the tyrosine substrates. It has already been shown that an isotope effect of the tritiated substrates is minimal [18], and will not be taken into account here. Examining the ^{14}C-labelled dihydroxyphenylalanine substrates first, one can see that the isotope with the highest percent of radioactivity in the insoluble fraction was the one labelled at the β-position. It has been previously shown [5] that less than 2.3% of carbon atoms in this position are lost upon the autooxidation of dihydroxyphenylalanine to melanin. One would expect from the classical reaction sequence of melanogenesis as presented in Scheme I, that for each monomer of indole-quinone formed that one carbon would be

lost in the decarboxylation step; thus leading one to expect that from the uniformly labelled precursor, that at most only 11% of the label would be lost. In fact however, about 25% of the label from uniformly labelled dihydroxyphenylalanine is lost, or approximately two carbon atoms for each monomer converted to melanin (mushroom tyrosinase, 19 ± 6%, $n = 4$; mammalian tyrosinase, 33 ± 2%, $n = 11$). This is also the case for the similarly labelled tyrosine substrates (mushroom enzyme, 32 ± 6%, $n = 3$; mammalian enzyme, 36 ± 3%, $n = 6$). The data from the carboxyl-labelled dihydroxyphenylalanine and tyrosine substrates revealed that very little, if any, of the carboxyl groups of these precursors are incorporated into melanin (for dihydroxyphenylalanine substrate, mushroom tyrosinase, 5 ± 1%, $n = 4$; mammalian, 4 ± 1%, $n = 11$; for tyrosine substrate, mushroom tyrosinase, 0.1 ± 0.1%, $n = 4$; mammalian, 0.9 ± 0.5%, $n = 5$). This is in contrast to some data previously reported; 9.5% was found by Chen and Chavin [29], and 20% carboxyl group retention was reported by Swan and Waggott [21]. On the other hand, Piattelli et al. [30] found that sepiomelanin retained approximately 5% of the original carboxyl group, and Mason [3] has reported that there is no incorporation of the tyrosine carboxyl group into melanin, and suggests that the free carboxyl groups demonstrated by other groups are derived from splitting of quinones during melanin formation. In fact, both Piattelli et al. [30] and Swan and Waggott [21] have reported that carboxylic acid formation is decreased dramatically by the presence of catalase, suggesting that hydrogen peroxide is generated during melanin production, and that the quinone site of the indole ring is cleaved as a result. Our data, using both ^{14}C-labelled tyrosine and dihydroxyphenylalanine precursors at cellular pH, are consistent with the notion that the carboxylic acid group is lost quantitatively, and that in addition, an average of one other carbon atom is lost per molecule of substrate converted to melanin. If, as seems most likely, this other carbon is lost by peroxide cleavage of the indole at the quinone site, resulting in the loss of two carbon atoms per monomer cleaved, 50% of the indole-quinones must be attacked in this manner. It should be noted that these experiments were carried out in vitro under ideal conditions, with only substrates and enzyme present in any quantity. This is far removed from the situation within the melanocyte, where a variety of other interfering substances are present. As one example, it is known that cysteine groups of proteins can be incorporated into melanin to a high degree when available [31] (this is thought to provide the means of formation of melanin-protein complexes), and it seems likely that due to the highly reactive nature of the melanin pigment and its immediate precursors, that many other compounds might be incorporated into the polymer, including perhaps non-cyclized precursors.

To determine if incomplete substrate utilization similar to that seen for [^{14}C]tyrosine (Fig. 1) occurred with [^{14}C]dihydroxyphenylalanine, dihydroxphenylalanine was isolated after varying degrees of reaction with mammalian tyrosinase and no such effect was discerned; all the dihydroxyphenylalanine isotopes examined were completely converted into melanin, and the specific activities remained constant. Thus, if it is assumed that the results found for the [^{14}C]dihydroxyphenylalanine substrates labelled at the β-position reflect most accurately the true value for melanin formation, a comparison of the label

TABLE V

^3H RETAINED IN MELANIN COMPARED TO THAT RELEASED AS H_2O

Assays were set up with varying amount of dihydroxyphenylalanine cofactor and with the listed tritiated substrates as per Materials and Methods; after 60 min incubation at 37°C, the assays were removed and aliquots taken to determine the formation of ^3H_2O and tritiated acid-insoluble products. The results are presented as the mean ± S.E. of the percent tritium retained in the melanin fraction compared to the total amount of tyrosine metabolized; the remainder of isotope was released as ^3H_2O. Number of experiments in parentheses. DOPA, dihydroxyphenylalanine.

Labelled substrate	Cofactor dihydroxy-phenyl-alanine (μM)	Enzyme source		
		Mushroom	B-16	C57
L-[α,β-^{3}H]DOPA	0	48.5 ± 4.7 (5)	61.1 ± 2.1 (15)	56.3 ± 2.3 (10)
L-[2,5,6-^{3}H]DOPA	0	31.9 ± 2.2 (9)	48.5 ± 2.6 (16)	51.1 ± 1.4 (19)
L-[α,β-^{3}H]Tyrosine	50	53.5 ± 9.0 (3)	55.8 ± 1.7 (6)	47.7 ± 2.8 (8)
	5	37.8 ± 6.4 (2)	33.2 ± 2.9 (8)	65.1 ± 3.1 (4)
	0	48.9 ± 6.3 (6)	—	—
L-[3,5-^{3}H]Tyrosine	50	53.3 ± 2.5 (3)	42.2 ± 4.1 (5)	38.0 ± 3.7 (7)
	5	56.0 ± 1.8 (2)	29.9 ± 3.8 (6)	44.7 ± 1.7 (3)
	0	55.0 ± 3.6 (5)	—	—
L-[2,6-^{3}H]Tyrosine	50	25.9 ± 2.0 (2)	47.3 ± 0.9 (2)	43.3 ± 2.1 (3)
	5	23.6 ± 2.2 (2)	26.9 ± 3.9 (6)	53.4 ± 1.7 (3)
	0	19.6 ± 2.0 (2)	—	—

retained in the melanin fraction with the titriated substrates reveals an interesting fact. Approximately the same percent of label is found in the melanin fraction from the titriated dihydroxyphenylalanine substrates (either labelled at the α,β-positions or the 2,5,6-positions) as with the [β-^{14}C]-dihydroxyphenylalanine. This would seem to indicate that all the ^3H remains in the acid-insoluble fraction, although this is not the case. With the mammalian enzyme, an additional 50—75% of the tritium is released as H_2O. The ratio of the production of ^3H_2O and melanin formation from each isotopic form of substrate is constant and is discussed below. Similarly, the tritiated tyrosine isotopes showed an analogous behavior; in each case the amount of label present in the melanin and ^3H_2O fractions added to a greater percentage than that found in the β-labelled [^{14}C]melanin fraction. These rates of reaction of the various isotopic forms of tyrosine and dihydroxyphenylalanine have been accumulated for the various enzyme preparations and are shown in Tables V and VI.

The fate of tritium label at various points on the melanogenic substrates of tyrosine and dihydroxyphenylalanine that was retained in the melanin have been studied in more detail. When the label is at the α,β-position of dihydroxyphenylalanine (Table V), it might be expected from the reactions shown in Scheme I, that if all of the substrate went through the reaction pathway to indole-5,6-quinone (VIII), that 25% of the label would be lost at the decarboxylation step (since only one of the two hydrogens in the β-position is labelled). Any percent of tritium lost greater than 25% must reflect the subsequent polymerization of the monomers to form melanin. With dihydroxyphenylalanine as substrate, very little difference was found between the percent tritium retention in melanins formed by tyrosinases from mushrooms, normal

TABLE VI

^3H_2O FORMATION COMPARED TO [U-^{14}C]MELANIN FORMATION

Assays were set up with varying amount of dihydroxyphenylalaine cofactor and with the listed tritiated substrates as detailed in Materials and Methods; after 60 min incubation at 37°C, the assays were removed and aliquots taken to determine the formation of ^3H_2O from tritiated substrates, and the formation of [^{14}C]melanin in analogous assays from uniformly ^{14}C-labelled substrates. The results are presented as the mean of the percent ^3H_2O formed/[^{14}C]melanin formed ± S.E. Number of experiments in parentheses. DOPA, dihydroxyphenylalanine.

Labelled substrate	Cofactor DOPA (μM)	Enzyme source		
		Mushroom	B-16	C57
L-[α,β-^{3}H]DOPA	0	13.9 ± 1.7 (4)	75.7 ± 6.5 (17)	104.5 ± 12.5 (8)
L-[2,5,6-^{3}H]DOPA	0	108.3 ± 24.2 (6)	109.0 ± 8.3 (13)	102.6 ± 8.4 (13)
L-[α,β-^{3}H]Tyrosine	50	50.5 ± 9.3 (4)	83.9 ± 9.6 (7)	127.8 ± 30.1 (6)
	5	34.7 ± — (1)	153.0 ± 18.1 (6)	138.1 ± 25.2 (4)
	0	50.4 ± 4.3 (5)	—	—
L-[3,5-^{3}H]Tyrosine	50	116.8 ± 28.3 (4)	196.0 ± 29.5 (6)	195.9 ± 29.5 (6)
	5	122.0 ± — (1)	233.4 ± 11.1 (4)	208.0 ± 34.4 (4)
	0	95.6 ± 15.1 (5)	—	—
L-[2,6-^{3}H]Tyrosine	50	122.7 ± 23.3 (2)	124.6 ± 5.8 (3)	256.7 ± 13.6 (2)
	5	81.6 ± — (1)	178.8 ± 28.9 (4)	221.5 ± 50.5 (3)
	0	100.8 ± — (1)	—	—

mammalian and melanoma mammalian sources, as well as from autooxidation (58.7 ± 8.6%, n = 9). Similarly, using tritiated α,β-tyrosine as substrate, the melanin formed by all three types of tyrosinase in the presence of 50 μM cofactor (or in the absence of cofactor by mushroom tyrosinase), was virtually identical to that formed with labelled dihydroxyphenylalanine as substrate. These data indicate that the amount of tritium remaining in the melanin that is released as the result of polymerization at either the α- or β-position ranges from 14 to 26%.

When the non-hydroxylated positions (2, 5 and 6) of the phenyl ring of dihydroxyphenylalanine are labelled with tritium, 33% of the label is expected to be lost upon creation of the indole ring at position 6 (or alternatively at position 2), as is assumed in the dihydroxyphenylalanine oxidase assay as described by Pomerantz [32]. In fact, however, it can be seen that rather than 66% of the label remaining in the melanin polymer, only about 32% remains in the melanin formed by the mushroom tyrosinase, indicating one of the remaining two tritiums is lost through polymerization, while only about 50% remains in the melanins formed by either the mammalian tyrosinases or by autooxidation (42.2 ± 5.2%, n = 7), which indicates that less extensive polymerization at these positions has occurred. When the tyrosine substrates which are labelled on the phenyl ring at either the 3,5-positions or the 2,6-positions are examined, it can be readily seen that the results are similar. In the presence of 50 μM cofactor, the mammalian tyrosinases are comparable, and retain about 38—47% of the label in the melanin. Examination of the same compounds utilize by mushroom tyrosinase reveals a slightly different picture: label in the 3,5-positions are retained to a slightly higher degree than the mammalian tyrosinases, either with or without cofactor. In contrast, mushroom tyrosinase yields melanins with much lower quantities of tritium

retained in the 2,6-positions, either with or without cofactor.

Specific labelling studies similar to these have been previously carried out by several laboratories. Hempel [2] used tritiated dihydroxyphenylalanine which was converted to melanin in vivo in murine melanomas; Swan [6] and Kirby and Ogunkoya [33] also used similarly labelled isotopes which were converted to melanin in vitro by mushroom tyrosinase. Each of these investigators arrived at data similar to ours presented in Table V. For the α,β-tritiated dihydroxyphenylalanine, the fraction of label retained in melanin was 0.47, 0.45, and 0.53 by Hempel [2], Swan [6] and Kirby and Ogunkoya [33], respectively. These numbers are certainly compatible with ours, although ours range slightly higher. Regarding the 2,5,6-tritiated dihydroxyphenylalanine, the fraction of label retained in melanin in their studies was 0.40, 0.26 and 0.29, respectively. Again, our data are very similar; with mushroom tyrosinase 0.32 was determined, and with mammalian enzyme, 0.49 was found. The reason for the significantly ($\rho < 0.05$) lower fraction of tritium retention by mushroom tyrosinase with both dihydroxyphenylalanine isotopes used is unclear, although most probably it reflects an increased polymerization of the melanins as directed by the enzyme.

A comparison of the amount of ^3H_2O formation to the amount of [^{14}C]-melanin formation for each of the enzymes is presented in Table VI. When labelled dihydroxyphenylalanine is used which is tritiated at the α- and β-positions, there is a significant (at $\rho < 0.05$) difference between each of the three enzymes used, i.e. mushroom, C57 normal and B-16 melanoma tyrosinases. For the C57 enzyme, approximately the same amount of ^3H_2O is formed as [U-^{14}C]melanin, while only about 75% ^3H_2O is formed by the B-16 enzyme, and still less (about 14%) is formed by mushroom tyrosinase. There was no significant difference between the mammalian enzymes and that by autooxidation (103.1 ± 21.2%, $n = 4$). Similar results can be seen when [α,β-^{3}H]tyrosine is used, although the percentages in each case are slightly higher. When the tritium label is on the phenyl ring of the dihydroxyphenylalanine (positions 2, 5 and 6), there was no significant difference between any of the enzyme preparations; each resulted in the release of slightly more than an equimolar amount of ^3H_2O over melanin formed; autooxidation yielded similar results (108.8 ± 21.5%, $n = 6$). When the tritium label is on the phenyl ring of tyrosine (either in positions 3 and 5, or 2 and 6), approximately the same values are found for the mushroom enzyme. By way of contrast, however, approximately twice as much ^3H_2O is formed as [^{14}C]melanin by each of the mammalian enzymes. This increased at the lower cofactor concentrations tested, and reflects a significant difference between these enzymes and that found in the mushroom. As has been pointed out in a previous paper [13], use of the [3,5-^{3}H]tyrosine isotope reveals that at the highest cofactor concentrations, almost twice as much ^3H_2O is produced as is melanin; at lower (and more catalytically optimized) concentrations of dihydroxyphenylalanine, less of the tyrosine which is hydroxylated within a given time is converted to melanin, and this is reflected in the data presented in this paper, with the [2,6-^{3}H]tyrosine isotope as well. This is due to the fact that mammalian tyrosinase has two catalytic sites which in the resting state are closely, but not precisely aligned. In the presence of increasing amounts of dihydroxyphenylalanine cofactor, there is a change in the nature of

the enzyme, probably allosteric, which results in the more precise alignment of the tyrosine-hydroxylating and dihydroxyphenylalanine-oxidizing sites. This in turn causes a more efficient transfer of nascent dihydroxyphenylalanine to the second catalytic site, and the net result is the higher efficiency of melanin production by the enzyme. Since this increase was not observed with the mushroom tyrosinase at the concentrations of cofactor examined, one might surmise that the arrangements of the catalytic sites might not be the same. There are several other pieces of evidence to suggest that although the melanin synthesized from these two types of enzymes may eventually turn out to be very similar, there are some differences in the control of melanogenic activity between them. It has been reported that the mushroom enzyme is a tetramer [34] for example, with a molecular weight of about 130 000, while it has been known for some time that mammalian tyrosinase is a single-chained molecule with a smaller total molecular weight (about 70 000). As another example, from the data presented in this paper alone, one can see that mushroom tyrosinase functions well in the absence of added dihydroxyphenylalanine cofactor, while mammalian tyrosinase has only minimal levels of tyrosine hydroxylation in the absence of dihydroxyphenylalanine cofactor.

In conclusion, we feel our data support the following: (1) Melanin synthesis from tyrosine by tyrosinase from either mushroom or mammalian sources proceeds primarily through the classic reaction sequence outlined in Scheme I. The proposed alternate reactions involving the production of trihydroxyphenylalanine must be either minimal or non-existent with these enzymic catalysts. (2) Mammalian tyrosinase is capable of hydroxylating tyrosine to dihydroxyphenylalanine; this enzyme alone is sufficient for the formation of melanin in the melanocyte; the importance of peroxidase in mammalian melanogenesis is suspect at best, since peroxidase could not be shown to be capable of forming dihydroxyphenylalanine or melanin with our methodology. (3) The assays currently used to measure the formation of acid-insoluble material (melanin) by tyrosinase, and the formation of ^3H_2O as a byproduct of tyrosinase action, accurately reflect these parameters in vitro. (4) Using labelled tyrosine substrates, it appeared that there was no incorporation of non-cyclized or carboxylated precursors into the melanin; however, using labelled dihydroxyphenylalanine, approximately 5% of the polymer consisted of carboxylated precursors, perhaps as the result of the more rapid formation of melanin. (5) Approximately 50% of the monomers incorporated into melanin have lost two carbon atoms, in addition to the carboxyl group; this probably occurs as the result of peroxide cleavage of the quinone site of the phenyl ring. (6) Hydrogens are lost from virtually every available position on the phenyl ring and side chain of tyrosine and dihydroxyphenylalanine, indicating extensive and varied polymerization of monomers into the melanin complex. (7) While the mammalian enzyme is dependent on dihydroxyphenylalanine as a cofactor, the mushroom tyrosinase does not have such a requirement, and a comparison of these enzymes' utilization of various substrates supports our earlier contention that mammalian tyrosinase uses dihydroxyphenylalanine as a cofactor to elicit a conformational change to enhance the efficiency of melanin formation, even though such an effect was not seen with mushroom tyrosinase. It would appear that control mechanisms over the expression of tyrosinase activity in these two systems must be different.

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