A STUDY OF TYROSINASE ACTIVITY IN NORMAL RAT SKIN

by

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A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

May, 1965

APPROVED [redacted]

..... (Professor in Charge of Thesis)

..... [redacted] ..

(Chairman, Graduate Council)

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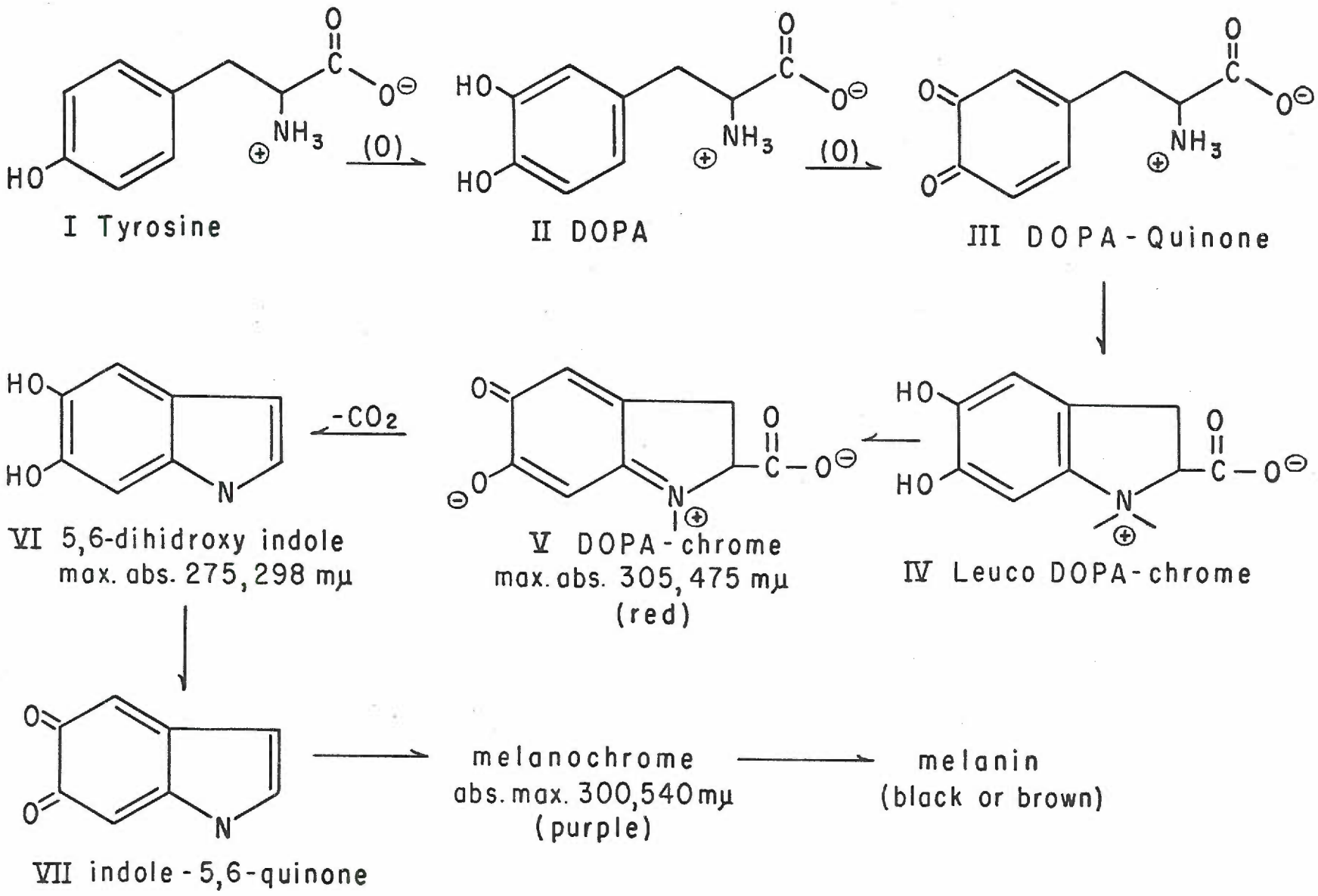
I. Preface

Tyrosinase activity, which plays a role in sclerotization of the insect cuticle, browning in plants, and in the integumentary pigmentation of chordates, constitutes an excellent example of the economy of tools used by living organisms. The phenolase complex, as it is termed by Mason (165), also plays a role in the formation of intermediates in flower pigments, tannins, lignins, phenolic alkaloids, quinones and tropolones. Tyrosinase has its maximum substrate specificity in mammals, where, among various phenols, it is believed to catalyze just the oxidation of tyrosine and dihydroxyphenylalanine (DOPA) to melanin (147, 163).

A distinction is made between tyrosinase of skin and hair and the tyrosine hydroxylase of the adrenal medulla. The latter is a tetrahydropteridine-linked iron containing mixed function oxidase which catalyzes just the hydroxylation of tyrosine to DOPA but shows no enzymic activity towards DOPA. Tyrosinase, on the other hand, is a copper containing mixed function oxidase which uses DOPA as the reducing agent and catalyzes the oxidation of both tyrosine and DOPA to melanin (186). In mammals, tyrosinase is present in a granular portion of the Golgi apparatus of melanocytes (26).


I Tyrosine
(O)
II DOPA
(O)
III DOPA-Quinone
IV Leuco DOPA-chrome
V DOPA-chrome
max. abs. 305, 475 mμ
(red)
-CO2
VI 5,6-dihidroxy indole
max. abs. 275, 298 mμ
VII indole-5,6-quinone
melanochrome
abs. max. 300,540mμ
(purple)
melanin
(black or brown)

II. Introduction

1. The Reaction Sequence Catalyzed by Tyrosinase

Since tyrosinase was first discovered by Berquelot and Bertrand (21) in 1895, it has become one of the most intensely studied enzymes. Early workers were in considerable doubt as to the nature of the reaction catalyzed by tyrosinase. The enzyme has been called a hydrolytic deaminase (1, 10) and a peroxidase (190) among others. Raper (214) noticed that the reaction catalyzed by tyrosinase occurs in several definite visible stages which are intermediate between tyrosine and melanin. The first of these stages involves the formation of a red substance which requires the presence of both oxygen and the enzyme. The second stage involves the spontaneous decolorization of the red pigment. The colorless substance is then oxidized to melanin during the third stage. While tyrosinase was observed to accelerate the last two steps, they can proceed in the absence of the enzyme.

Early theories on the action of tyrosinase were based on the assumption that deamination constitutes part of the reaction sequence (10, 190). Raper (215) effectively ruled out the possibility of deamination by demonstrating that a tyrosine-tyrosinase solution shows no increase in ammonia content during the oxidation and that the resulting melanin contains a slightly higher percentage of nitrogen than tyrosine. Furthermore, there was no indication of aldehyde formation as would be required by the deamination theory (102).

The early observation by Raper (214) that there are three visible stages in the oxidation of tyrosine when catalyzed by

tyrosinase was carried out using potato tyrosinase. Other workers (98, 99) using tyrosinase from the mealworm Tenebrio molitor did not observe a change from the red substance to the colorless one as had Raper. This led to the possibility that the red substances might be different in the two cases and that tyrosinases from different sources might have different modes of action on tyrosine. Raper (213) then obtained tyrosinase from different sources such as the mealworm Tenebrio molitor and the mushroom Agaricus dryophilus and observed the same visible stages as when potato tyrosinase had been used. These enzymes from different sources had activity over the same pH range and also had similar activity towards other substrates such as phenol, p-cresol, resorcinol, and catechol. Raper (210) was the first to demonstrate that DOPA is the first product of the action of tyrosinase on tyrosine. Others had suggested this, but had not provided experimental proof (102, 190). Raper purified and identified the DOPA after isolating it from the reaction mixture as the lead salt. Added DOPA was found to increase the rate of oxidation of tyrosine. That DOPA is the first oxidation product was later confirmed by Evans and Raper (72, 73). The amount of DOPA accumulation in the reaction mixture was markedly increased by the addition of ascorbate. Dopa-quinone is reduced to DOPA by the ascorbate, thereby increasing the accumulation of DOPA to about ten percent of the tyrosine oxidized.

Studies by Mason (164, 167) with O_2^{18} and H_2O^{18} indicate that the oxygen of the newly introduced hydroxyl group arises from molecular oxygen and not from solvent water. This study coupled

with the observation by Lerner (141) that tyrosinase is specific for L-tyrosine indicates that cresolase activity is enzymic rather than being due to nonenzymic oxidation by a quinone. Dawson (64) showed that hydrogen peroxide is not formed during the reaction.

Wood and Ingraham (249) have shown that there is a small but definite tritium rate effect during the tyrosinase catalyzed hydroxylation of dimethylphenol. From this evidence it was concluded that the second step in the hydroxylation reaction, proton removal, is rate limiting rather than the actual oxidation. The fact that the tritium rate effect is small has been taken to indicate that the tritium does not leave as a free ion, but forms a new bond. Wood and Ingraham suggest that this new bond is probably with a Lewis base on the enzyme.

Since tyrosinase is much more active toward DOPA than toward tyrosine, there was a question as to why there should be any DOPA accumulation at all. An examination of the oxidation-reduction potentials of the system concerned with melanin formation led Evans and Raper (73) to suggest that DOPA accumulates because of the reduction of DOPA-quinone by the dihydroindole as shown below. E_0's are also indicated.

DOPA-quinone + leukocompound ⟷ DOPA + DOPA-chrome

DOPA ⟷ DOPA-quinone E_0 = +0.511 V at pH 4.6 (11)

Leukocompound ⟷ DOPA-chrome E_0 = +0.170 V at pH 4.6 (92)

Evidence in support of this concept was gained by showing that the addition of 5,6-dihydroxy-2,3-dihydroindole-2-carboxylic acid to

reaction mixtures containing tyrosinase and tyrosine increased the amount of DOPA accumulation (73).

Not all workers have been able to demonstrate the formation of DOPA in the presence of tyrosinase. Kim (132) was unable to demonstrate the presence of C^{14}-DOPA after incubating C^{14}-tyrosine in the presence of goldfish tyrosinase. Added unlabeled DOPA did not decrease the amount of C^{14}-activity found in the resulting melanin. Kim therefore concluded that DOPA formed as an intermediate may remain bound to the protein and not be released into solution. Dressler and Dawson (67) also suggest that free o-diphenols may not be formed during the action of tyrosinase on monophenols.

Lissitzky and Rolland (153) described an intermediate derived from DOPA-quinone which is not an indole and gives rise to the formation of intermediate pigments characteristic of melanization in vitro. This substance was formed during the oxidation of tyrosine in the presence of highly purified polyphenoloxidase. It was first detected on chromatographs by spraying with ninhydrin. Later, it was shown to be formed from C^{14}-tyrosine. Accumulation of this intermediate was increased by the addition of ascorbate to the reaction mixture. The fact that the intermediate gives a grey-purple color with ninhydrin indicates that the nitrogen is not yet part of an indole ring. It gives a pink color with Arnow's reagent (sodium nitrite and molybdate in HCl) and reduces ammoniacal silver nitrate. The intermediate is also produced during the oxidation of tyrosine in nonenzymic systems containing ascorbate, ferrous ion, and oxygen. Lissitzky and Rolland suggest that it is a trihydroxy compound similar to that obtained by Senoh

(228) from brain. Such a trihydroxy compound would be the first intermediate detected between DOPA-quinone and the appearance of pigments.

Raper (211) found that tyrosinase-tyrosine mixtures contained a more immediate precursor of melanin than tyrosine or DOPA. In this precursor the nitrogen was no longer present as a free amino group. This material proved to be so easily oxidized that it could not be isolated directly. Instead, the red solution obtained from the action of tyrosinase on tyrosine was allowed to decolorize. Then it was treated with methyl sulfate in an atmosphere of hydrogen. After extraction with ether, two crystalline methylated products were obtained, one a weak base and the other an acid. The weak acid was obtained when decolorization took place in the absence of oxygen and the weak base primarily obtained when the decolorization took place in the presence of sulfurous acid. Both of these substances gave color reactions characteristic of indoles. During cyclization the nitrogen of the amino group potentially could attach at either the two or six position on the ring. However, from the work of Jones and Robinson (121) on orientation of substituted catchol ethers, it appeared likely to Raper (211) that the 5,6-quinone of indole-2-carboxylic acid (VIII, chemical structures given on following page) would be formed. Therefore, it was suspected that the methylated indole derivative with acid properties would be 5,6-dimethoxyindole-2-carboxylic acid (IX). Decarboxylation of this compound would give 5,6-dimethoxyindole (X), a weak base. Since the properties of these two compounds were unknown at the time, they

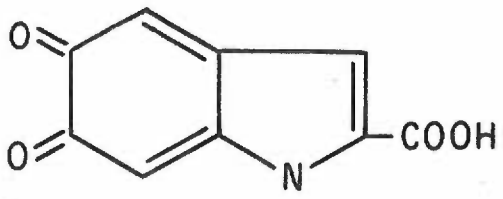
were synthesized and found to be identical in all respects with the methylated indole derivatives isolated from the oxidation products of tyrosine. Having established that the red substance, in the presence of sulfurous acid, gives rise to 5,6-dihydroxyindole-2-carboxylic acid (XI) led Raper (211) to suggest that the structure of the red substance is probably the 5,6-quinone of 2,3-dihydroindole-2-carboxylic acid (DOPA-chrome V). Tyramine and DOPA-amine were also found to be converted to 5,6-dihydroxyindole (VI) in the presence of tyrosinase (69). Although DOPA-chrome (V) was once thought to be identical to hallochrome, Bu'Lock showed that they are in fact two separate and distinct compounds.

Mason (161) later followed the enzymic oxidation of DOPA spectrophotometrically. The reaction sequence also takes place in several distinct stages when followed in this manner. The first stage is characterized by the appearance of absorption maxima at 305 mu and 475 mu, the second stage by absorption maxima at 300 mu and 540 mu, and the third stage by general absorption. The first stage corresponds to the formation of the red substance observed by Raper (211). Further inferences about the structure of the red substance were made by comparing spectra of known compounds with that of the red pigment. Two such compounds are rubreserine (XII) and adrenochrome (XIII), both of which contain a fixed 2,3-dihydro-indole-5,6-quinone nucleus. Both of these compounds have absorption spectra almost identical to that of the red substance prepared by the enzymic or silver oxide oxidation of DOPA. There are two possible tautomeric forms of this red pigment as shown on page eleven

The second phase of the reaction sequence as followed spectrophotometrically involves the appearance of absorption maxima at 300 mμ and 540 mμ (162). However, when the red pigment was allowed to undergo rearrangement at pH 5.6 in the absence of oxygen, maxima at 275 mμ and 298 mμ but none in the visible range were observed. This is in marked contrast to the spectrum of the second phase. Since there is generally only a slight difference between the maxima of undissociated phenols and their methyl esters (7, 120), the spectrum of 5,6-dihydroxyindole (XI) was compared with that of the rearrangement product of the red pigment (162). The spectra were found to be almost identical, suggesting the rearrangement product to be 5,6-dihydroxyindole (VI) or the 2-carboxylic acid derivative. Mason and Wright (170) later provided manometric evidence that rearrangement and decarboxylation occur simultaneously, and that the reaction is base catalyzed, and nonenzymic. When 5,6-dihydroxyindole obtained from the rearrangement and decarboxylation of 2-carboxy-2,3-dihydroindole-5,6-quinone (DOPA-chrome V) is oxidized in the presence of tyrosinase, a material appears whose absorption spectrum has maxima at 300 mμ and 540 mμ, the same as that obtained during the second phase of the reaction sequence. Because tyrosinase dehydrogenates catechols to the corresponding quinones (208) and in view of the catechol nature of 5,6-dihydroxyindole, Mason (162) suggested that the purple pigment with absorption maxima at 300 mμ and 540 mμ is indole-5,6-quinone (VII).

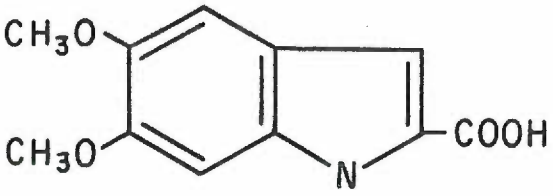
Bu'Lock (50) observed an intermediate in the oxidation of 5,6-dihydroxyindole which has an absorption maximum at 530 mμ.

Chemical structures of substances mentioned in the text



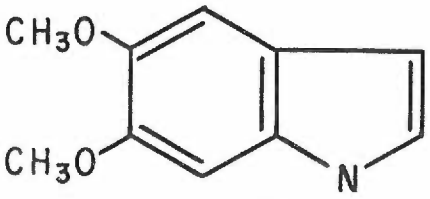
VIII

indole-5,6-quinone-2-carboxylic acid



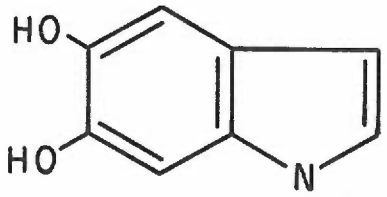
IX

5,6-dimethoxyindole-2-carboxylic acid



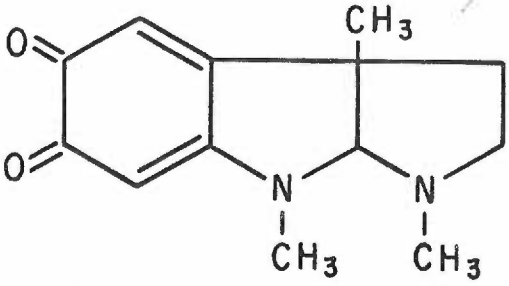
X

5,6-dimethoxy indole

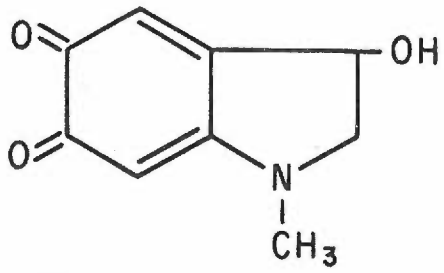


XI

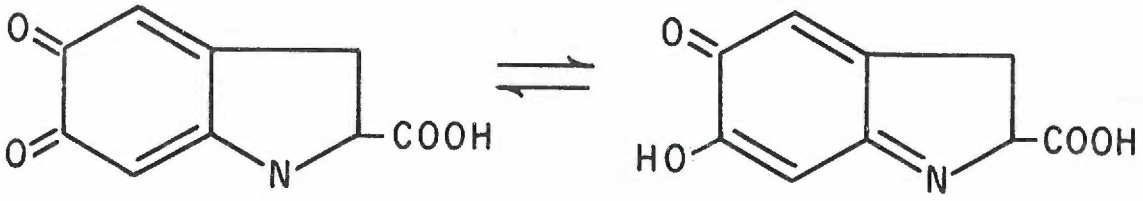
5,6-dihydroxyindole



XII rubreserine



XIII adrenochrome



V

2-carboxy-2,3-dihydro indole-5,6-quinone (DOPA-chrome)

2-carboxy-2,3-dihydro-6-hydroxy indole-1,5-quinonimine

Evidence based on the absorption and fluorescence spectra suggests that this intermediate is a 5,6-dihydroxyindolylindole-5,6-quinone. Further oxidation of this dimer gives a pigment with a broader absorption maximum at 540 mu. Forsyth (85) identified three tetrahydroxydiphenyl intermediates formed during the oxidation of catechol in the presence of tyrosinase. These same intermediates were formed as a result of ferric chloride or silver nitrate oxidation of catechol.

Spectrophotometric studies by Beer (18) indicate that the formation of purple melanochromes is preceded by the appearance of a yellow intermediate which he considers to be the true indole-5,6-quinone. Beer suggests that the melanochromes are smaller and less complex polyindoles than melanin. Melanin itself could be formed by crosslinking between the melanochrome chains.

The final step in melanin synthesis involves the polymerization of indole-5,6-quinone or related compounds. Possible structures resulting from such polymerizations have been extensively reviewed by Mason (166) and Swan (235). The earlier structures proposed for melanins all involve highly conjugated ring systems. Melanin has been a term loosely used to describe pigments formed by oxidative polymerization of various phenolic substances. Somewhat greater precision has been obtained by defining melanins as pigments formed by the action of tyrosinase on tyrosine, DOPA, and related substances (236). However, this is still not satisfactory. Mason (168) points out that the proper description of a melanin should include an identification of the monomer units,

the length of the chain, the nature of the interunit links, degree of branching, redox state of the polymer, and the nature of the protein and its link to the pigment. As yet, no melanin has been isolated as a single chemical compound having a definite chemical composition (235). The probability that melanins are irregular polymers makes it doubtful that any will be isolated as a single chemical compound.

Model systems have been used to study the nature of the condensation reactions. Mohlau et al (181) found that 2-methylindole condenses with p-benzoquinone to give 3'-(2'-methylindolyl)-1,4-benzoquinone. The reaction was interpreted as occurring in two steps. First, there was the addition of 2-methylindole to the quinone with the transfer of two hydrogen atoms giving the indolyl-hydroquinone. The second reaction involves the oxidation of the hydroquinone by p-benzoquinone. Electron density of the indole nucleus is greatest in the 3- position (158). Therefore, cationic reagents would be expected to react primarily at the 3- position. Bu'Lock and Harley-Mason (51) have suggested that a similar scheme may apply to reactions of indoles with quinones. They found the reaction to be acid catalyzed.

The autoxidation of various methylated 5,6-dihydroxyindoles has been studied in an attempt to determine which of the positions on the indole nucleus are involved in the interunit links. Cromartie and Harley-Mason (59) found that 5,6-dihydroxyindole and its 1-, 2-, 4-, and 7-methyl derivatives all give black insoluble pigments when allowed to autoxidize in buffered solutions at pH 6.85. However,

the 3-methyl- and 4,7-dimethyl derivatives gave intensely colored pigments soluble in ethanol. These pigments were considered to be oligomeric in nature. Differences in molecular weight may be due to a smaller amount of crosslinking in the polymers formed from the methylated indoles. Similar results were obtained using methylated DOPA derivatives. Beer (18) found that 3-methyl substitution of indoles did not prevent polymerization but altered the course of the reaction. Beer concluded that the 3- and 7-positions of the indole nucleus are essential for normal melanin formation. Experiments with labeled substrates led Swan and Wright (236) to conclude that melanin polymers are composed of 3,7- rather than 3,4- linkages. Cromartie and Harley-Mason (59) also concluded that the 3- position is essential for normal melanin formation and that either the 4- or the 7- position must be free. According to our present concepts, indole-5,6-quinone has more than two reactive centers at which polymerization can occur (53, 54). In addition, observations based on an induction period and the formation of hydrogen peroxide led to the suggestion that a free radical mechanism is involved in polymerization. A free radical mechanism could result in a macromolecular structure with many possible variations. End groups, side chains, and types of cross links would be dependent on the environment in which they were formed.

More recent work indicates that melanin polymers may consist, in part, of monomer units other than indole-5,6-quinone. Clemo (56), using carboxyl labeled tyrosine and DOPA, found that some of the

carboxyl units are incorporated into the melanin. Carboxyethyl residues are retained in melanin when the ethyl ester of tyrosine is oxidized in the presence of tyrosinase (55). Furthermore, about half the carbon dioxide evolved during the conversion of labeled tyrosine or DOPA to melanin arises from carbon atoms other than carboxyl groups (56). Carbon dioxide is also evolved from DOPA-amine and 5,6-dihydroxyinole during their conversion to melanin. Swan and Wright (236), using 2-(3,4-dihydroxyphenyl)-ethylamine labeled with C^{14} in the 3-, 4-, or 5- positions of the aromatic ring, showed that carbon dioxide arising during the oxidation of these compounds comes from oxidative fission of the benzene ring. Cromartie and Harley-Mason (59) suggest that this may be the result of oxidation by hydrogen peroxide formed during the polymerization. The numerous pyrrolic acids found on degradation of Sepia melanin are probably the result of oxidative breakdown of the benzeneoid portion of the indole quinone units during polymerization (24, 197). The suggestion is also made that quinones formed earlier in the reaction sequence may be included in the polymer (197, 198). It was once thought that indole-5,6-quinone formed the only monomeric unit in melanin (107).

The variety of substances obtained on degradation of melanin (24, 197, 198, 199) and the observation of unpaired electrons in melanin (32, 57, 168) form the basis for the suggestion that polymerization proceeds by way of a free radical mechanism. Such free radicals would be expected to lead to the formation of a variety of monomer units and bond types (32). The ESR spectrum of

o-benzosemiquinone has also been observed during the tyrosinase catalyzed oxidation of catechol (169). Piattelli et al (198) have found evidence of an ether linkage in a catechol melanin from Ustilago. They interpret this as further evidence indicating that polymerization proceeds by a free radical mechanism.

Unpaired electrons in a highly conjugated system would be expected to show a marked lowering of the g-value (32). The absence of such a lowering has been interpreted to mean that the melanin monomers are mostly unconjugated. Unconjugated condensed ring structures could occur if an atom were present between condensed rings or if the rings were not coplanar as in the case of 3,4-diindolyl-indoles (59). The inability to detect a hyperfine structure in the ESR signal is interpreted to mean that the unpaired electrons are present on a variety of monomer units (32).

Numerous investigators (58, 106, 154, 254) have observed the ability of tyrosinase to catalyze the oxidation of tyrosyl groups in peptide chains. These oxidations have been studied in RNA-ase, insulin, denatured pepsin, partial hydrolysates of pepsin and insulin, tyrosine ethyl ester, N-formyltyrosine, α-lactalbumin, and β-lactoglobulin (106). Variability in the action of tyrosinase towards these substrates has been attributed to differences in both the source and methods of preparation of tyrosinase and the proteins used as substrates. The reactions have been followed spectrophotometrically (58) and by measuring the oxygen uptake (254). Tyrosyl residues in peptide chains are oxidized primarily to DOPA-quinone residues (154). DOPA has been identified in protein hydrolysates

after oxidation in the presence of ascorbate. Tyrosyl groups in peptide chains are not found to form DOPA-chrome or black pigment (106).

2. Induction period.

Numerous workers have observed an initial induction period followed by an increased rate of tyrosinase activity with a variety of substrates (19, 41, 126, 134, 145). This induction period can be shortened or eliminated by the addition of diphenols such as DOPA or catechol or by other reducing agents such as ascorbate to the reaction mixtures. D,L-DOPA is about 75% as effective as L-DOPA in shortening the induction period. Oxidation products of catechol, however, do not decrease the length of the induction period (19). The length of the induction period is proportional to the negative logarithm of the DOPA concentration. Tyrosine also influences the length of the lag period, increasing concentrations tending to increase the lag (145).

The question of how tyrosinase can initiate the oxidation of a monohydric phenol if it first has to be activated by a catechol is an important one. The presence of an induction period suggests that some product which will permit the oxidation of monophenols must first be formed. The addition of a catechol to the reaction mixture decreases the length of the induction period and suggests that this product is a catechol. Initial formation of catechol is postulated to be the result of nonenzymic oxidation of the phenol (19). More recently, Pomerantz (205) has suggested that, in the case of mammalian tyrosinase, another reducing agent such as a tetrahydropteridine may be required for the initial hydroxylation

of tyrosine. Some insect polyphenoloxidases are inhibited by pteridines (117, 193).

3. A protein inhibitor of tyrosinase.

Recent attempts at purifying mushroom tyrosinase on DEAE-cellulose have demonstrated that the enzyme is accompanied by a protein inhibitor (91). The most highly purified mushroom tyrosinase lacks the characteristic lag and shows a decreased K_m using tyrosine as the substrate (91, 122). Addition of this protein inhibitor to a highly purified tyrosinase preparation results in a return of the characteristic lag period and a decrease in enzyme activity. Adding the inhibitor to crude tyrosinase preparations increases the lag period. With tyrosine as a substrate the inhibitor both increases the length of the induction period and decreases the reaction rate once it is started. When using DOPA as the substrate, the inhibitor decreases the rate of reaction without inducing a lag period.

Karkhanis and Frieden (123) later found that the protein inhibitor may be the apoprotein of tyrosinase. This inhibitor serves as a precursor of tyrosinase activity when incubated with cupric ions. Also, the apoenzyme of tyrosinase, prepared by treating tyrosinase with cyanide, shows inhibitory activity similar to that of the protein inhibitor. The transformed enzyme, obtained by incubating the protein inhibitor of tyrosinase with cupric ions, had a specific activity of 8,100 units per milliliter and contained 0.22% copper. Its K_m was 4×10^{-5}. By way of comparison, tyrosinase prepared directly from mushrooms had a specific activity of 10,000 units per milliliter, contained 0.20% copper and had a K_m of 5×10^{-5}.

Although the possibility that the protein inhibitor of tyrosinase may be an artifact of the preparative procedure is not excluded, Karkhanis and Frieden suggest that the competition between an apoenzyme and a holoenzyme at the metal ion site may constitute a naturally occurring biochemical regulatory mechanism. In other words, an apoenzyme could modify the activity of the parent enzyme by direct inhibition.

4. Protyrosinase.

The existence of protyrosinases from a variety of sources has been demonstrated by numerous workers (6, 35, 36, 37, 38, 39, 40). Bodine and coworkers (38, 39, 40) described the presence of protyrosinase, an activator, and substrate in grasshopper eggs. The protyrosinase could be activated by urea and detergents such as sodium oleate, duponol, and dioctyl sodium sulfosuccinate as well as by the naturally occurring activator. Of the synthetic detergents, duponol was the most effective, having an optimal concentration of 0.02%. The inactive form of the enzyme is particle bound (40). Detergents are bound to the enzyme during solubilization and activation. When the detergents are precipitated with barium chloride, the enzyme remains in the active form, suggesting the fragmentation of an enzyme protein complex during activation.

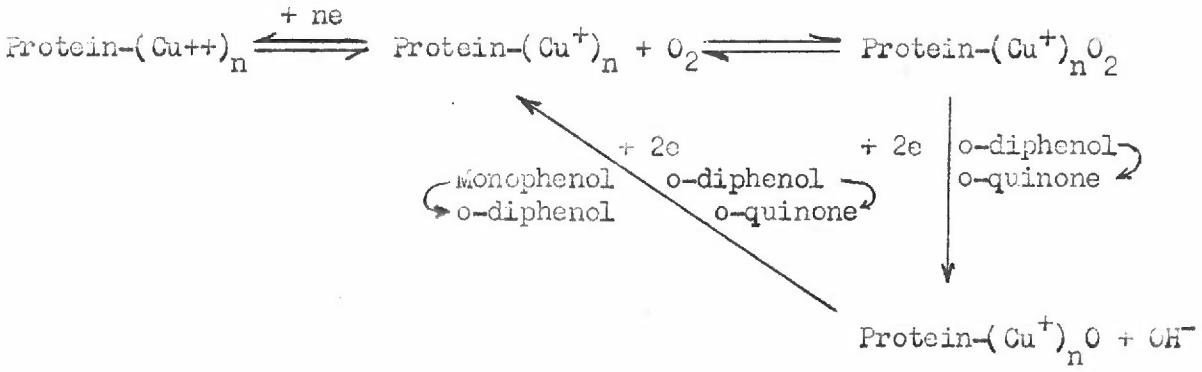
5. Copper content of tyrosinase.

Tyrosinase has long been known to contain copper. Keilin and Mann (126) found the copper content of tyrosinase preparations to be proportional to the enzyme activity. Also, numerous investigators have found the enzyme to be inhibited by copper chelating

agents (20, 79, 113, 129, 130, 140, 146, 185, 195). However, little is known about the actual role of copper in this enzyme. Copper in purified tyrosinase preparations is tightly bound to the enzyme. The enzyme retains virtually all its copper during dialysis against copper free water (66). Furthermore, when tyrosinase is incubated in the presence of Cu^{64} in the absence of substrate, there is little or no incorporation of radioactive copper by the enzyme. The very low exchange in some experiments has been attributed to nonspecific binding to the protein molecule. This tends to be substantiated by the observation that such binding decreases with increasing purity of the enzyme preparation. A somewhat different situation exists when the exchange experiments are carried out in the presence of substrate. During the oxidation of catechols, a much higher exchange rate was observed (67). A much lower rate of exchange was noted when using monophenols as the substrate. Similarly, high catecholase preparations showed a much higher rate of exchange of Cu^{64} than did the high cresolase preparations. This has been interpreted as further evidence for two active sites on the enzyme, one having catecholase and the other having cresolase activity. The studies mentioned above do not indicate whether actual oxidation of the substrates is necessary for exchange of copper to take place or if simply complexing of enzyme and substrate are sufficient.

Tyrosinase shows an absolute requirement for copper (140). Diets deficient in copper lead to depigmentation (90, 100, 125, 230). This depigmentation is due to the loss of melanin forming ability of new skin and hair rather than a loss of pigment from

existing hair and skin which are already pigmented. Silver, mercury, and gold compete with copper for the activie site on the enzyme. However, metals other than copper can not restore activity to copper-free tyrosinase preparations. The yellow color of tyrosinase preparations was taken by Kertesz (129, 130, 131) to indicate that the copper is present only in the cuprous state. Kubowitz (135) found the cupric form of tyrosinase to be inactive. Bouchilloux et al (42) found both cupric and cuprous copper in their tyrosinase preparations. Mason (165) proposed the following conceptual model for the role of tyrosinase copper.



Because solutions of phenols and catechols buffered above pH 7.0 are subject to considerable autoxidation, it has not been feasible to investigate tyrosinase activity in alkaline solutions (3). Between pH's of 5 and 7 there is little change in the rate of tyrosinase activity toward catechols. At lower pH's of 3 - 4, mushroom tyrosinase preparations show a rapid loss of copper. Between the pH's of 5.5 and 7.0, the optimum varies both with the source of tyrosinase employed (176) and the catechol used as substrate (65).

6. Reaction inactivation.

One of the characteristic features of the oxidation of catechols in the presence of tyrosinase is the inactivation of the enzyme during the early course of the reaction. Tyrosinase activity of melanin granule suspensions decreases with increasing melanization (222). Asimov and Dawson (9) have studied this reaction inactivation in considerable detail. Their kinetic studies indicate that the inactivation rate is not constant, but rather is 3/2 order with respect to the active enzyme. The results of these experiments have been interpreted in terms of a model in which there is either an inactivation in a stepwise manner through a series of increasingly stable intermediates, or two active sites which lose activity, each according to first order kinetics, but at different rates. The possibility that products of catechol oxidation such as o-benzoquinone, the semiquinone intermediate, or decomposition or condensation products of these are responsible for the inactivation was considered by Asimov and Dawson. The kinetic studies were performed using the chronometric method (176) in which ascorbate is always present for determining tyrosinase activity. Therefore, one would not expect decomposition or condensation products of benzoquinone to be formed. Benzoquinone could exist only momentarily at the enzyme surface in reaction mixtures of the type used in these experiments. Whether this is sufficient to cause reaction inactivation is uncertain. Inclusion of gelatin in the reaction mixtures protects tyrosinase from the type of inactivation described (3, 157, 177). Using tyrosinase from Psalliota campestri, Ludwig and Nelson

(157) found that when small amounts of enzyme were employed, it became inactivated before the catechol was completely oxidized. This inactivation occurred after a given oxygen uptake per unit of enzyme, and was independent of the rate of oxidation, oxygen concentration, hydrogen ion concentration between the pH's of 5.0 and 7.5, and the catecholase to cresolase ratio. When excess enzyme was present, two atoms of oxygen were used per mole of substrate. Mayberry and Mallette (172) observed that catechol inhibits its own oxidation by a competitive process. Phenol, on the other hand, inhibits catechol oxidation by a process which is neither competitive nor noncompetitive, but a mixture of the two types. Mushroom tyrosinase contains four atoms of copper per molecule (159). Asimov and Dawson (9) suggest that the stepwise inactivation may involve the progressive loss of copper atoms. If the catecholase centers are preferentially inactivated, the catecholase to cresolase ratios should decrease as inactivation continues. It is interesting to note, therefore, that preparations having low catecholase to cresolase ratios are more resistant to reaction inactivation than are high catecholase preparations.

7. The multiple components of tyrosinase.

The enzyme tyrosinase has the unusual property of being able to catalyze two quite different types of chemical reactions. One of these involves the hydroxylation of a monophenol to the corresponding catechol. The other reaction involves the dehydrogenation of a catechol to the corresponding quinone. These types of activity are generally referred to as cresolase and catecholase activities after

the substrates most often used in their measurement. Over the years, numerous workers have observed varying ratios of catecholase to cresolase activity in tyrosinase preparations (3, 113, 159, 160, 229, 231, 255). These ratios have ranged from a low of about 2 (160) to a high of about 240 (255). More commonly, however, these ratios range from about 10 to 80 (231). The catecholase to cresolase ratios of mushroom tyrosinase vary markedly depending on the method of purification (159, 160).

Hogeboom and Adams (113), who first demonstrated tyrosinase activity in mammalian melanoms, thought that two enzymes were present, one which oxidized just DOPA and another which oxidized tyrosine. Using the enzyme from Psalliota campestri, Mallette and coworkers (159) were unable to obtain a complete separation of the two activities and concluded that tyrosinase activity can best be explained on the basis of a single enzyme having two distinct types of activity. Mallette postulated that the different ratios of catecholase to cresolase activities arose as a result of fragmentation of the protein molecule during the preparation and from unfolding of the protein chains to expose the cresolase centers. None of the copper was lost in the process, the most highly purified preparations still containing four atoms of copper per molecule. The tyrosinase preparations were highly homogenous in the ultracentrifuge and during moving boundry electrophoresis in a Tiselius cell, even though they possessed widely differing ratios of catecholase to cresolase activity. There was no separation of the two activities during the electrophoresis. Based on this evidence, Kendal (127) similarly concluded that tyrosinase is a single enzyme with two active sites.

Many types of treatment are found to vary the ratio of catecholase to cresolase activity of tyrosinase preparations including heat (3), charcoal treatment (191), adsorption on kaolin or alumina (3), or by increasing the degree of purification (3, 61, 126, 157). No preparations with only monophenolase activity have been reported (65, 187). In highly purified high cresolase preparations, both activities are proportional to the copper content (194).

In recent years, as improved techniques for separation of proteins have been developed, enzyme preparations once thought to be highly homogeneous have been fractionated into several components. An example of this type of work is that of Smith and Krueger (231) who, using column chromatography on hydroxylapatite and DEAE-cellulose, starch gell electrophoresis, and calcium phosphate gel adsorption, have separated mushroom tyrosinase into five separate components. Their results suggest that the different components are separate and distinct entities. After separation none of the fractions was observed to change into another. The catecholase to cresolase ratio of a particular fraction was not changed by recycling through a column or by passage over a different adsorbent. Also, the high catecholase and high cresolase fractions have different K_m values for catechol and p-cresol, further indicating the distinctive character of the different fractions. The component having a high cresolase activity showed a much greater resistance to reaction inactivation than did the high catecholase fraction. Using similar techniques, Bouchilloux, McMahill and Mason (42) confirmed the results of Smith and Krueger (231) by showing that

mushroom tyrosinase exists in several forms. Sodium dodecyl sulfate was also found to bring about the dissociation of the enzyme into its subunits with a retention of enzyme activity (42). It was suggested that tyrosinase may exist in a state analogous to that of the isozymes.

Some workers have found tyrosinases of varying thermostabilities in Neurospora crassa (87, 88, 95, 234). Horowitz (114) reports the presence of four neurospora tyrosinases of varying thermostability and electrophoretic mobility. These different forms of the enzyme are interconvertible in crude preparations, but not in purified preparations. Horowitz suggested that some additional factor is required for the interconversion. All forms of the enzyme from Neurospora crassa have the same pH optimum and the same Km toward L-tyrosine, L-DOPA, and D-DOPA. It was concluded that the different forms of the enzyme differ mainly in their secondary and tertiary structures and not in their active centers.

Of particular interest is the work of Brown and Ward (45, 46) who obtained the first soluble preparation of mammalian tyrosinase from the Harding-Passey melanoma. The enzyme was brought into solution using a combined ammonium sulfate-acetone fractionation procedure (45). Using starch gel electrophoresis and ion exchange chromatography on DEAE-cellulose, Brown and Ward separated their tyrosinase preparation into three active fractions (46). In so doing, they provided the first indication that mammalian tyrosinase is made up of several active components. All three of the components had activity toward both tyrosine and DOPA. Seiji

et al (227) observed two active tyrosinase components following electrophoresis of soluble tyrosinase from B-16 mouse melanoma. Pomerantz (201) separated tyrosinase from hamster melanoma into two active fractions.

8. The mammalian melanocyte system.

The first indication that a tyrosinase is responsible for the formation of pigment in mammalian skin was provided by Bloch (27, 28, 77) in 1917. He incubated samples of rabbit skin in the presence of buffered DOPA solutions and observed the deposition of pigment in the melanocytes. Pigment deposition was observed when the skin preparations were incubated in the presence of DOPA, but not when incubated with tyrosine. Therefore, Bloch gave the name DOPA-oxidase to the enzyme responsible for pigment formation in normal skin. Later he showed that the DOPA reaction is positive only in the skin cells which form melanin and only at the time when they are capable of forming it (30). It was not possible at the time to demonstrate pigment formation from tyrosine or from catechols other than DOPA. Later work showed that melanomas from a variety of sources had both tyrosinase and DOPA-oxidase activities (113, 145). This provided the first conclusive evidence of a mammalian tyrosinase. Fitzpatrick (75) was able to demonstrate tyrosinase activity in melanocytes of normal skin using histochemical techniques. The enzyme proved to be present in an inactive or partially inhibited state in unirradiated human skin. Tyrosinase activity was greatly increased by previous irradiation with ultraviolet light. After irradiation and

incubation in the presence of tyrosine, melanin appeared in both the dendritic processes and cell bodies of the melanocytes. Unirradiated control samples from the same donor showed no dendritic melanocytes containing tyrosinase activity. Tyrosinase from normal human skin is inhibited by diethyldithiocarbamate, indicating that normal tyrosinase is a copper containing enzyme, as was demonstrated earlier using the enzyme from melanoma (145).

The observation that pigment is found in cells other than melanocytes led early investigators to suggest that the basal cells have the ability to produce melanin and to become dendritic following the appropriate stimulation (29, 192, 196). However, the microscopic studies of Becker (16) and Masson (171) indicate that the dendritic cells belong to a specific cell type. Several different groups of investigators have since concluded that the ability to produce melanin is an exclusive property of the melanocytes (17, 22, 174, 223). Variations in degree of pigmentation in vitiligous, caucasian, Negro, and albino skins were found to be due to variations in physiologic function and not to the number of melanocytes (17). Evidence based on the spectral reflectance and absorption curves of human skin indicates that the same pigment is found in the hair and skin of all races (2, 49, 71).

The only site of melanin formation in the mammal is the pigment cell or melanocyte (223). This is a specialized and distinctive cell characterized by the presence of two or more dendritic cell processes and containing numerous pigmented cytoplasmic granules. Mammalian tyrosinase was first shown by Herman

and Boss (110) in 1945 to be localized in the particulate portion of the melanocyte. Although the enzyme was shown to be present in particles of about mitochondrial size (145), the nature of the tyrosinase containing particles was not determined for many years. Early electron microscope studies (14, 26) indicated that the melanin containing particles are distinct cytoplasmic entities while biochemical assays indicated that they might be modified mitochondria (68). Correlation between biochemical and electron microscope studies by Seiji (223) support the concept that the melanin granules are distinct cytoplasmic particles and different from mitochondria. In man, melanocytes are present in relatively few sites: at the epidermal-dermal junction (13), rarely in the dermal connective tissue, in the hair bulb (13, 25, 26), in the leptomeninges (78), the uveal tract (248), and in the retinal pigment epithelium (5). Because of the great similarity of the melanocytes from the different sites, they are grouped under the designation melanocyte system. The cells comprising this system arise in the neural crest (63, 70, 216, 217) with the exception of the retinal melanocytes which arise in the outer layer of the optic cup (183, 253). All of the melanocytes of the human melanocyte system have been found to be primary sites of malignant melanoma, except for those from the retinal epithelium and hair bulb. In mammalian skin, the melanocytes are found primarily at the epidermal-dermal junction. In the hair bulb they are found attached to the basement membrane in the upper portion of the papilla. Dendritic cells containing a black pigment within

cytoplasmic granules are also found in the substantia nigra of higher primates (84). The nature of this pigment has not yet been determined.

When sections of pigmented skin are observed under the light microscope, pigment granules are seen in ordinary epidermal cells as well as in melanocytes (171). Also, cortical cells of hair often contain melanin granules, either in small bundles or individually. As the cortical cells move up the hair follicle, the bundles of pigment granules become dispersed. They then lie in strings between and parallel to keratin fibers. It has been shown that whenever melanin granules are found in the cytoplasm of basal cells they are invariably located close to a point on the cell wall which is adjacent to the button like terminal of a dendritic cell process (22). Birbeck et al (26) report that ordinary epidermal cell membranes form folds, enveloping the terminal of a melanocyte dendritic process, and suggest that a phagocytosis-like mechanism may account for the presence of melanin granules in cells other than melanocytes. These findings together with the observation that the dendritic cells are the only ones in skin or hair to give a positive DOPA reaction (22) has led to the conclusion that although melanin granules may be found in cells other than melanocytes they are not of endogenous origin. Rather, the melanin granules originate in melanocytes and are passed on to adjacent cells through the dendritic processes of the melanocytes (237).

Melanocytes have a characteristically dendritic shape, and when viewed under the electron microscope, a distinctively granular

cytoplasm (13, 14, 26). Because of their larger size, dendritic shape, granular cytoplasm, and absence of keratin fibers in their cytoplasm, the melanocytes are readily distinguished from the keratinizing cells of skin and hair. The main body of the cell contains a relatively structureless nucleus, many mitochondria, and a well developed endoplasmic reticulum and Golgi apparatus. This latter structure also distinguishes the melanocytes from other cells of skin and hair. The distinctive secretory character of the melanocytes is retained by malignant melanomas (60, 246).

Melanocytes of the hair bulb have been the subject of considerable investigation by electron microscopists. Birbeck and Barnicott (25, 26) have been two of the most active workers in this field, and have described the fine structure of the melanocyte in considerable detail. Thin sections of melanocytes from hair follicles show the presence of a cytoplasmic organization characteristic of secretory cells. There is an endoplasmic reticulum at the end of the cell nearest the papilla. On the side of the nucleus opposite the basement membrane is a region with many vesicles and mitochondria, but containing few pigment granules. This region is analogous to the Golgi region of other secretory cells. Surrounding the Golgi region are many partly developed granules in intermediate stages of melanization as well as numerous large round vesicles believed to be the immediate precursors of the melanin granules. Melanin granules are distributed throughout the cell body and its dendritic processes except for the Golgi region. Between the Golgi region and portions of

the cell containing mature melanin granules are other granules in intermediate stages of developemnt. These latter observations by Birbeck and Barnicott (26) suggest that the melanin granules originate in the Golgi apparatus. The evidence for the origin of melanin granules in the melanocyte is based on the spatial arrangement between the small Golgi vesicles and the immature granules and the arrangement between the immature granules and the mature granules.

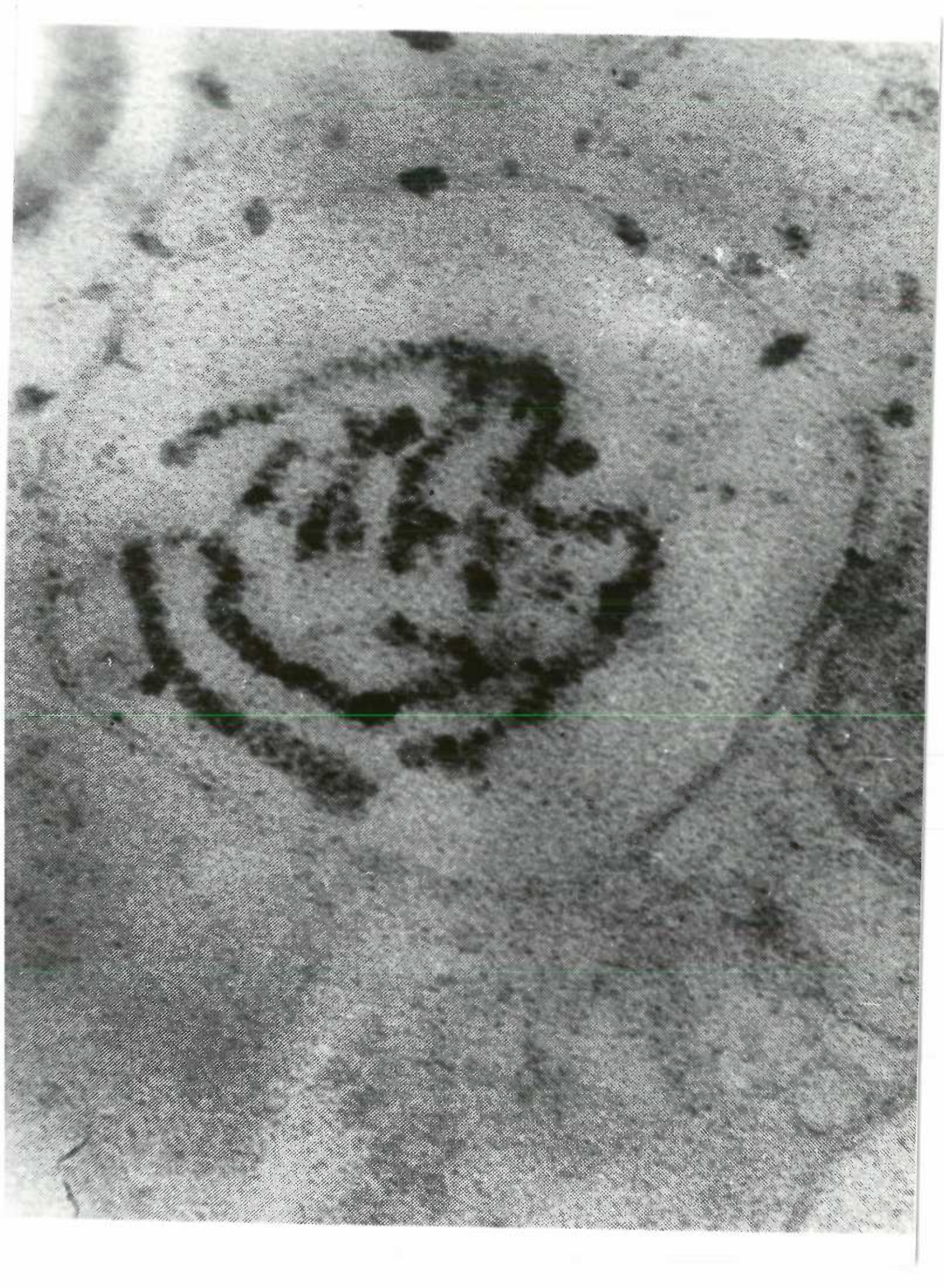
Intact pigment granules when viewed under the electron microscope appear as dense elongated structures with little visible internal structure. The internal structures are made visible only when the granules are sectioned. These are best seen in particles which are partly melanized. Cross sections of the melanin granules, when viewed under the electron microscope, show internal membranes arranged concentrically or as irregular spirals in transverse section, or as parallel membranes in longitudinal section. The internal structure of the melanin granule differs markedly from that of mitochondria, and is the basis for the conclusion that the melanin granules are distinct cellular particles and not modified mitochondria. Furthermore, intermediate stages between melanin granules and mitochondria have never been observed.

The earliest morphological structures observed are small vesicles about 0.05 μ in diameter, apparently formed by a pinching off of a portion of the Golgi apparatus. An increase in size of these particles is then believed to occur either by

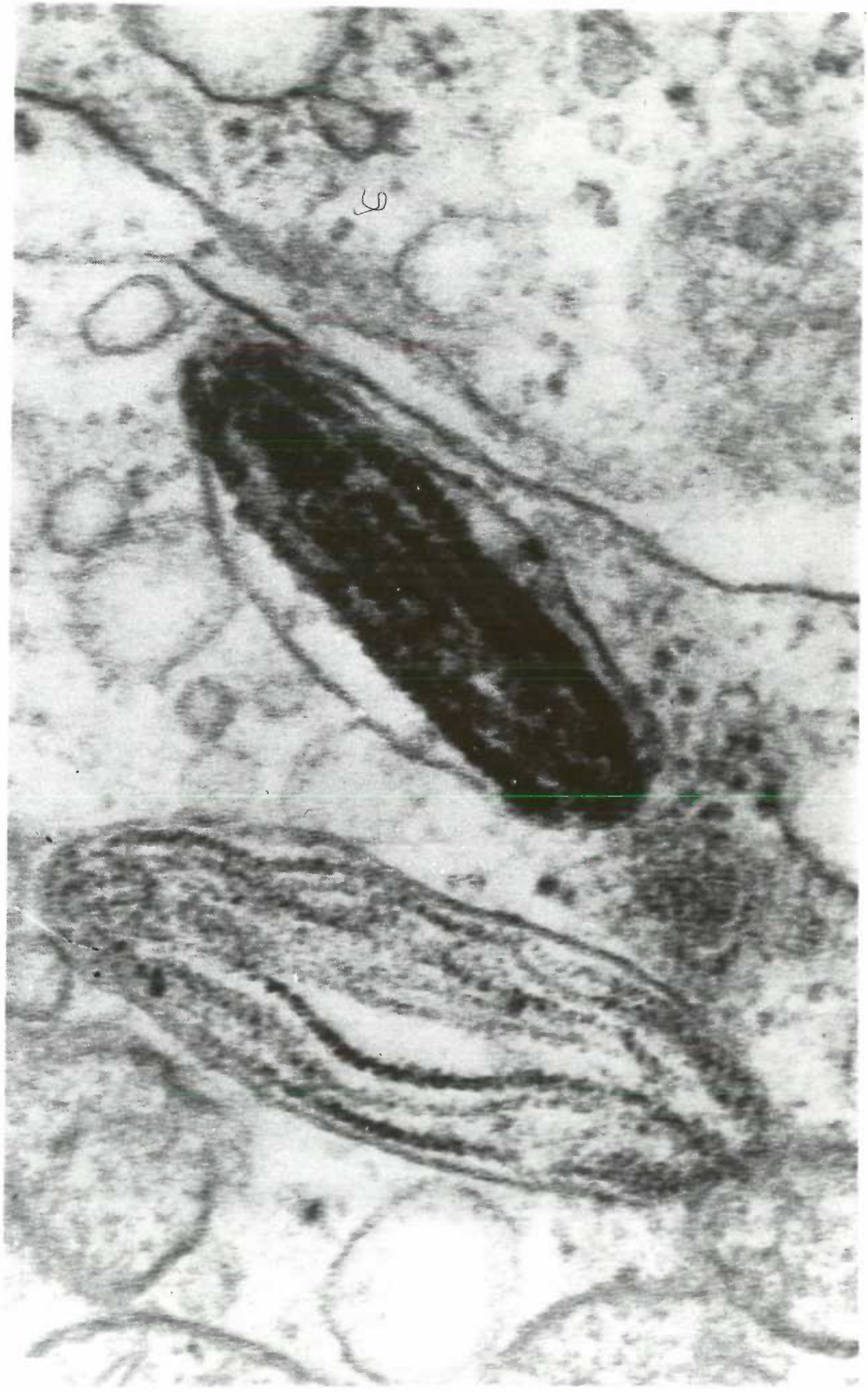
growth or by fusion, forming large round vesicles approximately the size of the mature melanin granule. At the same time, internal membranes develop within the vesicles. The outer membrane of this structure and of mature melanin granules can be resolved by the electron microscope into two dense lines about 70 Å apart. Internal structures consist of just a single membrane. During early stages of development, the premelanin granules have membranes of about the same density as other structures in the cell. A gradual increase in density occurrs as melanin is deposited on the internal membranes. As already mentioned, the inner structures of the premelanin granule consist of either several concentric membranes or of a single membrane wrapped in an irregular spiral. Using the electron microscope, a fine structure, related to the sites of melanin deposition, can be detected on the inner membranes. This fine structure is more readily resolved after a small amount of melanin has been deposited on the internal membrane. However, as melanin deposition proceeds, the fine structure eventually becomes obscured. The appearance of this fine structure depends on the orientation of the internal membrane to the plane of sectioning. It is most readily seen when sections are made parallel to and above the long axis of the granule. In such a case the section may contain a portion of one of the inner membranes. When examined under the electron microscope, the membrane may be seen to consist of small particles in orthogonal array about 80 Å apart on the long axis and 45 Å apart at right angles. When membranes are viewed at right angles, melanin is seen to be deposited on both sides of the membrane. These sites

A cross section of an early melanosome. The double structure of the outer membrane may be seen.

frm Birbeck (25)



Two longitudinal sections of melanosomes; one very early and one in which melanization is almost complete. In the early one melanin may be seen depositing on either side of a membrane, and the helical thread appearance may be seen.
From Birbeck (25)



of melanin deposition alternate, rather than being directly opposite one another. These electron micrographs have been interpreted in terms of a model composed of units having dimensions of 80 Å x 30 Å and arranged so that alternate rows are reversed. Each such unit is thought to be a site of melanin biosynthesis. A protein having such dimensions would have a molecular weight of about 80,000. Such calculations, however, are of little value because of the difficulty of making accurate measurements. Since the fine structure can be seen in albino melanocytes, its formation must be independent of melanin formation. Fractional centrifugation (224, 225) of mouse melanoma cells and attempts to solubilize tyrosinase with deoxycholate (226) indicate that tyrosinase is either firmly bound to the internal membrane of the melanosome or a constitutional element of that membrane.

In accordance with our present concepts of protein formation, the tyrosinase molecule is thought to be synthesized on RNA particles and then transferred in some way through the endoplasmic reticulum to the vesicles of the Golgi apparatus which eventually become the melanin granules (223, 226, 227, 246). Normally however, tyrosinase does not catalyze the formation of melanin until it is activated in the mature melanosome. This concept is supported by evidence showing that C^{14}-leucine is first incorporated into tyrosinase of the small granule fraction described by Seiji (227). The time course of the incorporation suggests a precursor product relationship between the small granules and the large granules in terms of tyrosinase activity. Electron microscope studies of Wellings

and Siegel (246) show that the Golgi apparatus is continuous with the endoplasmic reticulum.

The pigmented dendritic cells from human skin are very similar to those found in other animals (23). In samples of white human skin, however, the melanocytes can not be visualized without the aid of the DOPA-reaction due to the scarcity of pigment. In deeply pigmented human skin, as in the skins of darkly colored animals, there is a high level of intrinsically maintained melanogenic activity. In white human skin, on the other hand, there is normally little or no pigment forming ability. White human skin contains the necessary tyrosinase (75). However, it is present in an inhibited form. Tyrosinase of white human skin can be activated by certain stimuli such as ultraviolet light. There is also an inhibited tyrosinase in the skin of the albino guinea pig which can be activated by cold (22, 96). In light of this it is surprising to note that there is no known physiological stimulus which will initiate melanogenesis in white skin of the black and white spotted guinea pig (151). In this latter case, the melanocytes, although present, apparently do not possess the necessary enzyme system for pigment formation.

Greenstein (105) studied the melanin granules from mouse melanoma from a chemical standpoint rather than with the electron microscope. By subjecting the combination of melanin and protein in these granules to the action of pancreatin, he found that the sulfur containing amino acid cyteine remained attached to the melanin. This led to the conclusion that melanin combines with protein of the

membrane via -S- linkages. Studies with the electron microscope have not been able to demonstrate the site of tyrosinase in the melanin granule. Greenstein postulated that tyrosinase may be attached to the internal membranes in a manner analogous to the enzymes of mitochondria which are attached to cristae.

9. Albinism.

Albinism is an inborn error of metabolism in which there is a lack of pigment in skin, eyes, and hair due to the absence of the enzyme tyrosinase (133). Barnicott et al (13) observed melanocytes in hair plucked from albinos. In the albinos, the melanocytes are present and may be identified by their characteristically dendritic shape and by their granular cytoplasm (14, 99). These properties make the melanocytes readily distinguishable from ordinary cortical cells of the hair bulbs. Granules of the albino melanocyte are much like those of normal melanocytes. However, there is little or no melanin in evidence. Structurally these granules resemble the immature granules of normal melanocytes in which melanin has not yet been deposited. The albino melanocyte is capable of synthesizing the granule and its internal membranes, but not of forming the melanin polymer within it. Furthermore, these granules are more numerous in albino melanocytes than in those of normally pigmented skin (15). These cytoplasmic granules are about 0.5 microns in diameter and have the appearance of immature granules in normally pigmented skin and hair. They differ from the latter, however, in that the internal membranes are less dense due to the absence of pigment (11). The most interesting feature here

is that albinos' skin contains melanocytes which appear normal in every respect when viewed under the electron microscope except that they lack the melanin which is normally deposited within the cytoplasmic granules.

Although the albinos are said to be lacking the enzyme tyrosinase, it is generally found that in the human albino Negro there is usually a small amount of pigment deposited in the melanocyte granules (12, 89, 200).

The skin of human albinos has been the subject of considerable investigation. A particularly interesting feature is the observation that in the human Negro albino the existence of some skin pigment seems to be the rule rather than the exception (12). This pigment is found generally distributed throughout the skin, concentrated in freckles, in the iris, and in hair (12, 89, 200). Caucasion albinos may show no skin pigment except for occasional freckles. The situation in human albinos then, appears to differ markedly from albinos of other species. Hu (115) states that complete albinism, as far as we know, does not occur in humans. Skin from patients with incomplete generalized albinism all give a positive DOPA reaction. Morphologically the melanocytes from human albinos resemble those from normal persons of fair complexion. When albinos are exposed to ultraviolet light their melanocytes increase in size and become more dendritic. But, there is no visible increase in pigmentation. However, after irradiation some of the melanocytes become DOPA positive. This has led to the suggestion that the tyrosinase is defective or inhibited rather than completely absent.

The melanocytes of normal fair skinned individuals can not be visualized directly under the light microscope due to the scarcity of pigment and the fact that it is light brown rather than intensely black as in some other species. The normally light brown melanin can be made visible, however, by staining it with silver. Silver ion is rapidly reduced to metallic silver by melanin. Metallic silver deposited on the melanin granules renders them readily visible under the light microscope.

10. Premelanin.

There is an as yet unidentified substance called premelanin which reduces ammoniacal silver nitrate but not ordinary silver nitrate in melanoblasts from ten day old fetuses, albino melanocytes, and in melanocytes from xanthic goldfish - cells which do not ordinarily become melanized. Melanocytes from xanthic goldfish will form melanin under certain physical stimuli (178). In the xanthic goldfish this may be an example of interrupted melanogenesis. Localization of premelanin is highly specific. It is found in melanin granules but not in mitochondria, nuclei, or cell membranes. Also, other cells such as epidermal cells, bone, or the basement membrane at the epidermal-dermal junction do not stain.

Attempts have been made to demonstrate tyrosinase activity in albino mice, rabbits, and humans (115, 136). Darkening of albino hairs was observed when they were incubated in the presence of DOPA or tyrosine solutions.

11. Sulfhydryl inhibition.

That the natural regulation of melanin formation may depend on organic sulfhydryl compound inhibition of tyrosinase activity was

first suggested by the observation that aqueous extracts of rabbit and guinea pig skin can inhibit mammalian tyrosinase (219, 220). Rothman et al (221) demonstrated the presence of this inhibitory activity in human skin, and showed that its activity can be prevented by sulfhydryl reagents. The sulfhydryl inhibitor was found to be localized in the pigment forming regions of the skin (220). Rothman concluded that this constitutes a naturally occurring regulatory mechanism in pigment formation. According to this theory, lightly pigmented skin remains lightly pigmented because a sulfhydryl compound or compounds keep tyrosinase in an inactive state. It should be noted that tyrosinase is inhibited by a large number of sulfhydryl compounds (20, 146, 195).

Inflamatory stimuli which lead to hyperpigmentation such as ultraviolet or X-ray irradiation, and some inflamatory skin diseases bring about a decreased sulfhydryl content of skin. The hyperpigmentation resulting from ACTH administration is also accompanied by a decreased content of sulfhydryl compounds in the skin. The skin of Negroes and other darkly pigmented humans contains lower levels of sulfhydryl compounds than does the skin of their less intensely pigmented counterparts. Conversely, skin from vitiligous lesions contains a higher level of sulfhydryl compounds than does the surrounding normally pigmented skin (244).

Flesch and coworkers (80, 82) have partially characterized the sulfhydryl inhibitor of mammalian skin and found it to be a heat stable, dialyzable, nonprotein-like compound. Its inhibitory activity is directly proportional to the logarithm of the sulfhydryl

concentration. Flesch also found that ultraviolet light causes an immediate decrease in the amount of water extractable sulfhydryl compounds of skin from colored rabbits. Since the epidermal extracts inhibit the action of cupric ions on melanin formation in nonenzymic systems as well as inhibiting tyrosinase, Flesch suggested that the sulfhydryl compound acts directly on the copper of tyrosinase (81, 82).

That melanocyte tyrosinase of humans is normally present in an inactive form was also demonstrated by Fitzpatrick and coworkers (75). Using histochemical techniques they showed that melanocytes of normal human skin will not convert tyrosine to melanin unless the tyrosinase is first activated. The activation was accomplished by irradiating the skin with ultraviolet light. Following this irradiation, melanin was deposited in the melanocyte cell bodies and dendritic processes when the melanocytes were incubated in the presence of buffered tyrosine solution. Fitzpatrick demonstrated that while the tyrosinase of benign pigmented lesions is present in an inhibited form, the free uninhibited enzyme can be demonstrated in malignant melanoma (79).

Even though the work just described has shed some light on the regulatory mechanism controlling pigment formation, our knowledge about the normal regulation of melanin formation is far from complete. For instance, it has not been possible as yet to reconcile the immediate drop in the level of sulfhydryl compounds following the ultraviolet irradiation of skin (80) with the prolonged period required for the demonstration of an increased tyrosinase activity (79).

12. Effect of sunlight.

Early workers alleged that skin pigment is able to protect against visible rays (97, 101), ultraviolet rays (111), and against heat rays (15, 175). Moderate amounts of ultraviolet light have two grossly observable effects. The first of these to become evident is an erythema (33, 34). Blum (33) postulates that the erythema is a result of direct injury to cells of the epidermis. As a result of this injury, the cells give off a variety of materials which have specific physiological action. These include a dilator substance, leucotaxine, and melanotic factors. The former are responsible for the erythema and swelling which are the first visible results of sunburn. Ultraviolet light between 290 mμ and 310 mμ is most effective in promoting the erythemal response (155, 156, 179). Because of the strong absorption of UV light by protein (34, 239), most of the light is thought to be absorbed by the stratum corneum and underlying epidermal cells. Little of the light passes through the capilaries whose dilation produces the reddening of sunburn. The stratum corneum of caucasians is more transparent to ultraviolet light than that of Negroes (239). Yet, the thickness of the horny layer is about the same in both cases. The higher optical density of the stratum corneum of Negroes is attributed to the greater pigment content of the skin. By way of comparison, stratum corneum from albino Negroes has about the same transparency to ultraviolet light as does the skin of caucasians.

The erythema of sunburn fades over a period of several days and is accompanied by a gradual increase in the pigment content

of the irradiated skin. Studies of the spectral reflectance curves of tanned skin led Edwards and Duntley (71) to conclude that the new pigment is melanin. Shortly after the rate of melanin formation increases as a result of UV irradiation, there is a migration of pigment through the epidermis towards the skin surface (119). The new pigment is distributed to a large number of epidermal cells as well as melanocytes (171). Experiments with C^{14}-labeled substrates indicate that melanin does not undergo further metabolic turnover (31). In addition to promoting the formation of new pigment, which requires several days, ultraviolet light also causes an immediate darkening of existing melanin (108, 109). This is the result of oxidation of existing melanin. Pigment darkening begins a few minutes after exposure to ultraviolet light and is over in approximately one hour. Pigment darkening is greatest in skins which have been previously exposed to ultraviolet light and therefore contain greater amounts of melanin (34). Formation of new melanin, on the other hand, occurrs to a greater extent in skin not previously exposed to ultraviolet light. Pigment darkening is stimulated maximally by light over a broad range from 300 mμ to 400 mμ. Erythema and new pigment formation are stimulated mainly by light below 320 mμ.

Attempts to explain the effects of ultraviolet light on skin have included direct photochemical oxidation of tyrosine to DOPA (8), and a decrease in sulfhydryl inhibitors of tyrosinase (75). No adequate explanation has yet been presented to account for the delay of several days between exposure to ultraviolet light and the

formation of new melanin. Blum (34) points out that increased melanization is a common response of skin to trauma, and may have no direct relation to the agent that caused the injury. Thus we see that in addition to exposure to ultraviolet light, increased melanization occurrs as a result of burns, prolonged rubbing, mechanical trauma, and inflammatory skin diseases.

The observation that melanin contains stable free radicals has changed our concept of the protective function of this pigment (32, 57, 62, 168). Despite their apparent heterogeneity, many natural products containing stable unpaired electrons have a common structural characteristic. They all contain polymeric condensed ring structures (57). The extraordinary stability of free radicals in melanin has led Commoner to suggest that the melanin acts as a free radical trap which sequesters photon-generated unpaired electrons in a chemically inactive state. Mason (168) suggested that melanin may protect tissues against oxidizing and reducing conditions which could give rise to reactive free radicals having a disruptive influence on metabolism. The finding that melanin in mammalian skin is present mostly as discrete aggregates in the basal layer of the epidermis may be more consistent with a chemical than an optical defense mechanism, particularly since a large portion of the melanin is beneath the cells thought to be damaged by ultraviolet light in the sunburn reaction (62). Thompson (239) found that melanin in the stratum corneum explained the greater protective effect of Negro skin against sunlight. Exposure of human black hair to ultraviolet light from a mercury arc light increases the free

radical concentration of the hair (168). Exposure of the hair to sunlight has a similar but less marked effect. Dark hair contains a higher concentration of free radicals than light hair. The free radical in hair was ascribed to a semiquinonoid form stabilized by resonance in a highly conjugated polymer. The free radical character of sepia melanin was lost on reduction. Other workers have observed an electron spin resonance signal which they interpreted as being due to an electron localized on a single semiquinone monomer (32). This data supports the conclusion that melanin is composed of several monomers and bond types which are not extensively conjugated. A free radical polymerization mechanism might be expected to lead to the formation of such an irregular polymer. Ferric and cupric ions and water, but not oxygen, decrease the strength of the ESR signal.

13. The influence of hormones on the melanocyte system.

Melanin formation and dispersion within melanocytes is under the influence of pituitary hormones (142, 149). The first successful isolation of melanocyte stimulating hormone (MSH) from hog pituitary glands was accomplished by Landgrebe in 1954 (137) and then by Porath in 1955 (203). Lerner and Lee (139, 148) subsequently showed that there are actually two MSH's, designated α- and β-MSH. α-MSH accounts for about 75% of the activity isolated from the hog pituitary, and has an isoelectric pH of about 10.5 - 11.0. β-MSH has an isoelectric pH of 5.5, and accounts for the remaining 25% of the activity. The amino acid sequence of these polypeptide hormones has been found to be very similar, both to each other and to ACTH (93, 94, 103, 104). MSH causes a dispersion of pigment granules in melanocytes, thereby

rendering them less transparent, and causing the skin to darken (142, 149). This dispersion does not occur in the absence of oxygen (150).

The pineal gland secretes melatonin which counteracts the action of MSH by causing an aggregation of melanin particles in the body of the melanocytes (143). Epinephrine and norepinephrine also bring about movement of melanin particles from the dendrites into the main body of the cells (142, 233) as does ACTH (142). The effect of ACTH is thought to account, at least in part, for the hyperpigmentation observed in persons experiencing adrenal cortical insufficiency (Addison's disease). As yet, no direct influence of a hormone on tyrosinase activity has been demonstrated (149).

Tyrosinase is inhibited by phenylalanine, phenylacetate, phenylpyruvate, and p-hydroxyphenyllactate (180). The increased concentration of these substances in the blood plasma of phenylketonurics is believed to account for the decreased pigmentation in the skin and hair of these individuals (180, 242).

14. Comparison of the properties of tyrosinase and tyrosine hydroxylase.

It should be noted that there are at least two enzymes capable of hydroxylating tyrosine in mammalian tissues, tyrosinase and tyrosine hydroxylase. A comparison of the properties of the enzymes shows that they are two separate and distinct entities. Tyrosine hydroxylase is a tetrahydropteridine-linked, iron containing mixed function oxidase which catalyzes just the hydroxylation of tyrosine to DOPA, but shows no enzymic activity towards DOPA (186).

Tyrosinase, on the other hand, is a copper protein which uses DOPA as the reducing agent and catalyzes the oxidation of both tyrosine and DOPA to melanin. In addition to having different cofactor requirements and substrate specificities, these enzymes also have different K_m's and pH optima. A comparison of the properties of these enzymes is gives in the following table.

15. Nonenzymic hydroxylation

During their studies of tyramine hydroxylation by adrenal medullary slices, Udenfriend and coworkers (43, 243) observed a nonspecific and nonenzymic hydroxylating system. Further studies were made using a model hydroxylating containing ascorbate, ferrous sulfate, and EDTA in a phosphate buffer at pH 5.5. A variety of aromatic substances were hydroxylated when incubated with the model system at 35° C in the presence of oxygen. Ascorbate was found to increase the rate of oxidation, as did many other compounds containing the ene-diol structure. EDTA, while not essential for oxidation, also increased the oxidation rate. Transition metal ions, on the other hand, are necessary for hydroxylation to proceed. Ferric and ferrous ions are the most active. Cupric, cuprous, and cobaltous ions were found to be about 5 - 10% as effective as iron. Hydroxylation of a wide variety of compounds occurred over a wide range of pH's from 2 - 7.5, the optimum depending on the individual substance. Evidence that ascorbate is not the primary oxidant was obtained by preincubating the mixture untill nearly all the ascorbate had didappeared before adding the substrate. Hydroxylation then proceeded rapidly.

Table 1.

Comparison of Mammalian
Tyrosinase and Tyrosine Hydroxylase*

Property	Tyrosinase	Tyrosine hydroxylase from Brain Particles	Tyrosine hydroxylase from Adrenal Medulla
pH optimum	7.0	6.2	6.0
Substrates:			
L-tyrosine	+	+	+
D-tyrosine	+	0	0
tyramine	+	0	0
DOPA	+++	0	0
K_m	6×10^{-4}	ca. 10^{-5}	1×10^{-5}
Activators:			
DOPA	+++	0	0
Inhibitors:			
DOPA	0	+	+
thiourea	++	0	0
diethyldithio-carbamate	++	0	0
α,α'-dipyridyl	0	++	++
Pigment formation	+++	0	0

*From Nagatsu (186).

Although ascorbate is not the primary oxidant, substitution of hydrogen peroxide in amounts equivalent to ascorbate resulted in negligible activity. Maximum activity was observed when both ascorbate and hydrogen peroxide were present in the molar ratio of 1:2. In a nitrogen atmosphere the system is inactive. However, the aromatic substances are hydroxylated very rapidly when hydrogen peroxide is added to the anaerobic system. This led Udenfriend to suggest that hydroxylation is brought about by a reaction product of hydrogen peroxide and ascorbate. The reaction was thought to occur in two steps. The first step involved the oxidation of ascorbate to dehydroascorbate. The second step was thought to be the reaction of hydrogen peroxide with dehydroascorbate to form a hydroperoxide. However, when dehydroascorbate was used to replace ascorbate in the anaerobic system, no hydroxylation occurred. Furthermore, synthetic hydroperoxides, when incubated in the presence of ferrous ion, EDTA, and substrates under anaerobic conditions, possessed no hydroxylating activity. Identification of the hydroxylated products shows that the entering hydroxyl group is directed to electronegative sites on the ring. Although the hydroxylating intermediate has not been identified, Mason (67) points out that since it does attack electronegative sites on an aromatic ring it is probably a cationic iron-oxygen complex.

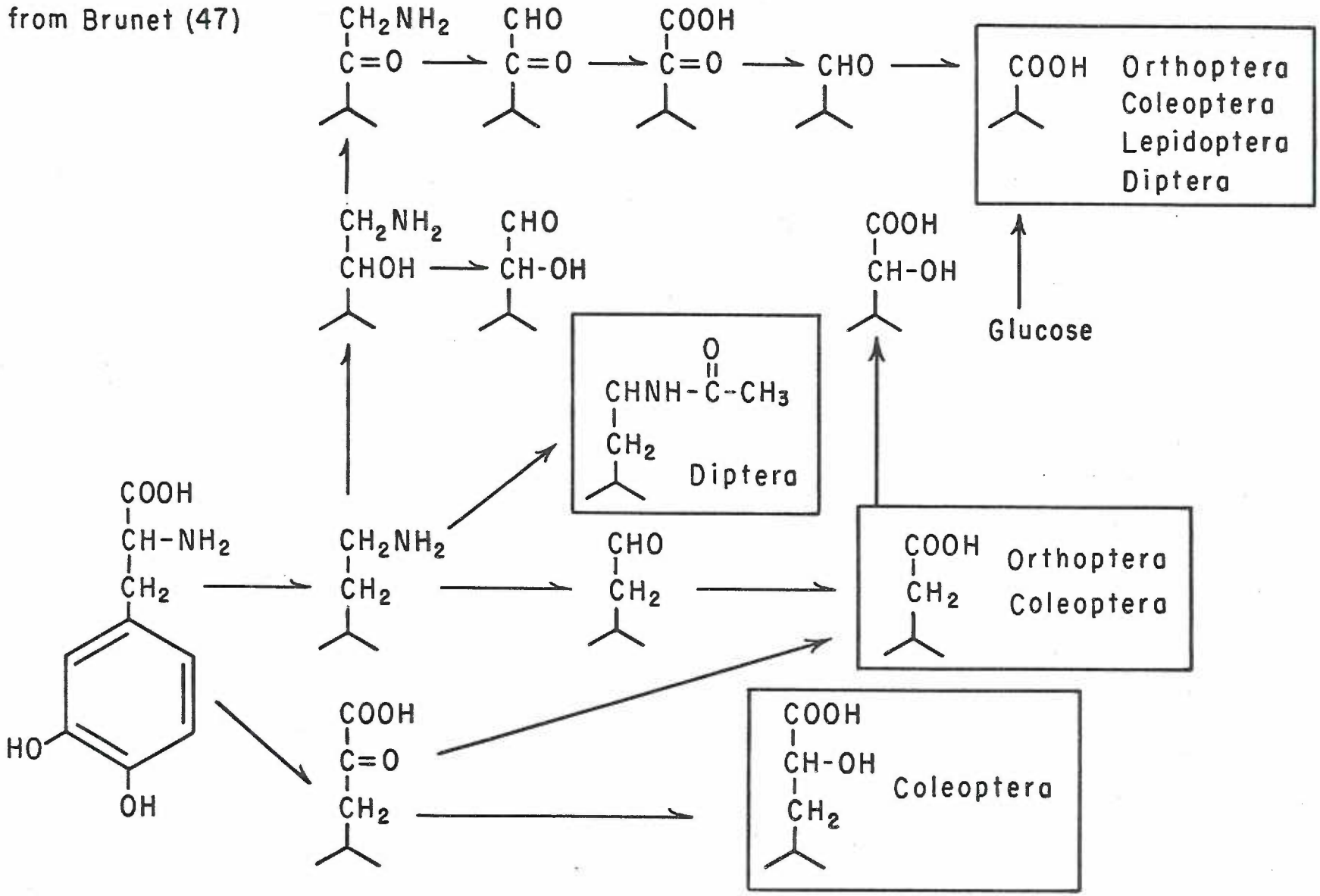
Various tissues contain proteins which bind DOPA and the catechol amines thereby protecting these catechols from autoxidation (116, 209).

16. Insect phenolases.

During the process of sclerotization, a soft colorless proteinaceuos material is converted to a hard, inert, and usually brown substance called sclerotin as a result of tanning by a quinone (48, 204). Quinone tanned proteins are of very wide occurrence in both the vertebrate and invertebrate animal kingdoms (44). Many phenolic and quinoid substances are reported to occur in insects (47). These are thought to be derived from tyrosine and phenylalanine which are found in high concentration in the hemolymph of many types of insects (83). Phenolic substances thought to be involved in hardening of the insect cuticle include 3,4-dihydroxyphenyllactate, 3,4-dihydroxyphenylacetate, and 3,4-dihydroxyphenylbenzoate (47, 204, 205, 206). Although the origin of these catechols is not certain, their structures and collective appearance suggest a common origin from DOPA (107).

In their studies of a phenoloxidase from Periplaneta americana, Whitehead et al (247) described a copper containing enzyme active toward protocatechuic acid, 3,4-dihydroxyphenylpropanoic acid, 3,4-dihydroxybenzaldehyde, catechol, and p-phenylenediamine. The enzyme was inactive toward D,L-DOPA, L-o-tyrosine, L-p-tyrosine, p-hydroxybenzoic acid, DOPA-amine, or resorcinol. On the basis of its substrate specificity, the enzyme should be classed as a laccase rather than a tyrosinase. The enzyme was present in the particulate secretion of the colateral glands of the cockroach in association with the protein precursor of sclerotin. A different phenolase from silkworm pupae has been found which shows high activity toward N-acetyltyramine (47).

from Brunet (47)



Eggs of Periplaneta americana are surrounded by a hard brown capsule, the ootheca (128). The walls of this capsule are composed of sclerotin. Secretions from two colateral glands are responsible for the formation of this hard shell (48, 204, 205). The left gland secretes the 4-O-β-glucoside of protocatechuic acid and the structural protein which becomes sclerotin when tanned. The glucoside of protocatechuic acid is not acted upon by the phenolase of the cockroach. The inert glucoside must first be hydrolyzed to the free protocatechuic acid and glucose by a β-glucosidase secreted by the right colateral gland. The free acid is oxidized to a quinone in the presence of phenolase and then tans the protein, forming sclerotin.

Although polyphenoloxidases are widely distributed in insects, its location in the cuticle has not been established with certainty (165). Brunet (47) suggests that there may be two cuticular oxidases, one responsible for sclerotization and the other for melanization. Hackman (107) suggests that phenols diffuse outward through the cuticle to the epicuticle where they are oxidized to quinones in the presence of a polyphenoloxidase. The quinones then diffuse inward to tan the proteins by reacting with terminal amino groups and with ε-amino groups of lysine.

17. Plant phenolase.

The suggestion has been made that the phenolase complex can serve as a terminal oxidase in plants (167). Its link to the usual metabolic pathways involving TPNH and DPNH may be through the enzyme quinone reductase. This enzyme catalyzes the reduction of quinones by TPNH and DPNH (251). The ease with which quinones can

be involved in reversible oxidation and reduction reactions makes them well suited as carriers in terminal electron transfer (250). Studies of the role of quinones in plant respiration are made difficult by the fact that large increases in respiration result when plant tissues containing phenolases are injured (165, 238). The wide distribution of quinone reductase in plants plus the fact that it can be coupled to the mechanisms of hydrogen transport have led Wosilait and coworkers (252) to suggest a generalized function connected to respiration for this enzyme. However, Mason (165) points out that there is as yet little direct evidence that quinones are involved in terminal respiration in plants.

18. Statement of problem.

Tyrosinase from mushroom and melanoma has become one of the most intensely studied enzymes. Over the years, however, there have been relatively few studies of normal mammalian tyrosinase as compared to the very large number of experiments using the enzyme from mushroom and melanoma. One example of work with tyrosinase from normal human skin is that of Fitzpatrick (75) who demonstrated the presence of and partially characterized the enzyme using histochemical techniques. However, no reports have been found in the literature of anyone having applied quantitative chemical techniques successfully to the study of mammalian tyrosinase of normal skin or hair. It would appear that the very low tyrosinase activity normally present in skin coupled with the difficulty in obtaining suitable enzyme preparations are responsible. In contrast to this is the very high activity of uninhibited enzyme found in

melanotic tumors (75). However, cellular regulatory mechanisms are markedly different in tumor cells. In melanotic tumors this is expressed in part by the production of large amounts of tyrosinase which exists in an uninhibited state and results in intense pigmentation of the melanoma. By way of contrast, the production of melanin in normal skin is very carefully controlled. In humans, for instance, the pigment content of the skin can be maintained within a very narrow range over a period of many years. It should be readily apparent, therefore, that any experimental attempt to describe the mechanisms regulating pigment formation should be carried out using normal skin. Such experiments are dependent upon the development of quantitative chemical techniques sufficiently sensitive to measure the low activity of normal skin tyrosinase. The recent development of such techniques in the study of tyrosinase (205) and tyrosine hydroxylase (184, 185, 186) now makes a study of normal skin tyrosinase feasible. The application of these techniques to the study of normal skin tyrosinase and the characterization of the enzyme were the problems undertaken in the experimental work reported here. It has been suggested that tyrosinase is in a partially inhibited state in normal skin (219). However, earlier investigators were restricted to the use of melanoma tyrosinase in testing the inhibitory activity of mammalian skins. With the more sensitive analytical methods reported here, it has been possible to study the inhibition of tyrosinase in normal rat skin, as well as tyrosine hydroxylation by albino rat skin. Initially it was not known that normal rat skin contained an active tyrosinase. Therefore, the

analytical methods were tested using rat adrenals, a tissue known to possess enzymic tyrosine hydroxylating activity (185).

The analytical methods are based on the incubation of melanocyte containing preparations with either L-tyrosine-U-C^{14} or L-tyrosine-3,5-H^{3}. In those cases where U-C^{14}-tyrosine was used, the reaction mixtures were analyzed for C^{14}-DOPA after incubation. When the tritiated tyrosine is oxidized to DOPA, one of the tritium atoms is replaced and appears in the reaction mixture as tritiated water which can be measured after separation from labeled tyrosine and DOPA.

A discussion of the results and a correlation of the observations reported here with those of previous investigators is also presented.

III. Methods

1. Incubation of adrenal homogenates

Female Sprague-Dawley rats weighing between 215 and 250 grams were sacrificed by decapitation. Adrenal glands were removed, trimmed free of visible fat pads and connective tissue, weighed, and then stored in 5 ml of iced 0.1 M phosphate buffer pH 6.4. One adrenal from each rat was used as a heated blank and the other as the experimental sample. Right and left adrenals were used alternately as the heated controls. These were heated for ten minutes at 76 - 80° C. The adrenals were homogenized individually for one minute in 2 ml of iced 0.1 M phosphate buffer pH 6.4 with a teflon-glass Potter-Elvehjem homogenizer. The homogenates were poured into Warburg flasks kept cold in a tray of crushed ice. The homogenizer was rinsed with 0.5 ml of buffer and the washings added to the homogenates in their respective Warburg flasks. Marsilid (isopropylisonicotinylhydrazine) to make a final concentration of 2×10^{-3} M, 0.2 µc of L-tyrosine-U-C^{14} having a specific activity of 35 µc/mg, and sufficient buffer to make the final volume 3 ml were added to each flask. All flasks were incubated in the Warburg apparatus for one hour at 37° C. At the end of the incubation period, the flasks were removed from the Warburg and the reaction stopped by the addition of 3 ml of 20% trichloroacetic acid (TCA). After being allowed to stand for ten minutes at room temperature, the acid mixture was centrifuged at 3,000 rpm for fifteen minutes at 4 - 5° C. The supernatant was decanted and then stored frozen in the refrigerator for later determination of radioactivity in DOPA and the catecholamines.

2. Preparation of skin scrapings.

The choice of laboratory animals for use in the study of skin pigmentation was a difficult one. Among the rats, guinea pigs, and mice examined, only piebald rats had an appreciable amount of skin pigment. The skin of the other animals, even though the hair was intensely black, contained virtually no visible pigment.

Female Sprague-Dawley and piebald or hooded rats were used in all these experiments. The rats were anesthetized by the intraperitoneal injection of 7.5 mg Nembutal per one hundred grams of body weight. Ten minutes after the injection, a section of skin was removed from the back and sides of the animals. The skin was stretched and pinned to a board hair side down. Connective tissue and fat were removed with forceps and a round bladed scalpel by both cutting and scraping. Considerable care was taken to clean the skin as thoroughly as possible without damaging the skin itself. Skin from piebald rats was scraped with the round edge of a scalpel until the melanocyte layer had been removed. The pigmented layer of cells served as a handy marker for indicating the depth to which scrapings were taken. In the case of the albino rat, there is no such marker indicating the depth or location of the melanocyte layer. Therefore, the experience gained with hooded rats was used as a guide to the depth of scrapings taken from the albino skins. In the case of hooded rats, scrapings were taken only from deeply pigmented regions of skin.

A great deal of care was taken not to cut too deeply into the skin. Otherwise, numerous small strands of keratinized skin would

have been included in the scrapings. These strands could not be homogenized under the conditions employed and interferred with homogenization of the rest of the scrapings, particularly if the keratinized strands were present in considerable quantity.

3. Homogenization of scrapings.

Skin scrapings were homogenized in 5.5 ml of 0.1 M phosphate buffer pH 6.8 with a wide tolerance teflon-glass Potter-Elvehjem homogenizer. Narrow tolerance homogenizers were clogged by the few strands of keratinized skin and connective tissue which were invariably included in the skin scrapings. Homogenization was carried out in three, one minute periods with about one minute between each to prevent warming of the homogenizer which was immersed in an ice bath throughout the entire operation. Before incubation of the homogenate, unhomogenized strands were removed by centrifuging at 500 rpm for three minutes at 4 - 5° C and the supernatant removed with a pipette.

4. Incubation of skin scrapings.

A. Whole scrapings.

Whole scrapings from pigmented regions of hooded rat skin were divided into two approximately equal portions which were then weighed individually. The tissue samples were then placed in separate 25 ml Erlenmeyer flasks along with 2.6 ml of 0.1 M phosphate buffer pH 6.8. One of the flasks was heated to 100° C for three minutes in a boiling water bath and served as the heated control. Only the tissue preparations were heated. Other additions were made after the flasks had been cooled. After the three minute

C. Granular preparations from pigmented regions of hooded rat skin

One milliliter aliquots of the granular preparations were incubated with 3.4×10^{-4} M DOPA and L-tyrosine-3,5-H^3 in the same manner as the homogenates of the skin scrapings.

5. $(NH_4)_2SO_4$-acetone fractionation

Weighed skin scrapings from pigmented regions of hooded rat skin were homogenized in 5 ml if iced 0.1 M phosphate buffer pH 6.8 with a Potter-Elvehjem homogenizer immersed in ice water. The homogenate was then centrifuged at 400 - 500 rpm for five minutes at 4 - 5° C. After decanting the supernatant, the precipitate was resuspended in phosphate buffer, homogenized again, and recentrifuged. Supernatants were combined and mixed thoroughly. The homogenate was then made one tenth saturated with ammonium sulfate previously adjusted to pH 7.0. The saturated solution of ammonium sulfate was added dropwise while the homogenate was stirred continuously and kept cold in an ice bath. A volume of ice cold acetone equal to that of the ammonium sulfate mixture was then added slowly and with continuous stirring. After centrifuging at 2,000 rpm for ten minutes at 4 - 5° C, a black melanin containing precipitate was obtained. The supernatant was decanted and a second volume of cold acetone added to it with continuous stirring. The solutions were kept cold in an ice bath throughout the entire procedure. A white precipitate was obtained by centrifugation and the supernatant decanted. Both precipitates were washed with cold acetone and recentrifuged. Supernatant acetone was discarded and the precipitate

was centrifuged and resuspended in the phosphate buffer. Both of these suspensions were then assayed for tyrosinase activity by incubating 1 ml aliquots in the presence of 3 μc of L-tyrosine-3,5-H^3 having a specific activity of 330 μc/mg, and 3.4×10^{-4} M L-DOPA. The final volume was 3.0 ml.

6. Fractionation of skin scrapings

In order to determine the cellular location of the activity measured with the scrapings from albino skin, scrapings from six different rats were taken in order to have sufficient material with which to work. The scrapings were weighed, suspended in 0.25 M sucrose, and then homogenized in several batches. The amount of material was too great to homogenize at one time. The Potter-Elvehjem homogenizer used was immersed in ice water throughout the entire procedure. Each batch of scrapings was homogenized during three periods, each lasting about one minute. A short interval of aproximately one minute was allowed between each homogenizing period to prevent warming of the homogenizer and its contents. Homogenates were combined and centrifuged at 400 - 500 rpm using head number 269 of the International centrifuge to remove collagenous strands and other unhomogenized material. The supernatant was decanted and stored in an ice bath while the precipitate was resuspended in 0.25 M sucrose and rehomogenized. This second homogenate was centrifuged again at 400 - 500 rpm and the supernatant combined with the one previously obtained. The supernatants were then thoroughly mixed. A portion of the whole homogenate was set aside in an ice bath for later determination of

the tyrosine hydroxylating activity. The remaining combined supernatants were then layered over 10 ml of 0.34 M sucrose and centrifuged at 700 rpm for ten minutes at 4 - 5° C. After decanting the supernatant, the precipitate was resuspended in 0.25 M sucrose and recentrifuged. The supernatant was combined with the one previously obtained and then centrifuged at 5,000 xg for ten minutes at 4 - 5° C. Meanwhile, the nuclear fraction from the 700 xg centrifugation was resuspended in 5 ml of 0.1 M phosphate buffer pH 6.8 and stored in an ice bath for later determination of the tyrosine hydroxylating activity. The pellet containing mitochondria from the 5,000 xg centrifugation was suspended in 5 ml of 0.25 M sucrose, and recentrifuged at 24,000 xg for ten minutes. the resulting precipitate was suspended in 5 ml of 0.1 M phosphate buffer pH 6.8 for later estimation of the tyrosine hydroxylating activity. Supernatant from the first 5,000 xg centrifugation was centrifuged at 24,000 xg for two hours at 4 - 5° C. This precipitate was also suspended in phosphate buffer after decanting the supernatant. The whole homogenate, nuclear fraction, mitochondrial fraction, microsomal fraction, and the soluble supernatant were all assayed for tyrosine hydroxylating activity according to the procedure used with homogenates of skin scrapings.

7. Preparation of granule suspensions from pigmented regions of hooded rat skin

Scrapings from pigmented regions of hooded rat skin were homogenized in 5 ml of 0.1 M phosphate buffer pH 6.8. This homogenate was centrifuged at 5,000 rpm for five minutes at 4 - 5° C.

The supernatant was decanted and the precipitate resuspended in 3 ml of buffer and centrifuged again. The two supernatants were combined then made 10% saturated with ammonium sulfate pH 7.0. A volume of ice cold acetone equal to that of the ammonium sulfate solution was added and the resulting mixture allowed to stand ten minutes at room temperature and then centrifuged at 600 rpm for ten minutes at 4 - 5° C. The supernatant was decanted and the precipitate washed with cold acetone then recentrifuged. The granular portion of the cells was washed two times by resuspending in cold 0.1 M phosphate buffer pH 6.8 with the aid of a Potter-Elvehjem homogenizer and then centrifuged as before. The precipitate was finally resuspended in 10 ml of cold buffer. It could then be stored several days in the refrigerator without loss of activity.

8. Measurement of tyrosinase inhibitory activity in skin.

Scrapings from pigmented and nonpigmented regions of hooded rat skin were homogenized in 5 ml of 0.1 M phosphate buffer pH 6.8 and allowed to stand overnight at 5° C, then centrifuged at 25,000 xg for two hours at 4 - 5° C. The supernatant was decanted and used as a source of the inhibitor.

Trichloracetic acid extracts were also used as a source of the inhibitor immediately after preparation. Scrapings from pigmented regions of hooded rat skin, obtained in the usual manner were homogenized in 5 ml of 10% TCA in the cold, then centrifuged at 3,000 rpm for ten minutes at 4 - 5°C. TCA was extracted four times with volumes of ether approximately equal to that of the TCA extract. Dissolved ether was then removed by bubbling nitrogen through the

solutions for about two minutes. The pH was then readjusted to 6.8. 1 ml aliquots of the protein free filtrates were then tested for their ability to inhibit tyrosinase activity of the granule suspensions by incubating them in reaction mixtures containing the granule suspensions from hooded rat skin, 3 μc of L-tyrosine-3,5- H^3 having a specific activity of 330 μc/mg, and 3.4×10^{-4} M DOPA in a total volume of three ml of 0.1 M phosphate buffer pH 6.8. Aliquots of the granule suspensions were incubated both with and without the added inhibitor. A separate heated control was prepared for each sample. After a two hour incubation at 37° C on a Dubnoff shaker, the reaction mixtures were analyzed for tritiated water in a manner to be described.

Attempts were made to identify the inhibitor by means of paper chromatography. Scrapings from pigmented regions of hooded rat skin were homogenized in 5 ml of distilled water. 5 ml of 20% TCA were add, the sample mixed, and then allowed to stand ten minutes at room temperature. The mixture was centrifuged at 3,000 rpm for ten minutes at 4 - 5° C and the supernatant decanted. Trichloroacetic acid was extracted four times with ether. Dissolved ether was removed by bubbling nitrogen through the solution for two minutes. The protein free filtrates were frozen in the form of a conical shell inside a test tube and then lyophilized while still in the tube. The residue remaining after lyophilization was dissolved in 0.1 ml of distilled water and spotted on Whatman #1 paper for chromatography. Butanol : acetic acid: water :: 4:1:5 was used as the solvent.

The solution for developing the sulfhydryl spots (241) was prepared by dissolving 1.5 gm of sodium nitroferricyanide in 5 ml

of 2 N sulfuric acid and diluting to 100 ml with methanol, then making the solution alkaline by the addition of 10 ml of concentrated ammonium hydroxide. After filtering to remove insoluble salts, the solution was ready for use. When paper chromatograms were dipped in the developing solution, sulfhydryl compounds appeared as red spots on a colorless background. Disulfide spots were made visible by the addition of 2 gm of sodium cyanide to the above solution.

9. Electrophoresis

It was necessary to determine whether the radioactivity measured in the eluates from the alumina was due to C^{14}-DOPA alone. Since the incubation mixtures contained a large amount of C^{14}-tyrosine of high activity and only a small amount of labeled DOPA, a very efficient separation of the two is required. Sufficient eluates were combined to result in a pooled sample containing several thousand counts per minute. The pooled sample was lyophilized in a test tube and the resulting residue redissolved in 0.1 ml of 4% formic acid. 0.05 ml of the formic acid solution of C^{14}-DOPA were electrophoresed for two hours in a Spinco Electrophoresis Cell at 500 V using 4% formic acid as the medium according to the procedure of Nagatsu (185). The paper strips were then air dried and taped end to end. A marker of C^{14}-tyrosine was added near the end of one of the strips. The strips were then scanned for radioactivity in a Packard radiochromatogram scanner model 7200. After being scanned, the strips were sprayed with diazotised sulfanilic acid to locate the position of the DOPA spots.

10. Determination of radioactivity in DOPA and catecholamines.

When the radioactivity was to be determined, the samples were thawed and 5 ml aliquots of each added to 50 ml screw capped test tubes. The aliquots were diluted to 10 ml with distilled water. Two drops of phenolphthalein, 2.5 ml of 10% EDTA, and one gram of chromatographic grade alumina were added to each tube. The resulting mixture was neutralized to the faint pink of phenolphthalein with 5 N NaOH while continuously shaking the tubes. The test tubes were then capped and placed on a mechanical shaker for ten minutes. After the first five minutes, the pH of the tubes was adjusted if necessary and the shaking continued for an additional five minutes. The tubes were then removed from the shaker and allowed to stand until all the alumina had settled. Supernatant was removed by aspiration and discarded. The alumina was washed by shaking with approximately 15 ml of distilled water. The washing was repeated four times. The alumina was allowed to settle after each washing, and the wash water removed by aspiration and discarded. After the final washing, DOPA and the catecholamines were eluted by shaking the alumina with 3.5 ml of 0.5 N acetic acid for fifteen minutes. The acid solution containing C^{14}-DOPA and catecholamines was separated by decantation, and a 1 ml aliquot assayed for C^{14}-DOPA using the scintillation solution described below.

11. Determination of radioactivity in tritiated water.

At the end of the two hour incubation period, 1 ml aliquots were removed from each of the reaction vessels and placed directly

on a column 1 cm in diameter containing 2 gm of the hydrogen form of Dowex 50. Samples were drawn slowly through the column at the rate of one drop every three to five seconds using suction from an aspirator. A 5 ml volumetric flask inside the suction flask was used as the receiving vessel. When the meniscus just disappeared into the surface of the resin, the lower walls of the column were washed down with about 1 ml of distilled water and the slow suction continued. When the second meniscus had disappeared, one more milliliter of water was added and the suction continued at the slow rate until the water was drawn into the column. When the meniscus disappeared again, the suction was increased to its maximum and the column sucked dry. The 5 ml volumetric was then removed from the suction flask, filled to the mark with distilled water, and thoroughly mixed. One ml aliquots of the eluate from the Dowex columns were added to scintillation vials containing 19 ml of scintillator solution, thoroughly mixed, and then counted. The scintillator solution (138) contained 100 gm of naphthalene, 7 gm of 2,5-diphenyloxazole, and 50 gm of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of 1,2-dimethoxyethane.

12. Determination of noncollagenous protein.

This protein determination is based on the method of Weichselbaum (245) and Lilienthal (152). Protein from the tissue homogenates was precipitated by adding an equal volume of 20% TCA. The mixture was then centrifuged at 3,000 rpm for ten minutes at 4 - 5° C and the supernatant decanted and discarded. The pellet of protein was washed by squirting water from a wash bottle into the test tube.

After recentrifuging, the wash water was removed by aspirating with a cotton tipped pipette. The cotton with some adhering protein was added to the precipitate. Aproximately 1 ml of 0.05 N sodium hydroxide was added for every 10 mg of protein. The test tubes were corked and shaken to disperse the tissue into the solution. Extraction of the noncollagenous protein was allowed to continue for eighteen to twenty-four hours with the tubes in a horizontal position, and then filtered using Whatman #2 filter paper.

Since the samples are turbid, a separate turbidity control must be set up for each sample to be analyzed. For each determination the following tubes are prepared:

a. 2 ml of alkali extract from the unknown sample, 3 ml of 0.05 N sodium hydroxide, and 5 ml of the biuret copper reagent.

b. A reagent blank with 5 ml of the biuret copper reagent, and 5 ml of 0.05 N sodium hydroxide.

c. A turbidity control containing 3 ml of 0.05 N sodium hydroxide, 2 ml of the alkali extract, and 5 ml of the biuret copper free blank reagent.

d. A turbidity blank with 5 ml of 0.05 N sodium hydroxide, and 5 ml of the biuret copper free blank reagent.

An Aloe Lyophilized Protein standard was used to establish the standard curve. After mixing the samples thoroughly, all tubes were incubated for thirty minutes at 37° C in a water bath. The optical density of the solutions was read on a Beckman Model B at 555 mu.

13. Preparation of Special Reagents.

A. Purification of labeled tyrosine.

1. Commercially obtained samples of L-tyrosine-U-C^{14} (New England Nuclear) contained appreciable amounts of a radioactive material which behaved like DOPA on adsorption to alumina and on paper electrophoresis. Since C^{14}-DOPA is the product measured in the assay for tyrosinase activity, it must be excluded rigorously from the starting material. The pH of the labeled tyrosine solutions was adjusted to pH 8.3 - 8.5 after the addition of approximately 1 gm of chromatographic grade alumina (Woelm). The mixture was shaken for ten minutes on a mechanical shaker, and then centrifuged at 2,000 rpm for ten minutes at 4 - 5° C. The supernatant was decanted and the procedure repeated. The tyrosine solutions were then adjusted to pH 6.8 with concentrated hydrochloric acid and stored frozen in the refrigerator.

2. L-tyrosine-3,5-H^3 (New England Nuclear) solutions contained a volatile radioactive material which was probably tritiated water. Since tritiated water is the product to be determined in reaction mixtures containing L-tyrosine-3,5-H^3, it was also necessary to exclude it rigorously from the starting material. Tritiated water in solutions of L-tyrosine-3,5-H^3 was removed by lyophilizing the material in a test tube. The residue after lyophilization was dissolved in 2 ml of distilled water and acidified with one drop of concentrated hydrochloric acid. Before use, appropriate dilutions were made with 0.1 M phosphate buffer pH 6.8 to give a solution containing 10 μc per ml. The amount of

dilution required was first determined by assaying an aliquot of the acidified solution. The acidified solution was found to be stable when stored frozen in the refrigerator.

B. L-dihydroxyphenylalanine (Nutritional Biochemical Corp.).

10 mg of L-DOPA were dissolved in 10 ml of 0.1 M phosphate buffer pH 6.8. A fresh solution was prepared each week.

C. Iodoacetic acid (Matheson Coleman and Bell).

100 mg of iodoacetic acid were dissolved in 10 ml of 0.1 M phosphate buffer pH 6.8.

D. α,α'-dipyridyl (Matheson Coleman and Bell).

9.4 mg of dipyridyl were dissolved in 10 ml of 0.1 M phosphate buffer 6.8 and stored frozen in the refrigerator.

E. Diethyldithiocarbamate.

10.2 mg of diethyldithiocarbamate were dissolved in 10 ml of 0.1 M phosphate buffer pH 6.8. A fresh solution was prepared just before each experiment.

F. Biuret Reagent.

The biuret copper reagent was prepared by dissolving 18 gm of sodium potassium tartrate, 6 gm of cupric sulfate, and 10 gm of potassium iodide in 2 L of 0.2 N sodium hydroxide. A copper free reagent was prepared in the same way, except that the cupric sulfate was omitted.

G. EDTA (Eastman Kodak).

10 gm of ethylenediaminetetraacetic acid and one drop of 1% phenolphthalein in ethanol were added to approximately 25 - 30 ml

of distilled water in a glass stoppered 100 ml graduated cylinder. The EDTA was dissolved by the progressive addition of 5 N sodium hydroxide until all the EDTA had dissolved. The pH of the solution was then adjusted to the faint pink of phenolphthalein and the solution diluted to 100 ml with distilled water.

IV. Results

Once having decided to undertake a study of tyrosinase in normal skin, the first and most immediate problem became one of chosing suitable laboratory animals and experimental techniques. It soon became obvious that most laboratory animals are unsuited for this type of experiment. In the literature (22), it is reported that the skin of black and white spotted guinea pigs contains an appreciable amount of pigment, implying the existence in this tissue of an active and intrinsically maintained melanogenic activity. However, of the animal skins examined, which includes those of mice, rats, and guinea pigs, only the skins of hooded or piebald rats contained an appreciable amounts of pigment. Skin from the other animals proved to be virtually lack in pigment even though the hair covering that skin was intensely pigmented.

Initially it was not certain that there would be a detectable tyrosinase activity in normal rat skin. Therefore, the first tests of the method were carried out using the rat adrenal, a tissue known to posses tyrosine hydroxylating activity (184, 185, 186). After having seen that such activity could be detected in the adrenal (Table 2), the method was applied to scrapings from the pigmented regions of hooded rat skin. In the first experiments attempted, 1.0×10^{-3} M tyrosine was included in the reaction mixtures in addition to the substances listed in the section on methods. Under these conditions, the experimental samples did not have a greater ability to hydroxylate tyrosine than did their corresponding heated controls (Table 3).

Table 2.

Tyrosine Hydroxylating Activity

of Adrenal Homogenates

Exp.[1]	Production of catechols mμmoles/gm/hr
1.	4.08
2.	1.22
3.	.58
4.	.86
5.	.08
6.	4.00
7.	.70
8.	.56
9.	.32
10.	1.02
11.	.16

1. Incubation mixtures contained the adrenal homogenate, 1×10^{-3} M Marsilid (isopropylisonicotinylhydrazine), and 0.2 μc of L-tyrosine-U-C^{14} having a specific activity of 35 μc/mg in a total volume of 3 ml of 0.1 M phosphate buffer pH 6.4. Results are expressed as the difference between an experimental sample and its corresponding heated blank.

Table 3.

Effect of Added Unlabeled L-Tyrosine (1×10^{-3} M) on Tyrosinase Activity of Granule Suspensions Prepared from Pigmented Regions of Hooded Rat Skin

Exp.[1]	DOPA production[2] mμmoles/gm/hr
1.	zero[3]
2.	.003
3.	.004
4.	.007
5.	zero

1. Incubation mixtures contained the granule suspensions, 0.2 μc of L-tyrosine-U-C^{14} having a specific activity of 35 μc/mg, 1×10^{-3} M L-tyrosine, and 3.4×10^{-4} M L-DOPA in a total volume of 3 ml of 0.1 M phosphate buffer pH 6.8

2. Results are expressed as the difference between an experimental sample and its corresponding heated blank.

3. The heated blanks of experiments #1 and #5 produced more DOPA than did the experimental samples.

Frequently the heated controls were able to hydroxylate tyrosine to a greater extent than did the experimental samples. Only when this unlabeled tyrosine was omitted from the reaction mixtures was it possible to detect such a difference (Table 4). When the samples were run using only the labeled tyrosine, The unheated samples were consistently observed to form more DOPA than the heated controls (Table 4). In other words, the heated preparations lost their activity. This is at least indirect evidence of enzymic activity. All measurements of tyrosinase activity are reported in terms of the difference in the production of either C^{14}-DOPA or tritiated water between a sample and its heated blank.

The observations reported here differ from those of Kim (132) who was unable to detect the formation of C^{14}-DOPA after incubating C^{14}-tyrosine in the presence of goldfish tyrosinase. Once having observed tyrosinase activity in scrapings from hooded rat skin, it became desirable to obtain a soluble preparation of the enzyme for further studies. The only suitable method known for solubilizing this enzyme from mammalian sources is the combined ammonium sulfate-acetone precipitation procedure of Brown and Ward (45, 46). When solubilization was attempted by this method, over 90% of the total activity remained in the granular precipitate (Table 5). Less than 10% of the activity was brought into solution. These results are very similar to those of Brown and Ward (45) and of Pomerantz (205). The melanoma is a source of relatively large amounts of tyrosinase. Since the enzyme is available in much greater quantities, it is possible to work with preparative

Table 4.

Tyrosine Hydroxylating Activity of Various Rat Skin Preparations[1]

Preparation	N[2]	DOPA production mμmoles/gm/hr
Whole scrapings from hooded rat skin	4	0.35[3] (0.13 - 0.66)[4]
Homogenates of scrapings from hooded rat skin	4	0.56 (0.42 - 0.73)
Homogenates of scrapings from albino rat skin	8	0.04 (0.004 - 0.16)
		H^3HO production mμmoles/gm/hr
Homogenates of scrapings from hooded rat skin	5	0.57 (0.55 - 0.64)
Granule suspensions prepared from homogenates of scrapings from hooded rat skin	8	28.0 (11.7 - 59.2)
Homogenates of scrapings from albino rat skin	3	0.44 (0.24 - 0.62)

1. Incubation mixtures contained the indicated tissue preparation, 0.2 μc L-tyrosine-U-C^{14} having a specific activity of 35 μc/mg or 3.0 μc of L-tyrosine-3,5-H^3 having a specific activity of 330 μc/mg and 3.4×10^{-4} M L-DOPA in a total volume of 3 ml of 0.1 M phosphate buffer pH 6.8.

2. number of animals

3. Results are expressed in terms of the average difference between the experimental samples and their corresponding heated blanks.

4. range

Table 5.

Attempted Solubilization of Tyrosinase from Normal Skin

According to the Procedure of Brown and Ward (45)

	cpm minus bkgrd.	cpm/gm/hr	difference cpm/gm/hr	DOPA production mμmoles/gm/hr
A. precipitate from[1] $(NH_4)_2SO_4$-acetone	375	3,472	2,944	0.68
heated blank	57	528		
B. soluble supernatant	73	676	222	0.05
heated blank	49	454		

1. Incubation mixtures contained 1 ml of the tissue preparation, 0.2 μc of L-tyrosine-U-C^{14} having a specific activity of 35 μc/mg, and 1.7×10^{-4} M L-DOPA in a total volume of 3 ml of 0.1 M phosphate buffer pH 6.8.

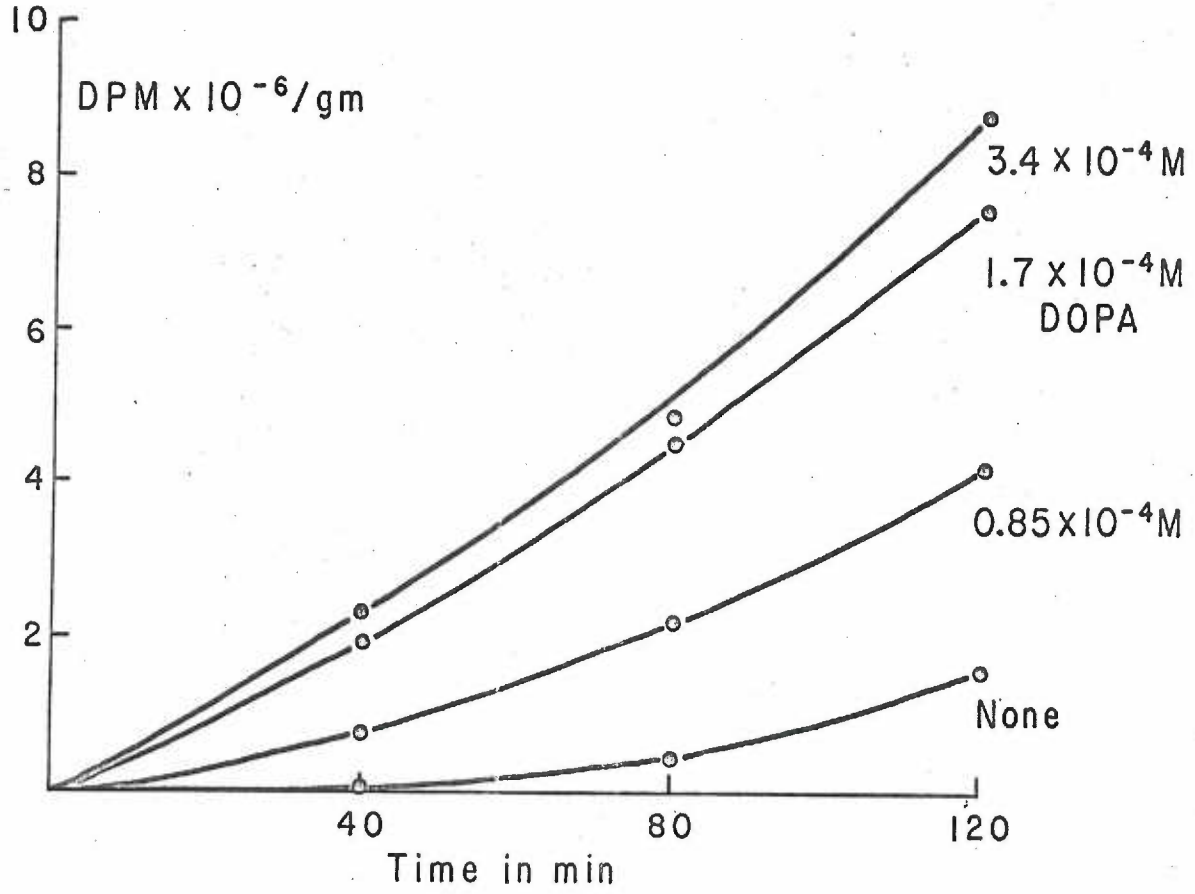
methods which make available only about 10% of the total activity. When rat skin is used as a source of the enzyme, one works nearer the limits of detectability. Therefore, a process which decreases the available enzyme to less than 10% of its initial activity is not suitable for preparing tyrosinase from normal skin. However, the granular precipitate retained most of the enzyme in a much higher specific activity than in the whole homogenates (Table 4). Therefore, this granular portion of the homogenate proved to be a satisfactory preparation for the studies reported here, even though it constitutes only a crude fraction of the cells.

Although DOPA formation can be detected by incubating C^{14}-tyrosine in the presence of skin preparations, its accumulation is not necessarily a measure of the overall process. DOPA is an intermediate in the reaction sequence catalyzed by tyrosinase. In order to measure the total tyrosine hydroxylation, L-tyrosine-3,5-H^3 was used. Unlike DOPA, the tritiated water is not acted upon further by the enzyme nor is it converted to other products by nonenzymic processes. Thus, the use of tritiated tyrosine is suitable for measuring the time course of the reaction.

One of the properties of the enzyme tyrosinase is its characteristic dependence upon a reducing agent such as DOPA. Therefore, the effect of varying the DOPA concentration was determined (Figure 1). Measurement of the time course of the reaction using tyrosinase from the granular preparation with L-tyrosine-3,5-H^3 as the substrate in the absence of DOPA, showed that very little tyrosine hydroxylation occurred even after two

figure 1

Effect of varying DOPA concentration on tyrosinase activity of granule suspension



Incubation mixtures contained the granular suspensions prepared from pigmented regions of hooded rat skin, 3 uc L-tyrosine-3,5-H^3, and the indicated amount of L-DOPA in a total volume of 3 ml of 0.1 M phosphate buffer pH 6.8. The incubation was carried out at 37° C in a Dubnoff type shaker. 0.75 ml aliquotes were removed from the reaction mixture at the stated intervals and analyzed for tritiated water in the usual manner.

hours of incubation. This was preceded by a lag period of at least 40 minutes during which there was virtually no DOPA production. When small amounts of DOPA were added to the reaction mixtures, an increased rate of tyrosine hydroxylation occurred. This increased rate was accompanied by a decreased lag period. A near maximal effect was noted at a DOPA concentration of 1.7×10^{-4} M. Doubling this concentration resulted in little further increase in tyrosine hydroxylation.

Commercially obtained samples of C^{14}-labeled tyrosine contained radioactive contaminants which behaved like catechols in being adsorbed on alumina at a pH of 8.3 - 8.5 and eluted from the alumina by 0.5 N acetic acid. Addition of carrier DOPA to dilutions of the commercially obtained C^{14}-tyrosine, followed by electrophoresis indicated that most of the radioactive contamination present was due to DOPA. Since C^{14}-DOPA is one of the products measured in the determination of tyrosine hydroxylating activity, it must be excluded rigorously from the starting material. This was accomplished by treating the solutions with alumina before using them in the experiments. When the solutions were electrophoresed after this treatment, only one radioactive peak was observed. Its position on the paper electrophoresis strips coincided with that of added carrier tyrosine. In addition, tritium labeled tyrosine contained a volatile radioactive contaminant, probably water. The radioactive contaminant was satisfactorily removed by lyophilization before use. These observations are in agreement with those of Pomerantz (205). Electrophoresis followed by scanning of the paper

strips was chosen to check the purity of the labeled substrate because the procedure can be performed quickly, easily, and provides a good separation of tyrosine from DOPA and DOPA-amine.

Measurement of the radioactive products of the reaction, C^{14}-DOPA and tritiated water, involves the separation of small amounts of product from much larger amounts of labeled substrate of high specific activity. Analysis of C^{14}-DOPA makes use of the property of catechols of being strongly adsorbed to alumina at alkaline pH's (232). Multiple washings remove labeled tyrosine and permit the elution and assay of C^{14}-DOPA. An important requirement of the assay procedure is that tyrosine be excluded rigorously from the eluate which is finally counted. Even a small amount of labeled tyrosine accompanying the C^{14}-DOPA would invalidate the results. Since any single eluate contained only a small amount of activity due to C^{14}-DOPA, enough samples were combined to result in a pooled sample containing several thousand counts per minute. This would markedly increase the chances of detecting any contamination of the samples by C^{14}-tyrosine. The pooled sample was then reduced in volume by lyophilization. The residue was dissolved in 4% formic acid and electrophoresed. When the paper electrophoresis strips were scanned for radioactivity, only one radioactive peak was found. In every case, the radioactive peak coincided with the DOPA spot. No detectable activity was ever found to coincide with added carrier tyrosine. Separation of the tyrosine and DOPA spots was sufficiently great to make certain that small amounts of tyrosine activity would not be obscured by or appear as shoulders on the DOPA peak. These

experiments serve two purposes. First, the product measured is identified as a catechol on the basis of its adsorption on alumina and later elution with dilute acetic acid. A more specific identification of the radioactive material is obtained by noting that it migrates identically with added carrier DOPA. Secondly, one is assured that the radioactivity measured is due to DOPA alone and not to contamination by tyrosine.

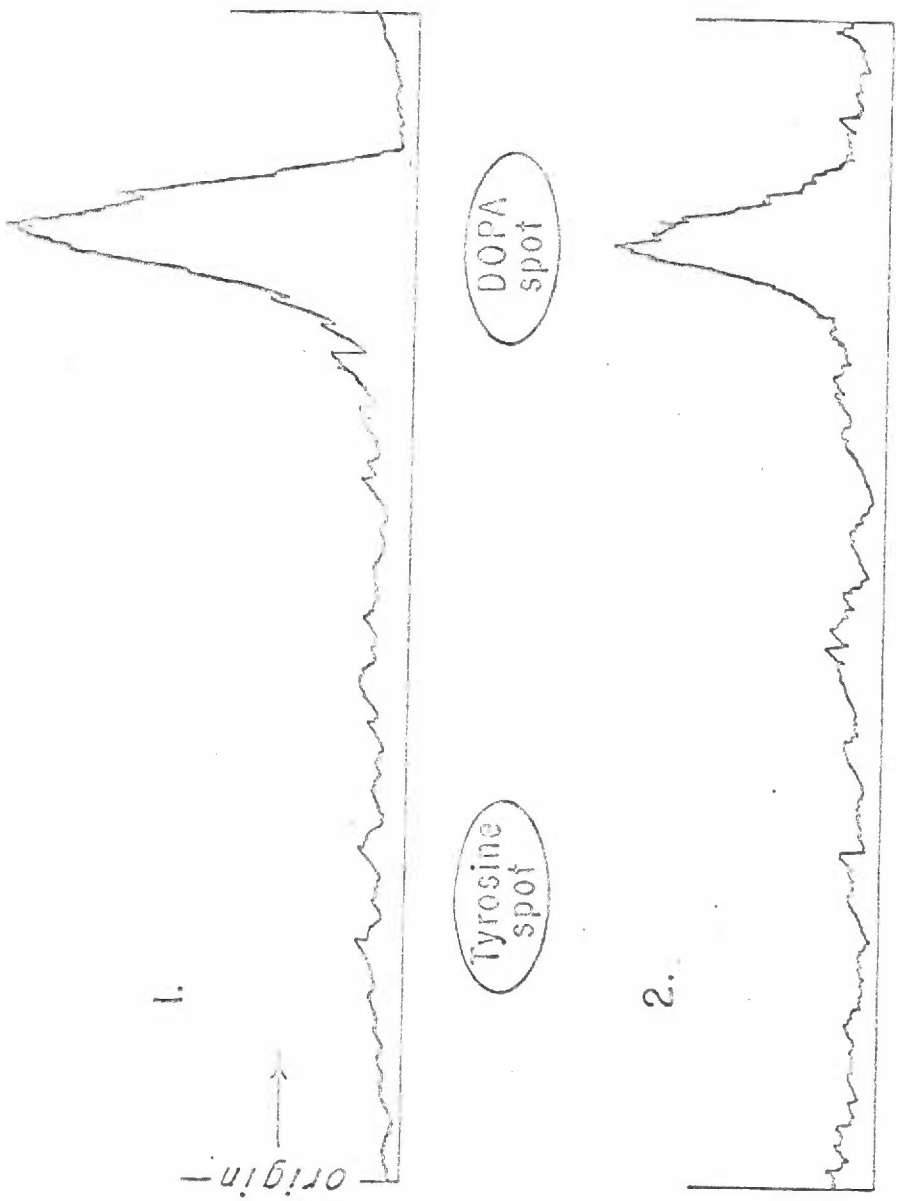
The method of Pomerantz (205) using tyrosine-3,5-H^3 was not suitable for the study of skin tyrosinase without modification. However, the fundamental principle remains the same. During the hydroxylation, one of the tritium atoms on the tyrosine is displaced and appears in the form of tritiated water. The use of charcoal-celite columns as an adsorbent, while useful for the more highly purified tyrosinase preparations seemed a poor choice for the present work because of the very slow flow of fluid through the columns. Also, the high enzyme activities found in melanoma permitted the use of large quantities of wash water. Similar amounts of wash water diluted the smaller amounts of activity obtained during studies with rat skin too much to permit its accurate measurement. Therefore, the hydrogen form of Dowex 50 was substituted. Tyrosine and DOPA are adsorbed to the resin permitting the tritiated water to be washed on through the column and collected. Both tyrosine and DOPA are adsorbed very strongly by this resin. This permits the use of a small quantity of resin and much less wash water than is needed with charcoal columns. Sucking the resin dry and catching the eluate directly in a small

1. C^{14}-DOPA produced during the incubation of C^{14}-tyrosine with homogenates of scrapings from hooded rat skin.
2. C^{14}-DOPA produced during the incubation of homogenates of scrapings from albino rat skin with C^{14}-tyrosine.

Several eluates from alumina were pooled to increase the possibility of detecting any C^{14}-tyrosine which might be present as a contaminant. After reducing the volume of the pooled sample by lyophilization, the residue was dissolved in 0.1 ml of 4% formic acid, spotted on paper in a Spinco Electrophoresis Cell, and electrophoresed at 500 V for two hours using 4% formic acid as the medium. Spots of carrier tyrosine and DOPA were made visible by spraying with diazotized sulfanilic acid. Radioactivity was never found to coincide with the tyrosine spot. The scanning was performed on a Packard Radiochromatogram Scanner at 1 inch per minute with a ten second time constant and a full scale deflection equal to 1,000 cpm.

Figure 2.

Identification of C^{14}-DOPA by Electrophoresis



volumetric flask were further steps required to hold dilution of the sample to a minimum. Catching the eluate directly in a small volumetric flask eliminates the need for making quantitative transfers of the eluates at a later time. Suction is also required for another reason. Aliquots from the reaction vessels are placed directly on the columns without first being deproteinized. Protein precipitates at the surface of the column, forming a cake which prevents further flow of fluid if gravity alone is depended upon to provide the driving force. The resulting eluate is protein free. No precipitate formed upon heating the eluate to 100° C, making it 10% saturated with trichloroacetic acid, or adding a few drops of 20% sulfosalicylic acid.

That the procedure is adequate for separating tritiated water from tritiated tyrosine and DOPA was determined by the same process of lyophilization and electrophoresis used to check the purity of the labeled tyrosine and identify the C^{14}-DOPA. Pooled eluates from the Dowex 50, H^+ columns did not contain any detectable radioactivity coinciding with carrier tyrosine and DOPA added to the eluate. In fact, no peaks above background were found following this procedure even though the starting pooled sample contained several thousand counts per minute. These results indicate that the procedure is effective in separating the tritiated water from labeled tyrosine and DOPA.

Since early work had shown that extracts of human and animal skins contain a sulfhydryl inhibitor which is capable of inhibiting both tyrosinase from melanoma and the cupric ion oxidation of

DOPA (80, 82, 219, 220, 221), it was very interesting to try and determine whether or not tyrosinase from normal skin is similarly inhibited. Two types of experiments were designed to test for this possibility. First, homogenates of hooded rat skin were incubated both in the presence and absence of iodoacetate, an alkylating agent which reacts with sulfhydryl groups. The second type of experiment involved testing extracts of rat skin for inhibitory activity toward the tyrosinase of the granular preparations. Granule preparations from pigmented regions of hooded rat skin were incubated both with and without skin extracts prepared by one of two methods. The simplest method for the preparation of a skin extract was patterned after that of Flesch, who simply allowed split skin preparations to stand for a period in distilled water. Similarly, homogenates of scrapings from pigmented regions of hooded rat skin were allowed to stand overnight in the refrigerator. The next day they were subjected to high speed centrifugation and the supernatant used as a source of the inhibitor. In two such experiments, the tyrosinase activity was inhibited to the extent of only 2.3 and 5.4% (Table 6). When viewed in light of normal experimental variation, this can not be considered evidence for the presence of an inhibitor. Since it was felt that a sulfhydryl compound might have been oxidized on standing overnight, experiments were devised to test the inhibitory activity of protein free filtrates from pigmented regions of hooded rat skin immediately after their preparation. These preparations proved to be inhibitory (Table 7), suggesting that the inhibitor had

Table 6.

Inhibitory Activity of Aqueous Extract of Scrapings from Hooded Rat Skin[1]

cpm as H^3HO

	counts 30 min.	cpm	cpm - bkgrd	difference	% of control
control[2]	14,365	479	451	392	---
heated blank	2,624	87	59		
+ extract from white skin	13,498	449	421	383	97.7
heated blank	1,978	66	38		
+ extract from black skin	14,516	484	456	371	94.6
heated blank	3,380	113	85		
background	844	28			

1. Scrapings from both white and pigmented skin were suspended overnight in distilled water at 5° C then centrifuged two hours at 25,000 xg at 4 - 5° C. The supernatant was used as a source of the inhibitor.

2. All flasks contained a 1 ml aliquote of a granular suspension, 3 µc of L-tyrosine-3,5-H^3 having a specific activity of 330 µc/mg and 3.4×10^{-4} M L-DOPA in a total volume of 3 ml of 0.1 M phosphate buffer pH 6.8.

Table 7.

Inhibitory Activity of Protein Free Filtrates[1]

cpm as H^3HO

	Exp.[2]	counts / 100 min. heated	Exp.	heated	difference	% inhibition
control	50,128	7,301	501	73	428	
+ inhibitor	33,841	8,901	338	89	249	41.8
control	47,029	6,542	470	65	405	
+ inhibitor	40,677	6,918	407	69	338	16.5
control	58,712	5,789	587	58	529	
+ inhibitor	37,427	6,692	374	67	307	51.9
control	62,473	5,429	624	54	570	
+ inhibitor	24,712	6,619	247	66	181	68.3
control	37,094	9,657	371	96	275	
+ inhibitor	35,748	10,006	357	100	257	6.6

1. Scrapings from the pigmented regions of hooded rat skin were obtained and homogenized in the usual manner. Protein was precipitated by the addition of an equal volume of 20% TCA, the TCA extracted four times with ether, the ether removed by bubbling nitrogen through the solution, and the pH adjusted to 6.8. This solution was used immediately as a source of the inhibitor.

2. All flasks contained 1 ml of a granule suspension, 3 µc L-tyrosine-3,5-H^3 having a specific activity of 330 µc/mg, and 3.4×10^{-4} M L-DOPA in a total volume of 3 ml of 0.1 M phosphate buffer pH 6.8.

indeed been oxidized during the prolonged standing. A further suggestion that skin tyrosinase is normally in a partially inhibited state is indicated by the fact that the enzyme activity is stimulated by the addition of iodoacetate to the reaction mixtures (Table 8).

The results obtained upon adding iodoacetate to granule suspensions provide a very interesting contrast to those obtained with the homogenates. Here, it can be seen that the iodoacetate has no stimulatory effect (Table 9).

Attempts to isolate and identify the sufhydryl inhibitor from pigmented skin by means of paper chromatography were unsuccessful.

When homogenates of scrapings from albino rat skin were incubated with L-tyrosine-U-C^{14} the unheated homogenates of those scrapings catalyzed the formation of C^{14}-DOPA at a greater rate than did the heated controls (Table 4). DOPA from several such experiments was identified by the process of lyophilization and electrophoresis previously described. At no time were the counted samples found to be contaminated by C^{14}-tyrosine. Two experiments were also carried out using white skins from hooded rats. In both cases, the heated controls produced more C^{14}-DOPA than did the unheated samples. In contrast to the white skin of hooded rats, experimental samples of albino rat skin consistently produced more DOPA than the corresponding heated controls. Later experiments using L-tyrosine-3,5-H^3 gave the same results as those employing L-tyrosine-U-C^{14} (Table 4). Therefore, an attempt was made to determine the nature of the agent catalyzing the formation of DOPA from tyrosine in the albino skin. The first observation, showing the catalyst to be heat inactivated,

Table 8.

Effect of Added Iodoacetate on Tyrosine Hydroxylating Activity of Homogenates Of Scrapings from Pigmented Regions of Hooded Rat Skin

	Tyrosine hydroxylation[1] mμmoles/gm/hr				
Flask	A	B	C	D	E
control[2]	0.51	0.59	0.38	0.34	0.46
+ iodoacetate (3.5×10^{-3} M)	0.93	0.84	0.62	0.60	0.65
total increase	0.42	0.25	0.24	0.26	0.18
% increase	83	41	64	77	39

1. Tyrosine hydroxylation was determined by measuring the accumulation of H^3HO in the reaction mixtures.

2. All incubation mixtures contained 1 ml of a homogenate of scrapings from pigmented regions of hooded rat skin, 3 μc of L-tyrosine-3,5-H^3, and 3.4×10^{-4} M L-DOPA. The labeled tyrosine had a specific activity of 330 μc/mg.

Table 9.

Effect of Iodoacetate on Tyrosinase Activity of Granule Suspensions

cpm as H^3HO

	counts / 30 min. exp.	heated	difference	activities in flasks with iodoacetate as % of control
control[1]	5,426	1,721	3,705	
+ iodoacetate	5,834	1,688	4,146	112
control	2,648	1,562	1,086	
+ iodoacetate	2,389	1,475	914	84
control	3,365	1,146	2,219	
+ iodoacetate	3,354	1,217	2,137	96
control	3,559	2,617	942	
+ iodoacetate	3,652	2,594	968	103
			average	99

1. All incubation mixtures contained a 1 ml aliquote of a granule suspension, 3 µc of L-tyrosine-3,5-H^3, having a specific activity of 330 µc/mg, and 3.4×10^{-4} M L-DOPA. The concentration of iodoacetate was 3.5×10^{-3} M.

indicated that it was enzymic. There are at least two mammalian enzymes capable of hydroxylating tyrosine, tyrosinase and tyrosine hydroxylase. These enzymes have quite different properties. Therefore, it should be possible to distinguish between them. The most marked and easily demonstrated difference lies in their metal cofactor requirements. The activity measured in albino rat skin is inhibited by diethyldithiocarbamate but not by α,α'-dipyridyl (Table 10). This indicates that the enzyme under study is a copper enzyme, eliminating the possibility that it might be a tyrosine hydroxylase of the type found in the adrenal medulla. Also, when the reducing agent DOPA was replaced by 6,7-dimethyl-5,6,7,8-tetrahydropteridine, the results became highly variable with heated controls often being higher than the unheated samples. The only constant feature observed under these conditions was an increased hydroxylation rate of about 50% in the heated controls. While the highly variable results have no meaning of themselves, the fact that stimulation of the tyrosine hydroxylating activity in albino skin was not observed on addition of a tetrahydropteridine plus the observation that this activity is inhibited by diethyldithiocarbamate provides a strong argument that the enzyme activity measured is not due to a tyrosine hydroxylase, but rather is a copper containing enzyme.

Because the albino skin was found to be capable of catalyzing the formation of DOPA from tyrosine, attempts were made to determine whether or not DOPA was initially present in the skin scrapings. Protein free filtrates, freed of TCA by extraction with ether, were

Table 10.

Effect of Metal Chelating Agents on Tyrosine Hydroxylating Activity of Homogenates of Scrapings from Albino Rat Skin

cpm as H^3HO

	counts / 30 min. exp	heated	difference	% of control
control[1]	13,950	2,431	11,519	---
+ 2 x 10^{-4} M DDC[2]	7,698	2,249	5,449	47.3
+ 2 x 10^{-4} M a-dip.[3]	12,970	1,580	11,390	98.9
control	14,400	1,972	12,428	---
+ 1 x 10^{-3} M DDC	2,923	1,755	1,168	9.4
+ 1 x 10^{-3} M a-dip.	12,017	1,528	10,489	84.4

cpm as C^{14}-DOPA

control	8,942	2,297	6,645	---
+ 2 x 10^{-4} M DDC	5,683	2,265	3,418	51.4
+ 2 x 10^{-4} M a-dip.	8,529	2,006	6,523	98.2
+ 1 x 10^{-3} M DDC	2,763	2,105	658	9.9
+ 1 x 10^{-3} M a-dip.	8,240	1,954	6,286	94.6

1. Scrapings from two albino rat skins were used in each experiment. In the experiments with tritiated tyrosine, combined scrapings weighing 1.610 and 1.842 gm were homogenized in separate 10 ml portions of 0.1 M phosphate buffer pH 6.8. The experiment with C^{14}-tyrosine had 2.104 gm of scrapings homogenized in 12 ml of buffer. In addition to the indicated amounts of metal chelating agents, the flasks also contained 3 µc of L-tyrosine-3,5-H^3 having a specific activity of 330 µc/mg or 0.2 µc of L-tyrosine-U-C^{14} having a specific activity of 35 µc/mg, and 3.4 x 10^{-4} M L-DOPA in a total volume of 3 ml.

2. DDC = diethyldithiocarbamate

3. a-dip. = a,a'-dipyridyl

lyophilized, and the residues redissolved in minimal volumes of water then chromatographed. No DOPA spots were found. Tyrosine spots were just barely visible. If DOPA were present at a concentration much lower than that of tyrosine, it could easily have gone undetected.

Further attempts at characterizing the tyrosine hydroxylating activity of albino rat skin by fractionation of the tissue indicated that most of the activity is located in the soluble supernatant after centrifugation at 25,000 xg for two hours (Table 11).

Table 11.

Fractionation of Scrapings from Albino Rat Skin[1]

fraction	total activity[2] cpm as H^3HO	cpm/gm of protein/hr	tyrosine hydroxylation mμmoles/gm/hr
whole homogenate[3]	580	2,569	0.48
nuclear fraction	252	1,875	0.35
mitochondrial fraction	---	----	----
microsomal fraction	125	2,800	0.52
supernatant	1,159	38,633	7.26

1. The method used was the $(NH_4)_2SO_4$ fractionation procedure of Hogeboom (112).

2. Activity as measured using a 1 ml aliquote of a fraction multiplied by the volume of that fraction.

3. Reaction mixtures contained a 1 ml aliquote of the indicated tissue preparation, 3 μc of L-tyrosine-3,5-H^3 having a specific activity of 330 μc/mg, and 3.4×10^{-4} M L-DOPA in a total volume of 3 ml of 0.1 M phosphate buffer pH 6.8.

V. Discussion

In the years since Bloch (28) demonstrated tyrosinase to be present in skin, no suitable methods have been devised for making quantitative studies of the enzyme from normal skin. Fitzpatrick's (75) fine work in partially characterizing tyrosinase of normal skin is one of the few successful attempts at studying this enzyme *in situ*. The availability of quantitative chemical techniques is one of the fundamental prerequisites in an attempt to study any enzyme. In the case of normal skin tyrosinase, such techniques have not been available, primarily because of the very low activity and the difficulty in obtaining suitable enzyme preparations from a tissue which is difficult to homogenize. Also, the enzyme is particle bound and in a partially inhibited state. By way of contrast, the very high enzyme activity in an easily obtained tissue has made the melanoma a very desirable source of mammalian tyrosinase.

The problem of measuring tyrosinase activity in skin is complicated by the fact that tyrosine is subject to nonenzymic hydroxylation in the presence of heated tissue extracts (43, 212, 243). This has been a source of difficulty to investigators attempting to study tyrosine hydroxylase. Nagatsu (185, 186) found that since tyrosine hydroxylase shows an absolute stereospecificity, the D-amino acid may be used as a control, eliminating the need for a heated blank. Unfortunately this is not possible in the case of tyrosinase since the enzyme shows appreciable activity toward D- as well as L-tyrosine (186). Nevertheless, it has been possible to show that preparations from pigmented regions of hooded rat skin

have a greater ability to hydroxylate tyrosine than do their corresponding heated controls (Table 4). This is of particular significance since, if anything, the heated control has a higher rate of nonenzymic hydroxylation than the unheated tissue. Earlier attempts to demonstrate tyrosinase activity in normal skin using quantitative chemical techniques have produced equivocal results due to the use of inadequate controls and high substrate concentrations (86, 189, 207).

The presence of labeled DOPA in the reaction mixtures indicates that free DOPA is in fact liberated from the protein before further conversion to DOPA-quinone. This finding differs from that of Kim (132) who did not find free C^{14}-DOPA to be liberated from the surface of goldfish tyrosinase during the conversion of C^{14}-tyrosine to melanin in the presence of carrier DOPA.

It should be noted that earlier attempts to demonstrate tyrosinase activity in normal skin without previous stimulation of enzyme activity with ultraviolet light were unsuccessful (74, 75, 78). These attempts were made using histochemical and manometric techniques which are much less sensitive than the experimental methods reported here. Also, it was not possible to demonstrate stimulation of tyrosinase activity by incubating various skin preparations in the presence of iodoacetate, sodium arsenite, or p-chloromercuribenzoate (86).

Numerous investigators have encountered difficulty because of the ability of heated tissues to catalyze the hydroxylation of aromatic substances (118, 214, 173, 185, 242). Udenfriend and

coworkers (243) have demonstrated that transition metal ions in the presence of a chelating agent such as versene and a reducing agent such as ascorbate are very active in catalyzing nonenzymic hydroxylations. The most active transition metal ions are those of iron and copper. In a living cell, transition metal ions are not in a free ionic state or randomly bound to protein (4, 182). Rather, they are selectively bound to specific sites on an enzyme or other protein moecule. The structural specificity shown by enzymes probably renders transition metal ions at the active centers of most such enzymes unavailable for reaction with tyrosine. The denaturation of proteins upom heating destroys this structural specificity, freeing the metal ion for reaction with tyrosine, and thereby increases the rate of nonenzymic hydroxylation. All of the substances necessary for nonenzymic hydroxylation are present in skin and other tissues, and are undoubtedly responsible for the formation of DOPA from tyrosine by heated tissues and tissue extracts.

The use of very low substrate concentrations on the order of 1×10^{-5} M has considerable precedent in the work of Nagatsu (185). In fact, such low substrate concentrations are required for the detection of enzymic hydroxylating activity, particularly in crude tissue preparations. Failure to detect tyrosine hydroxylating activity has been attributed to the use of substrate concentrations which were too high and to the lack of sufficiently purified substrates. The use of high substrate concentrations results in relatively large amounts of nonenzymic hydroxylation which obscures the enzymic processes. A dilution of the labeled substrate may also affect the ability to

detect enzymic hydroxylating activity when unlabeled tyrosine is added to the reaction mixtures. Similarly, in experiments with rat skin, no difference between experimental samples and their corresponding heated controls was noted when as little as 0.5 mg of unlabeled L-tyrosine was added to the reaction mixtures (Table 3).

The low concentration of substrate and reducing agent which gave optimal results in these experiments differ markedly from the high concentrations reported to give optimal performance in the model or nonenzymic hydroxylating system of Udenfriend (43, 243). The heat inactivation, characteristic lag period, dependence on DOPA, and the low substrate requirements all support the suggestion that the activities measured in normal rat skin are enzymic.

Although the techniques described can be used for making quantitative studies of tyrosinase activity in normal skin, it should be recognized that there are numerous factors which tend to increase the variability of the results. The first such factor lies in the anatomical distribution of the melanocytes. They are normally present as a single layer of cells at the epidermal dermal junction. Any scraping procedure, therefore, must necessarily include a relatively large and variable proportion of cells other then melanocytes. Also included in these scrapings are collagenous strands and lipid from the skin. Fat from subcutaneous depots probably does not contribute significantly to the nonmelanocyte material, since it can be removed easily from the inner surface of the skin. It can be seen that the melanocytes, which are the only source of tyrosinase, constitute only a small fraction of the

total cell population included in the scrapings. Therefore, any variation in the quantity of such extraneous material will affect the final outcome of the experiment. Also, once the inner surface of the skin has been cleaned free of fat and connective tissue, a variable amount of water can be lost by evaporation from the skin depending on the length of time required to obtain the scrapings.

The finding that tyrosinase from melanoma can be inhibited by a sulfhydryl compound extracted from skin is not of itself sufficient evidence that such a mechanism constitutes a naturally occurring regulatory mechanism controlling pigment formation in normal skin. However, at the time the earlier observations were made, there were no means available for making quantitative studies of tyrosinase from normal skin. Using tritium labeled tyrosine, it has been possible to show that tyrosinase from normal rat skin is in a partially inhibited state. Stimulation of tyrosinase activity by iodoacetate suggests that this inhibition is probably due to a sulfhydryl compound. Consistent increases in tyrosinase activity were found by incubating homogenates of scrapings from pigmented regions of hooded rat skin in the presence of iodoacetate. Previous investigators have been unable to demonstrate iodoacetate activation of tyrosinase when oxygen consumption was measured (74). Iodoacetate also inhibits enzymes whose activity depends on the presence of sulfhydryl groups. Tyrosinase is not inhibited by this alkylating agent. Otherwise an experiment using sulfhydryl inhibitors would not be possible. The observation that tyrosine contains no sulfhydryl groups was of course carried out using the enzyme from

melanoma. It is, however, not unreasonable to expect that the enzyme from normal skin will show the same properties. That this is true is borne out by the observation of an increased tyrosinase activity in the presence of iodoacetate (Table 8). These increases ranged from 39 to 83%, and were observed on every occasion that the skin homogenates were incubated in the presence of iodoacetate. It is of significance that the granule suspensions, when similarly incubated with iodoacetate, did not show an increased tyrosinase activity (Table 9). The granule suspensions were washed several times in the course of their preparation. Any low molecular weight soluble molecules may be expected to have been washed away. In the absence of a soluble inhibitor there were no increases in tyrosinase activity in the granule preparation when incubated in the presence of iodoacetate. The tyrosinase in such a preparation may be at or near its maximum activity. This then could account, at least in part, for the much higher specific activity of tyrosinase found in the granule suspensions as compared to the homogenates and whole scrapings of skin (Table 4). These experiments, when viewed in light of Rothman's (220) observation that the inhibitory activity is present in the pigment forming regions of skin strengthen the argument that sulfhydryl inhibition of tyrosinase activity constitutes a naturally occurring regulatory mechanism controlling pigment formation in skin.

The product of the reaction catalyzed by tyrosinase is different from that of most biosynthetic pathways in that it is an insoluble polymer. Therefore, the usual mechanisms of feedback or product

inhibition may not be available for this pathway. This could be the reason for the requirement of a soluble inhibitor of tyrosinase activity in controlling melanin formation. There is, however, a reaction inactivation of the enzyme through tanning of the protein during the reaction (222). Another method of regulating enzyme activity is through control of new protein synthesis. Direct effects of hormones on tyrosinase activity are not known. At the present time, one can only speculate about the relative importance of these mechanisms in regulating pigment formation in normal skin. However, the availability of a technique which can be used for the quantitative determination of tyrosinase activity now makes a study of these factors possible.

Even though tyrosinase from N. crassa, mushrooms, and melanoma have been shown to consist of several different components, none of these has been found to have activity toward just tyrosine but not toward DOPA. The reasons for this may lie in the methods used for the detection of tyrosinase activity, which usually depend on the appearance of pigment. Since Raper (210, 211) demonstrated that DOPA is an intermediate in the conversion of tyrosine to melanin, it is probably not reasonable to expect a component of tyrosinase to be capable of oxidizing tyrosine to DOPA and subsequently oxidize the DOPA to melanin but have no activity toward DOPA alone. Bouchilloux, McMahill, and Mason (42) have suggested that the components of tyrosinase may be analogous to the isozymes. Unlike the isozymes, all components of which have the same type of enzyme activity, individual tyrosinase subunits may be responsible for

different types of activity. It should be remembered that tyrosinase does not catalyze just one type of reaction, but two markedly different ones, a hydroxylation and a dehydrogenation. If a subunit of tyrosinase exists which is responsible for hydroxylating tyrosine to DOPA, but not of oxidizing DOPA further, it would not be detectable by any method which depends on the appearance of pigment for the detection of tyrosinase activity. Rather, the presence of such a component would have to be demonstrated by showing an enzyme activity leading to the formation of just DOPA from tyrosine. Furthermore, the enzyme would have to be a copper protein rather than an iron containing one. The results of the experiments reported here seem to show just such an activity (Tables 4 and 10).

The finding that homogenates of scrapings from albino rat skin are able to catalyze the formation of DOPA from tyrosine can not be ascribed to the presence of tyrosinase as we have come to know it. Pigment formation normally does not occur in the albino rat. In this manner, the Sprague-Dawley albino rat differs from humans where incomplete generalized albinism is common (115).

Both the melanocytes and the adrenal medullary cells are derived embryologically from the neural crest. Since the adrenal medulla has been shown to contain an active tyrosine hydroxylase, it was necessary to determine whether or not the tyrosine hydroxylating activity of albino rat skin is due to this enzyme. Tyrosine hydroxylation in the albino rat skin was inhibited by diethyldithiocarbamate, but not by α,α'-dipyridyl, indicating that the activity is due to a copper rather than an iron containing

enzyme. Also, no stimulation of activity could be demonstrated by the addition of a tetrahydropteridine. This would seem to eliminate the possibility that the tyrosine hydroxylating activity of the albino rat skin is due to a tyrosine hydroxylase of the type found in the adrenal medulla.

Numerous workers have attempted to demonstrate the presence of tyrosinase in albinos (115, 136). These attempts were carried out by incubating the skin in buffered solutions of tyrosine or DOPA. After a prolonged incubation at 37° C, pigment was observed in the melanocytes. Occasionally this resulted in generalized darkening of the cells, but more often the pigment was localized in the pigment granules (115). The appearance of pigment and the specificity of its localization in the melanocytes was taken to be indicative of the presence of tyrosinase. As far as can be determined, the structure of the albino melanosomes is the same as that of their normal counterparts. The structures upon which pigment is normally deposited remain and should be capable of reacting with quinones. As Greenstein (105) has shown, these reactive sites are probably sulfhydryl groups on the protein forming the internal membranes of the granules. These sulfhydryl groups would remain capable of coupling with quinones, whether the quinones are the result of enzymic or nonenzymic oxidations. It should be remembered that high concentrations of tyrosine and DOPA were used in the earlier attempts to demonstrate tyrosinase in albino skins. Incubations with tyrosine were carried out at a pH of 6.8 and with DOPA at a pH of 7.4. In the latter case, especially during prolonged

incubations, it would not be surprising to find melanin formation as a result of nonenzymic oxidations. One would in fact expect this to be the case, especially where generalized pigmentation of the cells was observed. These early findings then do not offer an adequate explanation of the present finding that albino rat skin is able to form DOPA from tyrosine.

The conversion of tyrosine to melanin requires a lengthy series of consecutive reactions. The condition of albinism could result, at least theoretically, if any one of the reactions in this sequence were blocked. Experimentally it has been determined that albino rat skin does not possess DOPA-oxidase activity (133). In other words, albino rat skin can not oxidize DOPA to melanin. The first step in the reaction sequence after the oxidation of DOPA is irreversible (144). Also, subsequent intermediates in the reaction sequence are readily autoxidizable (211). It seems, therefore, that the metabolic block in the albino must occur before the cyclyzation of DOPA-quinone. Any reaction sequence leading to the formation of DOPA-quinone or subsequent intermediates would almost certainly lead to the formation of pigment. This leaves two possible sites at which the metabolic block could occur, hydroxylation of tyrosine or the dehydrogenation of DOPA. The results of the experiments reported here show that albino skin possesses an enzymic tyrosine hydroxylating capability. This observation along with the knowledge that albinos lack DOPA-oxidase activity leads to the suggestion that the metabolic defect in the albino is an inability to convert DOPA to DOPA-quinone. It is not yet known whether this is due to the absence of or to a

defect in that portion of the tyrosinase molecule responsible for the oxidation of DOPA.

In view of the recent work showing that tyrosinase from mushroom and melanoma is composed of several different components (42, 45, 46, 201, 227), it has been suggested that the enzyme exists in a state analogous to that of the isozymes (42). However, two types of enzyme activity are involved. If there are two types of protein subunits, each being responsible for a different type of activity, and the two are under separate genetic control, the loss of or a defect in the protein subunit responsible for DOPA-oxidase activity could leave the albino unable to form melanin from DOPA, yet be able to convert tyrosine to DOPA. Cases of incomplete generalized albinism might then be viewed in a different light. It is in the Negro albino that incomplete generalized albinism is most common. Normally one may expect that a relatively high degree of intrinsically maintained melanogenic activity is responsible for the dark skin color of the Negro. Tyrosine hydroxylation is the slow or rate controlling step in pigment formation. If this type of activity remains at a comparatively high level in the Negro albino, nonenzymic oxidation of the resulting DOPA could well account for the small amounts of pigment in the skin of those experiencing incomplete generalized albinism.

The finding that after homogenization most of the tyrosine hydroxylating activity of albino rat skin is found in the soluble supernatant seems, at first, to be inconsistant with previous observations on the location of tyrosinase activity in melanocytes

from melanoma. There is, however, some evidence indicating that already melanized melanin granules can be fragmented during homogenization (145). It does not seem unreasonable to suggest that the fragile unmelanized granules from albino melanocytes may have been similarly disrupted. It is immediately recalled, of course, that tyrosinase from melanoma is very difficult to solubilize. Preparative procedures to date have brought only about 5 - 10% of the enzyme into solution. In addition to whatever forces bind the enzyme to the membranous structure of the melanin granule, the protein, during melanin synthesis, becomes bound to insoluble melanin through covalent bonds formed by coupling of the protein with quinones. If this binding takes place on a portion of the protein molecule in such a manner that it does not interfere with the active center, then the still active enzyme will be bound firmly to the insoluble polymer, a stable form from which it would not be brought into solution by any of the usual methods. Seiji (226) found that treatment of melanin granules from mouse melanoma with deoxycholate brought the internal membranes from those granules into a stable suspension from which they could not be precipitated by centrifugation at 105,000 xg for two hours. This treatment did not separate the tyrosinase molecule from the membrane. Seiji suggested that the tyrosinase molecule is either very firmly attached to or an integral part of the membrane. In the albino melanocyte there is no melanin to which an enzyme could be bound by covalent bonds. The forces holding the enzyme on the internal membrane then are just those by which it was initially attached. If this structure

is disrupted by the forces of homogenization, it could explain the presence of the tyrosine hydroxylating activity in the soluble supernatant.

VI. Summary

1. A survey of the literature related to the enzyme tyrosinase and to the mammalian melanocyte system has been presented.

2. Of the animal skins examined, only those from hooded rats contained appreciable amounts of visible pigment.

3. Methods for the determination of tyrosinase activity based on incubation of melanocyte containing preparations with L-tyrosine-U-C^{14} or with L-tyrosine-3,5-H^3 have been described.

4. Tyrosinase from normal skin is activated by DOPA. In the absence of DOPA, there was little measurable tyrosinase activity and a prolonged induction period using granule suspensions prepared from homogenates of scrapings from pigmented regions of hooded rat skin. Addition of DOPA to the reaction mixtures markedly increased the reaction rate and decreased the length of the induction period.

5. The heat inactivation, characteristic lag period, dependence on DOPA, and the low substrate requirements indicate that the activities measured in normal rat skin are enzymic.

6. Normal skin tyrosinase is in a partially inhibited state. The inhibitor is inactivated by iodoacetate and remains soluble in 10% TCA, indicating that it is probably a low molecular weight sulfhydryl compound. Tyrosinase activity in granule suspensions prepared from homogenates of scrapings from hooded rat skin was not in an inhibited state, suggesting that the inhibitor is soluble and was removed by the washings to which the granule suspensions were subjected.

7. The inhibitor is not stable on prolonged standing. Attempts at isolating and identifying it by means of paper chromatography were unsuccessful.

8. The attempted solubilization of tyrosinase from hooded rat skin brought less than 10% of the total activity into solution. The solubility of tyrosinase from normal rat skin is very similar to that of tyrosinase from melanoma. As in the case of tyrosinase from melanoma, normal skin tyrosinase is firmly bound to insoluble particles, probably through covalent bonds with melanin.

9. By employing the same techniques with albino rat skin that had been used with hooded rat skin, it was possible to show that the albino rat skin possesses a tyrosine hydroxylating capability which is heat inactivated. This activity was inhibited by diethyldithiocarbamate but not by α,α'-dipyridyl. Results obtained by substituting a tetrahydropteridine for DOPA as the reducing agent were highly variable. This evidence indicates that the tyrosine hydroxylating activity of albino rat skin is due to a copper protein and is not due to a tyrosine hydroxylase of the type found in the adrenal medulla and brain.

10. Tyrosine hydroxylating activity was not found in white skin from hooded rats.

11. When scrapings from albino rat skin were subjected to the fractionation procedure of Hogeboom and Adams, most of the hydroxylating activity was found in the soluble supernatant.

12. The suggestion that tyrosinase exists in a state analogous to that of the isozymes seems to be a simple unifying concept by which one can explain a) the multiplicity of tyrosinases from various sources, b) the ability of albino rat skin to hydroxylate tyrosine but not to form melanin, c) the possible site of the metabolic block in albinos and d) the common appearance of incomplete generalized albinism.

I would like to express my sincerest gratitude to Dr. Jack Fellman and the other members of the Department of Biochemistry who have given me their advise, support, and encouragement during the years I have spent as a student at the University of Oregon Medical School.

Special thanks are also extended to Dr. Alfred. J. Ramponе for allowing me to use his scintillation counter on so many occasions.

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