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Dopachrome Conversion: A Possible Control Point in Melanin Biosynthesis

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Melanin biosynthesis involves the enzymatic conversion of tyrosine, via dihydroxyphenylalanine (dopa), to melanin through a number of intermediate steps. It is generally assumed that the major rate-limiting factor in this process is the amount of active tyrosinase (monophenol monoxygenase; monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) available since when dopa has been oxidized by tyrosinase to dopa quinone the succeeding steps can occur spontaneously through autooxidation. We now present evidence that a later step, namely the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid may also be under regulatory control. We have found a factor(s) from 3 different melanomas (Cloudman, B16, and Greene) which catalyzes this step. This activity is also present in mushrooms, which contain a potent pigment-synthesizing system, but not in a variety of cell lines of nonmelanocytic origin. We have not yet identified the factor but it is of relatively low molecular weight (less than 1000 daltons by Sephadex G10 gel filtration), negatively charged at pH 6.8, and stable to boiling. The factor contains no detectable sulfhydryl groups. In crude cell extracts the factor appears to be complexed to tyrosinase since it cochromatographs with tyrosinase through the early steps of purification. The factor binds tightly to QAE-Sephadex anion exchange resin and can be separated from tyrosinase by differential salt elution. We have not yet demonstrated that the factor has a physiological role in melanogenesis but we feel such a role is likely since the activity was found only in cells with melanin-synthesizing systems.

Melanin biosynthesis from tyrosine proceeds through a series of oxidation-reduction reactions (Fig 1). There is good evidence that the first 2 steps of this pathway (tyrosine→ dihydroxyphenylalanine (dopa)→ dopa quinone) are catalyzed by the bifunctional enzyme tyrosinase [2,3]. Since dopa quinone and later intermediates can spontaneously form melanin through autooxidation it has generally been felt that these steps are not under regulatory control. We were surprised to find, therefore, that extracts of Cloudman S91 melanoma cells could rapidly convert dopachrome to a colorless compound. The subsequent formation of melanin occurred at a far greater rate than that which could be accounted for by autooxidation. When we investigated this phenomenon in more detail we found that a factor could be purified from the cells that catalyzed the conversion of dopachrome to melanin. It appears that in the presence of this factor dopachrome is converted into 5,6-dihydroxyindole-2-carboxylic acid, followed by a spontaneous decarboxylation reaction to 5,6-dihydroxyindole. The factor is specific for dopachrome, having no effect on tyrosine or dopa. We found it in 2 different mouse melanomas, a hamster melanoma, and mushrooms, but it was absent in Friend Erythroleukemia cells, neuroblastoma cells, myeloma cells, and L cell fibroblasts. The factor seems to be complexed to tyrosinase but it can be separated from tyrosinase on purification. In this report we describe the partial purification and some properties of this factor, which we call dopachrome conversion factor or "DCF."

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Abbreviations:
DCF: dopachrome conversion factor
HPLC: high-pressure liquid chromatography

MATERIALS AND METHODS

Cells

Cloudman S91 mouse melanoma cells were cultured in monolayer by routine procedures as described previously [4]. A number of melanotic and amelanotic variants of this line, derived in our laboratory, were assayed for the dopachrome conversion factor. Tumors of the Cloudman cells were grown in DBA 2/J mice. NIE 115 mouse neuroblastoma cells were provided by Dr. Xandra Breakefield, mouse Friend leukemia cells by Dr. Bernard Forget, mouse myeloma cells by Dr. David Ward, Lan 2 fibroblasts (derivatives of mouse A9 L cells) by Dr. Jerome Eisenstadt, and mouse B16 melanoma cells by Dr. John Lazzo. The Greene melanoma line was adapted to culture from a spontaneous tumor isolated from a Syrian Golden hamster by Dr. Harry Greene [5]. Mushrooms were purchased from a local food store. Cultured cells were harvested with Joklik's medium containing EDTA (1 mM), except for Lan 2 fibroblasts which were similarly exposed to the Joklik's medium but removed from the culture flasks with a rubber policeman. After harvesting, cells were spun for 10 min at 2000 $\times g$ and lysed in ice cold 0.5% Triton X 100 in sodium or potassium phosphate buffer (50 mM, pH 6.8) at a concentration of 2×10^7 cells per ml of lysis buffer. The lysate was then centrifuged (5000 g, 10′) and the pellet, which contained little DCF activity, was discarded. Protein content was determined by the Bradford method [6] and averaged between 2 to 4 mg/ml for various lysate preparations.

Synthesis of Dopachrome

Dopachrome was synthesized by exposure of dopa to silver oxide as described by Mason [7]. Ice cold dopa (0.5 mg/ml 0.1 M sodium phosphate pH 6.8) was mixed with solid Ag_2O (30 mg Ag_2O: 1 mg dopa) for about 1 min and filtered through Gelman Acrodisc Disposable Filter 4192 (0.2 μm diameter pore). ^{14}C-dopachrome was synthesized by the same procedure using ^{14}C-carboxy-labeled dopa (New England Nuclear Corp. NEC-245, 50 μCi/μM) as starting material.

Purification

DEAE BioGel and QAE Sephadex anion exchange resins were prepared by washing extensively with NaCl (1 M) in sodium phosphate (10 mM, pH 6.8) and then equilibrating with sodium phosphate only. The purification procedure for dopachrome conversion factor (DCF) was as follows: Cells (3×10^8) were lysed with 15 vol of sodium phosphate (10 mM, pH 6.8) containing Triton X 100 (0.5% vol/vol). The lysate was centrifuged (30,000 *g*, 15 min) and the supernatant fraction was stirred with an equal volume of calcium phosphate gel (Bio-Gel HT, Bio-Rad Laboratories) overnight at 4°C. The slurry was centrifuged (5000 *g*, 10 min) and the supernatant fraction, containing both tyrosinase and DCF activities but less than 10% of the total starting protein, was dialyzed against 10 vol sodium phosphate (10 mM, pH 6.8) and applied to a DEAE Bio Gel column (20 × 2.3 cm). DCF and tyrosinase were eluted from the column with a salt gradient of 0 to 0.5 M NaCl in sodium phosphate (10 mM, pH 6.8). About 60 fractions (2.4

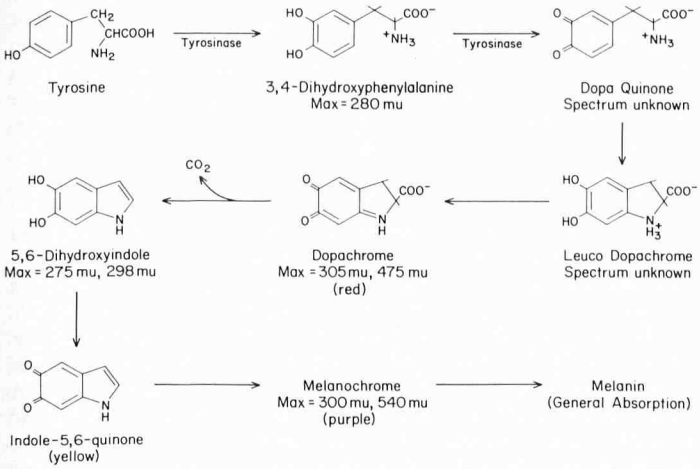

FIG 1. Stages in the oxidation of tyrosine by tyrosinase. A slight modification of the scheme of Mason and Raper [1]. Note that while this scheme depicts melanin biosynthesis as a polymerization of indole-5,6-quinone alone, it has been shown that mammalian melanins are heteropolymers, containing many of the precursors shown [12].

ml) were collected and aliquots (50 μl) were assayed for tyrosinase activity by a modification of the Pomerantz method [8–9]. DCF activity was located by mixing aliquots (50 μl) of the fractions with dopachrome (50 μl) and observing color changes visually. The DCF peak, which co-chromatographed with the tyrosinase peak on DEAE Bio Gel, was dialyzed exhaustively against sodium phosphate (10 mM, pH 6.8) and applied to a QAE Sephadex column (18 × 1.2 cm). DCF and tyrosinase were eluted with a salt gradient from 0 to 1.0 M NaCl in sodium phosphate. About 60 fractions (1.4 ml) were collected and tyrosinase and DCF were located as described for the DEAE Bio Gel step. The DCF peak was again dialyzed, but at this stage DCF passed through the dialysis tubing. The retentate was therefore discarded and the dialyzate was concentrated either by lyophylization or by re-elution from an anion-exchange column.

Detection of Sulfhydryl Groups

Sulfhydryl groups were detected with 5,5′ dithiobis (2-nitrobenzoic acid) [10].

$^{14}CO_2$ Release

$^{14}CO_2$ release from ^{14}C-carboxy-labeled dopachrome was monitored by 2 techniques. ^{14}C-dopachrome (0.1 μCi) was incubated with buffer, cell extracts, or DCF (37°C) in a total volume of 100 μl. At various times the tubes were mixed vigorously with a Vortex Genie Mixer and aliquots (10 μl) were removed, mixed with NaOH (0.1 M, 100 μl), and counted in a scintillation counter. A disappearance of ^{14}C counts with time was assumed to be due to the evolution of $^{14}CO_2$. The alternative method involved incubating tubes that were sealed with natural corks soaked in NaOH (0.1 M). At various times corks were removed and counted in a scintillation counter. ^{14}C counts from the corks were assumed to be due to $^{14}CO_2$ which had been trapped in the form of $Na_2{}^{14}CO_3$. There was good agreement between the 2 methods but the first was chosen for its simplicity.

Detection of 5,6-dihydroxyindole

5,6-dihydroxyindole was detected by high-pressure liquid chromatography (HPLC). An Altex Model 330 Isocractic Liquid Chromatograph equipped with a Model 110A pump and a 20 μl sample loop was used. Absorbance of the eluent was monitored at 254 mμ with an 8 μl Analytical Optical Unit. Experiments were performed with an Ultrasil ODS Column (Altex Instruments, Inc.), 0.46 cm (i.d.) × 25 cm. The column was packed with octadecylsilica particles of 5 μm average diameter. Mobile phase was methanol: sodium phosphate (50 mM, pH 6.5) 1:9. Flow rate was maintained at 1.8 ml/min.

RESULTS

DCF Activity in Crude Cell Extracts

We were initially surprised to find that an extract of amelanotic Cloudman S91 melanoma cells [11] could convert orange-red solutions of dopachrome to a colorless form. Dopachrome absorbs in the visible spectrum with a maximum at 475 mμ and we found that extracts of amelanotic cells promoted a rapid reduction of this absorbance at room temperature (Fig 2). Since tyrosinase activity is absent from these cells we felt that the ability to catalyze dopachrome conversion must have been due to a factor other than tyrosinase.

We assayed a number of extracts of different types of cells for dopachrome conversion by visually determining their ability to turn dopachrome solutions colorless. Extracts of mouse L cell fibroblasts (Lan 2), neuroblastoma, myeloma, and erythroleukemia cell lines were unable to bring about this conversion, but extracts of Cloudman S91 amelanotic and melanotic melanoma cell lines, Cloudman S91 melanotic tumors, B16 mouse melanoma cells, and Greene hamster melanoma cells all possessed this ability. Comparisons were made using extracts from equal numbers of cells containing similar amounts of protein but the DCF-positive lines showed activity even when cell extracts were diluted 10-fold. Mushrooms, which possess a potent pigment synthesizing system, proved to be a rich source of DCF activity, indicating a widespread biological occurrence of this factor.

Purification of DCF

The purification scheme for DCF is shown in Table I. Aliquots of column fractions were incubated with dopachrome. The elution point of DCF was determined by noting the fractions which converted dopachrome into melanin. During the purification procedure the physical characteristics of DCF went through considerable changes. In crude cell extracts DCF was labile to heating (90°C, 2 min) and remained inside dialysis tubing even after exhaustive dialysis. DCF activity and tyrosinase activity eluted from DEAE Bio Gel columns at identical

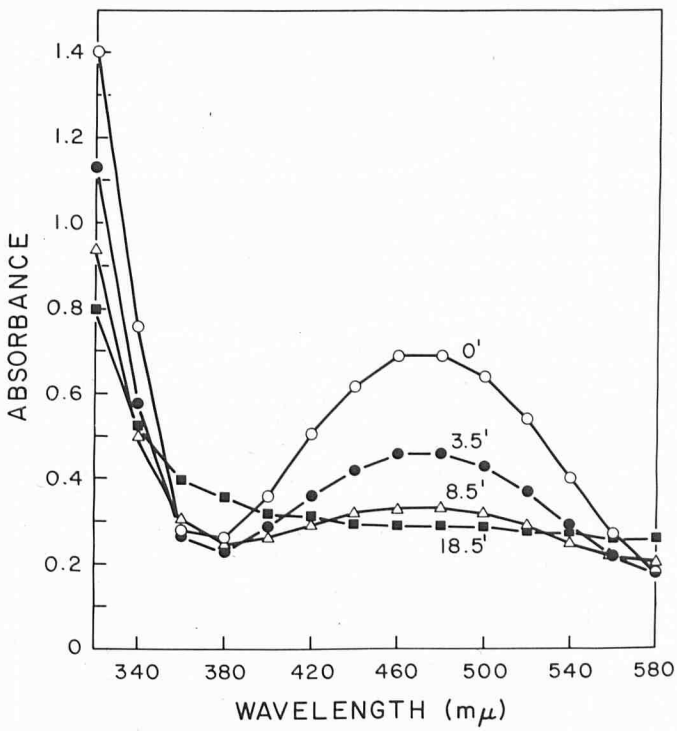

FIG 2. Effect of a melanoma cell extract on dopachrome absorbance. A lysate of amelanotic cells [11] lacking tyrosinase activity was prepared as described in Methods. Lysate (0.05 ml, 0.14 mg protein) was mixed with 0.45 ml of dopachrome which had been diluted for absorbance measurements. Dopachrome absorbance at various wavelengths is shown after different incubation periods at room temperature. Absorbance due to the lysate alone was negligible and dopachrome incubated in the absence of lysate showed little change in absorbance during the experiment (see Fig 5, *upper*). Dopachrome was prepared by mixing dopa with silver oxide (see Methods).

salt concentrations. After recovery of DCF activity from DEAE Bio Gel, it was still heat labile and resistant to dialysis. When the DEAE fraction that contained DCF and tyrosinase activity was applied to QAE Sephadex, the 2 activities were readily separated. DCF had a considerably higher affinity than tyrosinase for the QAE resin (Table II). Furthermore, the DCF recovered from the QAE column was no longer heat labile, in that it remained active after boiling for 5 min, and it no longer remained inside the dialysis tubing upon dialysis. Gel filtration through Sephadex G10 of the DCF activity recovered from QAE Sephadex indicated an apparent molecular weight less than 1000 daltons, assuming that the factor had no ionic interaction with the Sephadex beads.

TABLE I. *Purification scheme for dopachrome conversion factor (DCF)*[a]

A. Cells lysed with Triton X 100 in hypotonic saline.
B. Lysate centrifuged 30,000 *g*, 15′. DCF in supernatant fraction.
C. 30,000 *g* supernatant fraction mixed with $CaPO_4$ gel.
D. $CaPO_4$ supernatant applied to DEAE Bio Gel column.
E. DCF and tyrosinase co-eluted with NaCl. Fractions pooled and dialyzed extensively. DCF and tyrosinase remain inside dialysis tubing.
F. Dialyzed fractions applied to QAE Sephadex column.
G. DCF and tyrosinase elute separately with NaCl. DCF fractions pooled and dialyzed extensively. DCF passes through dialysis tubing.
H. Dialysate containing DCF concentrated by lyophilyzation or by eluting from an anion exchange resin.

[a] See Methods for details. All procedures were carried out at 4°C.

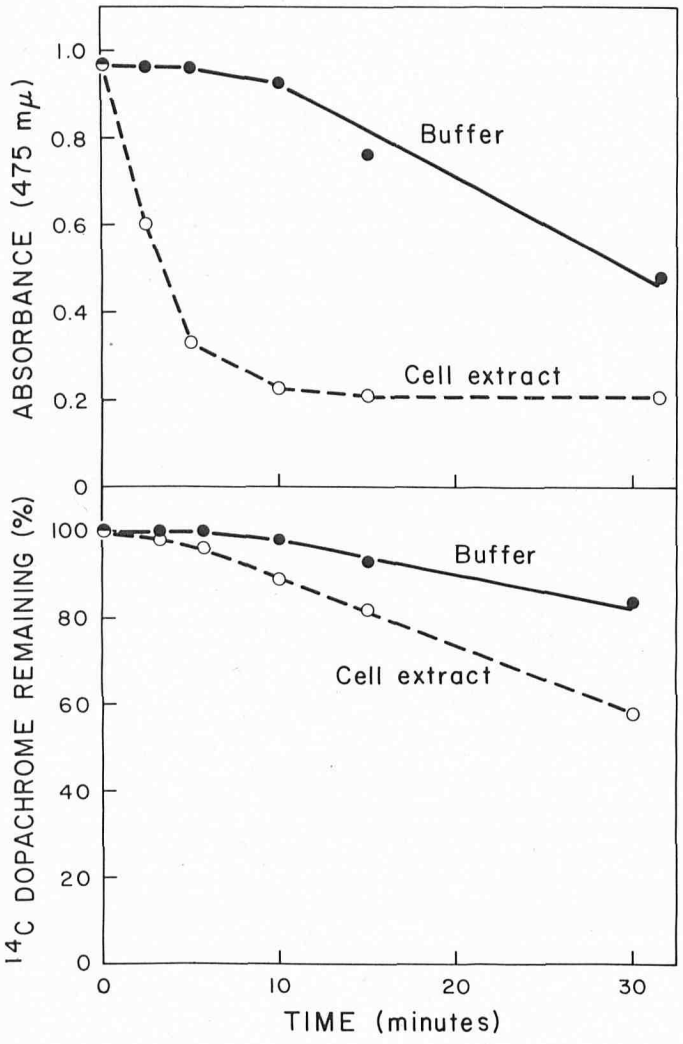

FIG 3. Effects of a melanoma cell lysate on dopachrome absorbance at 475 mμ and on release of $^{14}CO_2$ from ^{14}C-carboxy-labeled dopachrome. A lysate of amelanotic cells (see Fig 2 legend) was incubated at room temperature with unlabeled dopachrome (*upper*) or ^{14}C-labeled dopachrome (*lower*) at the same dopachrome concentrations. Assays are described in Methods.

Detection of Sulfhydryl Groups

We carried out experiments to determine if DCF contained any reactive sulfhydryl groups since it is known that dopachrome can be converted to 5,6-dihydroxyindole by reducing compounds such as glutathione and cysteine. We were unable to detect any sulfhydryl groups in solutions of purified DCF even though we could readily detect the sulfhydryl groups of glutathione and cysteine at concentrations as low as 10^{-6} M. A solution of DCF which contained no detectable sulfhydryl groups was able to convert dopachrome (10^{-4} M) to a colorless compound in 5 min at 30°C. Glutathione and cysteine were also able to promote this conversion but only at concentrations in the millimolar range (data not shown). These results make it unlikely that DCF promotes dopachrome conversion through the action of sulfhydryl groups.

The Dopachrome Conversion Reaction

The Mason-Raper scheme of melanogenesis depicts the conversion of dopachrome to 5,6-dihydroxyindole (Fig 1). This

TABLE II. *NaCl concentrations for elution of tyrosinase and DCF from anion exchange resins*[a]

Resin	NaCl Concentration for Elution	
	Tyrosinase	DCF
DEAE Bio Gel	.082 M	.082 M
QAE Sephadex	.095 M	.360 M

[a] See methods for procedures. Salt concentrations were determined with a conductivity meter (Radiometer, Copenhagen, Type CDM 2c).

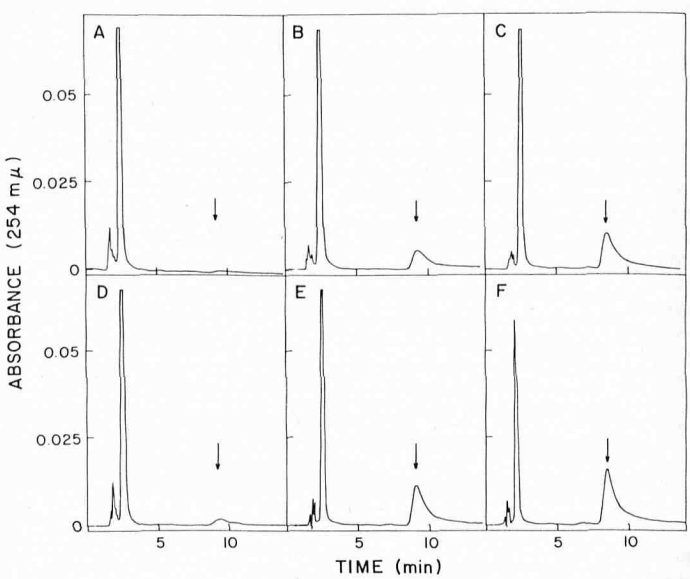

FIG 4. Analysis of the products of the DCF reaction by high-pressure liquid chromatography. Details of the HPLC procedure are described in Methods. Dopachrome (10^{-4} M) was incubated at room temperature with either sodium phosphate (pH 6.8, 0.05 M, *A–C*) or purified DCF (*D–F*) for 0 (*A,D*), 15 (*B,E*), and 30 (*C,F*) min. *Arrows* mark the elution point of 5,6-dihydroxyindole. The large, off-scale peak at the origin is due to absorbance of a mixture of dopachrome and, presumably, 5,6-dihydroxyindole-2-carboxylic acid. The elution point of dopachrome was determined by authentic standard. The structures of 5,6-dihydroxyindole-2-carboxylic acid and dopachrome are such that they would have eluted together. However, no authentic standard of the carboxylic acid was available to verify this.

conversion must involve the evolution of CO_2 from 5,6-dihydroxyindole-2-carboxylic acid. In order to determine the molecular events that occurred during the DCF-mediated conversion reaction, we compared the evolution of $^{14}CO_2$ from ^{14}C-carboxy labeled dopachrome to the reduction in absorbance at 475 mμ (Fig 3, *upper* and *lower*). We found that the color change was complete after 10 min exposure of dopachrome to DCF (Fig 3, *upper*) whereas under the identical conditions only 10% of the ^{14}C had been liberated as $^{14}CO_2$ (Fig 3, *lower*). These results suggest that the initial step catalyzed by DCF is the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid. To determine whether 5,6-dihydroxyindole was formed during the DCF reaction, we analyzed the products by high-pressure liquid chromatography after various incubation periods at room temperature (Fig 4, *arrows* mark elution point of 5,6-dihydroxyindole). We found that 5,6-dihydroxyindole was generated spontaneously from dopachrome incubated in phosphate buffer (Fig 4, *A–C*). However, the rate of production was increased by more than a factor of 2 when dopachrome was incubated with purified DCF (Fig 4, *D–F*). The rates of production of 5,6-dihydroxyindole were in close agreement with the rates predicted from Fig 3 (*lower*). Together our results indicate that DCF catalyzes the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid, and this step is followed by a spontaneous decarboxylation to 5,6-dihydroxyindole.

DISCUSSION

We have discovered a factor which catalyzes the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid thereby causing an increased rate of melanin formation. The factor, "DCF," appears to have a physiological role in melanin biosynthesis since it has been found only in cells with melanin-synthesizing systems, and seems to be associated with tyrosinase. While the Mason-Raper scheme (Fig 1) presents melanin synthesis as a polymerization of indole-5,6-quinone alone, it has been shown that mammalian melanins are heteropolymers, containing many of the intermediates shown in Fig 1 [12–15]. However, our finding of DCF activity suggests that melanin biosynthesis might be a more ordered process than previously suspected. When visually observing the DCF reaction we often see the systematic color changes depicted in the Mason-Raper scheme (red-orange→ colorless→ yellow (occasionally)→ purple→ black). It is our feeling that although many of the intermediates probably polymerize as shown by Hempel [12] the Mason-Raper scheme nonetheless represents a preferred pathway in melanin synthesis, at least in Cloudman melanoma cells. On the other hand we realize that our studies have been with cell extracts and the results do not necessarily apply to melanin synthesis *in vivo*. More experiments are necessary before a physiological role can be assigned to DCF with certainty and to this end we are trying to ascertain the molecular structure of the compound.

It has been assumed up to now that the amount of tyrosinase activity is the only rate-limiting factor in melanogenesis and that once dopa quinone is formed the remaining steps in the pathway occur spontaneously through autooxidation [e.g., 16]. However, Logan and Weatherhead [17] have provided recent evidence for post-tyrosinase inhibition of melanogenesis by melatonin in hair follicles of Siberian hamsters, suggesting the involvement of a second regulatory site. Control of melanization by additional factors could be significant for the understanding of disorders such as albinism and vitiligo, the cytotoxicity of melanin precursors [18], spontaneous regression of melanomas, and questions regarding variations in skin and coat pigmentation throughout the animal kingdom.

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