diazepam, are only slowly eliminated from the body (*14*); thus, the rate at which the benzodiazepine receptors are left unoccupied is relatively slow, thereby producing a gradual change in the functional status of the central nervous system. On the other hand, by virtue of its ability to displace diazepam from the benzodiazepine receptor, Ro 15-1788 produces a relatively rapid change in the central nervous system. A similar phenomenon has been identified for the opioid 1-α-acetylmethadol (LAAM). This opioid has long-acting metabolites (*15*) that delay the onset of abstinence signs after spontaneous withdrawal (*16*). Immediate and intense withdrawal effects, however, were observed after LAAM-treated animals received the opioid receptor antagonist naloxone (*16*).

The clinical significance of the relatively rapid development of physical dependence to diazepam observed in our study is unclear. Several clinical case reports indicate that withdrawal signs and symptoms have been observed after relatively short-term administration (2 weeks to 4 months) of diazepam, even at therapeutic doses (*17*). The low frequency of such reports, however, has led some investigators to conclude that the risk of significant benzodiazepine withdrawal is very low if patients have been taking diazepam for long periods at therapeutic doses for 6 months or less (*18*). On the other hand, it has been argued that benzodiazepine withdrawal may be missed or underreported because anxiety is the cardinal symptom of both benzodiazepine withdrawal and of the original condition for which the drug was prescribed (*19*). Carefully conducted clinical studies will be necessary to determine the minimum dose and treatment interval after which significant benzodiazepine withdrawal occurs.

A major implication of this study is that benzodiazepines may produce clinically relevant functional changes in the central nervous system more rapidly than heretofore expected. The procedure used in this experiment of exploring the development of physical dependence on benzodiazepines with antagonist-precipitated withdrawal will be useful in investigating biochemical, neurophysiological, and behavioral aspects of benzodiazepine physical dependence.

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8. The suspending agent was Agent K (BioServ, Inc., Frenchtown, N.J.) at a concentration of 4.0 g/liter.
9. There was one exception to this procedure. Subject PH received a continuous infusion with a peristaltic pump of the suspended drug at the same daily dose (20 mg/kg).
10. There were several exceptions to this procedure. Subject AL was evaluated after the administration of 10 mg/kg, intragastrically, on day 7; subject PH was evaluated at day 14 (rather than day 7) at 10 mg/kg, intragastrically, and again at day 35 at 10 mg/kg, intramuscularly. The Ro 15-1788 was dissolved in a propylene glycol–alcohol–water vehicle in ratios of 40:10:43.5, respectively, the remaining percentage being buffers and preservatives (Valium vehicle) and administered in a volume of 2 ml per injection.
11. Observations were carried out for 10 minutes every 3 hours during the peak of withdrawal (days 9 to 10) and at least once daily (12 noon) on all other days.
12. In addition to these observational data, additional behavioral data were collected for subject AL. During daily sessions this animal responded on a lever which produced a 1-g food pellet after every 50th response. Response rates, which were progressively depressed over the first 4 days of diazepam treatment, gradually returned to predrug control levels by day 15 of diazepam administration.
13. Although daily therapeutic doses of diazepam are commonly in the range of 0.1 to 0.5 mg/kg, diazepam is apparently not infrequently abused at doses in excess of 3 mg/kg, and occasionally doses of 15 to 20 mg/kg have been reported [M. L. Stitzer, R. R. Griffiths, A. T. McLellan, J. Grabowski, J. W. Hawthorne, *Drug Alcohol Depend.* **8**, 189 (1981)].
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Mammalian Tyrosinase Catalyzes Three Reactions in the Biosynthesis of Melanin

Abstract. *The biosynthesis of melanin is initiated by the catalytic oxidation of tyrosine to dopa by tyrosinase in a reaction that requires dopa as a cofactor. Tyrosinase then catalyzes the dehydrogenation of dopa to dopaquinone. The subsequent reactions can proceed spontaneously in vitro. Tyrosinase, purified from murine melanomas and the skins of brown mice, has now been shown to catalyze a third reaction in mammalian melanogenesis, namely the conversion of 5,6-dihydroxyindole to melanochrome. This reaction requires dopa as a cofactor and is inhibited by tyrosine. Conversely, 5,6-dihydroxyindole inhibits the oxidation of tyrosine to dopa, so that the relative concentrations of tyrosine and 5,6-dihydroxyindole within the mammalian pigment cell are capable of regulating melanogenesis in a previously unrecognized fashion. Tyrosinase has the unusual property of catalyzing three distinct reactions within a single biochemical pathway: the hydroxylation of a monophenol, the dehydrogenation of a catechol, and the dehydrogenation of a dihydroxyindole. The first and third of these reactions require dopa as a cofactor; in the second reaction, dopa is a substrate.*

Melanin is a biopolymer found throughout the animal and plant kingdoms. Regulation of melanin biosynthesis has been under investigation for more than 80 years, and the intermediate chemical reactions, known as the Mason-Raper pathway, were determined in the 1920's (*1*). The pathway is initiated by the conversion of tyrosine to dihydroxyphenylalanine (dopa), then of dopa to dopaquinone. Both steps are catalyzed by the enzyme tyrosinase (E.C. 1.14.18.1), and, in mammals, dopa is a cofactor for the oxidation of tyrosine to dopa. Melanotropin (MSH) causes a marked increase in tyrosinase activity and a concomitant increase in melanin formation (*2*). Until recently it appeared

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that, after the first two reactions were completed, no further regulatory controls existed since melanin can form spontaneously from dopaquinone in the test tube:

tyrosine $\xrightarrow[\text{(dopa)}]{\text{tyrosinase}}$ dopa $\xrightarrow{\text{tyrosinase}}$

dopaquinone → leucodopachrome →

dopachrome → 5,6-dihydroxyindole →

indole 5,6-quinone →

melanochrome → melanin

The first indication that regulatory controls are exerted distal to the initial two reactions came from the work of Logan and Weatherhead (*3*), who showed that when hair bulbs of Siberian hamsters are exposed to low levels of melatonin, melanogenesis is inhibited even though tyrosinase activity remains high. Their findings could explain how melatonin regulates seasonal changes in the coat color of these animals.

Using variant Cloudman S91 melanoma cells in culture, we showed that at least three reactions, in addition to the initial two oxidation steps, are under regulatory control (*4, 5*). Dopachrome conversion factor accelerates the conversion of dopachrome to 5,6-dihydroxyindole (DHI). Indole conversion factor accelerates the conversion of DHI to melanochrome. Indole blocking factor inhibits melanogenesis from DHI (*5*). Melanotropin and other agents that increase levels of adenosine 3′,5′-monophosphate (cyclic AMP) in Cloudman cells cause an increase in the activity of indole conversion factor and a decrease in the activity of indole blocking factor. However, increased levels of cyclic AMP do not appear to affect the activity of dopachrome conversion factor. During these studies, we noticed that the activity of the indole conversion factor migrated with tyrosinase activity, even after a variety of purification steps were completed (*5*). Also, it was shown that highly purified isozymes of tyrosinase retained an activity that accelerated formation of melanin from dopachrome (*6*). We now report that highly purified tyrosinase from Cloudman S91 melanoma cells catalyzes the conversion of DHI, which is colorless, to melanochrome, which is purple and absorbs light at 540 nm. The reaction proceeds more readily if dopa is included as a cofactor in the reaction (Fig. 1).

Tyrosinase is a copper-containing protein. Phenylthiourea and sodium diethyldithiocarbamate inhibit tyrosinase activity by combining with the copper in the enzyme (*7*). Both of these compounds inhibit the conversion of dopa to dopaquinone and of DHI to melanochrome to the same extent. For example, 70 percent inhibition of either reaction is achieved by $5 \times 10^{-6} M$ phenylthiourea or $1.5 \times 10^{-5} M$ diethyldithiocarbamate.

We tested the effect of tyrosine on the conversion of DHI to melanochrome and the effect of DHI on the conversion of tyrosine to dopa (Fig. 2). L-Tyrosine inhibited the conversion of DHI to melanochrome with a half-maximal concentration for inhibition of $1 \times 10^{-4} M$ (Fig. 2A). D-Tyrosine had no effect on the conversion reaction (not shown), whereas DHI inhibited the conversion of tyrosine to dopa with a half-maximal concentration for inhibition of $3 \times 10^{-4} M$ (Fig. 2B). The half-maximal concentrations for inhibition, although of the same order of magnitude, were clearly different, suggesting that different active sites on the tyrosinase molecule were involved in the two reactions. Using highly purified ty-

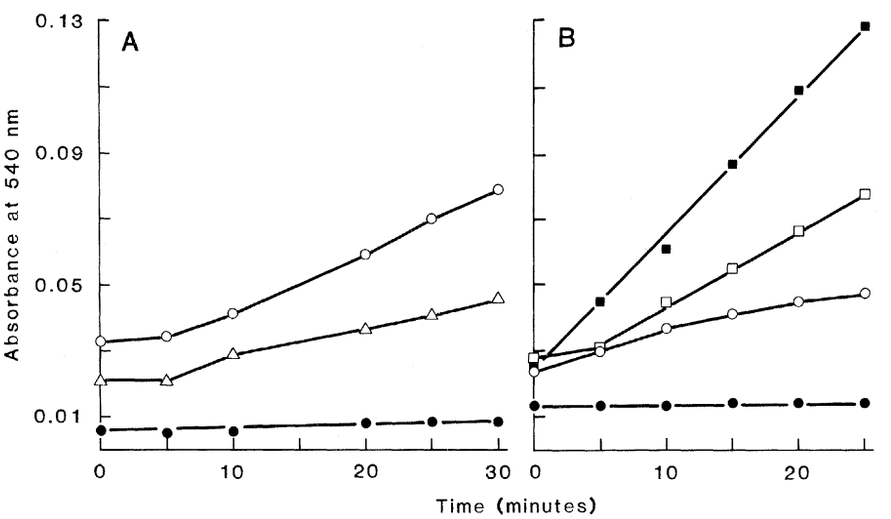

Fig. 1. Conversion of DHI into melanochrome is catalyzed by tyrosinase, and dopa is a cofactor for the reaction. The reactions were monitored spectrophotometrically as absorbance at 540 nm. Each reaction was performed in a plastic cuvette at 25°C in a total volume of 0.4 ml of sodium phosphate (10 m*M*, *p*H 6.8). (A) (●) DHI alone ($2.5 \times 10^{-5} M$); (△) DHI ($1.25 \times 10^{-5} M$) and tyrosinase (100 μg); (○) DHI ($3.75 \times 10^{-5} M$) and tyrosinase (100 μg). (B) (●) DHI ($2.5 \times 10^{-5} M$) and dopa ($3.4 \times 10^{-5} M$); (○) dopa ($3.4 \times 10^{-5} M$) and tyrosinase (100 μg); (□) DHI ($2.5 \times 10^{-5} M$), dopa ($1.7 \times 10^{-5} M$), and tyrosinase (100 μg); (■) DHI ($2.5 \times 10^{-5} M$), dopa ($3.4 \times 10^{-5} M$), and tyrosinase (100 μg). In the reactions where DHI, dopa, and tyrosinase were mixed (B), the data have been corrected for absorbance due to the oxidation of dopa to dopachrome. Tyrosinase was prepared as follows. All procedures were carried out at 4°C. Cloudman S91 melanoma tumors (200 g) were removed from DBA/2J mice, washed with NaCl (146 m*M*), and homogenized with a Virtis stainless steel homogenizer in 1 liter of Triton X100 (0.5 percent). Calcium phosphate gel [20 mg/ml in 1 liter of sodium phosphate (10 m*M*, *p*H 6.8); Bio-Rad] was added to the homogenate. The mixture was stirred for 2 days, centrifuged (10,000*g*, 20 minutes), and the supernatant fraction, which contained more than 90 percent of the starting activity of tyrosinase, but less than 10 percent of the total protein, was retained and dialyzed overnight against H_2O (10 volumes). The dialyzed material was applied to a DEAE column (BioGel A, 100 to 200 mesh, 40 by 6 cm; Bio-Rad). Tyrosinase was eluted with a linear salt gradient [0.0 to 0.5*M* NaCl in 2 liters of sodium phosphate (10 m*M*, *p*H 6.8)]. The fractions containing tyrosinase activity were pooled, dialyzed overnight against H_2O, and applied to a QAE-Sephadex column (90 by 1.5 cm; Pharmacia). Tyrosinase was eluted with the same linear salt gradient that was used for the DEAE column. The fractions containing tyrosinase activity were pooled, dialyzed exhaustively, and lyophylized to dryness. The lyophylized material was applied to a Sephadex G-150 column (90 by 1.5 cm; Pharmacia), and tyrosinase was eluted after the void volume. The Sephadex G-150 was equilibrated with sodium phosphate (10 m*M*, *p*H 6.8), containing NaCl (0.1*M*). The tyrosinase was dialyzed and applied to an Affi-Gel Blue affinity column (90 by 1.5 cm, 100 to 200 mesh; Bio-Rad) and eluted with H_2O. The tyrosinase activity eluted in the void volume. The tyrosinase was dialyzed and lyophylized to dryness. The final yield of material was about 5 mg. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed major bands at 66,000 and 55,000 daltons, which comprised more than 90 percent of the total protein. It was assumed that these major bands were isozymic forms of tyrosinase, but no attempt was made at further purification. Tyrosinase activity was measured as the release of ^3H_2O by a modified method of Pomerantz (*14*), or by spectrophotometric measurement at 475 nm of conversion of dopa to dopachrome in Sarstedt plastic cuvettes in a Beckman model 24 spectrophotometer. The DHI was synthesized as described by Axelrod and Lerner (*15*). Conversion of DHI to melanochrome was measured spectrophotometrically at 540 nm.

rosinase from Cloudman S91 melanomas, with pure DHI and dopa as substrate and cofactor, respectively, we analyzed the nature of the inhibition of melanochrome formation by L-tyrosine. Double reciprocal plots of the rate of formation of melanochrome from DHI versus a range of concentrations of DHI, at different concentrations of L-tyrosine, intersected on the abscissa (not shown). A similar result was obtained with electrophoretically pure isozymes from B16 melanomas. These results suggest that the inhibition of the conversion of 5,6-dihydroxyindole to melanochrome by L-tyrosine is noncompetitive and that the tyrosinase molecule may have separate active sites for binding tyrosine and DHI (*8*).

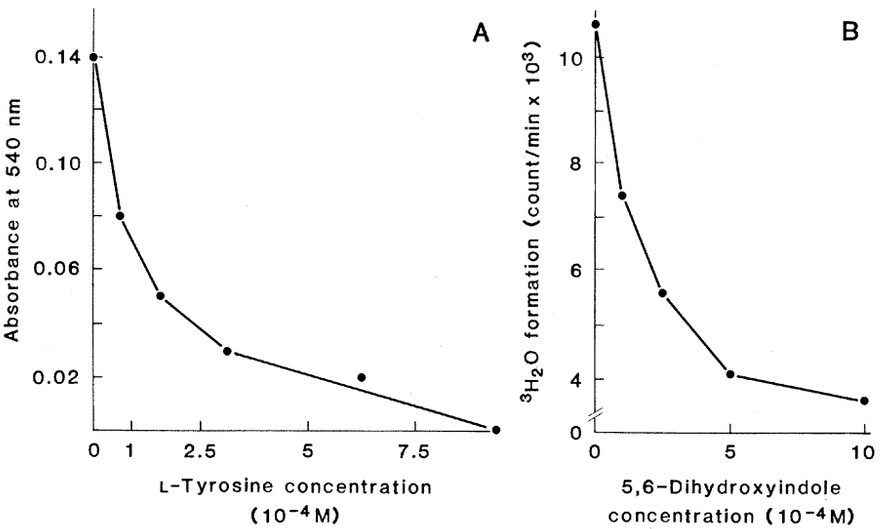

Fig. 2. (A) The inhibition by L-tyrosine of melanochrome synthesis from DHI. Each reaction mix contained DHI ($2.5 \times 10^{-5}M$), dopa ($2.5 \times 10^{-5}M$), tyrosinase (100 μg/ml), and L-tyrosine at the concentrations noted. D-Tyrosine had no effect on the conversion of DHI to melanochrome (not shown). Each reading has been corrected for absorbance due to oxidation of dopa and for spontaneous oxidation of DHI. Reactions were measured after 25 minutes at 25°C. (B) Inhibition by DHI of the oxidation of [^{3}H]tyrosine to dopa. Each reaction mix contained L-[3,5-^{3}H]tyrosine (0.25 μCi, New England Nuclear, 50.1 Ci/m*M*), dopa ($2.5 \times 10^{-5}M$), tyrosinase (100 μg/ml), and DHI at the concentrations noted, in a total volume of 165 μl of sodium phosphate (10 m*M*, *p*H 6.8). Reactions were stopped after 30 minutes of incubation at 37°C by the addition of charcoal. Conversion of tyrosine to dopa was measured as the amount of ^3H_2O released (*15*). Tyrosinase was purified from Cloudman S91 melanomas. Similar results were obtained with highly purified tyrosinase isozymes from B16 melanomas and with partially purified tyrosinase from skins of newborn brown mice.

Our results, in combination with earlier findings, demonstrate that mammalian tyrosinase catalyzes three reactions in the Mason-Raper pathway: conversion of tyrosine to dopa, conversion of dopa to dopaquinone, and conversion of DHI to melanochrome. The first two reactions are well documented [see, for example, (*9*)]. Proof for the third is as follows.

1) There is histochemical evidence suggesting that melanocytes convert DHI to melanin (*10*).

2) Crude extracts of Cloudman melanoma cells can convert DHI to melanin, and the conversion is enhanced when the cells have been exposed to MSH (*11*).

3) The factor that catalyzes the conversion of DHI to melanochrome migrates with tyrosinase through a variety of purification steps (*5*).

4) Highly purified tyrosinase isozymes from B16 melanomas and the hair follicles of C57BL/6J mice stimulate conversion of ^{14}C-labeled dopachrome or ^{14}C-labeled DHI, or both, into acid-precipitable form (*6*).

5) The same isozymes, as well as highly purified tyrosinase from Cloudman melanomas, or partially purified tyrosinase from skins of brown mice, convert DHI to melanochrome.

6) The conversion of DHI to melanochrome by tyrosinase requires dopa as a cofactor and is inhibited by L-tyrosine; DHI inhibits the conversion of tyrosine to dopa.

7) Phenylthiourea and diethyldithiocarbamate inhibit both the conversion of dopa to dopaquinone and of DHI to melanochrome.

8) Amelanotic variants of Cloudman melanoma cells, extracts of skins from two different strains of albino mice, as well as mice with deletions at the albino locus, are unable to convert tyrosine to dopa, dopa to dopaquinone, or DHI to melanochrome (*12*).

The discovery that tyrosinase regulates three rather than two steps in mammalian melanogenesis suggests that tyrosinase is a very unusual enzyme, having the ability to catalyze three separate reactions within a single pathway. Each of the reactions is distinct, namely: the hydroxylation of a monophenol, the dehydrogenation of a catechol, and the dehydrogenation of a dihydroxyindole. The first and third of these reactions require dopa as a cofactor; and in the second reaction, dopa is a substrate.

The findings that DHI inhibits the initial steps in melanogenesis and that L-tyrosine inhibits the conversion of DHI to melanochrome suggest the possibility of feedback regulation through subtle changes in precursor-product concentrations. Precursors to melanin are toxic to pigment cells, probably because of the generation of free radicals by the quinone intermediates; in particular, tyrosine, dopa, dopachrome, and DHI are toxic to melanoma cells (*5*, *13*). Exposure of murine melanoma cells to MSH increased their sensitivity to melanin precursors. Hence, normal pigment cells must have some protective mechanism against the toxicity, since they are able to synthesize melanin actively without suffering cytotoxic side effects. The relative concentrations of tyrosine and DHI may be important in the regulation of this protective mechanism.

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