GENETIC CONTROLS OVER ACTIVITIES OF TYROSINASE AND DOPACHROME CONVERSION FACTOR IN MURINE MELANOCYTES

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ABSTRACT

We evaluated the three catalytic activities of tyrosinase and one activity of dopachrome conversion factor (DCF) in extracts made from skins of 6-day-old yellow and nonyellow mice. At least one of the catalytic activities of tyrosinase and of DCF correlate with the color of pigment being produced in the hair follicles of the mice. We use these data to evaluate existing hypotheses about the mechanism of the interacting genetic controls over melanogenesis.

THREE or more genetic loci interact to control the branched biochemical pathway that results in melanogenesis (production of yellow pheomelanin or nonyellow eumelanin pigment) in differentiated murine follicular melanocytes (pigment cells) (Figure 1). Active tyrosinase, controlled by the albino (C) locus, catalyzes the first two steps in melanogenesis, which lie before the branch point of the pathway. Tyrosinase also catalyzes a distal step in the process of eumelanogenesis. Another enzyme, dopachrome conversion factor (DCF) reportedly has two catalytic activities that influence the eumelanin branch of the pathway. The gene locus responsible for the activity of DCF has not been identified. Direct catalytic controls over the pheomelanin branch of the pathway have not been identified. However, the unlinked agouti (A) and extension (E) loci determine whether eumelanin or pheomelanin will be produced in the melanocyte; that is, they control the branch point of the pathway.

The agouti locus influences melanogenesis within melanocytes through the mesodermal tissues that surround the ectodermally derived hair follicle, presumably via production of a chemical messenger to which the melanocyte responds. The extension-locus alleles apparently influence the ability of the melanocytes to respond to the mesodermal environment. Allelic substitution at the agouti or extension loci determines whether the follicular melanocytes will produce yellow or nonyellow melanin, apparently by influencing intracellular levels of cyclic AMP (cAMP). It is not clear how these changes influence the branch point of the melanogenic pathway (Figure 1) to direct the process toward the production of pheomelanic (yellow) or eumelanic (nonyellow) pigment. BARBER et al. (1984, 1985) have shown that extracts made from pheo-
melanic hair follicles of yellow mice lack dopachrome conversion (DC) activity of an enzyme with DCF activity that they call dopachrome oxidoreductase (DCOR), one of the enzymes known to catalyze eumelanogenesis. When they injected alpha-MSH into mice which were yellow because of agouti-locus alleles, the melanocytes responded by production of black pigment in newly activated follicles. Recessive-yellow mice did not so respond. Extracts made from the hair follicles of the responsive mice did have DC activity similar to that in extracts from genetically black mice.

In addition to the agouti and extension loci, a third locus is known to have direct influence over melanogenesis. The albino locus controls production of tyrosinase, the other enzyme known to catalyze melanogenesis. Without active tyrosinase, no pigment forms in the melanocyte. Tyrosinase is known to exert three catalytic activities that influence melanogenesis in vitro (KORNER and PAWELEK 1982; MURRAY, PAWELEK and LAMOREUX 1983). BARBER et al. (1985) studied one of these activities, tyrosine hydroxylase (TH) activity, in yellow and nonyellow mice. This leaves several of the catalytic controls that are known to influence melanogenesis yet to be evaluated in terms of their influence over the branch point of the melanogenic pathway. The known enzymatic controls over melanogenesis are shown in Figure 1 and Table 1 and are as follows.
MURINE MELANOCYTES

TABLE 1

Genetic controls over melanogenesis

<table>
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<tr>
<th>Enzyme</th>
<th>Gene locus</th>
<th>Catalytic function</th>
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<tr>
<td>Tyrosinase</td>
<td>Albino (C)</td>
<td>TH, tyrosine hydroxylase—may be similar in eumelanin and pheomelanin tissues</td>
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<tr>
<td></td>
<td></td>
<td>DO, dopa oxidase and high in eumelanin tissues</td>
</tr>
<tr>
<td>Dopachrome conversion factor</td>
<td>Not known</td>
<td>DC, dopachrome conversion—absent from pheomelanin tissues</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IB, indole blocking?—measurable only in unpigmented tissues</td>
</tr>
<tr>
<td>Not known</td>
<td>Agouti (A)</td>
<td>Not known—determine whether melanocyte will make pheomelanin or eumelanin. May modulate intracellular levels of cAMP</td>
</tr>
<tr>
<td>Not known</td>
<td>Extension (E)</td>
<td></td>
</tr>
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Tyrosinase catalyzes two steps in the part of the melanogenic pathway that is common to both eumelanogenesis and pheomelanogenesis. In vitro, tyrosinase is required for the first step, conversion of tyrosine to 3,4-dihydroxyphenylalanine (dopa), (TH activity), and speeds the second, conversion of dopa to dopaquinone (dopa oxidase, DO, activity). Dopaquinone spontaneously converts to dopachrome. Tyrosinase also influences conversion of 5,6-dihydroxyindole (DHI) to melanochrome (DHI conversion activity).

DCF also influences eumelanogenesis. In vitro, DCF speeds conversion of dopachrome to DHI (DC activity). In absence of tyrosinase activity, it reportedly slows conversion of DHI to melanochrome. This latter DHI blocking activity is not measurable in pigmented tissues and, therefore, cannot be evaluated in this study.

No specific enzymatic controls have been identified that catalyze pheomelanogenesis distal to the branch point of the pathway.

The overall purpose of this research is to clarify the interacting controls that the albino, agouti and extension loci of the laboratory mouse exert over melanogenesis in terms of the catalytic activities of tyrosinase and DCF. In this paper we describe the catalytic activities of tyrosinase and DCF expressed in extracts made from skins of yellow and nonyellow 6-day-old mice, with or without previous injection of MSH. The DO and DC activities were found to be reduced in pheomelanin tissues. We discuss the significance of these observations in evaluating several hypotheses that attempt to explain the eumelanin/pheomelanin switch mechanism.

MATERIALS AND METHODS

Mouse stocks: Mice were maintained under standard conditions. A/a E/E (lethal yellow) and a/a E/E (black) mice were full sibs and were co-isogenic to the C57BL/6J inbred strain. a/a e/e (recessive yellow) mice were congenic to C57BL/6J. Unpigmented mice used as controls included the following: (1) C57BL/6J-Mi°/Mi° mice, and mi°/mi° mice which were in the fifth generation of backcrossing onto C57BL/6J. These "microphthalmia-white" and "microphthalmia-black-eyed-white" mice have few or no

"microphthalmia-white" and "microphthalmia-black-eyed-white" mice have few or no
melanocytes in their skins; (2) co-isogenic C57BL/6J-<sup>c<sub>1</sub></sup>/<sup>c<sub>2</sub></sup> standard albino mice that originated with a mutation in C57BL/6J; (3) <sup>c<sub>6H</sub></sup>/<sup>c<sub>400</sub></sup> mice of the fourth backcross generation onto C57BL/6J. These "albino-deletion" mice are the result of the presence, in heterozygous conditions, of two overlapping deletions, each of which includes the albino locus. We chose these animals as controls because, although none produces pigment, each has a different genetic defect. "White" mice have no pigment cells, albino mice do have pigment cells but do not produce pigment, albino-deletion mice lack the albino locus, but do have pigment cells.

**Preparation of skin extracts:** Mice were uninjected, sham-injected or MSH-injected at 3 and 5 days of age. Each sham-injected mouse received 50 μl of peanut oil per injection. MSH-injected mice received approximately ½–1 μg of alpha-MSH in 50 μl of peanut oil per injection, a sufficient quantity to cause A<sup>y/a</sup> mice to produce black pigment. At 6 days of age the mice were sacrificed by decapitation, and their skins were removed with dissecting scissors. The skins were homogenized in 0.5 ml of ice-cold sodium phosphate buffer (pH 6.8, 0.05 M) containing 0.5% Triton X-100, for every 100 mg of skin. The homogenates were centrifuged at 38,000 × g for 30 min, and the supernatant was used as skin extract. Protein concentration in the skin extracts was determined by the BRADFORD (1976) method, with bovine serum albumin as standard. Extracts were adjusted to 4 mg protein/ml. For one assay, low MW proteins were removed by filtration in Amicon Centricon microconcentrators, following the manufacturer's recommendations. The filtered extracts were reconstituted with lysis buffer. All extracts were used within 1 wk of collection and were thawed no more than one time. DCF assays were performed before freezing.

**Enzymatic assays:** TH activity was measured by counting the tritium released as tritiated tyrosine was converted to dopa, using PAWELEK's (1979b) modification of the method of POMERANTZ (1963). DO activity was measured spectrophotometrically by following the increase in absorbance at 475 nm, using the method of MURRAY, PAWELEK and LAMOREUX (1983). The decrease in absorbance at 475 nm, as the result of conversion of dopachrome to DHI (DC activity), was also measured spectrophotometrically at 475 nm using the method of MURRAY, PAWELEK and LAMOREUX. Conversion of DHI to melanochrome was measured spectrophotometrically at 540 nm using a modification of the technique of KORNER and PAWELEK (1980). DHI blocking activity of DCOR cannot be measured in the presence of active tyrosinase.

**RESULTS**

Because these results report data that relate to at least nine interacting variables, there is no one best sequence in which to report the data. We have chosen first to confirm previous observations regarding DC activity, then to present our data which show that DO activity is also reduced in extracts from pheomelanic tissues. The testing of these data includes a discussion of TH activity. DHI conversion activity does not contribute to our discussion, but we made some original observations that may be useful and time-saving for other investigators, and so we include this information toward the end of the results.

Figure 2 shows DC activity in extracts of skins taken from mice which were producing yellow pigment or black pigment, either as a result of their genotypes or as a result of MSH injection. The data confirm previous reports that DC activity is absent in mice that are producing yellow pigment (BARBER et al. 1985). In skin extracts made from mice producing nonyellow (black) pigment (A<sup>y/a</sup> E/E mice which had been injected with alpha-MSH or a/a E/E mice), DC activity was high.
FIGURE 2.—Conversion of dopachrome to 5,6-dihydroxyindole, measured at 475 nm on a Beckman DU-8 spectrophotometer. Extracts from skins of yellow (A'/a) mice which had been injected with alpha-MSH did have the dopachrome conversion activity of DCF, whereas those from lethal-yellow mice which had been sham injected or uninjected did not. Extracts from skins of black (a/a) mice had dopachrome conversion activity. Reaction mixes contained 400 μl of buffer, 400 μl of dopachrome solution, and 400 μl of extracts or, in the blanks, lysis buffer. The dopachrome solution still contained about 30% of the dopa that was used to synthesize the dopachrome. Therefore, a parallel set of control cuvettes that contained about this amount of dopa in buffer was measured, and the absorbance of each control was subtracted from the experimental. The experiment was repeated at least three times, with similar results each time. ○, A'/a sham-injected; ●, blank; Δ, A'/a not injected; ■, A'/a MSH-injected; □, a/a sham-injected; ▲, a/a MSH-injected.
Figure 3.—Conversion of dopa to dopachrome was measured at 475 nm. Reaction mixes contained 400 μl of dopa (0.003 M) plus 400 μl of skin extract at 2 mg of protein per milliliter. Data points represent averages of two replicates. The experiment was repeated at least three times, with similar results each time. The curve shown for the mi<sup>bw</sup>/mi<sup>bw</sup> mouse, which lacks pigment cells, is similar to curves resulting from measurements using skin extracts from either type of albino mouse. ○, Genotype of skin extract a/a E/E Mi/Mi; ●, genotype of skin extract a/a e/e Mi/Mi; ■, genotype of skin extract A'/a E/E Mi/Mi; □, a/a E/E mi<sup>bw</sup>/mi<sup>bw</sup>; ○, blank.

Figure 3 shows the increase in absorbance at 475 nm, an absorbance maximum of dopachrome, when dopa is present as substrate. In the presence of control skin extracts from white mice which contain very few or no functional melanocytes, there is no increase in absorbance. Results are similar when skins from albino mice are used. These skin extracts will block conversion of dopa to dopachrome indefinitely, as shown in Figure 4. This photograph was taken after the reaction mixes had been at room temperature for several days. Apparently, in the presence of skin extracts, oxidation of dopa is prevented by a nonenzymatic blocking action. This blocking action is not melanocyte-specific, as extracts from “white” skins, which lack melanocytes, were used in the reaction mix shown in Figure 4. The buffer blank does eventually become pigmented, as shown in Figure 4. Extracts made from skins of recessive-yellow
(a/a e/e) mice are between black and lethal-yellow in their ability to catalyze formation of dopachrome from dopa. Recessive-yellow mice at 6 days of age are producing substantial amounts of nonyellow pigment in their skin and hair follicles (POOLE AND SILVERS 1976a).

Skin extracts from lethal-yellow (A'/a E/E) mice catalyze a small increase in absorbance. The initial lag and dip in the curve generated by lethal-yellow skin extracts, and shown in Figure 3, are characteristic of the activity curves of skin extracts from lethal-yellow mice. The reaction mixes containing extracts from yellow mice remain relatively unpigmented for an indefinite period of time; presumably, this results from an interaction between the low DO activity of the extract and the unrelated dopa-blocking capacity of all skin extracts that lack tyrosinase activity.

Figure 5 shows that the relative lack of DO activity in lethal-yellow extracts is true even when the extract of yellow skin is twice the protein concentration of the extract of black skin.

Figure 6 illustrates the results of combining different amounts of skin extract from lethal-yellow mice with a constant amount of extract from black mice in a reaction mix of constant total volume. The typical high rate of conversion normally catalyzed by black extracts is seen, but is preceded by a short lag period. A similar lag results from the addition of extracts made from skins of
FIGURE 5.—Conversion of dopa to dopachrome measured at 475 nm. Varying the concentration of the extract from black mice resulted in a marked difference in rate of production of dopachrome. Extract from lethal-yellow mice was not capable, at any concentration tested, of catalytic activity similar to that of extracts from black mice. The blank has been subtracted, but was similar to that shown in Figure 3. ■, Reaction mix contains dopa solution, buffer and extract made from skins of a/a mice (2 mg protein/ml); □, same as above, but skin extract at 1 mg/ml; ●, dopa solution, buffer and extract from A/y skin at 2 mg/ml protein; ○, same as above, but skin extract 1 mg/ml protein.

recessive-yellow mice (data not shown) or albino mice (data not shown) or from mice with no functional melanocytes (Figure 7).

Figure 8 illustrates the DO activity of skin extracts that had been filtered so that low MW substances were removed. The extract from black skin was still higher in DO activity than that from yellow skin. However, the usual lag period was absent from the reaction catalyzed by the filtered extract from yellow skin. Evidently the lag period is influenced by the low MW substances, but the rate difference in DO activity between yellow and nonyellow extracts is not. Thus, the difference in DO activity between yellow and nonyellow extracts is due neither to difference in concentration nor to the presence of low MW substances.

Having described the differences between skin extracts made from mice which were genetically different at the agouti and extension loci, we then assayed extracts from co-isogenic mice which had or had not been injected with MSH, in order to determine whether the catalytic activities would correlate with the manipulated phenotype or with the constant genotype of the mice. Table 2 shows the TH activities of skin extracts made from mice which had increased TH activity compared with those from uninjected controls. Recessive-yellow mice did not respond to MSH injection by increasing TH activity. Whether MSH-injected, sham-injected or uninjected, all extracts had TH activity that correlated with the phenotypes of the mice. This information confirms previous observations (GESCHWIND and HUSEBY 1972).

We then wished to compare the TH activities of skin extracts from respon-
FIGURE 6.—Conversion of dopa to dopachrome measured at 475 nm. Adding extract of lethal-yellow mouse skins to the extract of black mouse skins does not cause a major change in the rate of production of dopachrome, but does cause a lag in its initiation. ■. Reaction mix contains equal amounts of dopa solution, buffer and extract from A/y/a mice; ○, dopa solution, a/a extract and A/y/a extracts in equal amounts; ●, dopa solution, a/a extract and buffer in equal amounts; □, dopa solution, a/a extract and A/y/a extract, the latter at half the normal protein concentration. All tubes contained the same total volumes of reaction mix.

Sive yellow and black mice which had been maximally stimulated by injection of MSH. Extracts from the MSH-injected A/y/a mice contained approximately 80% the TH activity of the extracts from MSH-injected black mice. Possibly, this moderate difference results from a smaller number of melanocytes in the lethal-yellow than in the black mice (Silvers 1979). Differences in the DO
FIGURE 7.—Conversion of dopa to dopachrome measured at 475 nm. Extracts from skins of recessive-yellow mice or extracts from skins of mice which have no pigment cells are added to extracts of skins from black mice in the standard reaction mix. Each of the extracts that has been added results in a lag before synthesis of dopachrome is initiated, but does not greatly influence the rate at which dopa is converted to dopachrome by extracts of skins from black mice. All extracts are identical at the agouti and extension loci; therefore, the observed differences cannot be caused by differences at these loci. Since "white" mice have no pigment cells, the lag also cannot be caused by a melanocyte-specific process or compound. ●, Blank; □, a/a E/E m1/m1 and buffer; ■, a/a E/E M1/M1 and a/a E/E m1/m1; ○, a/a e/e and buffer; ●, a/a E/E and a/a e/e.

activity, however, are much greater than differences in the TH activity (Figure 9). Apparently, there is a genuine difference in the ability of tyrosinase to
FIGURE 8.—Dopa oxidase activities in extracts of the same skins that have been or have not been filtered to remove low molecular weight substances. It is clear that the low molecular weight substances do influence the lag portion of the curve. Probably they do not influence the rate of the reaction, and the altered slopes of the curves generated by the filtered extracts represent removal of some of the tyrosinase during the filtering process. Clearly, the low molecular weight substances that were removed do not account for the difference in slope of the curve generated by extracts from black or from yellow mice. △, C57BL/6J-u/u extract not filtered; ○, filtered; □, C57BL/6J-Ay/a extract not filtered; △, filtered.

convert dopa to dopachrome in tissues that are producing pheomelanin, compared with those producing eumelanin.

The graph of DHI conversion activities (Figure 10) shows similarities with dopa oxidase activities when comparing relationships among the slopes of the curves. In the presence of skin extract, DHI was converted to melanochrome more slowly than it was in the blank that contained lysis buffer instead of skin extract. The two absorbance maxima of melanochrome are 300 nm and 540 nm. Because dopachrome has an absorbance maximum of 305 nm, the extracts were evaluated at 540 nm. The conversion of DHI to pigment, unlike the
TABLE 2
Tyrosine hydroxylase activities of extracts made from skins of C57BL/6J-Ay/a (yellow), C56BL/6J-e/e (yellow), C57BL/6J-a/a E/E (nonyellow, black) 6-day-old mice which (1) had not been injected, (2) had been injected with alpha-MSH in peanut oil, (3) had been injected with peanut oil alone

<table>
<thead>
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<th>Black</th>
<th>Yellow</th>
<th>Yellow</th>
<th>Date</th>
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<tr>
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<td>C57BL/6J-Ay/a e/e</td>
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<td>(2) Injected</td>
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Extracts were adjusted with lysis buffer to 4 mg/ml protein. Figures represent cpm of tritium after subtracting the blank.

conversion of dopa to dopachrome, proceeds to completion in the presence or absence of skin extract. The presence of unpigmented melanocytes, the presence of skin extract (with or without unpigmented melanocytes) and the presence or absence of active tyrosinase each influence the rate of increase in absorbance, as is seen when the various controls are compared (M. L. LAMOREUX, unpublished results). However, a more detailed analysis of controls over DHI conversion activity was thought not to be a productive enquiry to pursue in relation to our primary goal, to evaluate controls over the branch point of the melanogenic pathway, because of (1) the similarity of activity curves between DHI conversion activity and DO activity; (2) the complexity of interactions; and (3) the fact that DHI conversion activity of tyrosinase influences a step in eumelanogenesis far distal to the branchpoint in the melanogenic pathway.

DISCUSSION

PROTA (1980) suggested, based on a long series of experiments conducted by himself and others, the branching biochemical pathway for melanogenesis, which is essentially the same as that shown in Figure 1. Our data are consistent with this branching pathway.

It is clear from the phenotypes of mice which are mutant at the C, A or E loci that the C (albino) locus determines whether or not pigment will form, and the A (agouti) and E (extension) loci determine which color it will be. GESCHWIND and HUSEBY (1972) showed that the system can be artificially manipulated. Injection of alpha-MSH (melanocyte simulating hormone, melanotropin) into lethal-yellow (Ay/-), but not recessive-yellow (e/e), mice will
induce follicular melanocytes to discontinue pheomelanin production and switch to eumelanin production. The question is, how do the agouti and extension loci control the branch point of the biochemical pathway and direct the follicular melanocytes to produce eumelanin or pheomelanin?

Before considering the immediate control over the pathway, it is useful to review some characteristics of the series of reactions apparently controlled by the agouti and extension loci. The agouti locus directs melanogenesis as a result of its gene action in the mesodermally derived cells that surround and nourish the hair follicle (Silvers and Russell 1955; Mayer and Fishbane 1972; Poole and Silvers 1976b). The gene action of the extension locus is autonomous to the ectodermally derived pigment cell (Lamoreux and Mayer 1975; Poole and Silvers 1976a). Tamate and Takeuchi (1984) cultured skins taken from 7.5-day-old mice. They showed that addition of alpha-MSH to the culture medium induced A<sup>+/a</sup>, but not e/e, mouse skins to initiate synthesis of eumelanin, as had been shown in vivo. Addition of dibutyryl cyclic AMP (dbcAMP) to the medium, however, caused a switch from pheomelanogenesis

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**FIGURE 9.**—Conversion of dopa to dopachrome measured at 475 nm. Extracts from yellow mice which had been injected with MSH had dopa oxidase activities similar to those from black mice. ■, Extract from skins of uninjected lethal-yellow (A<sup>+/a</sup>) mice; ○, from sham-injected lethal-yellow mice; —, from lethal-yellow mice which had been previously injected with α-MSH; Δ, sham-injected black (a/a) mice; ●, α-MSH-injected black mice; ▲, blank.
**FIGURE 10.**—Indole conversion activity. Reaction mixes contained the same dopachrome solution and skin extracts as is the case for the DC assay (Figure 2). However, instead of water, 0.1 M phosphate buffer, pH 8, is added, to rapidly convert the dopachrome to DHI. Then, the conversion of the DHI to melanochrome is followed spectrophotometrically. Controls are subtracted as in the DC assay. Each data point represents the average of two cuvettes. At least three assays were run with similar results. The slopes of the curves, relative to each other, of the extracts from the yellow and nonyellow tissues are very similar to those seen in the DO assay. The blocking activity which has been attributed to DCF cannot be seen in this graph, but in similar assays that compare extracts from “white” mice with extracts from albino mice, the albino extracts do slow the conversion of DHI to melanochrome more than do the extracts from white mice, which contain no melanocytes and no evidence of DCF activity. 0, Blank; O, C57BL/6J-a/a, MSH-injected; □, C57BL/6J-A/y/a, MSH-injected; ■, C57BL/6J-a/a, not injected; □, C57BL/6J-A/y/a, not injected; □, C57BL/6J-a/a e<sup>0</sup>/c<sup>0</sup>, uninjected albino control.

...to eumelanogenesis in skins from yellow mice of both genotypes. TAMATE and TAKEUCHI therefore concluded, based on all the above information, that the **agouti** locus controls an extracellular message, and the **extension** locus controls some element necessary to reception of the message by the melanocyte. Thus, the result of the interactions of the functions of the **agouti** and **extension** loci is presumed to be control over levels of cyclic AMP (cAMP) within the melanocyte, the cAMP levels in some way determining whether eumelanogenesis or pheomelanogenesis will take place in competent melanocytes.

cAMP is known to activate protein kinases that influence enzymatic activities. In melanoma cells, fluctuations in levels of cAMP may cause changes of the activities of protein kinases (PAWELEK 1979a). But whether the **agouti** and **extension** loci exert their influence over the branch point of the melanogenic
pathway by controlling levels of cAMP, or through some other mechanism, the result is a modulation in catalytic activities of both tyrosinase and DCF. The known modulations in catalytic activities are that (1) skin extracts (Figure 2) or hair follicle extracts (Barber et al. 1985) taken from yellow mice have no DC activity, and (2) they also have low DO and TH activities compared with the extracts taken from black mice (Figures 3, 5, 6; Table 2). The relatively low TH activity may result from the yellow mice having fewer melanocytes.

In an effort to clarify the relationships among the genotype, the phenotype and the catalytic activities, we injected alpha-MSH into lethal-yellow mice at 3 and 5 days of age, then we prepared skin extracts from these genetically yellow mice that had been induced to produce eumelanin. The TH activity of these skin extracts was approximately 80% of the TH activity in similarly injected genetically black (a/a) littermates (Table 2). This fact suggests that black mice have no more than 20% more melanocytes in their skin than do yellow mice. There may, of course, be a difference between the amount of tyrosinase produced in pheomelanic melanocytes compared with eumelanic melanocytes. Immunoprecipitation assays are under way to evaluate that question. But, even without knowing the exact amount of tyrosinase present, we can still determine that DO activity is reduced in yellow extracts. The fourfold dilution of skin extracts from black mice is more than sufficient to reduce the TH activity to that observed in skin extracts from yellow mice; it is not sufficient to replicate the reduced DO activity of the skin extracts of the yellow mice (see Figure 5). Thus, although yellow mice may contain fewer melanocytes than nonyellow mice, it seems unlikely that the relatively low rate of DO activity in their tissue extracts can be adequately explained in terms of quantity of tyrosinase present in the extracts.

It has been proposed that the presence of cysteine, glutathione or other low molecular weight inhibitors of tyrosinase activity may be responsible for the differences between pheomelanogenesis and eumelanogenesis. However, the presence of low molecular weight inhibitors of tyrosinase activity, although apparently causing a lag phase in the curve, does not seem to influence the differences in the rate at which dopa is converted to dopachrome (Figure 8). Taking all these observations into consideration, we conclude that there is a genuine difference between pheomelanic and eumelanic tissues in the DO activity of their tyrosinase, as well as in the DC activity of their DCF.

It is useful to relate these observations to the three hypotheses that have been proposed to explain A-locus and E-locus control over the branch point of the melanogenic pathway in murine melanocytes. Prota (1980) and Quevedo, Fleischmann and Holstein (1981) proposed the first hypothesis, that the branch point is controlled by increased availability of cysteinyldopa, a low molecular weight substance, as a substrate in cells that then produce yellow pigment. If this were the primary cause of the difference between a/a and A'/a mice, then one would expect an equal mixture of skin extracts from a/a and A'/a mice either to have an activity approximately midway between the two, which is contrary to observations presented here (Figure
6), or to exhibit an initial lag until the cysteinyldopa or other substrate is consumed, followed by increased activity, as was observed. However, a similar lag is seen when extracts from a/a E/E (black) mice were combined with skin extracts from mt/heterozygote mice which were also a/a E/E (Figure 7). Thus, the observed lag cannot be attributed specifically to any characteristic of melanocytes, because mt/heterozygote mice have essentially no melanocytes. Because the agouti locus is known to exert its influence via the tissues that surround the melanocyte, it might conceivably be that the nonmelanocytic tissues of A/y mice could cause the lag. However, neither the agouti locus nor the extension locus can be responsible for the lag in these experiments, because all the extracts used were identical in their agouti and extension loci genotypes. Thus, there is no evidence from these data that suggest agouti-locus or extension-locus control over differences in substrate that influence rate of eumelanogenesis in vitro.

PRADE and TERENCEI (1982) found that removal of low-molecular-weight substances from Neurospora crassa tissue extracts eliminated the lag phase from the curve that illustrates dopa to dopachrome conversion as catalyzed by tyrosinase, and it increased the slope of the curve. While removal of low-molecular-weight substances from extracts of yellow mouse skin also removed the lag phase from the curve, it did not increase the slope of the curve (Figure 10). Work of other investigators also bears on the question of tissue milieu on the catalytic activities controlling melanogenesis. HOLSTEIN, BURNETT and QUEVEDO (1967; HOLSTEIN et al. 1973) showed that electrophoretically separated isozymes of tyrosinase in hair follicle extracts of yellow mice differ from those of black mice.

The differences suggest that the DO activity of the tyrosinase of the yellow mice differs from that of the black mice, or that there are different quantities of the various isozymes. The only further information available regarding the catalytic activities of the various tyrosinase isozymes comes from nonyellow tissues. The purified T4 isozyme (the isozyme found in the pigment granule) of black mice has an increased ability to convert dopa or dopachrome to pigment, compared with other isozymes (HEARING, KORNER and PAWELEK 1982). If these differences among the isozymes, or the differences between the isozymes of yellow and nonyellow mice, were due to lack of substrate, or to the presence of molecules that prevent the spontaneous conversion of dopaquinone to dopachrome, or to the presence of an enzyme that uses dopaquinone as substrate in the production of pheomelanin, then electrophoresis should separate the tyrosinase isozymes from the molecules that influence them, leaving tyrosinase free to perform its catalytic functions similarly whether it was obtained from yellow or from nonyellow skin extracts. The fact that this is not the case suggests the differences in DO activity between skin or hair follicle extracts of yellow mice and those of nonyellow mice reflect a property of tyrosinase itself or of a molecule coupled with tyrosinase.

A second hypothesis was proposed by BARBER et al. (1985), who suggested that the level of "DCOR" (DC activity) in melanocytes determines whether or not yellow pigment will form in the melanocyte and that the E locus may "be
the DCOR locus." If this is meant to suggest that a structural mutation at the E locus results in the inability of e/e melanocytes to respond to normal stimuli, then the explanation is not sufficient. This hypothesis does not explain why DO activities of a different enzyme, tyrosinase, are also consistently reduced in pheomelanic tissues or why the follicular melanocytes are functionally different from those outside the hair follicles, which continue to produce eumelanin.

The third hypothesis, that of TAMATE and TAKEUCHI (1984), falls short of clarifying the same two questions. They suggest that the E locus controls the responsiveness of a cell-surface receptor. However, even the e/e melanocyte, while it does not respond to MSH, does respond differentially to the follicular and extrafollicular environments; the e/e melanocyte is capable of response to environmental stimuli that control the eumelanin/pheomelanin switch mechanism.

Obviously, control over the eumelanogenesis/pheomelanogenesis branch point in the biochemical pathway is complex, and probably a portion of the true picture is explained by each of the above hypotheses. Melanocytes obviously must recognize and respond to a "signal" from the mesodermally derived tissues, a signal controlled by the agouti locus. The signal is not MSH. Melanocytes probably respond by modulation of intracellular levels of cAMP, DO activity and DC activity. At least in the case of tyrosinase, as discussed above, the change in activity cannot be explained by a difference in the quantity of enzyme. In our opinion, an explanation that most adequately accounts for all these observations is that the modulation of catalytic activities of both enzymes is posttranslational and is influenced by intracellular fluctuations in cAMP levels that are responding to a signal other than MSH. It is tempting to speculate that the specific change that causes modulation of DC and DO activities involves a reversible physical interaction between tyrosinase and DCOR in the pigment granule.

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