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# 5,6-Dihydroxyindole-2-carboxylic acid is incorporated in mammalian melanin

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The role of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) in the biosynthesis of melanins has been studied by using the incorporation of specifically radiolabelled melanogenic precursors into melanins formed by melanocytes growing *in vitro* and *in vivo*. Extracts of mouse melanocytes and intact viable melanocytes were found to incorporate into melanin from 25% to more than 60% of [1-$^{14}$C]tyrosine. Melanins from melanoma tumours grown in mice were radiolabelled with 3,4-dihydroxy[1-$^{14}$C]phenylalanine, purified and chemoselectively decarboxylated. Determination of the $^{14}CO_2$ evolved showed that at least 20% of the precursor incorporated *in vivo* retains the label in the form of non-aminoacidic aromatic-type carboxyl groups. These results provide the first unambiguous demonstration that DHICA is incorporated in physiologically relevant amounts in mammalian melanins.

## INTRODUCTION

Among the natural pigments, the melanins occupy a unique position because of their involvement in human pigmentation (Prota & Thomson, 1976; Fitzpatrick, 1981; Pawelek & Körner, 1982; Hearing & Tsukamoto, 1991), and the intrinsic importance of related phenomena at the biological and physiological levels (e.g. skin photoprotection, suntanning, hair-greying and aging). Additional interest in these pigments derives from their occurrence in malignant melanoma (Nicolaus, 1968), a tumour whose increasing incidence and resistance to conventional therapeutic regimens represents a challenge to the whole scientific community.

Despite intensive investigations carried out since the turn of the century, the structure and origin of melanins is still far from being well understood (Riley, 1974; Swan, 1974; Prota, 1988). In mammals and other vertebrates, pigment formation is known to take place in a lineage of unique cells, termed melanocytes (Quevedo *et al.*, 1987), which are localized mainly at the epidermal–dermal junction and in hair bulbs. These cells contain the copper-containing enzyme tyrosinase (EC 1.14.18.1) (Hearing & Ekel, 1976; Körner & Pawelek, 1982; Hearing & Jiménez, 1987; Lerch, 1988), which catalyses the initial events of melanogenesis, namely the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and its conversion into DOPAquinone. According to the classical studies *in vitro* by Raper (1927) and Mason (1959), the subsequent steps of the pathway proceed spontaneously and involve the oxidative cyclization of DOPAquinone to afford the relatively stable intermediate DOPAchrome. At physiological pH values, DOPAchrome undergoes rearrangement with concomitant decarboxylation to give 5,6-dihydroxyindole (DHI), which is then converted into melanin by way of 5,6-indolequinone: hence Mason's (1959) view that melanins were homopolymers consisting of DHI units (Scheme 1).

Evidence that has accumulated in recent years, however, suggests that *in vivo* the rearrangement of DOPAchrome is under the regulatory control of a specific enzyme, DOPAchrome tautomerase (EC 5.3.2.3) (Pawelek *et al.*, 1980; Körner & Pawelek, 1982; Barber *et al.*, 1984; Leonard *et al.*, 1988; Aroca *et al.*, 1990; Palumbo *et al.*, 1991), which diverts the normal course of the reaction towards the non-decarboxylative formation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA). A similar effect on the rearrangement of DOPAchrome is exerted by some transition-metal cations, e.g. $Cu^{2+}$, $Zn^{2+}$, which are commonly found in pigmented tissues (Palumbo *et al.*, 1987, 1988, 1991). The intervention of these regulatory factors in the melanogenic pathway has been taken to account for the occurrence of DHICA in certain subcellular melanogenic compartments within melanocytes, including notably the coated vesicles and the premelanosomes (Hatta *et al.*, 1988; Chakraborty *et al.*, 1989), as well as in physiological fluids (Rorsman & Pavel, 1990). Yet, whether and to what extent DHICA is also involved in the build-up of the melanin polymer has so far been more a matter of surmise than of direct proof, owing to the notorious difficulties in the isolation, chemical degradation and spectral analysis of natural pigments (Riley, 1974; Swan, 1974; Prota, 1988). We now report the results of biosynthetic experiments measuring the incorporation of specifically radiolabelled melanogenic precursors into mouse melanomas which provide the first conclusive demonstration that DHICA is integrated in physiologically significant amounts into melanins *in vivo*.

## EXPERIMENTAL

### Materials

Specifically radiolabelled compounds used in this study were as follows: [U-$^{14}$C]tyrosine (sp. radioactivity 498 mCi/mmol) and [1-$^{14}$C]tyrosine (sp. radioactivity 48 mCi/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.); [1-$^{14}$C]DOPA (sp. radioactivity 6.2 mCi/mmol) and [3-$^{14}$C]-DOPA (sp. radioactivity 12 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). The specific radioactivities of all labelled precursors were adjusted to 6 mCi/mmol before use. DHI and DHICA were prepared by standard procedures (Benigni & Minnis, 1965). All other chemicals used were of the highest purity available. Soluene 100 was from Packard. When necessary, distilled deionized water was

Abbreviations used: DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DOPA, 3,4-dihydroxyphenylalanine.

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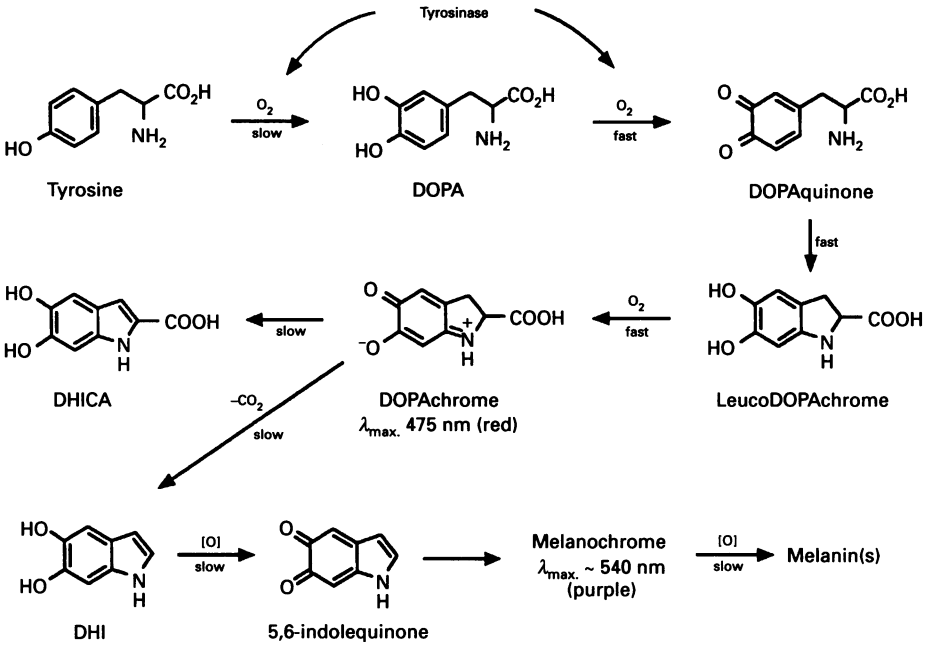


**Scheme 1. Early stages of melanogenesis, according to Raper (1927) and Mason (1959)**

All steps following DOPAquinone formation are envisaged as proceeding spontaneously. Under the reported conditions *in vitro*, the rearrangement of DOPAchrome is slow and gives a DHI/DHICA ratio greater than 49:1 (Palumbo *et al.*, 1987; 1988).

made $CO_2$-free by boiling and was then stored under $CO_2$-free $N_2$. Saturated $Ba(OH)_2$ was filtered under $N_2$ and stored under $N_2$.

### Cell lines and tissue culture

The derivations of the immortalized mouse melanocyte cell lines melan-a (black) and melan-b (black) (Bennett *et al.*, 1987, 1989) and the mouse melanoma cell lines B16 F10 (black) and S91 (brown) (Hearing *et al.*, 1985; Law *et al.*, 1987) used in this study have been previously reported. Cells were routinely grown *in vitro* in medium containing 10% (v/v) fetal-bovine serum and other supplements as detailed in the original references and by Jiménez *et al.* (1988, 1991). Cells were harvested with brief trypsin treatment, and all tissue-culture media and supplies used were obtained from GIBCO Corp. (Grand Island, NY, U.S.A.), unless otherwise noted. C57B16 mice used were pathogen-free 6-week-old females obtained from the NIH Small Animal Section.

### Melanogenic intermediates produced by melanocyte extracts *in vitro*

Cells grown in tissue culture as noted above were harvested by trypsin treatment, and then solubilized with 1% Nonidet-P40/0.01% SDS/0.1 M-Tris/HCl (pH 7.2) aprotinin (1 μg/ml)/100 μM-phenylmethanesulphonyl fluoride for 60 min at 4 °C. Protein concentrations of the extract supernatants after centrifugation to 12000 *g*-min were measured (Bramhall *et al.*, 1969), adjusted to 1 mg/ml, and then assayed for melanin production at 37 °C by using [U-$^{14}$C]tyrosine or [1-$^{14}$C]tyrosine (Hearing & Ekel, 1976; Hearing, 1987). At various times (noted in Table 1 and Figs. 1 and 2), samples were removed to 3MM filter-paper discs, air-dried and measured for radioactivity in a liquid-scintillation counter after washing as detailed by Jiménez *et al.* (1988). In some experimental protocols, identical samples were removed and analysed for DHI and DHICA production by h.p.l.c. All h.p.l.c. analyses were performed on a Beckman model 344 instrument with a $C_{18}$ 5 μm-pore column (4.6 mm × 250 mm). The mobile phase was aq. 0.15 M-$Na_2B_4O_7$/25% (v/v) methanol, adjusted to pH 2.5 with HCl, as previously described (Palumbo *et al.*, 1987, 1988). Compounds were detected by their $A_{280}$ and/or by liquid-scintillation counting of the eluted radioactive peaks. Measurement of retention times, peak areas and radioactivity, and comparison with external calibration curves for DHI, DHICA and for radioactive precursors, allowed quantitative analysis of melanogenic indoles. Assays were routinely performed in triplicate (except where noted), and results are presented as means of the independent assays ± S.E.M.

### Incorporation of radiolabelled tyrosine into intact melanocytes growing *in vitro*

Melan-a (black) and melan-c (albino) melanocytes were seeded into sterile 96-well microtitre plates and allowed to grow to confluence. The cells were then washed twice in tyrosine-free medium containing 1 μg of cycloheximide/ml and incubated (times noted in Fig. 3) in tyrosine-free medium containing cycloheximide and specifically labelled [U-$^{14}$C]tyrosine or [1-$^{14}$C]tyrosine. At the end of the pulse-labelling period, the cells were washed in Hanks balanced salt solution, harvested with trypsin, and solubilized in the detergent buffer as detailed above. Samples of the extracts were then removed to 3MM filter-paper discs, air-dried and assayed for melanin that had been produced.

### Incorporation of radiolabelled DOPA into melanoma melanin *in vivo*

**(a) Isolation of melanoma melanin.** B16 F10 melanomas were grown subcutaneously in the rear flanks of C57B1 mice. When the tumours were ~ 1 cm in diameter, the mice were treated with daily intravenous inoculations of 3 μCi of [1-$^{14}$C]DOPA or [3-$^{14}$C]DOPA per day for 5 days. The mice were then killed and the tumours excised and weighed. Melanin from the tumours was prepared after homogenization in 70% (v/v) ethanol and centrifugation at 17300 *g* for 15 min; the precipitates were re-extracted three times with water. The washed melanins were then further purified by sucrose-density-gradient (30%, 25%, 20% and 15%) ultracentrifugation at 150000 *g* for 60 min. The melanosomal fraction obtained at the bottom of 30% sucrose was washed with water and treated three times with 1% Triton X-100/0.1% SDS in 0.1 M-Tris/HCl, pH 7, overnight at 23 °C. The melanins were

then again extensively washed with water, dried in a silica-gel desiccator, and equilibrated in the presence of saturated $CaCl_2$ until a constant weight was obtained. Weighed samples of the labelled melanins were dissolved in Soluene and counted for radioactivity in a liquid-scintillation counter.

**(b) Decarboxylation of melanoma melanin.** For decarboxylation experiments, ~ 50 mg of each labelled melanin was suspended in 6 M-HCl and heated under reflux with bubbling $N_2$ gas (made $CO_2$-free by passage through KOH). Evolving $CO_2$ was collected in a tube containing saturated $Ba(OH)_2$; after 24 h, the $BaCO_3$ formed was collected by centrifugation (10000 *g*, 40 min) under $CO_2$-free $N_2$, rapidly washed with boiled water, collected on 3MM filter discs, washed with ethanol, dried and counted for radioactivity. The melanin remaining in the HCl suspension was centrifuged (150000 *g*, 60 min). The supernatant (soluble fraction) was collected and counted for radioactivity; the precipitate (decarboxylated melanin) was washed with water, dried over $P_2O_5$ and weighed, then dissolved in Soluene and measured for radioactivity.

## RESULTS AND DISCUSSION

Initially, we used [U-$^{14}$C]tyrosine to investigate the production of DHICA by extracts of melanoma cells growing *in vitro*. In the experiment reported in Table 1, it is shown that the synthesis of

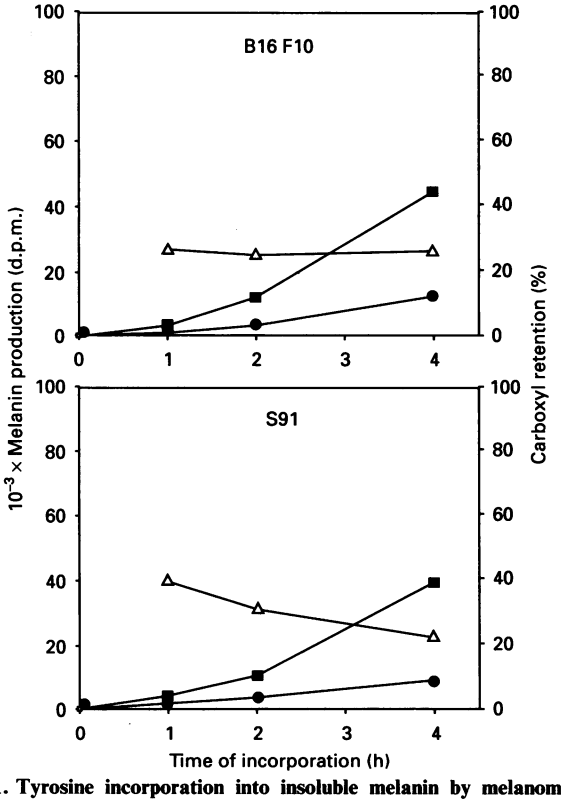


**Fig. 1. Tyrosine incorporation into insoluble melanin by melanoma-cell extracts**

Extracts of B16 F10 or S91 melanoma cells grown *in vitro* were prepared and assayed for melanin production by using [$^{14}$C]tyrosine, labelled either uniformly (■) or at the carboxyl (c-1) position (●), as detailed in the Experimental section. At the times noted, samples were removed to filter-paper discs, air-dried and assayed for melanin production. The results are shown as means of three samples taken at each time point (S.E.M. was < ±5%). 'Carboxyl retention' (△) indicates the relative production of carboxylated melanin compared with total melanin.

**Table 1. Melanogenic-intermediate production by melanocyte extracts**

B16 F10 or S91 melanoma cells growing *in vitro* were harvested and solubilized as detailed in the Experimental section, and protein concentrations of the extract supernatants were adjusted to 1 mg/ml before assay for melanogenic-intermediate production by using [U-$^{14}$C]tyrosine. At the times noted, samples were removed for analysis by h.p.l.c. and liquid-scintillation counting of eluted radioactive peaks. The results are presented as pmol of substrate or product remaining in the reaction mixture at the times specified. At those same times, samples were removed to filter-paper discs, air-dried and counted for radioactivity to measure melanin production; '% $CO_2$' reports the DHICA generated as a percentage of the total melanin produced.

| Melanoma | Incubation time (h) | Substrate or product (pmol) | | | % $CO_2$ |
|---|---|---|---|---|---|
| | | Tyrosine | DHI | DHICA | |
| B16 F10 | 0 | 750.2 | 0.0 | 0.0 | — |
| | 1 | 751.4 | 5.1 | 1.1 | 22 |
| | 2 | 721.0 | 11.7 | 3.2 | 27 |
| | 4 | 621.0 | 21.5 | 4.6 | 21 |
| | 8 | 550.6 | 25.0 | 7.3 | 29 |
| | 20 | 493.9 | 37.5 | 13.5 | 36 |
| S91 | 0 | 817.9 | 0.0 | 0.0 | — |
| | 1 | 716.0 | 3.8 | 0.3 | 8 |
| | 2 | 709.0 | 7.6 | 1.7 | 22 |
| | 4 | 628.0 | 15.1 | 3.3 | 22 |
| | 8 | 538.0 | 20.0 | 5.3 | 27 |
| | 20 | 428.6 | 32.2 | 10.6 | 33 |

DHICA relative to DHI by extracts of B16 F10 cells is approximately constant at 27% (S.E.M. ±3%, $n = 5$) over the time course of a 20 h incubation. Extracts of S91 melanoma cells also produced similar amounts of DHICA and DHI throughout the whole course of incubation (22 ± 4%, $n = 5$), although the rates of indole formation were slightly decreased compared with the rates of B16 F10 cells.

Specifically radiolabelled [1-$^{14}$C]tyrosine and [U-$^{14}$C]tyrosine were then used as probes to detail the rates of incorporation of DHI and DHICA into the insoluble melanin pigments (Fig. 1). The radiolabelled carboxyl (at position 1) is preserved when tyrosine is converted into DHICA, but is lost if DHI is formed instead, so that the relative radioactivity of melanins from [1-$^{14}$C]tyrosine and [U-$^{14}$C]tyrosine gives a rough yet reliable estimate of the degree of incorporation of DHICA with respect to total incorporation of the precursor. Carboxylated DHICA-derived units were shown to be incorporated into melanin to similar degrees by extracts of B16 F10 cells (26 ± 1%, $n = 5$) and of S91 cells (31 ± 4%, $n = 3$). These ratios are highly consistent with those determined in separate experiments using other protocols (cf Table 1). Note again the overall rates of melanin formation by S91 cells were significantly below those by B16 F10 cells.

Parallel experiments performed on immortalized normal melanocytes, which have defined genotypes of black and brown (melan-a and melan-b respectively) (Bennett *et al.*, 1987, 1989), demonstrated similar characteristics of incorporation (Fig. 2). Melan-a cells incorporated carboxylated derivatives into melanin as approx. 18% (±2%, $n = 4$) of the total pigment produced, compared with 23% (±5%, $n = 4$) incorporation by melan-b melanocytes. Though the levels of carboxylated precursors incorporated into melanin by both types of brown cells used in this study (S91 and melan-b) are somewhat higher than those of their black counterparts (B16 F10 and melan-a), the difference is not statistically significant, and is unlikely to be the underlying cause of the phenotypic differences of the melanins.

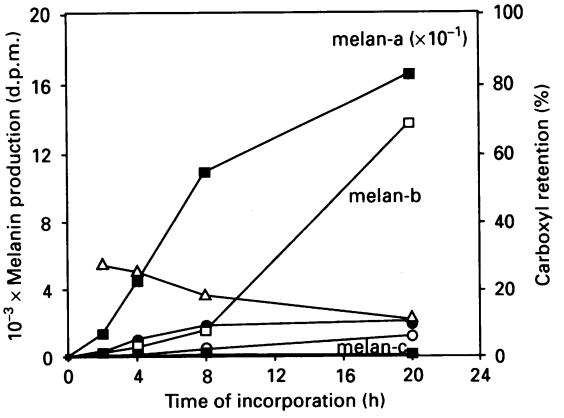


**Fig. 2. Tyrosine incorporation into melanin by normal melanocyte-cell extracts**

Melan-a (■, ●), melan-b (□, ○) and melan-c (■, □) melanocytes grown *in vitro* were prepared and assayed for melanin production by using specifically labelled [$^{14}$C]tyrosine (■, □, ■, U-$^{14}$C, ●, ○, □, 1-$^{14}$C), as detailed in the Experimental section. At the times noted, samples were removed to filter-paper discs, air-dried and assayed for acid-insoluble melanin production. Data presented are the means of assays performed in triplicate (S.E.M. was $< \pm 10\%$).

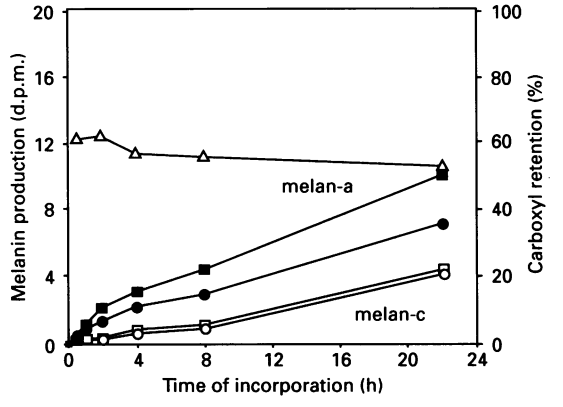


**Fig. 3. Melanins produced by normal melanocytes during growth in culture**

Melan-a (■, ●) and melan-c (□, ○) melanocytes were grown to confluence in microtitre plates, washed twice and incubated for the times noted in tyrosine-free medium containing cycloheximide and specifically labelled [$^{14}$C]tyrosine (■, □, U-$^{14}$C; ●, ○, 1-$^{14}$C), as detailed in the Experimental section. At the end of the pulse-labelling period, the cells were washed in Hanks balanced salt solution, harvested with trypsin, and solubilized. Samples of the extracts were then removed to filter-paper discs, air-dried and assayed for melanin production. 'Carboxyl retention' (△) indicates the percentage of carboxyl group incorporated into acid-insoluble melanin, after subtraction of incorporation (d.p.m.) into the amelanotic melan-c cells (which represents endogenous protein synthesis).

Thus far, we have dealt with detergent extracts of melanocytes grown in tissue culture, which might not be entirely physiologically relevant, in view of the multienzymic nature of the melanogenic pathway, the confined reaction environment afforded by the melanosome in which these processes occur *in vivo*, and the complex interactions involved. As a next step, we therefore examined the incorporation of DHICA-derived units into melanin, using intact and viable melanocytes, where the alignment of the melanogenic enzymes in the melanosomal membrane has not been altered. Since tyrosine is incorporated into proteins which are in part also insoluble, we have used the unpigmented melan-c cells (albino-mutant melanocytes) as an internal control. Interestingly, the incorporation of carboxylated units into melanins in viable melanocytes was significantly higher than the incorporation measured with detergent extracts of those same cells *in vitro*. The percentage carboxyl-group incorporation of the melan-a cells averaged 62% ($\pm 3\%$, $n = 6$) and 66% ($\pm 8\%$, $n = 6$) in two independent experiments (Fig. 3). In this experiment we have measured only the incorporation of radioactivity into insoluble melanin, and thus the rate of DHICA formation may actually be underestimated, since some of the DHICA may remain unpolymerized and would not be measured using this protocol.

**Table 2. DOPA incorporation into melanin by melanomas *in vivo***

B16 F10 melanomas were grown subcutaneously in the rear flanks of C57Bl mice until ~ 1 cm in diameter. The mice were then treated with daily intravenous inoculations of 3 $\mu$Ci of [1-$^{14}$C]DOPA or [3-$^{14}$C]DOPA for 5 days, and melanin was then purified as detailed in the Experimental section. The results from two independent experiments are reported as the specific radioactivity of the purified melanins labelled with the precursor indicated. Recoveries of radioactivity (%) in the soluble and insoluble melanin fractions after reflux in HCl are indicated, as is the percentage of radioactivity liberated in the $CO_2$ evolved after specific decarboxylation.

| Expt. no. | Melanogenic precursor | Sp. radioactivity (c.p.m./mg) | Recovery of radioactivity (%) | | $CO_2$ evolved (%) |
|---|---|---|---|---|---|
| | | | Soluble | Melanin | |
| 1 | [1-$^{14}$C]DOPA | 2700 | 47 | 41 | 11 |
| | [3-$^{14}$C]DOPA | 2900 | 45 | 55 | 0.1 |
| 2 | [1-$^{14}$C]DOPA | 500 | 65 | 20 | 15 |
| | [3-$^{14}$C]DOPA | 650 | 68 | 30 | 1 |

In the experiments presented above, we have demonstrated the active production of DHICA and DHI by extracts of melanocytes and by intact melanocytes, although the incorporation of these subunits into the melanins synthesized has not been definitively shown. Thus, in a conclusive series of experiments, a procedure was developed to probe on a chemical basis the presence of DHICA-derived units in melanoma melanins *in vivo*. Carboxyl-radiolabelled [1-$^{14}$C]DOPA was injected *in situ* to B16 melanomas growing in mice. As a control, specifically labelled [3-$^{14}$C]DOPA was used. After 5 days, the tumours were excised, and the radioactive melanins were isolated and carefully purified by a mild non-hydrolytic procedure involving sucrose-density-gradient ultracentrifugation and repeated extraction with detergents. The intact pigments thus obtained were analysed for total radioactivity, and were then subjected to a chemoselective acid decarboxylation reaction in boiling 6 M-HCl for 24 h (Ito, 1986). Under these conditions, control experiments showed that only those carboxyl groups linked to aromatic units, as in DHICA, are lost, whereas amino acidic and other aliphatic carboxylic groups remain virtually unaffected. The $CO_2$ evolved from the melanins was collected as $BaCO_3$ and counted. The data shown in Table 2 indicated consistently that the reference melanins derived from [3-$^{14}$C]DOPA did not contain labelled carboxyl groups. By contrast, the pigment(s) originating from [1-$^{14}$C]DOPA gave a lower total recovery of the label as compared with the reference melanin, owing probably to a partial loss of the C-1 carboxyl group after the rearrangement of DOPAchrome to DHI, but liberated on decarboxylation a significant fraction of the total radioactivity. These data provide unambiguous proof that part of the labelled carboxyl groups of melanoma melanin belong to cyclized aromatic-type units, i.e. to DHICA units.

Given the intrinsic limitations of the method, it is difficult to make a reliable estimate of the relative proportion of DHICA units. Yet, if the radioactivity found in the soluble fraction is assumed to be derived from the DOPA which has not been converted into melanin, an incorporation of DHICA of about 20% (Expt. 1) and about 40% (Expt. 2) can be deduced from the remaining values, by using the data for the reference melanin(s) as a basis of comparison. Such percentage values, however, do not take into account the fraction of DOPA which is decarboxylatively converted into DHI, which is roughly expressed by the relative decrease in the specific radioactivity of the carboxyl-labelled melanin with respect to the reference melanin. Residual radioactivity in decarboxylated melanins labelled with [1-$^{14}$C]DOPA may be due to uncyclized DOPA units present in the polymer. The observed discrepancies in the data from the two experiments might reflect a different degree of incorporation of the precursors into the melanocytes growing as subcutaneous tumours, but also might result from other variable parameters such as rate of growth of the tumours.

It should be cautioned that the results obtained in this study demonstrate that DHICA is incorporated into melanins *in vitro* and *in vivo*, but do not provide information as to the mode of such incorporation, i.e. whether the pigment formed consists of an intimate copolymer of DHI and DHICA or a mixture of DHI and DHICA homopolymers which otherwise remain separated. In most accounts of melanin pigmentation, the copolymer concept is generally accepted (Hempel, 1966; Nicolaus, 1968; Körner & Pawelek, 1982; Quevedo *et al.*, 1987; Hearing & Jiménez, 1987), and it has been suggested that up to 50% of the total monomeric units are derived from DHICA (Ito, 1986). However, evidence in support of this is largely circumstantial, or involves the determination of trace amounts of degradation fragments of questionable structural significance (d'Ischia *et al.*, 1985). In this connection, recent experiments *in vitro* (O. Crescenzi, A. Napolitano & G. Prota, unpublished work) indicate that co-oxidation of DHI and DHICA under biomimetic conditions gives mainly mixtures of the corresponding homopolymers, in keeping with their markedly different redox potentials.

After decades of studies on the structure and origin of melanin, DHICA is the first melanogenic intermediate to be conclusively demonstrated to be incorporated into the biopolymer *in vivo*. This result furnishes a direct proof that the balance of DHI versus DHICA is actively regulated in mammalian melanocytes, probably on a constitutive basis through the activity of DOPAchrome tautomerase and/or through the intervention of metal ions. Another important implication from our study is that natural melanins are conclusively shown to be different from synthetic pigments, which are known to consist almost exclusively of decarboxylated DHI units (Hearing *et al.*, 1980; Ito, 1986; Palumbo *et al.*, 1986). Such a difference, which is too often underlooked, represents a caveat to the widespread tendency to extend results of biophysical experiments from one type of pigment to the other. The incorporation of carboxylated DHICA units into natural melanins is also well consistent with the known property of these pigments to bind nitrogenous substances (Larsson & Tjälve, 1979) and heavy metals (Sarna *et al.*, 1976), and provides a new biochemical basis for the selective targeting of malignant melanoma by utilizing melanin-affinic drugs and radioisotopes (Link *et al.*, 1989).

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