Quantitative Measurement of Melanin as Tyrosine Equivalents and as Weight of Purified Melanin

ATSUSHI OIKAWA AND MICHIE NAKAYASU

Biochemistry Division, National Cancer Center Research Institute, Chuo-ku, Tokyo 104, Japan

Previously we reported stoichiometric studies on melanin synthesis in cultures of mouse melanoma cells (1). In this system there was no accumulation of intermediates in the Raper-Mason-Nicolaus pathway for melanin synthesis and the amount of hydroxylated tyrosine was quantitatively related to the release of hydrogen as water from positions 3 and 5 of the benzene ring of tyrosine during the series of reactions involved in melanin synthesis. Therefore, the amount of newly synthesized melanin could be expressed as tyrosine equivalents.

The present paper describes a colorimetric method for determination of melanin, using protein-free melanin as standard, and values are compared with those expressed as tyrosine equivalents. For this method, a new procedure was developed to solubilize melanin.

MATERIALS AND METHODS

Chemicals. "Soluene 100," a 0.5 N solution of dimethyl n-undecyl n-dodecyl ammonium hydroxide in toluene, is a product of Packard Instrument Co. L-Tyrosine-3,5-3H was purchased from the Radiochemical Centre, Amersham and L-3,4-dihydroxy-phenylalanine (dopa) and mushroom tyrosinase, grade III, from Sigma Chemical Co. Eagle's minimum essential medium and calf serum were obtained from the Serum Institute, Chiba.

Cells and tumors. Stable melanotic and amelanotic clonal cell lines, C2M (2) and C3W (3), respectively, of mouse melanoma B 16 were used. Culture conditions were as reported previously (1,4). C2M cells were implanted into C57BL mice and the resulting black tumors (B 16–C2M) were used for preparation of melanoma melanin.

Preparation of Standard Melanins

i. Autoxidation of dopa. A solution of 51 mg of L-dopa in 40 ml of 50 mM sodium phosphate buffer (pH 8.1) was vigorously aerated for 48 hr at room tem-
perature. The solution was acidified with 1 ml of concentrated HCl and centrifuged and the precipitate was washed with 10 ml vol of 6 N HCl, distilled water (four times), acetone (three times), ether, and air-dried. The black powder was finely ground in an agate mortar and dried in vacuo overnight.

ii. Synthesis of melanin using mushroom tyrosinase. A mixture of 47 mg of L-dopa and 1 mg of purified mushroom tyrosinase in 20 ml of 0.1 M sodium phosphate buffer (pH 6.8) was incubated for 4 hr at 37°C with shaking and then left overnight at room temperature. The black pigment was precipitated by addition of 1 ml of concentrated HCl and was washed with distilled water. It was then refluxed with boiling 6N HCl for 48 hr with occasional renewal of the HCl. The insoluble pigment was washed and dried as described above.

iii. Synthesis of melanin with a melanoma cell extract. A mixture of 5 mg of L-dopa and a cell-free extract of C57M cells (3.8 mg as protein) in 25 ml of 0.1 M sodium phosphate buffer (pH 6.8) containing 50 µg of chloramphenicol per ml was incubated for 24 hr at 37°C with occasional shaking. The black pigment was treated with HCl as described above but was refluxed for 72 hr. The purified melanin was washed and dried as described above.

iv. Melanin from melanoma and black hair of mice. A B 16–C57 melanoma was homogenized in distilled water and filtered through four layers of gauze. The filtrate was mixed with 2 vol of concentrated HCl, and stood at room temperature for about 24 hr, and then the mixture was diluted with water. The black pigment was collected by centrifugation, refluxed with boiling 6N HCl for 72 hr and then washed and dried as described above. The hair of C57BL mice was washed with lukewarm water containing neutral detergent and then with distilled water. The hair was then suspended in water and treated with concentrated and with 6N HCl in the same way as with the melanoma homogenate. The hair readily decomposed and gave a black precipitate, which was washed and dried.

This procedure is a slight modification of that of Nicolaus (5).

Protein Content of Melanin Preparations

A weighed sample was hydrolyzed with redistilled azeotropic HCl for 24 hr at 110°C in an evacuated, sealed tube and the hydrolyzate was filtered through a Millipore Filter, HA. It was analyzed with an automatic amino acid analyzer, Model JLC-5AH (Japan Electron Optics Lab. Co.) with a 10 mm flow cell using the highest sensitivity. Protein content was calculated as the sum of the weights of individual amino acid residues.

Determination of Tyrosinase Activity and Melanin Content

The tyrosinase activities of cells in cultures and in cell-free extracts were determined as reported previously (1), and expressed as the amount of tyrosine hydroxylated (ΔTy) during the indicated time.

Fractions of washed cells or cell-free extracts precipitated with 5% trichloroacetic acid were dissolved in “Soluene 100” and their absorbance at 400 nm was determined with a Hitachi Spectrophotometer, model 124. Melanin purified from melanoma tissue by HCl treatment was used as a standard. The amounts of melanin were expressed as the product of the absorbance at 400 nm and volume of the solution, or the weight of the standard melanin.
RESULTS

_Solubilization of purified melanin preparations._ In contrast to crude and wet preparations of melanoprotein, purified melanin preparations treated with boiling 6 \(N\) HCl are almost insoluble in 1 \(N\) KOH. But they can be solubilized in “Soluene 100.”

Purified preparations were suspended in an appropriate volume of “Soluene 100” and sonicated for 2 min. The suspension was kept for several hours at 37°C, and then sonicated again for 1–5 min. The resulting preparation was judged to be completely solubilized since there was no further increase in absorbance at 400 nm on further sonication.

Crude melanin preparation and packed cells of cultured melanoma became solubilized within 1 hr.

_Effect of HCl-treatment on the extinction coefficient._ As shown in Fig. 1, the extinction coefficient of melanin after 24-hr treatment with boiling 6 \(N\) HCl increased slightly during further acid-treatment and had almost reached a plateau after 72 hr, when the contaminating substances, mainly protein, had almost completely been eliminated (0.2% protein by weight).

_Absorption characteristics of solubilized melanin._ The absorption spectrum of solubilized melanotic melanoma cells C\(_2\)M is shown in Fig. 2A with that of amelanotic melanoma cells C\(_2\)W. Under the conditions used, cellular components other than melanin had no adsorption at 400 nm.

The absorption spectra of purified melanin preparations from various sources were similar, with a monotonic increase in absorbance from 700 to 300 nm, but differed slightly in gradient and melanoma melanin had a slight shoulder around 400 nm (Fig. 2B). In relation to the gradients it should be noted that natural melamins and melamins synthesized from dopa \textit{in vitro} differed in their \(A_{500}/A_{400}\) and \(A_{360}/A_{400}\) ratios (Table 1).

The change in the absorption pattern of solubilized melanin during incubation at 37°C showed an apparent isosbestic point around 360 nm, as seen in Fig. 3. At 400 nm the decrease in absorbance with time was slight and quantitative deter-

![Fig. 1. Effect of acid treatment on the extinction coefficient of melanin. A tumor homogenate or hair of mice was treated with concentrated HCl. Then insoluble material was refluxed with boiling 6 \(N\) HCl for the indicated times and washed and dried as described in the Methods. The resulting black powder was solubilized in “Soluene 100” and the absorbance was determined at 400 nm. Ordinate: extinction coefficient in cm\(^2\)/mg.](image-url)
QUANTITATIVE MEASUREMENT OF MELANIN

Fig. 2. Absorption spectra of melamins. Absorbance of melanin solubilized in "Soluene 100" was recorded with a Hitachi Spectrophotometer, model 124, using the same solvent as the reference. A: Aqueous suspensions of melanotic (a) and amelanotic (b) melanoma cells (each 2.39 mg protein/ml) were solubilized with 10 vol of "Soluene 100." B: Purified melamins solubilized in "Soluene 100" prepared from melanoma B 16–C2M (a), by oxidation of dopa with a cell-free extract of melanoma cells C2M (b) and with mushroom tyrosinase (c), and by autoxidation of dopa (d). Measurements were made about 5 hr after addition of "Soluene 100," when solubilization was complete. The absorption curves at the bottom are those of solvent identical with that of the reference.

Solubilization was possible if carried out between 3 and 5 hr after the start of solubilization.

Melanin solutions followed Beer’s law, at least up to an absorbance of 1.6 at 400 nm.

Extinction coefficients. The extinction coefficients at 400 and 360 nm of melanin preparations are given in Table 1. Natural melanins produced by mouse melanoma

TABLE 1

| Preparation                          | $A_{400}/A_{400}$ | $A_{360}/A_{400}$ | Extinction coefficient at 400 nm | Extinction coefficient at 360 nm |
|--------------------------------------|-------------------|-------------------|---------------------------------|---------------------------------|
| Natural melanin                     |                   |                   | $(\text{cm}^2\text{mg}^{-1})$    |                                 |
| Melanoma B 16–C2M                    | 0.71              | 1.07              | 1.9 ± 1.7                       | 21.8 ± 1.8 (5)                  |
| Black hair of C57BL mice             | 0.79              | 1.09              | 19.8; 20.0                      | 21.6; 21.7                     |
| C2M melanoma cells$^b$               | 0.70              | 1.11              | —                               | —                               |
| Melanin synthesized from dopa        |                   |                   | (cm$^2$mg$^{-1}$)               |                                 |
| Catalyzed by cell-free extract of C2M cells | 0.61            | 1.19              | 20.1; 18.1                      | 24.2; 22.1                     |
| Catalyzed by purified mushroom tyrosinase | 0.63            | 1.18              | 27.4; 28.2                      | 32.9; 33.9                     |
| Autoxidation                         | 0.56              | 1.20              | 28.7 ± 0.4                      | 34.6 ± 0.5 (3)                  |

$^a$ Procedures for preparation are described under Methods. Determinations were carried out immediately after complete solubilization, within 5 hr after addition of "Soluene 100." Figures in brackets are numbers of preparations.

$^b$ Cultured C2M cells were washed, packed and immediately solubilized.
and hair bulb melanocytes had the same extinction coefficient. The extinction coefficient of melanin synthesized from dopa by a cell-free extract of cultured cells of mouse melanoma was the same as that of natural melamins of mouse origin at 400 nm. However, preparations of melanin produced by autoxidation of dopa or by oxidation of dopa with mushroom tyrosinase definitely had higher values.

Amount of melanin synthesized by cell-free extracts and cultured cells estimated photometrically and as tyrosine equivalents. The amount of tyrosine converted to melanin (tyrosine equivalents of melanin, ΔTy) and the amount of melanin estimated photometrically (ΔA_{400}V, defined in the legend to Table 2) using whole cells and extracts of C_{2}M melanoma cells were compared. As shown in Table 2, these values were proportional and the ratio, ΔA_{400}V/ΔTy, the molar extinction coefficient of monomer units of melanin, was fairly constant in both reaction systems, being 3000–3100 cm⁻¹M⁻¹ at 400 nm.

The value ΔA_{400}V was converted to the increase in weight of melanin (ΔMel) using the extinction coefficient of purified melanin from melanoma (Table 1). The ratio ΔMel/ΔTy should give the mean molecular weight of monomer units of melanin. The values given in the last column in Table 2 are slightly higher than the molecular weight of indole quinone, which is thought to be the main component of melanin.

DISCUSSION

Protein-free melanin was solubilized in a tissue solubilizer “Soluene 100,” 0.5 N solution of dimethyl n-undecyl n-dodecyl ammonium hydroxide in toluene. “Soluene 100” is probably effective because it is basic, and its lipophilic residues as well as toluene may also act in dispersing melanin. Using this procedure for solubilization, it will be possible to use milder treatments in structural studies on melanin than those used previously for insoluble melanin (5, 6).
TABLE 2
TYROSINE EQUIVALENTS AND INCREASE IN ABSORPTION OF MELANIN SYNTHESIZED BY CULTURED C2M MELANOMA CELLS

| Expt. no. and preparation | Melanin synthesized | "Molar"° increment coefficient at 400 nm |
|---------------------------|---------------------|------------------------|
|                           | Tyrosine equivalents | Increase, Increase | Extinction coefficient at 400 nm |
|                           | (ATy)                | in A₄₀₀V, in weight | (ΔA₄₀₀V/ΔTy) |
|                           | (nmole/tube)         | (cm⁻¹)                 | (g/mole)      |
|                           | ml/tube              | (μg/tube)      | (cm⁻¹M⁻¹) × 10⁻³ |
|                           |                      |                        |               |
| 1. Cell-free | 26.7 | 0.080 | 4.0 | 3.00 | 150 |
| extract        | 26.2 | 0.076 | 3.8 | 2.90 | 145 |
| 2. Cell-free | 127  | 0.380 | 19.1 | 2.99 | 150 |
| extract        | 127.5 | 0.403 | 20.2 | 3.16 | 158 |
| 3. Living cells in  | 63.9 | 0.195 | 9.8 | 3.05 | 153 |
| culture            | 67.0 | 0.217 | 10.9 | 3.24 | 163 |
| 74.7 | 0.231 | 11.6 | 3.09 | 155 |
| 70.1 | 0.211 | 10.6 | 3.01 | 151 |

Mean | 3.10 | 156 ± SD | 0.09 ± 5 |

a In Expts 1 and 2, reaction mixtures contained 2.50 nmole of L-tyrosine-3, 5-³H, 25 nmole of L-dopa and a cell-free extract of C2M cells in 250 aliters of 0.08 M sodium phosphate buffer (pH 6.8). In Expt 1 and 2 the amounts of protein were 0.17 and 0.22 mg and the specific activities of tyrosinase were 156 and 381 nmole tyrosine hydroxylated/mg protein/hour, respectively. Incubation was continued for 60 min in Expt 1, and for 90 min in Expt 2, at 37°C. The amount of tyrosine hydroxylated (ATy) was calculated from the radioactivities of water, dopa and melanin (1). Material was precipitated with 5% trichloroacetic acid and dissolved in "Soluene 100" and its absorbance was measured at 400 nm. The increase in weight of melanin during the incubation (ΔMel) was calculated from ΔA₄₀₀V using the extinction coefficient of melanoma melanin (Table 1).

In Expt 3, 4-day old cultures of C2M cells in TD-15 culture flasks (11.7 ± 4.1 × 10⁵ cells/culture) were used. Each culture received 4 ml of culture medium containing 8 μCi of L-tyrosine-3,5-³H at 0-time and cultivation was continued for 2 days, changing the medium after 24 hr. ATy was determined from the radioactivity released as water in the culture medium (1). Absorbance of cells was determined as described above after removal of culture medium.

b A₄₀₀V is defined as the product of the absorbance at 400 nm and volume of melanin solution. The values at 0-time were 0.106 and 0.095 cm⁻¹ ml/tube and 0.105 cm⁻¹ ml/culture in Expts 1, 2, and 3, respectively.

c Molar extinction coefficient of monomer units of melanin, defined as the absorbance of a melanin solution, at a concentration of 1 M tyrosine equivalents using a 1 cm-light path.

When suspensions of melanin from melanoma and hair of mice had been refluxed with 6 N HCl, their extinction coefficients both approached a value of about 20 cm⁻¹mg⁻¹ (Fig. 1). The convergence if the values for melanin from the two sources was mainly due to elimination of contaminating protein, although the structural change reported by Swan and Waggott (6) has not been ruled out as a minor cause.

The presence of an apparent isosbestic point in the absorption curve of protein-free melanin (Fig. 3) seems favorable for photometric determination of melanin, because at the "isosbestic point" the absorbance is stable enough to allow overnight incubation of preparations to obtain complete solubilization. But (1) tissues or
crude preparations of melanin usually contain substances which show absorption at 360 nm, the "isosbestic point" of melanin (Fig. 2A), and (2) these crude preparations are usually readily solubilized by "Soluene 100." So, it is better to determine the amount of melanin from the absorbance at 400 nm.

It should be pointed out that the weight of melanin calculated from the absorbance and extinction coefficient given in Table 1, is not the true weight of natural melanin, but the weight equivalent of a standard preparation of melanin obtained by acid treatment. The weight of natural melanin in situ can not be defined on the basis of the present knowledge of melanin. The chemical composition and structure of the final products obtained after HCl treatment under the present conditions are not known, but are probably similar to those reported by Swan and Waggott (6). They calculated from chemical analyses that the mean molecular weight of monomer units of melanin obtained by autoxidation of dopa was 163. Our value of 150–160 as the mean molecular weight of monomer units of HCl-treated melanoma melanin (ΔMel/ΔTy, in Table 2) is consistent with Swan's results and a little higher than the molecular weight of indole quinone. Comparison of the molecular weight of dopa-quinone (195), dopa-chrome (193) and indole quinone (147), suggests that acid-treated preparations of melanocyte melamins and probably synthetic melamins from dopa with melanocyte-extracts have largely lost the carboxyl group originating from tyrosine or dopa, mainly during melanin synthesis and also partly during acid-treatment (6).

The results in Table 2 clearly show that the tyrosine equivalents of newly synthesized melanin are proportional to the increase in A_{400} of melanin in both cell-free and whole-cell systems. The ratio ΔA_{400} V/ΔTy, the molar extinction coefficient of monomer units of melanin (defined as the absorbance in melanin solution at a concentration of 1 M tyrosine equivalents with a 1 cm-light path) was calculated to be 3000–3100 cm^{-1}M^{-1}. It should be emphasized that this definition for the "molar" extinction coefficient of melanin does not include the extinction coefficient of acid-treated melanin samples, which may differ from that of natural melanin in situ as discussed above. Using the "molar" extinction coefficient in Table 2 or the extinction coefficient in Table 1 it is possible to estimate the amount of melanin as tyrosine or dopa equivalents or as the weight of a standard melanin preparation, respectively, from the absorbance of a solubilized sample.

The extinction coefficients of melanin preparations produced by autoxidation or with mushroom tyrosinase were definitely higher than those of melamins produced by melanocytes (Table 1). From the slopes of their absorption curves natural melamins can be distinguished from those formed from dopa in vitro (Table 1). These results suggest that these melamins may differ with respect to the species of their monomer units and/or the ratio of monomer units in the respective melamins. To elucidate these differences, further studies on the chemistry and physico-chemistry of melanin are required and for these studies the new procedure for solubilizing melanin described here should be useful.

SUMMARY

Melansins were purified from melanoma tissues and black hair of mice by HCl-treatment. This treatment hydrolyzed protein and other macromolecules leaving melanin as insoluble material. These melanin preparations were dissolved in 0.5 N dimethyl n-undecyl n-dodecyl ammonium hydroxide in toluene. Their extinction coefficients were about 20 cm^2mg^{-1} at 400 nm. Melansins produced by autoxidation
of dopa or oxidation of dopa with mushroom tyrosinase had a distinctly higher extinction coefficient of about 28 cm$^2$mg$^{-1}$.

The molar amount of tyrosine converted to melanin by cell-free extracts and whole cells of a cultured line of melanoma cells was measured. The increase in the melanin content during the incubation was assayed colorimetrically. From the results the molar extinction coefficient of monomer units of melanin produced by melanocytes was calculated to be 3000–3100 cm$^{-1}$M$^{-1}$. The mean molecular weight of monomer units of purified melanin was calculated as 150–160. This is slightly higher than that of indole quinone, which is thought to be the main component of melanin.

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ADDENDUM

Under the solubilizing conditions described, observed "absorbances" (semi-integral attenuances, $\mu E_r = \log (l_0/l_r)$ for natural melanins at 400 nm were about 7% higher than the respective integral attenuances, $\mu E_r = \log (l_0/l_r + l_r)$, which were measured with a Shimadzu Multi-purpose Spectrophotometer, MSP-50L, equipped with two 2pi-photomultipliers (K. Shibata, In "Methods in Biochemical Analysis" (D. Glick, Ed.) Vol. 7, 77–109 and Vol. 9, 217–234, Interscience Publishers, Inc., New York, 1959, 1962). So, the terms "absorbance" and "extinction coefficient" should be replaced by "semi-integral attenuation" and "semi-integral attenuation coefficient."

However, during solubilizing incubation the semi-integral attenuation reaches the plateau earlier than the integral attenuation reaches the same plateau, probably because the scattering compensates the lower absorbance due to incomplete dispersion of melanin in the early phase of solubilization, and then both attenuances decrease gradually as shown in Fig. 3. Thus, the procedure, although it measures semi-integral attenuation but not integral attenuation, is valid for the practical purpose discussed in the paper. The effect of scattering in synthetic melanin was about 2% under the same conditions.

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