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Incorporation of [^{125}I]-5-Iodo-2-thiouracil in Cultured Hamster, Rabbit, and Human Melanoma Cells[1]

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ABSTRACT

The incorporation of [2-^{14}C]-2-thiouracil and a series of [^{125}I]-5-iodo-2-thiouracils ([^{125}I]ISUra(s)) into cultured Greene hamster melanoma cells was determined in order to establish their properties as false precursors in the melanin-biosynthetic pathway. The cold trichloroacetic acid-precipitable incorporation of [2-^{14}C]-2-thiouracil as well as [^{125}I]ISUra into melanoma cells after a 24- to 48-hr labeling period proved to be completely tyrosinase dependent (more than 99.5% inhibition could be achieved by 0.5 mM phenylthiourea). [^{125}I]ISUra incorporation was 3-fold higher than was [2-^{14}C]-2-thiouracil incorporation and was enhanced by 1 mM theophylline treatment. [^{125}I] ISUra incorporation into hamster, rabbit, and human melanoma cells showed a linear relationship with cell melanin content. Methylation of the sulfur completely prevented the incorporation, while propylation but not methylation at position 6 resulted in lower incorporation. [^{125}I]ISUra proved to be a marker for melanogenesis and may be useful in studies on the differentiation of cultured melanoma cells.

INTRODUCTION

Cell cultures of murine or hamster melanomas have been used widely in studies on the regulation of melanocyte differentiation (7, 19, 23). The synthesis of black or brown melanin pigment from L-tyrosine is a specialized differentiated function in both normal and neoplastic melanocytes. Although the details of melanogenesis have not yet been elucidated, it seems that the rate-limiting steps in melanin synthesis occur at the level of the oxidation of tyrosine and dopa, catalyzed by tyrosinase (monophenol dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) (15). Some recent evidence, however, points to a more complex regulation of the melanin-biosynthetic pathway (16).

Although the fundamental changes in the regulation of the cell cycle in the transformation of normal to malignant melanocytes are not known, it has been noticed that the processes of growth and melanogenesis in melanoma cells are related. As in a variety of other cell types, the expression of the differentiated function, in this case melanin synthesis, is often associated with a reduction in replicative capacity. This has been demonstrated after treatment of melanoma cells with the tumor promoter phorbol-12-myristate-13-acetate (6, 12), α-melanocyte-stimulating hormone (15), cyclic adenosine 3′:5′-monophosphate or theophylline (15, 21). However, the coupling between cell proliferation and expression of melanin synthesis does not seem to be fundamentally antagonistic (8), as may also be inferred from studies on the effects of various retinoids on melanogenesis (12, 13).

As differentiation markers in studies on melanogenesis, either the amount of synthesized melanin as absorbance at 400 nm or the tyrosinase activity as liberated ^3H_2O during L-[^{3}H]tyrosine incorporation has been determined. The former, however, has appeared to be a rather insensitive measure and has the drawback of unknown protein-melanin interactions (20), while the latter necessitates rather laborious column procedures. Whittaker showed that [2-^{14}C]SUra[3] may serve as a false precursor in melanin synthesis in chick embryo retinal pigment epithelium (22) and in melanoma cells (5) and suggested that its incorporation in melanin be used as a convenient method for measuring melanin synthesis. We have studied the usefulness of such a method using Greene hamster melanoma cells. As an important extension, a series of [^{125}I]ISUras were compared to [2-^{14}C]SUra with respect to degree of incorporation into melanin. Because of its γ- and β-radiation, the former may be useful for scintigraphic or therapeutic purposes in malignant melanoma (3), but as far as we know, reports dealing with the incorporation of this compound into melanin have not been published. Our studies show that incorporation of [^{125}I]-ISUra may provide an assay for differentiation in melanoma cells.

MATERIALS AND METHODS

Cells and Culture Techniques. A cell line was established from a melanotic Greene hamster melanoma. Tumor-bearing hamsters were originally obtained from Dr. S. Packer (Brookhaven National Laboratory, Upton, N. Y.). The melanoma was excised from a male Syrian golden hamster about 12 days after transplantation. Black superficial pieces of melanoma tissue were cut from the tumor and treated with 0.33% trypsin (Difco Laboratories, Inc., Detroit, Mich.) in Ca^{2+}- and Mg^{2+}-free PBS. The obtained single-cell suspension was dispersed in growth medium supplemented with 10% FCS (Flow Laboratories, Inc., Irvine, Scotland). The medium was composed of Eagle's minimal essential medium (Flow Laboratories) with added L-glutamine (BDH Chemicals, Ltd., Poole, England; 0.06%, w/v); D-biotin (Difco; 1 mg/liter); glucose (Bacto-dextrose, 0.4%; Difco); amino acids, nonessential amino acids, and vitamins (Flow Laboratories); sodium penicillin G (Gist-Brocades, Delft, The Netherlands; 100 IU/ml); and streptomycin (Specia, Paris, France; 100 μg/ml). The cells were grown in 9-cm-diameter tissue culture dishes (Nunc, Roskilde, Denmark) placed in a

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[3] The abbreviations used are: SUra, 2-thiouracil; ISUra, 5-iodo-2-thiouracil; PBS, phosphate-buffered saline; FCS, fetal calf serum; PHTU, 1-phenyl-2-thiourea; TCA, trichloroacetic acid; 2S-methyl-ISUra, 2S-methyl-5-iodo-2-thiouracil; 6-methyl-ISUra, 6-methyl-5-iodo-2-thiouracil; 6-*n*-propyl-ISUra, 6-*n*-propyl5-iodo-2-thiouracil.

humidified atmosphere of 5% CO_2:95% air at 37°. Subculture occurred each 3 to 4 days by resuspending cells using PBS containing 0.05% trypsin and 0.4 mM EDTA (Analar-BDH, Poole, England). Cell numbers were obtained with a hemocytometer, and viability was determined by exclusion of trypan blue. The cells have now been grown routinely for over 1 year; after about 6 months, FCS supplement was reduced to 5%. Cells have a doubling time of about 24 hr and may reach high densities of more than 2.5×10^5/sq cm, since the cells form colony-like aggregates on top of a monolayer of cells. Tumorigenicity, as determined by injecting 10^6 cells s.c. into hamsters, was maintained. Melanin production is dependent on factors such as growth rate and cell density as in S91 murine melanoma cells (11) and medium tyrosine concentration as in B16 murine (9) and RPMI 3460 hamster melanoma cells (5) and increased after theophylline addition (15).

By the same methods, a cell line was established from an amelanotic Greene melanoma growing in the eye of an albino New Zealand rabbit. It was originally obtained from Dr. L. H. S. Liu, Harvard Medical School, Boston, Mass.

A cloned human melanoma cell line was obtained from Dr. D. Ruiter, Department of Pathology, Leiden University, Leiden, The Netherlands. It was derived from a lymph node metastasis of a breast melanoma occurring in a 35-year-old man.

As a control amelanotic cell line, baby hamster kidney (BHK_{21}-C_{13}) cells were used and were maintained in Eagle's minimal essential medium supplemented with L-glutamine (0.03%), biotin (1 mg/liter), glucose (0.1%), antibiotics, and 5% FCS.

Radioisotopes. [2-^{14}C]SUra (27 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, England). [^{125}I]ISUra, 2S-methyl-[^{125}I]-ISUra, 6-methyl[^{125}I]ISUra, and 6-*n*-propyl[^{125}I]ISUra were prepared and purified as described (1).

Incorporation Experiments. Cells from stock cultures were seeded in 3 ml medium plus 5% FCS at a density of 5×10^4 to 5×10^5 cells/ml in plastic Petri dishes (5 cm diameter; Nunc). Hamster melanoma cells had been passaged >50 times; rabbit and human melanoma cells were of passages 5 to 15. After 2 to 3 days, the medium was changed, and L-tyrosine was added to a final concentration of 0.6 mM, giving, as expected (5), a visual increase in melanin synthesis. Twenty-four hr after refeeding the cells, the radioactive compounds were added in 0.3 ml PBS to the final concentrations and specific activities stated. PHTU (Sigma Chemical Co., St. Louis, Mo.) in 0.3 ml PBS was added to some dishes in order to inhibit tyrosinase activity (concentration as required). At the end of the incubation period, the medium was aspirated, and the cells were collected by trypsinization. In some cases, cells were scraped from the plates with a rubber policeman; this procedure did not lead to differences in incorporation values compared to trypsinized cells. Cell numbers were obtained by counting parallel cultures with the use of a hemocytometer. Viability determined by exclusion of trypan blue was ≥97%. Melanin content of the cells was monitored spectrophotometrically as material absorbing at 400 nm after solubilization of the cells in Soluene-350 (14). A_{400} was measured against an amelanotic standard (BHK_{21}-C_{13} cells). All reported experiments were performed separately at least twice, with qualitatively similar results.

Assay of Incorporated Material. Cells were pelleted by centrifugation and extracted with 0.5 ml of 5% TCA for at least 30 min at 4°, followed by centrifugation (5 min, 2000 × *g*). ^{125}I measurements were carried out directly in a Packard 5120 gamma counter. Samples were counted for 10 min (background, 30 cpm; efficiency, 65%). For ^{14}C measurements, 500-μl samples of medium were added to 10 ml of Instagel scintillation fluid. TCA extracts were solubilized in 1 ml of Soluene-350 at 45°, while TCA supernatants were mixed with 1 ml of Soluene-350, and both were added to 10 ml of Instagel. Samples were counted in a Packard Model 2450 liquid scintillation counter for 10 min (background, 20 dpm; efficiency by the external standard method, 78 to 84%). Results are expressed as percentage of incorporation of added radioactivity or as nmol incorporation in TCA-precipitated cells per culture dish or per 10^6 cells, counted at the end of the labeling period.

RESULTS

Incorporation of [2-^{14}C]SUra. The incorporation of [2-^{14}C]SUra into TCA-insoluble material was studied as a function of substrate concentration by adding a constant amount of [2-^{14}C]SUra to the cells (0.025 μCi/dish) and varying the concentration of SUra (Chart 1).

The melanoma cells incorporated radioactive SUra to an extent which turned out to be inversely linear with log(SUra). Normalizing the incorporation values to a constant specific activity shows that the amount of SUra incorporation increased with a factor of 4 at the highest concentration used (Chart 1). In this and all other experiments, we assume cold TCA-insoluble incorporation to represent melanin synthesis-related incorporation. This is justified because: (*a*), SUra has been shown not to be incorporated into nucleic acids, while the label was retained after a 3-step purification of melanin (22); (*b*) the melanin polymer is highly stable, and there is no significant degradation of melanin for at least 48 hr after its synthesis (5); (*c*) [2-^{14}C]SUra was not incorporated into a series of nonpigmented tissues *in vitro* (22). We included BHK_{21}-C_{13} cells in all of our experiments. These always showed an acid-insoluble incorporation essentially equal to a zero time incorporation in melanoma cells (about 0.005 to 0.01%; not shown).

PHTU Inhibition of Incorporation. Table 1 shows that PHTU did not affect cell proliferation, even at the concentrations which reportedly inhibit protein synthesis (8, 17). The slight increase in cell numbers might be related to protection to melanoma cells from the toxic effects of melanin precursors when tyrosinase is inhibited (15). The [2-^{14}C]SUra incorporation was reduced to 0.2% of control values (without PHTU) with 10^{-3} M PHTU, which is similar to zero time incorporation (about 0.1%). A similar "zero incorporation" could be obtained by the addition of 0.01% mercaptoethanol to the growth medium (not shown). In further experiments, we chose a 99.5% inhibiting concentration (5×10^{-4} M PHTU).

Incorporation of [^{125}I]ISUras. The exploration of the possible incorporation of [^{125}I]ISUra derivatives was undertaken within the framework of our studies on potential melanoma-seeking

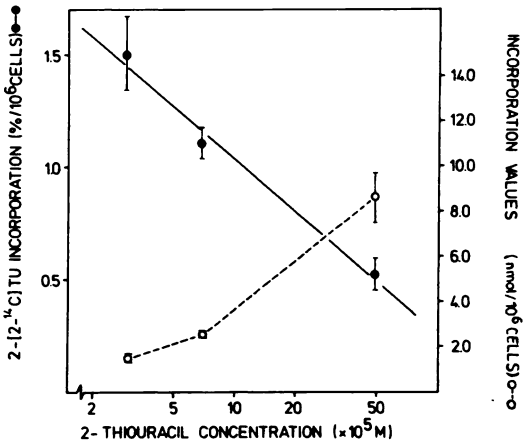

Chart 1. Concentration dependence of [2-^{14}C]SUra (*TU*) incorporation into hamster melanoma cells. Cells were plated in 5-cm dishes at 1.5×10^5 cells/dish in normal medium and refed after 3 days with medium containing 0.6 mM L-tyrosine. Twenty-four hr later, SUra was added at indicated concentrations. Cells were collected 48 hr later, and ^{14}C incorporation was determined. Values are averages of triplicate cultures. ——, calculated by the method of least squares ($r = 0.991$). *Bars*, S.D.

Table 1

Effect of PHTU on cell proliferation and [2-^{14}C]SUra incorporation

Hamster melanoma cells were plated in 5-cm dishes at 1.0 × 10^5 cells/dish in normal growth medium and refed after 3 days with medium containing 0.6 mM L-tyrosine. Twenty-four hr later, PHTU was added; 5 min later, [2-^{14}C]SUra (3 × 10^{-4} M; specific activity, about 0.3 mCi/mmol) was added. Cells were grown for another 48 hr, collected and counted, or processed for ^{14}C incorporation determination.

PHTU concentration (mM)	Viable cells/dish (× 10^{-6})	Inhibition of ^{14}C incorporation (%)
0	5.64 ± 0.12[a]	
0.1	5.82 ± 0.34	93.7
0.3	6.21 ± 0.51	99.0
0.5	6.05 ± 0.21	99.5
1.0	5.97 ± 0.21	99.8

[a] Average ± S.D. of triplicate cultures.

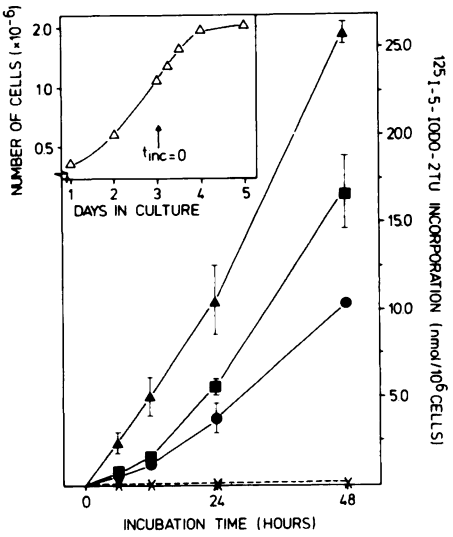

Chart 2. Time course of [^{125}I]ISUra (*^{125}I-5-IODO-2TU*) incorporation into hamster melanoma cells. Cells were plated in 5-cm dishes at 3.3 × 10^5 cells/dish in normal medium. The medium was changed on Day 2 for medium supplemented with L-tyrosine to a final concentration of 0.6 mM. Twenty-four hr later, ISUra (about 0.05 μCi) was added to the cultures (*inset, arrow*) to a final concentration of 3 × 10^{-5} M (●), 7 × 10^{-5} M (■), or 5 × 10^{-4} M (▲). To some cultures of the 3 × 10^{-5} M series, PHTU (0.5 mM) was added (×– – –×). At the indicated times, cells were harvested. Values are means of duplicate cultures. *Bars*, S.D.

compounds. Chart 2 shows that the introduction of ^{125}I into the SUra molecule did not affect its ability to be incorporated in the melanoma cells. When the time course of incorporation of [^{125}I]ISUra was followed, the retention of ^{125}I showed an inverse relationship with the growth rate of the cells (Chart 2 and *inset*), with the greatest incorporation apparent when the cells had entered a stationary phase. This finding is in accordance with the visual observation that during this phase the cells produced the most melanin. However, the [^{125}I]ISUra incorporation may not become maximal until after a longer incubation period, regardless of the rate of melanin production of the cells. Therefore, an experiment was included to study the time dependence of incorporation *versus* state of melanin production. The melanin production in hamster melanoma cells was enhanced by adding 1 mM theophylline to the growth medium (15). Table 2 shows that, during the first 24 hr, this treatment resulted in an [^{125}I]ISUra incorporation which was even greater than the 48-hr incorporation in control cells. This excludes the involvement of time dependence as a major factor in the [^{125}I]ISUra incorporation. This experiment also shows that the concentration of [^{125}I]ISUra used (1.5 × 10^{-5} M) is not at or below saturation level for incorporation into control hamster melanoma cells, since a much higher incorporation level can be achieved by manipulating the melanin production. Nevertheless, the concentration dependence of [^{125}I]ISUra incorporation showed characteristics very similar to that of SUra itself (Chart 3). Under the present conditions, an optimal incorporation was seen in the concentration range of 2 × 10^{-4} to 5 × 10^{-4} M, which is in the same order as that reported for [2-^{14}C]SUra incorporation in RPMI 3640 hamster melanoma cells (5).

To establish the quantitative relationship between [^{125}I]ISUra incorporation and melanin content of melanoma cells, an experiment was performed with 3 melanoma cell lines which vary considerably in melanin production. The amelanotic rabbit melanoma cells appear microscopically as white cells with very few melanotic cells, while the human melanoma cells are filled with black melanin granules. A linear relationship was found between [^{125}I]ISUra incorporation and melanin content of the cells (Chart 4).

Some experiments were directed at determining the structural requirements for incorporation. [^{125}I]ISUra was oxidized according to the method of Visser and Klootwijk (18). It was found that the products formed by this method were incorporated at about 25% of the efficiency of ISUra. Since this method

Table 2

Time dependence of [^{125}I]ISUra incorporation into hamster melanoma cells

Cells were plated in 5-cm dishes at 3.0 × 10^5 cells/dish in normal growth medium or medium supplemented with 1 mM theophylline and refed after 3 days with medium containing 0.6 mM L-tyrosine. Twenty-four hr later, [^{125}I]ISUra (1.5 × 10^{-5} M) was added. Cells were grown for another 24 or 48 hr and collected, and incorporation was determined.

Theophylline (1 mM)	Incubation time (hr)	[^{125}I]ISUra incorporation (nmol/10^6 cells)
–	24	0.95 ± 0.27[a]
+	24	3.31 ± 0.11
–	48	2.74 ± 0.17
+	48	4.06 ± 0.08

[a] Average ± S.D. of triplicate cultures.

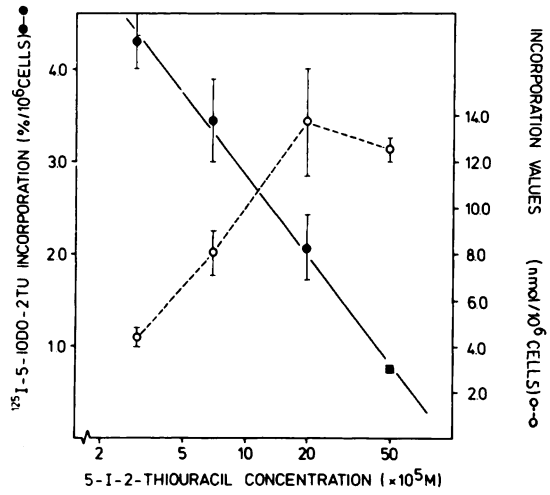

Chart 3. Concentration dependence of [^{125}I]ISUra (*^{125}I-5-IODO-2TU*) incorporation into hamster melanoma cells. Cells were plated in 5-cm dishes at 1.5 × 10^5 cells/dish in normal medium and refed after 2 days with medium containing 0.6 mM L-tyrosine. Twenty-four hr later, ISUra was added at indicated concentrations. Cells were collected 48 hr later, and ^{125}I incorporation was determined. Values are averages of triplicate cultures. ——, calculated by the method of least squares (r = 0.998). *Bars*, S.D.

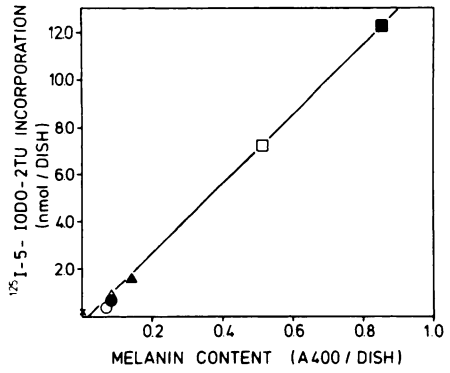

Chart 4. Relationship between [^{125}I]ISUra (*^{125}I-5-IODO-2TU*) incorporation and melanin content of melanoma cells. Hamster (△, ▲), rabbit (○, ●), and human (□, ■) melanoma cells and BHK_{21}-C_{13} cells (×) were plated in 5-cm dishes at 1.5 × 10^5 M cells/dish in normal medium and were refed after 3 days. Twenty-four hr later, [^{125}I]ISUra (3 × 10^{-5} M) was added. The cells were collected 24 or 48 hr later, and ^{125}I incorporation was determined in 5 dishes. In 3 parallel dishes, A_{400} was monitored. △, ○, □, 24-hr incubation period; ▲, ●, ■, 48-hr incubation period. ——, calculated by the method of least squares (r = 0.998).

Table 3

Incorporation of some [^{125}I]ISUras into hamster melanoma cells

Cells were plated in 5-cm dishes at 3.0 × 10^5/dish in normal growth medium and refed after 3 days with medium containing 0.6 mM L-tyrosine. Twenty-four hr later, [2-^{14}C]SUra or [^{125}I]ISUra or its 2*S*-methyl, 6-methyl, or 6-propyl derivative were added (3 × 10^{-5} M). Cells were grown for another 24 hr and collected, and incorporation was determined.

Compound	[^{125}I]ISUra incorporation (nmol/dish)
[2-^{14}C]SUra	1.31 ± 0.19[a]
[^{125}I]ISUra	4.19 ± 0.17
6-Methyl[^{125}I]ISUra	3.62 + 0.39
6-Propyl[^{125}I]ISUra	1.40 ± 0.38
2S-Methyl[^{125}I]ISUra	0.01 ± 0.00

[a] Average ± S.D. of triplicate cultures.

causes oxidation of more than 95% of ISUra (18), which we confirmed by thin-layer chromatography, it seems likely that some of the incorporated products represent sulfinic and sulfonic acids. Desulfurated products are not incorporated into melanin (3).

The incorporation of [2-^{14}C]SUra, [^{125}I]ISUra, 2S-methyl-[^{125}I]ISUra, 6-methyl[^{125}I]ISUra, and 6-*n*-propyl[^{125}I]ISUra was compared in one experiment. We found (Table 3) that [^{125}I] ISUra and the 6-methyl compound were incorporated 2 to 3 times more effectively than was [2-^{14}C]SUra, while the 6-propyl derivative was incorporated to the same extent as [2-^{14}C]SUra. On the other hand, the 2*S*-methyl compound was not incorporated at all.

DISCUSSION

The properties of SUra as false precursor in melanin synthesis have been well documented for normal melanocytes (4, 22) and melanoma cells (3, 5). Because of the selective incorporation of SUra into melanin but not into nucleic acids or protein, the use of properly labeled SUra derivatives has been suggested to be of value for diagnostic and therapeutic purposes in the management of malignant (ocular) melanoma (4, 5).

In this study, the practical usefulness of SUra and its 5-iodo derivatives as substrates that are incorporated into melanin in melanoma cells was investigated, the [^{125}I]ISUras being potential *in vivo* melanin-seeking compounds.

We found that a high percentage of TCA-precipitable incorporation of [2-^{14}C]SUra could be achieved in melanoma cells during a 24- to 48-hr labeling period and was inhibited for more than 99% by addition of 0.5 mM PHTU, a tyrosinase inhibitor. The low residual incorporation may be accounted for by the reported incomplete inhibition of [2-^{14}C]SUra incorporation after short incubation times (5). Longer incubation periods are more appropriate when drug effects on melanin synthesis are to be studied. The finding that cold TCA-insoluble [2-^{14}C]SUra or [^{125}I]ISUra incorporation is completely tyrosinase dependent makes further purification of melanin from other macromolecules unnecessary. However, if desired, such purification is possible (20). The use of a high-specific-activity [2-^{14}C]SUra provides a high incorporation rate of radioactivity. When a higher concentration of SUra or ISUra (mM range) is used, a higher amount of ISUra is incorporated, as is the case with increasing concentrations of the physiological precursor L-tyrosine (5). Whether this also means that ISUra, like L-tyrosine, can stimulate melanin synthesis by a higher precursor availability (5) is not apparent from the present experiments. The concentration dependence of incorporation, however, is not simply caused by a shortage in availability of ISUra for the labeling of melanin at lower concentrations (Table 2). More complex mechanisms, as well as the possible existence of different precursor pools of L-tyrosine, may be involved (5), such that a higher labeling index of melanin might be achieved at higher SUra concentrations. The elucidation of these problems requires more experimentation.

[^{125}I]ISUra proved to be incorporated into melanoma cells; this incorporation (at 3 × 10^{-5} M) showed a quantitative relationship with the melanin content of the cells. Hence, the concentration dependence of [^{125}I]ISUra incorporation forms no problem for its use as marker for melanin synthesis at a particular concentration. Further evidence for the melanin specificity of [^{125}I]ISUra incorporation are its tyrosinase dependence and the finding that incorporation was maximal when the culture was in the stationary phase, confirming a certain inverse relationship between cell proliferation and melanogenesis. The results imply that the introduction of a radiolabeled iodine into the SUra molecule does not inhibit its oxidative incorporation into the melanin polymer, as seems to be the case for the tyrosine molecule (2). The even greater incorporation percentage of [^{125}I]ISUra compared to [2-^{14}C]SUra may result from an increased nucleophilicity at the sulfhydryl group, which would facilitate condensation with quinones. The requirement for a free sulfhydryl group to serve as a precursor in the melanin synthesis follows from the nonincorporation of the sulfur-methylated derivative, although oxidation of ISUra did not completely prevent the incorporation. Hence, the requirement for a sulfhydryl group is not absolute. However, whether the sulfur was alkylated or not, *in vivo* short-term accumulation in hamster melanoma of iodinated SUras was not very different (10), suggesting that other factors besides melanin synthesis may contribute to the *in vivo* accumulation. Such factors may account for non-TCA-precipitable accumulation (10). The 6-propylated ISUra derivative showed a somewhat lower percentage of incorporation than did the parent compound, but this compound too may be regarded as a fairly good false melanoma precursor.

In conclusion, this method may be useful for studies on differentiation of melanoma cells in culture. It may find appli-

cation in comparative studies on melanogenesis in various cell lines and in investigations into drug effects on melanogenesis and its relation to cell proliferation. Furthermore, it is worth testing the use of the γ-ray-emitting species in *in vivo* research on melanomas, as is being done in our laboratory.

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