Biosynthesis of a Thiouracil Pheomelanin in Embryonic Pigment Cells Exposed to Thiouracil*

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SUMMARY

[2-14C]Thiouracil and [6-35S]thiouracil are incorporated into the melanin of chick retinal pigment epithelium incubated in vitro. This synthesis of a thiouracil pheomelanin (thiol-containing melanin) can be used to measure the rate of melanin synthesis in living cells.

Thiouracil incorporation is dependent on tyrosinase activity: incorporation is inhibited by phenylthiourea, a tyrosinase inhibitor, and there is a proportional relationship between thiouracil incorporation and the level of tyrosinase activity in pigment tissues. Other chick embryo tissues do not incorporate thiouracil, but small quantities are incorporated by embryo tissues containing tyrosine hydroxylase. Radioactivity is retained in melanin prepared from isolated melanosomes of pigment tissues incubated in [2-14C]thiouracil. A comparison of [35S]thiouracil and [2-14C]tyrosine incorporations into melanosomes indicates that 1.5 moles of thiouracil are incorporated for each mole of tyrosine.

These results are consistent with the hypothesis that thiouracil condenses with the quinone intermediates in the melanin pathway and is incorporated into the melanin polymer as part of the subunit structure.

Sulfhydryl compounds, such as thiourea (1, 2), cysteine (3, 4), and glutathione (3, 4-7) react in vitro with an intermediate product of melanin synthesis—probably one of the quinone intermediates, since thiol compounds are known to react with quinones (8-11). Thiouracil (4-oxo-2-thiopyrimidine), a well known antithyroid drug, must react similarly in vitro, since it alters the color of pigment granules in the cells of chick melanoocyte cultures (12) and changes the size and color of melanin granules which develop in the pigment cells of ascidian embryos (13). Radioactive thiouracil is incorporated into the pigment granules of ascidian embryos (13), indicating that a melanin-thiouracil complex is formed.

The yellow and red pigments of hair and feathers referred to as pheomelans (14) result from the incorporation of large quantities of a thiol (perhaps cysteine) into the melanin of certain melanocytes (15, 16). Chemical studies of isolated red feathers also suggest that pheomelans derive biogenetically from tyrosine and cysteine (17). Since pheomelans and the black or brown eumelans are synthesized in similar subcellular melanosome structures (18), pheomelanin and eumelanin may be alternate activities of the same cells and organelles.

The melanin-thiouracil product which forms in vitro in otherwise eumelanin-producing cells (13) is a kind of pheomelanin (by definition) and may resemble natural pheomelans in many important details of biosynthesis and structure. This report describes the biosynthesis of a thiouracil pheomelanin in chick embryo pigment cells.

MATERIALS AND METHODS

Tissue Incubations and Cell Culture—Incorporation experiments were carried out with retinal pigment epithelium (tapetum nigrum) from the eyes of White Leghorn chick embryos incubated 7 days to the Hamburger and Hamilton (19) embryonic Stages 29, 30, and 31. These pigment tissues were obtained completely free of other cells by a method described previously (20, 21). Pieces of retinal pigment epithelium were incubated in radioactive medium for 4 hours. Some pigment tissues were disaggregated (20, 21) into single cells and small clumps of cells and cultured in monolayer for different periods of time prior to the addition of radioactive medium. Other embryonic tissues studied were dissected from the appropriate organs and cut into fragments not exceeding 1 mm in thickness.

In some of the experiments, tissues were incubated in a complex medium consisting of equal parts of Tyrode’s solution, horse serum (Baltimore Biological Laboratory, Baltimore, Maryland) and 12-day chick embryo extract (20). This medium also contained potassium penicillin G and streptomycin sulfate (50 i.u. each per ml) and 0.005% phenol red indicator. The culture vessels were previously coated on the bottoms with a 0.5-ml layer of plasma clot, consisting of equal parts of reconstituted chicken plasma (Hyland Laboratories, Los Angeles, California) and 12-day embryo extract. Pigment cells or tissues from two eyes were added to the flask in 0.5 ml of medium and maintained at 37° under 5% CO2 in air. Since the plasma clots diute substances in the medium by 1:1, all concentrations of chemicals and radioactive compounds stated for these culture conditions are the concentrations to which the cells were actually exposed, i.e. one-half the concentration of reagents in the medium. Under these conditions, tissue pieces flattened against...
the plasma clot and adhered to it; single cells and clumps attached to the clot and gradually proliferated into monolayer sheets (20, 21). Tissues or cells were recovered from the culture vessels by treatment with 0.03% EDTA (disodium salt) and 2% trypsin (20).

In other experiments, retinal pigment tissues were incubated at 37° in Eagle's minimum essential medium (22) (Flow Laboratories Inc., Rockville, Maryland) with 10% fetal bovine serum and a gas phase of 5% CO₂ in air. In this medium the pigment tissues adhered slightly to the wall of the glass tubes in which they were incubated, but could be detached easily.

Tests with the MEM and fetal bovine serum showed that disaggregated retinal pigment cells could form monolayer sheets and slowly proliferate when seeded in this medium on plasma clots (clotted with 10 units of thrombin per ml of chicken plasma) and could also proliferate colonies in this medium on the surface of Falcon Plastic Petri dishes. Cells growing in this medium did not look as healthy as those growing in embryo extract medium, but there is no question that MEM with 10% fetal bovine serum is a completely satisfactory maintenance medium for chick retinal pigment tissues.

**Incorporation of Radioactive Compounds**—The following radiotracer compounds were used: [2-¹⁴C]thiouracil (Amersham-Searle; ICN); [³⁵S]methionine and [³⁵S]creatinine (Amersham-Searle); dl-[2-¹⁴C]tyrosine (Tracerlab); L-[²-¹⁴C]tyrosine (Calbiochem); and L-[¹⁴C]leucine (New England Nuclear). These compounds were all obtained at greater than 98% radiochemical purity.

After incubation in the isotopic-containing media, recovered cells and tissues were washed with a large quantity of Tyrode's solution, frozen briefly in a Dry Ice-95% ethanol freezing mixture, and then extracted by the method of Siekevitz (23). The hot (90°) trichloroacetic acid step in this procedure (23) was used and a gas phase of 5% CO₂ in air. In this medium the pigment tissues adhered slightly to the wall of the glass tubes in which they were incubated, but could be detached easily.

Tests with the MEM and fetal bovine serum showed that disaggregated retinal pigment cells could form monolayer sheets and slowly proliferate when seeded in this medium on plasma clots (clotted with 10 units of thrombin per ml of chicken plasma) and could also proliferate colonies in this medium on the surface of Falcon Plastic Petri dishes. Cells growing in this medium did not look as healthy as those growing in embryo extract medium, but there is no question that MEM with 10% fetal bovine serum is a completely satisfactory maintenance medium for chick retinal pigment tissues.

**Measurements of Radioactivity and Protein**—The acid-insoluble fractions (23) were dissolved in 1 N NaOH by heating for 10 min in a boiling water bath, and the protein content was measured in samples of the digest (24). Samples (100 μl) of the digest were added to 18 ml of 30% methanol in toluene containing 4 g per liter of 2,5-diphenyloxazole and 50 mg per liter of β-bis[2-(5-phenyloxazolyl)]benzene. Radioactivity was measured in a Beckman liquid scintillation counting system. Disintegrations per min were calculated from the cpm, with efficiency measurements based on the addition of external standards to the vials. Radioactivity was expressed as disintegrations per min per mg of cell protein in the acid-insoluble cell fraction.

**Inhibition Studies in Vitro**—Dopachrome formation was measured in 3-mI reaction mixtures (3) with a Beckman recording spectrophotometer. These mixtures contained 0.1 M sodium phosphate buffer (pH 6.8), 0.2 mM 3,4-dihydroxy-L-phenylalanine, 50 catecholase units of mushroom tyrosinase (Nutritional Biochemicals) per ml, and various concentrations of thiouracil up to 3.0 mM. The enzyme reactions were carried out at 26°.

**Melanin Isolation and Purification**—Pigment granules (melanosomes) were isolated from groups of 20 retinal pigment tissues (25). After the final ultracentrifugation step, 5% trichloroacetic acid was added to the black pellet and an acid-insoluble fraction prepared (23). The method for purifying the melanin was based on the solubility of melanin in hot aqueous NaOH and its insolubility in water and HCI (26). The acid-insoluble pellet was heated for 10 min at boiling water temperature in 1 ml of 1 N NaOH and centrifuged. The insoluble residue was heated with 2 further volumes of 1 N sodium hydroxide, and the final insoluble residue was discarded. To the combined alkaline supernatants was added a quantity of carrier melanin, prepared from the embryo extract medium (27). The specific activity, as disintegrations per min per mg of melanin, was measured by the radioisotope assay procedure outlined above, and a spectrophotometric determination of melanin content (20). The alkaline supernatant was then acidified with concentrated HCI and the pigment precipitated at 5° for 24 hours or longer; the precipitate was washed twice with acidified water. Solution in 1 N NaOH and precipitation with acid was repeated twice. After the third precipitation, the melanin was redissolved in 1 N NaOH and the specific activity measured once again.

**RESULTS**

**General Effect of Thiouracil**—Thiouracil apparently has no adverse effect on chick retinal pigment cells in culture. When disaggregated cells were seeded in embryo extract medium and exposed to 0.75 mM thiouracil, the pigment cells underwent attachment to the plasma clot, flattening and spreading, increase in cell size, cell division, and formation of monolayer sheets. This confirms previous observations that thiouracil has no toxic effect on differentiation and development of ascidian embryos (13), and shows that over short periods of time thiouracil causes no significant metabolic disturbance in cells.

**Incorporation of [²-¹⁴C]Thiouracil**—After preliminary observations established that [²-¹⁴C]thiouracil is incorporated into the acid-insoluble fraction of cultured retinal pigment tissues, some of the properties of the incorporation reaction were studied in detail. The concentration of [²-¹⁴C]thiouracil in the culture medium which saturates the incorporation during a 4-hour incubation period was determined. In these experiments the concentration was changed, but the specific activity of [²-¹⁴C]thiouracil in the medium was kept constant (1 mCi per mmole). Saturation occurred at a concentration of 0.75 mM thiouracil in the embryo extract medium (Fig. 1A) and of 1.5 mM thiouracil in the MEM with fetal bovine serum (Fig. 1B). There is no obvious reason why a higher concentration of thiouracil is needed in the MEM to saturate the reaction. However, the maximum 4-hour rate of incorporation achieved in each medium is approximately the same. Thiouracil was used in all subsequent experiments at saturating concentrations.

As illustrated in Fig. 2, thiouracil incorporation is not linear with time over a 4-hour period, but decreases gradually. MEM was used in the experiment shown; similar results were obtained with the embryo extract medium. Experiments in which the reaction was measured at 10-, 20-, and 30-min intervals likewise showed a gradual decrease in the rate of incorporation. This change is probably caused mainly by the loss of melanotic activity which occurs when pigment cells are maintained in vitro (21). Previous measurements of melanotic change by other techniques have suggested that loss of activity begins very early in culture, but these methods were not sufficiently sensitive to demonstrate this change over such a short time interval.

Although thiouracil incorporation is not linear with time,
Fig. 1. The effect of different concentrations of thiouracil on the rate of incorporation of [2-14C]thiouracil (specific activity, 1 mCi per mmole) into the acid-insoluble fraction of chick retinal pigment epithelium. Each point is the mean incorporation value of two cultures. A, series of tissues incubated for 4 hours at 37° in embryo extract medium. B, series of tissues incubated for 4 hours at 37° in MEM with 10% fetal bovine serum.

Fig. 2. Incorporation of [2-14C]thiouracil into the acid-insoluble fraction of chick retinal pigment epithelium as a function of time. Tissue was incubated at 37° in MEM with 10% fetal bovine serum containing 4.0 mM [2-14C]thiouracil (specific activity 1 mCi per mmole). Cultures were recovered at the times indicated. The points are mean incorporation values for three cultures.

There is reasonably close agreement between 4-hour incorporation values for tissues from many different batches of embryos of the same approximate age. The closest agreement is obtained when tissues are selected from precisely the same embryonic stage. Four different experimental series are shown in Table I in which Stage 29 tissues were carefully selected for each series.

Another indication of uniformity in the incorporation reaction besides the close agreement of the incorporation rates is the observation that isotope incorporation is proportional to the quantity of tissue used. Fig. 3 shows that in cultures prepared with one, two, or three retinal pigment tissue pieces per culture tube the incorporation of [2-14C]thiouracil per culture is proportional to the quantity of cell protein per culture.

Thiouracil itself may have a direct effect on limiting further incorporation by some gradual reaction-inactivation mechanism.

<table>
<thead>
<tr>
<th>Experimental series</th>
<th>Specific activity (mCi/mmol)</th>
<th>Incorporation (dpm/mg protein)</th>
<th>Incorporation (nmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.75</td>
<td>21,800</td>
<td>13.1 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>0.75</td>
<td>22,000</td>
<td>13.7 ± 1.0</td>
</tr>
<tr>
<td>3</td>
<td>0.76</td>
<td>23,400</td>
<td>13.8 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>0.76</td>
<td>23,000</td>
<td>13.6 ± 0.9</td>
</tr>
</tbody>
</table>

a The figures are mean incorporation values for six cultures in each experimental series.

This could account for the early nonlinearity of the incorporation reaction. However, when [2-14C]thiouracil incorporation in cultures is started at successive 1-hour intervals, thiouracil incorporation (a 4-hour period of measurement) still declines with time in the absence of prior exposure to thiouracil (Table II).

Tyrosinase-dependent Thiouracil Incorporation—Two methods were chosen to demonstrate that thiouracil incorporation is dependent on tyrosinase (EC 1.10.3.1) activity of the pigment tissues. The first method involved measuring the rate of [2-14C]thiouracil incorporation during culture periods at which tyrosinase activity became progressively lower; the second method involved the effect of phenylthiourea, an inhibitor of tyrosinase activity, on [2-14C]thiouracil incorporation. If thiouracil is incorporated into melanin by reacting with an intermediate compound in the oxidative pathway of tyrosine to melanin, such as dopaquinone or 5,6-dihydroxyindolequinone, it should not be incorporated in the absence of tyrosinase activity, since tyrosinase catalyzes the first steps in the oxidative pathway.

Previous studies of chick retinal pigment cells grown in culture
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3. Incorporation of [2-14C]thiouracil into the acid-insoluble fraction of chick retinal pigment epithelium as a function of tissue quantity (protein per culture). A series of cultures was incubated at 37°C for 4 hours in MEM with 10% fetal bovine serum. The medium contained 4.0 mM [2-14C]thiouracil (specific activity 1 mCi per mmole). Each point represents the radioactivity and protein of one culture. The number next to the points indicates the number of tissue pieces per culture.

Table II
Effect of prior time in culture on incorporation of [2-14C]thiouracil into acid-insoluble fraction of chick retinal pigment tissues

<table>
<thead>
<tr>
<th>Time in culture prior to thiouracil treatment</th>
<th>Isotope incorporation period</th>
<th>Incorporation (dpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td>hrs</td>
<td>Experimental Series 1</td>
</tr>
<tr>
<td>0</td>
<td>0–4</td>
<td>18,000</td>
</tr>
<tr>
<td>1</td>
<td>1–5</td>
<td>16,400</td>
</tr>
<tr>
<td>2</td>
<td>2–6</td>
<td>15,800</td>
</tr>
</tbody>
</table>

showed that the rate of melanin biosynthesis and the level of tyrosinase activity declined rapidly over 36 hours of culture time (21). Similar studies in this investigation with retinal pigment cells and 4-hour incubation periods with [2-14C]thiouracil gave results (Fig. 4) which parallel exactly the decline in tyrosinase activity observed previously (21). This correlation suggests that there is a proportional relationship between thiouracil incorporation and tyrosinase activity.

1-Phenyl-2-thiourea is remarkably effective in vivo as an inhibitor of tyrosinase activity and melanin synthesis at low inhibitor concentrations (29). Phenylthiourea was added at various concentrations to retinal pigment tissues during 4 hours of incubation in [2-14C]thiouracil (Fig. 5). A plateau was reached at a concentration of approximately 0.15 mM phenylthiourea; at this concentration about 80% of thiouracil incorporation is inhibited. This result (Fig. 5) suggests that most of the thiouracil incorporation is dependent on tyrosinase activity. However, it is important to know whether the remaining 20% of thiouracil incorporation is caused by some reaction not directly dependent
on tyrosinase activity, or whether 80% inhibition is simply the maximal inhibition of tyrosinase activity possible with phenylthiourea under these experimental conditions. Chemical groups able to react with or incorporate thiouracil might be present in the pigment cells at the time phenylthiourea and labeled thiouracil are first added to the tissues. Actively synthesizing pigment cells may have a modest pool of intermediate substances in the melanin pathway, including the quinone intermediates with which thiouracil is presumed to react. If significant quantities of reactive intermediate substances are present in freshly prepared retinal pigment tissues, stopping tyrosinase activity for 1 hour in these tissues (with phenylthiourea) prior to the addition of thiouracil should permit some of these substances to become depleted. Such tissues should incorporate less [2-14C]thiouracil in the presence of phenylthiourea. The effect of phenylthiourea on thioracil incorporation for the first 4 hours (hours 0 to 4) of culture was compared to its effect on incorporation of isotope for 3 hours (hours 1 to 4) after an initial 1-hour exposure to 0.15 mM phenylthiourea. Since the hourly rate of [2-14C]thiouracil incorporation was approximately the same in both sets of cultures (Table III), there is probably not a very large pool of intermediate melanin metabolites in pigment cells.

The plateau effect seen with phenylthiourea may simply indicate that 20% of the tyrosinase activity remains unaffected by phenylthiourea. Experimental results with another tyrosinase inhibitor are consistent with this explanation. Sodium diethyl-dithiocarbamate seems to inhibit tyrosinase activity by virtue of its properties as a copper chelator, and there is some possibility that its inhibitory action is slightly different from that of phenylthiourea (90). At a concentration of 0.15 mM, diethyl-dithiocarbamate inhibits 50% of [2-14C]thiouracil incorporation into retinal pigment tissues during 4 hours. When 0.15 mM diethyl-dithiocarbamate is combined with 0.15 mM phenylthiourea in culture, the 79% inhibition caused by phenylthiourea alone is increased to 87%. Apparently some of the remaining 20% of tyrosinase activity can be inhibited by a second tyrosinase inhibitor.

An additional observation also suggests that phenylthiourea does not inhibit tyrosinase completely in chick cells. Chick retinal pigment tissue becomes pigmented in the embryo during the 4th day of incubation. When 3-day retinal pigment epithelium pieces are placed in organ culture on Millipore filter rafts in embryo extract medium, these tissues start to become pigmented within 2 to 3 days of culture. Phenylthiourea at 0.45 mM concentration only retards pigmentation in these organ cultures, and the tissues slowly become pigmented.

Phenylthiourea has an effect on L-[2-14C]tyrosine incorporation similar to its effect on thiouracil incorporation. Inhibition of tyrosine incorporation into the acid-insoluble fraction of pigment epithelium pieces reaches a plateau of inhibition beginning at a concentration of 0.15 mM phenylthiourea (Fig. 5). Apparently, at least 65% of tyrosine incorporation is tyrosinase-dependent and undoubtedly represents melanin synthesis. The remaining 35% of incorporation is probably incorporation of tyrosine into protein, although the studies with thiouracil suggest that a part of this incorporation is also melanin synthesis that is unaffected by phenylthiourea. The 65% figure could be a considerable overestimation of melanin synthesis if phenylthiourea partially inhibits protein synthesis as well as tyrosinase activity. Since phenylthiourea does not inhibit L-[14C]leucine incorporation into acid-insoluble cell protein (Fig. 5), there is no reason to suppose that it inhibits any of the tyrosine incorporation into protein.

**Effect of Phenylthiourea on Tyrosinase-Dependent Tyrosine Incorporation**—There is evidence which suggests that thiouracil might be a direct inhibitor of tyrosinase activity (31, 32) and thus cause a substantially lower rate of melanin synthesis in tissues exposed to it. Measurements of radioactive thiouracil incorporation indicate that any such inhibition of melanin synthesis must reach a maximum at concentrations of thiouracil above 1.5 mM (Fig. 1B).

The question has been answered by examining the effect of 2.0 mM thiouracil on tyrosinase-dependent incorporation of L-[2-14C]tyrosine into retinal pigment tissues. Tyrosinase dependence is defined as inhibition of tyrosine incorporation caused by 0.30 mM phenylthiourea. Results (Table IV) show that thiouracil causes a 90% inhibition of melanin synthesis under conditions of incubation in MEM. This change is significant and would have to be included in any calculations of thiouracil incorporation purported to represent an actual rate of melanin synthesis.

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**Table III**

Effect of phenylthiourea on incorporation of [2-14C]thiouracil into acid-insoluble fraction of chick retinal pigment tissues

<table>
<thead>
<tr>
<th>Experimental series</th>
<th>Tissue Isotope Incorporation period</th>
<th>Incorporation</th>
<th>dpm/mg protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0-4</td>
<td>1200</td>
<td></td>
</tr>
<tr>
<td>0.75 mM</td>
<td>1-4</td>
<td>1170</td>
<td></td>
</tr>
</tbody>
</table>

**Table IV**

Effect of thiouracil on tyrosinase-dependent (phenylthiourea-sensitive) incorporation of L-[2-14C]tyrosine into acid-insoluble fraction of chick retinal pigment tissues

<table>
<thead>
<tr>
<th>Experimental series</th>
<th>Tyrosinase-dependent incorporation</th>
<th>Inhibition caused by thiouracil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Thiouracil</td>
</tr>
<tr>
<td></td>
<td>dpm/mg protein</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>91,000</td>
<td>73,200</td>
</tr>
<tr>
<td>2</td>
<td>100,900</td>
<td>82,000</td>
</tr>
<tr>
<td>3</td>
<td>86,200</td>
<td>70,100</td>
</tr>
</tbody>
</table>

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Fig. 6. Effect of thiouracil on dopachrome formation in 0.1 M phosphate buffer (pH 6.8) containing 0.2 mM L-dopa and mushroom tyrosinase. Optical density measurements of the dopachrome absorption band (480 nm) were recorded at the time of maximum color development (7 to 12 min at 26°C).

**TABLE V**

Radioactivity of melanin isolated from chick retinal pigment tissue melanosomes

Tissues were incubated for 4 hours at 37°C in embryo extract medium and exposed to one of the following: (a) 0.75 mM [2-14C]thiouracil, specific activity 1 mCi per mmole; (b) 0.2 mM [U-14C]leucine, specific activity 5 mCi per mmole; and (c) 0.4 mM DL-[2-14C]tyrosine, specific activity 5 mCi per mmole.

<table>
<thead>
<tr>
<th>Labeled compound in medium</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-14C]Thiouracil ...........</td>
<td>10,900</td>
</tr>
<tr>
<td>[2-14C]Thiouracil plus 0.15 mm phenylthiourea</td>
<td>730</td>
</tr>
<tr>
<td>L-[14C]Leucine ............</td>
<td>11,200</td>
</tr>
<tr>
<td>DL-[2-14C]Tyrosine ........</td>
<td>17,500</td>
</tr>
<tr>
<td>DL-[2-14C]Tyrosine plus 0.15 mm phenylthiourea</td>
<td>2,700</td>
</tr>
</tbody>
</table>

**Inhibition of Dopachrome Formation by Thiouracil**—The oxidation of L-dopa catalyzed by mushroom tyrosinase provides clear evidence that thiouracil prevents the formation of red dopachrome in vitro (Fig. 6). Dopachrome has an absorption band maximum at 480 nm, and optical density changes at this wave length should give a reliable measurement of interference with dopachrome formation. L-Dopa was selected as the substrate to establish that thiouracil does not inhibit only tyrosine hydroxylolation. However, the results in the previous section imply that thiouracil is not a major inhibitor of either tyrosine hydroxylation or of the conversion of dopa to dopaquinone. Since dopaquinone rearranges spontaneously to form dopachrome (32), the inhibitory effect of thiouracil supports the hypothesis that thiouracil combines with dopaquinone to prevent dopachrome formation.

**Incorporation of Thiouracil into Melanin**—In the foregoing incorporation experiments, [2-14C]thiouracil incorporation into the acid-insoluble cell fraction was assumed to represent incorporation into melanin. This is a reasonable assumption considering the tyrosinase dependency of the incorporation. Direct proof of this was provided by isolating melanosomes (pigment granules) from tissues after [2-14C]thiouracil incorporation, and purifying the melanin from these particles. The specific activity of melanin (disintegrations per min per mg of melanin) in dissolved melanosomes plus carrier was measured before purification and then again after three cycles of purification. The specific activity measurements (Table V) indicate that at least three-quarters of the radioactivity remains with the melanin during purification and is presumably an indestructible part of the melanin structure. For comparison, other pigment tissues were incubated in L-[U-14C]leucine; the melanosomes were isolated from these tissues and treated similarly. Most of the leucine label disappeared during purification, indicating that it was probably located in contaminating protein and not part of the melanin structure. [2-14C]Thiouracil incorporated in the presence of 0.15 mm phenylthiourea is also apparently incorporated into melanin, since melanin purified from such incubations likewise retained much of the label during purification (Table V).

In comparable experiments with DL-[2-14C]tyrosine incorporation, the label was actually concentrated to some extent by the purification procedure. A comparison with the thiouracil result suggests that some thiouracil is lost during the purification process. [2-14C]Tyrosine labeling of the pigment granules in the presence of 0.15 mm phenylthiourea is likewise retained after purification (Table V), offering additional support for the contention that phenylthiourea does not totally inhibit melanin synthesis.

**Specificty of Thiouracil Incorporation**—Another assumption in the incorporation experiments is that [2-14C]thiouracil is only incorporated into melanosome particles and not into other cell parts. As a corollary to this idea, only melanin-producing cells should incorporate labeled thiouracil into the acid-insoluble fraction. The results (Table VI) show that only retinal pigment
epithelium and skin with pigmented feather germs (Barred Rock embryos) incorporate large quantities of thiouracil. There was no incorporation into five other nonmelanotic tissues. Three tissues (heart, adrenal gland, and liver) incorporated small amounts of label, probably as a result of the activity of a tyrosine hydroxylase (EC 1.14.3.4), which these tissues are known to contain (33). This enzyme acts to produce dopa, which is a precursor in the synthesis of norepinephrine (33). Presumably, under tissue culture conditions some of this dopa becomes oxidized to form quinones which could then react with thiouracil. This suggestion is supported by observations of Gross and Riedel (34) that heart cells maintained in monolayer culture accumulate tiny asphalt-colored vacuoles; these vacuoles probably contain diffuse melanin material resulting from the oxidation of some of the dopa formed through tyrosine hydroxylase activity.

Interestingly, thyroid gland and older skin tissues do not incorporate thiouracil. Thiouracil is an effective antithyroid compound (35), which probably binds to a protein sulfenyl iodide (36). Under the conditions used here, no [2-14C]thiouracil was found in the acid-insoluble fraction of active embryonic thyroid tissue. Thiouracil might also be expected to react with sulfhydryl groups that occur in 15-day chick skin (37), but label was not observed in the acid-insoluble fractions.

Many nonmelanotic tissues may not be permeable to thiouracil and therefore would be unlikely to incorporate labeled thiouracil into cell components. However, experiments with neural retina tissues contradict this idea. Neural retina tissues (7 day), which do not incorporate [2-14C]thiouracil into the acid-insoluble fraction, were incubated in [2-14C]thiouracil for 4 hours (under the conditions given in Table VI) and the tissue samples were washed and lyophilized. The radioactivity in these lyophilized samples was about one-sixth the amount of [2-14C]thiouracil occurring in the acid-insoluble fraction of retinal pigment tissues. Although the trichloroacetic acid steps in the Siekevitz procedure (23) for preparing the acid-insoluble fraction probably remove most of the nucleic acids, it was of some interest to know if [2-14C]thiouracil might become incorporated into nucleic acids of cells. Accordingly, neural retina tissues incubated in [2-14C]thiouracil under the conditions given in Table VI were processed with cold perchloric acid and cold ether alcohol to preserve the nucleic acids (38), but there was negligible radioactivity in the samples.

Incorporation of [35S]Thiouracil—Since thiol compounds are believed to react with the aromatic nucleus of quinones through the sulfhydryl group, an important question about thiouracil incorporation into melanin is whether the sulfur atom is retained in the melanine-thiouracil complex that forms. Its retention would be consistent with this mechanism of action. Incorporation of [35S]thiouracil was compared to that of [2-14C]thiouracil in three experimental series (Table VII). Surprisingly, on a molar basis, about twice as much sulfur-labeled thiouracil was incorporated as carbon-labeled thiouracil. Possibly, half of the pyrimidine rings become detached from the thiouracil-melanin complex, leaving behind the sulfur atoms. If the label is on the pyrimidine ring, as with [2-14C]thiouracil, then the loss of pyrimidine rings would appear as less incorporation of [2-14C]thiouracil than of [35S]thiouracil. The reproducibility of this ratio in different experimental series (Table VII) probably has significance in reference to the structure of the thiouracil-melanin complex; perhaps molecules in certain structural positions are more labile.

Evidently, the greater accumulation of [35S]thiouracil into the acid-insoluble tissue fraction is not caused by chemical reactions unrelated to melanin synthesis. [35S]Thiouracil incorporation is tyrosinase-dependent (phenylthiouracil-inhibitable) to about the same extent as [2-14C]thiouracil incorporation, and [35S]thiouracil is not incorporated into the acid-insoluble fraction of 7-day neural retina tissues.

**Table VII**

<table>
<thead>
<tr>
<th>Experimental series</th>
<th>Labeled compound in medium</th>
<th>Specific activity</th>
<th>Incorporation</th>
<th>Molar ratio mCi/mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[35S]Thiouracil</td>
<td>1.12</td>
<td>48,500</td>
<td>19.5</td>
</tr>
<tr>
<td>2</td>
<td>[2-14C]Thiouracil</td>
<td>1.12</td>
<td>23,000</td>
<td>9.2</td>
</tr>
<tr>
<td>3</td>
<td>[35S]Thiouracil</td>
<td>1.12</td>
<td>50,600</td>
<td>20.3</td>
</tr>
</tbody>
</table>

* Figures are mean incorporation values for three cultures.

Fig. 7. The effect of different concentrations of L-tyrosine on the rate of incorporation of L-[2-14C]tyrosine (specific activity 2.13 mCi per mmol) into the acid-insoluble fraction of chick retinal pigment epithelium. A series of tissues was incubated for 4 hours at 37°C in MEM with 10% fetal bovine serum. The points are mean incorporation values for two cultures.
Comparison of tyrosinase-dependent (phenylthiourea-sensitive) incorporation of [35S]thiouracil and L-[2-14C]tyrosine into acid-insoluble fraction of chick retinal pigment tissues

Tissues were incubated for 4 hours at 37°C in MEM with 10% fetal bovine serum. The medium contained both 2.0 mM thiouracil and 0.4 mM L-tyrosine. In each experimental series (12 cultures), six cultures were treated with [35S]thiouracil and six with L-[2-14C]tyrosine; the medium in three cultures of each of these two sets contained 0.3 mM phenylthiourea.

<table>
<thead>
<tr>
<th>Experimental series</th>
<th>Labeled compound in medium</th>
<th>Specific activity</th>
<th>Tyrosinase-dependent incorporation</th>
<th>Molar ratio thiouracil/tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>dpm/mg protein</td>
<td>nmol/mg protein</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>[35S]Thiouracil</td>
<td>121,900*</td>
<td>25.9</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>L-[2-14C]Tyrosine</td>
<td>83,000</td>
<td>17.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>[35S]Thiouracil</td>
<td>138,200</td>
<td>26.4</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>L-[2-14C]Tyrosine</td>
<td>89,000</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>[35S]Thiouracil</td>
<td>128,900</td>
<td>28.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-[2-14C]Tyrosine</td>
<td>81,500</td>
<td>17.4</td>
<td></td>
</tr>
</tbody>
</table>

*a Figures are the differences obtained by subtracting the mean incorporation values of the cultures with phenylthiourea from those without the drug.

Comparison of incorporation of [35S]6-n-propylthiouracil and [35S]thiouracil into acid-insoluble fraction of chick retinal pigment tissues

Tissues were incubated in embryo extract medium and exposed to a 0.75 mM concentration of labeled compound for 4 hours at 37°C.

<table>
<thead>
<tr>
<th>Experimental series</th>
<th>Labeled compound in medium</th>
<th>Specific activity</th>
<th>Incorporation</th>
<th>Molar ratio propylthiouracil/thiouracil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>dpm/mg protein</td>
<td>nmol/mg protein</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>[35S]Propylthiouracil</td>
<td>53,600*</td>
<td>17.0</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>[35S]Thiouracil</td>
<td>45,100</td>
<td>23.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>[35S]Propylthiouracil</td>
<td>34,600</td>
<td>17.0</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>[35S]Thiouracil</td>
<td>47,400</td>
<td>24.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>[35S]Propylthiouracil</td>
<td>31,100</td>
<td>15.3</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>[35S]Thiouracil</td>
<td>42,700</td>
<td>22.3</td>
<td></td>
</tr>
</tbody>
</table>

*a Figures are mean incorporation values for three cultures.

was defined as phenylthiourea-sensitive (tyrosinase-dependent) incorporation. As shown in Fig. 5, maximal inhibition of labeled thiouracil and labeled tyrosine incorporations occurred at 0.30 mM phenylthiourea. Although phenylthioureas apparently does not inhibit melanin synthesis completely, the maximal phenylthiourea-sensitivity undoubtedly defines comparable quantities of melanin synthesis in both incorporation reactions.

Saturating amounts of labeled thiouracil and L-tyrosine were selected for these experiments on the assumption that optimal rates of reaction occur at these saturating levels, and that the two incorporation reactions are most likely to be comparable at optimal reaction rates. An experiment similar to that in Fig. 1B has illustrated that [35S]thiouracil incorporation in MEM is likewise saturated at a concentration of 2.0 mM. Experiments with L-[2-14C]tyrosine show that incorporation of labeled tyrosine by pigment tissues in MEM is saturated (for both melanin and protein synthesis) at a concentration of 0.4 mM L-tyrosine (Fig. 7). The experimental design for comparing tyrosinase-dependent thiouracil and tyrosine incorporations is shown in Table VIII.
Thiouracil at 2.0 mM is included in both incubation media so that thiouracil and tyrosine incorporation can be compared directly without making corrections for the inhibitory effect of thiouracil on tyrosinase activity. The results indicate that 1.5 moles of thiouracil are incorporated into melanin for each mole of tyrosine incorporated.

Incorporation of [tS]Propylthiouracil—Studies with [tS]6-n-propylthiouracil suggest that thiouracil derivatives can also be incorporated into melanin. Incorporation of this compound was compared directly to the incorporation of [tS]thiouracil in three experiments with embryo extract medium. Table IX shows that on a molar basis, incorporation of propylthiouracil is about 70% that of thiouracil incorporation. Propylthiouracil incorporation may be saturated at the concentration (0.75 mM) used in these experiments, but a saturation difference could easily account for the 30% discrepancy between the two incorporations.

Although the evidence suggests the probable incorporation of propylthiouracil into melanin, these experiments prove only that the sulfur atom is retained in acid-insoluble material. The pyrimidine moiety of incorporated propylthiouracil may be much more labile than the pyrimidine of thiouracil (Table VII). The answer to this question must await the availability of 14C-labeled propylthiouracil.

**DISCUSSION**

The data presented support the hypothesis that a thiol-containing melanin, or pheomelanin, is synthesized in live pigment cells exposed to thiouracil. This melanin must be a thiouracil-containing pheomelanin since [tS] of the sulphydryl group and [14C] of the pyrimidine ring of thiouracil are both incorporated, and the incorporation is tyrosinase-dependent. Thiouracil forms an integral part of the melanin polymer as shown by the insolubility of thiouracil pheomelanin in trichloroacetic acid and ethanol, and the purification of highly radioactive melanin from tissues exposed to [2-14C]thiouracil.

The 4-hour incorporation of thiouracil by retinal pigment tissues of a specific age is highly reproducible, although the incorporation reaction does not follow a straight line relationship with time. The reaction can be saturated with external thiouracil and incorporation is proportional to tissue mass. Obviously, thiouracil incorporation can be used to measure relative rates of melanin synthesis in pigment cell cultures or in pigment cells incubated in vitro. Melanin synthesis in small aquatic organisms can be studied directly by this method (18). Because of the specificity of the incorporation reaction, a tritiated thiouracil could be used in conjunction with autoradiography to investigate melanotic activity at the subcellular level.

Two quinone intermediates appear in the oxidation of tyrosine to melanin: a dopaquinone (Fig. 8a) formed early in the pathway, and a 5,6-dihydroxydopaminequinone which appears later. The in vitro experiment with mushroom tyrosinase indicates that thiouracil reacts with dopaquinone. Clearly, thiols can also condense with the indolequinone (11, 40). The tyrosinase dependence of thiouracil incorporation strongly supports the idea that thiouracil condenses with these reactive intermediates in the melanin pathway. Presumably, thiouracil becomes part of the melanin structure when these reactive intermediates subsequently polymerize.

Very little is known about which intermediates polymerize and what kind of basic melanin structure is formed. Mason (40) believes that melanin structure is largely a homopolymer of dihydroxyindole units linked in regular series between aromatic ring positions 4 to 7 (Fig. 8b). However, results of degradation studies (41) and biosynthetic experiments (42) support a structure based on less regular and more complex condensation of various oxidative intermediates.

Given the evidence that thiols react through their sulphydryl groups with free positions on the aromatic rings of quinones (8-11), there are three positions, 2, 5, and 6, at which thiouracil could condense with dopaquinone (Fig. 8a) and two positions, 4 and 7, at which it could react with the 5,6-dihydroxydopaminequinone. The Mason model (39) is inconsistent with the finding (Table VIII) that 3 moles of thiouracil occur in melanin for each 2 moles of tyrosine oxidation products; the structure in Fig. 8a is impossible if either position 4 or 7 of the indolequinone is occupied by thiouracil. If thiouracil reacted only with dopaquinone, a tetramer would be the largest structure that could have the necessary thiouracil to tyrosine ratio and still involve the kind of chain linkage proposed by Mason (39). Tetramers could not possibly account for the highly polymerized (viscous and gelatinous) melanin observed visually in the giant pigment cells of ascidian embryos reared in thiouracil (13), nor would tetramers be likely to be retained by the purification process used in Table VI. Obviously, there is some other kind of linkage between the melanin subunits, at least in thiouracil pheomelanin.

Thiouracil pheomelanin, and possibly some natural pheomelains, may have a primary structure similar to eumelains. The thiouracil results are consistent with this view. A model of eumelanin structure devised by Hempel (42) accommodates thiouracil incorporation without requiring any change in the primary structure of melanin. In this model (Fig. 9) the number of potential thiouracil locations compared to subunit number gives a ratio (1.3) reasonably close to the ratio (1.5) actually measured in Table VIII. The Hempel model may prove to be a good approximation of the real structure of vertebrate eumelanin, but there is still no general agreement on this point (39, 43).

Synthesis of thiouracil pheomelanin concentrates the drug in a specific differentiated cell type, the melanocyte. Propylthiouracil experiments suggest that some thiouracil derivatives would be similarly concentrated. Perhaps thiouracil and its derivatives can be used to destroy malignant and abnormal melanocytes. For example, [tS]thiouracil of high specific activity incorporated into pigment-producing cells might inflict sufficient radiation damage to be destructive to the cells or inhibitory to growth. Possibly thiouracil derivatives with toxic side groups would be concentrated into pheomelanin by synthetically active melanocytes. Thiouracil and its derivatives might, therefore, find many applications in chemotherapeutic, diagnostic, and cosmetic procedures with normal and malignant pigment cells.

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J. R. Whittaker


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