SUPPRESSION OF PIGMENTATION IN MOUSE MELANOMA CELLS BY 5-BROMODEOXYURIDINE

Effects on Tyrosinase Activity and Melanosome Formation

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ABSTRACT

Low concentrations (1–3 μg/ml) of 5-bromodeoxyuridine (BrdU) reversibly suppress pigmentation in a highly pigmented clone ($B_5$59) of cultured B16 mouse melanoma cells. We have found that unpigmented cells (clone $C_3$471), derived by long-term culture of $B_5$59 cells in 1 μg of BrdU/ml, were completely amelanotic with no biochemically or cytochemically detectable tyrosinase activity or ultrastructural evidence of premelanosomes. The process by which pigmentation is suppressed was studied in $B_5$59 cells during a 7-day period of growth with BrdU (3 μg/ml). Assays of tyrosinase activity showed that activity was reduced after 1 day and decreased progressively, approaching zero by 7 days. A quantitatively minor part of this reduction was directly attributable to the appearance of a dialyzable inhibitor of tyrosinase activity. Acrylamide gel electrophoresis revealed two bands of activity corresponding in Rx values to the T_1 and T_2 forms of soluble tyrosinase. Both were progressively reduced during growth with BrdU but one form (T_1) was consistently affected earlier than the other (T_2). Ultrastructural-cytochemical studies also showed an early effect on the localization of tyrosinase reaction product. At day 3, reaction product was no longer present in Golgi saccules and Golgi-associated smooth surfaced tubules, but was still seen within premelanosomes, compound melanosomes, and occasional Golgi-associated vesicles. By 7 days tyrosinase reaction product was usually not demonstrable. The number of premelanosomes was progressively decreased during growth with BrdU. Premelanosomes became concentrated in the juxtanuclear region and at day 3 many were contained within abnormally large and numerous compound melanosomes. Premelanosomes and compound melanosomes were rarely seen at 7 days, by which time the cultures were nearly amelanotic. The coordinated suppression of melanogenesis by BrdU may provide a useful model in which to study the normal regulation of this process.

Low concentrations of 5-bromodeoxyuridine (BrdU) have been shown to suppress differentiated functions preferentially in a variety of cell types (1–9). Silagi and Bruce (5) reported that treatment of melanotic melanoma cells (clone $B_5$59) with BrdU altered cellular morphology and suppressed both pigmentation and tumorigenicity while cell proliferation continued at a near normal level. These effects appeared dependent upon incorporation of the BrdU into cellular DNA as an analogue of thymidine. The effects upon morphology, pigmentation, and tumorigenicity were reversible upon growth of the cells in normal medium (5, 10). The molecular basis of this reversible effect is

not understood but it serves to provide a useful system in which cellular control of both differentiated function and tumorigenicity may be studied. We now report more detailed information on the manner in which treatment with BrdU results in loss of pigmentation in cultured melanoma cells (clone $B_5$59).

Tyrosinase (EC 1.10.3.1; *O*-diphenol:oxygen oxidoreductase) catalyzes the conversion of tyrosine to melanin. The enzyme contains copper which is required for activity (11, 12). The abbreviated reaction scheme below is based on the studies of Raper (13) and Mason (14).

Tyrosine $\xrightarrow[1]{+O}$ 3,4-dihydroxyphenylalanine

(DOPA)

$\xrightarrow[2]{+O}$ dopaquinone $\rightarrow$

2,3-dihydro-5,6-dihydroxyindole-2-carboxylic acid

(leucodopachrome)

$\rightarrow$ 2,3-dihydroindole-5,6-quinone-2-carboxylic acid

(dopachrome)

$\xrightarrow[\text{steps}]{\text{several}}$ melanin

Reactions *1* and *2* occur enzymatically. The subsequent reactions proceed in the absence of tyrosinase but the reaction rates are increased in the presence of the enzyme (11).

Vertebrate melanin synthesis occurs in vivo within specialized tyrosinase-containing organelles, the melanosomes. On the basis of combined cell fractionation and ultrastructural investigations, the following scheme was proposed in 1963 for melanosome formation (15). Tyrosinase is synthesized on ribosomes and then may be transported through endoplasmic reticulum (ER) to the Golgi area. Here tyrosinase-containing vesicles are released. These develop into membrane-limited particles (premelanosomes) with a distinctive ordered matrix. The matrix of the premelanosomes is progressively obscured during melanin synthesis, resulting in the formation of the fully melanized mature melanosomes in which tyrosinase activity is no longer detected. Subsequent investigations into the role of the Golgi and associated elements in premelanosome formation have indicated that new premelanosomes are formed from dilated cisternae of smooth ER (16, 17). Novikoff et al. (16) have suggested that this ER is part of a specialized system of Golgi-associated smooth ER (GERL) which is involved in the packaging of secretory proteins in many cell types (18).

Although tyrosinase activity was cytochemically demonstrable within GERL and small vesicles near the Golgi apparatus, melanin was not evident in these structures (16, 17). Cell fractionation revealed tyrosinase activity associated not only with the large particulate fraction containing premelanosomes and melanosomes, but also with the microsomal and soluble fractions (15). The cellular mechanisms preventing in vivo melanin synthesis by extra-melanosomal tyrosinase are not understood. Soluble tyrosinase has been separated by chromatographic (12) and electrophoretic (19, 20) means into multiple forms. The origin of these multiple forms and their relative place in melanin synthesis in vivo have not been determined. The present studies were undertaken in order to explore an experimental system which we believe could be useful for investigating these and other unresolved questions concerning normal melanogenesis.

MATERIALS AND METHODS

Cells

The melanoma cell line was derived from the B16 mouse melanoma by Hu (21). Pigmented clones were produced from this line by Silagi (22) and the highly melanotic clone, $B_5$59, was used in this investigation. Clone $C_3$471 (5, 10, 23), derived from $B_5$59 cells grown continuously and cloned in medium containing 1 μg of BrdU/ml, was also studied. The $C_3$471 cells used in this study had been exposed to BrdU (1 μg/ml) for a total of approximately 9 mo. Cells were maintained as monolayer cultures in Eagle's minimal essential medium with 10% fetal calf serum and antibiotics as described previously (22). Cells were tested for *Mycoplasma* by Hayflick's procedure (24) and found free from contamination.

$B_5$59 and $C_3$471 cells were grown in 32 oz prescription bottles, seeded at 2×10^5 viable cells/bottle. Replicate cultures for observation of living cells and for light and electron microscopy were grown in 35 mm Falcon plastic petri dishes, either directly on the plastic or on sterile glass cover slips. Cultures were seeded at 10^4 viable cells/dish. For time course experiments, cultures of $B_5$59 cells were seeded 2 days before the start of an experiment, then changed to fresh regular medium or medium containing BrdU (Nutritional Biochemicals Corp., Cleveland, Ohio) at the final concentration of 3 μg/ml. For each point in the time course studies, replicate cultures of cells

grown in medium without and with BrdU were compared (e.g., 3 day controls vs. 3 day treated).

Enzyme Assays

Cells were harvested with 0.25% trypsin-0.1% EDTA (ethylenediaminetetraacetate, disodium salt) in phosphate-buffered saline (PBS). The cells were washed by centrifugation with PBS and the washed pellet was frozen at −20°C. All subsequent operations were performed at 0–4°C. The frozen pellet was homogenized in 0.25 M sucrose with a Dounce homogenizer using 50 strokes of a tight pestle. In some experiments this homogenate was fractionated by differential centrifugation. The homogenate was centrifuged at 650 *g* for 10 min. The pellet was gently resuspended in sucrose and recentrifuged. The resulting pellet resuspended in sucrose was termed the "nuclear fraction". The combined supernatants were centrifuged at 30,000 *g* for 30 min. The pellet from this centrifugation was resuspended in sucrose as the "large particulate" fraction. The 30,000 *g* supernatant was termed the "supernatant fraction". In other experiments the supernatant fraction was prepared without preliminary fractionation. Usually a sample of the supernatant fraction was dialyzed overnight at 4°C against 0.05 M barbital buffer (pH 8.6).

Tyrosinase activity was assayed spectrophotometrically by following the oxidation of L-DOPA to dopachrome at 475 nm (25). At this absorption maximum of dopachrome, the molar absorbancy coefficient is 3.7×10^3 (14). The reaction mixture contained 3.8 μmol of L-DOPA (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), up to 200 μl of enzyme extract and 0.1 M phosphate buffer, pH 6.8, to a final volume of 2.2 ml. Assays were performed at 30°C in a Gilford recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Rates were measured during the first few minutes of the reaction while they were linear. Correction for DOPA autoxidation was made. One unit of activity is defined as that amount of enzyme catalyzing the production of 1 μmol of dopachrome per minute and is equal to an increase in absorbance of 1.6818 A per minute under these conditions. Specific activity equals units per milligram protein. Protein was estimated by the method of Lowry et al. (26), using Protein Standard Solution (Armour Pharmaceutical Co., Chicago, Ill.) of crystalline bovine serum albumin standardized by the Kjeldahl method.

Electrophoresis

Soluble tyrosinases were separated by acrylamide gel electrophoresis. The method of Ornstein (27) and Davis (28) was modified by the use of more concentrated (5 ×) reservoir buffer and the omission of sample and stacking gels. The samples mixed with 25% sucrose were layered directly onto the running gel. Bromphenol blue was mixed with the sample as a tracking dye and the Rx values were calculated relative to the migration of this dye. The position of bands containing tyrosinase was revealed by incubation with L-DOPA, according to the method of Burnett et al. (20). Immediately after electrophoresis the gels were neutralized in 0.5 M phosphate buffer (pH 6.6) for 30 min, then incubated for 1 h in 0.15% L-DOPA in 0.1 M phosphate buffer (pH 6.8) at 37°C. The incubation mixture was changed twice during this period. Gels were fixed in 7.5% acetic acid. Densitometric tracings at 475 nm were made by scanning fixed gels in a Gilford recording spectrophotometer.

Light Microscopy

Cover slip cultures were fixed at 4°C either overnight in 4% formaldehyde-1% calcium chloride (29) or for 10 min–3 h in 3% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.) in 0.2 M cacodylate buffer (pH 7.4). For demonstration of tyrosinase activity the formaldehyde-fixed cells were rinsed in buffer and directly incubated in DOPA-containing medium (16, 30) while glutaraldehyde-fixed cells were rinsed overnight in buffer, containing 7% sucrose, before incubation. After incubation, the cover slips were mounted with glycerin jelly on glass slides.

Electron Microscopy

Monolayer cultures of cells were rinsed in physiological saline, fixed for 1 h at 4°C in 3% buffered glutaraldehyde, rinsed in buffer, and left overnight at 4°C in buffer containing 7% sucrose. Some cultures were incubated for cytochemical detection of tyrosinase activity as described previously (16, 30). All of the cultures were postfixed for 1 h at 4°C in 1% osmium tetroxide in 0.2 M cacodylate buffer, dehydrated through a graded series of alcohols, propylene oxide, and embedded as a monolayer in Epon 812. After polymerization, the petri dish was removed, and suitable areas were selected for electron microscopy.

Cell pellets were prepared from cultures which had been changed to calcium-free medium 16–24 h before trypsinization in order to facilitate removal of the cells without the use of EDTA. Trypsinized cells were washed by centrifugation in physiological saline. The cell pellet was fixed for 1 h in 3% buffered glutaraldehyde at 4°C, and processed essentially as described for the monolayers.

Thin sections were cut from the monolayers and pellets with a diamond knife on a Porter-Blum MT-1 microtome (Ivan Sorvall, Inc., Newtown, Conn.), collected on uncoated copper grids, stained lightly with lead citrate (31), stabilized with carbon (32),

TABLE I

Distribution of Tyrosinase Activity in Subcellular Fractions

	Tyrosinase		Protein			
	U/10^9 cells	% of total	Mg/10^9 cells	% of total	Specific activity	Percent control*
Control‡						
Nuclear§	0.62	12.0	66.50	17.3	0.0093	—
Large particulate	1.98	38.4	153.63	39.9	0.0129	—
Supernatant	2.55	49.5	165.25	42.9	0.0154	—
Total	5.15		385.38		0.0134‖	
BrdU-Treated‡						
Nuclear§	0.09	24.3	152.69	47.1	0.0006	6.5
Large particulate	0.10	27.0	46.19	14.2	0.0022	17.1
Supernatant	0.18	48.6	125.50	38.7	0.0014	9.1
Total	0.37		324.38		0.0011‖	

* (Specific activity of BrdU-treated/specific activity of control) × 100.

‡ Replicate cultures of $B_5$59 cells were grown for 3 days in regular medium (Control) or medium containing 3 μg of BrdU/ml (BrdU-treated).

§ Contained melanin granules sedimenting with the nuclei.

‖ Total units/total mg protein.

and examined in a Siemens Elmiskop 101 electron microscope.

A number of tests were made to determine the specificity of the cytochemical reaction for tyrosinase activity in both light and electron microscopic preparations. Some cells were incubated in a substrate-free medium. Other cells were left overnight at 4°C in 0.2 M cacodylate buffer containing 10^{-2} M Na diethyldithiocarbamate (DECA, a copper chelator), 2 mg DOPA/ml and 7% sucrose, rinsed thoroughly in buffer, and then incubated with DOPA. In some experiments, the DECA-treated cells were rinsed in buffer, left for 1 h in 1% aqueous copper sulfate, and rinsed again in buffer before incubation.

RESULTS

Enzyme Assays

Tyrosinase activity in control $B_5$59 cells was present in various cell fractions separated by differential centrifugation (Table I). Growth with 3 μg of BrdU/ml for 3 days caused a reduction in activity of all fractions. In addition the pattern of distribution of both tyrosinase activity and cell protein was altered. The BrdU-treated cells showed an increased proportion of tyrosinase units sedimenting with the nuclei at the expense of activity in the large particulate fraction. Protein distribution was altered in a similar direction but to a greater degree. Phase microscopy revealed that the crude nuclear fraction of both control and BrdU-treated cells contained large numbers of melanin granules to which we attribute the tyrosinase activity of this fraction. Visual comparison of the fractions indicated less melanin in the large particulate fraction and more melanin in the nuclear fraction of the treated cells than in these fractions of the controls.

As tyrosinase activity of all cell fractions was found to be affected by growth with BrdU, the

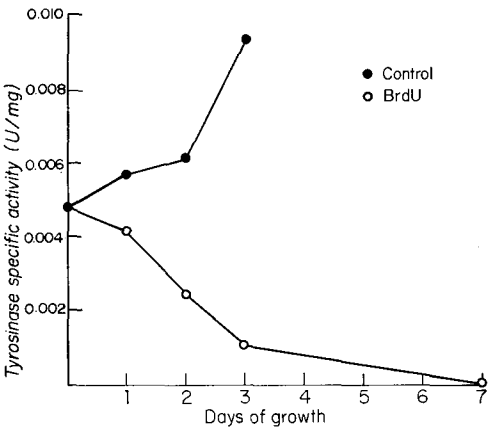


FIGURE 1 Changes in tyrosinase specific activity of the supernatant fraction of $B_5$59 cells grown in regular medium (●) and replicate cultures grown in medium containing 3 μg of BrdU/ml (○). Data from one time course experiment.

supernatant fraction was chosen for time course studies because of greater ease in studying enzyme which is primarily in the soluble form.

The results of a time course experiment are shown in Fig. 1. In each experiment tyrosinase specific activity increased during the culture cycle of untreated $B_5$59 cells, while growth with BrdU (3 μg/ml) caused a progressive reduction in activity with time. This decrease in activity was accompanied by the appearance of a lag period in the assays of supernatant fraction of BrdU-treated cells, during which DOPA autoxidation was inhibited. Representative assays are shown in Fig. 2. This lag period tended to increase with time of treatment (Table II). Dialysis of the supernatant fraction of control cells against barbital buffer (0.05 M, pH 8.6) caused a reduction in Lowry-positive material and concomitant modest increase in tyrosinase specific activity. Dialysis of the supernatant fraction of treated cells resulted in a similar decrease in Lowry-positive material but there was also a large increase in detectable units of tyrosinase. The specific activity was often increased more than 100%. There was complete loss of the lag period in the assays of dialyzed material.

Fig. 3 summarizes data from 10 experiments showing the time course of the effect of BrdU (3 μg/ml) treatment on tyrosinase activity of the supernatant fraction. A reduction was evident after only 24 h of culture in the presence of BrdU. By 2 days the activity of the supernatant fraction

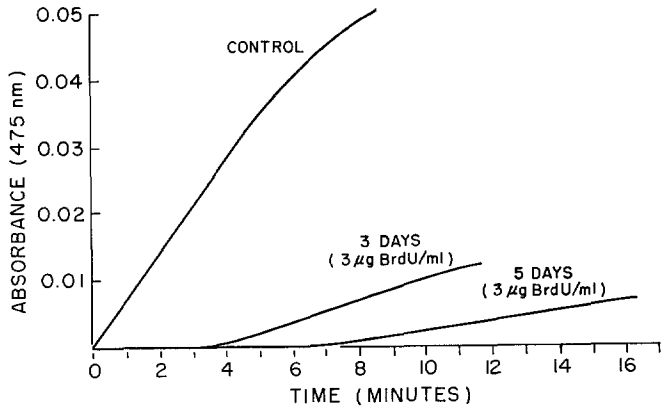


FIGURE 2 Representative assays of tyrosinase activity of the supernatant fraction of untreated $B_5$59 cells (control) and $B_5$59 cells grown in medium containing 3 μg of BrdU/ml for 3 and 5 days.

TABLE II

*Lag Periods in Tyrosinase Assays**

Days of Growth	Regular medium		Medium with BrdU (3 μg/ml)	
	Mean lag	Range	Mean lag	Range
	min		*min*	
1	0.7	— (1)‡	0.6	— (1)
2	0.6	0.5–0.9 (3)	3.2	2.1–5.4 (3)
3	0.5	0.0–1.3 (7)	6.1	0.9–13.1 (7)
5	0.6	0.3,0.8 (2)	14.4	— (1)§
7	0.9	0.5–1.6 (3)	19.1	9.1,29.0 (2)§

* Assays of undialyzed supernatant fraction. After dialysis, the mean lag period was 0.1 min (range 0.0–0.8) with no difference in lag between control and experimental dialyzed supernatant fractions.

‡ Number in parentheses denotes number of extracts assayed.

§ Remaining extracts contained no detectable tyrosinase activity (see Fig. 3).

of treated cells was less than 50% of the control activity. This reduction continued with longer periods of growth in medium containing BrdU, and tyrosinase activity approached zero by 7 days of treatment. Variation in the time course of effect of BrdU in separate experiments should be noted. For example, the activity of supernatant fractions of cells treated for 3 days ranged from 7% to 28% of control activity. In each individual experiment, dialysis of the supernatant fraction resulted in a marked increase in tyrosinase activity of BrdU-treated cells. However, this increase was somewhat overshadowed by the variability in activity of the undialyzed supernatant fractions from different experiments. In three of five experiments in which cells treated for 7 days with BrdU were studied, no tyrosinase activity was detectable in the supernatant fraction. In two of these three experiments the supernatant fractions were dialyzed and reassayed. No tyrosinase activity was detectable even after dialysis.

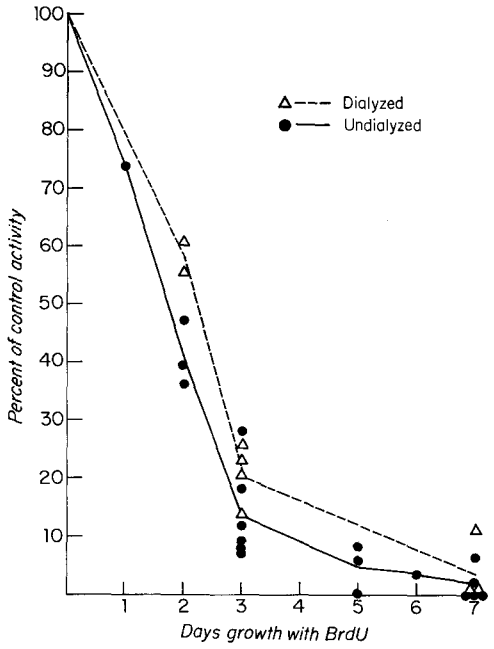


FIGURE 3 Time course of effect of growth with BrdU (3 μg/ml) on tyrosinase specific activity. Summary of 10 experiments. Values for the cultures grown with BrdU are expressed as a percentage of the specific activity of a replicate control culture grown in regular medium for the same period. Activities determined on supernatant fractions before (●—) and usually also after dialysis (△---) overnight at 4°C against 0.05 M Barbital buffer (pH 8.6). Lines are drawn between the arithmetic means of multiple experimental points.

No detectable tyrosinase activity was found in undialyzed or dialyzed extracts of C_3471 cells grown continuously in medium containing 1 μg of BrdU/ml. Undialyzed supernatant fraction of these cells inhibited DOPA autoxidation but this inhibition was eliminated by dialysis. Mixing experiments with extracts of C_3471 cells (see Table III) demonstrated: (*a*) inhibition of the tyrosinase activity of untreated $B_5 59$ cells, proportional to the amount of C_3471 material present; (*b*) elimination of this inhibition by dialysis of C_3471 extracts; and (*c*) the heat stability of inhibitory material in C_3471 extracts. Dialysis against distilled water also removed inhibitory activity but preliminary attempts to recover inhibitory material for further study by flash evaporation of the water used for dialysis were unsuccessful.

TABLE III

Inhibition of Tyrosinase Activity by Supernatant Fraction of C_3471 Cells

			Mixtures*		
	$B_5 59$ (25 μl)	C_3471 (200 μl)	(50 μl)	(100 μl)	(200 μl)
Untreated	100‡	0	75.4	51.1	31.5
Heated 10 min at 100°C	0	0	—	46.7	36.8
Dialyzed 0.05 M barbital, pH 8.6	104.1	0	—	100.9	—

* C_3471 supernatant fraction (volume and treatment as indicated) was added to 25 μl untreated $B_5 59$ supernatant fraction immediately before assay.

‡ Activity expressed as percent of units detected in untreated $B_5 59$ supernatant fraction.

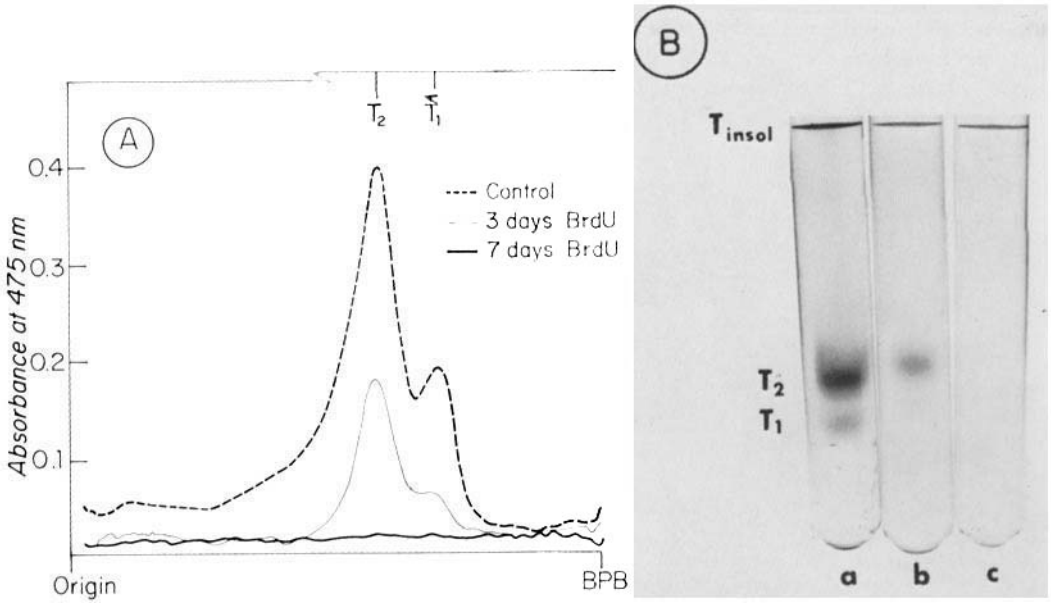


FIGURE 4 Effect of growth with BrdU (3 μg/ml) on soluble tyrosinase pattern, as shown by acrylamide gel electrophoresis. Dialyzed supernatant fraction containing ~ 188 μg protein was applied to each gel. Fig. 4 A, densitometric tracings of gels scanned at 475 nm with a Gilford recording spectrophotometer equipped with a linear transport attachment; migration of bromphenol blue dye marker (BPB). Fig. 4 B, photograph of same gels. *Gel a*, control culture grown for 3 days on regular medium. *Gel b*, replicate culture grown for 3 days with 3 μg of BrdU/ml. *Gel c*, replicate culture grown for 7 days with 3 μg of BrdU/ml.

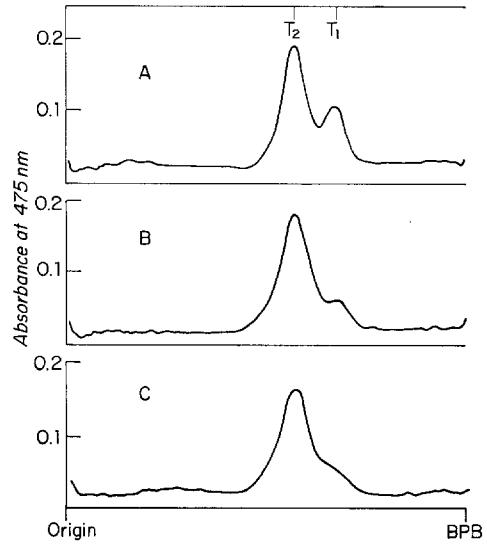


FIGURE 5 Effect of growth with BrdU (3 μg/ml) on T_1 and T_2 forms of soluble tyrosinase. Dialyzed supernatant fraction containing approximately 0.0006 U of tyrosinase activity was applied to each gel. Fig. 5 A, control $B_5$59 cells grown for 3 days on regular medium. Fig. 5 B, cells of replicate culture grown for 3 days with BrdU. Fig. 5 C, cells grown for 7 days with BrdU.

Electrophoresis

Electrophoresis of the supernatant fraction of untreated $B_5$59 cells revealed the presence of two soluble forms of the enzyme as well as DOPA-reactive material unable to penetrate the 7% acrylamide gels. The latter may correspond to insoluble (small particle-bound) tyrosinase (20) not sedimented at 30,000 *g*. The soluble tyrosinase activity was seen in bands with Rx values of approximately 0.68 (T_1) and 0.57 (T_2). The activity of T_2 appeared greater than that of T_1 in untreated $B_5$59 cells.

Treatment with BrdU caused reduction in insoluble and soluble activity, without affecting the Rx values. Fig. 4 depicts the progressive reduction of T_1 and T_2 during treatment with 3 μg of BrdU/ml. In this experiment 7-day treated cells had no detectable activity by assay, and no bands of tyrosinase activity were seen upon electrophoresis.

The multiple forms of tyrosinase appeared to be affected unequally by treatment with BrdU. Although the insoluble tyrosinase was difficult to quantitate, it appeared to be greatly reduced in

cells treated for 2–3 days. The relative effects on T_1 and T_2 are shown in Fig. 5. During the course of treatment with BrdU, T_1 was reduced earlier than T_2. In experiments in which the supernatant fraction of 7-day treated cells retained some activity, that activity was almost exclusively in the T_2 band. It thus appeared that the order of effect was $T_{insol} \geq T_1 > T_2$.

Electrophoresis of supernatant fractions of C_3471 cells confirmed the absence of tyrosinase activity in these cells.

Light Microscopy

The untreated melanoma cells grew in a reticulated, piled manner (Fig. 6). The various control cultures presented much the same picture except for some crowding by day 7, and will be described as a group. Cells were generally spindle-shaped with melanin granules dispersed throughout the cytoplasm (Fig. 7). Heavily pigmented cells growing within densely piled regions contained occasional enlarged melanin granules which were identified by electron microscopy as compound melanosomes (Fig. 21, inset). The number of such cells containing compound melanosomes increased as the cultures became more crowded. In the control cultures, a darkening of the melanin granules was seen after incubation in the DOPA-containing medium but because of the high degree of pigmentation normally present within these cells, it was not possible to document any increase in pigmentation or localization of reaction product in the Golgi apparatus. The staining reaction as well as autoxidation of DOPA could be inhibited by adding DECA (a copper chelator) to the incubation medium. In order to evaluate whether the darkening of the melanin granules was due to enzyme activity or absorption of oxidized DOPA, the cultures were first exposed to a solution containing both DECA and DOPA overnight, then rinsed thoroughly and incubated in DOPA-containing medium without DECA. When this was done, although autoxidation of DOPA occurred in the incubation medium, no significant staining of the cells was observed. The inhibition of tyrosinase activity by DECA could be partially reversed by treating the cells with a solution of copper sulfate before incubation in DOPA-containing medium.

Growth in medium containing 3 μg of BrdU/ml caused alterations in morphology which were discernible by 2 days and obvious by 3 days (Fig. 8). The cells began to disassociate and individual cells flattened and enlarged. Melanin granules became concentrated near the nucleus, with a concomitant loss of pigmentation from the cytoplasmic processes. Additionally, compound melanosomes were frequently seen in the 3-day treated cells (Fig. 9). Incubation of the cells for tyrosinase activity showed staining of the melanin granules. With longer periods of growth with BrdU, the cells became more flattened, exhibited "contact inhibition", and the juxtanuclear melanin granules were reduced, with many cells appearing amelanotic by light microscopy at 7 days (Fig. 10). Except for staining of the few melanin granules present in these cells, there was no cytochemically demonstrable tyrosinase activity. Continuous growth in 1–3 μg of BrdU/ml resulted in the production of completely amelanotic cell lines (5, 10). One of these, clone C_3471, is shown in Fig. 11. C_3471 cells grew as a flattened fibroblastic monolayer and appeared completely amelanotic. Cells incubated for tyrosinase activity were completely negative.

Electron Microscopy

Examination of the control cultures showed that the melanin granules seen by light microscopy were premelanosomes and melanosomes in all stages of development. Because of the difficulty of distinguishing late premelanosomes from mature melanosomes we will follow the terminology of Novikoff et al. (16) in referring to all such granules as premelanosomes. The control cells were spindle shaped and contained large numbers of premelanosomes distributed throughout the cytoplasm. A well developed Golgi apparatus was present in control cells (Fig. 12). Premelanosomes in the early stages of development were seen frequently near the Golgi apparatus. Occasional compound melanosomes were found near the nucleus. Tyrosinase reaction product was localized in the inner Golgi saccule, Golgi-associated smooth surfaced tubules and vesicles, premelanosomes and compound melanosomes (Fig. 16). Pretreatment of the cells with DECA prevented the formation of reaction product (Fig. 17).

After 3 days of growth with 3 μg of BrdU/ml the major morphological alteration was the absence of premelanosomes from the cytoplasmic processes and their concentration near the nucleus (Fig. 20). Compared with the controls, the treated cells con-

tained fewer premelanosomes in total but there was an increased concentration of premelanosomes within the Golgi region (Fig. 13). Additionally, there was an increase in the number of large compound melanosomes which appeared to contain greater numbers of early and intermediate premelanosomes (Fig. 21). Many of the premelanosomes contained structural alterations, such as paracrystalline inclusions (Fig. 22). Examination of the 3-day treated cells for tyrosinase activity showed that reaction product was absent from the Golgi saccules and Golgi-associated smooth surfaced tubules (Fig. 18). However, the cells still showed reaction product in premelanosomes, compound melanosomes, and in some of the Golgi-associated vesicles.

Those cells which were exposed to 3 μg of BrdU/ml for 7 days contained very few premelanosomes (Figs. 14 and 19). Most of those seen were fully pigmented late premelanosomes, usually located at the periphery of the Golgi region (Fig. 19). Small dense bodies were observed which exhibited no tyrosinase activity or recognizable elements of premelanosomal structure. Based on this evidence, we did not consider these to be premelanosomes. Except for those few cells which still retained some pigmentation, compound melanosomes were rare. Incubation for tyrosinase activity revealed no reaction product within most of the cells examined (Fig. 19). Only the most pigmented cells showed slight amounts of reaction product in premelanosomes and small vesicles. Even in these cells reaction product was usually not seen in the Golgi saccules or Golgi-associated smooth surface tubules.

The C_3471 cells, grown continuously in the presence of 1 μg of BrdU/ml, were completely amelanotic. A poorly developed Golgi apparatus was present, but no normal or abnormal premelanosomes were observed within these cells (Fig. 15). The dense bodies noted in 7-day treated cells were not observed in these cultures.

DISCUSSION

We found that the growth of highly pigmented melanoma cells in the presence of BrdU resulted in the complete loss of the melanin-synthesizing system of the cells. The process of suppression of pigmentation by BrdU appeared to involve four major effects. These were: (*1*) progressive reduction in tyrosinase activity; (*2*) suppression of the formation of premelanosomes; (*3*) alteration in the cellular distribution of premelanosomes; and (*4*) loss of melanin-containing structures from the cells.

Reduction in tyrosinase activity was detected as early as 24 h after the cultures were changed to medium containing BrdU (3 μg/ml). The progressive reduction with continued treatment was documented by both biochemical and ultrastructural-cytochemical studies. Tyrosinase activity approached zero by 7 days with 3 μg of BrdU/ml and was undetectable in cultures grown continuously with BrdU (1 μg/ml). At least four possible mechanisms may be considered to account for the reduction in tyrosinase activity: (*a*) an inhibitor of enzyme activity may be produced; (*b*) an altered, enzymatically inactive form of the tyrosinase molecule may be produced due to BrdU-induced mutations; (*c*) active tyrosinase may be produced but degraded at an increased rate; or (*d*) the synthesis of normal tyrosinase may cease.

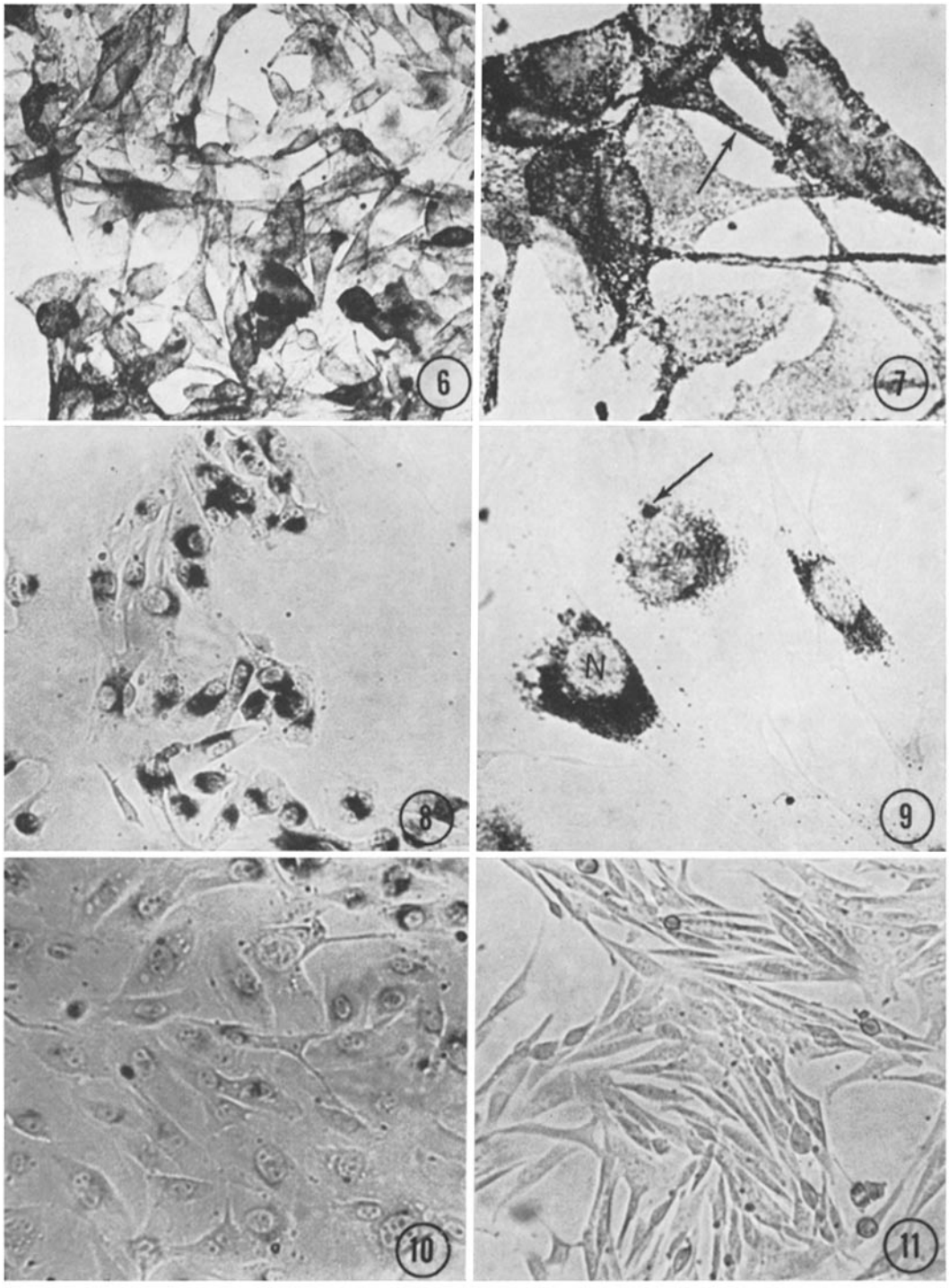
The reduction in tyrosinase activity was partially attributable to dialyzable inhibitory material detected in extracts of BrdU-treated cells. Inhibitors

FIGURES 6 and 7 B_559 melanoma cells grown in regular medium. Fig. 6, Living cells. The heavily pigmented cells grow in a piled reticulated fashion. × 180. Fig. 7, Formal-calcium fixed cells. Melanin granules are distributed throughout the cytoplasmic processes (arrow). × 500.

FIGURES 8 and 9 B_559 cells grown for 3 days with 3 μg of BrdU/ml. Fig. 8, Living cells. The cells have disassociated and melanin granules are concentrated in a juxtanuclear position. × 180. Fig. 9, Formal-calcium fixed cells. The enlarged melanin granules (arrow) have been identified by electron microscopy as compound melanosomes (see Fig. 21). × 500.

FIGURE 10 B_559 cells grown for 7 days with 3 μg of BrdU/ml. Living cells. Cells are nearly amelanotic. × 180.

FIGURE 11 C_3471 cells derived from long-term culture of B_559 cells with 1 μg of BrdU/ml. Living cells. Completely amelanotic cells grow in a fibroblastic manner. × 180.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of tyrosinase have previously been reported in extracts of mammalian skin (33–36), melanomas (37–40), amelanotic melanoma cells in culture (22), and amelanotic cells resulting from the hybridization of pigmented melanoma cells to L cells (41). Our preliminary characterization of the inhibitory material in C_3471 cells indicates the presence of heat-stable, dialyzable material capable of inhibiting both DOPA autoxidation and tyrosinase-catalyzed production of dopachrome. These results are consistent with the characteristics of inhibitors prepared from hamster melanoma by Satoh and Mishima (39) and from an amelanotic mouse melanoma by Chian and Wilgram (37). In both of these cases kinetic studies indicated competitive inhibition. The report that homogenates of a variety of mammalian organs are capable of inhibiting in vitro tyrosinase activity (42) indicates the need for caution in ascribing specific regulatory functions to all detectable inhibitors. In our experiments in which an inhibitor of tyrosinase activity was detected in cells at a time when pigment production was being suppressed, it seems reasonable to postulate a correlation. The production of inhibitory material may facilitate the suppression of melanogenesis and/or the maintenance of the amelanotic state. However, our data on dialyzed supernatant fraction indicated that the direct effect of the inhibitory material on tyrosinase activity was quantitatively minor compared to the total decrease of activity in BrdU/treated cultures.

If most of the decrease of tyrosinase activity cannot be accounted for by the presence of inhibitory material, then the remaining three postulated mechanisms must be considered. The reversible nature of BrdU suppression of pigmentation (5) rules out the possibility that inactive tyrosinase resulted from BrdU-induced mutations. A transitory increase in degradation of tyrosinase may occur within the large numbers of compound melanosomes noted in cells during the early stages of treatment with BrdU. However, increased degradation seems an unlikely mechanism for the production of long-term amelanotic tyrosinase-negative cell lines by BrdU.

We think the most reasonable hypothesis to account for the eventual absence of tyrosinase activity in cultures grown in the presence of BrdU is that it causes the synthesis of tyrosinase to cease. Stellwagen and Tomkins (8) demonstrated that growth with BrdU (3 μg/ml) for a 30 h period caused a reduction in tyrosine aminotransferase activity of hepatoma cells by reducing the rate of synthesis of this enzyme. BrdU may act similarly in melanoma cells to decrease and eventually stop synthesis of the enzyme tyrosinase.

An additional aspect of the reduction of tyrosinase activity during BrdU treatment is the preferential effect on the T_1 form of soluble tyrosinase. We detected two forms of soluble tyrosinase in preparations of untreated melanoma cells with Rx values (0.68 and 0.57) similar to the T_1 and T_2 forms reported in studies of normal melanocytes from a variety of mammals (43) and both mouse (20) and human (44) melanomas. We found no evidence of a third form (T_3) which has been detected in preparations of hair bulk melanocytes of some mammals (43) but not in either mouse (20) or human (44) melanomas.

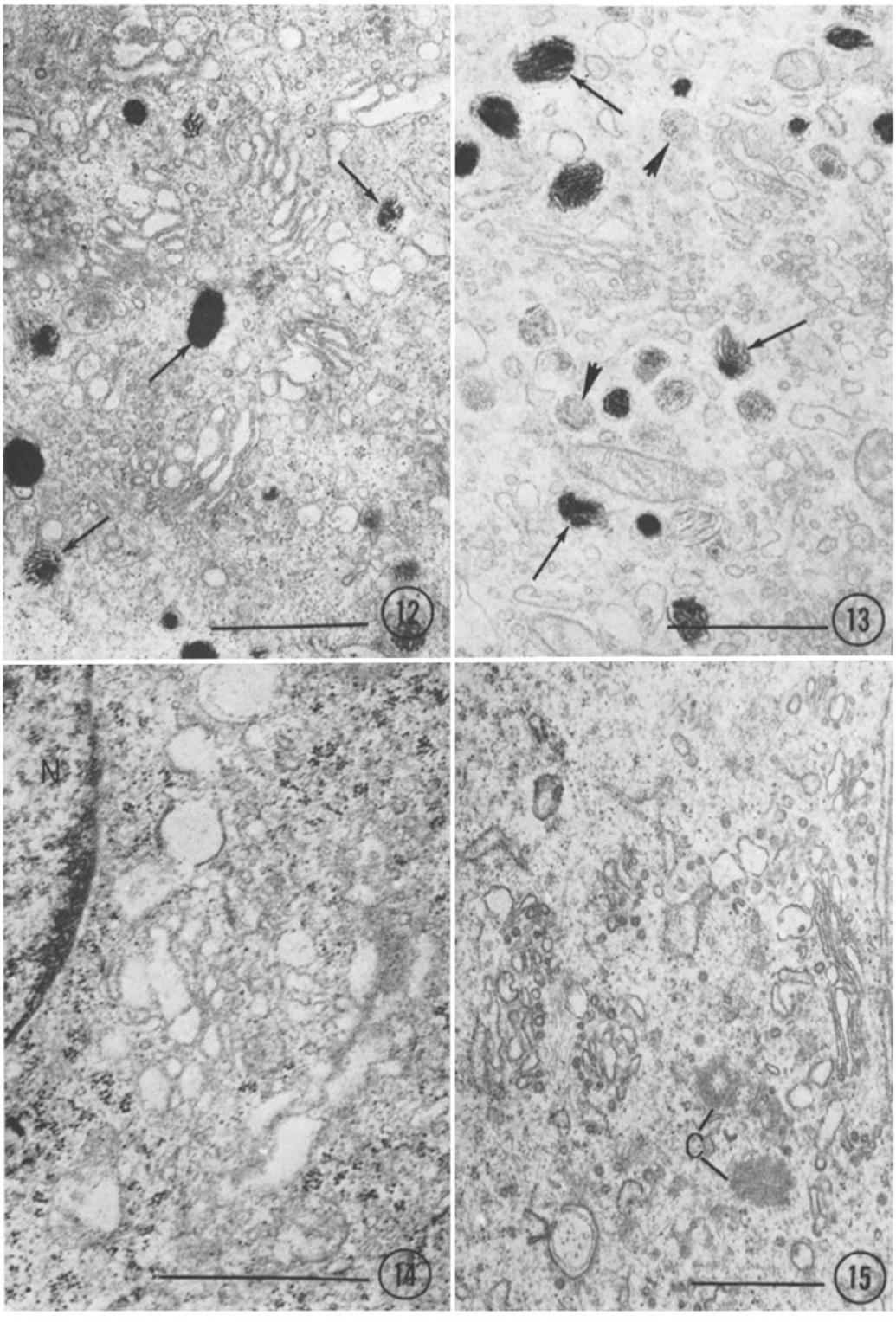
The origin of these multiple soluble forms of tyrosinase is obscure. The C locus in the mouse apparently contains the structural gene for tyrosinase (43, 45) and the multiple soluble forms probably result from secondary modifications of the product of the C locus (43). Variations have been reported in the relative activity and Rx values of the multiple forms of tyrosinase in ex-

FIGURE 12 $B_5$59 cell grown in regular medium. A portion of the Golgi apparatus is shown. Premelanosomes (arrows) in various stages of development are associated with it. × 24,000.

FIGURE 13 $B_5$59 cell grown for 3 days with 3 μg of BrdU/ml. A portion of the Golgi apparatus with associated premelanosomes (arrows) is shown. Increased numbers of premelanosomes in the early stages of development (arrowheads) are present in this area. × 25,000.

FIGURE 14 $B_5$59 cell grown for 7 days with 3 μg of BrdU/ml. No premelanosomes are associated with the Golgi apparatus. (*N*, nucleus). × 33,600.

FIGURE 15 $C_3$471 cell grown continuously with 1 μg of BrdU/ml. No premelanosomes are associated with the Golgi apparatus. (*C*, centrioles). × 20,000.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tracts of melanocytes from different mammals (43) and different genotypes of the mouse (19, 43). However, the consistent presence and Rx value of the T_1 form has been noted in all preparations of melanotic melanocytes (43). T_1 seems to be a key form and could be the original, or only slightly modified, product of the C locus which is subject to secondary modification.

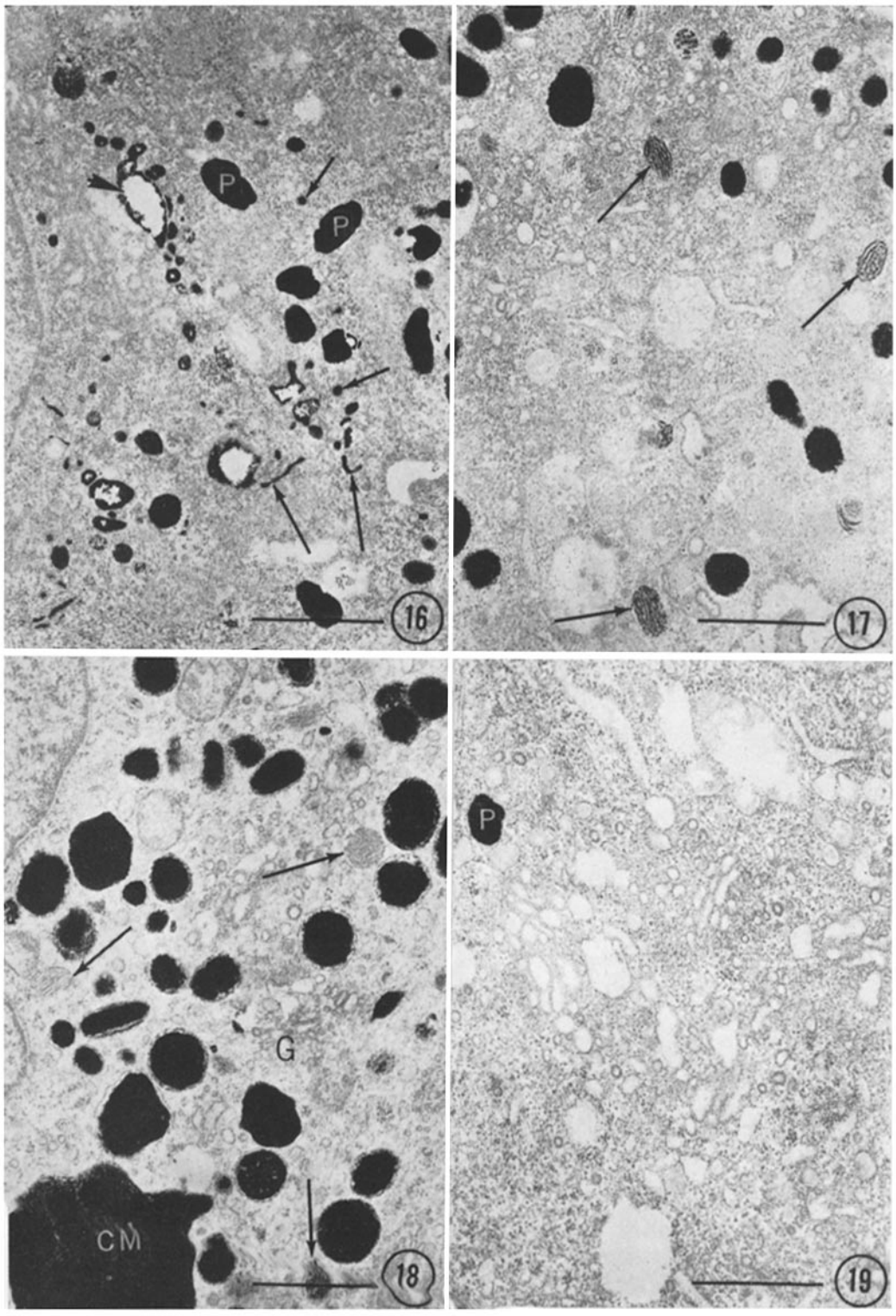
Tyrosinase has been solubilized from both melanosomal (46) and smooth membrane (47) fractions of Harding-Passey mouse melanoma. Only a single form of tyrosinase, corresponding to the T_1 soluble form, was revealed by acrylamide electrophoresis of the purified preparations, although both T_1 and T_2 are present in the soluble fraction of this melanoma (20, 46). The absence of detectable T_2 in the melanosomal preparation raises doubt that this form of tyrosinase is active in melanin synthesis in vivo. The apparent absence of T_2 in the smooth membrane fraction suggests to us that it is the T_1 form of tyrosinase which has been cytochemically demonstrated in the GERL (16). This suggestion is strengthened by our finding that the early reduction in the T_1 form of soluble tyrosinase during suppression of pigmentation by BrdU was correlated with the early loss of tyrosinase activity from the Golgi saccules and Golgi-associated smooth surfaced tubules.

On the basis of our finding that T_1 decreased before T_2 during BrdU-induced suppression of melanogenesis, and the evidence of other authors described above, we suggest that T_1 may be a precursor form from which T_2 (and T_3 where present) is produced by secondary modification. As the molecular weight of the T_2 (and T_3) form is lower than that of T_1 (43, 48), this modification may involve the removal of a portion of the T_1 molecule. T_2 could be a degraded form of tyrosinase which is not destined to be incorporated into, or has been released from, melanosomes. If T_2 is actually derived from the T_1 form, then the onset of melanogenesis in previously amelanotic cells may show evidence of T_1 before the appearance of the T_2 form. We are studying cultures reversing after BrdU treatment to determine whether this is the case.

The reduction of tyrosinase activity by BrdU was coordinated with a suppression of premelanosome formation. Extensive formation of premelanosomes in control cells was indicated by the presence of large numbers of premelanosomes in all stages of development and the detection of tyrosinase reaction product in Golgi saccules, Golgi-associated vesicles, and smooth surfaced tubules. These tubules probably correspond to the GERL from which premelanosomes are considered to arise in B16 melanoma (16). Tyrosinase reaction product was not seen in the Golgi saccules of B16 melanoma (16), in contrast to our findings in $B_5$59 cells. This may indicate physiological differences between these cultured cells and the solid tumor from which they were originally derived.

The normal formation of premelanosomes was suppressed by culture of melanoma cells in medium containing BrdU. The suppression of pigmentation by BrdU did not result in an albino phenocopy. In albino (c/c) mice tyrosinase activity is absent but amelanotic melanosomes are produced (49). We have found no evidence of premelanosomes, normal or altered, in amelanotic cells (clone $C_3$471) produced by long-term culture in the presence of 1 μg of BrdU/ml. Reduced premelanosome formation was indicated in cells treated for 3 days with 3 μg of BrdU/ml by the general absence of tyrosinase reaction product in the Golgi saccules and Golgi-associated smooth surfaced tubules of these cells. Cells treated for 7 days did not show tyrosinase reaction product in the Golgi saccules, Golgi-associated smooth surfaced tubules, or vesicles, and the absence of early forms of premelan-

FIGURES 16–19 Thin sections of cells incubated for tyrosinase activity. Fig. 16, $B_5$59 cell grown in regular medium. Tyrosinase reaction product is localized in the inner Golgi saccule (arrowhead), Golgi-associated smooth surfaced tubules (long arrows) and vesicles (short arrows), and in premelanosomes (*P*). × 20,000. Fig. 17, $B_5$59 cell grown in regular medium. Treated before incubation with a tyrosinase inhibitor, DECA. The absence of reaction product in intermediate premelanosomes (arrows) and associated structures indicates the specificity of the tyrosinase reaction. × 20,000. Fig. 18, $B_5$59 cell grown for 3 days with 3 μg of BrdU/ml. Tyrosinase reaction product is localized primarily in the compound melanosome (*CM*). Many premelanosomes (arrows) contain no reaction product. × 19,000. Fig. 19, $B_5$59 cell grown for 7 days with 3 μg of BrdU/ml. No reaction product is present in the Golgi saccules or any associated structure. *P*, premelanosome. × 20,000.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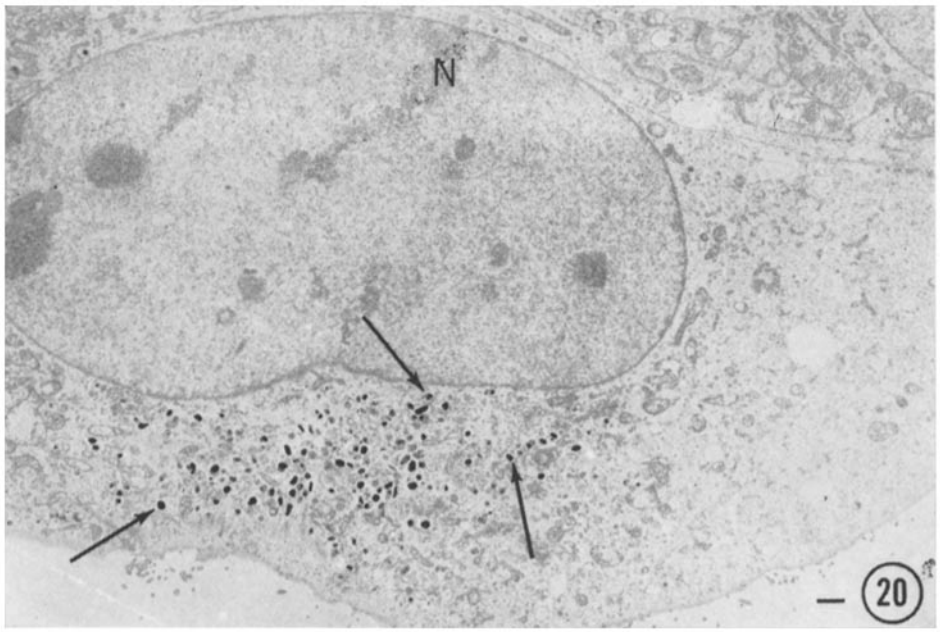

FIGURE 20 B_559 cell grown for 3 days with 3 μg of BrdU/ml. Individual premelanosomes (arrows) are concentrated near the nucleus (N). × 3,200.

osomes in these cells indicated that new formation had ceased. It thus appears that treatment with BrdU causes a progressive reduction of premelanosome formation.

The most dramatic morphological alteration observed during culture of melanoma cells in medium containing BrdU was the juxtanuclear concentration of pigment first seen in cells at 2 days. Electron microscopic studies of 3-day treated cultures showed that this juxtanuclear pigment consisted of a high concentration of individual premelanosomes and compound melanosomes and confirmed the absence of premelanosomes in the cytoplasmic processes of these cells. We have no direct evidence for the mechanism(s) which cause this effect but our ultrastructural studies indicated decreased numbers of premelanosomes in cells treated for 3 days; thus some loss does appear to occur during production of this altered pigment distribution. However, the concentration of premelanosomes in the juxtanuclear region also appeared to be greater than in control cells, inferring that either absence of outward movement or actual inward movement may also be involved.

The production of amelanotic cells by BrdU requires the loss of melanin-containing structures as well as suppression of new melanin production. We found some decrease in number of premelanosomes during the first 3 days of treatment with BrdU, and a marked reduction between the 3rd and 7th day of culture in medium containing 3 μg of BrdU/ml. The abnormal number and composition of compound melanosomes in cells treated for 3 days may facilitate this loss. The formation of compound melanosomes has been considered as a possible catabolic process, with these structures serving as autophagic vacuoles (16). The compound melanosomes of 3-day treated cells contain unusually high numbers of premelanosomes in early stages of development; this may represent an altered manifestation of a process which occurs normally in untreated melanoma cells. We have not, however, obtained evidence that pigment is lost by actual degradation *in situ* within compound melanosomes. If this occurred, residual bodies should have been seen in 7-day treated cells. Cell division would per se cause a dilution of melanin, but we believe that the extent of loss indicates there is also elimination of compound melanosomes and individual premelanosomes from cells grown with BrdU.

The four major effects of BrdU on pigmentation in melanoma cells which we have discussed separately occurred together in the cells. The evi-

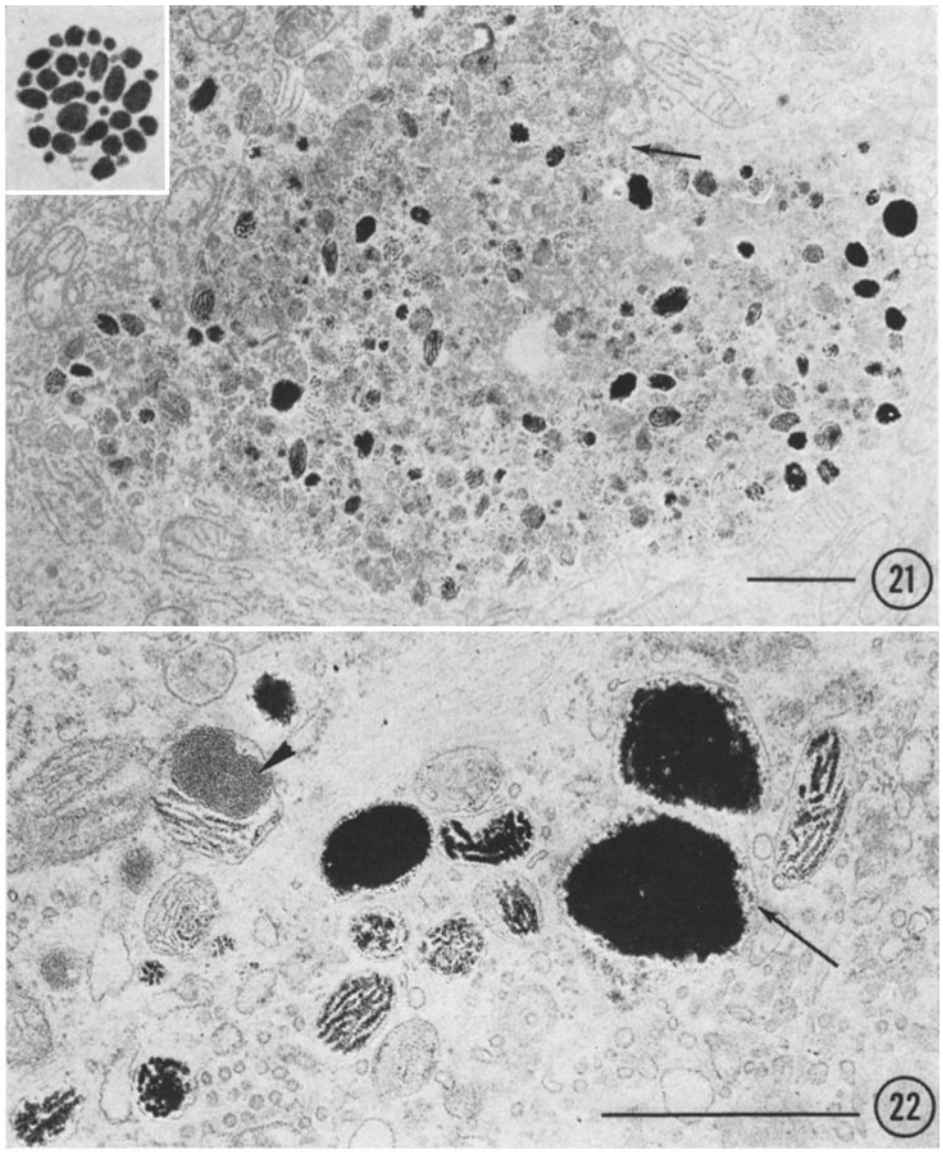

FIGURE 21 $B_5 59$ cell grown for 3 days with 3 μg of BrdU/ml. The large, membrane-limited, compound melanosome (arrow) contains primarily premelanosomes in the early stages of development. × 14,400. *Inset,* A compound melanosome from a control cell is composed of late, well pigmented premelanosomes. × 10,000.

FIGURE 22 $B_5 59$ cell grown for 3 days with 3 μg of BrdU/ml. A paracrystalline inclusion is present in one premelanosome (arrowhead). A small compound melanosome delimited by a unit membrane is also shown (arrow). × 34,400.

dence of coordinated reduction of tyrosinase activity and suppression of premelanosome formation is particularly significant. Tyrosinase, the product of the C locus, is thought to have a structural as well as enzymic function within melanosomes (49). Melanosomal structure appears to be under complex genetic control, as fine structural alterations in melanosomal morphology have also

been described for mutants at the B and P loci (49). The complex genetics indicates that a number of primary gene products are probably needed for the normal production of functional melanosomes. BrdU appears, directly or indirectly, to affect all of these products. We believe that the coordinated effects of treatment with BrdU on the melanin-synthesizing apparatus of these melanoma cells may provide a useful experimental system in which the normal regulation of melanogenesis can be probed.

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REFERENCES

1. Stockdale, F., K. Okazaki, M. Nameroff, and H. Holtzer. 1964. 5-Bromodeoxyuridine: effect on myogenesis *in vitro*. *Science* (*Wash. D C.*). **146**:533.
2. Lasher, R., and R. D. Cahn. 1969. The effects of 5-bromodeoxyuridine on the differentiation of chondrocytes *in vitro*. *Dev. Biol.* **19**:415.
3. Coleman, A. W., J. R. Coleman, D. Kankel, and I. Werner. 1970. The reversible control of animal cell differentiation by the thymidine analog, 5-bromodeoxyuridine. *Exp. Cell Res.* **59**:319.
4. Bischoff, R., and H. Holtzer. 1970. Inhibition of myoblast fusion after one round of DNA synthesis in 5-bromodeoxyuridine. *J. Cell Biol.* **44**:134.
5. Silagi, S., and S. A. Bruce. 1970. Suppression of malignancy and differentiation in melanotic melanoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **66**:72.
6. Mayne, R., J. W. Sanger, and H. Holtzer. 1971. Inhibition of mucopolysaccharide synthesis by 5-bromodeoxyuridine in cultures of chick amnion cells. *Dev. Biol.* **25**:547.
7. Miura, Y., and F. H. Wilt. 1971. The effects of 5-bromodeoxyuridine on yolk sac erythropoiesis in the chick embryo. *J. Cell Biol.* **48**: 523.
8. Stellwagen, R. H., and G. M. Tomkins. 1971. Preferential inhibition by 5-bromodeoxyuridine on the synthesis of tyrosine aminotransferase in hepatoma cell cultures. *J. Mol. Biol.* **56**:167.
9. Turkington, R. W., G. C. Majumder, and M. Riddle. 1971. Inhibition of mammary gland differentiation *in vitro* by 5-bromo-2′-deoxyuridine. *J. Biol. Chem.* **246**:1814.
10. Silagi, S. 1971. Modification of malignancy by 5-bromodeoxyuridine. Studies of reversibility and immunological effects. *In Vitro.* **7**:105.
11. Lerner, A. B., and T. B. Fitzpatrick. 1950. Biochemistry of melanin formation. *Physiol. Rev.* **30**:91.
12. Pomerantz, S. H. 1963. Separation, purification, and properties of two tyrosinases from hamster melanoma. *J. Biol. Chem.* **238**:2351.
13. Raper, H. S. 1928. The aerobic oxidases. *Physiol. Rev.* **8**:245.
14. Mason, H. S. 1948. The chemistry of melanin. III. Mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase. *J. Biol. Chem.* **172**:83.
15. Seiji, M., K. Shimao, M. S. C. Birbeck, and T. B. Fitzpatrick. 1963. Subcellular localization of melanin biosynthesis. *Ann. N. Y. Acad. Sci.* **100**:497.
16. Novikoff, A. B., A. Albala, and L. Biempica. 1968. Ultrastructural and cytochemical observations on B-16 and Harding-Passey mouse melanomas. The origin of premelanosomes and compound melanosomes. *J. Histochem. Cytochem.* **16**:299.
17. Maul, G. G. 1969. Golgi-melanosome relationships in human melanosomes *in vitro*. *J. Ultrastruct. Res.* **26**:163.
18. Novikoff, P. M., A. B. Novikoff, N. Quintana, and J. Hauw. 1971. Golgi apparatus, GERL, and lysosomes of neurons in rat dorsal root ganglia, studied by thick section and thin section cytochemistry. *J. Cell Biol.* **50**:859.
19. Holstein, T. J., J. B. Burnett, and W. C. Quevedo, Jr. 1967. Genetic regulation of multiple forms of tyrosinase in mice: action of a and b loci. *Proc. Soc. Exp. Biol. Med.* **126**:415.
20. Burnett, J. B., H. Seiler, and I. V. Brown. 1967. Separation and characterization of multiple forms of tyrosinase from mouse melanoma. *Cancer Res.* **27**:880.

21. HU, F., and P. F. LESNEY. 1964. The isolation and cytology of two pigmented cell strains from B_{16} mouse melanoma. *Cancer Res.* **24**:1634.
22. SILAGI, S. 1969. Control of pigment production in mouse melanoma cells *in vitro*. *J. Cell Biol.* **43**:263.
23. SILAGI, S., D. BEJU, J. WRATHALL, and E. DEHARVEN. 1972. Tumorigenicity, immunogenicity and virus production in mouse melanoma cells treated with 5-bromodeoxyuridine. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3443.
24. HAYFLICK, L. 1965. Tissue cultures and mycoplasmas. *Tex. Rep. Biol. Med.* **23**:285.
25. POMERANTZ, S. H., and J. P. LI. 1970. Tyrosinases (hamster melanoma). *Methods Enzymol.* **17A**:622.
26. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
27. ORNSTEIN, L. 1964. Disc electrophoresis. I. Background and theory. *Ann. N. Y. Acad. Sci.* **121**:321.
28. DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**:404.
29. BAKER, J. R. 1944. The structure and chemical composition of the Golgi element. *Q. J. Microsc. Sci.* **85**:1.
30. OLIVER, C., and E. ESSNER. 1972. Protein transport in mouse kidney utilizing tyrosinase as an ultrastructural tracer. *J. Exp. Med.* **136**:291.
31. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
32. DEHARVEN, E. 1958. A new technique for carbon films. *J. Biophys. Biochem. Cytol* **4**:133.
33. ROTHMAN, S., H. F. KRYSA, and A. M. SMILJANIC. 1946. Inhibitory action of human epidermis on melanin formation. *Proc. Soc. Exp. Biol. Med.* **62**:208.
34. FLESCH, P. 1949. Inhibitory action of extracts of mammalian skin on pigment formation. *Proc. Soc. Exp. Biol. Med.* **70**:136.
35. FLAWN, P. C., and P. F. WILDE. 1970. Isolation and partial characterization of the naturally occurring inhibitor of Dopa autoxidation in skin. *J. Invest. Dermatol.* **55**:153.
36. FLAWN, P. C., M. I. BIOL, and P. F. WILDE. 1970. A study of the mechanism of action and role of a natural inhibitor of Dopa autoxidation isolated from guinea pig skin. *J. Invest. Dermatol.* **55**:159.
37. CHIAN, L. T. Y., and G. F. WILGRAM. 1966. Tyrosinase inhibition: its role in suntanning and in albinism. *Science (Wash. D. C.)*. **155**:198.
38. SEIJI, M., T. YOSHIDA, T. SAKURANE, and R. OGURA. 1967. Sulfhydral content in subcellular fractions of mouse melanomas. *J. Invest. Dermatol.* **48**:230.
39. SATOH, G. J., and Y. MISHIMA. 1967. Tyrosinase inhibitor in Fortner's amelanotic and melanotic malignant melanoma. *J. Invest. Dermatol.* **48**:301.
40. HAMADA, T., and Y. MISHIMA. 1972. Intracellular localization of tyrosinase inhibitor in amelanotic and melanotic malignant melanoma. *Br. J. Dermatol.* **86**:385.
41. DAVIDSON, R., and K. YAMAMOTO. 1968. Regulation of melanin synthesis in mammalian cells, as studied by somatic hybridization. II. The level of regulation of 3,4-dihydroxyphenylalanine oxidase. *Proc. Natl. Acad. Sci. U. S. A.* **60**:894.
42. MENON, I. A., and H. F. HABERMAN. 1971. Widespread occurrence of inhibitors of melanoma tyrosinase in plant and mammalian tissues. *Experientia (Basel)*. **27**:644.
43. HOLSTEIN, T. J., W. C. QUEVEDO, JR., and J. B. BURNETT. 1971. Multiple forms of tyrosinase in rodents and lagomorphs with special reference to their genetic control in mice. *J. Exp. Zool.* **177**:173.
44. BURNETT, J. B., and H. SEILER. 1969. Multiple forms of tyrosinase from human melanoma. *J. Invest. Dermatol.* **52**:199.
45. COLEMAN, D. L. 1962. Effect of genic substitution on the incorporation of tyrosine into the melanin of mouse skin. *Arch. Biochem. Biophys.* **96**:562.
46. MIYAZAKI, K., and M. SEIJI. 1971. Tyrosinase isolated from mouse melanoma melanosome. *J. Invest. Dermatol.* **57**:81.
47. SEIJI, M., H. ITAKURA, and T. IRIMAJIRI. 1972. Tyrosinase in the membrane system of mouse melanoma. *In* Pigmentation—Its Genesis and Control. V. Riley, editor. Appleton-Century-Crofts, New York. 525.
48. BURNETT, J. B. 1971. The tyrosinases of mouse melanoma. Isolation and molecular properties. *J. Biol. Chem.* **246**:3079.
49. MOYER, F. H. 1963. Genetic effects on melanosome fine structure and ontogeny in normal and malignant cells. *Ann. N. Y. Acad. Sci.* **100**:584.