

Enhanced Melanization of Harding-Passey Mouse Melanoma Cells Following Treatment with Exogenous Melanosomes in Monolayer Culture

H. B. Leising and D. O. Schachtschabel

Institut für Physiologische Chemie der Philipps-Universität Marburg

Z. Naturforsch. **34 c**, 124–130 (1979) ; received September 29/November 9, 1978

Dedicated to Prof. Dr. P. Karlson on the Occasion of His 60th Birthday

Melanoma Culture, Melanosomes, Phagocytosis, Melanogenesis

Purified melanosomes isolated from subcutaneously growing Harding-Passey melanomas of NMRI-mice were labeled either *in vitro* with [^{14}C]tyrosine or [^{14}C]DOPA in the melanin portion, or *in vivo* in the melanin and protein portion following i. p. injection of [^{14}C]tyrosine. Treatment of monolayer cultures of Harding-Passey melanoma cells (HPM-73 line) with such labeled melanosomes resulted in rapid uptake of label during the first 4 h which leveled off thereafter. A portion of the “incorporated” label could be removed by a 15 min chase with unlabeled melanosomes.

Uptake of labeled melanosomes by HPM-73 cells was followed by increased cellular melanization which was not only due to melanin derived from incorporated melanosomes but primarily to newly formed melanin. Tyrosinase activity was elevated in melanosome-treated cells. Tyrosinase activity of control cells was significantly reduced following a 24 h exposure to actinomycin D or cycloheximide. On the other side, the same inhibitor treatment of melanosome-pretreated cells resulted in less inhibition of tyrosinase activity.

The present findings suggest “melanophagic” properties of cultured melanoma cells resulting in enhanced melanogenesis after phagocytotic uptake of functionally active exogenous melanosomes.

Introduction

Melanosomes are specialized cytoplasmic organelles of melanocytes or melanocyte-derived melanoma cells within which tyrosinase-catalysed synthesis and deposition of melanin occurs (for review: see ref. 1). A distinct feature of mammalian epidermal melanocytes is their ability to “transfer” melanosomes to neighbouring non-melanocytic cells (keratinocytes): the recipient keratinocyte engulfs and detaches a tip of a melanocyte dendrite containing melanosomes, the plasma membrane remaining intact (for review see ref. [1–3]). After disintegration of both membranes (of the melanocyte dendrite and keratinocyte), the melanosomes are dispersed in the cytoplasm either singly or in aggregates.

It is not known whether melanocytes or melanoma cells per se might also have the property to “inoculate” each other with melanosomes by the above described mechanism or by “simple” phagocytosis following cellular release of melanosomes as e. g. by exocytosis or by cell necrosis-associated processes. If melanoma cells would have a “melanophagic ability”, then such a property might perhaps

ultimately be utilized for trials in melanoma treatment because of the potential application of isolated melanosomes as vehicles for delivering antitumor drugs.

Further, if melanosomes are found to be phagocytized by melanoma cells the question arises with respect to the functional integrity and activity, especially in regard to the possible function of ingested melanosomes in melanogenesis. Cell culture systems offer the possibility to answer such questions. The present investigation utilizing cultured Harding-Passey melanoma cells [4, 5] which are exposed to purified, radioactively labeled melanosomes isolated from an *in vivo* Harding-Passey melanoma [6] is concerned with the following objectives: Does uptake of exogenous melanosomes occur? Does exposure to melanosomes result – possibly after ingestion by the cells – in changes of cellular melanin synthesis?

Materials and Methods

Cell culture: The Harding-Passey melanoma cell line (HPM-73 line) used in the present study was isolated in culture in 1973 according to methods previously described [4] and kept in continuous culture since that time. The morphology, ultrastructure, growth pattern and melanogenic properties

Requests for reprints should be sent to Prof. Dr. D. O. Schachtschabel, Institut für Physiologische Chemie der Philipps-Universität, Lahnberge, D-3550 Marburg.



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.

have also been described [3, 5, 7]. Eagle's basal medium (BME; GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (GIBCO), 100 IU/ml penicillin-G-sodium, 135 µg/ml streptomycin sulfate and 4×10^{-4} M L-tyrosine (Merck AG, Darmstadt) was used as culture medium. Experimental cultures were prepared by distributing equal volumes (5 ml, ca. 200 µg cell protein) in Falcon plastic tissue culture flasks with a growth area of 25 cm². These subcultures were made by dislodging the cells from stock cultures by means of a rubber scraper. Cultures were incubated at 37 °C in a CO₂-incubator containing 7% CO₂ in air. Following a medium change after one day, further medium renewals without ("control") or with addition of melanosome suspension (see below; 0.1 ml per culture) were performed every 2 days, thereafter.

Preparation and labeling of isolated melanosomes

The subcutaneous *in vivo* melanoma used for isolation of melanosomes was routinely passaged in NMRI-mice since 1973 in our laboratory. It was originally obtained from Dr. Gericke (Hoechst AG, Frankfurt) and the HPM-73 monolayer cell culture line employed in the present study was derived from the same melanoma in 1973. After aseptical removal of the melanoma tissue (about 3 g) and rinsing (2 ×) with Earle's salt solution it was homogenized immediately in a solution of 0.25 M sucrose and 1 mM CaCl₂ (0 °C, 8 ml per 1 g tissue) by means of a Potter-Elvehjem homogenizer (with teflon pestle, 20 strokes) at 0 °C. The homogenate was centrifuged 10 min at 700 × g, the resultant supernatant for 10 min at 11 000 × g and thereafter the resuspended pellet 10 min at 15 000 × g. The final pellet was resuspended in 1.8 ml 0.25 M sucrose containing 1 mM CaCl₂, and 1 ml of this suspension was applied on top of a density gradient (1.5–2.6 M sucrose). Following centrifugation (1 h at 103 000 × g) and separation according to Seiji *et al.* [8], 0.5 ml of the lowest gradient fraction containing nearly exclusively melanosomes (for electron microscopical examination see ref. 3 and 9) was washed twice with serum-free Eagle's basal medium. For radioactive labeling, the pellet was suspended in 5 ml of sodium phosphate buffer (pH 6.8, 0.1 M) containing 500 IE penicillin-G-sodium, 675 µg streptomycin sulfate, 4×10^{-4} M L-tyrosine, 4×10^{-5} M L-DOPA (both Merck, Darmstadt) and either 5 µCi of L-[U-¹⁴C]tyrosine (spec. act. 522 mCi/

mmol; Amersham-Buchler, Braunschweig) or 5 µCi of L-3,4-dihydroxyphenyl[3-¹⁴C]alanine (L-[¹⁴C]-DOPA; spec. act. 8.4 mCi/mmol; Amersham-Buchler, Braunschweig) and incubated 4 to 16 h at 37 °C. After centrifugation (10 min, 15 000 × g) the labeled melanosomes were washed by suspension and centrifugation 4 times with serum-free BME containing 4×10^{-4} M L-tyrosine (or 4×10^{-5} M L-DOPA in case of ¹⁴C-DOPA labeling), and finally suspended in 7.5 ml BME containing 3 mg of Polymyxin B (Pfizer, Karlsruhe). This suspension was used for addition to cultures.

In vivo labeling of melanosomes was performed by 3 i.p. injections of L-[U-¹⁴C]tyrosine (Amersham-Buchler, Braunschweig; each time: 5 µCi and 215 µg tyrosine in 0.5 ml Earle's salt solution) into a melanoma (ca. 3 g) bearing mouse on three consecutive days. Following aseptical removal of the melanoma on the 4th day, melanosomes were isolated according to the procedure described above.

Incorporation studies with labeled melanosomes

Following the respective incubation periods of cultures with labeled melanosomes (0.1 ml melanosome suspension per culture) the medium was decanted and the cell layer treated consecutively with Earle's salt solution (4 × 10 ml, 30 sec, 0 °C), 5% trichloroacetic acid (2 × 10 ml, 5 min, 0 °C) and ethanol (96%, 4 × 10 ml, 2 min 0 °C). The air-dried monolayer was dissolved in 1.5 ml 1 N NaOH and aliquots used for scintillation counting, *protein-* and *melanin* determination [5, 10].

Tyrosinase activity of control and melanosome-treated monolayer cultures was determined following 3 washes with regular growth medium (in order to remove the non-incorporated melanosomes) according to Oikawa *et al.* [11] using 20 µCi per 10 ml medium of L-[3,5-³H]tyrosine (spec. act. 42 Ci/mmol, Amersham-Buchler, Braunschweig). Following an incubation period of 24 h 500 µl of the culture medium were collected and stored deep-frozen until determination of ³H₂O. After thawing, addition of 500 µl of 5% trichloroacetic acid and centrifugation (2 min, 12 000 × g), 600 µl of the supernatant were treated with 100 µl of a charcoal (Aktivkohle, Merck AG, Darmstadt) suspension in aqua bidest (1 : 1) and recentrifuged. An aliquot of the supernatant was used for scintillation counting.

Tyrosinase activity of isolated melanosomes alone was measured under the same conditions as that of cell cultures (0.1 ml melanosome suspension in 10 ml of culture medium, 20 μ Ci of [3 H]tyrosine, etc.).

Chemicals: Cycloheximide was obtained from Sigma Chemical Co., München and Actinomycin D from Boehringer AG, Mannheim.

Results

Isolated melanosomes: As revealed by electron-microscopic examination the melanosome fraction used for labeling and addition to cultures was not significantly contaminated by other cell organelles [3, 9]. The protein/melanin ratio was 0.4. The melanosome preparation had a tyrosinase activity of $4 - 5 \times 10^3$ cpm per 100 μ l (see under Materials and Methods). However, among 12 preparations there were five which did not show a significant enzyme activity. Following *in vitro* labeling of (tyrosinase-active) melanosomes with radioactively labeled tyrosine or L-DOPA it was found (after repeated washings and hydrolysis for 20 h with 6 N HCl at 105 °C and N₂) that incorporated radioactivity was nearly exclusively in the melanin portion. On the other side, following *in vivo* labeling with [14 C]tyrosine (see under Materials and Methods) the isolated melanosomes contained incorporated radioactivity in both the melanin and protein portion (the ratio was about 3 between melanin and protein radioactivity).

Uptake of isolated melanosomes by cell cultures: Exposure of HPM-73 monolayer cultures in exponential growth-phase to isolated and radioactively labeled melanosomes (with the label in the melanin portion) during an incubation-period of up to 4 days — with medium change and renewed addition of melanosomes after 2 days — resulted in a rapid uptake of radioactivity during the first 4 hours and a remarkably slower incorporation, thereafter (Fig. 1). This uptake was about 10-fold higher at 37 °C than at 0 °C during an incubation-period of 5 h. The incorporated cellular radioactivity could not be removed by repeated washings with Earle's salt solution or treatment with 5% trichloroacetic acid or ethanol. However, it should be mentioned that following a short preincubation (2 h) with labeled melanosomes a 15 min "chase" with "cold" melanosomes (5 x) resulted in 30–40% loss of radioactivity indicating that part of the "incorporated" melanosomes are not irreversibly "engulfed" by the cells. On the other side, the same "chase" with cold melanosomes of cultures which were preincubated for 24 h with labeled melanosomes resulted only in 5–10% loss of "incorporated" radioactivity.

In order to obtain an indication whether the whole melanosomes (or perhaps only *e.g.* their melanin part) were incorporated into HPM-73 cells, cultures were incubated with *melanosomes labeled in vivo* (by i.p. injection of [14 C]tyrosine into a melanoma bearing mouse; see under Materials and Methods) which contained the label both in the melanin and protein part. Following an incubation

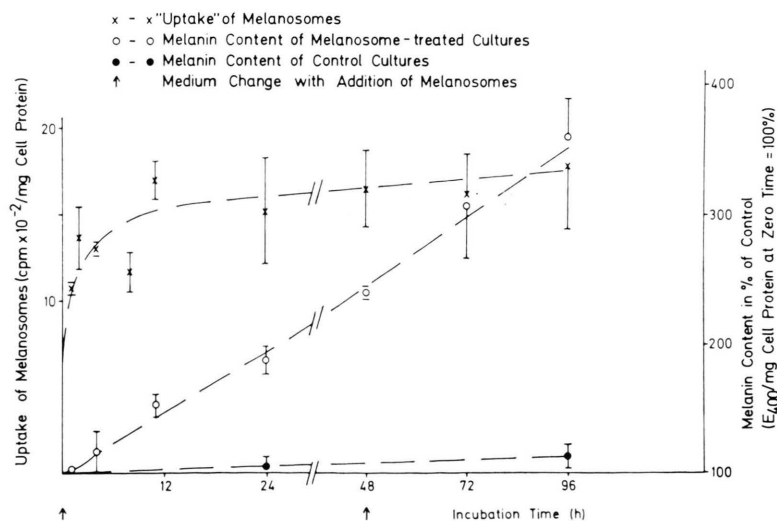


Fig. 1. "Uptake" of exogenous (tyrosinase-active) melanosomes by cultured Harding-Passey melanoma cells (HPM-73) and changes in melanin content during incubation times of up to 4 days. Cultures were incubated without ("control") or with exogenous (labeled) melanosomes (0.1 ml suspension/culture) for the respective times and after several washes analysed for incorporated radioactivity (cpm/mg cell protein) and melanin content (E_{400} /mg cell protein) (see Material and Methods). Melanin content of incorporated melanosomes was subtracted. Results: mean (\pm SD) of 14 cultures for each point.

period of 4 days (with medium change and read-
dition of melanosomes after 2 days), incorporated
radioactivity was determined in the acid hydro-
lyzable ("protein") and acid resistant (6 N HCl,

20 h, 105 °C, N₂) portion ("melanin"). The isolat-
ed *in vivo* melanosomes contained ca. 25%, the
melanosome treated cells ca. 20% of acid hydro-
lyzable ("protein") radioactivity.

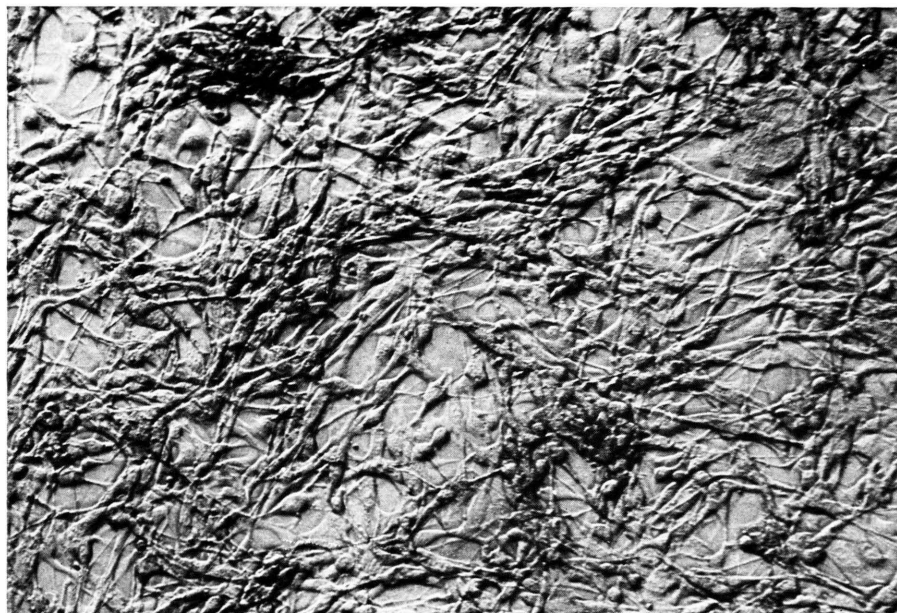


Fig. 2.



Fig. 3.

Figs 2 and 3. Harding-Passey melanoma cells (HPM-73) cultured without (control, Fig. 2) and with (Fig. 3) isolated melanosomes (for 5 days in the medium). After establishing the subcultures medium changes of control and treated cultures were performed after 1, 3, 5 and 7 days. Melanosomes (0.1 ml melanosome suspension per culture; see under Materials and Methods) were added after 3, 5 and 7 days.

Note that treated cultures contain a higher proportion of pigmented cells (some are rather large) and the presence of melanized patches. (Photographed in living state, $\times 170$.)

Cellular melanin content and tyrosinase activity following melanosome treatment

As shown by light microscopy melanosome treated cultures revealed a striking increase of pigmented cells (Figs 2, 3). Some are arranged in melanized patches. Also, the presence of several rather large, less branched and heavily pigmented cells following melanosome exposure is noteworthy (Fig. 3). As shown in Fig. 1, this increased melanin content of melanosome treated cultures seems to be not only the result of melanin uptake derived from "incorporated" melanosomes, but in addition due to newly formed melanin. After a short lag-phase of about 2 hours a nearly linear increase of melanin content was observed during an incubation-period of 4 days (Fig. 1).

Following melanosome exposure, tyrosinase activity of treated cells is increased (Table I). However, in the course of 24 h a gradual decrease of the activity occurs; but still after 48 h tyrosinase activity is elevated in comparison with control cells (Table I).

Also, [^3H]DOPA incorporation into melanosome-treated cells during a period of 24 h was found to be about twice of that into control cells (results not shown). In this case, incorporation occurred only into cellular melanin. Further support of the concept that the enhancement of melanin synthesis (Fig. 1) is caused by ingested melanosomes was derived from negative findings in regard to melanin formation if the utilized melanosomes did not exhibit tyrosinase activity. About 40% of melanosome

preparations (isolated from *in vivo* melanomas) did not show tyrosinase activity. The reason for this "loss" is not known, yet. It is possible that inactivation of tyrosinase occurred in the course of the melanosome labeling procedure *in vitro*. In any case, treatment of cultures with tyrosinase-inactive melanosomes did not result in induction of melanin formation, though these melanosomes were "incorporated" by the cells (Table II).

In order to find out whether new protein synthesis was required for the increased melanogenic properties of melanosome-treated cells (Fig. 1, Table I), the effect of cycloheximide (0.1 $\mu\text{g/ml}$) on tyrosinase activity of melanosome-pretreated cultures (for 24 h, thereafter removal of the melanosomes by washing and further incubation for 24 h in the presence of cycloheximide) was measured. Under these conditions protein synthesis as measured by [^{14}C]leucine incorporation was inhibited by ca. 80%. As seen in Table III, cycloheximide treatment resulted in a more than 50% inhibition of tyrosinase activity in control cells, while the activity of melanosome exposed cells was less inhibited. This additional activity appears to be due to the activity derived from the ingested melanosomes. Also, actinomycin D (0.05 $\mu\text{g/ml}$) treatment for 24 h of melanosome pre-incubated cultures resulted in significantly less inhibition of tyrosinase activity as compared to control cells (Table III).

Discussion

The present study shows that cultured melanoma cells take up radioactivity from exogenously supplied and radioactively labeled melanosomes. Especially the finding that cells exposed to *in vivo* labeled melanosomes (which contained radioactivity in both the melanin and protein portion) contained the label in a similar proportion (between protein and melanin) as it was present in the isolated melanosomes, supports the concept that whole melanosomes are "ingested". The slight loss of label from the protein portion following cellular uptake could indicate a partial intracellular alteration (perhaps due to some lysosomal attack?). The enhanced melanin formation (Fig. 1), the increased tyrosinase activity (Table I) and the striking increase of pigmented cells following exposure to melanosomes (Figs 2 and 3) favor the interpretation of an uptake of functionally active melanosomes. Since much less cellular incorporation of radioactivity (present in

Table I. Tyrosinase activity of cultured HPM-73 cells following treatment with isolated melanosomes (0.1 ml melanosome suspension per culture, see under Materials and Methods) for increasing time-periods (2, 8, 24 and 48 h). Following the respective incubation-times, the monolayer cells of melanosome-treated and untreated (control) cultures were washed several times with culture medium and tyrosinase activity was determined as described under Materials and Methods. Results are expressed in % of control (=100%). Determinations were carried out with 2 control and 2 treated cultures in case of each incubation-period.

Incub.-Time [h]	Relative tyrosinase activity of melanosome-treated cultures (cpm/mg cell protein of control=100%)
0	100 \pm 5
2	210 \pm 26
8	152 \pm 10
24	123 \pm 8
48	126 \pm 15

Table II. Lack of melanogenic response of cultured HPM-73 cells following uptake of *tyrosinase-inactive* melanosomes. Duplicate cultures (for each point) were treated without (controls) or with isolated, radioactively labeled melanosomes exhibiting *no* tyrosinase activity (see text) for 24 or 48 h. Since the melanin content (E_{400}) and the radioactivity of the utilized melanosomes (1000 cpm correspond to an E_{400} of 0.042) are known, melanin formation of melanosome-treated cells, which was not due to melanin derived from incorporated melanosomes, was calculated by subtracting the respective E_{400} corresponding to incorporated radioactivity from the total melanin content of the culture.

Incubation-time [h]	Uptake of melanosomes [cpm/mg cell protein]	Melanin content (E_{400} /mg cell protein)		
		Control	After treatment with melanosomes	
			Total	Total E_{400} minus E_{400} of "incorporated" melanosomes
24	1256 ± 205	0.369 ± 0.02	0.385 ± 0.01	0.330
48	1678 ± 139	0.510 ± 0.01	0.596 ± 0.03	0.525

the melanin portion of [^{14}C]tyrosine- or [^{14}C]DOPA-labeled melanosomes) occurred at 0 °C than at 37 °C and since most of the incorporated label was resistant to washing (with Earle's salt solution or with culture medium), "physical" adsorption does not appear to be the main reason for this "uptake" phenomenon. However, it should be mentioned that part of the "incorporated" melanosomes (30–40%) following a short-term (2 h) preincubation with labeled melanosomes could be removed by a "chase" (for 15 min) with cold melanosomes, though these cell-attached melanosomes could not be removed by "simple" washings with balanced salt solutions. This observation could be interpreted as indicating a "reversible" interaction (binding) of melanosomes with the cell surface before their final uptake.

As described in detail elsewhere [3], electron microscopic studies revealed strong support for phagocytic uptake of exogenous melanosomes by HPM-73 cells following melanosome exposure. Thus, invaginations of the plasma membrane containing exogenous melanosomes were observed [3]. The enhanced melanogenic activity of melanosome treated cells as demonstrated by increased melanin formation (Fig. 1) and enhanced tyrosinase activity (Table I) is probably caused by the melanogenic properties of the incorporated melanosomes and not by induction of cell-indigenous melanin synthesis. In support of this assumption are inhibitor studies with cycloheximide or actinomycin D revealing a fraction of tyrosinase activity in melanosome exposed cells which was refractory to this inhibitor treatment and which might be accounted for tyrosinase derived from ingested melanosomes (Table III). The significant time-dependent decline of tyrosinase

activity of melanosome-treated cells (Table I) could be the result of a gradual (but not complete) intracellular degradation (due in part to lysosomal attack?) or of an inactivation of tyrosinase during the process of melanin formation within the melanosomes [12].

The results (Table III) about a 80–90% decrease of tyrosinase activity of cultured melanoma cells following a 24 h treatment with actinomycin D indicate a rather short half-life (in the range of several hours) of cell-indigenous messenger RNA for tyrosinase. Also, the finding about a marked reduction of tyrosinase activity after incubation in the presence of cycloheximide (Table III) indicates that expression of cell-indigenous tyrosinase activity is subject to *de novo* protein synthesis. These results

Table III. Influence of cycloheximide or actinomycin D on tyrosinase activity of HPM-73 cultures preincubated without or with exogenous melanosomes.

Duplicate cultures (for each point) were treated for 24 h without (control) or with isolated melanosomes (0.1 ml suspension/culture). Thereafter, the monolayers were washed with medium (3×) and then incubated for another 24 h without or with addition of cycloheximide (0.1 µg/ml) or actinomycin D (0.05 µg/ml) in the presence of 20 µC [^3H]tyrosine (for determination of tyrosine activity, see under Materials and Methods). Each result (expressed in % of control) represents the mean of duplicate cultures. Controls: cultures incubated in media without addition of melanosomes, cycloheximide or actinomycin D.

Addition	Relative tyrosinase activity (cpm/mg cell protein of control = 100%)	
	– Melanosomes	+ Melanosomes
–	100 ± 4	122 ± 13
Cycloheximide (0.1 µg/ml)	43 ± 9	69 ± 14
Actinomycin D (0.05 µg/ml)	14 ± 7	40 ± 6

are in good agreement with those of other authors [13, 14]. On the other side, at least in short-term experiments melanogenesis of hamster melanoma cells was reported to be resistant to cycloheximide treatment [15, 16].

In conclusion, the present findings strongly support the concept of phagocytic uptake of functionally

active, exogenous melanosomes by cultured melanoma cells. We regard it as possible that also under suitable *in vivo* conditions melanoma cells might exhibit this property of "melanophages". It will be of considerable interest to find out, whether such a property might be used to influence or inhibit melanoma cell growth, *e.g.* by utilisation of melanosomes as carriers for cytostatic drugs.

- [1] P. A. Riley, Melanin and melanocytes. In: The Physiology and Pathophysiology of the Skin (A. Jarrett ed.) Vol. 3, pp. 1101–1130, Academic Press London, New York 1974.
- [2] K. Okazaki, M. Uzuka, F. Morikawa, K. Toda, and M. Seiji, J. Invest. Dermatol. **67**, 541–547 (1976).
- [3] D. O. Schachtschabel, H. B. Leising, O. A. Schjeide, and D. V. Molsen, Cytobios, in press.
- [4] D. O. Schachtschabel, Virchows Arch. Abt. B Zellpath. **7**, 27–36 (1971).
- [5] H. B. Leising and D. O. Schachtschabel, Z. Naturforsch. **32c**, 567–572 (1977).
- [6] H. E. Harding and R. D. Passey, J. Path. Bact. **33**, 417–427 (1930).
- [7] O. A. Schjeide, D. O. Schachtschabel, and D. V. Molsen, Cytobios **17**, 87–102 (1976).
- [8] M. Seiji, K. Shimao, M. S. C. Birbeck, and T. B. Fitzpatrick, Ann. N. Y. Acad. Sci. **100**, 497–533 (1963).
- [9] D. O. Schachtschabel and H. B. Leising, Pigment Cell, Vol. 5, S. Karger, Basel 1978, in press.
- [10] D. Schachtschabel, R.-D. Fischer, and F. Zilliken, Hoppe-Seyler's Z. Physiol. Chem. **351**, 1402–1410 (1970).
- [11] A. Oikawa, M. Nakayasu, M. Nohara, and T. T. Tchen, Arch. Biochem. Biophys. **148**, 548–557 (1972).
- [12] M. Seiji and T. B. Fitzpatrick, J. Biochem. **49**, 700–706 (1961).
- [13] I. Werner, Yale J. Biol. Med. **46**, 444–463 (1973).
- [14] J. A. Brumbaugh and D. G. Schall, J. Exp. Zool. **202**, 163–170 (1977).
- [15] Y. Kitano and F. Hu, Exp. Cell Res. **64**, 83–88 (1971).
- [16] R. A. Farishian and J. R. Whittaker, Biochim. Biophys. Acta **496**, 20–28 (1977).