THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 57, No. 1
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DEGRADATION OF MELANOSOMES BY LYSOSOMES*

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ABSTRACT

The degradation of melanosomes by the lysosomes of the mouse liver was studied *in vitro*. Mice with melanoma received intraperitoneal injections of a ^{14}C-amino acid mixture and ^{14}C-dopa separately. Radioactive mitochondria and melanosomes were isolated from these mouse melanomas. Digestion of the ^{14}C-amino acid labeled melanosomes and mitochondria was carried out with lysosomes isolated from the mouse liver. The progressive degradation of the protein moiety of the mitochondria was observed and also the slow but steady degradation of melanosomes. Digestion of ^{14}C-dopa labeled melanosomes was attempted with liver lysosomes. There was no significant amount of degradation. Melanin appears to be very resistant to lysosomal digestion *in vitro*. It is assumed, therefore, that in the compound melanosomes, the melanosomes can be degraded by lysosomes at the protein moiety but not at the melanin moiety.

The compound melanosomes resemble structures known as compound granules (1), lysosomes or phagocytic vacuoles (2), which are ordinarily seen in macrophages. It is now believed that compound melanosomes are formed either as an expression of phagocytic or autophagic activities (3–7). By electron microscopic observation, it has been clearly demonstrated that melanosomes, present in the form of compound melanosomes, can be disintegrated. Such disintegrating types of compound melanosomes can be found in the aged melanoma melanocyte (3–6), dermal melanophages (8) and keratinocytes (7, 9, 10). The melanosomes found in compound melanosomes are in various stages of degradation. In some the outer membranes are not visible; in others the internal structures have become disorganized, have disintegrated into small pieces or have tended to disperse (6, 7). Thus, the degradation of melanosomes is well shown morphologically, but the biochemical mechanisms for the degradation of melanosomes are not known. A series of *in vitro* experiments was carried out in order to study the problem mentioned above. Melanosomes isolated from B-16 mouse melanoma were digested with mouse liver lysosomes.

This work was supported by research grants from the Ministry of Education, Japan, Westwood Pharmaceuticals, Buffalo, New York, and the Japan O'Leary Pigment Research Funds, Tokyo.

Received October 2, 1970; accepted for publication March 24, 1971.

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MATERIALS AND METHODS

Preparation of lysosomes from mouse liver. Lysosomes were prepared from male D-D mice weighing about 20 g. After fasting for 24 to 48 hours, they were killed by decapitation and bled. The livers were quickly taken out, weighed, washed free of blood with 0.25 M ice cold sucrose and finely minced. Homogenization was done in 0.25 M sucrose (10 V/W) with a Potter-Elvehjem homogenizer for 30 sec. The homogenate was successively centrifuged in the same way as described by Sawant *et al.* (11), and the lysosomal fraction was isolated.

Enzyme activities, *i.e.* acid phosphatase, cathepsin, β-glucuronidase, of the homogenate and of the lysosomal fraction were measured. Acid phosphatase was estimated by the method described by Berthet and de Duve (2). β-glucuronidase was determined by the Nimmo-Smith method (3) and cathepsin was done by Schamberger's modification (4) of Anson's method.

Preparation of radioactive melanosomes. A group of male Swiss mice with B-16 or Harding-Passey melanoma (1 to 2 cm in diameter) was given intraperitoneal injections of 10 μCi/10 g body weight (BW) of ^{14}C-amino acid mixture (^{14}C-chlorella protein acid hydrolysate; 8.0 mCi/mMC; Institute of Applied Microbiology, University of Tokyo, Tokyo) in 0.05 ml of distilled water 3 times every other day. The other group of male mice was given ^{14}C-dopa of 5 μCi/10 g BW (DL(3,4 - dihydroxy phenyl)alanine - 2 - ^{14}C; 54.5 mCi/mM; the Radiochemical Center, Amersham) in 0.05 ml of distilled water in the same way.

On the second day after the last injection, the animals were killed. The melanomas were then taken out and immediately homogenized in 0.25 M ice cold sucrose. Melanosomes and mitochondria were prepared by Seiji's method (5).

TABLE

Enzyme activities of liver homogenate and lysosomal fraction

	Acid phosphatase μg-p/10 min/mg protein	β-Glucuronidase μg-p-nitrophenol/30 min/mg protein	Cathepsin μg-tyrosine/30 min/mg protein
Homogenate	3.4	10.7	55.93
Lysosomal fraction	27.52	63.64	1403.51

Digestion of the labeled melanosomes and mitochondria by liver lysosomes. The labeled melanosomes and mitochondria to be digested were suspended in 0.25 M sucrose solution. Three tenths ml of each suspension was mixed with 0.3 ml of 0.1 M sodium acetate and acetic acid buffer pH 5.0 and incubated at 37° C for 5 min. Then 0.4 ml of lysosome suspension was added to the reaction mixtures. Incubation was carried out for 0, 15, 30 and 60 min. with continuous shaking. The amounts of these cell particles used in the individual experiments are indicated in the legends. At the end of each incubation, the reaction mixture was centrifuged at 11,000 × g for 15 min. Then an aliquot of the supernatant obtained was transferred to vials containing 10 ml of a scintillating solution (methanol 150 ml; toluene 350 ml; dioxane 350 ml; POPOP 0.5 g; PPO 5 g; naphthalene 25 g) and the radioactivity was measured in the Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co.).

RESULTS

Liver lysosomes. The Table shows the lysosomal enzyme activities of the liver homogenate and the lysosomal fraction. The specific activities of various enzymes in the lysosomal fractions were 8 to 25 times more than those of the original homogenate. The lysosomal fraction thus obtained was used for the digestion of labeled melanosomes.

Digestion of ^{14}C-amino acid labeled melanosomes and mitochondria by liver lysosomes. The radioactivity of the melanosomes and mitochondria isolated from Harding-Passey mouse melanoma was 582 cpm/mg protein and 549 cpm/mg protein, respectively. The counting efficiency of the melanosome suspension was 83%. The radioactivity of the supernatants of the digestion mixtures was determined and the degradation rate was expressed as a percentage of the total radioactivity of melanosomes or mitochondria used. The acid phosphatase activity of the lysosomes used was 13.4 μg-p/10 min/mg protein.

As shown in Figure 1, the lysosomes caused a progressive solubilization of the protein component of mitochondria. At the end of 60 min about 40% of the total radioactivity had become soluble, whereas in the case of melanosomes, it was only 7% for the same period of time. Melanosomes proved to be somewhat more resistant to the hydrolytic attack of the lysosomal hydrolase than were the mitochondria. Mitochondria incubated without lysosomes for the same period of time also showed about 18% solubility. In the case of melanosomes, the incubation effect was negligible.

Degradation of ^{14}C-amino acid labeled melanosomes and ^{14}C-dopa labeled melanosomes by

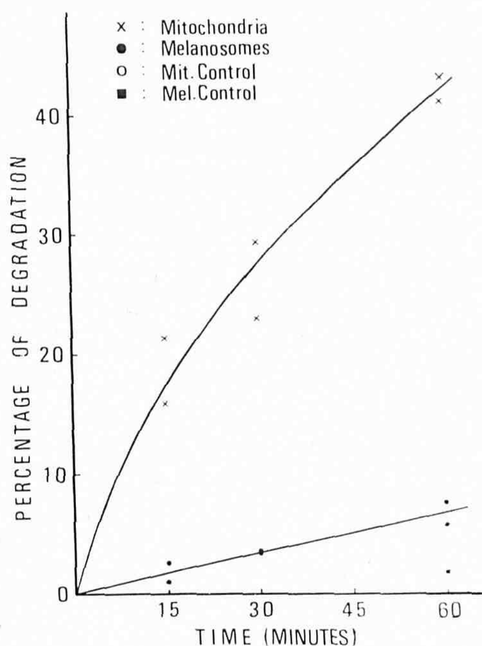

FIG. 1. Time courses of digestion of mitochondria and melanosomes labeled with ^{14}C-amino acid by the action of liver lysosomes. Three tenths of a ml of mitochondria suspension (1.2 mg protein) and of melanosomes suspension (1.1 mg protein) isolated from Harding-Passey mouse melanoma that received the intraperitoneal injection of a ^{14}C-amino acid mixture were incubated separately with 0.4 ml of liver lysosomes suspension (1.9 mg protein) in a 0.1 M acetic acid buffer, pH 5.0 at 37° C. After incubation for the indicated periods of time, the mixture was centrifuged at 11,000 × g for 15 min, and the radioactivity of the supernatant was determined. The amount of radioactivity liberated in the supernatant was expressed as a percentage of the total radioactivity used. The controls were incubated in the absence of added lysosomes. ×——×, Mitochondria-lysosomes mixture; ●——●; melanosomes-lysosomes mixture; ○, mitochondria alone; ■, melanosomes alone.

liver lysosomes. The radioactivity of melanosomes, isolated from B-16 mouse melanoma, that received an intraperitoneal injection of the ^{14}C-amino acid mixture was 298 cpm/mg protein, and the radioactivity of those that received an injection of ^{14}C-dopa was 455 cpm/mg protein. The acid phosphatase activity of the lysosomes resulted in the formation of 13.6 μg inorganic phosphate/10 min/mg protein.

Figure 2 shows the time course of digestion of melanosomes labeled with ^{14}C-amino acids and ^{14}C-dopa. A slightly greater degradation of melanosomes labeled with ^{14}C-amino acids was observed in the B-16 mouse melanoma than in the melanosomes of the Harding-Passey mouse melanomas. On the other hand, the control incubated in the absence of added lysosomes for the same period of time showed only negligible change. The melanosomes labeled with ^{14}C-dopa did not show any significant change even at the end of 60 min. Melanin appears to be very resistant to lysosomal digestion *in vitro*.

DISCUSSION

Naturally occurring melanin pigments are strongly resistant to chemical and physical exposures *in vitro*. The best known function of melanin in man is to serve as a light-absorbing mantle for the epidermal cells and protect them against the harmful effects of UV light (6). For these reasons, it has been generally considered that melanosomes *per se* were not changed in the epidermal cells but were discarded from the skin surface with the epidermal cells to which they were transfered.

As mentioned in a previous paper (6), recent electromicroscopic work has shown that melanosomes are disintegrated in the aged melanoma melanocytes (3–6), melanophages in the dermis (8, 9) as well as in the epidermal cells (7, 10, 18, 19). In particular, malignant melanoma cells are known to undergo spontaneous ulceration (3, 8, 20, 21). These degradation processess are related to the formation of lysosomes and to the degradation of melanoma cells themselves (3).

Using mouse melanoma, an *in vitro* biochemical study on the degradation of melanosomes by lysosomes was carried out to clarify the degradation mechanisms. Because of the tissue specificities in lysosomal enzymes (22, 23), the authors first attempted to isolate the lysosomes from the mouse melanoma by the method described by Wattiaux *et al.* (24) and Leighton (25), using the Triton WR 1339. However, the harvest of the lysosomal fraction, having high activities of various enzymes, was too small to allow the performance of a series of *in vitro* experiments (26). This might be due to the paucity of primary lysosomes in the melanoma cells, as seen under the electron microscope. Liver lysosomes were used instead. They were prepared by the method described by Sawant *et al.* (11). With respect to the specific activities of various lysosomal enzymes, the lysosomes isolated showed about a 25 fold increase over the homogenates.

The radioactivity of mitohcondria isolated is assumed to be due to the ^{14}C-amino acids incorporated into a protein moiety of mitochondrial constituents. The digestion was carried out at pH 5.0 in order to minimize the control values.

The experimental results show that mitochondrial protein components appear to be easily degraded by lysosomes. The exact nature of the disintegrated components is not known, but

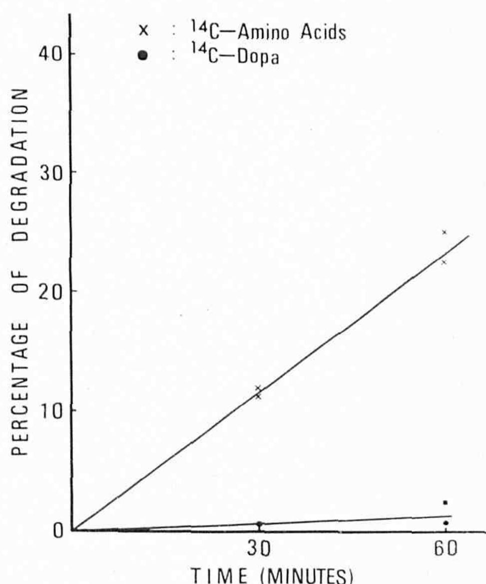

FIG. 2. Time courses of digestion of melanosomes labeled with ^{14}C-amino acids and ^{14}C-dopa by the action of liver lysosomes. Three tenths of a ml of melanosomes suspension (0.44 mg protein) isolated from B-16 mouse melanomas that received intraperitoneal injections of ^{14}C-amino acid mixture and ^{14}C-dopa were incubated separately with 0.4 ml of liver lysosomes suspension (2.6 mg protein) in a 0.1 M acetic acid buffer, pH 5.0 at 37° C. The control was incubated in the absence of added lysosomes. ×——×, ^{14}C-Amino acid melanosomes-lysosomes mixture; ●——●, ^{14}C-dopa melanosomes-lysosomes mixture; ■, ^{14}C-amino acid melanosomes alone.

supposedly, they are soluble proteins and small membranous components which contain ^{14}C-amino acids. When the reaction mixtures were centrifuged at 105,000 × g for 60 min instead of at 11,000 × g for 15 min, the radioactivity of the supernatant thus obtained was the same as with low speed centrifugation. The relatively high value of the control which was incubated in the absence of added lysosomes is thought to be due to the contaminated lysosomes in the mitochondrial fraction isolated from mouse melanoma. The melanosomal preparation isolated by the sucrose density gradient centrifugation does not contain any other cell particles except melanosomes, but the mitochondrial fraction isolated by the same method is supposed to contain lysosomes (15).

Melanosomal protein components also were degraded by lysosomes at a steady rate. The control experiment with melanosomes did not show any significant degradation. The net degradation percentage of melanosomes by lysosomes was approximately one third of that of mitochondria. In electronmicroscopic histochemical studies, Seiji *et al.* (27) and Olson *et al.* (7) have shown that individual melanosomes in the melanocyte exhibit lysosomal enzyme activities. It was, however, most unlikely that lysosomal enzymes found in the individual melanosomes participated in the digestion of melanosomes in this particular experiment since the control experiments did not show any significant degradation.

According to Seiji *et al.* (28), when ^{14}C-dopa was injected into mice with melanoma, the ^{14}C-dopa incorporation occurred specifically in the newly synthesized melanin of melanosomes. Experimental results obtained with ^{14}C-dopa showed that there was no significant degradation of the melanin moiety in melanosomes. It should, however, be pointed out that in our experiments we were estimating only the released radioactivity of the supernatant solution after centrifugation and since melanin exists in a highly polymerized form and is a high molecular weight substance, the degraded moiety of the melanin polymer may still be sedimenting and not released in the supernatant solution.

According to the experimental results presented in this study it appears that lysosomes isolated from liver cells did not seem to degrade melanin *per se*, but they could be subjected to hydrolytic action at the protein moiety of the melanosome by the lysosomal enzymes. Melanosomes basically consist of outer membranes and an inner membranous matrix, which is presumably mainly protein, and on which the deposition of the polymerized indole 5,6-quinone and other intermediates of the tyrosine to melanin reaction takes place. Therefore, it is assumed that in the compound melanosomes, there can be degradation by lysosomal enzymes at the protein moiety.

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