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New insights into the physicochemical effects of ammonia/peroxide bleaching of hair and *Sepia* melanins

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Synopsis

Chemically unaltered melanosomes from black hair were isolated using a mild enzymatic procedure reported by Novellino *et al.* (1) involving sequential treatment of a homogenized hair sample with different protease enzymes. Time-dependent fluorescence studies show, under identical conditions, that the rate of bleaching upon NH_3/H_2O_2 treatment of hair melanosomes is twice that of *Sepia* melanosomes. The structure and morphology of hair melanosomes are compared to *Sepia* eumelanin using ESEM and TEM imaging studies. Black hair melanosomes are aggregates of rice-shaped ellipsoidal particles (0.8–1.0 μm in length and 0.2–0.6 μm in width) surrounded by an amorphous material suspected to be made of non-proteinacious materials. *Sepia* eumelanin aggregates are larger (2–5 μm) particles with a "doughnut" shape comprised of 100–150-nm spherical particles. Time-dependent TEM imaging studies of ammonia-treated (pH 10) hair melanosomes showed an initial breakdown of melanosomal aggregates followed by rupture of the melanosomal membrane, releasing melanin nanoparticles and leaving a ghost membrane behind. After prolonged treatment with aqueous NH_3, a total loss of characteristic melanosome morphology was observed leading to an amorphous material. By contrast, *Sepia* melanosomes under identical conditions of ammonia treatment did not show such changes, probably due to different surface properties and aggregation behavior. Sodium hydroxide or sodium carbonate at identical pH did not show similar changes to ammonia, suggesting that the changes are not merely due to alkaline pH, but, rather, are specific to ammonia. Co-treatment with ammonia and peroxide induced a faster disintegration of the melanosomes, resulting in a complete dissolution and discoloration of melanin in 30 minutes. The data suggest that ammonia helps to release melanin nanoparticles out of melanosomes, making them more susceptible to oxidative attack by H_2O_2.

INTRODUCTION

Melanins are a class of highly heterogeneous biological pigments found in animals, plants, and humans, and are responsible for the color of skin, eyes, and hair. The color of hair and wool in mammals and feathers in birds is mostly determined by the quantity and quality of melanin that is synthesized in follicular melanocytes and transferred to keratinocytes. It is generally known that hair pigmentation is due to two types of

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melanin: black-to-brown eumelanin and yellow-to-red pheomelanin (2). Eumelanin is believed to be a polymer derived from oxidative copolymerization of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (3,4). Pheomelanin is composed of tyrosine and cysteine-derived units constructed into benzothiazine monomers that make the polymer (5). Extensive research work has been conducted by various groups to elucidate the structure of melanin and the melanogenesis pathway. Despite the vast literature on natural and synthetic melanins, the molecular structure of this class of pigments remains unknown. This is due to the lack of adequate methods to isolate melanin from biological sources, their insolubility at neutral pH, and the heterogeneity in their structural features. Melanins are typically isolated under harsh hydrolytic conditions such as prolonged treatment with concentrated hydrochloric acid or sodium hydroxide. Recent studies demonstrate that melanin, when exposed to boiling mineral acids, suffers profound structural alterations, particularly extensive decarboxylation (3,6). The isolation of melanin from hair or fur is more complex because of the compactness of the keratin in which the pigment is encapsulated. As a result, most of the literature work used *Sepia* melanin or synthetic eumelanin as a model for black hair melanin.

Regardless of the source and type of melanin, various physical and chemical methods have been established in the last fifty years towards characterizing the structure of eumelanin and pheomelanin at the microscopic and macroscopic levels. Both X-ray and mass spectroscopic studies gained insight into the structural units of eumelanin and suggest that synthetic eumelanins and natural eumelanin from *Sepia* have similar building blocks (7–9). Based on wide-angle X-ray diffraction measurements of eumelanin, a fundamental particle consisting of a graphite-layered structure with four to eight connected monomers per layer has been proposed. Both MALDI-mass spectroscopy and X-ray diffraction studies identified oligomeric units of masses in the range of ~500–1000 amu for *Sepia* eumelanin and synthetic eumelanin (8–10). Scanning electron microscopy studies of size- selected *Sepia* melanin samples suggest that the pigment of eumelanin is an aggregated structure with subunits of ~150 nm, and that smaller ~15-nm-size particles adhere to these larger subunits (11). Recent AFM studies on *Sepia* melanin also confirmed that the 100–200-nm-size spherical particles are not a fundamental structural unit of eumelanin, but are composed of smaller 5–15-nm-size particles (12).

A review by Prota (13) claims the color of black hair is due to intact or poorly degraded eumelanin, whereas brown hair contains a degraded variant of eumelanin. Prota also claims that only red hair types contain pheomelanins. The study by Borgers *et al.* (14) uses sensitive HPLC methods for the identification and quantification of eumelanin and pheomelanin from human hair by measuring product yields produced by alkaline hydrogen peroxide degradation of melanin. This study suggests that color determination for black, brown, or blonde hair depends more on melanin quantity than on eu-/pheomelanin composition, while red hair may arise through production of pheomelanin.

Wolfram and Albrecht (15) studied the oxidation of human hair with and without melanin pigment and the oxidation of melanin granules isolated from human hair. They found that hair with pigment degrades hydrogen peroxide at a much faster rate than hair without the pigment. Thus, they conclude that peroxide reacts with melanin much faster than with proteins. In another study, the same authors compared the chemical and photobleaching of brown and red hair and concluded that pheomelanin is more resistant

than eumelanin to chemical or photodegradation (16). It is also known from the literature that the ammonia/hydrogen peroxide combination is an effective melanin-bleaching agent, whereas hydrogen peroxide at neutral conditions bleaches melanin very slowly. Studies by Gallas *et al.* (17), using scanning tunneling microscopy (STM), showed a dramatic decrease in the STM-measured apparent heights for mildly bleached melanin compared to untreated melanin. These authors interpret the observed decrease as a result of oxidative conversion of the multilayered stacked sheets of melanin to largely destacked melanin sheets.

There are no detailed studies in the literature on the ultrastructural features of hair melanosomes or on the mechanism of hair melanin bleaching induced by ammonia and peroxide. In order to study the mechanism of human hair melanin bleaching, intact hair melanosomes were isolated using an enzymatic procedure (1) to digest hair keratin under neutral conditions. The present study, aimed at understanding the mechanism of hair melanin bleaching, presents the first report on the direct demonstration by TEM of morphological and structural changes undergone by isolated hair melanosomes as a result of ammonia and ammonia/peroxide treatments. The study reveals that ammonia has a specific role in the bleaching process by breaking the melanosomal membrane, unlike other alkalizing agents such as sodium hydroxide and sodium carbonate at identical pH conditions. In addition, we also show by TEM that the micron-sized hair melanosomes are composed of smaller constituents of melanin (30–60 nm) encapsulated in a membrane sac.

EXPERIMENTAL

MATERIALS

Untreated Asian black hair was purchased from International Hair Importers and Products Inc., Bellerose, NY. *Sepia* melanin and all protease enzymes were purchased from Sigma Biochemicals (St. Louis, MO). All other chemicals and reagents used in the study were analytical grade. All solutions were prepared using milli-Q-water, and melanin suspensions were prepared freshly by sonication prior to the experiments.

METHODS

Isolation of hair eumelanin from Asian black hair. The isolation of black hair melanin was achieved by the procedure reported by Novellino *et al.* (1), using the following enzymatic treatments: (1) An Asian black hair sample, after removal of sebum and surface lipids, was treated with DTT (dithiothreitol) for 18 hr at pH 7.4 and 37°C. After centrifugation, the pellet was treated with Proteinase K and DTT and stirred under argon at 37°C for another 18 hr. The mixture was centrifuged for 20 min (3500 × g, 4°C) in an Eppendorf centrifuge. (2) The pellet was extensively rinsed with water, suspended in 30 ml of 0.1 M phosphate buffer, and treated with papain and DTT at 37°C under argon for 18 hr. The mixture was centrifuged as above. (3) The black pellet collected after sixfold washing with water was suspended in 30 ml of buffer and treated with protease and DTT at 37°C under argon for 18 h. (4) The dark residue obtained after centrifugation was suspended in 40 ml of phosphate buffer deaerated with argon for 30 min. An oxygen-free solution of 2% Triton X-100 was added and stirred at room temperature for 4 hr under argon. The mixture was centrifuged for 20 min (106,000 × g, 4°C). After

washing with water:methanol (1:1) and four times with water, the black pellet was again treated with protease and DTT as described in step 3. The final pigment collected by centrifugation was dried over NaOH overnight and equilibrated with saturated aqueous $CaCl_2$ for 24 hr. The final yield of melanin was 600 mg from 15 g of Asian black hair (4%).

Environmental scanning electron microscopy studies (ESEM). ESEM studies were conducted using the FEI ESEM (model E-3) equipped with a lanthanum hexaboride (LaB_6) filament. The microscope was equipped with a Peltier cooling stage (used to maintain the moist environment), which was operated at 2.0°–3.0°C while the chamber pressure was maintained between 4 and 5 Torr. The sample chamber was pumped down to 5 Torr and then increased to 9 Torr. This was repeated eight times while maintaining a Peltier temperature of 2°–3°C. Samples were then viewed at 10–24 kV while maintaining a chamber pressure of 4–5 Torr. Occasionally when the liquid precluded the viewing of melanin, the pressure was decreased to remove surface liquid and reveal the melanin. The ESEM was also equipped with the Image Acquisition and Archive System (IAAS), which allowed the results to be recorded and stored digitally as TIF files.

Transmission electron microscopy studies (TEM). A Jeol 1200-EX transmission electron microscope equipped with a tungsten filament was used for all imaging studies. The vacuum in the chamber was maintained at 10^{-3} Pa. Samples were viewed at 100 keV with a low beam current of 63–65 uA. An AMT digital camera system was attached to the port of the TEM. The images were captured, and the signal was sent to a PC for image storage and analysis.

The specimen support grids used in the experiments were 200-mesh gold finder grids. Finder grids were used to provide an ease of relocation in interesting areas of the grid. The gold grids had a formvar support film and a carbon coat. The specimens were observed under the following sample preparation: A 5-μl drop of melanosome suspension was placed on a formvar/carbon-coated gold grid. The sample was allowed to settle for one minute, excess liquid was gently drained off with filter paper, and the sample was allowed to dry.

UV-Vis and fluorescence spectroscopic studies. UV-Vis spectral studies were performed using a Shimadzu Multispec-1501 UV-Vis spectrophotometer with diode array detection. Time-dependent spectral measurements were recorded in the scan mode. The fluorescence studies were performed using a Cary Eclipse fluorescence spectrophotometer.

RESULTS

UV-VIS ABSORPTION PROPERTIES OF HAIR AND *SEPIA* MELANIN

The absorption spectra of aqueous suspensions (0.1 mg/ml) of hair and *Sepia* melanin at neutral pH are shown in Figure 1. The absorption of hair melanin exhibits a structureless spectrum with absorptivity decreasing monotonically with decreasing wavelength. *Sepia* melanin exhibits a higher absorption at longer wavelengths, indicating that the particles are bigger than that of hair melanin. The longer wavelength absorption is attributed to scattering and absorption due to higher-molecular-weight melanin polymers. It is true that the spectral features of melanin change with size and that a sonicated suspension of hair melanin filtered through a micron-size filter displays an absorption spectrum with a decrease in longer wavelength absorptivity (data not shown).

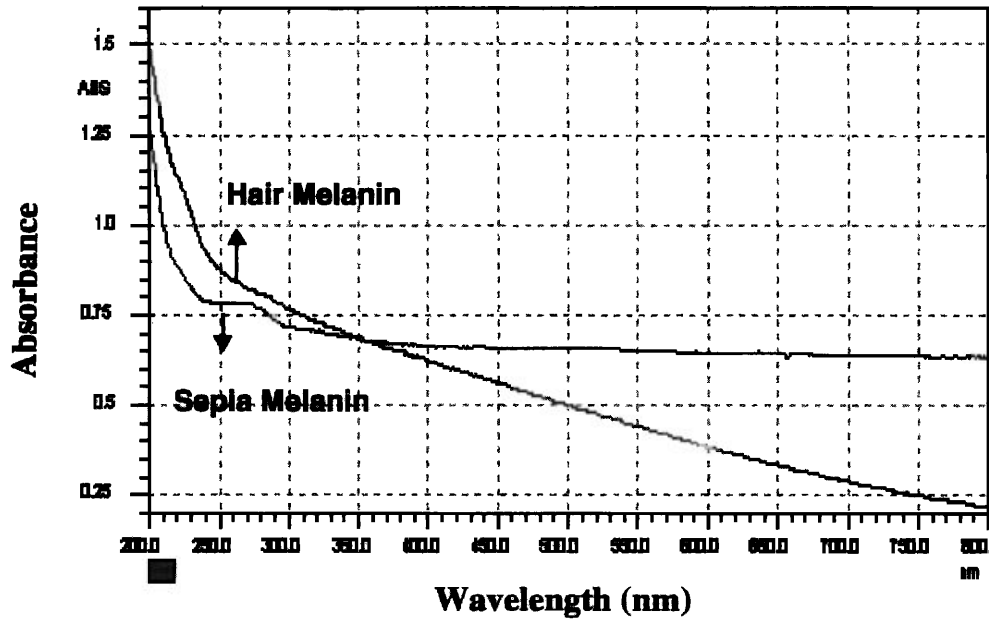

Figure 1. Absorption spectra of aqueous suspensions of hair and *Sepia* melanin at pH 7.0. Sonicated suspensions of 0.1 mg/ml of melanosomes in water were used for the absorption spectral measurements.

FLUORESCENCE PROPERTIES OF HAIR AND *SEPIA* MELANIN

Both isolated hair melanosomes and *Sepia* melanin at pH 10.0 did not show any fluorescence before bleaching. However, after bleaching with NH_3 and H_2O_2 at pH 10, both melanins exhibited fluorescence properties. Figure 2 shows the fluorescence emission (λ_{ex} = 350 nm) and excitation spectra (λ_{em} = 450 nm) of hair melanin (0.5 mg/ml) after 45 min of treatment with 2% H_2O_2 and 0.75% NH_3 at pH 10.0. The emission maximum is at 468 nm for any excitation wavelength from 300–350 nm. No other emission band is observed for hair melanin. These results agree with the finding by Kayatz *et al.* (18) that both synthetic melanin and isolated bovine melanosomes fluoresce only after oxidation. However, the fluorescence emission band is reported to be at 548 nm for bovine melanosomes, while hair melanosomes did not show any fluorescence emission in this region.

Figure 3 shows the emission and excitation spectra of *Sepia* melanin under identical conditions of treatment with ammonia and peroxide. The fluorescence emission spectrum of *Sepia* melanin showed a maximum at 460 nm (λ_{ex} = 350 nm) and an excitation maximum at 349 nm (λ_{em} = 450 nm). The excitation spectra of both *Sepia* and hair melanin exhibited maxima around the 350 nm region, suggesting that a yellow chromophore is responsible for the fluorescence emission at 460 nm.

The rates of ammonia/peroxide bleaching of both *Sepia* and hair melanins were studied by time-dependent fluorescence measurements under identical conditions. Suspensions of 0.2 mg/ml of melanin samples were treated with 1% NH_3 and 2% H_2O_2 at pH 10.0 and 25°C. Oxidation of melanins results in fluorescence emission at 450 nm, as shown above. The intensity of fluorescence emission increases with increase in time. Figure 4

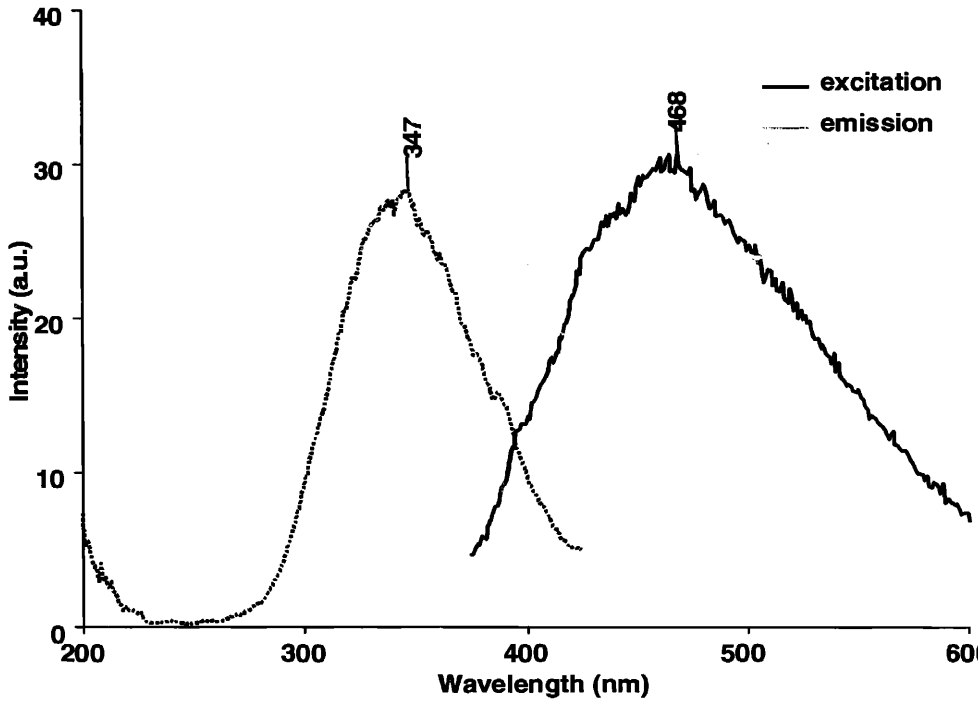

Figure 2. Fluorescence emission and excitation spectra of isolated hair melanosomes (0.5 mg/ml) after 45 min of oxidation with 2% H_2O_2 and 0.75% NH_3 at pH 10.0. Fluorescence intensity is shown in arbitrary units.

shows the plot of fluorescence emission intensity at 450 nm as a function of time for both hair and *Sepia* melanosomes at pH 10.0.

The rate of bleaching of hair melanin at pH 10 is 2.1×10^{-4} s^{-1}, nearly twice as fast as that of *Sepia* melanin, 1.2×10^{-4} s^{-1}, under identical conditions. The fluorescence emission intensity reached a saturation value after complete oxidation, resulting in a clear solution for hair melanin and a yellow solution for *Sepia* melanin. The rates of bleaching of *Sepia* and hair melanin are different, possibly due to differences in morphological and aggregation behavior of the melanosomes. These aspects were verified using ESEM and TEM imaging studies.

ESEM AND TEM STUDIES: COMPARISON OF *SEPIA* AND HAIR MELANOSOMES

ESEM and TEM imaging studies were performed to understand the structural and morphological properties of isolated hair melanosomes. For comparison, *Sepia* melanin was also studied, since it is widely used as a model for black eumelanin. Figure 5 shows the ESEM image of a suspension of *Sepia* melanin in water. *Sepia* melanosomes are micron-sized particles with a characteristic "doughnut" shape. Magnification of the micron-size particles revealed that they are comprised of aggregates of nanometer-size particles (100–150 nm). It is also noted that the micron-size particles of *Sepia* melanin could be easily broken down to nanoscopic particles upon sonication. These results are consistent with the previous work by Nofsinger *et al.* (11) on SEM imaging studies of

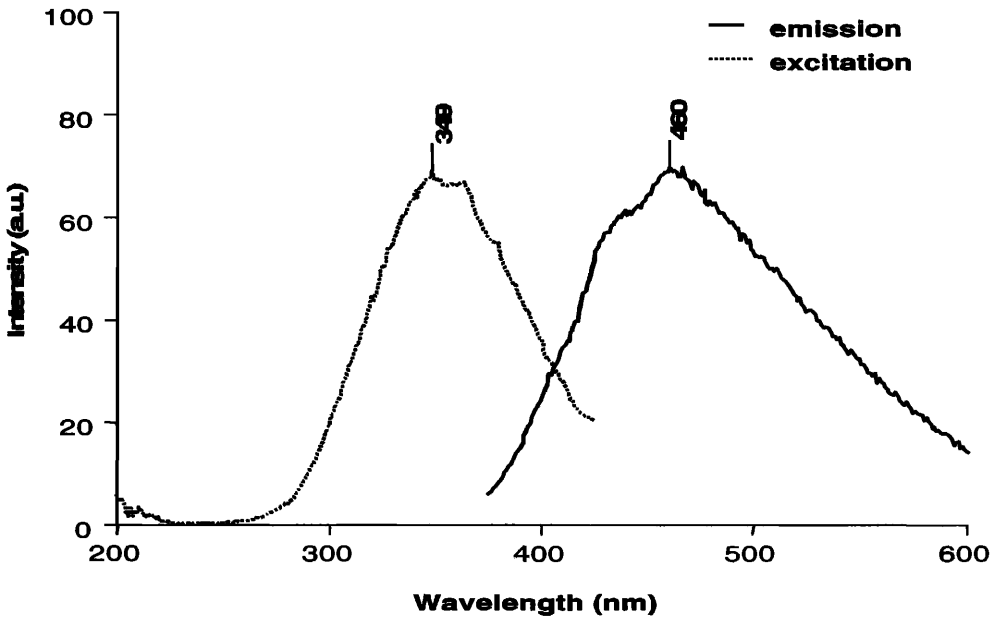

Figure 3. Fluorescence emission and excitation spectra of *Sepia* melanin (0.5 mg/ml) after 45 min of oxidation with 2% H_2O_2 and 0.75% NH_3 at pH 10.0. Fluorescence intensity is shown in arbitrary units.

Sepia melanosomes. Figure 6 shows a representative image of a suspension of isolated hair melanosomes in water. It is evident from the pictures that black hair melanosomes are elongated in shape, typically 0.8–1.2 μm in length and 0.3–0.6 μm in width. Isolated hair melanosornes retain their morphology, as in hair, except that the granules form larger aggregates of rice-shaped particles in aqueous suspensions. Interestingly, it is found that these aggregates are surrounded by an amorphous material clearly evident in the TEM image. Since the melanosomes were isolated using protease enzymes, this material may not be a simple protein. Unlike *Sepia,* suspension of hair melanosomes did not break into nanoparticles upon sonication. Ultrastructural features were also not clearly evident in the TEM images of hair melanosomes.

TEM STUDIES ON THE EFFECT OF AMMONIA

A time-dependent TEM imaging study was performed to monitor the morphological and structural changes of hair melanosomes in the presence of aqueous ammonia. This study was designed to understand the specific role of ammonia in the bleaching process of hair. Isolated hair melanosomes (0.05 mg/ml) were treated with 1% NH_3 (pH 10.0) in aqueous solution. Aliquots were taken every 30 min for TEM analysis.

TEM images show that ammonia treatment removes the amorphous material surrounding the melanosomes. As a result, the melanosomes appear very well dispersed. Figure 7 shows a TEM image of ammonia-treated hair melanosomes after 30 min. It is evident from Figure 7 that ammonia treatment ruptures the melanosomal membrane, releasing melanin nanoparticles (30–60 nm) out of the sac. This study also demonstrates that hair melanosomes are comprised of melanin nanoparticles encapsulated in a membrane sac. However, a similar melanosomal membrane sac is absent in *Sepia* melanosomes. Sodium

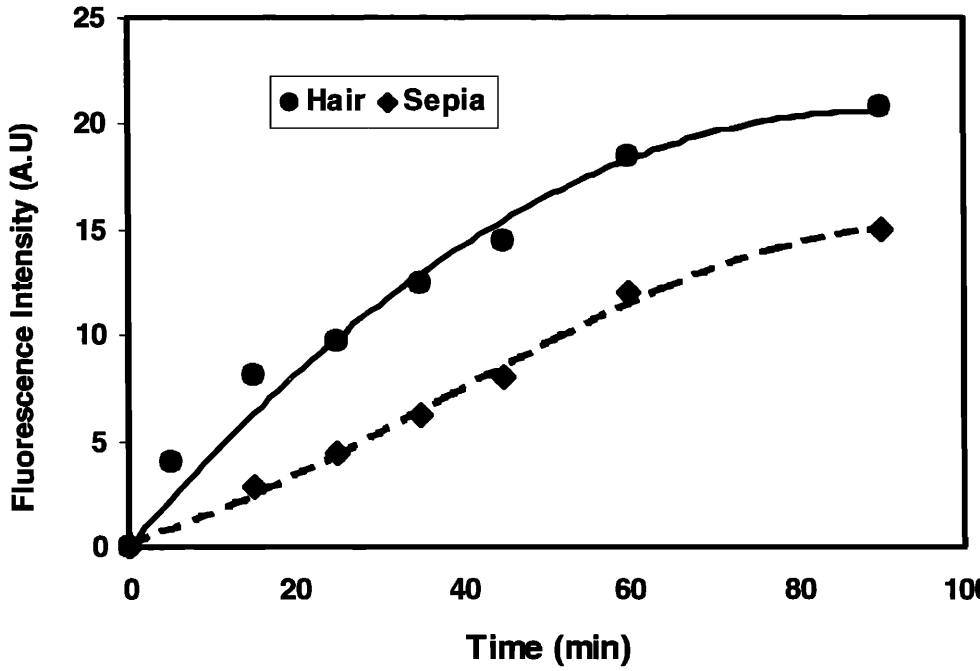

Figure 4. Time-dependent fluorescence study for the bleaching of hair and *Sepia* melanin using NH_3/H_2O_2 at pH 10.0. A suspension of melanosomes (0.5 mg/ml) was treated with 2% H_2O_2 and 1.0% ammonia, and aliquots were taken every 5 min for time-dependent measurements. Intensity of fluorescence emission is plotted as a function of time.

hydroxide or sodium carbonate treatment at pH 10 does not effect similar structural changes to hair melanosomes as ammonia. Thus, it is confirmed that the observed melanosomal changes are not merely due to alkaline pH but are, rather, specific to ammonia. *Sepia* melanosomes under identical conditions of ammonia treatment did not show such changes, probably due to a slow rate of bleaching, as evident from the time-dependent fluorescence studies.

Prolonged treatment of hair melanosomes with NH_3 induced a complete destruction of characteristic melanosome morphology and a shrinking of the granule size (Figure 8). As shown in Figure 8, the melanosomes appeared as fibril-type structures devoid of the melanosomal membrane sac. After 3 hr of treatment, no particles were seen by TEM, except an amorphous material. The ammonia-treated melanin suspension showed a lighter color compared to the untreated suspension, and a decrease in longer wavelength absorbance in the UV-Vis spectrum. Thus, it is evident that ammonia could possibly induce lightening of hair melanosomes. However, the overall process of particle breakdown leading to an amorphous material is slow and took more than 3 hr.

TEM STUDIES ON THE EFFECT OF HYDROGEN PEROXIDE

Isolated hair melanosome suspension was treated with 2% H_2O_2 solution at pH 7.0 and pH 8.0 to see how peroxide alone degrades melanosomes. The degradation of melanosomes by H_2O_2 at neutral pH is a very slow process, and even after 60 min of treatment,

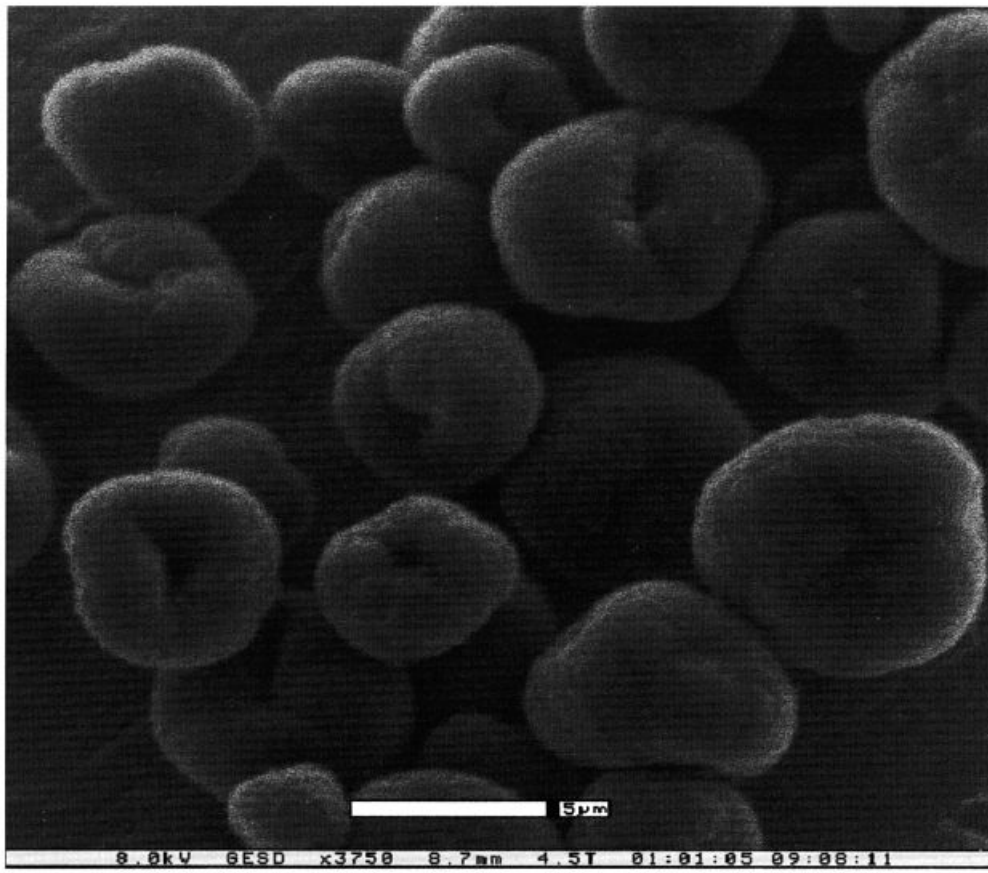

Figure 5. ESEM image of a suspension of *Sepia* melanin (×4000). A suspension of 0.05 mg/ml of *Sepia* melanosomes was used for the ESEM imaging studies.

intact melanosomes were visible by TEM image analysis. However, after 2–3 hr of treatment, structural changes leading to the dissolution of melanin granules were observed. The changes were slightly faster at pH 8.0, and prolonged treatment led to a clear solution, indicating that bleaching has occurred. The study clearly suggests that neutral H_2O_2 is less efficient than NH_3 in inducing morphological and structural changes to hair melanosmes.

TEM STUDIES ON THE EFFECT OF AMMONIA/PEROXIDE

Time-dependent TEM imaging studies were performed on isolated hair melanosomes (0.05 mg/ml) after treatment with NH_3 (1%) and H_2O_2 (1%) at pH 10. Co-treatment with NH_3 and H_2O_2 induced a faster disintegration of melanosome particles, and structural changes were visible even after 10 min of treatment. Figure 9 shows the TEM image of ammonia/peroxide-treated hair melanosomes after 15 min of treatment.

In contrast to the effect of ammonia, co-treatment with ammonia and peroxide induced a simultaneous disintegration and dissolution of melanosomes. The overall disintegration, dissolution, and discoloration process is much faster than ammonia treatment due

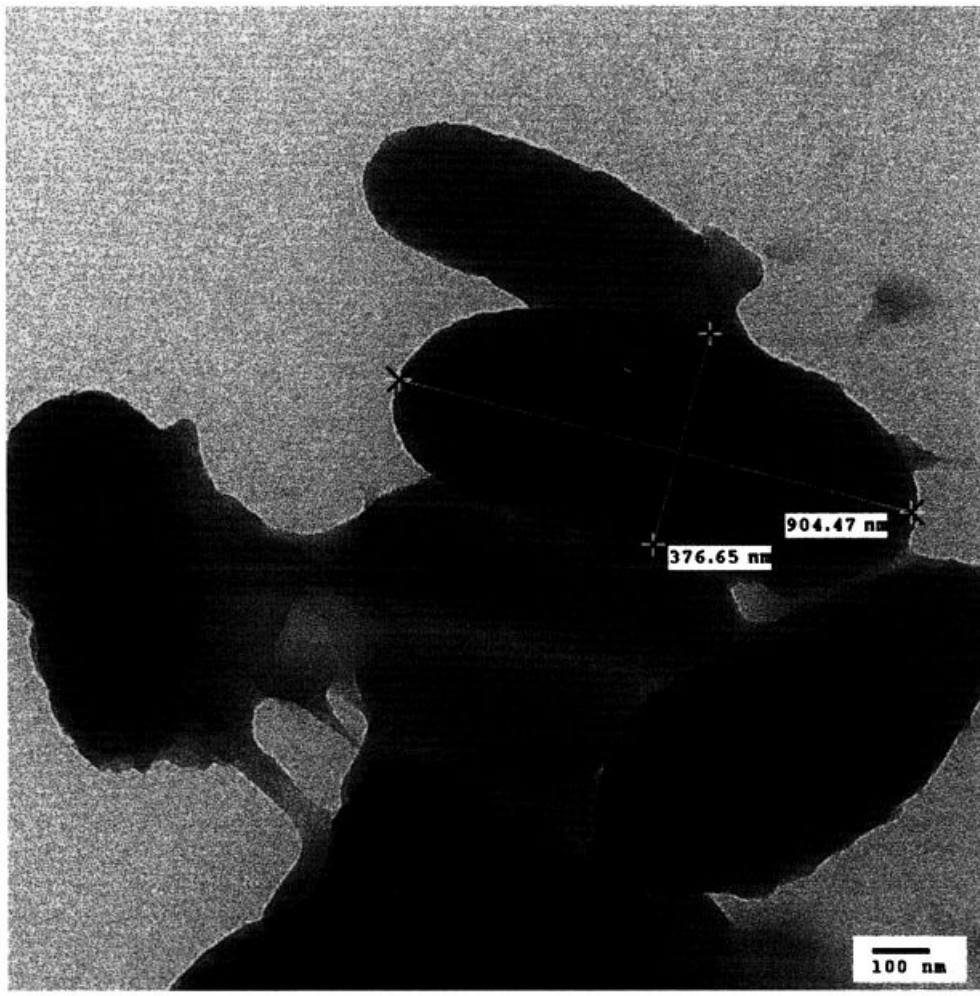

Figure 6. TEM image of isolated hair melanosomes from Asian black hair (×75,000). A sonicated suspension of hair melanosomes (0.05 mg/ml) in water at neutral pH was used for the TEM imaging study.

to oxidative attack by hydrogen peroxide. Thus, it is clear from the study that the role of ammonia is to help the release of melanin nanoparticles from the melanosomal sac, making it more susceptible to attack by peroxide. At the end of 30 min, the ammonia/peroxide-treated melanosome suspension gave a colorless solution devoid of any particles, while ammonia treatment gave a yellow-colored suspension, clearly indicating that the role of peroxide is to decolorize the degraded melanosomes.

DISCUSSION

Chemically unaltered melanosomes from Asian black hair were isolated using a mild enzymatic procedure involving sequential treatment of a hair sample with different protease enzymes (1). The UV-Vis absorption spectrum of hair melanosome suspension in water showed a structureless spectrum with absorptivity increasing monotonically with decreasing wavelength, characteristic of natural and synthetic melanins. Both *Sepia*

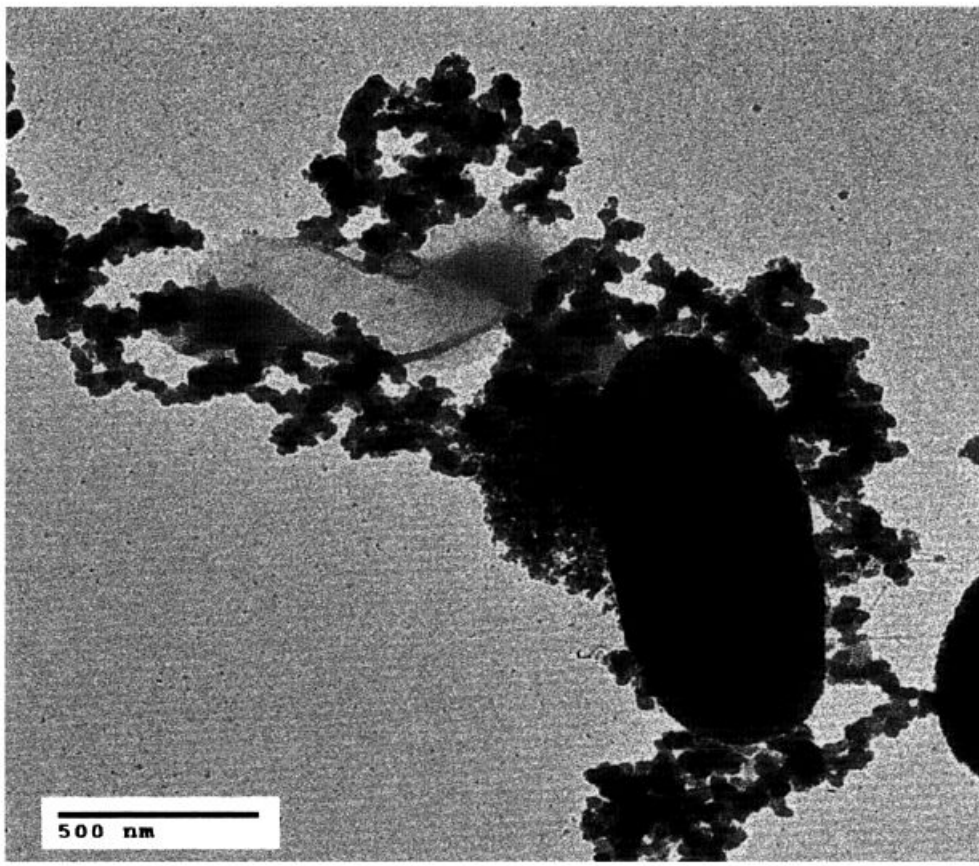

Figure 7. TEM image of hair melanosomes after 30 min of ammonia treatment at pH 10.0 (×75,000). A suspension of 0.05 mg/ml of hair melanosomes was treated with 1.0% ammonia at pH 10.0.

and hair melanosomes did not show fluorescence before bleaching, while after oxidation with NH_3 and H_2O_2, the fluorescence properties of both melanins exhibited identical emission and excitation spectra. From the excitation spectra, it is clear that a yellow chromophore is responsible for the fluorescence emission properties of melanin after oxidation. This yellow choromophore could be indoquinone or similar quinoid structures resulting from the breakdown and degradation of polymeric melanin as a result of oxidation. The fluorescence data also suggest that *Sepia* and human hair melanosomes have the same molecular constituents. Time-dependent fluorescence studies show that, under identical conditions, the rate of bleaching upon NH_3/H_2O_2 treatment of hair melanosomes is twice that of *Sepia* melanosomes. The rates of bleaching of *Sepia* and hair melanin are different, possibly because of differences in morphological and aggregation behavior of the melanosomes.

The morphological behavior of isolated hair melanosomes was compared to that of *Sepia* melanosomes using ESEM and TEM imaging studies. Black hair melanosomes are aggregates of rice-shaped ellipsoidal particles (0.8–1.0 μm in length and 0.2–0.6 μm in width) surrounded by an amorphous material suspected to be made of non-proteinacious

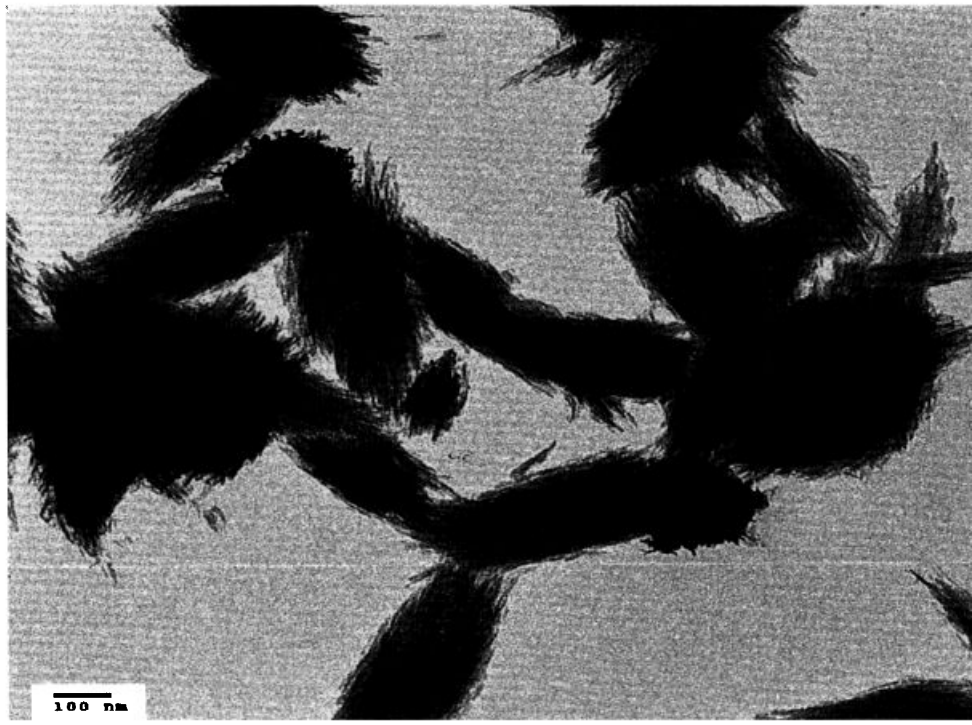

Figure 8. TEM image of isolated hair melanosomes after 120 min of ammonia treatment at pH 10 (×75,000). A suspension of 0.05 mg/ml of hair melanosomes was treated with 1.0% ammonia at pH 10.0.

materials. Since the isolation of melanosomes involved sequential treatments with different protease enzymes, this amorphous material could not be ordinary proteins, but, rather, highly resistant glycolipids or glycoproteins. A report by Swift (19) indicates a closer association between melanin granules and the cell membrane complex in matured hair samples. Thus, the amorphous material seen in isolated melanosomes could possibly be the cell membrane complex. More work needs to be done to identify the nature of the waxy material surrounding the melanosomes.

Sepia eumelanin aggregates are larger (2–5 μm) particles with a "doughnut" shape comprised of 100–150-nm spherical particles, and do not show the waxy material. The ESEM data on *Sepia* melanin correlates very well to the published SEM data by Nofsinger *et al.* (11). Magnification of the micron-size particles of *Sepia* melanosomes showed smaller particles of 100–150 nm in size, indicating that the bigger spherical particles are aggregates of smaller nanoparticles. The reported literature also confirms that the self-assembly of *Sepia* melanin is a hierarchical process (12). Similar subunits were not seen in isolated hair melanosomes under identical magnification, probably due to the fact that hair melanin nanoparticles are tightly encapsulated in a membrane sac that acts as a protective barrier.

Time-dependent TEM imaging studies of ammonia-treated hair melanosomes help us to understand the early events occurring in the bleaching process of isolated hair melano-

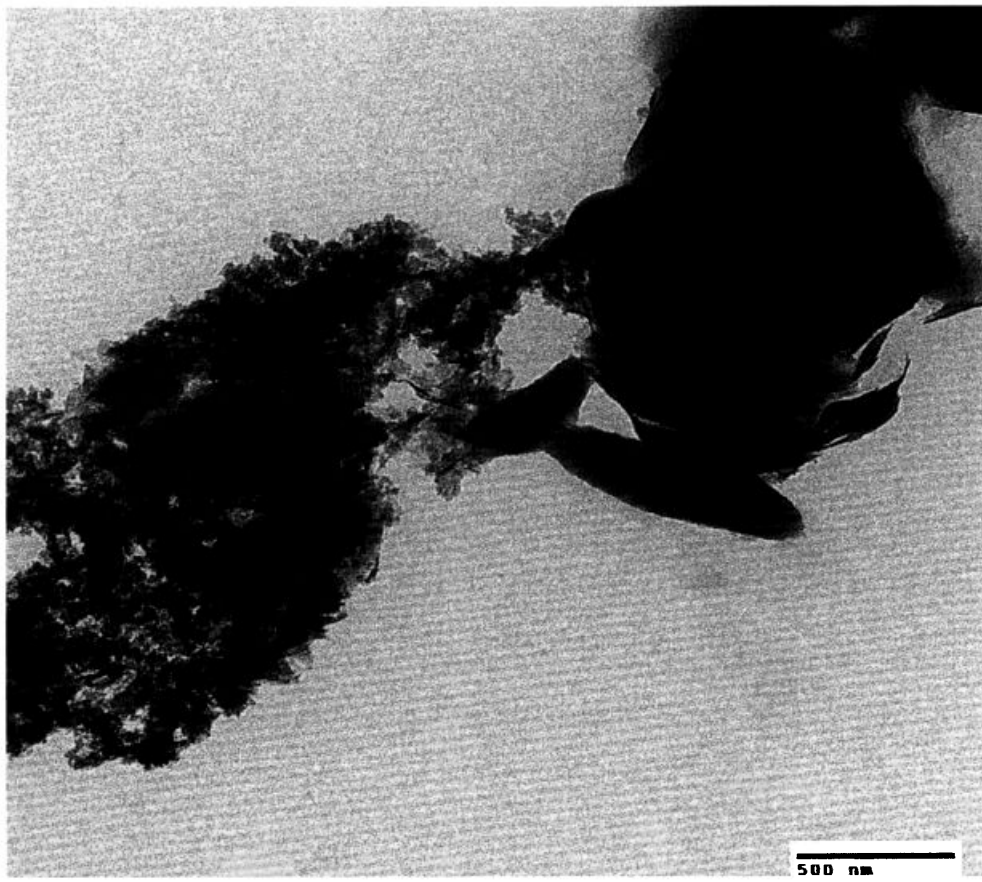

Figure 9. TEM image of hair melanosomes after 15 min of treatment with NH_3/H_2O_2 at pH 10.0 (×40,000). A suspension of 0.05 mg/ml of hair melanosomes was treated with 1.0% ammonia and 1.0% hydrogen peroxide at pH 10.0.

somes and provide new insights into the role of ammonia in the bleaching process. Aqueous ammonia treatment results in an initial breakdown of melanosome aggregates by removal of the surrounding amorphous material. This is followed by rupture of the melanosomal membrane sac, releasing melanin nanoparticles (Figure 7). The observation of melanin nanoparticles after ammonia treatment suggests that the micron-size hair melanosomes are comprised of 30–50-nm-size particles. In addition, these studies also provide evidence that, unlike *Sepia,* hair melanin particles are encapsulated in a membrane sac. Prolonged treatment of hair melanosomes with aqueous ammonia induced a complete destruction of characteristic melanosome morphology and a shrinking of the granule size, resulting in an amorphous material (Figure 8). By contrast, *Sepia* melanosomes under identical conditions of ammonia treatment did not show such changes. This might be due to differences such as metal content and the nature and type of metals present or differences in surface properties and aggregation behavior. Other alkalizing agents such as sodium hydroxide or sodium carbonate at pH 10 did not induce changes to melanosomes similar to those from aqueous ammonia. Thus, it is clear that ammonia-

induced morphological and structural changes to hair melanosomes are not merely due to alkaline pH, but, rather, specific to ammonia.

Hydrogen peroxide at neutral pH is found to be less efficient in bleaching hair melanosomes. However, TEM images of hair melanosomes treated with NH_3 and H_2O_2 revealed a complete distortion of the melanosomal structure immediately after treatment. The overall dissolution process is faster compared to independent treatments with either ammonia or peroxide. After 15 min of bleaching, the nanoparticles released from the melanosomes had undergone a complete dissolution, and at the end of 30 min, a clear solution devoid of any particles was obtained. We believe that ammonia as a neutral species can diffuse across the melanosomal membrane and that, by a still unidentified mechanism, facilitate the rupture of the melanosomal membrane. Possible ways include an increase in intra-melanosomal osmotic pressure and/or inducing membrane protein conformational changes. TEM data clearly suggest that ammonia helps to release melanin nanoparticles from the melanosomes, making them more susceptible to oxidative attack by H_2O_2. This effect of ammonia does not preclude the likelihood that ammonia may mobilize or chelate transition metal ions present in hair melanosomes, facilitating the peroxide-induced bleaching.

CONCLUSION

In summary, the TEM data presented here provide direct evidence for the structural and morphological changes undergone by isolated melanosomes as a result of ammonia and ammonia/peroxide treatments. Our study clearly shows that ammonia has a specific role in the bleaching process, unlike other alkalizing agents, and that it facilitates the peroxide-induced bleaching of melanosomes.

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