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Ultrasonic Measurement of Melanosomes for Characterization of Their Physicochemical Structure in B16 and Harding-Passey Melanomas[1]

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ABSTRACT

Ultrasonic measurement of the two different forms of melanosomes was carried out at 20 to 300 MHz on B16 and Harding-Passey mouse melanomas which produce ellipsoidal-lamellar and spherical-granular melanosomes, respectively. We found that the structure of the two forms is basically amorphous and copolymeric in the molecular dimension of a segment composed of 5 to 6 zigzag units. A marked difference in particle wave resonance was found around 200 MHz in the two melanosomes. Based on the chemical structure of melanin proposed by Hempel, it was indicated that the physical structure of Harding-Passey melanosomes is a copolymer in which melanin and protein moieties run parallel to each other but may bind together at the sites of planar groups, while that of B16 melanosomes is a double-helix polymer of melanin and protein moieties with a screw symmetry of $N = 6$. This type of one-dimensional cyclic ordering, commonly known as the Born-Karman periodic boundary condition in semiconductive band theory, may be related to the formation of the lamellar structure seen in B16 melanosomes.

INTRODUCTION

Synthesis of melanin and melanosomes is a unique biological property which can be a potential tool for development of rational approaches for diagnosis and treatment of malignant melanoma (9). In this regard, B16 and HP[3] mouse melanomas have been most commonly used as experimental models, inasmuch as B16 melanosomes are ellipsoidal-lamellar and brown-black, while HP melanosomes are spherical-granular and reddish brown. In our previous biochemical studies (6–8), we have shown that (*a*) B16 and HP melanosomes possess tyrosinase identical in molecular weight and common to antigenic site; (*b*) the contents of lipid, which is responsible for the functional differentiation of melanosomes, are significantly different in the 2 melanosomes; and (*c*) the size and number of polypeptide proteins responsible for structural differentiation of melanosomes are similar, although their relative contents are significantly different. Furthermore, our chemical and physical studies (4, 5, 9) have indicated that (*a*) B16 and HP melanosomes primarily consist of eumelanin, the amount of which is, however, markedly different in the 2 melanosomes, and (*b*) the spectra of electron spin resonance, IR, and X-ray small-angle scattering are basically similar between them.

Extending our previous studies (11),[4] this study clarifies the physical structure of B16 and HP melanosomes by ultrasonic measurement at frequencies from 20 to 300 MHz as compared with those of sepia melanosomes, which are known as "pure" natural eumelanosomes. It will be shown that the structure of B16 and HP melanosomes is basically amorphous and copolymeric in the molecular dimension of a segment composed of 5 to 6 zigzag units and that a marked difference of particle wave resonance (10) is present around 200 MHz in the 2 melanosomes.

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[3] The abbreviation used is: HP, Harding-Passey.

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MATERIALS AND METHODS

Preparation of Melanosomes. B16 and HP melanomas were grown in C57BL/6 and ICR mice, respectively. The details of melanoma preparation and melanosome isolation followed our previously described methods with a minor modification (6, 7). The isolated melanosomes were treated with Brij-35 in 4 mM phosphate buffer by an ACE tissue homogenizer (Nihon Seiki Kaisha, Ltd., Japan) at 15,000 rpm for 3 min in ice and then underwent sucrose density gradient ultracentrifugation (1.0 to 2.0 M sucrose) to remove external contaminants as reported previously (7). The purity of melanosomes was checked by electron microscopy after fixing the melanosome pellets with 1% osmium tetroxide and was described in our previous report (9). The purified melanosomes were washed extensively with distilled water and lyophilized.

The sepia melanosomes were collected from the ink sac of a cephalopod, *Sepia* (*Acanthosepion*) *subaculecta* Sasaki, kindly supplied by Dr. Y. Fujinuma (Shiseido Cosmetic Co., Yokohama, Japan).

Apparatus for Analysis. An apparatus consisting of an ultrasonic pulse generator and a receiver (Matec, Inc.), which generated ultrasonic sound from 1 to 300 MHz, was used for the entire experiment and is schematically shown in Chart 1. For longitudinal wave measurement, electrical oscillations were fed to the transmitting crystal of the pulse generator which generated a pulsed ultrasonic wave that traveled into the suspension. This wave was picked up by the receiving crystal of the receiver, and the resultant signal was amplified and fed into a synchroscope. When the path of the beam was changed, the wave pattern on the synchroscope altered in amplitude, and attenuation against the path length in the melanosomal suspension was obtained.

Ultrasonic Measurement. Ultrasonic measurement was carried out at frequencies between 20 and 300 MHz. Sound absorptions of the melanosomes changed slowly after water was added to the lyophilized materials, and it usually required about 24 hr to equilibrate. Thus, measurement was made a few days after the melanosomes were suspended. A conventional pulse method was used to measure the sound velocity of the suspension (V) and the attenuation coefficient α at a fundamental frequency of 1 MHz as well as at higher harmonics up to the 321st, with a continuous stirring to avoid settling of the melanosomes by gravity.

RESULTS

Sound Attenuation in Melanosomes: In a dilute suspension of the melanosomes, α was shown in the form of

$$\alpha = \alpha_1 + \alpha' \quad \text{(A)}$$

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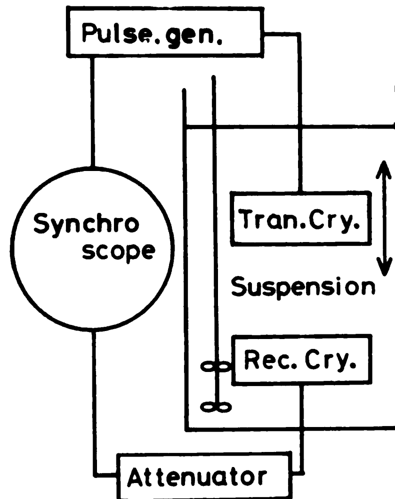

Chart 1. Schematic drawing of an ultrasonic pulse-generator (*Pulse. gen.*) and a receiver which generated ultrasonic sound from 1 to 300 MHz. *Tra. Cry.*, transmitting crystal; *Rec. Cry.*, receiving crystal.

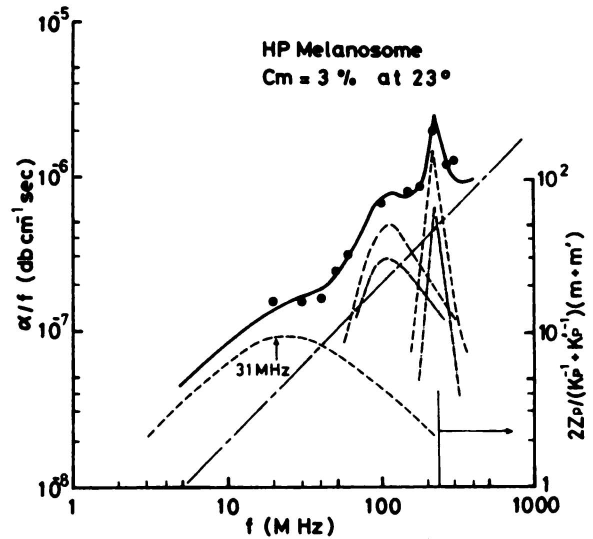

Chart 2. Longitudinal attenuation per frequency plotted against frequency for dilute suspension of HP melanosomes. ——, composite behavior based upon the relaxation and resonances using the parameters in Tables 1 and 2. - - -, sepia melanosomes.

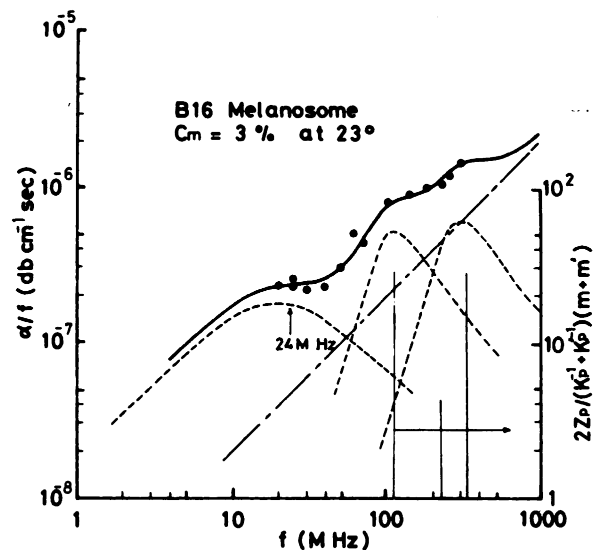

Chart 3. Longitudinal attenuation per frequency plotted against frequency for dilute suspension of B16 melanosomes. ——, composite behavior based upon the relaxation and resonances using the parameters in Table 1.

where α and α' were the attenuation coefficients of water and the melanosomes, respectively. It was found that absorptions below 300 MHz, referred to as scattering and viscous drag effects, were negligibly small. Noting that c is the volume fraction of the melanosomes in water, a ratio of α to the frequency of f was expressed in the form of (23)

$$\alpha'/f = cK''\pi/V^3 \quad \text{(B)}$$

where V was approximately equal to the sound velocity of solvent, and K'' was the imaginary part of the bulk modulus of the melanosomes.

Attenuations per frequency in a weight fraction of $c_w = 0.03$ for HP and B16 melanosomes, which were plotted against frequency in Charts 2 and 3, showed 3 peaks at 30, 100, and 220 MHz. Experimental errors in these measurements were less than 5%. The measured values of α_1/f^2 were 208×10^{-17} dB cm^{-1} sec at 23° and 400×10^{-17} at 3° and frequencies between 100 and 310 MHz, respectively. α_1/f was plotted as a thin chain line in Charts 2 and 3. The α/f curve was subtracted from the α_1/f curve and yielded 2 resonant components at 100 and 220 MHz for HP melanosomes. It was found that the ultrasonic spectra of HP melanosomes are very close to those of sepia melanosomes, which are composed of 3 terms: Term 1, a particle wave resonance at 220 MHz resulting from a stiff chain mode motion; Term 2, stacking mode resonance; and Term 3, the principal relaxation due to microbrownian motion in the main c-c bond at 56 MHz (13). The theoretical curve of α/f in terms of the additive contribution in a comparison of the 3 spectra corresponded well with the data of sepia melanosomes. It can thus be seen from Charts 2 and 3 that HP and B16 melanosomes may also be composed of the 3 spectra.

Analysis. Terms 1 and 2 resulted in a resonant equation (12), which could be shown in the form of

$$K'' = (1/\omega Kr_1)[(1Kr_0 - 1/\omega^2 Kr_2)^2 + (1/\omega Kr_1)^2]^{-1} \quad \text{(Ca)}$$

with a quality factor of

$$Q = 2\pi fr Kr_1/Kr_0 \quad \text{(Cb)}$$

where Kr_0 was the elastic constant associated with resonance, Kr_1 was the viscosity, Kr_2 was the inertial term, and ωr was a radian resonant frequency.

The Kr_0 was expressed as

$$Kr_0 = (\omega' K''_m/\omega r)(1 - \omega r/\omega'^2)(2\omega'/\omega r - 1)^{-1/2} \quad \text{(D)}$$

where K''_m was the maximum value of K'' at $\omega = \omega r$ and ω' was a radian frequency at $K''_m/2$. To adequately fit the data with Equation B using Equation Ca, empirical values were chosen and tabulated in Tables 1 and 2. The final subtraction presented a single relaxation curve.

Term 3 of the spectrum followed in the form of

$$K'' = Kr_0\omega\tau(1 + \omega^2\tau^2)^{-1} \quad \text{(E)}$$

with a single relaxation time of $\tau = (2\pi fc)^{-1}$. The 2 melanosomes around $fc = 30$ MHz followed Equation E, indicating that the

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Table 1

Parameters of particle wave resonance of stiff chain mode in HP and B16 melanosomes at 23° suspended in water with comparison to sepia melanosomes

	c_w	b_r (MHz)	cK_{r0} (dyne/sq cm × 10^8)	cK_{r1} (poise)	cK_{r2} (g/cm)	Q	Mode
Sepia	0.03	220	2.28	0.612	1.20×10^{-10}	3.7	Standing
HP melanosomes	0.03	220	3.05	1.22	1.60×10^{-10}	5.5	Standing
B16	0.03	333	8.83	0.302	2.02×10^{-8}	1.1	Running
		100	4.74	0.796	1.20×10^{-9}	1.1	Running
		220					Standing

Table 2

Parameters of particle wave resonance of stacking mode in HP and B16 melanosomes at 23° suspended in water with comparison to sepia melanosomes

	c_w	b_r (MHz)	cK_{r0} (dyne/sq cm × 10^8)	cK_{r1} (poise)	cK_{r2} (g/cm)	Q
Sepia	0.03	95	2.91	0.611	8.18×10^{-10}	1.0
HP melanosomes	0.03	100	3.98	0.772	1.00×10^{-9}	1.1
B16	—0.03	~100	Unmeasurable	Unmeasurable	Unmeasurable	Unmeasurable

principal relaxations of HP and B16 melanosomes are similar. From the present and previous observations,[4] it appears that the conformational structure of the 2 melanosomes is basically amorphous and copolymeric in the molecular dimension of a segment composed of 5 to 6 zigzag units. This linear structure in a short range was further examined in the particle wave resonance shown below.

Particle Wave Resonance in Stiff Chain Mode. Kono *et al.* (14) recently proposed an extension of Fitzgerald's theory (2) of particle wave resonance in the equation for 2 fixed end groups. The *fr* for the standing mode with respect to the stiff chain mode in the amorphous state was shown in the form of

$$fr = f_{qN} = f_0 \sin^2(q\pi/2N) \quad \text{(Fa)}$$

$$f_0 = h/2\pi^2(m + m')d^2 \quad \text{(Fb)}$$

where $q = 1, 2, 3, \ldots$ N-1, and the *fr* for the running mode was shown in the form of

$$fr = f_0 \sin^2(q\pi/N) \quad \text{(Fc)}$$

where $q = 1, 2, 3, \ldots N/2$.

The Kr_0 for the standing mode was expressed in the form of

$$Kr_0 = \omega^2 \rho d^2 N^2/q^2\pi^2 \quad \text{(Ga)}$$

$$= (m + m')K'_p/mm' \quad \text{(Gb)}$$

where m ($=M/N_A$) was the mass of the planar group in the main lattice, M was the molecular weight, N_A was Avogadro's number, m' was the mass of the water molecule, h was Planck's constant, K'_p was a force constant of the optical mode for the particle wave, q was the mode number of the stiff chain constant for the definite *fr*, $N = Z - 1$ and the integer Z ($\geqq 3$) were the numbers of planar group. A reciprocal Q was related to a half-width *fr* in the form of

$$1/Q = \Delta fr/fr = \Delta[(m + m')d^2]/(m + m')d^2 \quad \text{(H)}$$

A distribution function of the mode for the frequency was shown by the form of

$$Zp(f) = N(Kp + K'p)(m + m')/[2K_pK'_p \sin(q\pi/2n)\cos(q\pi/2N)] \quad \text{(I)}$$

where K_p was the force constant for the acoustic mode. It was found that $Zp(f)$ possesses a maximum at $q = N - 1$, indicating that the particle wave resonated markedly at the highest mode. $Zp(f)$ was therefore proportional to the maximum value of α'/f by Equations B and D. When $(m + m')d^2$ was distributed with a constant N, the observable *fr* was expressed in the form of

$$fr = (h/2\pi^2)\sin^2(q\pi/2N) \cdot \sum[1/(m + m')_i d_i^2]/\sum N_i \quad \text{(J)}$$

where N was the number of species.

It can be seen in Table 1 that the *fr* and the Q for HP melanosomes are the same as those for sepia melanosomes, indicating that the physical structure in the short range is identical between the 2 melanosomes through the equality in N (=2) and, hence, in $\sum(m + m')_i d_i^2$ and $\sum\Delta(m + m')_i/(m + m')_i$. It was thus assumed that the structure of HP melanosomes may be a linear copolymer in which melanin and protein moieties run parallel to each other but in which they may bind together at the sites of every other group. Both the melanin and protein moieties should be the rigid molecules responsible for the stiff chain mode motion. To test this assumption, the values of $(m + m')_i d_i^2$ were evaluated according to the chemical structure of melanoma melanin proposed by Hempel (3). Assuming $m' = 3\ H_2O$, *i.e.*, one water molecule associated with $-COOH$ and 2 water molecules acting as plasticizers, Equation F indicated a value of $fr = 220$ MHz, which was completely consistent with the data shown in Table 1. If the torsion angle of the planar groups against the main axis is identical at every sixth group, which means ($N = 6$), the running mode takes place and results in $f_{qN} = 332$ MHz, at $q = 2$ and 110 MHz at $q = 1$ with $Q = 1.4$, as calculated from Equation F. Consistency with the data in Table 1 was well within experimental accuracy.

The present model was further examined in a different manner. The relative intensity of $N/\sin(q\pi/2N)\cos(q\pi/2N)$ was plotted as the solid lines in Charts 2 and 3. The proportionality to the maximum value of α'/f, which was equivalent to Kr_0 in Equation D, was generally plausible. On the other hand, the Q of HP melanosomes was much higher than the calculated value of Q = 1.4. This discrepancy may arise from the small quantity of melanin involved in HP melanosomes. The value of *fr* was basically independent of the concentration changes in the planar groups because the melanin and protein moieties had the same fr_0. In contrast, the small number of planar groups in the melanin moiety might suppress the width of the melanosomes, since the protein moiety exhibited a very narrow width. Roughly speaking, this suppression might be proportional to the decrease in the

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quantity of groups contained. In our previous study (8), it was indicated that the melanin content in HP melanosomes was about one-third of that in B16 melanosomes. Then $\Delta(m + m')/3$ in Equation H became $Q = 4.2$.

Particle Wave Resonance in Stacking Mode. Our previous study of X-ray small-angle scattering in HP and B16 melanosomes (8) showed distinct peaks which appeared to result from differences in the stacking profiles of the indol rings or analogous ring substances in the chemical bindings of melanin with structural protein moieties. The present acoustic data presented a similar development in this regard. The resonant frequency at 100 MHz indicated the number of stacking layers in the planar groups in HP melanosomes. In this case, Equations F and G may be applied, but with a different sense of the parameters, *i.e.*, m is the mass of the segment composed of several monomer units involving structural protein moiety, m' is the mass of the associated molecule, d is the distance between parallel layers, and Z is the number of segments to be stacked. Putting $Z = 2.9$ and $d = 4.5$ Å, obtained through the previous study of X-ray scattering (17), into Equation Fa resulted in $f_0 = 200$ MHz. Equation Fb was the molecular weight of the segment $M + M' = 500$. The $Z = 1.5$ of B16 melanosomes was too small to account for the stacking profile. Actually, the running mode contributed to the absorption as indicated above (Table 2).

DISCUSSION

The physicochemical structure of melanin and melanosomes in malignant melanoma still remains unsettled because no single physical method has led to anything approaching a complete understanding of their detailed structures. Furthermore, most of previous studies have been conducted on the structure of melanin. This study, therefore, appears to be the first to characterize the structure of melanosomes in malignant melanoma by ultrasound measurement.

Current concepts of melanin structure have been largely based on the analytical studies of Nicolaus (18) and the isotope tracer study of Hempel (3). Nicolaus (18), using degradative methods and chromatographic analysis, concluded that sepia melanin is not a homopolymer of indole quinone units linked through a single-bond type, as indicated by the classic Raper-Mason scheme of enzymic melanin formation from tyrosine and dopa (15, 20) but that it consists of several different types of monomer including uncyclized aromatic compounds and pyrrolic acids in addition to indoles. Nicolaus *et al.* (19) further proposed that melanin is linked to proteins within squid melanosomes by means of cystein units and that it consists of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid moieties at various oxidative levels. The detailed study of melanin structure using specifically labeled precursors which Swan (21) conducted *in vitro* was subsequently extended to HP melanoma cells by Hempel (3). The latter, in combination with Nicolaus' study, indicated that melanin is a copolymer of considerable complexity with several types of bonds being involved and proposed that the structure of melanoma melanin is a copolymer of dopa quinone, 5,6-indolequinone, and 5,6-indolequinone-2-carboxylic acid in a ratio of 3:2:1. At the same time, Blois *et al.* (1), on the basis of electron spin resonance studies, reached a similar conclusion that melanoma melanin is a highly irregular, 3-dimensional polymer of several types of monomers joined by different covalent bonds. In addition, X-ray diffraction studies of Thathachari (22) indicated that monomers of melanin are either planar molecules like catechol and indolequinone or molecules like dopa having planar portions. All of these previous studies, however, clarified only the structure of melanin, not the 3-dimensional structure of melanin and protein moieties, as having been done in the present study.

Ultrasonic measurement appears to be a useful means for studying the structure of melanin and melanosomes because of the general transmittivity of sound waves through suspended media and because of an unusually efficient resonant transfer mechanism for absorption of ultrasound by melanosomes (16). In our previous study (14), we first clarified the structure of synthetic diethylamine melanin because synthetic melanin can be polymerized without the lipid and protein fractions which are present in native melanosomes and which complicate the physical structure arising as a result of incorporation of even less-well-defined molecules. Our measurement of shear impedance at high frequencies resulted in the conclusion that a difference in the stacking of planar groups makes an essential difference in the mechanical properties of hydrated melanins. Our subsequent experiment of sepia melanin and melanosomes with comparison to hydrated diethylamine melanin indicated that sepia melanosomes possess an amorphous structure in which protein moieties are bound to a rod-like form of melanin and that the conformations of the protein moieties govern the secondary structure of the melanin moieties.[4] In addition, we found that the structure of sepia melanin is also amorphous in a middle range of ~50 Å, consisting of a rod-like rigid molecule in a short range of ~10 Å. This study, based on a comparison of sepia melanosomes and Hempel's chemical structure of HP melanoma-melanin, further clarified the structure of B16 and HP melanosomes. We found that the structure of HP melanosomes is a copolymer in which melanin and protein moieties run parallel to each other, but in which they may bind together at the sites of planar groups, while that of B16 melanosomes is a double-helix polymer of melanin and protein moieties with a screw symmetry of $N = 6$. This type of one-dimensional cyclic ordering, commonly known as the Born-Karman periodic boundary condition in semiconductive band theory, appeared to be related to the formation of the lamellar structure seen in B16 melanosomes. The above model may, however, need to be confirmed by other physical methods such as by means of X-ray high- or intermediate-angle scattering. Nevertheless, the application of ultrasonic measurement will provide not only a new modality for characterization of melanosome structure but also a deeper insight into the mechanism of the action of ultrasound which is currently being used for the treatment of malignant melanoma since it has the advantage of providing localized deep heating (hyperthermia) and efficient energy transduction to the melanosomes (16).

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