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# Modification of Membrane Fluidity in Melanin-Containing Cells by Low-Level Microwave Radiation

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The treatment of a B16 melanoma cell line with 2.45-GHz pulsed microwaves (10 mW/cm$^2$, 10-μs pulses at 100 pps, 1-h exposure; SAR, 0.2 W/kg) resulted in changes of membrane ordering as measured by EPR (electron paramagnetic resonance) reporter techniques. The changes reflected a shift from a more fluid-like phase to a more solid (ordered) state of the cell membrane. Exposure of artificially prepared liposomes that were reconstituted with melanin produced similar results. In contrast, neither B16 melanoma cells treated with 5-Bromo-2-Deoxyuridine (3 μg/day × 7 days) to render them amelanotic, nor liposomes prepared without melanin, exhibited the microwave-facilitated increase of ordering. Inhibition of the ordering was achieved by the use of superoxide dismutase (SOD), which strongly implicates oxygen radicals as a cause of the membrane changes. The data indicate that a significant, specific alteration of cell-membrane ordering followed microwave exposure. This alteration was unique to melanotic membranes and was due, at least in part, to the generation of oxygen radicals. 

**Key words: microwaves, cell membrane, order, melanin, oxygen radicals**

## INTRODUCTION

The role of melanin in inducing cell-membrane alterations, particularly in response to non-ionizing radiation, has been a question of great interest. Melanin is a ubiquitous polymeric pigment that occurs in membrane-bound organelles or melanosomes of epidermal cells and several cell types in the eye. The major biological function of melanin is thought to be its role in photoprotection from harmful ultraviolet radiation, but melanin may also become a source of damage if its protective capabilities are overloaded [Pathak et al., 1976].

Melanin can function as a redox polymer, and it forms a paramagnetic species that is detectable by electron paramagnetic resonance (EPR). In addition to the per-

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sistent free radicals contained in melanin, transient free radicals are formed in melanin by a variety of chemical reactions, as well as by exposure to ultraviolet and visible light [Sarna and Sealy, 1984; Sealy et al., 1984]. The photo-reactions of melanin have been an area of particular interest with regard to interactions with oxygen, or in melanin's production and subsequent reaction with superoxide anion radicals [Sarna et al., 1980; Felix et al., 1978]. Using electron paramagnetic resonance spin-trapping techniques, Felix et al. [1978] demonstrated the capability of melanin to scavenge oxygen during photo-irradiation along with a further reduction of oxygen to hydrogen peroxide via a one-electron reduction that yielded superoxide anion. Korytowski et al. [1986] investigated the reaction of superoxide anion with melanin by spin-trapping techniques, and they demonstrated the formation of a transient, melanin, free-radical species. Studies have been performed to examine the interaction of melanin with electromagnetic radiation at various wavelengths: ultrasonic, visible, and ultraviolet [Sarna and Sealy, 1984; Sealy et al., 1984].

Little information has been gathered to examine the interaction between microwave radiation and melanin or other pigment-containing biological systems. In related studies, investigators have applied microwave energy to melanotic cells in the hope of inducing a thermal toxicity specific to tumor cells. These studies commonly employ B16 melanoma cell lines and evaluations of cell development [Santini et al., 1988], and antigenicity [Santini et al., 1986]. However, studies have not been performed to investigate possible interactions of microwave energy with the membrane bilayer of melanin-containing cells.

Studies of microwave effects on membranes of amelanotic cell lines and liposomes have become controversial. Liburdy and Vanek [1985] demonstrated that microwave radiation (2,450 MHz) reversibly increased membrane permeability to $^{22}Na$ in rabbit erythrocytes at a temperature that coincides with an apparent membrane phase-transition temperature. Several free-radical modulators were found to exert an influence on this effect [Liburdy and Vanek, 1985]. Similar studies in liposomes demonstrated a microwave-induced enhancement of permeability to $^{22}Na$, at or near an apparent phase-transition temperature of the liposomal membrane [Liburdy and Magin, 1985]. Of interest is that addition of cholesterol to the liposomal membrane obliterated both the apparent phase-transition as well as the microwave induction of increased membrane permeability. Conversely, Rafferty and Knutson [1987] found no evidence of microwave-induced membrane alterations in liposomes of the same composition, but exposed at a different microwave frequency (0.93 GHz). Liu and Cleary [1988] further investigated the possibility of frequency-dependent alterations in liposome permeability. They demonstrated that differences between the results of Rafferty and Knutson [1987] and those of Liburdy and Magin [1985] were not due to differences in microwave frequency. They concluded that differences in protocols of microwave exposure may have accounted for the discrepancies between the studies. Further investigations must be performed to determine the factors that are pivotal in causing divergent results.

Our investigations centered on structural changes in membranes that are produced by low-level microwaves, which may be unique to melanin-containing cells, or to artificially prepared liposomes containing melanin. These membrane changes were assessed by EPR reporter techniques. With a series of EPR doxyl stearate probes, differing in the attachment site of the doxyl reporter group to the fatty acid (stearic acid), various locations within the membrane can be examined. The stearic acid acyl

chain of the reporter orients itself in membranes parallel to the phospholipid fatty acids. Varying the position of the doxyl substituent (reporter group) on the fatty-acid chain allows for the examination of various depths of the membrane. Order parameters ($S_{\parallel}$) calculated from the spectral characteristics of the reporters are an indication of membrane fluidity or order. Changes in membrane order have previously been correlated with alterations in protein function [Sinensky et al., 1979] and lipid peroxidation [Bruch and Thayor, 1983]. We report data that indicate a depth-dependent membrane interaction with microwave radiation that may be unique to melanotic cells, and that appears, in part, to be due to the generation of superoxide anions.

## MATERIALS AND METHODS

### Culture of B16 Melanoma Cell Line

The B16 cell line, line BL6 (DCT Tumor repository, Frederick, MD) originally isolated from C57BL/6 mice as described by Fidler [1973], was maintained as attached cells grown in culture. The melanoma cells were maintained on Minimal Essential Medium (MEM with Earles salts) containing 10% fetal bovine serum (MEM/10); at confluence they were passaged with 0.1% bovine pancreatic trypsin (Sigma) and maintained in T75 cell-culture flasks (Corning) as a cell culture with MEM/10 containing pen/strep under standard conditions (95% air, 5% $CO_2$, 37°C, 90% relative humidity). All tissue-culture-media reagents and PBS (phosphate buffered saline) were acquired from Gibco (Grand Island, NY), and serum was obtained from Sterile Systems (Logan, UT). Once grown to confluence or slightly subconfluent, melanoma cells were microwave- or sham-exposed (serum free). Following exposure, they were trypsin-quenched with fetal-calf serum, pelleted, washed with PBS (pH 7.4), and labeled with reporter agents as described in the EPR section below.

### Treatment of B16 Cells with BrdU to Produce Amelanotic Cells

B16 cells were made amelanotic by adding 3 μg of 5-Bromo-2-Deoxyuridine (BrdU, Sigma Chemical Co., St. Louis, MO) per ml of medium per day for 7 days. Medium was changed on days 3, 5, and 7 after washing the cell layer. B16 cells shed melanin and BrdU prevents melanin production, resulting in amelanotic cells in 7 days [Wrathall et al., 1973]. The cells were then handled like non-BrdU-treated B16 cells for microwave exposures and EPR reporter techniques.

### Liposome Preparation

Liposome vesicles reconstituted with melanin prepared from the persulfate oxidation of L-tyrosine (Sigma Chemical Co., St. Louis, MO) were formed by a modified procedure described by Van Rooijen and Van Nieuwmegen [1980]. A total of 100 mg of phosphatidylcholine was used. It was prepared from egg yolk (99% pure, Sigma Chemical Co., St. Louis, MO) and contained a variety of saturated and unsaturated acyl chains. To this phospholipid preparation 30% mol/mol cholesterol (Sigma Chemical Co.) was added. This mixture was suspended in 25 ml of chloroform/methanol 2:1 vol/vol and evaporated under vacuum in a 500-ml round-bottom flask on a rotary evaporator at 37°C for 2 hr. To this thin film, a 10-ml solution of melanin (1 mg/ml) in PBS (pH 7.4) was added and shaken vigorously for 15 min under nitrogen. The resulting suspension was sonicated under nitrogen for 1 min at

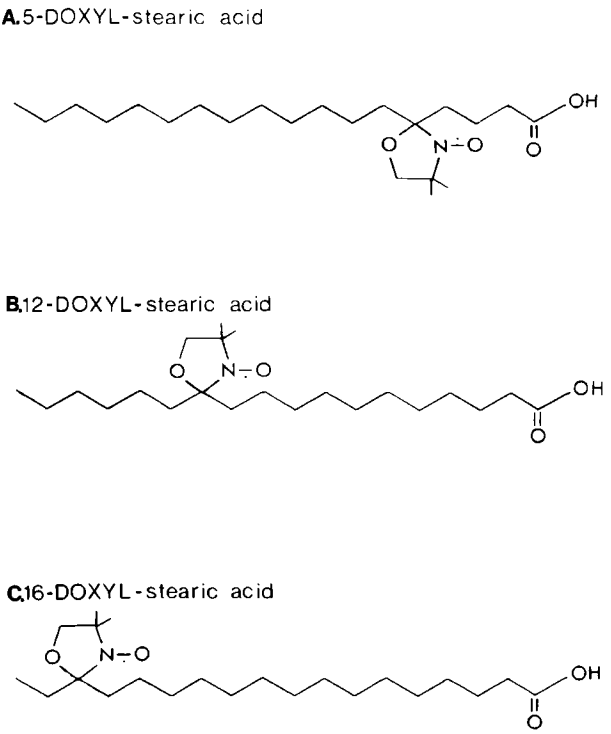


Fig. 1. Representative spin labels. **A:** 5-Doxyl stearic (5-DS) acid with paramagnetic nitroxide label on 5th carbon from carboxyl group (polar head group). **B:** 12-Doxyl stearic (12-DS) acid with nitroxide label on 12th carbon (middle of acyl chain) from carboxyl group. **C:** 16-Doxyl stearic (16-DS) acid with nitroxide label on 16th carbon (end of acyl chain) from carboxyl group.

4 °C by a Branson cell disruptor delivering 60 watts and equipped with a microprobe adapter. Remaining melanin not encapsulated inside the liposome was removed by centrifugation for 10 min at 1,000*g*. Empty liposomes (liposomes not containing melanin) were prepared identically with the exception of the melanin addition.

## Spin Labeling and EPR Measurements

Manipulations of B16 melanoma cells and liposomes for EPR fluidity studies were similar. Liposomes and B16 cells were placed or grown in 5-ml MEM (serum free) in T-75 cell-culture flasks and arranged in a suitable configuration in the microwave chamber as described below. Following microwave exposure, liposomes were removed and the B16 cells were trypsinized for removal. To these membranes were added 30 μM of a doxyl stearic (DS) acid reporter with a nitroxyl-labeled paramagnetic moiety at the 5, 12, or 16 carbon on the stearic acid chain, labeling from the polar head-group region to the terminal end of the acyl chain (Fig. 1). Following incubation with the reporter (10 min at 37 °C), the liposomes or cells were washed with PBS 3 times to remove unbound label (11,000*g*, 10 min). The final pellet was transferred into a 25-μl micropipet, sealed, and placed into a quartz tube. The sample was supported vertically and placed in the cavity of a Bruker ER 200D-SRC EPR spectrometer, in a central field with a density of 3,350 G (335 mT), a

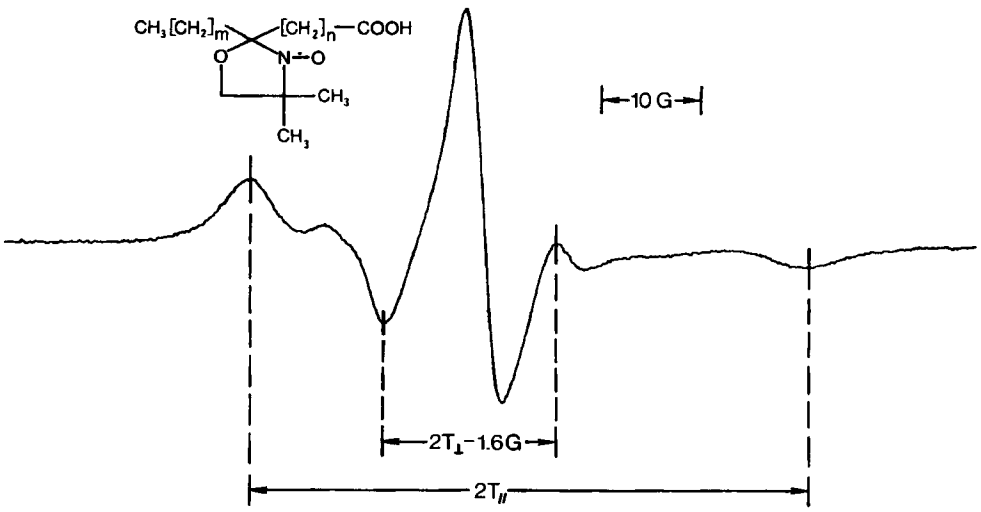


Fig. 2. Representative spectra of 5-doxyl stearic acid (5-DS) incorporated into a biological membrane. The figure demonstrates the spectral parameters $2T'_{\parallel}$ and $2T'_{\perp}$ which are used to calculate order parameters. In addition, the chemical structure of the doxyl stearic acids is shown with the following (M,N) values: 5-DS (12,3); 12-DS (5,10); and 16-DS (1,14).

sweep width of 100 G (10 mT), modulation frequency of 100 kHz, modulation amplitude of 2 Gpp (peak to peak), microwave power of 4.7 mWatts, time constant of 1.0 s, and a scan time of 1 ks. Order-parameter ($S_{\parallel}$ value) calculations were employed for the estimation of membrane fluidity. The spectral parameter $2T'_{\parallel}$ (Fig. 2) was employed to calculate $S_{\parallel}$ values. Computer-assisted signal averaging (IBM-PC with software (EPRDAS = 1.00 B/2.89) supplied by Adaptable Laboratory Software, Rochester, NY), curve smoothing, and amplifications were employed. The $S_{\parallel}$ values were calculated in accord with the technique of Sauerheber et al. [1977] and Gordon et al. [1983]. The order parameter calculations from the spectral characteristics are

$$S_{\parallel} = \frac{1}{2}\left[\frac{3(2T'_{\parallel} - T_{xx})}{(T_{zz} - T_{xx})} - 1\right].$$

$T_{xx}$ and $T_{zz}$ are splitting elements determined from host crystal studies. $T_{zz}$ and $T_{xx}$ splitting elements for 5- and 12-DS were taken from Seelig [1970]. Because $T_{xx}$ and $T_{zz}$ were not available for 16-DS, we utilized the splitting elements for 5-DS. Temperature was regulated by a nitrogen-controlled, variable temperature unit and spectra were obtained at 296 K.

Data presented are means of three or more samples for each group. Following spectral acquisition, order-parameter ($S_{\parallel}$) values were calculated according to the method described by Sauerheber et al. [1977]. $S_{\parallel}$ values allow for the expression of membrane fluidity independent of lateral phase separations and associated probe-probe interactions, which can be misconstrued as alterations in membrane fluidity. Briefly, values of $S_{\parallel}$ approaching unity indicate a completely hindered mobility (anisotropy) of the spin label in the membrane (Fig. 3e); in contrast, values approaching zero (isotropy) indicate the spin label tumbling freely in all dimensions in space (Fig. 3a). Those values between 0 and 1 (Fig. 3b–d) correspond to intermediate mobilities of the reporter molecules.

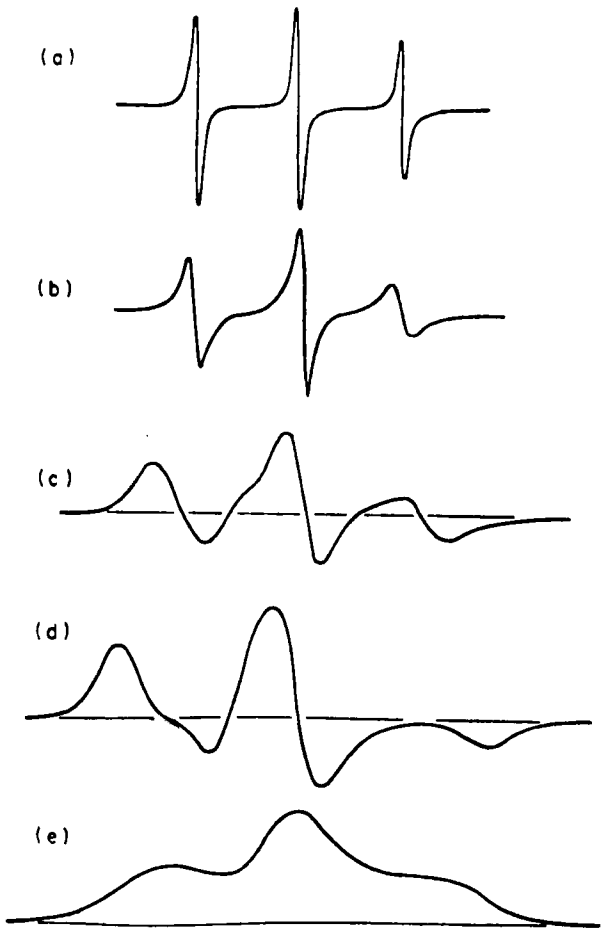


Fig. 3. Anisotopy of a nitroxide spin-label ESR spectrum under various conditions of motion. **a:** Isotropic spectrum from a nitroxide spin label randomly tumbling in a non-viscous solution. **b:** Lipid-dispersion spectrum from a nitroxide label undergoing anisotropic motion in a randomly oriented lipid dispersion. **c,d:** Lipid-dispersion spectra of a nitroxide label undergoing an increasingly hindered mobility. **e:** Powder spectrum from a nitroxide label randomly and rigidly oriented in a frozen solution (from Jones [1980]; printed with permission from Academic Press, Inc., New York).

## Microwave Exposure Protocol

Cell-culture flasks were placed on a Styrofoam platform in an anechoic chamber lined with AN-77 and SPY-12 absorbing material. The flasks were positioned with the medium's surface perpendicular to the direction of energy propagation. Pulsed microwave energy ($TE_{102}$) at 2.45-GHz (10 μs at 100 pps) was generated by a Epsco PH40K generator and transmitted through a ferrite isolator (LS110LCI) and an HP-360D low-bandpass filter (> 30 dB at 2.45 GHz). The microwaves were transmitted by coaxial cable (RG-214/U) through a bidirectional coupler (Narda-30V) that provided measures of forward and reflective power. Final coupling of microwaves was through a custom-designed horn antenna (Narda). The Sytrofoam platform containing the cell flask was positioned beyond the $2d^2/\lambda$-defined, far-field boundary.

The power-density of incident microwaves (10 $mW/cm^2$, rms) at the cell culture's media surface was determined by a calibrated, 2.45-GHz Narda 8201 electromagnetic monitor. A specific absorption rate (SAR) of 0.2 W/kg was calculated for the power density of 10 $mW/cm^2$ based on temperature measurements during microwave exposure with a non-perturbing Fluoroptic (1000B system) thermal probe [Durney et al., 1980].

Control (sham) culture flasks were placed in the anechoic chamber with the irradiated flasks during the 1-h exposure. The control flasks, however, were shielded from the microwave field by AN-77 microwave absorbing material. To exclude the possibility of a temperature-only effect, several 4-h exposures were conducted in which a fluoroptic 1000B temperature measurement system was used to monitor both the exposed and non-exposed cell flasks, as well as the internal temperature of the anechoic chamber. The difference between exposed and non-exposed flasks was within ± 0.2 °C during exposures. Although sham-exposed flasks examined with identical EPR parameters were used as controls, it should be noted that magnetic-field interactions from the EPR spectrometer cannot fully be ruled out as a component in these experimental results. Studies that permit comparison of results from EPR probes and fluorescent probes could be performed to rule out possible magnetic/microwave interactions. One drawback in the use of fluorescent probes for membrane-order determinations is the lack of depth-dependent probes, thus eliminating the evaluation of regional membrane alterations with these probes.

## STATISTICS

Group comparisons conducted by Student's t-test or ANOVA, where significant differences were obtained for $P < .05$ [Steel and Torrie, 1980].

## RESULTS

Figure 4 shows that microwave irradiation of B16 melanoma cells significantly altered membrane characteristics reported by two of the three reporters that were used to measure membrane fluidity. The 5-doxyl stearic acid probe, which sampled the polar head-group region, reported a 5–10% increase in membrane order following microwave radiation that was 90% inhibitable by the addition of superoxide dismutase (SOD, 1,500 units/ml). Similarly, the 12-doxyl stearic acid reporter (sampling the middle-membrane region) reported a dramatic increase (> 20%) in membrane order following microwave exposure over the order of sham-exposed cells (Table 1). The application of SOD (1,500 units/ml) was unable to completely inhibit (~ 15%) the alteration in membrane order produced by microwave radiation. The inability of SOD to inhibit the membrane changes reported by 12-doxyl stearic acid may be related to the difficulty of SOD to cross biological membranes. If both extracellular and intercellular free radicals are being formed, then SOD will inhibit only the extracellular component. Of interest was the apparent inability of microwave radiation to alter membrane fluidity characteristics reported by 16-DS. This may be secondary to the relative absence of double bonds on acyl chains in this region, or a function of the high mobility of the acyl chains at this depth in the bilaminar leaflet.

Figure 5 demonstrates that B16 cells treated with 5-bromo-2-deoxyuridine to produce amelanotic cells showed results that contrast dramatically with those found

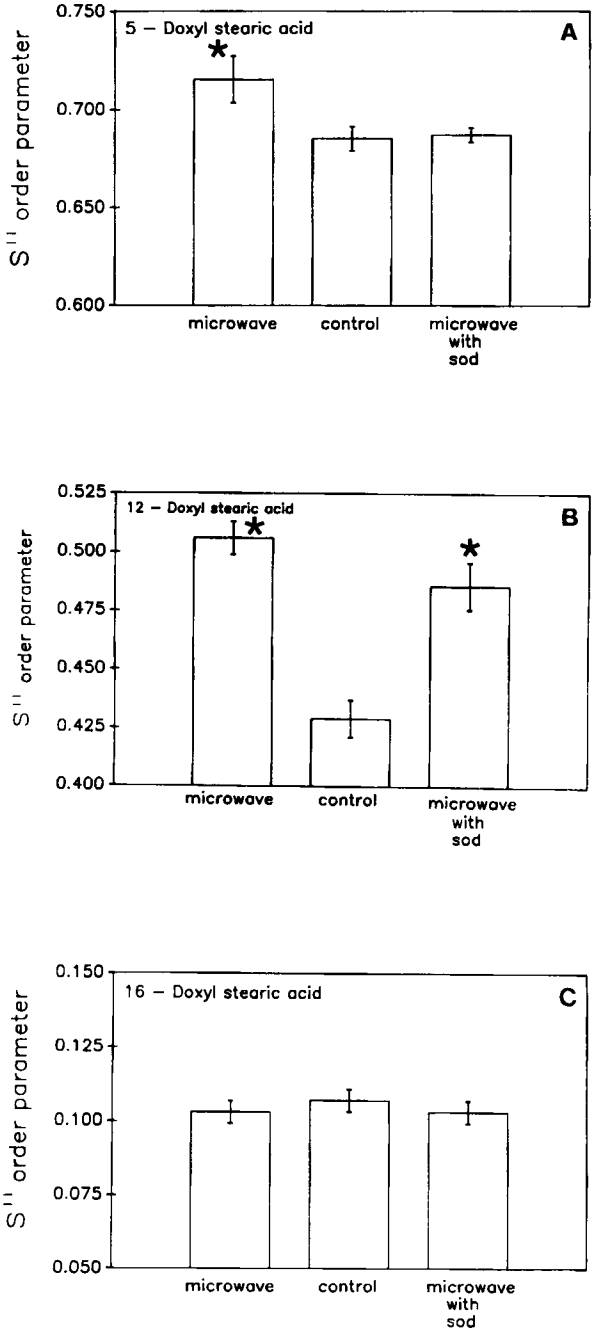


Fig. 4. The effect of microwave radiation [2.45 GHz, 10 mW/cm$^2$ (10-μs pulses, 100 pps), 1 h exposure] on membrane fluidity characteristics of B16 melanoma cells in the presence and absence of superoxide dismutase (SOD, 1,500 units/ml). **A:** Membrane fluidity reported by 5-DS, near the polar head-group region. **B:** Membrane fluidity reported by 12-DS, near the middle of the lipid acyl chains. **C:** Membrane fluidity reported by 16-DS, near the terminal region of the acyl chain. (*$P < .05$, ANOVA.)

**TABLE 1. Summary of Order Parameters ($S_{||}$) Following B16 Melanoma Microwave Irradiation [2.45 GHz, 10 mW/cm$^2$ (10-μs pulses, 100 pps); 1-h exposure]**

| | B16 melanoma cells—no BrdU treatment | | |
|---|---|---|---|
| Reporter | Sham exposed (N) | 10 mW/cm$^2$ × 1 h (N) | 10 mW/cm$^2$ × 1 h + SOD (N) |
| 5-doxyl stearate (5-DS) | .6839 ± .0063 (4) | .7153 ± .0118 (4)* | .6871 ± 0.037 (4) |
| 12-doxyl stearate (12-DS) | .4295 ± .0041 (4) | .4969 ± .0058 (4)* | .4858 ± .0145 (3)* |
| 16-doxyl stearate (16-DS) | .1032 ± .0038 (3) | .1072 ± .0038 (3) | .1033 ± .0038 (3) |

| | B16 melanoma cells treated with 5-bromo-2-deoxyuridine (BrdU), 3 μg/day × 7 days to produce amelanotic cells | |
|---|---|---|
| Reporter | Sham exposed (N) | 10 mW/cm$^2$ × 1 h (N) |
| 5-doxyl stearate (5-DS) | .6841 ± .0044 (5) | .6643 ± .0062 (4) |
| 12-doxyl stearate (12-DS) | .4214 ± .0242 (3) | .3932 ± .0244 (3) |
| 16-doxyl stearate (16-DS) | .0996 ± .0002 (3) | .1033 ± .0038 (3) |

*$P < .05$, compared to sham (ANOVA).

in melanotic cells. Microwave exposure [2.45 GHz, 10 mW/cm$^2$ (10-μs pulses, 100 pps), 1 h] caused a slight but insignificant decrease in order as reported by 5-DS and 12-DS. The observed effect indicates a mechanism different from that seen in the melanotic B16 cells (Table 1).

Figure 6 (Table 2) shows the effect of microwave irradiation (2.45 GHz, 10 mW/cm$^2$ (10-μs pulses, 100 pps), 1-h exposure) on artificial liposomal membranes reconstituted with melanin. Similar changes in membrane order were seen in this model as in the untreated B16 melanoma cells, with the exception of the 16-DS reporter. Both 5-DS and 12-DS reported an increase in membrane order following microwave exposure, with 12-DS showing the more significant effect. Conversely, the 16-DS reporter showed a small fluidization in the terminal end of the acyl chain. It is possible that peroxidation of the unsaturated bonds on the acyl chain located in the 5- and 12-DS region may provide an increase in rotational movement further down the acyl chain as reported by 16-DS.

Figure 7 (Table 2) presents the results on liposomal systems lacking melanin reconstitution. A small, fluidizing effect in the 5- and 12-DS reporter regions occurred, but no effect was seen in the 16-DS region of the amelanotic liposome membrane. As in the amelanotic B16 cell line, it appears that the membrane changes produced by microwave exposure in the amelanotic liposomes were different from those produced in melanotic liposomes.

## DISCUSSION

The results presented in this paper demonstrate the existence of a unique, field-induced alteration of membrane order associated with melanotic cells and with artificial membranes reconstituted with melanin. The membrane effects appeared to involve an interaction between relatively weak microwave fields and melanin. As we noted earlier, an interaction between the intense magnetic field produced by the EPR spectrometer and the melanotic end point cannot be ruled out as a factor in our experimental results. The mechanism(s) associated with these membrane alterations

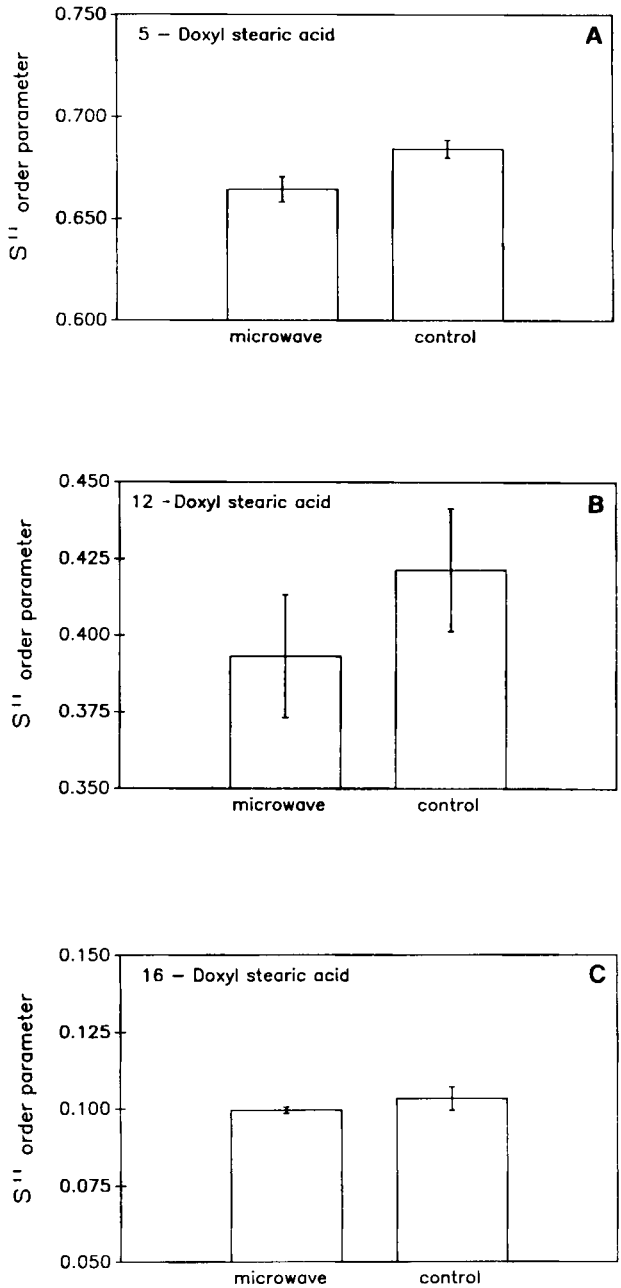


Fig. 5. The effect of the microwave radiation on membrane-fluidity characteristics of B16 melanoma cells treated with 5-bromo-2-deoxyuridine (BrdU), 3 μg/day × 7 days to produce amelanotic cells. **A:** Membrane fluidity reported by 5-DS near the polar head group region. **B:** Membrane fluidity reported by 12-DS near the middle of the lipid acyl chains. **C:** Membrane fluidity reported by 16-DS, near the terminal region of the acyl chain. (*$P < .05$, ANOVA.)

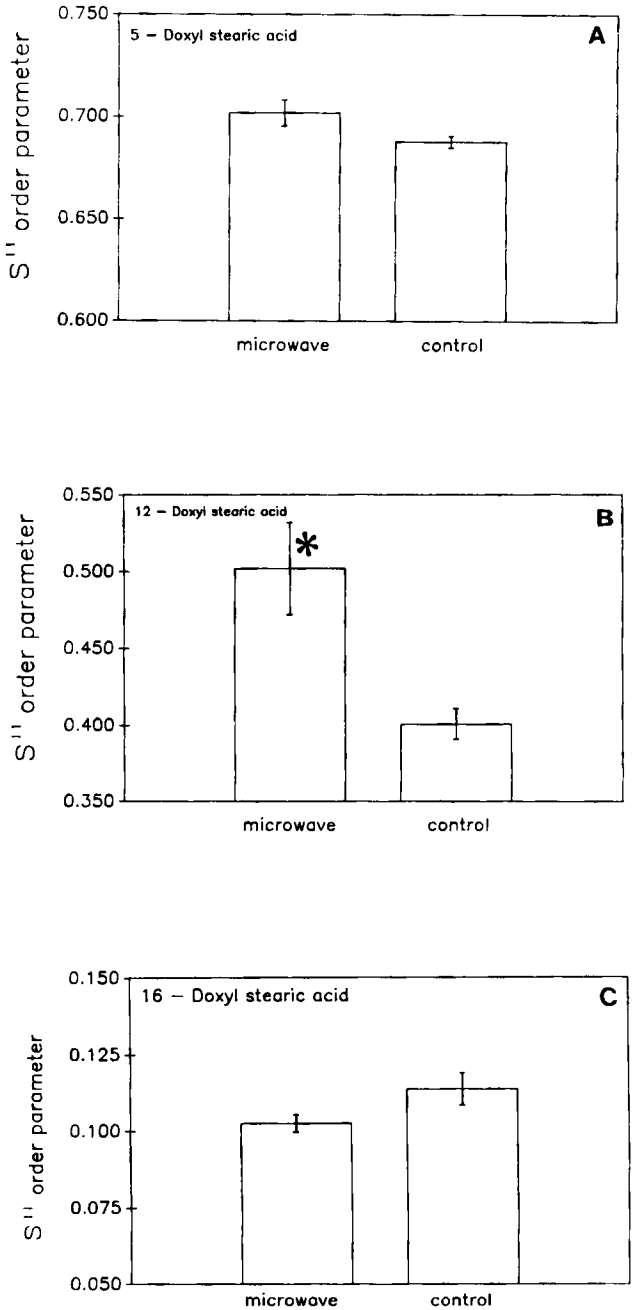


Fig. 6. The effect of the microwave radiation on membrane-fluidity characteristics of phosphatidylcholine/cholesterol liposomes reconstituted *with* melanin. **A:** Membrane fluidity reported by 5-DS, near the polar head-group region. **B:** Membrane fluidity reported by 12-DS, near the middle of the lipid acyl chains. **C:** Membrane fluidity reported by 16-DS, near the terminal region of the acyl chains. (*$P < .05$, ANOVA.)

**TABLE 2. Summary of Order Parameter ($S_{||}$) Following Microwave Irradiation [2.45 GHz, 10 mW/cm$^2$ (10-μs pulses, 100 pps), 1-h exposure] of Egg Lecithin Liposomes With or Without Reconstituted Melanin (1 mg/ml)**

| Egg lecithin liposomes reconstituted with melanin (1 mg/ml) | | |
|---|---|---|
| Reporter | Sham exposed (N) | 10 mW/cm$^2$ × 1 h (N) |
| 5-doxyl stearate (5-DS) | .6878 ± .0028 (4) | .7018 ± .0064 (4) |
| 12-doxyl stearate (12-DS) | .4008 ± .0103 (3) | .5020 ± .0426 (3)* |
| 16-doxyl stearate (16-DS) | .1025 ± .0028 (4) | .1137 ± .0053 (4) |
| **Egg lecithin liposomes—no melanin** | | |
| Reporter | Sham exposed (N) | 10 mW/cm$^2$ × 1 h (N) |
| 5-doxyl stearate (5-DS) | .6713 ± .0027 (3) | .6559 ± .0098 (3) |
| 12-doxyl stearate (12-DS) | .4035 ± .0060 (3) | .3825 ± .0088 (3) |
| 16-doxyl stearate (16-DS) | .1054 ± .0056 (3) | .1067 ± .0043 (3) |

*$P < .05$, compared to sham (ANOVA).

is (are) unclear. The evidence indicates the involvement of oxygen radicals, because superoxide dismutase inhibited field-induced effects. Treatment of the B16 melanoma cell line with an agent shown previously to produce amelanotic cells [Wrathall et al., 1973] completely abolished the effects seen in the B16 melanotic cell line, which strongly supports the involvement of melanin in these reactions. Artificial liposomes reconstituted with melanin showed similar alterations in bilayer structure following microwave exposure, but these effects were not duplicated in liposomes devoid of melanin.

The photochemistry and photobiology of melanin in relation to its involvement with free radicals have been extensively studied. Several investigators have related melanin to free-radical production [Felix et al., 1978, 1979], as well as to its ability to act as a free-radical scavenger [Goodchild et al., 1981]. Evidence has been obtained to demonstrate the production of superoxide during ultraviolet radiation of melanin systems [Persad et al., 1983], as well as documenting melanin free-radical scavenging properties [Korytowski et al., 1986]. Reactive oxygen species formed from the interaction of melanin systems with radiation may subsequently produce membrane alterations via lipid peroxidation [Kirsch et al., 1987; Bruch and Thayor, 1983; Phelan and Lange, 1991]. In our system, microwave radiation has been shown to produce an ordering effect in membranes of melanin-containing cells or liposomes. Inhibition of these ordering effects by superoxide (SOD) in the melanotic membranes following microwave exposure strongly indicates the involvement of oxygen-free radicals in these reactions. Oxygen-free radicals have been implicated in membrane lipid-peroxidation reactions associated with membrane ordering [Bruch and Thayor, 1983; Phelan and Lange, 1991]. The superoxide scavenger (SOD) has also been shown to inhibit the formation of lipid peroxidation product malondialdehyde (MDA) following ischemia–reperfusion reactions [Kirsch et al., 1987].

The membrane alterations seen in our melanin systems are similar to those found by Bruch and Thayor [1983] in non-melanin liposomal systems undergoing lipid-peroxidation reactions. Using similar EPR reporting techniques, Bruch and Thayor [1983] demonstrated that the region reported by 12-doxyl stearic acid (12-DS) was uniquely sensitive to lipid peroxidation reactions, most likely due to the abun-

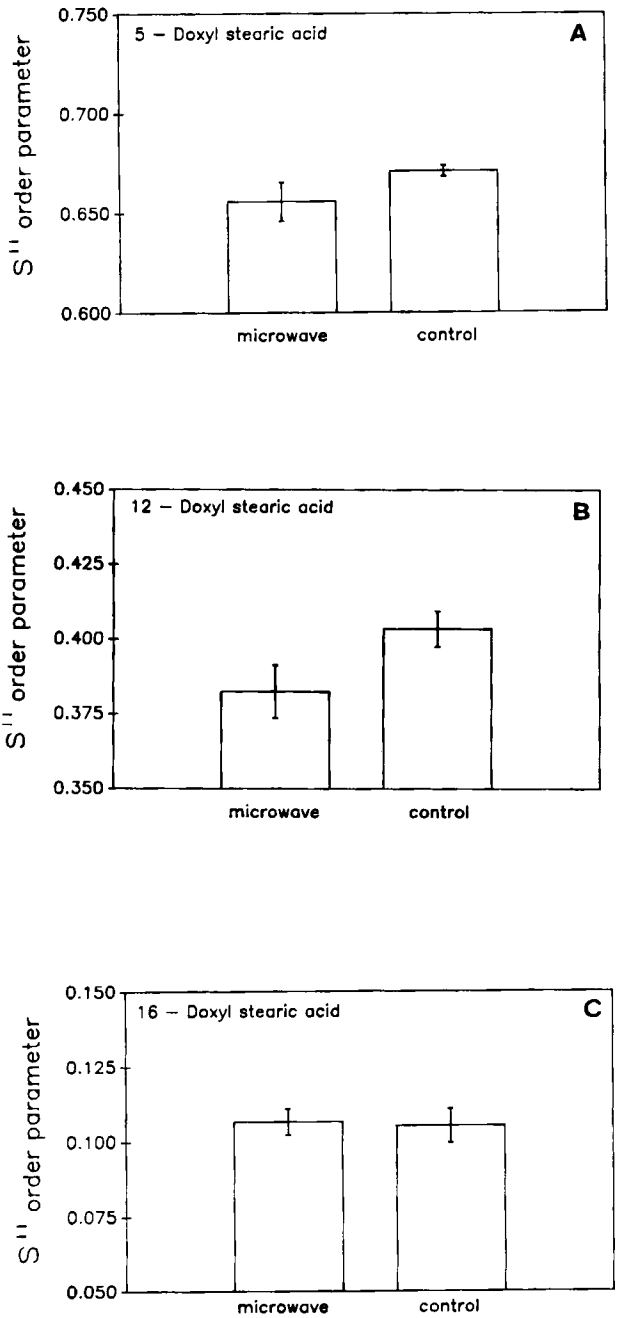


Fig. 7. The effect of microwave radiation on membrane-fluidity characteristics of phosphatidylcholine/cholesterol liposomes reconstituted *without* melanin. **A:** Membrane-fluidity characteristics of phosphatidylcholine/fluidity reported by 5-DS, near the head-group region. **B:** Membrane fluidity reported by 12-DS, near the middle of the lipid-acyl chains. **C:** Membrane fluidity reported by 16-DS, near the terminal region of the acyl chains. (*$P < .05$, ANOVA.)

dance of unsaturation in that area. Our results demonstrate a 25% increase in order in the region reported by 12-DS that may be related to lipid peroxidation. The increase in order found in this region correlated well with the degree of peroxidation found in Bruch's system. Further studies need to be performed to directly relate microwave-induced membrane ordering to lipid peroxidation in melanin-containing systems. This degree of membrane ordering in the 12-DS region is similar during active peroxidation, in liposomes producing between 15 and 20 nMole of malondialdehyde/mg lipid [Bruch and Thayor, 1983]. Changes in membrane order have been shown to alter protein function of integral proteins such as ATPase [Sinensky et al., 1979]. Thus, it is clear that the degree of membrane ordering (rigidification) produced can significantly affect biological systems.

The mechanisms by which melanin produces phototoxic reactions *in vivo* are still not well understood. Future studies need to be performed to determine the involvement of melanin's intermediates and metabolites in these reactions. Several biologically active derivatives and intermediates are formed during the biosynthesis of melanin [Koch and Chedekel, 1987; Graham et al., 1978]. Melanin contained in the subcellular melanosome of melanocytes is only one possible reactant in a series of precursors and metabolites found during melanogenesis. The autoxidation and photolysis of catechols during melanogenesis produce a number of highly reactive, free-radical species that have been demonstrated to form other free-radical species, including superoxide anion, hydroxyl radicals (via Harber Weiss or Fenton reactions), as well as semiquionones and indolyl nitrogen-based radicals [Chedekel et al., 1984; Kalyanaramah et al., 1982]. These intermediates and metabolites are shed actively by these cells, and many of them have been documented as photochemically unstable and products of free radicals [Koch and Chedekel, 1987; Rorsman et al., 1973]. The oxygen-free radicals produced by photolysis have been demonstrated to initiate lipid peroxidation, DNA strand breakage, and protein damage [Kirsch et al., 1987; Bruch and Thayor, 1983; Mason, 1979; Miranda et al., 1984].

Our data demonstrate the ability of microwave radiation to uniquely interact with melanin-containing cells and to produce membrane-order changes associated with the production of free radicals. There are several reaction schemes that could be hypothesized to account for these observations. Although melanin has previously been shown to scavenge free radicals, microwave interaction with this polymer may change its structure or excited state in a way that allows it to combine with oxygen to produce superoxide radicals. Alternatively, microwaves may directly or indirectly reduce oxygen to produce superoxide, which may interact with melanin to produce the membrane alterations seen. Membrane order can have biological implications with regard to alterations in protein function and permeability characteristics associated with normal physiological function. Work is currently in progress to evaluate protein alterations associated with these changes, in addition to extending these studies to *in vivo* evaluations of microwave interaction with highly melanotic tissues (i.e., iris, retina, and pigment epithelium). Future studies will involve investigations to determine what rôle these various components play in microwave-induced alterations of melanotic cell membranes, as well as in surrounding amelanotic tissue. These studies may aid in the explanation of changes observed in other membranes. Our findings support theories purporting the involvement of melanin in radiation-induced, free-radical formation, but further extend this theory to energies found in the low-level range of microwave energy.

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