

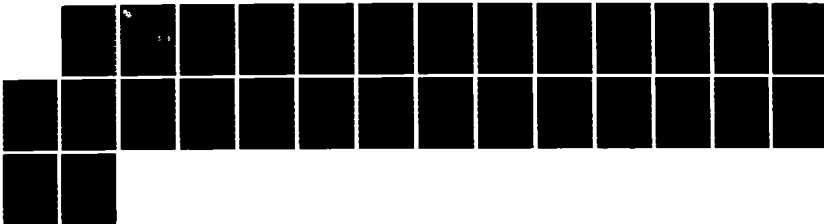
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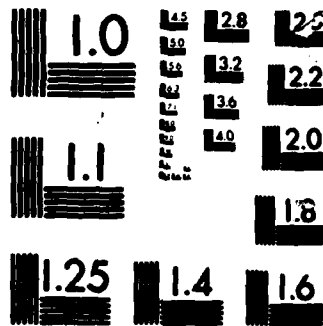
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INSTITUTE REPORT NO. 216

MELANIN. THE EFFECTS OF DIMETHYL SULFOXIDE ON THE SPECTRAL PROPERTIES

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LETTERMAN ARMY INSTITUTE OF RESEARCH
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Melanin. The Effects of Dimethyl Sulfoxide on the Spectral Properties
--Cooper et al

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Institute Report No. 216	2. GOVT ACCESSION NO. A168617	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Melanin. The Effects of Dimethyl Sulfoxide on the Spectral Properties	5. TYPE OF REPORT & PERIOD COVERED Final Report, 1985-1986	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Thomas M. Cooper, PhD, CPT, MSC; Steven T. Schuschereba, MA; Richard D. O'Connor, MS; David L. Bolton, PhD; David J. Lund, BS	8. CONTRACT OR GRANT NUMBER(s)	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Letterman Army Institute of Research, Division of Ocular Hazards and Comparative Medicine & Toxicol. Presidio of San Francisco, CA 94129-6800	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Project No. 3M161102BS10 WU 245, Physiological Basis of Laser Effects	
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research & Development Command Fort Detrick, Frederick, MD 21701	12. REPORT DATE January, 1986	13. NUMBER OF PAGES 27
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Melanin; Dimethyl Sulfoxide; Spectroscopy; Fluorescence		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Investigation of the spectroscopic properties of melanin dissolved in dimethyl sulfoxide (DMSO) is one part of a more expansive study of melanin. The absorption spectrum of melanin dissolved in DMSO differed from the spectrum in acidic or neutral solution, but was similar to the spectrum when melanin was dissolved in a basic solution. All absorption spectra fit to an amorphous semiconductor model. Infrared absorption spectra of melanin dissolved in DMSO and melanin suspended in a potassium bromide pellet were collected. Changes in the infrared spectrum were observed upon dissolution into DMSO. These changes had		

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spectral features of melanin that had been previously dissolved in basic solution and dried. Fluorescence spectra of melanin when excited at 310 nm in aqueous suspension differed from those collected in 10% (v/v) DMSO. In the presence of DMSO, the fluorescence was quenched by a factor of 4, and the fluorescence maximum shifted from 435 nm to 450 nm. Compared to the fluorescence properties of the water-inaccessible fraction of chromophores obtained from an iodide quenching experiment (fluorescence maximum at 415 nm), DMSO lowers the energy of the excited state more than water.

The results indicate that DMSO alters the micro-environment of the chromophore by changing its acid-base properties and altering the solvation of its excited state, perhaps through the formation of a hydrogen bond to melanin.

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ABSTRACT

Investigation of the spectroscopic properties of melanin dissolved in dimethyl sulfoxide (DMSO) is one part of a more expansive study of melanin. The absorption spectrum of melanin dissolved in DMSO differed from the spectrum in acidic or neutral solution, but was similar to the spectrum when melanin was dissolved in a basic solution. All absorption spectra fit to an amorphous semiconductor model. Infrared absorption spectra of melanin dissolved in DMSO and melanin suspended in a potassium bromide pellet were collected. Changes in the infrared spectrum were observed upon dissolution into DMSO. These changes had spectral features of melanin that had been previously dissolved in basic solution and dried. Fluorescence spectra of melanin when excited at 310 nm in aqueous suspension differed from those collected in 10% (v/v) DMSO. In the presence of DMSO, the fluorescence was quenched by a factor of 4, and the fluorescence maximum shifted from 435 nm to 450 nm. Compared to the fluorescent properties of the water-inaccessible fraction of chromophores obtained from an iodide quenching experiment (fluorescence maximum of 415 nm), DMSO lowers the energy of the excited state more than water.

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PREFACE

The authors thank Brian Pierce for useful suggestions and molecular orbital calculations on indole-quinone; Michael McCreery and Aaron Lewis for assistance in the interpretation of the spectral data; Ms. Christine L. Noah-Cooper for stimulating and useful discussions; Ms. Lottie B. Applewhite for editorial and technical assistance in preparing the manuscript.

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Spectral Effects of Dimethyl Sulfoxide on Melanin

Melanins are complex polymers found in hair, skin, the choroid and iris of the eye, in the brain, inner ear, and other sites containing pigments. The biological functions of melanin are not well understood. A study of its chemical and spectroscopic properties may give clues to its biological functions. It absorbs ultraviolet light intensely and contains free radicals under diverse conditions of illumination, pH and temperature (1). In the skin and eyes it protects against damage by ultraviolet light. Melanin is thought to be responsible for the photochemical (non-thermal) laser damage processes in the eye (2). Parkinson's disease apparently destroys preferentially melanin-containing cells in the substantia nigra of the brain (3). Melanin is an oxidation-reduction polymer (4). Ultrasound absorbed by melanin causes cytotoxicity in tumor cells (5). Melanin seems to be a general purpose energy absorber. It is capable of protecting cells in its immediate environment from ultraviolet light, for example, but it becomes cytotoxic under conditions where melanin has absorbed a great deal of energy.

Melanin, both synthetic and that obtained from biological samples, is a difficult substance to study spectroscopically. Natural melanin is an oxidized polymer of tyrosine complexed with protein. Synthetic melanin is protein-free, but its spectroscopic and chemical properties are similar to those of natural melanin. It is black sooty solid and its absorption spectrum shows no distinct bands, only an increasing molar absorptivity at shorter wavelengths. Wolbarsht et al (6) interpreted optical absorption by squid ink melanin as predominantly Rayleigh and Mie scattering. Van Woert & Ambani (7) observed weak shoulders in the ultraviolet absorption spectrum of neuromelanin, as well as a monotonically increasing absorbance at short wavelengths. Crippa et al (8) studied optical absorption and photoconductivity of melanins and observed increases in the optical absorption and photoconductivity in the ultraviolet. They interpreted their results in terms of a band model of amorphous materials. Strzelecka (9) measured optical absorption of melanin during its degradation in sodium hydroxide and interpreted her data in terms of amorphous semiconductor theory. McGinness & Proctor (10) speculated the black appearance of melanin resulted from efficient electron-phonon coupling.

Slawinski et al (11,12) observed fluorescence from melanin and humic acids. Their emission spectra revealed three main bands at 480-500 nm, 570 nm, and 615-630 nm. The positions and intensities of these bands were dependent upon illumination time, excitation wavelength, and the presence of singlet oxygen quenchers. They presented evidence that the 615-630 nm band resulted from singlet

oxygen emission. Gallas (13) studied the fluorescent properties of synthetic melanin under a variety of conditions, including the effect of temperature, viscosity, and added metal ions. He observed fluorescence with a maximum between 440 nm and 480 nm. He interpreted his results as a combination of two bands whose relative intensity and position were affected by his experimental conditions. Kozikowski et al (14) recently observed fluorescence of melanins from argon ion laser excitation.

Infrared absorption spectra of melanin compressed in KBr pellets were obtained by Bridelli et al (15). They were able to observe spectral features in the NH stretch, OH stretch regions, and the carbonyl stretch region. Their results indicated the existence of intramolecular hydrogen bonding and salt bridges. Recently, Wilczok et al (16) collected infrared and electron spin resonance spectra of chemically modified synthetic melanins. They correlated spectral changes with chemical modification procedures and found conditions influencing the free radical content of their samples. Oxidation increased the carboxyl content while reduction caused no noticeable change in its infrared spectrum.

Dimethyl sulfoxide (DMSO) has many biological effects, including an ability to penetrate skin and biological membranes. Its physical properties were reviewed by Kharasch & Thyagarajan (17). DMSO is a dipolar aprotic substance. It has a dipole centered on the electronegative oxygen of the sulfoxide bond and an unshared pair of electrons on sulfur localized in the fourth corner of a regular tetrahedron. It is a powerful hydrogen bond acceptor. DMSO has the ability to complex to metal cations, components of tissues, proteins, nucleic acids, carbohydrates, fats, and various drugs. Work has been done measuring the effects of DMSO on proteins (17). The hydration shell around the protein molecules is replaced by a shell of DMSO molecules, breaking up intramolecular and intermolecular hydrogen bonds and solubilizing some multisubunit proteins into their subunits. Depending upon the system studied, DMSO can either raise or lower enzyme activity.

As part of a study of solvent effects on melanin, we found that DMSO affects the spectroscopic properties of synthetic melanin. Changes in its ultraviolet, visible, near infrared absorption spectrum, infrared absorption spectrum, and fluorescence spectrum were observed. The data are consistent with DMSO behaving as a hydrogen bond acceptor to melanin, breaking up intermolecular and intramolecular hydrogen bonds and salt bridges. Since DMSO preferentially stabilizes cations and destabilizes anions, thereby increasing their basicity, DMSO causes an intramolecular acid-base reaction. This reaction may account for the observed spectral changes of melanin in the presence of DMSO.

Spectral Effects of Dimethyl Sulfoxide on Melanin

METHODS

Synthetic melanin, prepared from the persulfate oxidation of L-tyrosine, was purchased from Sigma Chemical Company and used without further purification. Samples of melanin were weighed out to make a 0.1 mg/ml suspension in buffer (Table 1). Solutions buffered from pH 4 to pH 9 were prepared, at an ionic strength $I = 0.05$ (18). Also, melanin solutions in 0.1 N HCl, 0.1 N NaOH, and DMSO were prepared. A 1 mg/ml melanin solution in DMSO was diluted ten fold in buffer to provide absorption spectra of melanin in DMSO combined with buffer. The absorption spectra were collected by a Varian 2300 spectrophotometer with 10 mm pathlength cells. Before actual data collection began, a baseline of solvent vs solvent was collected and stored in instrument memory. The spectrophotometer numerically subtracted the baseline from the observed melanin absorption.

The absorption spectra were digitized and fitted to an amorphous semiconductor absorption function (19).

$$(\alpha h\nu)^{1/2} = K (E_g - h\nu) \quad (1)$$

where α is the absorption coefficient (cm^{-1}), h is Planck's constant, $h\nu$ is the photon energy in electron volts, E_g is the optical band gap in electron volts, and K is a proportionality constant. A plot of the data using equation 1 yields two constants, E_g and K , which were further used to rationalize the experimental data.

In order to measure the scattering component of melanin optical absorption, diffuse reflectance spectra of melanin solutions were obtained by using the diffuse reflectance accessory of the Varian spectrophotometer. The procedure involved the measurement of optical absorption with this accessory and the measurement of diffuse reflectance with respect to TiO_2 .

To gain information about the rate samples approach equilibrium upon addition of solvent, a comparison of order of solvent addition (DMSO vs water) was made. A 0.1% melanin sample, after an absorption spectrum had been collected, was titrated with DMSO to a concentration of 10% (v/v). The reverse procedure was also performed; a DMSO solution of melanin was added to water at a final concentration of 10% (v/v).

Infrared spectra were collected by using a Perkin-Elmer 457 Grating Infrared Spectrophotometer. Infrared spectra of solid melanin and melanin dissolved in DMSO were collected. Solid melanin was mixed with KBr at a concentration of 0.60 mg/100 mg KBr. The sample was then dried 30 min under a vacuum with a liquid nitrogen trap and pressed into a 13 mm pellet with 20,000 lb (9072 kg) pressure. In order to measure the effect of pH, melanin solutions were incubated in

Table 1

Effect of pH, Presence of DMSO on the Absorption Spectrum of Synthetic Melanin

Solution ^a	pH	E _g ^b	K ^c
0.1 N HCl	1.18	<0	0.347
Acetate Buffer	3.78	<0	0.298
" "	5.05	<0	0.265
Phosphate Buffer	6.00	<0	0.304
" "	7.05	0.431	0.419
Tris Buffer	7.85	1.04	0.500
" "	8.80	1.06	0.547
0.1 N NaOH	12.70	1.30	0.734
100% DMSO	N/A	1.04	0.537
0.1 N HCl, 10% DMSO	1.10	1.29	0.614
Acetate Buffer, 10% DMSO	3.70	1.19	0.592
" " 10% DMSO	5.00	1.12	0.590
Phosphate Buffer, 10% DMSO	6.00	1.12	0.590
" " 10% DMSO	6.95	1.05	0.585
Tris Buffer, 10% DMSO	7.75	1.04	0.586
" " 10% DMSO	8.45	1.03	0.595
0.1 N NaOH, 10% DMSO	12.70	1.21	0.655

^a All buffers have an ionic strength $I=0.05$. The melanin concentration is 0.1 mg/ml in all samples.

^b Band gap, E_g was calculated by fitting the absorption spectrum to equation 1 and extrapolating to the x axis.

^c The proportionality constant, K was obtained from the slope in equation 1.

0.1 N HCl, distilled water, or 0.1 N NaOH for 10 minutes, dried at 60°C, pressed into KBr pellets, and dried under vacuum by a liquid nitrogen cold trap. To perform infrared spectroscopy of melanin dissolved in DMSO, a 20 mg/ml melanin solution was prepared and placed in a sample cell with KBr windows. The spectrometer was blanked and balanced by using a variable path length reference cell containing DMSO to subtract the effect of solvent.

In order to determine if the DMSO-induced spectral changes were reversible, a melanin sample dissolved in DMSO was diluted 1:1 with distilled water and centrifuged at 750 x g for 10 minutes. The supernatant was discarded and the pellet resuspended in distilled water. This procedure was repeated 5 times to extract all the DMSO. The sample was then dried by heating at 60°C. After drying, the sample was suspended in KBr and dried again under vacuum with a liquid nitrogen cold trap. An infrared absorption spectrum of the sample was then collected. For all data obtained, the spectral assignments are based on published data (20).

Fluorescence spectra were collected on a Perkin Elmer Model 310 S spectrofluorometer. The excitation monochromator was set at 310 nm with a 5 nm spectral band pass. The emission monochromator scanned from 320 nm to 700 nm with a slit width of 5 nm. The sample geometry was right angle detection. Grating ghosts at 620 nm created distortions in the signal in this spectral region. Accordingly, the emission spectrum in this spectral region is presented as an extrapolation. All data were collected at 25°C. Fluorescence spectra were collected from a 0.10 mg/ml melanin suspension in twice distilled water and a 0.10 mg/ml melanin solution in 10% (v/v) DMSO. The emission spectra were digitized and the solvent emission subtracted from the sample spectrum. Absorption spectra of the samples were collected and used to correct the emission spectra for self absorption (21).

To compare the solvent effect of water on melanin fluorescence to that of DMSO, an iodide quenching experiment was performed to determine the fluorescent properties of the water-inaccessible fraction of chromophores. The iodide concentration was varied from 0 to 7 molar. The quenching data were analyzed by equation 2 (22).

$$F_0/(F_0-F) = 1/(f_a K [Q]) + 1/f_a \quad (2)$$

where f_a is the fraction of initial fluorescence accessible to quencher, K is a fluorescence quenching constant, F_0 is the initial fluorescence intensity, and F is the fluorescence intensity in the presence of quencher. A plot of $F_0/(F_0-F)$ vs $1/[Q]$, a modified Stern-Volmer plot was used to measure f_a and K . The sample studied was a 0.001% melanin solution excited at 310 nm (5 nm spectral band pass) and the fluorescence intensity was measured at the wavelength of maximum fluorescence.

Spectral Effects of Dimethyl Sulfoxide on Melanin

RESULTS

Observations of the physical appearance of the melanin solutions demonstrated an interaction of melanin with the solvent. Solid melanin added to 0.1 N HCl or pH 4 buffer had a grey, cloudy appearance. Sonication of the solution was necessary to break up the melanin particles. At higher pH solutions, the melanin dissolved more readily and the solution appeared reddish brown with no turbidity. The order of addition of solvents affects the results of these experiments. Melanin dissolved in DMSO, then diluted tenfold in buffer, had a clear reddish brown color at all pH values. DMSO added to an aqueous solution of melanin to a final concentration of 10% (v/v) DMSO did not cause the spectral changes observed when a melanin solution in DMSO was added to water. Examples of typical melanin absorption spectra in the range 300-1000 nm are given in Figure 1. The absorption spectra were fit to equation 1.

Equation 1 is the standard expression for optical absorption of amorphous semiconductors, and our data can be fitted quite accurately with this expression. An example of this fit is given in Figure 2, where absorption data collected from melanin dissolved in 100% DMSO are plotted.

Table 1 presents band gaps and values of K calculated for melanin samples as a function of pH and in the presence of DMSO. The absorption spectra of melanin in acid, when fitted to equation 1, behave as if they had a negative "band gap." Diffuse reflectance spectra of aqueous melanin solutions at acidic to neutral pH (data not shown) indicated a significant scattering component to the optical absorption, demonstrating that the anomalous "band gap" values result from a contribution of Mie scattering to the overall absorption. At pH 7 and greater, the calculated band gap increases to around 1 eV. At pH 13, it increased again to 1.30 eV. Melanin in basic solution showed no measurable diffuse reflectance, indicating a small scattering component to the optical absorption.

The behavior of melanin in 10% DMSO solutions as a function of pH is different from aqueous solutions. At all pH values the measured optical band gap ranged from 1.03 eV to 1.29 eV. Also, no diffuse reflectance was detected from melanin solutions in the presence of DMSO, showing that the observed optical absorption results from the actual absorption of light and not a scattering process. Also, melanin dissolved in 100% DMSO resulted in a measured band gap of 1.04 eV. Melanin in the presence of DMSO behaves as if it were in a basic medium. The measured band gap is similar in DMSO and basic solution.

Table 1 lists calculated values of the proportionality constant, K , in solutions of various pH values and in the presence of DMSO. When melanin was dissolved in acid, K was near 0.3, approached a

Spectral Effects of Dimethyl Sulfoxide on Melanin

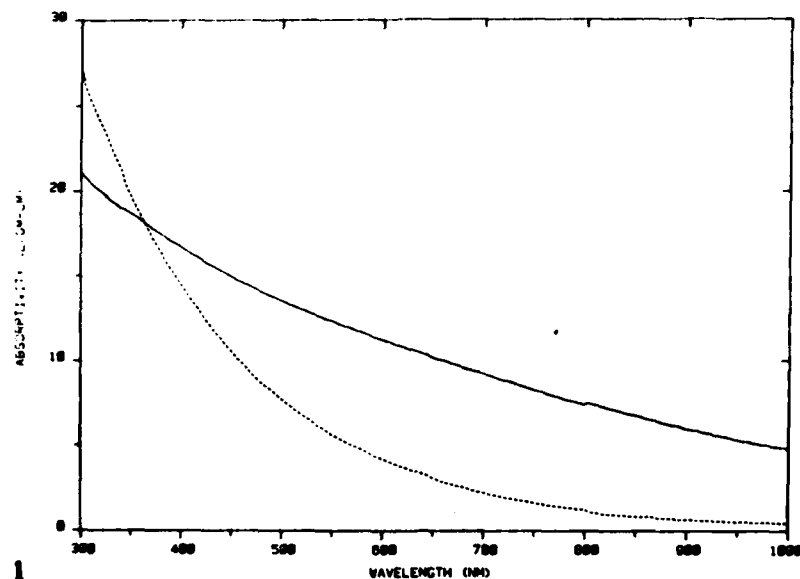


Figure 1. Ultraviolet/visible/near-infrared absorption spectra of melanin. The solid curve is the absorption spectrum of melanin in 1 N HCl. The dotted curve is the absorption spectrum of melanin in 10% (v/v) DMSO in 1 N HCl. The spectra were collected from 300 to 1000 nm. The ordinate is the melanin absorbance normalized to 1 mg/ml concentration.

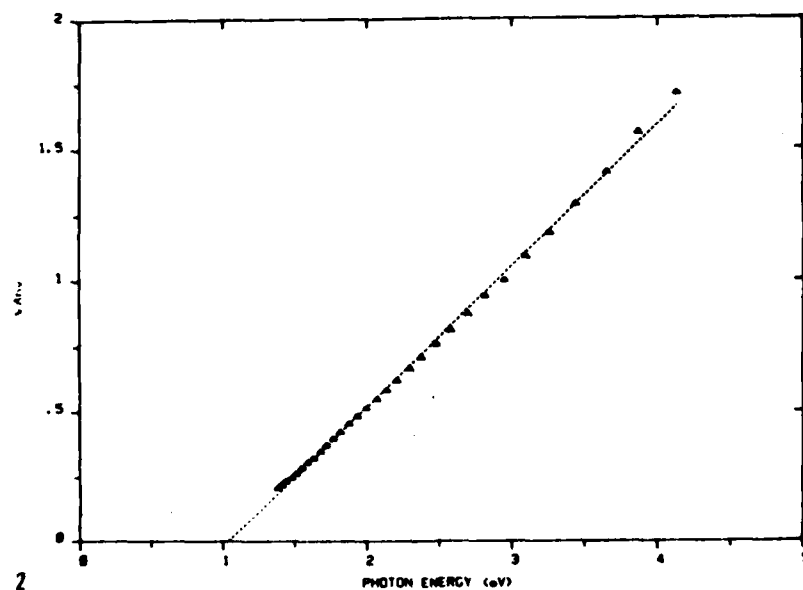


Figure 2. Plot of $(ahv)^{1/2}$ versus $h\nu$ according to equation 1. The abscissa gives the energy of excitation in electron volts, a is the absorbance of the melanin solution, a 0.1% melanin solution dissolved in DMSO.

Spectral Effects of Dimethyl Sulfoxide on Melanin

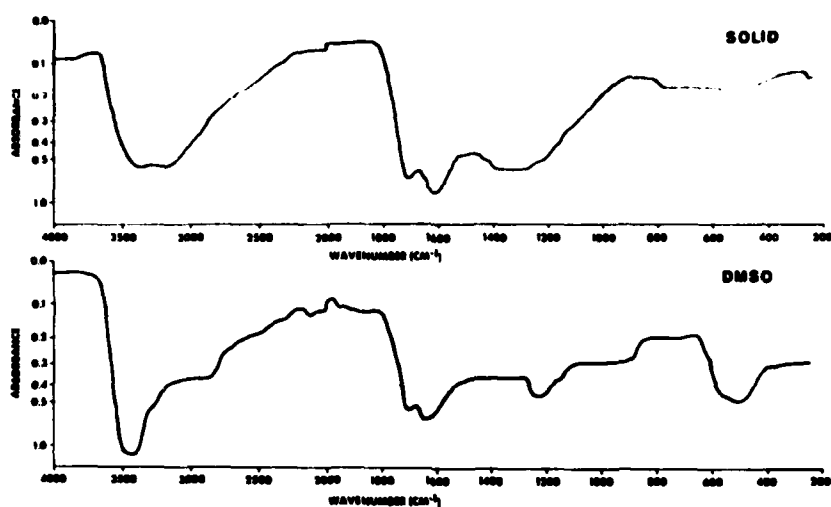
minimum at pH 5, and increased thereafter. Between pH 7 and 8 an acid base reaction occurred and the melanin dissolved. In contrast, melanin in the presence of 10% DMSO showed a value for the slope as if the solution had a pH 7 - 9. The behavior of K as a function of pH was similar to the behavior of the band gap as a function of pH. With these constants (band-gap, K) is a way to describe the absorption spectra.

Figure 3 presents infrared absorption spectra of solid melanin and melanin dissolved in DMSO. In both cases the spectra were broad and featureless. Distinct differences exist between the two spectra. Solid melanin showed major infrared absorption bands in the NH stretch, O-H stretch region and in the carbonyl stretch region. There was a broad absorption from 2000-3650 cm^{-1} , with peaks at 3400 cm^{-1} (unprotonated hydrogen bound N-H stretch), and 3200 cm^{-1} (ammonium ion N-H stretch). The absorption in this region also included OH stretch. In the carbonyl stretch region were two bands at 1715 cm^{-1} (protonated carboxylate carbonyl stretch) and 1615 cm^{-1} (carboxylate anion antisymmetric stretch). In the lower frequency domain was a broadened absorption from 1000-1500 cm^{-1} , resulting from carboxylate ion symmetric stretch (1400 cm^{-1}), and low frequency vibrations (400-800 cm^{-1}).

Several spectral changes occurred when solid melanin was dissolved in DMSO. These spectral changes were reversible. Melanin was dissolved in DMSO, washed in distilled water, and dried, then suspended in KBr. The resulting infrared absorption was identical to that of a solid melanin sample that had been washed and dried. The absorption band at 3400 cm^{-1} shifted to 3450 cm^{-1} and increased in intensity (amine N-H stretch). Intensity was lost at 3200 cm^{-1} and a band at 3280 cm^{-1} appears. These results were consistent with the ionization of ammonium ions to amino groups. At 2850 cm^{-1} a new band appeared (hydrogen bonded OH stretch, carboxylic acid dimer). In the carbonyl stretch region the 1715 cm^{-1} band remained unchanged. The 1615 cm^{-1} band shifted to a higher frequency, 1645 cm^{-1} . Intensity was lost at 1400 cm^{-1} . This loss in intensity resulted from the formation of dimerized carboxylic acid species. A C-O stretch band of a carboxylic acid dimer appeared at 1240 cm^{-1} and an out of plane bending vibration of a dimeric carboxylic acid appeared at 850-950 cm^{-1} . Finally, an intense band, out of plane, hydrogen bonded N-H vibration appeared at 400-650 cm^{-1} .

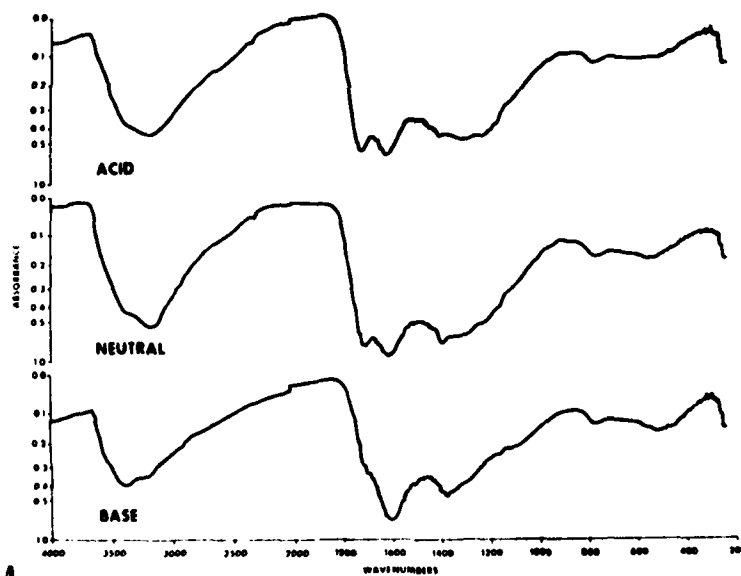
How do these spectral changes compare with the spectral changes observed when melanin has been dissolved in acid or base and subsequently dried? Figure 4 gives infrared absorption spectra of melanin that were equilibrated and dried in either 1N HCl, distilled water, or 0.1 N NaOH. These spectra can be compared to the spectrum of melanin dissolved in DMSO. The infrared absorption spectrum of melanin in DMSO appeared to have features common to melanin that had been treated with base and also with melanin that had been treated

Spectral Effects of Dimethyl Sulfoxide on Melanin



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Figure 3. Infrared absorption spectra of melanin. The upper curve gives the infrared absorption spectrum of solid melanin suspended in a KBr pellet, where the melanin concentration is 0.6% in weight. The lower spectrum is the infrared absorption spectrum of melanin dissolved in DMSO at a concentration of 20 mg/ml. To subtract the effect of solvent, the spectrometer was blanked and balanced using a variable pathlength reference cell containing DMSO.



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Figure 4. The effect of pH on the infrared absorption spectrum of melanin. The spectrum labeled "acid" is an infrared absorption spectrum of a melanin sample that had been incubated in 1N HCl for ten minutes. The spectrum labeled "neutral" is an infrared absorption spectrum of a melanin sample that had been incubated in distilled water. The spectrum labeled "base" is an infrared absorption spectrum of melanin that had been incubated in 0.1 N NaOH. All samples were incubated in their respective solvents for 10 minutes, dried at 60 °C and suspended in a KBr pellet at a concentration of 0.6%(w/w).

Spectral Effects of Dimethyl Sulfoxide on Melanin

with acid. Table 2 summarizes the spectral changes observed under these conditions. Column a lists the ratio of the intensity of the 3200 cm^{-1} band to the 3400 cm^{-1} band, which is a measure of the concentrations of protonated ammonium ions to free amino groups. The ratio dropped from 1.33 in melanin that had been equilibrated in acid to 0.90 in melanin that had been equilibrated in base showing a greater proportion of free amino groups in basic conditions. The infrared absorption spectrum of melanin in DMSO shows a dramatic drop in this ratio, to 0.37, indicating a low concentration of protonated amino groups. Column b is the ratio of intensity of the protonated carboxylate stretch band to the antisymmetric stretch band of the carboxylate ion. As the pH of the incubated solution increased, this ratio dropped, indicating the presence of greater numbers of free carboxylate ions at high pH incubation. Melanin in DMSO gave a ratio of these two peaks at 0.90, characteristic of melanin that had been equilibrated in an acidic solution. Also, melanin dissolved in base absorbed at $400\text{--}650\text{ cm}^{-1}$; melanin previously equilibrated in acid had only a faint evidence of this band. Upon dissolution in DMSO, absorption in this spectral region was very intense, again giving evidence of an intermolecular acid-base reaction. The behavior of melanin in DMSO was similar in some respects to previously acidified melanin and also to previously alkalized melanin.

Figure 5 shows fluorescence spectra of a melanin suspension in water and melanin dissolved in DMSO. Under these conditions the fluorescence was weak and self absorption had only a small effect on the spectrum. Along with the Raman band of water, there were two emission bands, one at 435 nm and a weaker shoulder at 530 nm. The emission spectrum of melanin in 10% DMSO changed dramatically. Interaction with solvent quenched the fluorescence and shifted the fluorescence to 450 nm.

To compare the effect of DMSO on melanin fluorescence with the effect of water, an iodide quenching experiment was performed on an aqueous melanin suspension. Table 3 lists the iodide concentration, the fluorescence intensity, and the wavelength of maximum fluorescence. Quenching of fluorescence occurred. A modified Stern-Volmer plot of the data (22) yielded a fluorescence quenching constant of 5.19 M^{-1} and from the intercept of the plot, it was calculated that 90% of the melanin chromophores were accessible to water and 10% were not. Also, as the iodide concentration increased, the fluorescence maximum shifted from 435 nm to 415 nm, revealing a solvent inaccessible fluorophore population.

Table 2

The Effect of pH on the Infrared Absorption Spectrum of Melanin

Preparation	a	b
Acid ^c	1.33	0.96
Neutral ^d	1.30	0.82
Solid ^e	1.00	0.80
Base ^f	0.90	0.49
DMSO ^g	0.37	0.90

a Ratio of the intensity of ammonium ion N-H stretch to unprotonated amine N-H stretch.

b Ratio of the intensity of protonated carboxylate carbonyl stretch to antisymmetric stretch, carboxylate anion.

c Solid melanin, suspended in 1 N HCl, dried at 60 °C for 10 minutes, pressed into a KBr pellet.

d Solid melanin, suspended in distilled water, dried at 60°C for 10 minutes and pressed into a KBr pellet.

e Solid melanin, untreated, pressed into KBr pellet.

f Melanin, dissolved in 0.1N NaOH, dried at 60°C for 10 minutes and pressed into KBr pellet.

g Melanin, 20 mg/ml in DMSO.

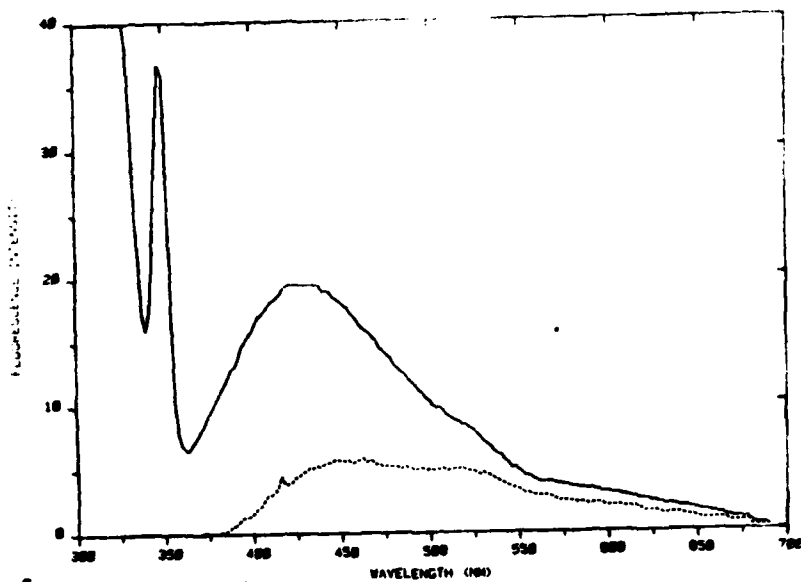
Table 3

The Effect of Iodide Ion on the Fluorescence of Melanin in
Aqueous Solution *

$[I^-](M)$	I_f	Fluorescence Maximum(nm)
0.0	80.0	435
1.0	19.5	425
2.0	15.0	424
3.0	11.5	420
4.0	11.5	420
5.0	10.8	418
7.0	9.5	415

*Melanin concentration was 0.001% in double distilled water thermostatted at 25°C. The excitation wavelength was 310 nm with a spectral band pass of 5 nm.

Spectral Effects of Dimethyl Sulfoxide on Melanin



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Figure 5. The effect of DMSO on the emission spectrum of melanin. The solid curve is an emission spectrum of melanin suspended in distilled water at a concentration of 0.1 mg/ml. The dotted curve is an emission spectrum of melanin dissolved in 10%(v/v) DMSO at a concentration of 0.1 mg/ml. Spectra presented have been corrected for solvent fluorescence and self-absorption. The excitation wavelength was 310 nm and the spectral band pass was 5 nm.

DISCUSSION

An amorphous semiconductor model explains most of the absorption spectral data. Close examination of Figure 1 reveals a slight curvature to the data, implying that the true mechanism of absorption is more complex than a simple amorphous semiconductor model. DMSO has

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a distinct effect on the optical absorption of melanin. Clearly, light scattering is a major component of its absorption spectrum at low pH, for the melanin particles are not appreciably ionized. Correspondingly, there is low solubility and a high degree of Mie scattering at this pH and the calculated band gap is meaningless. Diffuse reflectance spectra of aqueous melanin solutions at acidic to neutral pH (data not shown) indicated a significant scattering component to the optical absorption, demonstrating that the anomalous "band gap" values result from a contribution of Mie scattering to the overall absorption. As the pH of the melanin solution was increased, the band gap gradually increased. Acidic groups in the melanin granule became ionized at higher pH, allowing greater stabilization of the melanin particles by the solvent shell and increased repulsion of the negatively charged groups. When the pH was adjusted to 12.70, the band gap increased to its largest value, 1.30 eV.

In the presence of DMSO, similar effects were observed on the melanin absorption spectrum. At most pH values, the band gap remained relatively constant; the absorption spectra mimicked those at high pH with no DMSO present. The band gap measured for melanin in 10% DMSO was similar to that measured in 100% DMSO. These results allow us to conclude that melanin can be solubilized either in base or DMSO. The mechanism of solubilization parallels in these two cases, involving ionization and acid-base reactions. The fact that the band gap varies little with pH in a 10% DMSO solution and ten-fold dilution of a melanin solution dissolved in DMSO into water implies that melanin particles reside in a shell of DMSO and there is relatively little interaction with water. The properties of DMSO-water mixtures are a function of the mole fraction of DMSO present. Addition of DMSO to an aqueous solution of melanin to a final concentration of 10% (v/v) did not cause changes in its absorption spectrum. This effect most likely results from the statistical improbability of a DMSO molecule binding to a melanin particle in the presence of excess water. Any approach to a true thermodynamic equilibrium is slow. Bertoluzza et al (23) suggested that DMSO enhances the solvent structure of water at low mole fractions then disrupts the water hydrogen bond network at high mole fractions. Under the conditions of this experiment, the mole fraction of DMSO is 0.027. When melanin dissolved in DMSO is diluted into water, DMSO interacts strongly with the melanin particle, and thus shields the melanin from the aqueous environment and enhances the solvent structure of water.

The infrared spectrum of melanin, when dissolved in DMSO, shows several significant changes in the carbonyl stretch region, N-H stretch and the O-H stretch region. The infrared spectrum of solid melanin was broadened, a characteristic of polymers. The broadening of the spectrum makes precise assignment of the observed absorption bands difficult. Frequency shift of the 1615 cm^{-1} band to 1645 cm^{-1} is analogous to shifts in amide carbonyl bands when the solid amide is dissolved. Intramolecular hydrogen bonds, when broken upon

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dissolution into solvent, allow the frequency of this band to increase. In solid melanin, this band (carboxylate anion asymmetric stretch) has been affected by a nearby ammonium ion bound to the carboxylate anion as a salt bridge. The spectral shifts observed when melanin is dissolved in DMSO can be interpreted as the formation of carboxylic acid dimers and intramolecular acid-base reactions, as well as the breakup of intramolecular hydrogen bonds and salt bridges.

The occurrence of an intramolecular acid-base reaction upon dissolution of melanin into DMSO can be understood when the effect of DMSO on the acid-base properties of carboxylic acids and amines is considered. Buncel and Wilson (24) made a detailed discussion of the effect of DMSO on acid base properties. Because of the high polarity of DMSO and the electronegativity of its oxygen, DMSO preferentially stabilizes cations and destabilizes anions. For example, oxalic acid in water has pK_a values for its two ionizations of 1.27 and 4.27 respectively. When oxalic acid is dissolved in DMSO, these values rise to 6.2 and 14.9 respectively. In contrast, the acidity of protonated aromatic nitrogens increases in the presence of DMSO due to solution of free protons. For example, the pK_a of protonated pyridine drops from 4.60 to 3.50 upon change of solvent from water to DMSO. These alterations in acidity are directly applicable to melanin. Oxidation of indole-quinone subunits in melanin can lead to the formation of carboxylate residues (16). Carboxylate residues in close physical proximity will behave in a similar manner to oxalic acid and undergo large increases in pK_a in the presence of DMSO.

The results presented in Figure 5 illustrate that DMSO has a dramatic impact on melanin fluorescence. The 435 nm band shifted to 450 nm and the fluorescence intensity dropped. The shoulder at 530 nm appeared not to be affected by the presence of DMSO. From the iodide quenching experiments, we can measure the fluorescent properties of two populations of chromophores, one solvent accessible, the other solvent inaccessible. Assuming that the solvent inaccessible population of chromophores resides in a hydrophobic environment, then DMSO causes a larger spectral shift than water, when the solvent inaccessible chromophores are used as a reference (a spectral shift of 1874 cm^{-1} vs 1107 cm^{-1}). A phenomenological view of solvent effects is provided through linear solvation energy relationships. Kamlet et al (25) used linear solvation energy relationships to quantify solvent effects of some fluorescent probes. They were able to quantify solvent effects on fluorescence through the use of a linear combination of three indices: a measure of solvent polarity/polarizability, an index of hydrogen bond donation ability, and an index of hydrogen bond acceptor ability. We can compare the constants given for water and DMSO and note that the solvent polarity/polarizability indices for water versus DMSO are nearly identical (1.09 vs 1.00). DMSO is an aprotic solvent and water has a hydrogen bond donation parameter of 1.10. DMSO is a more potent hydrogen bond acceptor than water (0.76 vs. 0.18). From these

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differences we can conclude that the solvent effects observed in Figure 5 result from a competition of the hydrogen bond donation ability of water with the hydrogen bond acceptor ability of DMSO to melanin. DMSO is a stronger hydrogen bond acceptor than water, while water is also a hydrogen bond donor. Thus, hydrogen bond donors (O-H and N-H groups) form hydrogen bonds preferentially with DMSO, while hydrogen bond acceptors on melanin will bind to water. The observed changes in the emission spectrum result from interactions between melanin and DMSO.

Hydrogen bonding can have profound effects on emission spectra. Spectral shifts resulting from hydrogen bonding depend upon the magnitude and sign of the difference in bonding energies between the ground and excited state. A lowering in energy of the emission band, assuming a π to π^* transition, implies that the hydrogen bond stabilization energy is greater in the excited state than in the ground state. This results from a greater acidity of hydrogen bond donor protons in the excited state, ultimately the effect of an increase in dipole moment upon absorption of light into the excited state. The spectral shift in the presence of DMSO is 1874 cm^{-1} , a typical value for hydrogen bond spectral effects (26).

These spectral changes give insight into the factors responsible for the observed melanin fluorescence. The electronically excited state of an aromatic compound has a larger dipole moment than the ground state. The polar excited state perturbs its solvent shell leading to a solvent relaxation process. Increasing solvent polarity leads to a lowering in the energy of the excited state, resulting in a longer wavelength shift of the emission spectrum. The infrared spectroscopy results presented above indicate that hydrogen bonding is significant in both solid melanin and melanin dissolved in DMSO. In other compounds hydrogen bonding has profound spectral effects (27). In melanin, hydrogen bonding occurs in all experimental conditions. Hydrogen bonding probably accounts for the observed shift in the 435 nm band. Hydrogen bonding to the chromophore allows a new radiationless decay pathway for the excited state, quenching the fluorescence. Possibly the fluorescence lifetime of melanin is lengthened in the presence of DMSO increasing the probability of radiationless decay.

DMSO has significant effects on the properties of melanin. It solubilizes melanin by its hydrogen bond-accepting ability. DMSO breaks up intermolecular and intramolecular hydrogen bonds and causes changes in carboxyl group acidity. DMSO exerts a solvent effect, quenching fluorescence, and shifting its fluorescence maximum to a longer wavelength. DMSO affects the absorption spectrum of melanin in a manner similar to a basic solution at pH 8 or 9. The measured band gap in the presence of base or DMSO are similar. The values are different at acidic pH primarily because of the contribution of Mie scattering to the overall absorption of light. It appears that DMSO

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does not affect the absorption spectrum of melanin, meaning that the difference in energy between the ground and excited state is not affected by the change in solvent. In contrast, the emission spectrum of melanin in the presence of DMSO is different from that in the spectrum of water. These results imply that DMSO exerts its effects on the excited state by lowering its energy through a solvent relaxation process and the formation of a strong hydrogen bond to the excited state, and thereby promoting vibrational relaxation to the ground state, quenching the fluorescence.

The biological significance of these observations is unknown. DMSO has a number of biological effects. The present results indicate possible interactions between melanin and DMSO under physiological conditions. Because the behavior of melanin dissolved in DMSO is similar to that under basic conditions, it is likely that DMSO causes an increase in free radical concentration in melanin. Aside from photoprotection, the biological functions of melanin are unknown. It is important to know how DMSO affects melanin under physiological conditions. Since Parkinsonism damages the substantia nigra in the brain, perhaps DMSO might have a therapeutic application in such a situation. Similarly, melanomas in the skin may be affected by DMSO. The dramatic effects of DMSO on the properties of melanin provide a means for the further investigation of this complex substance.

CONCLUSIONS AND RECOMMENDATIONS

Spectroscopic results indicate that DMSO alters the micro-environment of the chromophore by changing its acid base properties and altering the solvation of its excited state, most likely through the formation of a hydrogen bond to melanin.

It is recommended that additional spectroscopic studies be performed to evaluate ultraviolet and coherent light effects on melanin with respect to free-radical generation in biological systems such as the eye.

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