ELECTRON SPIN RESONANCE STUDIES ON MELANIN

M. S. BLOIS, A. B. ZAHLAN, *and* J. E. MALING
From the Biophysics Laboratory, Stanford University, Stanford

ABSTRACT Electron spin resonance (e.s.r.) observations of squid melanin have been conducted over the temperature range 500°K to 4.2°K, and the effect of various chemical treatments of the melanin upon the e.s.r. spectrum has been studied. The findings have shown that the paramagnetism of this melanin follows the Curie Law from 500°K to 4.2°K, that the spin signal can be eliminated by the addition of Cu^{++} to the melanin, and that the optical and e.s.r. absorptions of melanin are independent since either can be reduced or eliminated without affecting the other. Similar studies on synthetic melanins produced by autoxidation or by enzymatic oxidation of a number of biphenols were carried out. It was found that the e.s.r. signals of these synthetic melanins were strikingly similar (with respect to line width, line shape, and *g*-value) with those of squid melanin. It is concluded that the unpaired electrons observed are associated with trapped free radicals in the melanin polymer, that the biosynthesis of melanin may involve a free radical mechanism, and that these physical data are in accord with the concept of Nicolaus that melanin is a highly irregular, three-dimensional, polymer.

1. INTRODUCTION

The term melanin refers to the black or brownish pigment which appears to occur at all phyletic levels of biological organization and is ordinarily considered to be an insoluble, nitrogen-containing polymer. The definition may be made more explicit by referring to the biological source of the pigment, or in the case of synthetic melanins, by specifying whether it was prepared by enzymatic or autoxidative means, and further defining the starting material and the experimental conditions. The existence of a multiplicity of acceptable definitions of melanin is a consequence of the fact that "no melanin, whether of natural occurrence or formed *in vitro,* has yet been isolated as a single chemical compound or definite composition . . ." (Swan, 1963).

Chemical studies on melanins have been rendered exceptionally difficult by their insolubility, and resistance to hydrolysis, and further complicated by what is recently becoming understood about their irregularity. The generally accepted concept of melanin synthesis and structure has until recently been essentially that

shown in Fig. 1 which has been taken from Lerner (1953) and is based upon the work of Raper (1928), as later extended by Mason (1948). The essential elements of this scheme are the stepwise oxidations and reductions of the initial substrate (in this instance tyrosine), the role of a bifunctional enzyme with cresolase and oxidase activity, and the polymerization finally of the indole 5,6-quinone to form melanin. This mechanism has led to the concept that melanin consists of a single monomer type linked by a single bond type to form a highly conjugated polymer.

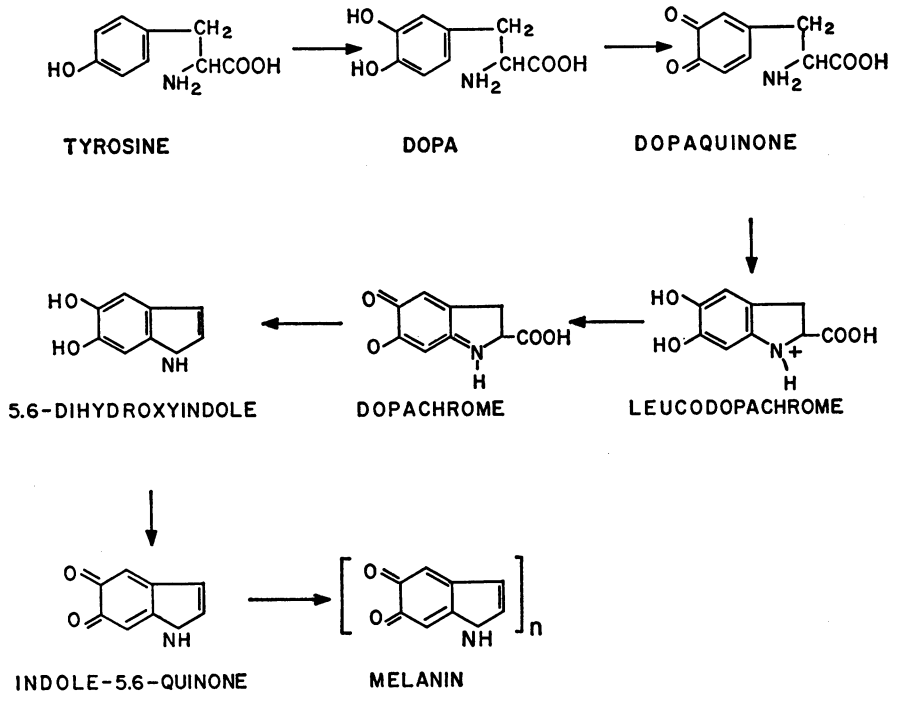

FIGURE 1 Pathway of melanin synthesis (Raper, 1928).

Physical studies of melanin have not in general been particularly helpful either in elucidating structural detail or confirming structures proposed upon the basis of chemical evidence. Optical absorption spectroscopy reveals only a structureless absorption in the ultraviolet and visible regions. Bonner and Duncan (1962) have, however, shown that while the infrared absorption spectra of melanins from several biological sources showed general similarities, there were detailed differences between the pigments from different species. X-ray diffraction examination has invariably revealed a lack of crystallinity. One quite interesting result, however, had been obtained by Commoner, Townsend, and Pake (1954) who had observed an electron spin resonance (e.s.r.) in natural melanin which they attributed to free

radicals trapped in the pigment. The entrapment and protection of a free radical by a growing polymer had been demonstrated with methacrylate (Fraenkel, Hirshon, and Walling, 1954) and one might have expected that autoxidized melanins prepared from suitable biphenols such as dihydroxphenylanlanine (DOPA) would show e.s.r. signals. Subsequent investigation in our laboratory of both natural melanins (squid, human hair, and Harding-Passey mouse melanoma), and melanins produced *in vitro* by both autoxidation and enzymatic oxidation revealed similar e.s.r. signals in all cases and suggested that at least for the autoxidized melanin the unpaired electron was located to at most a few monomer units (Vivo-Acrivos and Blois, 1958).

Mason, Ingram, and Allen (1960) have reported the results of an e.s.r. study of a number of natural melanin samples, including the finding that the spin density was increased upon irradiation by ultraviolet light. At the same time, to explain the paramagnetism of melanins, Longuet-Higgins (1960) suggested that melanin behaves as a one-dimensional semiconductor with the protons acting as electron traps.

2. EXPERIMENTAL METHODS

(*a*) *Preparation of Melanins by the Autoxidation of Quinols*

The starting materials were reagent grade: *p*-hydroquinone, *o*-hydroquinone (catechol), *m*-hydroquinone (resorcinol), L-dihydroxyphenylalanine (L-DOPA), D-dihydroxyphenylalanine (D-DOPA), and L-adrenalin. These compounds were freshly made up in aqueous solutions to a concentration of approximately 0.5 M (or to saturation) and then brought to pH 9-11 with concentrated ammonium hydroxide. The autoxidations were then allowed to proceed in the presence of air at room temperature for a period of 4 days. At the end of this period the polymeric material was dialyzed against several changes of distilled water over a period of a few days and finally lyophilized.

(*b*) *Enzymatic Polymerization of Quinols*

A commercial preparation of mushroom tyrosinase (Worthington Biochemical Corporation, Freehold, New Jersey), prepared by the method of Dawson and Magee (1955) through step 3, was employed. The same quinols as above were made up at one-fifth of the above concentrations in 0.1 M Sorensen buffer pH 7.0 in a volume of 500 ml. To each of these was added 12 ml of the enzyme solution containing 20 mg/100 ml in distilled water. These solutions were incubated at 35°C for 4 days in the presence of atmospheric oxygen. At the end of this period they were dialyzed against distilled water and lyophilized.

(*c*) *Purification of Natural Melanins*

Squid (*Loligo opalescens*) ink, drawn directly from the ink sac, was filtered, centrifuged, washed with distilled water, and dialyzed. This melanin gave a negative Nessler test. Pieces of mouse melanoma tissue (Harding-Passey) from nine different animals were separately homogenized, filtered, and differentially centrifuged until cellular particulates were removed. The pigment was then dialyzed against distilled water and lyophilized. Potato melanin was prepared by thinly slicing pared potatoes, exposing them to the atmosphere until maximum development of pigment had occurred, and then homogeniz-

ing the slices. The starch granules were separated by repeated washing and centrifuging, and the pigment was lyophilized. Human black hair, after rinsing, was treated with normal sodium hydroxide until dissolution had occurred when the preparation was neutralized with concentrated HCl, and the pigment was then dialyzed against distilled water. The suspension was then lyophilized. Samples of washed, but otherwise untreated, human hair were retained for comparison.

(*d*) *Examination of Melanin by e.s.r.*

Melanin samples, ordinarily in the form of the nominally dry powder, were examined in a Varian X-band e.s.r. spectrometer with a modulation frequency of 100 kc. The *g*-values of the samples were obtained by the method previously reported (Blois, Brown, and Maling, 1961) which involves a determination of the magnetic field intensity at resonance by performing a simultaneous proton resonance observation and precisely comparing the latter frequency with the microwave frequency. Melanin g-values were measured at the point of zero slope of the absorption curve, and the line width (ΔH) was measured between inflection points of the resonance absorption. Some measurements were made on copper-doped melanin as well; $g_{\perp}$ and $g_{\parallel}$ and the hyperfine coupling constant for the copper resonance were obtained in the same manner as Malmstrom and Vänngard (1960).

3. EXPERIMENTAL RESULTS

It has been found that melanins extracted from several organisms, produced enzymatically by tyrosinase in combination with one of several substrates, or produced by autoxidation of such substrates alone, given in each instance a single absorption line with *g*-values between 2.0044 and 2.0030.

(*a*) *Melanins Produced by Autoxidation*

The oxidations of simple diphenols to produce the corresponding semiquinones have been studied extensively by e.s.r. in recent years (Venkataraman and Fraenkel, 1955; Wertz and Vivo-Acrivos, 1955; Hoskins, 1955; Adams, Blois, and Sands, 1958). The reaction scheme of Fig. 1 suggests that the successive oxidations and reductions proceed *via* semiquinone intermediates provided that this reaction sequence is followed. Wertz, Reitz, and Dravnieks (1961) have reported on the autoxidation of 3,4-dihydroxyphenylalanine and have shown that a anumber of such intermediates can be observed.

When the autoxidations had in each case gone to completion, the e.s.r. of the pigments showed single, structureless lines having *g*-values as shown in the second column of Table I.

(*b*) *Melanins Produced with Tyrosinase*

The pigments produced by mushroom tyrosinase acting upon a variety of substrates showed in each instance a single absorption line having the *g*-values tabulated in the third column of Table I. It is of interest to note that *m*-hydroquinone (resorcinol)

TABLE I

g-VALUES OF MELANINS AND SEMIQUINONES

Quinol	Semiquinone*	Autoxidized melanin	Enzyme oxidized melanin
p-benzoquinol	2.0047	2.0040	2.0037
o-benzoquinol	2.0044	2.0038	2.0038
m-benzoquinol	2.0044	2.0036	No polymer formed
L-DOPA	2.0044‡	2.0038	2.0036
D-DOPA	—	2.0038	2.0038
L-adrenalin	2.0044	2.0038	2.004

* The semiquinone *g*-values are taken from Blois *et al.* (1961).
‡The semiquinone *g*-value was measured in the DL-DOPA.

does not form a pigment in the presence of this enzyme but does so autoxidatively under strongly alkaline conditions. Examination of the enzyme alone showed a copper resonance (in accord with the copper known to be present) and a very weak free radical line of unknown origin. Neither of these resonances was observed in the melanins because of the high dilution of the enzyme in the latter samples.

(*c*) *Melanins of Biological Origin*

A variety of natural melanins have been shown to give single, structureless absorptions, with line widths comparable to those of the autoxidized and enzymatically prepared melanins (4 to 8 gauss), and with *g*-values as shown in Table II.

TABLE II

g-VALUES OF SOME NATURAL MELANINS

Source	*g*-value
Squid (*Loligo opalescens*)	2.0030 ± 0.0005
Harding-Passey mouse melanome	2.0031 ± 0.0005
Human hair (alkaline extraction)	2.0043 ± 0.0001
Human hair (inact hair)	2.0037 ± 0.0001
Potato melanin	2.0040 ± 0.0001

As a consequence of the recent chemical evidence on the structure of sepia *(Sepia officinalis)* melanin (Nicolaus, 1962), we undertook a detailed study of the e.s.r. of melanin from the closely related squid, *Loligo opalescens*. Unless otherwise stated, it is upon this substance that the observation described below, were made. The fluid taken from the ink sac of this squid was purified as described above and prepared in one of two forms:

A-melanin. The natural melanin (Piatelli and Nicolaus, 1961) occurs as a

calcium-magnesium salt of the polyacid polyquinone. This is the melanin as obtained above and will be referred to as *A*-melanin. This melanin is hygroscopic (Piatelli and Nicolaus, 1961), and it was examined in both the dehydrated and hydrated forms. It was prepared in the dehydrated form by storing in a concentrated H_2SO_4 dessicator for about 2 months, and, in hydrated form, by simply exposing it to the atmosphere. The water content could be varied continuously from about 30 per cent by weight to zero. This was also true for the other form described below.

B-melanin. This was obtained from the *A*-melanin by removing the calcium and magnesium ions, which was accomplished by digesting with concentrated HCl in a sealed tube at 105°C for about 100 hours. Both *A*- and *B*-melanins are decarboxylated (Piatelli and Nicolaus, 1961) by heating above 140°C.

(i) *Line Shape, g-values, and Line Widths.* Fig. 2 shows the derivative

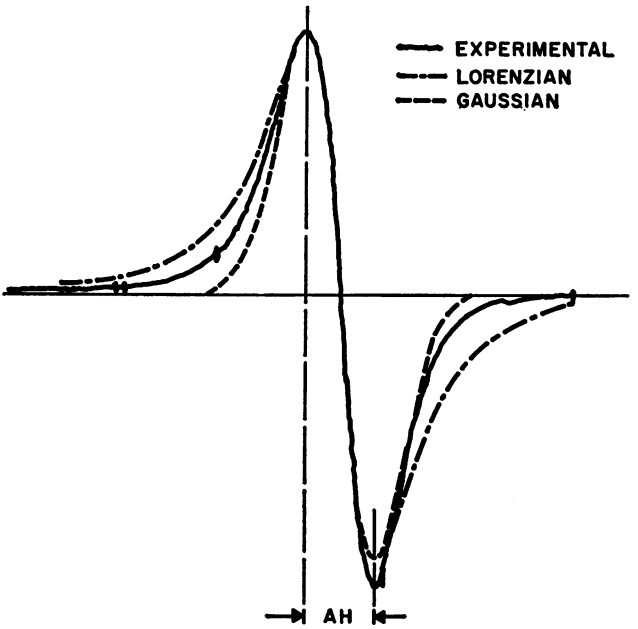

FIGURE 2 The e.s.r. absorption of squid melanin (first derivative trace).

of the e.s.r. absorption line for *A*-melanin along with the Lorentzian and Gaussian absorption line derivatives. The resonance is seen to be intermediate between these two line shapes and slightly asymmetric.

Table III lists the *g*-value and line width of the melanin after several different treatments. For two copper-doped samples, $g_\perp$ and $g_\parallel$, and in one sample the hyperfine coupling constant *A* is given for the copper resonance as well. The line width of the purified untreated melanin (*A*-melanin) is 4.8 ± 0.3 gauss. In order to per-

TABLE III

DATA ON THE e.s.r. OF MELANIN AND OF COPPER IN COPPER-DOPED MELANIN

Sample	$g_{\perp}$	$g_{\parallel}$	A
XI [1.4 mg $CuSO_4 5H_2O$ + 6.7 mg A-melanin]	≤ 2.13	≥ 2.20	–
VII [0.04 mg $CuSO_4 \cdot 5H_2O$ + 11 mg A-melanin]	2.073 ± 1	2.27–2.31	0.0116–0.126

Sample	g_0	ΔH
		gauss
A-melanin	2.00476 ± 10	4.8 ± 0.3
XI (see above)	2.00384 ± 10	8.4 ± 0.5
A-melanin in KBr pellet, $T = 295°K$	2.00374 ± 10	6.5 ± 0.5
A-melanin in KBr pellet, $T = 77°K$	2.00376 ± 10	7.2 ± 0.7
A-melanin in KBr pellet, $T = 77°K$		7.7 ± 0.7
A-melanin (ultraviolet irradiated, 55 min. unfiltered)	2.00375 ± 10	

form irradiation and optical absorption experiments, melanin was thoroughly mixed with KBr and pressed into a thin pellet. The e.s.r. line width increased after this treatment to almost double that of the original signal. The shape altered slightly; and the g-value shifted to a lower value which was very likely a direct result of the broadening of the asymmetric line. The presence of copper ions above a concentration of 1.4 mg $CuSO_4 \cdot 5H_2O$ per 7 mg of dry A-melanin caused the line to broaden and the g-value to shift to the same degree. At a sufficiently high concentration of added copper ions the melanin signal disappeared.

(ii) *Spin Density.* The determination of the spin density was made difficult by the long spin-lattice relaxation time T_1. For the range of incident microwave power employed for most of the measurements (4 to 100 mw), it was necessary to correct the resonance absorption for the effect of saturation. (See Appendix and Section iv for a detailed discussion of this correction and its measurement.) The absolute values of spin density so obtained are correct to within a factor 2. Relative values of spin density could be measured to an accuracy of $\pm$ 10 per cent, however.

The direct comparison of several A-melanin samples with a known DPPH (α, α-diphenyl-β-picryl hydrazyl) standard gave a value of 10^{-4} for the ratio of the spin density of melanin to that of DPPH. Using the conventional value of 10^{21} spins/gm for DPPH, gave 10^{17} spins/gm as the uncorrected value for A-melanin. This value, corected for the saturation effect, becomes 10^{19} spins/gm. Mason, Ingram, and Allen (1960) report:

Sepia ink	6.0×10^{17} spins/gm
Sepia ink reduced)	3.0×10^{17} spins/gm
Melanin from sepia	5.4×10^{18} spins/gm

Our value above is, therefore, in substantial agreement with theirs. The corrected spin density for the *B*-melanin was found to be 5×10^{18} spins/gm, again in agreement with the earlier report.

If one assumes a monomer molecular weight in the range 140–200, it follows that there is one spin per 100 to 200 monomers on the average. The melanin particle has a volume of about 10^{-3} μ^3 and would contain 10^6 such monomers. There are, therefore, an average of about 10^4 spins in each of these relatively massive particles. Because of the stability of these spins, it is concluded that they are embedded within the melanin particle.

It was found that the uncorrected signal intensity depended upon the state of hydration of the powder sample. Upon drying, the signal amplitude of *A*-melanin increased to a maximum of six times the original intensity and then decreased until it was about twice the initial value. This could be simply due to a variation in spin-lattice relaxation time, but a complete explanation for this behavior is not available. Unless otherwise stated, measurements discussed below aply to melanin samples in equilibrium with atmospheric moisture.

(iii) *Temperature Dependence of e.s.r. Signal Intensity.* It was important to determine the temperature dependence of the melanin resonance in order to distinguish between the possible origins of the unpaired electrons. The *A*-melanin had too long a relaxation time ($T_1 \sim 100$ μsec.) to accurately determine the saturation correction for the power range employed. However, *B*-melanin and copper-doped *A*-melanin had a sufficiently short T_1 ($\sim$30 μsec.) so that an accurate signal intensity could be determined over the temperature range investigated.

The intensity of the *B*-melanin resonance measured at 4.2°, 77°, 295°, and 500°K after correction for saturation gave a variation with temperature that deviated no more than 10 per cent from that expected for a system having a simple Curie Law dependence. The Cu^{++}-doped *A*-melanin resonance measured at 77°, 295°, and 450°K gave a similar result.

A few measurements were made in the microwatt range with a low power bridge in an effort to check the Curie Law dependence of a resonance without the necessity of correcting for saturation. The e.s.r. of *B*-melanin was measured at 295°C and 77°K, over the power range 10 to 1000 μw. The ratio of signal intensity at 77°K to that at 295°K at an incident power of 20 μw was 3.9. Under these conditions the power saturation was negligible and the agreement with the Curie Law was shown directly.

(iv) *Relaxation Times.* Using the methods described in the Appendix, the spin-lattice relaxation time T_1 and spin-spin relaxation time T_2 were determined for *A*- and *B*-melanins, for Cu-doped melanin, and for these materials after chemical or physical treatment.

For all samples, T_2 was found to be about 10^{-8} sec., and the line shape was found not to be strongly dependent upon the incident power level.

For example, the *A*-melanin resonance, with a $T_1 \sim 100$ μsec. at 100 mw and a saturation factor less than 10^{-2}, was only 20 per cent narrower at 4 mw than at 100 mw. The *B*-melanin resonance with $T_1 \sim 26$ μsec. and a saturation factor ~0.6, at 4 mw was 25 per cent narrower than the *A*-melanin resonance at 4 mw. The width of both the *A*- and *B*-melanin resonance increased about 30 per cent upon cooling from 500° to 4.2°K.

The spin-lattice relaxation time for the *A*-melanin was found to be shortened by copper-doping; this is due to a cross relaxation effect (Pake, 1962) due to the interaction between the copper and the melanin spin system. Heavy copper doping of the *A*-melanin (about 80 Cu^{++} ions per melanin spin) gave a $T_1 \simeq 30$ μsec. at 77°K, ≃20 μsec. at 295°K, and ≃6 μsec. at 450°K. It was found that the copper spin system in melanin powder did not saturate in the temperature range 77-300°K. However, when the copper ion-doped *A*-melanin was ground with KBr and a pellet formed under a pressure of 20,000 psi, the copper spin system partially saturated at 77°K and 100 mw.

For *B*-melanin T_1 (300°K) was found to be 20 ± 10 μsec.; T_1 (77°K) was 30 ± 20 μsec. Decarboxylation of this sample did not alter T_1.

Effects on relaxation time due to hydration have been observed. In *A*-melanin line intensity varied by as much as a factor 6 with the state of hydration; T_1 was, however, too long to be accurately measured as a function of hydration. An effect on T_1 and resonance intensity was noted in *B*-melanin; however, it was not pronounced. In copper-doped *A*-melanin resonance intensity varied with state of hydration but T_1 did not (Section v).

(v) *Resonance Intensity vs. Metal Ion Concentration.* It was observed that ferric and cupric ions in large concentrations (*e.g.*, 0.5 mg of $CuSO_4 \cdot 5\ H_2O$ per mg of melanin) completely eliminate the squid melanin e.s.r. signal. This behavior was also displayed by a pigment produced by the autoxidation of hydroquinone.

The effect was studied in detail only for the case of copper ion, which was added to the melanin suspension prior to lyophilization. The intensity of the melanin resonance was measured as a function of Cu^{++} ion concentration (doping was done with $CuSO_4$), and the results are plotted in Fig. 3. The effect can be described essentially as a titration of the melanin-free spins by the copper.

Becauuse of the ability of melanin to act as a reducing agent the density of cupric ions was estimated using the Cu^{++} spin resonance. A standard of 60 ppm Cu^{++} in ATP was used and at least at low concentrations (1 Cu^{++} ion per 2 melanin spins) the cupric ion concentration is equal to the total copper concentration with an estimated 20 per cent uncertainty.

In principle it would be possible to decide if some of the Cu^{++} ions were reduced to Cu^{+} by reaction with free radicals in the melanin. Such a reduction would mean that the Cu^{++} resonance intensity would be less than its stochiometric value. However, the elimination of the melanin signal required approximately 80 times as many

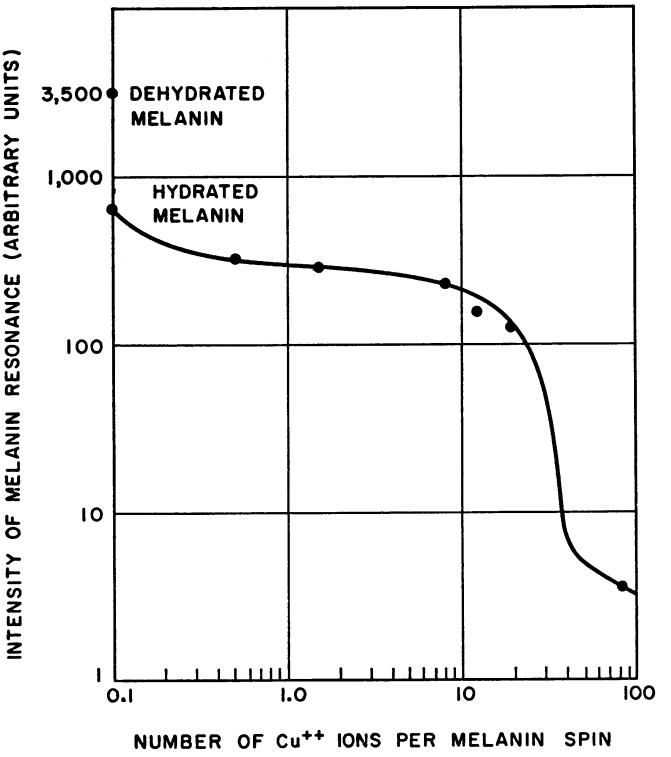

FIGURE 3 The effect of the addition of Cu^{++} upon melanin spin density.

Cu^{++} ions as there were melanin spins, and it was impossible to detect this small an effect if, indeed, it were present. The implication of the titration results is that the Cu^{++} ions react with or affect the spin sites only after complexing first with other sites; the most likely sites would be the groups—COOH and $\diagdown\!\!\diagup C = O$.

The e.s.r. signal intensity in copper-doped *A*-melanin was also found to be a function of the water content of the Cu^{++}-doped sample. Where water was removed slowly from the sample by pumping, the melanin resonance was observed to increase gradually to as much as 16 times the original intensity. Although T_1 for the melanin resonance was a decreasing function of Cu^{++} concentration, it was independent of the state of hydration of a given sample implying a true increase in the number of spins with dehydration. The copper resonance was found to decrease with dehydration on the other hand, either through a change of oxidation state or because T_1 becomes very short (Kozyrev, 1955). This effect was reversible. Rehydration of the sample caused the copper and melanin resonances to return to their former intensities. The titration curve that is shown in Fig. 3 represents the melanin signal for samples that are equilibrated with atmospheric moisture. If the cupric ion

oxidized the radical, the complex at or near the radical site is such that water can be a part of it and acts to moderate in a reversible manner the oxidation-reduction potential of the cupric-cuprous transition of the copper. This is not implausible since the oxidation-reduction potential of the $Cu^{++} \rightarrow Cu^{+}$ reaction is known to be a function of both other complexing ions and the pH (Kolthoff and Lingone, 1952; Brezina and Zuman, 1958).

In view of the quenching effect on the free radical resonance by the copper ions, a comparison of *g*-values was made with those of copper ion resonances in which the binding site was known. The *g*-values given in Table III fit most closely the values of Malmstrom and Vänngard (1960) for copper bound to EDTA, 1-10 phenanthroline, or to oxalate. Assuming a square, planar complex, the copper may then be bound to four oxygen atoms, four nitrogen atoms, or to two of each. According to the results of Kivelson and Neiman (1961), our data indicate an ionic rather than a covalent binding of the copper: that is, a less covalent character than, for example, that of copper in the copper-pthalocyanine complex. No hyperfine structure attributable to interactions with nitrogen has been observed in the copper resonances, but weak splittings would not have been detected.

(vi) *Effect of High Temperature upon Melanin.* HCl-digested melanin begins to be oxidized in an O_2 atmosphere above 200°C. During this process no char e.s.r. signal appeared. The melanin e.s.r. signal, however, decreased with the resulting decrease in the mass of the sample and did not disappear until the entire sample was burned away. This is evidence that O_2 does not react with the spin site (or sites).

(vii) *Optical Absorption.* A Cary model 14 recording spectrophotometer was used for determining the absorption spectra. A Dewar flask of conventional design contained the sample and was introduced into the light path. The absorption spectrum was determined in the region 2000 to 25,000 A, and over the temperature range 77-300°K. It was found that the melanin spectrum did not depend on temperature over the above range. Heavy doping with $CuSO_4$—which was more than enough to destroy the e.s.r. spectrum—did not alter the absorption spectrum.

For these measurements KBr pellets containing 0.3 to 1 mg of sample were employed and the absorption spectra of squid melanin, graphite, and charcoal black are compared in Figs. 4*a* and 4*b*. The infrared absorption spectrum (Fig. 5) was determined using a Perkin-Elmer model 221 spectrophotometer, and the samples were the same pellets described above.

It was found that mild treatment with ascorbic acid altered the color of the melanin but left the e.s.r. signal unchanged. The melanin was treated with an excess of ascorbic acid; the mixture was lyophilized without removing the excess ascorbic acid and the dry sample examined for e.s.r. in air. The melanin under this treatment turned a dark brown color that was stable, indicating that the e.s.r. signal is independent of optical absorption. The fact that destruction of the unpaired spins

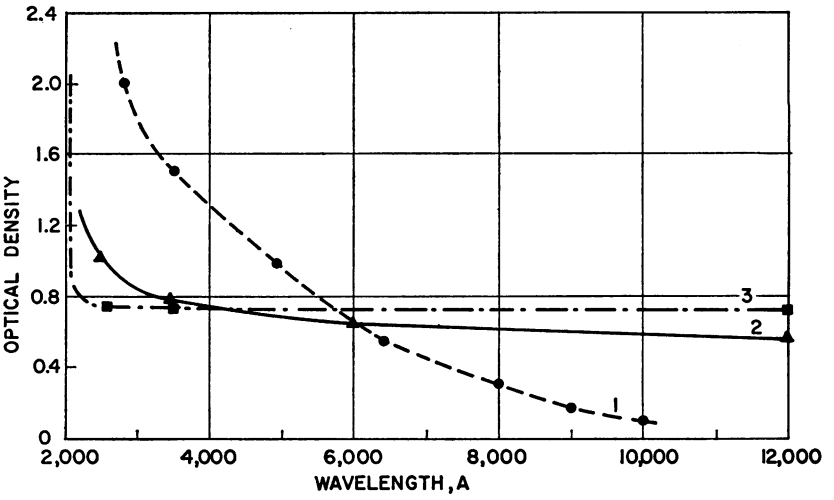

FIGURE 4*a* Optical absorption of (*1*) 0.1 mg squid melanin dispersed in a 300 mg KBr pellet compared with (*2*) 0.3 mg charcoal and (*3*) 0.1 mg graphite prepared similarly.

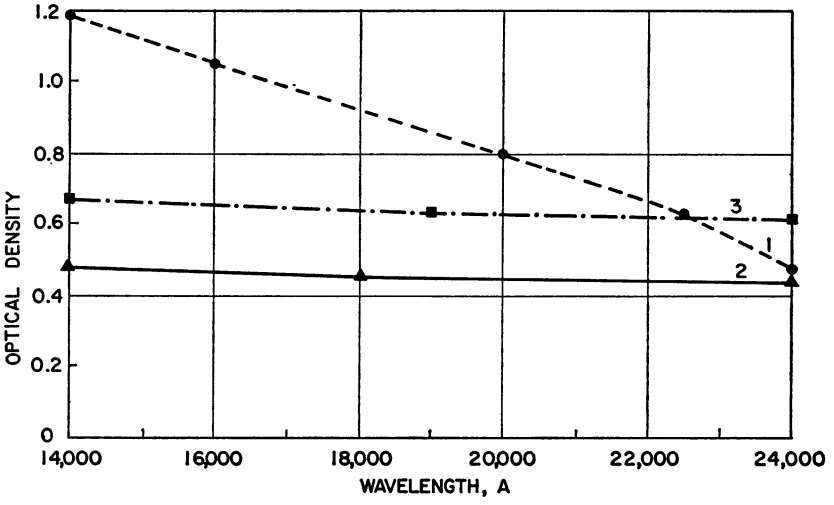

FIGURE 4*b* The near infrared absorption of (*1*) 2 mg squid melanin in a 300 mg KBr pellet. (*2*) and (*3*) are the same samples as in Fig. 4*a*.

with Cu^{++} leaves the melanin absorption spectrum unchanged, is further evidence in support of the conclusion that the melanin color and the melanin paramagnetism are independent of each other.

The absorption spectrum (Fig. 4*b*) of the melanins appears to decay asymptotically to zero at long wavelength but it is difficult to determine whether or not there is

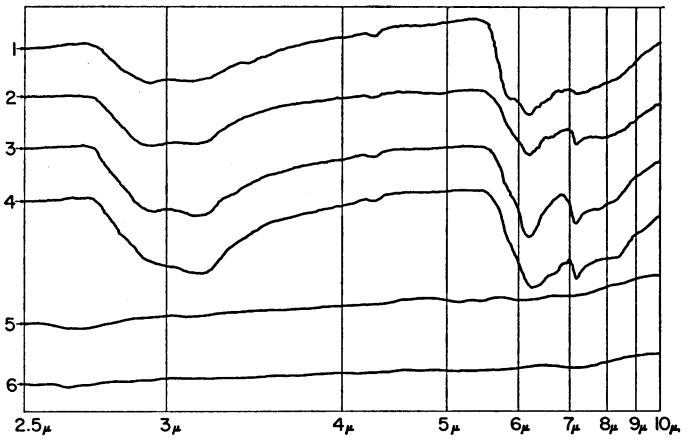

FIGURE 5 Infrared absorption spectra of (*1*) squid melanin, (*2*) catechol melanin—autoxidized, (*3*) L-DOPA melanin—autoxidized, (*4*) hydroquinone melanin—autoxidized, (*5*) graphite, and (*6*) charcoal. All samples observed in KBr pellets.

absorption at say 24,000 A since the small optical density at this wavelength could be due to scattering. The optical spectrum, when compared to that of graphite and charcoal, indicates that the degree of conjugation is lower in the melanins.

(viii) *Diverse Chemical Treatments.* The addition of concentrated (~98 per cent) H_2SO_4 to melanin and heating to 100°C for 1 hour did not destroy the e.s.r. signal. It was not possible, however, to compare precisely the spin density before and after the addition of the H_2SO_4 because of the resulting change in the Q of the sample. When concentrated HCl was added to a melanin suspension and taken to dryness, the e.s.r. signal was unaffected. Neither was it altered by the addition of alcoholic KOH, nor by treatment with Na_2CO_3 at pH = 11 followed by drying. In short, the melanin e.s.r. signal was stable against a variety of chemical treatments, as was the melanin itself.

4. DISCUSSION

The studies described above relate to three phases of the melanin problem:

(*a*) the origin of the paramagnetism,
(*b*) the polymetric structure of melanin, and the relationship between synthetic and natural melanins, and
(*c*) the role of free radicals in the biogenesis of melanin.

(*a*) *Origin of the Paramagnetism*

A matter of primary importance is the origin of the resonance in melanin. The occurrence of the e.s.r. with a g-value near 2 is consistent with a free radical origin,

but does not establish it. Possible alternatives include: transition element ions, melanin behaving as a semiconductor (Longuet-Higgins, 1960); or overlap of the first excited triplet state with the ground state singlet. (The latter is an extrapolation of Lyons' results, Lyons, 1962.)

The possibility of the observed resonances arising from contaminating transition element ions seems remote because of their appearance, *g*-value, and the Cu^{++}-quenching effects observed in natural melanins. Because of the striking similarities in their several paramagnetic properties, and infrared absorptions (see below) of the synthetic and natural melanins, a common origin for their unpaired electrons seems likely. Since the autoxidized melanins were prepared from highly purified starting materials, transition element ions are known not to be present in quantities sufficient to account for the resonances.

The intensity of the e.s.r. absorption of the squid melanin followed Curie's Law down to 4.2°K. If melanin is an intrinsic semiconductor, the energy gap associated with it can certainly be no greater than 10^{-2} ev. An energy gap of magnitude kT would cause a loss in resonance intensity below a temperature of the order of *T;* and in fact no such loss was observed down to a temperature of 4°K. The melanin spin density is not an exponential function of temperature; therefore, the resonance cannot be due to thermally excited electrons in a conduction band. The failure of both alkali and strong H_2SO_4 to affect the e.s.r. signal is further evidence against the model of proton traps which partially empty the valence bands (Longuet-Higgins, 1960).

The experimental discrimination between the trapped free radical model and the triplet-singlet band overlap model is a more difficult problem. It is well known that free radicals can be occluded or buried in free radical generated polymers. Some of these traps are so tight that the free radical is stable indefinitely. Hence, the fact that heating in O_2 at 200-500° does not destroy the signal, but simply burns up the sample, is understandable. The insensitivity to the action of ascorbic acid is also understandable for this reason. However, small metal ions capable of undergoing oxidation-reduction reactions (ferric and cupric ions) may be able to diffuse to these reactive sites and destroy the free radical. The effect of H_2O on the Cu^{++} and melanin e.s.r. signal implies it also can penetrate into the vicinity of these tight traps. The question as to why copper ions and water molecules do so and oxygen does not is unanswered. The observation that a large amount of Cu^{++} is adsorbed before the e.s.r. signal is quenched (Fig. 3), can be explained by assuming the existence of a large number of relatively exposed complexing sites which the Cu^{++} ions may favor to the free radical sites. According to the free radical model the unpaired electron cannot be exposed on the surface, since $O_2 + OH^-$ would oxidize it to a stable diamagnetic form.

All of this chemical evidence would be difficult to reconcile with a triplet-singlet band overlap model. First of all such a model depends on the presence of extensive

conjugation. A disturbance of this conjugation by reduction with ascorbic acid should destroy the signal, whereas we observe the signal to be unaffected. It is also difficult to see why so many cupric ions are needed to destroy the e.s.r. signal if they were simply splitting the overlapping singlet-triplet bands.

In addition to these difficulties both the g-value and T_1 are not what one would expect on the basis of such a delocalized electron model. The observed g-value is more characteristic of an electron localized on a single quinone monomer (Blois *et al.*, 1961). The fact that $T_1 > T_2$ implies that there is no exchange narrowing (Goldsborough *et al.*, 1960) and that the spins are localized and at high dilution (Singer and Kommandeur, 1961). The spin density leads to one spin per ~200 monomers. In the melanins, T_1 is a quantity that is only determined by spin-lattice interaction. The evidence is as follows: "packing" the melanin particle with KBr under pressure increases by an order of magnitude T_1; decarboxylation, removal of Ca^{++} and Mg^{++} (these ions induce cross-linking *via*——COO^-—Ca^{++}—^-OOC——) all result in a decrease of T_1 from a value in excess of 0.2 msec. to about 30 μsec. T_1 is also temperature-dependent, expecially so below 77°K. It is interesting to note that Goldsborough, Mandel, and Pake (1960) find for DPPH in polystyrene, at a spin concentration of 5×10^{18}/gm, $T_1 \simeq 20$ μsec. at 300°K and $T_1 \simeq 0.3$ msec. at 77°K. This 300°K value of T_1 is nearly identical with T_1 in *B*-melanin while the ~300 μsec. value at 77°K is much larger than for this melanin sample but similar to the values observed for the Ca^{++}-Mg^{++} salt. Finally, recent structural determinations for melanin (Nicolaus, 1962) indicate that the extensive conjugation required by the triplet-singlet overlapping band model, does not in fact exist.

The trapped free radical origin of the melanin e.s.r. resonances, which was first proposed by Commoner *et al.*, thus seems to be borne out by these results.

(*b*) *Structures of Natural and Synthetic Melanians*

It was hoped some years ago that e.s.r. would be useful in elucidating the structure of melanins: if melanins consisted simply of a single monomer and single bond type, and the paramagnetism were due to trapped free radicals, then one might have expected to resolve a hyperfine structure which in turn should reveal the molecular structure of the radical. This had already been done for the case of polymethylmethacrylate, for example, by Ingram, Symons, and Townsend (1958). Alternatively, if the melanin were highly conjugated and the unpaired electrons were delocalized, interaction with other magnetic nuclei would become extensive and the hyperfine structure would assume a complexity that might not be resolved. In this case, however, the effect of increased conjugation should have been revealed by a pronounced g-value lowering. Experimentally, it was found that neither effect was observed—there was no hyperfine structure and the g-values measured did not correspond to those of a highly conjugated system but instead, to an electron localized to an aromatic ring or two.

The e.s.r. evidence could not, therefore, support the earlier structures for melanin, (Raper's scheme), but is completely consistent with the proposals of Nicolaus (1962). He has suggested that sepia melanin is not a simple polymer of 5,6 indole quinone, but a highly irregular copolymer of 5,6 indole quinone, its quinol, and their 2′-carboxyl derivatives, and the various tautomers. He furthermore proposes that instead of polymerization proceeding through a single bond type, that the bonds may include —C—C—, —C—O—C—, —C—O—O—C—, and —C———H—N—. Such an irregular, cross-linked, copolymer bonded by means of various bond types, can indeed account for the observed e.s.r. results. The variety of monomer types and bonds can account for the inability to detect a hyperfine structure, the general similarity of monomer types accounts for the relatively narrow line, and the lack of conjugation accounts for the localization of the odd electron and the observed *g*-value. The e.s.r. data, while shedding no light upon the specific nature of the monomer or bond types, supports the concept that melanins are an irregular polymer of presumably several monomer and bond types, and that they are not extensively conjugated.

Inasmuch as the complete structure of a single melanin has yet to be determined, it is doubtful whether purely physical comparisons between natural and synthetic melanins should be counted on too heavily. However, it is interesting to note that in a number of specific e.s.r. experiments (spin density *vs.* temperature, quenching of the absorption by Cu^{++}, production of photosignals, range of *g*-values) the naturally occurring and autoxidatively-produced melanins behave similarly. The similarity of their infrared spectra, while perhaps a less sensitive tool in this particular application, is shown in Fig. 5.

(*c*) *The Mechanism of Melanin Biogenesis*

The highly irregular structure of melanin proposed by Nicolaus and supported by the present results is particularly intriguing when considered in a biological context. It is the first instance of such a degree of irregularity to be found in a biological macromolecule, and the interest is heightened by the finding that free redicals are stabilized therein *in vivo*.

The participation of free radicals as intermediates in biological redox reactions was proposed years ago by Michaelis, Shubert, and Smythe (1936) and their participation in several enzymatic oxidation-reduction reactions has been demonstrated by e.s.r. (for example, see Blois *et al.*, 1961). In each of these reported systems, and in accord with Michaelis' conception of biological two-electron transfers, the free radicals exist only as intermediates, and the final fully oxidized or reduced substrates are again diamagnetic. The situation with melanin is quite different, and free radicals are found trapped and stabilized in the macromolecule. Because of the similar behavior in the case or organic free radical polymerizations, the question arises naturally as to whether the free radicals demonstrated in melanins

play a role in their synthesis. In the case of the autoxidized melanins, a free radical synthesis is both expected and demonstrable. The similarities betwen the free radical properties of the natural and autoxidized melanins have already been commented upon, and the possibility that the biogenesis of melanin involves a free radical polymerization is not easily dismissed. The role of the enzymes traditionally associated with melanin formation has been reevaluated. Nakamura (1960) has shown in the case of laccase that while the enzyme catalyzes the oxidation of hydroquinone to the semiquinone, the further oxidation to the quinone does not require the enzyme. The reaction competing with the second oxidation, or the dismutation reaction, is the addition reaction between the free radical and either the quinone or quinol and conditions favoring this will initiate polymerization. Finally, if melanin were biologically synthesized in a free radical polymerization reaction, then the irregularity of the polymer and the cross-linking would at once become expected and a serious question arises as to the constancy of composition of melanin.

Merely to assert that melanin is a random cross-linked polymer understates the inherent complexity of this material as proposed by Nicolaus and fails to emphasize the paradox inherent in the chemistry of melanin. To completely describe a polymer it is necessary to:

1. determine the monomer(s), their sequence if more than one type is present, and whether the polymer is linear (or the spatial representation of it is two- or three-dimensional), and the molecular weight;
2. specify the bonding between each pair of monomers;
3. specify the location of all branch points or singularities (*i.e.*, determine the location of all polyfunctional monomers).

If natural melanin is synthesized by a free radical polymerization, with the several (polyfunctional) monomer and bond types proposed by Nicholaus, it is clear that a very high order of randomness may be involved since each of the above parameters will be statistically distributed. If the biosynthesis affords anything approaching this degree of freedom, then it is simple to show that the number of possible detailed structures (for very modest molecular weights) for melanin is much larger than the number of melanin molecules on earth. This is equivalent to asserting that there are probably no two molecules of melanin alike, and this conclusion alone would convert the statement of Swan, given at the onset, from a progress report to a permanent expectation.

The biological roles of melanin remain poorly understood, except for the employment of melanin in mimicry by lower forms and its photo-protective function in man.

5. SUMMARY

A series of studies by e.s.r. on natural melanins, enzymatically synthesized melanins, and autoxidized melanins have shown that all give e.s.r. absorptions quite similar in

their general properties. It appears that the origin of the observed paramagnetism arises from free radicals trapped in the polymer and stabilized. All samples of melanin which we have examined have failed to reveal hyperfine structure. This latter result is consistent with Nicolaus' proposed chemical structure for melanin, since the trapped radicals consisting of several structural types and localized in different molecular environments would give a resonance in which the individual hyperfine splittings would be lost during the superposition of the individual resonances. The lack of hyperfine structure could be equally well accounted for by the anisotropic hyperfine interactions effective in a solid sample. In summary, our results are in accord with Nicolaus' proposed structures and do not support the concept that melanin is a highly conjugated system.

From the finding of stabilized free radicals in all examined samples of biologically synthesized melanins, and the striking similarities in physical properties of these free radicals with those present in synthetic melanins prepared *in vitro* by a free radical polymerization, it is suggested that biological melanogenesis may proceed *via* a free radical mechanism and without essential participation of enzymes once the initial semiquinone form has been generated.

APPENDIX

The saturation method was used to determine T_1. The expression for the rate of absorption of power

$$I = \tfrac{1}{2}\omega''\chi''H_1^2$$

was employed where

$$\chi'' = \chi_0[\tfrac{1}{2}\omega_0 T_2]/[1 + (\omega - \omega_0)^2 T_2^2 + \tfrac{1}{2}\gamma^2 H_1^2 T_1 T_2.]$$

χ_0 is the Curie susceptibility; ω_0 is the microwave frequency at resonance; ω is the experimental microwave frequency; T_1 is the spin-lattice relaxation time, T_2 is the spin-spin relaxation time. T_2 was estimated from $\Delta H_{1/2}$, the line width of the e.s.r. at half-amplitude using the expression $\gamma\Delta H_{1/2} = 2/T_2 \cdot \Delta H_{1/2}$ was computed from the integrated first derivative of the absorption curve. For the present purposes the Lorentzian line shape relation $\gamma\Delta_{1/2} = 2/T_2$ and the Gaussian relation $\gamma\Delta H_{1/2} = 4 \ln 2/T_2$ are sufficiently close so that the former could be used for simplicity.

To obtain T_1, subject to certain conditions cited below, the derivative trace was integrated and the peak height, divided by the square root of the power, was plotted against H_1 on a log-log paper. The linear portion at the curve was used to determine T_1(at $\omega = \omega_0$) from the following relation

$$\chi_1''[H_1(1)]/\chi_2''[H_1(2)] = 1 + \tfrac{1}{4}\gamma^2 H_1^2(2) T_1 T_2/1 + \tfrac{1}{4}\gamma^2 H_1^2(1) T_1 T_2$$

According to Portis (1953) Equation (1) can be used for homogeneously and inhomogeneously broadened lines as long as $\omega_m T_1 < 1$, where ω_m = frequency of the

modulation envelope of the microwaves. However, when $\omega_m T_1 > 1$ more detailed analysis of the dependence of the signal intensity upon the power is necessary.

A plot of (signal amplitude) $(\text{power})^{1/2}$ *vs.* $(\text{power})^{1/2}$ is equivalent to the plot of $(\chi'' H_1)$ *vs.* (H_1). If $\chi'' H_1$ levels off for increasing H_1, the signal is homogeneously broadened. An inhomogeneously broadened line displays a decreasing $\chi'' H_1$ with increasing H_1 (above a critical value of H_1). (See Portis, 1953, Figs. 1 and 5). The results for melanin indicated an inhomogeneously broadened line. Furthermore when T_1 was estimated using Equation (1) it was found that $\omega_m T_1 > 1$. Again, according to Portis, there is a special case under which Equation (1) is adequate for the determination of T_1. The criterion is whether or not the observed signal fits a curve of the type $1/(1 + \frac{1}{4}\gamma^2 H_1^2 T_1 T_2)$. We found that the dependence of the observed signals upon the power fits such an expression closely. Since this relation is the closest of the four special cases discussed by Portis, one is justified in using Equation (1) at $\omega = \omega_0$ for the determination of T_1. Although the line was inhomogeneously broadened it did exhibit slight broadening at increased power. An estimate of the true line width can be made using Portis' expression for the line width of spin packets in an inhomogeneous population of dilute (< 0.01 per cent) spins in a cubic array. We find for $N = 10^{18}$ spins/cc, where N is the number of spins per unit volume of sample, $\Delta H \cong 0.5$ gauss which is an order of magnitude smaller than the measured line width. In view of the observed broadening at increased power, it is believed that the true line width may be nearer to one-fifth that of the envelope line width.

This will not affect the approximation that T_1 is large compared to T_2. Given the quantity $T_1 T_2$ determined from saturation measurements, and a T_2 in the range of 2×10^{-8} sec. to 10^{-7} sec. we conclude that $T_1 \gg T_2$ for all samples at all temperatures.

It should be noted that this uncertainty in the values of T_1 and T_2 does not lead to comparable uncertainty in the relaxation time corrections for the spin density computation because the saturation factor involves the product $T_1 T_2$ which is a measured quantity and has an uncertainty of $\sim$ 100 per cent, essentially the same accuracy that is to be expected from an e.s.r. measurement on an unsaturated spin system where such a correction is unnecessary.

In the determination of T_1 by the power saturation method it is desirable to work in a power range where $\gamma^2 H_1^2 T_1 T_2 \gtrless 1$. This condition is not generally fulfilled in the case of any of the melanin samples investigated (see, however, Section iii). It is estimated that with an incident power of 4mw, $\frac{1}{4}\gamma^2 H_1^2 T_1 T_2 \simeq 1$. This sets a limit on the accuracy of the method. In addition it should be noted that although T_1 seemed to depend upon the state of hydration in some cases, hydration was not a controlled variable in these experiments. The relaxation times were generally measured on samples equilibrated with the atmosphere and therefore contained 15 to 30 per cent moisture by weight.

It is a pleasure to record the invaluable participation, in various portions of this work, of Dr. J. Vivo-Acrivos, Mrs. Lina Taskovich, and Mr. Nicholas Pappas. We are grateful to Drs. P. Fensham and J. Miller of the Physics Department for the use of their e.s.r. spectrometer for the low power and low temperature (4.2°K) measurements.

This research was supported in part by the United States Air Force under contract AF 18(600)-1511, and by the United States Public Health Service under Grant PHS A-2304.

Dr. A. B. Zahlan is on leave of absence from the Physics Department, American University of Beirut, Beirut, Lebanon.

Received for publication, December 9, 1963.

REFERENCES

ADAMS, M., BLOIS, M. S., and SANDS, R. H., 1958, *J. Chem. Physics,* **28,** 774.

BLOIS, M. S., BROWN, H. W., and MALING, J. E., 1961, *in* Free Radicals in Biological Systems, (M. S. Blois, Jr., H. W. Brown, R. M. Lemmon, and M. Weissbluth, editors), New York, Academic Press, Inc., 117.

BONNER, T. G., and DUNCAN, A., 1962, *Nature,* **194,** 1078.

BREZINA, M., and ZUMAN, P., 1958, Polarography in Medicine, Biology and Pharmacy, New York, Interscience Publishers, Inc., 40.

COMMONER, B., TOWNSEND, J., and PAKE, G., 1954, *Nature,* **174,** 689.

DAWSON, C. R., and MAGEE, R. J., 1955, *Methods Enzymol.,* **2,** 822.

FRAENKEL, G., HIRSHON, J. M., and WALLING, C., 1954, *J. Am. Chem. Soc.,* **76,** 3606.

GOLDSBOROUGH, J. P., MANDEL, M., and PAKE, G. E., 1960, *Physic. Rev. Letters,* **4,** 13.

HOSKINS, R. H., 1955, *J. Chem. Physics,* **23,** 1975.

INGRAM, D. J. E., SYMONS, M. C. R., and TOWNSEND, M. G., 1958, *Trans. Faraday Soc.,* **54,** 409.

KIVELSON, D., and NEIMAN, R., 1961, *J. Chem. Physics,* **35,** 149.

KOLTHOFF, I. M., and LINGONE, J. I., 1952, Polarography, New York, Interscience Publishers, Inc., 227, 482.

KOZYREV, B. M., 1955, *Discussions Faraday Soc.,* **19,** 135.

LERNER, A. B., 1953, Metabolism of phenylalanine and tyrosine, *Advances Enzymol.,* **14,** 73.

LONGUET-HIGGINS, H. C., 1960, *Arch. Biochem. and Biophysics,* **86,** 225.

LYONS, L. E., 1962, Organic Crystal Symposium, Ottawa, 42, (abstracts).

MALMSTROM, B. G., and VÄNNGARD, T., 1960. *J. Mol. Biol.,* **2,** 118.

MASON, H. S., 1948, *J. Biol. Chem.,* **172,** 83.

MASON, H. S., INGRAM, D. J. E., and ALLEN, B., 1960, *Arch. Biochem. and Biophysics,* **86,** 225.

MICHAELIS, L., SHUBERT, M. P., and SMYTHE, C. V., 1936, *J. Biol. Chem.,* **116,** 587; *Science,* **84,** 139.

NAKAMURA, T., 1960, *Biochem. and Biophysic. Research Communications,* **2,** 111.

NICOLAUS, R. A., 1962, *Rass. Med. Sper.,* **9.** No. 1, suppl., 1.

PAKE, G. E., 1962, Paramagnetic Resonance, New York, W. A. Benjamin, Inc.

PIATELLI, M., and NICOLAUS, R. A., 1961, *Tetrahedron,* **15,** 66.

PORTIS, A. M., 1953, *Physic. Rev.,* **91,** 1071.

RAPER, H. S., 1928, *Physiol. Rev.,* **8,** 245.

SINGER, L. S., and KOMMANDEUR, J., 1961, *J. Chem. Physics,* **34,** 139.

SWAN, G. A., 1963, Chemical structure of melanins, *Ann. New York Acad. Sc.,* **100,** 1005.

VENKATARAMAN, B., and FRAENKEL, G. F., 1955, *J. Am. Chem. Soc.,* **77,** 2207; *J. Chem. Physics,* **23,** 588.

VIVO-ACRIVOS, J. F., and BLOIS, M. S., 1958, An electron spin resonance study of stable free radicals in natural and synthetic melanins, *in* Informal Discussions on Free Radical Stabilization, Sheffield, England, Faraday Soc., 93.

WERTZ, J. E., REITZ, D. C., and DRAVNIEKS, F., 1961, *in* Free Radicals in Biological Systems, (M. S. Blois, Jr., H. W. Brown, R. M. Lemmon, and M. Weissbluth, editors), New York, Academic Press, Inc., 183.

WERTZ, J. E., and VIVO-ACRIVOS, J. L., 1955, *J. Chem. Physics,* **23,** 2441; **24,** 470.