Electron Paramagnetic Resonance Studies on Melanins. I. The Effect of pH on Spectra at Q Band

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Abstract: Previous studies of melanin pigments with X-band epr revealed essentially characterless singlet spectra with a signal intensity which varied with pH and other environmental factors. Natural melanins (mostly from the bovine eye) and enzyme-synthesized or air-oxidized melanins demonstrate at Q-band broader asymmetrical spectra composed of two components with different pH dependencies. Melanins from indolamines and indolizable substrates give Q-band epr spectra at pH 10–12 which are very similar to each other and to those from animal melanin pigments, but that are significantly different from catechol melanin spectra, thus confirming the presumed indole-polymer nature of the former. The findings are explained by considering *g*-value *shifts* as unpaired electron density on oxygen increases with pH and by attributing the greater signal widths at Q band than at X band to *g*-value *anisotropy* due to electron delocalization over heterocyclic nitrogen atoms. The epr spectral changes with pH give further evidence that melanin polymers may not be chemically or biologically inert.

It is well known that the black pigments of skin, hair, eyes, and brain, the melanins, have an epr signal which is sensitive to pH changes and to other environmental manipulations.[3–7] Previous reports have shown the signal from natural melanins at X band (9.5 GHz, 3-cm wavelength) to be slightly asymmetrical,[5,8] the peak being broader on the high-field side. It was also pointed out that the signal increases in amplitude with increasing pH of the suspending medium.[5]

In this study we examined aqueous suspensions of natural melanins, obtained from the eye, at different values of pH and examined suspensions of several synthetic melanins prepared by air oxidation or by the action of tyrosinase on dopa (3,4-dihydroxyphenylalanine) and catechol or of mitochondrial suspensions on dopamine (3,4-dihydroxyphenylethylamine), norepinephrine, tryptamine, and serotonin (5-hydroxytryptamine). It was found that the characteristic epr signal of melanins changes in shape and amplitude, and it appears to have at least two distinct components at alkaline pH.

Experimental Section

Melanins. Bovine eyes were iced from the time of enucleation, and melanins were extracted by removal of the iris, choroid, and ciliary processes from each eye and digestion of these in concentrated (12 *N*) hydrochloric acid at room temperature for 1 week. The suspending acid was changed three times during the week by centrifuging the mixture, discarding the supernatant, and resuspending the melanin in fresh acid. The products of this digestion are designated as "mixed eye melanins."

The enzymatic and amphiuma liver melanins were provided by M. H. Van Woert, prepared by the method of Van Woert, Prasad, and Borg,[7] and the synthetic melanins were made by air oxidation with tyrosinase or with mitochondrial catalysis. Nonenzymatic air oxidation was carried out at pH 9–10 (usually in carbonate buffer), with the samples standing at room temperature for several days with intermittent shaking.

Epr Spectra. A Varian V4502 epr spectrometer operating with 100-KHz modulation frequency and fitted with a Varian V4561 Q-band (35 GHz, 8-mm wavelength) microwave bridge and reflection cavity (TE_{011} mode) was used. For X-band spectrometry a V4500-41A bridge and V4531 cavity (TE_{102} mode) were employed.

At Q band slurries of melanin particles were examined in precisely centered plastic tubing of 0.24-mm i.d., and *g* values were determined by intercalibration with a solution standard of Fremy's salt (peroxylaminedisulfonate, $g = 2.0055$). At X-band polycrystalline DPPH ($g = 2.0036$) was used as an intercomparison standard.

Melanin epr signals reveal microwave power saturation,[8] resulting in lower spectral resolution, so microwave power from the Q-band bridge usually was attenuated by 10–20 db (nominal output *ca.* 150 mW), depending on the signal strength. A 10-db attenuation produces barely detectable signal rounding and saturation broadening from eye melanins in comparison with an attenuation of 15–20 db, but signal-to-noise ratios are slightly decreased with the latter. Higher microwave power increases distortion and finally decreases signal amplitudes. Catechol and 5-hydroxyindole melanins reveal detectable Q-band saturation at −16 to −20 db. At X band gross saturation (decrease in signal amplitude with obvious broadening) occurs above about 37 mW (−6 db), but slight sharpening of signal shape is gained with attenuation up to 20 db (1.5 mW).

Results

For all of the melanins studied the Q-band epr signal at acid pH is nearly symmetrical (Figure 1). The signal begins to change in the range of pH 8–9 and, with the exception of air-oxidized 5-hydroxyindole melanin, is asymmetrical in basic solution (Figure 1). In the light of these findings, the mild asymmetry in alkali at X band (Figure 2) becomes more apparent.

At Q band the epr spectrum in alkali appears to represent a composite of the singlet seen in neutral or acid media plus a superimposed narrower singlet at lower magnetic field (Figure 2). With melanins made from catechol, catecholamines, or indolamines media of even higher pH (over 13) give rise to the low-field singlet only.[9,10] The natural eye melanins, however,

(1) To whom inquiries should be sent.
(2) Supported by the U. S. Atomic Energy Commission.
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(10) This was confirmed by epr absorption spectra run at 60 GHz by W. E. Blumberg (Bell Telephone Laboratories). At high alkalinity only a relatively narrow singlet was observed, but nearer neutrality this was superimposed on a broader absorption line of slightly lower *g* value.

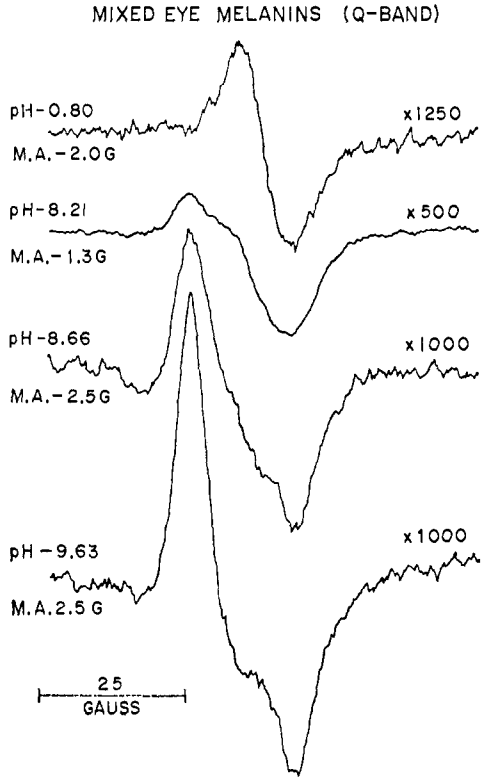

Figure 1. Epr spectra. M.A. = modulation amplitude, in gauss. Microwave power attenuation = 15 db. In these and subsequent spectra magnetic field increases from left to right.

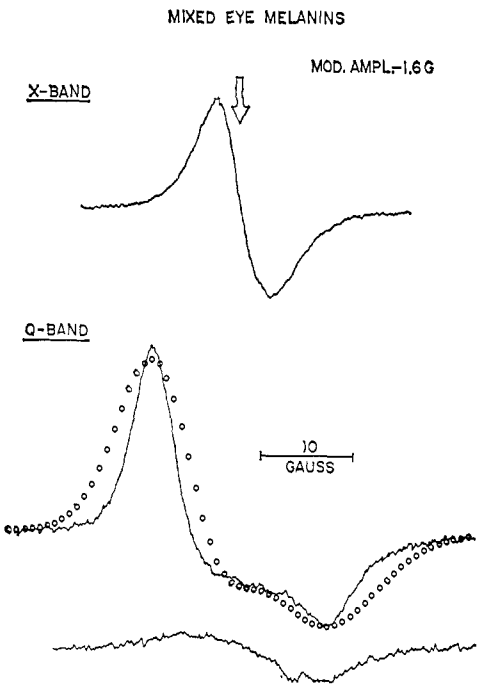

Figure 2. Epr spectra. X band: alkaline pH, power = −6 db (37 mW). The arrow is at $g = 2.0042$. Q band: alkaline (upper) and neutral (lower) pH, power = −15 db. Circles represent calculated values from a computer synthesis (see text).

never lose the high-field component, even in strongly alkaline pH. Furthermore, incubation of eye melanins for several weeks in D_2SO_4–D_2O or for several days in NaOD–D_2O gives rise to spectra indistinguishable from the usual Q-band signal (*e.g.*, see Figure 2).

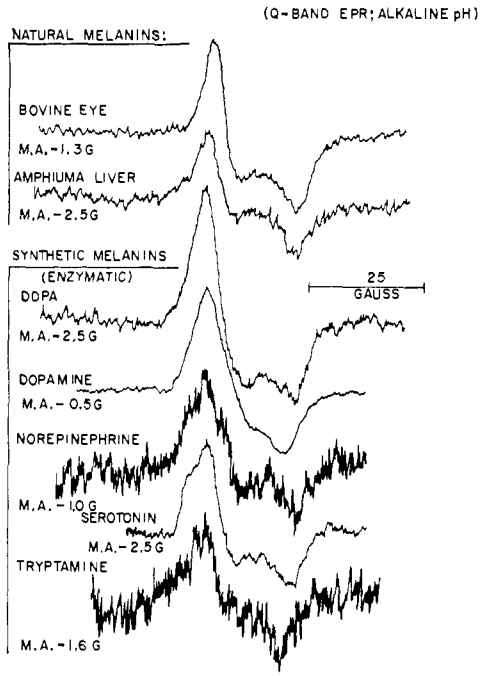

Figure 3. Epr spectra. M.A. = modulation amplitude, in gauss. Power = −15 db except for amphiuma and dopamine (−20 db) and for serotonin (−10 db).

At pH values below about 13, however, natural and synthetic melanins made from phenolic amino acids, phenolamines, or indolamines yield epr spectra that are qualitatively the same. This is exemplified by Q-band spectra taken from alkaline suspensions in the pH region 10–12, where the asymmetrical character of the melanin signal is most prominent (Figure 3). Despite different intensities, reflected in the signal-to-noise ratios, the spectral breadth and contour is similar for all examples in Figure 3.

This similarity is to be distinguished from a comparison with synthetic melanins derived from phenols alone, for example, the dihydric phenol, catechol. Even at X band, the signal from catechol melanin differs from that educed from indole melanins,[9] although a similar pH dependency obtains for both classes. At Q band, catechol melanin shows a singlet epr spectrum at pH values below neutrality, with a composite asymmetric structure appearing in weak alkali (Figure 4), and the low-field component alone persists at pH 14. However, in comparison with any of the typical indole melanins, the catechol melanin signal is much narrower at Q band, both in over-all width and in the width of any one component (Figure 4).

Although melanins formed from catecholamines and indolamines by *either* mitochondrial catalysis or simple autoxidation in weak alkali give epr spectra at Q band that are "typical" of natural melanins in the region of pH 10–12 (*e.g.*, Figure 5), a simple indole melanin appears different. Indole itself yielded no melanin upon attempted enzymatic synthesis or air oxidation, presumably because of the absence of a bridging substituent for facile polymerization. However autoxidation of 5-hydroxyindole gives rise to a melanin[9] whose Q-band epr spectrum reveals only a singlet throughout the pH range where the natural and other synthetic melanins exhibit a pH-sensitive composite spectrum,

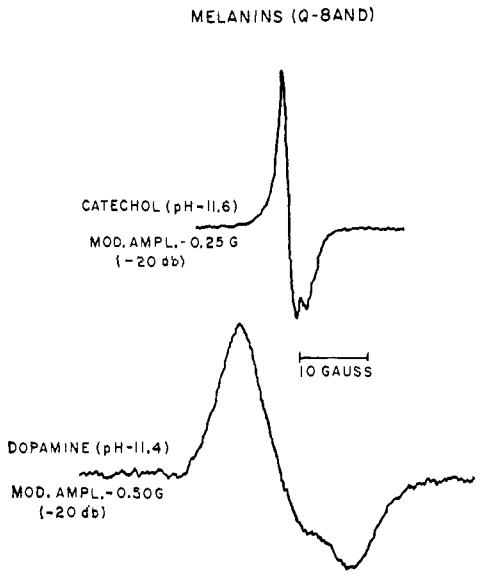

Figure 4. Epr spectra. Upper: catechol melanin from autoxidation in air-saturated alkaline solution. Lower: dopamine melanin from enzymatic oxidation.

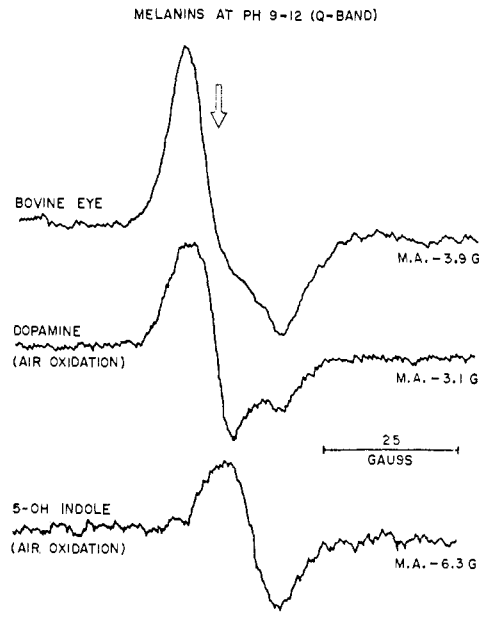

Figure 5. Epr spectra. M.A. = modulation amplitude, in gauss. Upper: mixed eye melanin (−10 db). The arrow is at $g = 2.0039$. Middle: autoxidized dopamine (−10 db). Lower: autoxidized 5-hydroxyindole (−16 db).

and this single broad (10–12 gauss) line coincides with the high-field component of the other melanin spectra (Figure 5).

Discussion

Interpretation of the asymmetrical envelope of melanins' Q-band epr spectra as a composite of two spectra with different average g values appears reasonable in light of the independent response of the two components to variations in pH. Furthermore, the general configuration of a Q-band epr spectrum at moderate alkalinity could be simulated by computer synthesis, assuming a line of Gaussian shape and 14-gauss width[11] shifted 8.6 gauss upfield ($\Delta g = -0.0014$) from a 9-gauss line 1.3 times as high (in terms of apparent signal amplitude) (Figure 2). Although these values provide an apparent peak-to-peak separation and relative height corresponding to the experimental spectrum, the contour of the actual spectrum is reproduced only qualitatively (Figure 2). No doubt this is because the melanin epr signal components are, in fact, not of pure Gaussian or Lorentzian shape nor intrinsically completely symmetrical[12] (see later discussion of g-value anisotropy), whereas the computer was programmed only for symmetrical lines of pure or mixed Gaussian and Lorentzian shapes.

One plausible explanation for the pH dependency of the melanin Q-band spectra is that dissociation of protons from free-radical center(s) in alkaline pH produces an anion radical of higher g value. Thus in 5-hydroxyindole melanin, where the single hydroxyl substituent of the monomer presumably has been converted to a bridging group during melanin polymerization, the absence of a dissociable moiety gives rise to no high-g signal (Figure 5). Probably in other melanins coulombic repulsion from a deprotonated hydroxyl group alters the delocalization of the unpaired electron so that in comparison with the undissociated radical there is increased spin density on a nitrogen or oxygen atom other than that of the dissociated hydroxyl group. The appreciable spin–orbit coupling of oxygen or nitrogen then raises the g value of the free-radical anion, resulting in the lower field singlet. At intermediate pH values both dissociated and protonated forms of the free-radical center(s) may coexist, while at low pH the protonated form predominates. Because g-value spectral shifts are linearly dependent upon magnetic field strength, the composite nature of the asymmetrical melanin epr signal in alkali is less apparent at X band than at Q band, where the resonance field is some 3.7 times greater.

Upon examination at Q band of synthetic melanins suspended in alkaline media, the same asymmetry seen with the natural melanins was demonstrated (Figure 3). The only exception found so far among indoles was the singlet obtained at Q band from 5-hydroxyindole melanin. Hence we conclude that the epr asymmetry is inherent in the nature of the polymer and is more a characteristic of the polymer and its linkages than of the basic subunits of which it is comprised. The melanin epr signal is generally attributed to free radicals (of one or of several different varieties) trapped in the polymer.[3,7,8] If, as Nicolaus[13] postulates, most melanins consist of highly irregular copolymers of indole quinones, quinols, 2′-carboxyl derivatives, etc., it is not unreasonable to expect that synthetic melanins formed from various monomers would behave similarly to each other and to natural melanins, as Blois, Zahlan, and Maling[8] and Van Woert, Prasad, and Borg[7] have found at X band, and as we show here at Q band. That melanin synthesis is not completely random, however, at least in its early stages, is indicated by X-band studies of melanogenesis which reveal precursor radicals with resolvable hyperfine structure, leading to a final melanin product with typical epr characteristics.[9]

The synthetic melanins discussed above are similar in that all derive from monomers which are indoles or

(11) Peak-to peak line width in the first derivative presentation; width at half-height in the absorption mode.

(12) Examine carefully the upper spectrum of Figure 1 or the lower spectrum of Figure 2 for examples of component asymmetry.

(13) R. A. Nicolaus, *Rass. Med. Sper.*, **9**, *Suppl. 1*, No. 1 (1962).

which can undergo oxidative indolization. When melanin is formed either enzymatically or by alkaline air oxidation from catechol, a monomer not containing nitrogen and therefore unable to indolize, the Q-band epr signal and its individual components are much narrower than with indole melanins (Figure 4). Furthermore, Figure 2 reveals that for indole or natural melanins the individual lines of the epr spectrum at Q band are wider than the full signal at X band. Since attempted deuterium substitution from the suspending medium did not alter the Q-band spectra, this intrinsic breadth of the spectral lines is not apt to result from unresolved hyperfine structure from exchangeable protons.

These findings may be understood if, in natural melanins (eye and amphiuma liver) and the synthetic melanins other than that from catechol, the unpaired electron distribution is delocalized to include the heterocyclic indole nitrogen. (The melanin from catechol polymerization contains no nitrogen.) Since electron density on nitrogen results, in most cases, in *g*-value anisotropy and because this will not average to zero in a suspension of insoluble (solid) melanin particles, there is broadening of the envelopes of even the individual components of the epr composite spectrum. Consistent with the explanation of *g*-value *shifts* offered above, the broadening effect of *g*-value *anisotropy* would be greater at Q band than at X band.

Coupling this reasoning to the previous discussion of higher *g* values attributed to dissociated forms of melanin's free-radical centers raises the possibility that the narrower intrinsic width of the low-field line in the Q-band spectra (*e.g.*, Figure 2) may denote a shift of unpaired electron density from indole nitrogens to oxygens of ether linkages or of uncharged hydroxyl groups. This would imply that greater spin–orbit coupling,[14] but lower *g*-tensor asymmetry on oxygen than on nitrogen, could lead to a higher average *g* value but with less anisotropic broadening of the spectral envelope for the former.

From the interpretation developed in these comments it would follow that the broad, asymmetrical Q-band epr spectra recorded from animal melanins in alkali (Figures 1–3) confirm the expectation[15,16] that the melanin chromophore of animal melanoproteins is a condensed polymer composed largely of indoles. The epr data alone do not clearly distinguish whether the indole monomer units are primarily of one kind or, as Nicolaus considers more likely,[13,15] of mixed types.[7] However, some identifying information is suggested by one plausible explanation of the observation that Q-band epr spectra of natural melanins remain asymmetrical at high pH, where the high *g* singlet alone characterizes the spectra of synthetic melanins. Should the natural melanins consist of indolamine units plus some admixture of unsubstituted indoles or other congeners lacking dissociable groups on the indole nucleus, then persistence of a low *g* spectral component refractory to high pH would be expected, as in the case of 5-hydroxyindole melanin (Figure 5) discussed previously. Alternatively, the natural melanins might be larger or more tightly meshed polymers than are the synthetic melanins such that some protons are shielded internally so that they are unable to dissociate, resulting in high-field epr signals that do not disappear completely in alkali.

Since melanogenesis from mitochondrial preparations has been attributed largely to the action of monamine oxidase,[7,17,18] it may appear inconsistent to ascribe an indole character to the melanins synthesized from catecholamines by mitochondrial catalysis, because oxidative deamination of these monomers would preclude indolization and could, at best, yield catechol-type melanin polymers. However, one of the main reasons for attributing melanogenesis to monamine oxidase is the marked inhibition of melanogenesis by monamine oxidase inhibitors, but this has been documented primarily with serotonin as the substrate,[18] and there is evidence that monamine oxidase manifests different substrate specificity for phenolamines than for indolamines[19] so that pigment formation from catecholamines, such as dopamine, is carried out predominantly by other enzyme systems in mitochondria.[20] Hence in our hands synthetic indole melanins probably were produced by mitochondrial catalysis from all the substrates used, monamine oxidase activity predominating in the oxidation of the preformed indolamines, serotonin, and tryptamine (Figure 3), and with other enzyme systems more active in melanogenesis from catecholamines.

It is believed by some that melanins not polymerized directly from L-dopa and its metabolites, *e.g.*, dopamine melanin[21] and norepinephrine melanin,[22] may indeed exist *in vivo* in the pigmented livers of certain amphibians[7] (Figure 3) and, especially, in the pigmented areas of the brain, the substantia nigra[7] and the locus coeruleus. Since melanins undergo relatively few chemical reactions, their paramagnetism may play a role in their function, if any, in the brain and eye. Alternatively, the behavior of melanins as weak acid ion exchangers in the binding of metal ions[23] and our present data demonstrating the sensitivity of their epr spectra to pH (Figure 1) reflect a responsiveness of melanin subunits to the surrounding environment. This reactivity, in conjunction with the random distribution within the condensed melanin chromophores of the free-radical centers responsible for their paramagnetism, is compatible with melanins reacting as loosely meshed redox polymers with appropriate substrates. Figge proposed a melanin redox system after noting reversible bleaching with dyes,[24] and epr observations of melanin interactions with hormonally active compounds,[25] with albumin,[26] and with electron-donating drugs[27] also support this speculation.

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