Ion-Exchange in Melanin: An Electron Spin Resonance Study with Lanthanide Probes

Abstract. *Changes are induced in the electron spin resonance signal amplitude and microwave power saturation of the naturally occurring free radical in melanin by bound paramagnetic ions. The changes serve as experimental observables in competition experiments between diamagnetic and paramagnetic metal ions for melanin binding sites and between melanin and ethylenediaminetetraacetic acid for paramagnetic metal ions. Evidence is presented for the existence of several specific types of metal binding sites. The interaction of copper with free radicals leading to loss of electron spin resonance signal amplitude is magnetic in nature and not, as has been supposed, chemical.*

Melanin is an irregular polymer consisting of quinoid-type monomeric subunits. It is the pigment found in hair, eyes, and skin, and also occurs in other tissues including the substantia nigra of the brain of primates. It is produced in quantity by the aggressive malignant melanoma tumor. It is opaque, insoluble, and has little order, making it unsuitable for many types of analysis.

Melanin contains phenolic hydroxyl, carboxyl, and amine groups, which provide a number of different potential binding sites for metal ions (*1*, *2*). Bruenger *et al.* (*3*) studied the metal binding properties of melanins by using radioactive isotopes. They showed that the reaction characteristics of melanin and of weak acid cation exchangers were similar. Natural melanins from many sources are known to contain metals, which has led to the speculation that one function of melanin is to serve as a trap for unwanted metals.

Commoner *et al.* (*4*) observed an electron spin resonance (ESR) signal from stable free radicals in natural melanins. The spectrum consists of a single, rather featureless line resembling that of a randomly oriented π-electron free radical of the semiquinone type. No other biological polymer has been found that contains a stable free radical.

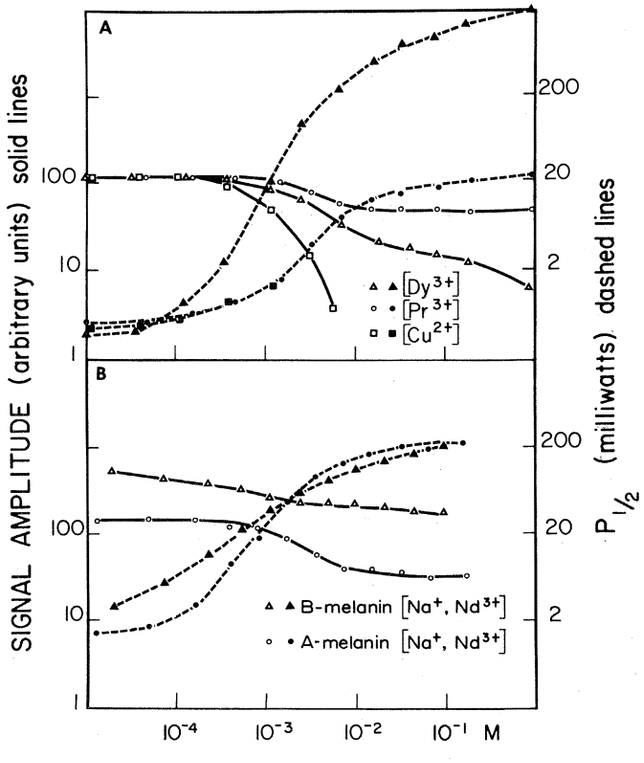
The free radical of melanin is quite nonreactive. An isolated exception to this generalization is the result of Blois *et al.* (*5*) that excess Cu^{2+} (in the ratio of 100 Cu^{2+} ions to one free radical) nearly eliminates the free-radical signal. They interpreted this as a chemical reaction between copper and free radical. This result has remained puzzling and inconsistent with the slowly developing understanding of melanin.

Blois *et al.* may have considered the possibility that the effect they observed was the consequence of a magnetic dipole-dipole interaction between paramagnetic copper and free radical, and rejected this possibility because no broadening of the ESR signal of the free radical was observed. However, Leigh (*6*) showed that there are circumstances under which a nearby paramagnetic center with a short spin-lattice relaxation time can appear to decrease the amplitude of a free-radical ESR signal with little change in the derivative line shape. It has occurred to us that the apparent reaction of melanin free radicals with paramagnetic copper could be a purely physical consequence of a magnetic dipolar interaction as described by Leigh.

The experiments described below confirm this hypothesis. In the course of the work, other paramagnetic transition-metal ions (Mn^{2+}, Fe^{3+}, Co^{2+}, and Ni^{2+}) and most of the lanthanides were investigated. Our point of view has gradually shifted until we now regard the free radical as a natural probe that enables us to detect the presence of paramagnetic metals on the surface of melanin granules.

Melanin was of type A, prepared from the choroid of bovine eyes according to the technique of Plumer and Kopac (*7*). This is a gentle extraction procedure that leaves protein still incorporated in the granules. It is considered that this material closely resembles native melanin. Samples were suspensions of about 10 mg of dried material per milliliter of distilled water.

Lanthanides have been widely used as shift reagents in nuclear magnetic resonance (NMR) (*8*). To a good approxima-



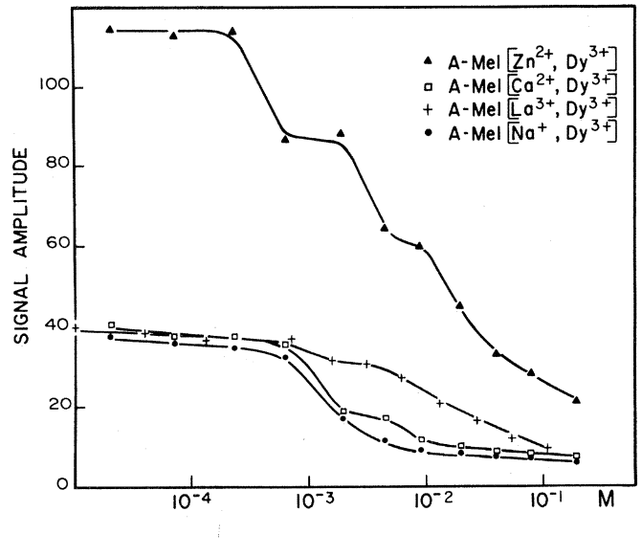


Fig. 1 (left). Signal amplitude and $P_{1/2}$ titration curves. (A) Type A melanin; (B) comparison of type A and type B melanin. Incubation in $10^{-1}M$ Na^+ before addition of Nd^{3+} was employed in order to maintain a constant ionic strength during the titration. Fig. 2 (right). Titration of paramagnetic metal ions competing with diamagnetic metal ions. For di- and trivalent ions, the *p*H and ionic strength do not change significantly during the titration.

tion, the chemical properties of the elements in the series are the same. Since the crystal field is a small perturbation, the magnetic properties are similar to those of the free ions. The range of spin-lattice relaxation times is large, but the times are so short that we observed no ESR signals from lanthanides except for Gd^{3+}.

Leigh (*6*) allowed the static dipolar interaction between metal and free radical to be modulated by spin-lattice relaxation of the metal at a rate that was comparable to the dipolar interaction (expressed in units of frequency). For an unordered solid, the integral for the free-radical line shape, which he solved by machine computation, is

$$L(H) = \int_0^{2\pi}\int_0^{\pi} \frac{\delta H}{\left[(H - H_0)^2 + \delta H^2\right]} \sin\theta d\theta d\varphi \quad (1)$$

where

$$\delta H = \gamma_f \mu_m^2 T_{1m} r^{-6}(1 - 3\cos^2\theta')^2 + \delta H_0 \quad (2)$$

Here θ' is the angle between the radial vector and the magnetic field H_0 and may be related to θ and φ (which express the angular dependence of the free-radical resonance) by geometry, T_{1m} is the spin-lattice relaxation time of the metal, μ_m is the effective dipole moment of the metal, δH_0 is the natural line width, and γ_f is the gyromagnetic ratio of the free radical. As $T_{1m} \to 0$, $L(H) \to \delta H_0$. The most rapidly relaxing lanthanides exert minimal effect on the free-radical line shape $L(H)$.

For very rapidly relaxing paramagnetic metals, the fluctuating magnetic dipole field seen by the free radicals provides a powerful spin-lattice relaxation mechanism. A calculation of Bloembergen (*9*) of the influence of paramagnetic metals in solids on relaxation of nearby nuclei can be used (*10*).

$$T_{1f}^{-1} = \frac{3\gamma_f^2\mu_m^2 \sin^2\theta' \cos^2\theta' r^{-6} T_{1m}}{1 + 4\pi^2\nu^2 T_{1m}^2} \quad (3)$$

A minimum occurs when $T_{1m}^{-1} = 2\pi\nu$, where ν is the microwave frequency. This condition is satisfied for some of the rapidly relaxing lanthanides.

Free radicals of melanin are found, on the average, once every 200 monomers. In the experiments described here, concentrations of metal ions bound to the surface of melanin range approximately from 1/100 to 100 times the free-radical concentration. Nothing is known about free-radical or metal binding site distributions.

We have studied the interaction of Cu^{2+} with melanin in some detail. The free-radical signal drops to 1 percent of its normal level when the copper concentration is $10^{-2}M$ (Fig. 1A). An essentially identical result is obtained with $10^{-2}M$ Gd^{3+}, but no change is observed when La^{3+}, which is diamagnetic, is added to melanin. Since La^{3+} and Gd^{3+} have similar chemical properties and Gd^{3+} and Cu^{2+} have similar magnetic properties, we conclude that magnetic interaction between copper and free radical is sufficient to explain the observed loss of free-radical signal.

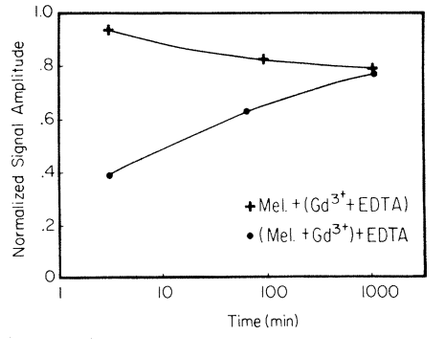


Fig. 3. "Associative competition" of melanin and EDTA for Gd^{3+}.

Both Gd^{3+} and Cu^{2+} exhibit the same degree of microwave power saturation at 35 Ghz and 77°K. No saturation of these ions could be observed at X-band, but it seems reasonable to assume that their relaxation times are generally similar. If one further assumes that the spatial distributions of the two metals with respect to the free-radical sites are the same, Eqs. 1 and 2 predict that they will have the same effect on the ESR signal. Other lanthanides have much shorter spin-lattice relaxation times, and Leigh's theory predicts a smaller reduction of signal amplitudes. Our data on other ions are collected in Table 1. Entries have been ordered by increasing signal amplitude (and in part by $P_{1/2}$ values, as described later). Assuming the validity of Eqs. 1 and 2, this ordering is with decreasing T_{1m}. It is similar to that obtained in high-resolution NMR with lanthanide shift reagents (*8*, p. 529).

Table 1. Values of $P_{1/2}$ and free-radical signal amplitude for various paramagnetic ions added to suspension of melanin (10 mg/ml). Concentrations of metal ions were all $5 \times 10^{-3}M$, except as noted. Experiments were at X-band and −150°C. Chloride salts (or sulfates in the case of Zn^{2+}, Cu^{2+}, and Ni^{2+}) were used.

System	Amplitude (%)	$P_{1/2}$ (mw)
Melanin in H_2O	100	1.3
Mn^{2+} ($5 \times 10^{-4}M$)	37 ± 8	22.5
Gd^{3+} ($5 \times 10^{-4}M$)	40 ± 10	16.0
($1.5 \times 10^{-3}M$)	5 ± 1	
Cu^{2+} ($5 \times 10^{-4}M$)	70	9.0
($1.5 \times 10^{-3}M$)	20	
Ni^{2+}	17 ± 3	11.0
Ho^{3+}	30 ± 3	112
Tm^{3+}	29 ± 3	400
Dy^{3+}	26 ± 5	315
Er^{3+}	35	200
Co^{2+}	33 ± 4	141
Nd^{3+}	40 ± 1	56
Sm^{3+}	38 ± 2	24
Pr^{3+}	48 ± 2	20

Bruenger *et al.* (*3*) observed reduced uptake of metal by melanin at low pH, as expected from a weak acid cation exchanger. The ESR spectrum of copper bound to melanin differs from that of free copper in water, and the amount of each can be readily determined. As the pH is lowered in a melanin-copper preparation, the amount of free copper increases, the amount of bound copper decreases, and the ESR free-radical signal increases. If the sample is then frozen and the initial concentration of Cu^{2+} is rather high, a decrease of ESR free-radical signal is observed. This is the consequence of magnetic dipolar interaction between bulk free copper in the ice and free radical in melanin. We find that the amount of added metal must be increased between one and two orders of magnitude at low pH in order to obtain the reduction of the free-radical signal that is observed at higher pH.

We have studied the ESR signal height of melanin-metal preparations as a function of temperature. One would predict that as the temperature is lowered there would be two effects on the ESR signal amplitude of opposite sign: a decrease because of increasing T_{1m} (see Eqs. 1 and 2) and an increase because of the Boltzmann factor. This has been observed, although the temperature dependence is complex since changes of T_{1m} result in changes of line shape as well as signal amplitude.

One expects spin-lattice relaxation times of lanthanides between 77° and 300°K to be very short. Equation 3 then predicts a strong reduction of the spin-lattice relaxation time of nearby free radicals. We express free-radical saturation characteristics in terms of the instrumental parameter $P_{1/2}$, which is the microwave power in milliwatts at which the signal amplitude is half of what it would be if no saturation occurred.

Referring to Table 1, values of $P_{1/2}$ are highest for Dy^{3+} and Tm^{3+}. We conclude that $2\pi\nu T_{1m} \approx 1$ for these ions at −150°C. For ions listed above these two entries in Table 1, the reduction of the ESR signal amplitude is greater and $P_{1/2}$ is reduced, which indicates that $2\pi\nu T_{1m} \gg 1$; similarly, for entries below Dy^{3+} and Tm^{3+}, $2\pi\nu T_{1m} \ll 1$.

One expects T_{1m} to increase when the temperature is lowered. If $2\pi\nu T_{1m} \ll 1$, lowering the temperature should then decrease T_{1f}, and $P_{1/2}$ should increase. This

was observed for Sm^{3+}, Nd^{3+}, Co^{2+}, Tm^{3+}, and Dy^{3+}, while the reverse was found for Cu^{2+}, Ni^{2+}, Gd^{3+}, and Ho^{3+} where $2\pi\nu T_{1m} > 1$.

Titration curves where the ESR signal amplitude and $P_{1/2}$ are plotted as functions of added metal concentration are shown in Fig. 1A. We waited about 15 minutes after each addition of metal before examining at 77°K. Of the ions studied, Pr^{3+} has the shortest relaxation time. At the highest concentration a reduction in ESR signal height of only a factor of 2 is observed. The ion Dy^{3+} is a very effective relaxation agent, changing $P_{1/2}$ by three orders of magnitude.

The S-shaped titration curves of Fig. 1A indicate a saturation of all metal binding sites at a metal concentration of $10^{-2}M$. This corresponds to 6×10^{20} metal binding sites per gram of dried material. The *p*H varies from 5.5 to 3.8 as metal is added and hydrogen ions are released.

Titration curves for type A and type B melanin are shown in Fig. 1B. Type B melanin was prepared from type A by digestion in 6*M* HCl in sealed tubes at 95°C for 120 hours, which removes protein (*2*). Figure 1B indicates that metal binding for type B melanin is less specific than for type A.

Figure 2 shows the result of metal-ion competition experiments. The sample was incubated for 2 to 3 hours in a $10^{-1}M$ solution of the first ion shown in brackets, which is diamagnetic. The free-radical ESR intensity was then plotted as a function of concentration of the second ion (usually Dy^{3+}). The incubation time in Dy^{3+} was 15 minutes for each experimental point. Data in Fig. 2 were obtained at room temperature (22°C).

The [Na^{+}, Dy^{3+}] curve is on Fig. 2 for reference. The chemical properties of La^{3+} and Dy^{3+} are similar, and the [La^{3+}, Dy^{3+}] curve indicates that the probability of Dy^{3+} replacing La^{3+} is determined primarily by the statistical abundance of the ions.

Incubation of melanin in zinc has been shown to increase the free-radical signal height (*11*). Thus the [Zn^{2+}, Dy^{3+}] titration curve starts from a higher level. We regard the well-defined steps in this curve as one of the most interesting results of this work. They apparently indicate the presence of several distinct types of binding sites. A similar step is observed in the [Ca^{2+}, Dy^{3+}] curve.

Since melanin binds metal very tightly, we compared the binding strength with that of a strong metal chelator, ethylenediaminetetraacetic acid (EDTA). We mixed $5 \times 10^{-3}M$ Gd^{3+} with a tenfold excess of EDTA and added it to melanin, observing the free-radical signal as a function of time after mixing. The results are shown in Fig. 3. Data are also plotted for a parallel experiment in which $5 \times 10^{-3}M$ Gd^{3+} was added to melanin and a tenfold excess of EDTA subsequently added. Equilibrium is reached in about 10^{3} minutes at an intermediate reduction of the ESR free-radical signal corresponding to the level obtained when $1.5 \times 10^{-4}M$ Gd^{3+} is added to melanin in the absence of EDTA. Melanin thus has some sites that bind metals more tightly than EDTA and some that bind them less tightly. Bruenger *et al.* (*3*) found that EDTA competed more successfully with metal ions at *p*H 7.6 than our results indicate at lower *p*H.

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Oncogenic Transformation of Human Embryo Lung Cells by Human Cytomegalovirus

Abstract. *Persistent infection of human embryo lung fibroblasts with a genital isolate of cytomegalovirus resulted in oncogenic transformation of these cells. Immunofluorescence techniques detected virus-specific antigens, while microcytotoxicity tests established that the transformed cells share a membrane antigen with hamster cells transformed by inactivated cytomegalovirus. The transformed human cells induced progressively growing tumors in weanling athymic nude mice.*

Human cytomegalovirus (CMV) can induce oncogenic transformation of hamster embryo fibroblasts (*1*), and can stimulate synthesis of host cell DNA and RNA (*2*). These properties are commonly associated with known oncogenic herpesviruses (*3*). We reported (*4*) that cells from human prostate tissue which apparently had been infected in vivo with CMV grew in vitro to passage levels well above those routinely attained by normal cells. After a number of passages virus was no longer rescuable, although CMV-specific antigens and nucleic acid continued to be detected in the cells. It is uncertain whether these cells, which exhibited loss of contact inhibition, were transformed by CMV or whether they were chronically infected and then released virus at levels below detection. Therefore, further studies of the transforming capacity of the isolated virus (designated Mj) were undertaken. Since this strain replicated quite slowly in human embryo lung (HEL) cells, the studies were performed without inactivating the virus.

We report evidence that infection of HEL cells with the Mj strain of CMV can lead to long-term persistent infection, and that occasional cell transformants can arise in the cultures. The transformed cells contain CMV-specific membrane and intracellular antigens, and share common antigens with CMV-transformed hamster cells. Furthermore, the cells induce nondifferentiated tumors when injected into weanling athymic nude mice.

Monolayers of HEL cells were initially grown in Dulbecco's medium supplemented with 10 percent fetal calf serum and 0.075 percent sodium bicarbonate. For transformation experiments, Ham's medium with 20 percent fetal calf serum and 0.075 percent sodium bicarbonate was used. Standard procedures for the indirect immunofluorescence test were used for the detection of CMV-specific antigens in the transformed cells (*4*). Human antiserums to CMV were obtained from hospital patients. Fluorescein isothiocyanate–conjugated goat antiserum against human immunoglobulin G was purchased from Cappel Laborato-