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Biophysics

Electron spin resonance absorption of tissue constituents

(cancer/methylglyoxal/crotonaldehyde/color of proteins)

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ABSTRACT **The electron spin resonance spectra at g = 2 of the structural and soluble proteins of mouse liver have been investigated separately. The structural materials have signals an order of magnitude stronger. Radicals with important similarities to those occurring in natural tissue can be induced by treating casein with methylglyoxal or crotonaldehyde. The structural proteins of cancer give little or no signal. The color of the proteins and their electron spin resonance signal seem closely related.**

It is generally assumed that life originated about three and a half billion years ago on a dark globe covered by dense water vapor. There was no light and no oxygen present at the surface. Life has left behind very few traces from this dark and anaerobic period. It seems reasonable to suppose that under those unhospitable conditions it could develop but the simplest forms, capable of performing only the simplest vegetative functions such as fermentation and proliferation. This first fermentative-proliferative anaerobic period has been termed the α-period (1). In this period the atmosphere had to be strongly reducing. In the resulting high electron tension or chemical potential the orbitals of protein molecules had therefore to be fully occupied by electron pairs.

It appears likely that life began to differentiate to complex and more functionally able forms when light could reach the surface of the globe. With its energy, life could decompose water into its elements, hydrogen and oxygen.

Oxygen was, and is today also, made available from the direct action of light on atmospheric water. The hydrogen, with its small mass and correspondingly high molecular velocity that is approximately equal to the escape velocity from earth's gravitational pull, continually escapes, leaving the earth's atmosphere continuously enriched in oxygen. As a result light, both directly, and through the medium of life, has brought us the present oxygen-rich atmosphere.

This second, aerobic phase of life's existence (that in the presence of appreciable amounts of oxygen) is termed the β-period. During the β-period life has succeeded in building more and more complex structures capable of performing increasingly complex functions. The appearance of oxygen meant the appearance of an electron acceptor capable of taking up single electrons and in so doing separating electrons of electron pairs, leading to the production of unpaired electrons, electron holes, and partially occupied orbitals. This lent a high and subtle reactivity to proteins, generating the unbalanced forces capable of linking molecules together to form integrated functional structures.

According to the concepts developed in this laboratory, the simple "vegetative" functions are still performed by soluble molecules with closed shells of electrons, while the more complex "animal" functions which produce work, W, and involve energy transformations are performed by the insoluble structures, characterized by their electronic disbalance. The present study was undertaken to see whether these concepts were borne out at the molecular level by the behavior of tissues.

Electron spin resonance (ESR) signals have been observed in living systems by many authors. It is a common experience that whatever living system is studied, it yields a signal. This signal has mostly been attributed to some unidentified metabolic process. The intensity of the signal seemed to depend on the intensity of the metabolism. In the present case the signal could not have been due to metabolic processes because the sediment in half-saturated ammonium sulfate could have no metabolism. The signal had to be due to the unpaired electrons of the material, in all probability to the structural proteins present.

The complexity of tissues makes the interpretation rather difficult. We have attempted to fractionate the material, hoping that the simplification will make interpretation easier.

EXPERIMENTAL

The livers of adult albino Swiss mice were cut out immediately after death. Then the livers were treated for half a minute on the Sorvall blender in 10 to 20 volumes of a half-saturated, ice-cold ammonium sulfate solution. We expected to extract this way the soluble proteins, and the soluble free radicals possibly produced in metabolism. Structures are by definition insoluble. We separated the insoluble structural part of the liver tissue by centrifugation after having strained our suspension through a double layer of cheesecloth. The soluble proteins of the supernate were later precipitated by saturating the solution with ammonium sulfate. They too were then separated by centrifugation of 6000 rpm on a table centrifuge (approximately $3000 \times g$).

Spin concentrations of the g = 2 line were determined using a Varian E-109 ESR system, provided with a flat silica-glass aqueous solution cell which held 0.10 ml of the precipitate to be examined in the region of the cavity. The spin concentrations were measured against an aqueous reference solution of manganese chloride, the practice of Mallard and Kent (2, 3) being followed to account for the fact that the Mn^{2+} ion has a spin of 5/2. Radical concentrations in the liver samples were calculated assuming a spin of 1/2 for the tissues.

Fig. 1 shows the ESR spectrum near g = 2 given by the structural proteins that had been precipitated by half-saturated ammonium sulfate. The signal was centered at g = 2.002. In a number of experiments spin concentrations between 1.7 and 3.2×10^{15} spins/g were found for the structural proteins. The intensity of the ESR signal exhibited by the structural proteins was also investigated as a function of the microwave power. It can be seen from Fig. 2 that the signal saturated quickly at a power of just a few milliwatts. It was found that the ESR signal disappeared completely if the samples were denatured by immersion in boiling water for as little as 30 sec. The signal had

Abbreviation: ESR, electron spin resonance.

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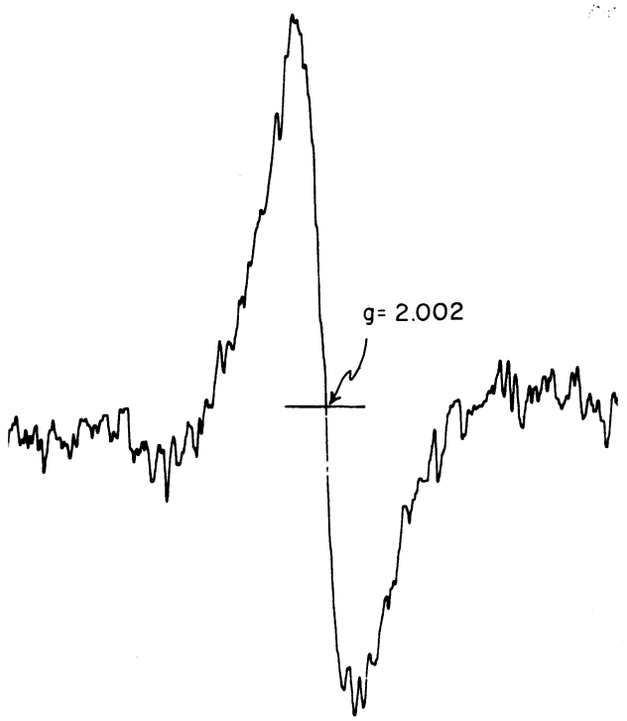

FIG. 1. ESR signal of normal mouse liver, "insoluble protein" fraction. The signal is centered at g = 2.002, and indicates a free radical concentration corresponding to 3.2×10^{15} spins/g.

therefore to be associated with the native state of the proteins present.

The soluble proteins, performing simpler vegetative functions *in vivo*, need no unbalanced electronic structure. The isolated soluble proteins precipitated by saturated ammonium sulfate gave no signal or gave only a weak one corresponding to 8×10^{13} spins. The signal of the soluble proteins was thus an order of magnitude weaker than the signal given by the structural proteins.

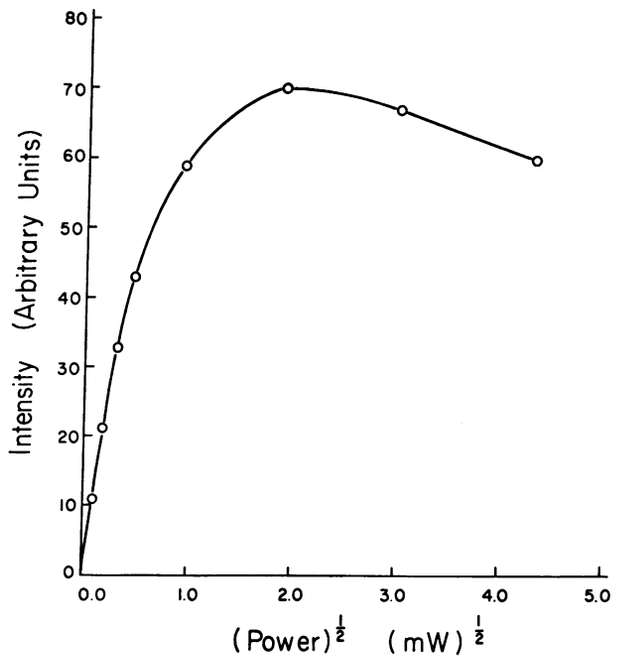

FIG. 2. The intensity of the ESR signal of Fig. 1 as a function of the square root of the microwave power.

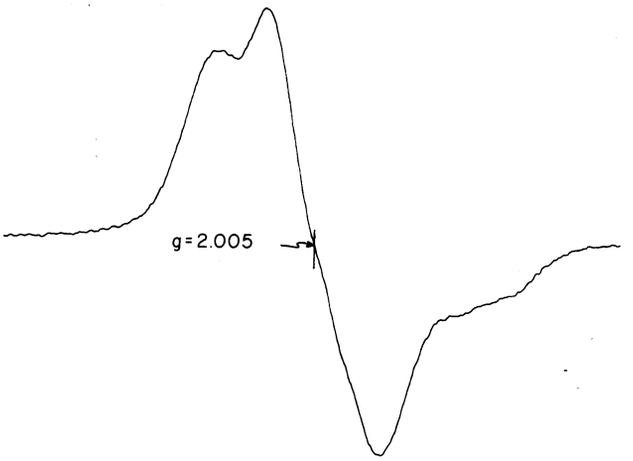

FIG. 3. ESR signal of casein treated with methylglyoxal. The signal is centered at g = 2.005, and indicates a free radical concentration corresponding to 8×10^{17} spins/g.

It has been emphasized in earlier papers of this laboratory that biologically active structural proteins such as the structural proteins of liver, kidney, or brain cells are colored, the color indicating the high reactivity of electrons, leading to an interaction with photons. It was also assumed that this highly reactive state of the protein, indicated by the electronic interactions with light, was due to the electronic desaturation of proteins by an electron acceptor containing an aldehydic C=O group and a conjugated double bond C=O in juxtaposition.

In order to see whether such electron acceptors as methylglyoxal or crotonaldehyde could actually induce color and ESR signals in protein, commercial casein was treated with these reagents (Figs. 3 and 4). Ten grams of casein powder was suspended in 10 volumes of methanol containing 10% commercial neutralized 40% methylglyoxal solution. The suspension was incubated overnight at 37°. Then the casein was separated on a filter, washed with methanol and acetone, and dried in the vacuum desiccator overnight. The white casein assumed a vivid brown color, similar to that of a blood-free liver (7). In the ESR spectroscope it gave the strong signal shown in Fig. 3, which corresponded to 8.0×10^{17} spins/g. In the ESR spectroscope this material gave a strong, structured signal and was shown to exhibit power saturation at a microwave power of a few milliwatts in a manner similar to that observed for the liver protein. This power saturation behavior, occurring both for the natural

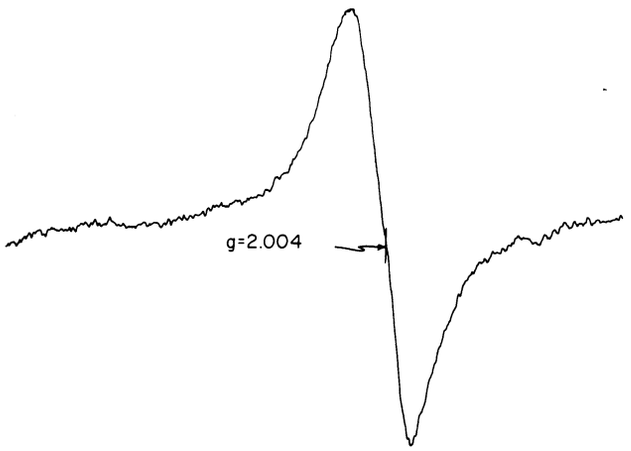

FIG. 4. ESR signal of casein treated with crotonaldehyde. The signal is centered at g = 2.004, and indicates a free radical concentration corresponding to 1.6×10^{16} spins/g.

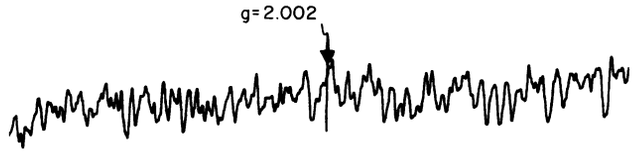

FIG. 5. ESR signal of rat hepatoma, "insoluble protein" fraction, indicating a radical concentration of less than 1×10^{14} spins/g. Similar "blank" traces were obtained for the heat-treated "insoluble protein" fraction of normal mouse liver, and for the saturated $(NH_4)_2SO_4$ solutions used.

and the charge-transfer-induced radicals, shows that the nature and microscopic environment of the two have important similarities.

In another experiment casein was dissolved in water at pH 7.4 to a final concentration of 5%, then 10% neutralized crotonaldehyde was added in a few ml of water (Fig. 4). The mixture was incubated 48 hr. It turned into a dark brown semisolid, corresponding to our experience that electron acceptors tend to make protein molecules link together to semisolids. The casein was precipitated with methanol, washed with acetone, and dried in the vacuum desiccator. It gave the signal shown in Fig. 4, which corresponds to 1.6×10^{16} spins/g. Dry, untreated casein also gave a weak signal, which was about 100 times weaker than the signal of the methylglyoxal-treated sample.

According to the concepts developed in this laboratory, cancer tissue is essentially a dedifferentiated tissue which has its equilibria shifted towards those of the α-state, in which the proteins have a low reactivity. Insoluble rat hepatoma proteins (Morris hepatoma 3924A) had no color and an undetectable ESR signal (Fig. 5), and differed from those of normal liver, which had a pink-brown color and gave a strong ESR signal. The signal given by the structure proteins of cancer was at least 10 times weaker than normal liver.

That the ESR signals of cancer tissues are weaker than those of the corresponding normal tissues has been observed by Commoner and his coworkers (4–6).

These experiments indicate that the ESR signal and color are closely related. They are in fact an expression of the same electronic disbalance (7). They also suggest that the malignant nature of cancer, due to its senseless proliferation, is intimately connected with the physical state that makes the cancer uncolored and makes it give a poor ESR signal. This makes it likely that the restoration of color and ESR signal to such cancer could also abolish malignancy.

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