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9. The term *in situ* is used here to indicate that the mitochondria were located inside of intact cells in their normal cellular milieu. However, it should be noted that technically speaking the chopped tissue was incubated and fixed for electron microscopy in vitro.
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14. Number 6 in the series. Supported in part by a program project grant No. GM-12847 and in part by NIH grant No. HE-06523, and in part by NIH training grant GM-88. C.H.W. is a postdoctoral trainee of the University of Wisconsin. We thank Mrs. T. Spennetta for skilled technical assistance and Mr. D. Silver for photographic preparations.

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Electron Spin Resonance Signals in Injured Nerve

Abstract. *Under certain conditions nerves (such as the frog sciatic) exhibit electron spin resonance signals with several unusual properties: (i) variable g value and linewidth, (ii) anisotropic g tensor, and (iii) g value dependence on temperature. Such a signal must be due to a small ferromagnetic crystal formed when the nerve is subjected to pressure, such as that due to mechanical injury.*

Although a number of normal animal tissues exhibit electron spin resonance (ESR) signals due to free radicals associated with enzymatic oxidation-reduction systems (*1*) in nerves, such signals are either absent or at least undetectable by means of present spectrometers. During investigations designed to determine whether ESR signals might arise in excised frog sciatic nerves during conduction of impulses, the absence of a normal ESR signal was confirmed under conditions of maximum available instrument sensitivity, both in the resting nerve and during conduction. However, in the course of these investigations, intense ESR signals at *g* values which varied between 2.05 and 2.30, with rather wide linewidths (100 to 400 gauss), appeared in several nerve preparations. Accordingly, a study was undertaken to determine the origin and properties of these signals. The results show that the signals arise in both frog sciatic nerve and lobster ventral nerve cord as a result of mechanical injury of the nerve, such as that due to pinching by means of forceps.

Most of the experiments were conducted with the excised sciatic nerve, dissected from cold-stored specimens of the bullfrog *Rana catesbeiana*. A few experiments were performed with the ventral nerve cord of the lobster *Homarus americanus*.

The excised nerves were examined in an ESR spectrometer operating at 9000 Mhz (modulation frequency, 100 khz), specially designed for maximum sensitivity in the presence of liquid water. The nerves were placed in either flat quartz cells (5 by 1 mm) or in a special cell consisting of a U-shaped glass tube which permitted initiation and detection of nerve impulses while the nerve was in place in the spectrometer cavity. Frog sciatic nerves were maintained in Ringer solution, and the lobster nerves were maintained in artificial seawater. The spectrometer was equipped with a temperature-regulating system which maintained the sample temperature to within ± 1°C of the indicated value. The spectrometer was operated in conjunction with a signal-averaging computer.

After the appearance of ESR signals during the handling of frog sciatic nerves, it became apparent that they frequently arose in association with mechanical injury, in particular, pressure such as that exerted by pinching with forceps. Examples of this effect are shown in Fig. 1, which illustrates that an intense ESR signal arises in the frog sciatic nerve after it has been pinched or stretched to the breaking point. By cutting off the injured portion we have observed that the signal is localized only in the injured portion of the nerve; the remainder of the nerve, like the original uninjured nerve, lacks an ESR signal.

Other treatment which elicits such signals in frog sciatic nerve is crushing (by means of pressure from a glass rod pressing against a flat glass surface), homogenization in a glass homogenizer, and splitting of the original nerve into two or more bundles (which inevitably involves stretching of the nerve). While studying 116 nerve preparations, we found that a signal could be elicited by these means in 80 samples.

One of the features of these signals is the considerable variability of shape and *g* value. In an effort to analyze

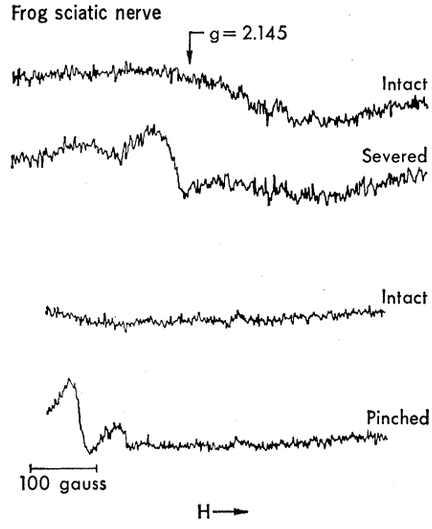

Fig. 1. Electron spin resonance signals from two different frog sciatic nerves (contained in a flat quartz ESR sample cell) before and after being severed by stretching (upper spectra) and before and after being pinched in forceps (lower spectra). Modulation amplitude: 16 gauss; temperature 15°C. Each of the signals represents a readout from a computer of average transients after summation by the computer of 50 successive spectra.

the source of this variability, we conducted a series of ESR determinations on single nerve preparations at different temperatures from −40° to +80°C. The signal is observed over this wide range of temperatures, but its *g* value is markedly dependent on temperature (Fig. 2). This figure shows that the position of the ESR signal shifts toward higher magnetic fields (lower values of *g*) as the temperature is increased (approximately linearly) and that the effect is completely reversible.

In order to study the origin of the ESR signal in the injured nerve, a number of nerves which exhibited the signal were sequentially cut in half (transversely, until very small); each time the two halves were studied for free radical content. If the nerve originally had a single ESR signal, the entire signal was always found in one half or the other—that is, the signal was never divided between the two nerve segments. By this means it has been possible, in some cases, to localize the ESR signal originally observed in the whole nerve in a segment as small as 0.3 mm on a side. When a small portion of an injured nerve which contains the ESR signal is isolated in this way, the signal intensity is essentially equal to that observed in the whole nerve. Hence the signal produced by the original injury is concentrated in a very

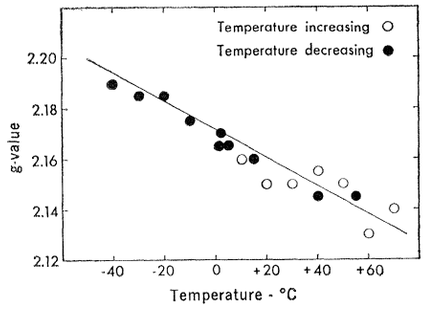

Fig. 2. The effect of temperature on the g value of the ESR signal exhibited by a pinched frog sciatic nerve. Instrument conditions as described in the legend to Fig. 1.

small region of the nerve. If the entire nerve exhibits two resolved ESR signals (that is, at different g values) after being injured, the cutting process usually isolated one of the two signals into a single segment.

During these studies we noticed that the apparent g value of the signal sometimes shifted when the nerve segment was taken out of the ESR cell and later replaced in it. This effect turned out to be a result of the dependence of the g value on the orientation of the nerve segment with respect to the direction of the magnetic field. Figure 3 shows the ESR signals obtained with a very small nerve segment

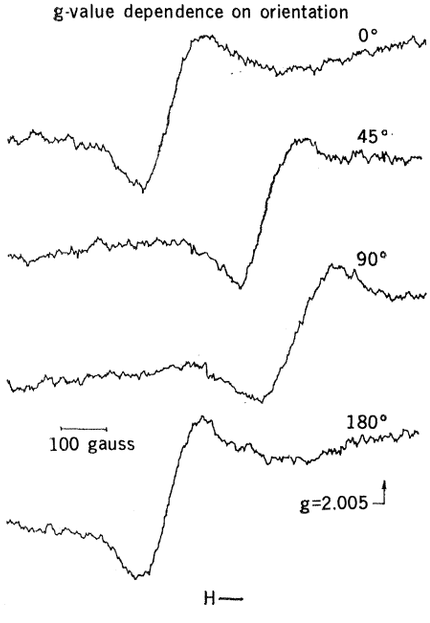

Fig. 3. The angular dependence of the ESR signal from a small segment (about 3 mm on a side) isolated from a pinched nerve. The segment was fastened to the end of a glass rod and inserted into the spectrometer cavity. The rod was rotated on its axis from an arbitrary starting position (0°) in 45° steps, and ESR signals were determined in each position. Modulation amplitude, 16 gauss.

mounted on the end of a glass rod which could be turned in the microwave cavity. The signal moves to the right (higher field) on the first 45° rotation (from an arbitrary starting position) and somewhat more in the next 45° rotation. Another 90° rotation brings it back to its original g value. We thus see that the g tensor of the signal exhibits 180° rotational symmetry. This type of behavior is that expected from molecules which are in some way aligned, as in the lattice of a single crystal.

The foregoing results show that the ESR signal elicited in nerve by injury has the following properties: (i) the g value is considerably in excess of that of the free electron (2.0023); (ii) the g value is dependent on temperature; and (iii) the g tensor is anisotropic.

The only mechanism that can account for these properties observed in the ESR signals from injured nerve, particularly that of the dependence of g value on temperature, is ferromagnetism. Ferromagnetism in the sample will cause an apparent shift in g value because the magnetization of the sample changes with temperature. The phenomenon of ferromagnetism requires the close proximity of iron (or other ferromagnetic metals such as cobalt or nickel) atoms to obtain the necessary exchange interaction. This close proximity is not possible for single iron atoms bound to large organic molecules, such as hemoglobin, but is present in many simple inorganic iron compounds such as γ Fe_2O_3 or Fe_3O_4. Such compounds give rise to signals similar to that observed here.

Ferromagnetic constituents have been found in samples of purified DNA, RNA, and nucleotides (*2–4*). Blois and Maling (*3*) have shown that such signals are produced by the application of pressure to the powders of these substances. Shulman *et al.* (*4*) have concluded that these ferromagnetic iron oxide-hydroxides are precipitated during extraction of the nucleic acid. The ferromagnetic crystals appear to be bound to the nucleic acid and cannot be separated with ethylenediaminetetraacetate or by filtration. These ferromagnetic samples have ESR properties similar to those in injured nerve, except that in most cases the signals in nerve are much narrower. This is probably due to the fact that in nerve the ferromagnetic part is a single crystal instead of being polycrystalline, as in some of the nucleic acid samples. Thus, pinching of a nerve results in the formation of a single crystal (or perhaps several single crystals) of a ferromagnetic iron oxide or hydroxide.

The formation of the crystal does not appear to be due to the aggregation of iron atoms randomly distributed in the tissue because no such crystals are observed when other organs, such as liver, are subjected to pressures even if they have been incubated in Fe^{2+} or Fe^{3+} salts. This suggests that the inorganic iron crystal is formed in association with some intracellular structure unique to nerve. The only clue to the identity of this site is the fact that the ferromagnetic signals are much more readily produced in nerves with a yellowish color than in white nerves. We have observed a considerable variability in the color of nerves from frogs of apparently equivalent age. The yellow color may be due to the lipid pigment lipofuscin, which contains iron (and requires iron for its formation from unsaturated fats). If the iron is bound to some ordered molecular structure (such as lipofuscin in a fat body) the pressure that occurs during injury could result in the aggregation of the iron into a ferromagnetic crystal. The yellow color may, however, be due to some iron compound which is made ferromagnetic if subjected to pressure.

The detection of such iron-containing crystals may find application in the study of certain kinds of diseases. For example, iron granules are sometimes seen in nerve cells from patients with Alzheimers disease. It is apparent from our results and from theoretical considerations [Shulman *et al.* (*4*) point out that about 10^{17} or fewer spins per gram are detectable in ferromagnetic substances] that such microscopic ferromagnetic crystals are readily detectable by means of ESR spectrometry.

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