NATURE OF FREE RADICALS IN FREEZE-DRIED FISHERY PRODUCTS AND OTHER LIPID-PROTEIN SYSTEMS

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ABSTRACT

The electron paramagnetic resonance spectrometer makes it possible to detect and study radicals which are produced during lipid peroxidation in freeze-dried lipid-protein systems. In such systems, two general types of resonances are observed. By studying effects of added antioxidants, added autoxidizable lipid, and type of substrate, it is possible to differentiate and characterize the two signals which are observed. It is postulated that the immobilized radicals observed in dry systems are the same as those that are responsible for damage at the molecular level in oxygenated lipid-protein-water emulsions, stored meats of normal moisture content, and in antioxidant deficiency states in man and animals.

Fish from the sea are rich in polyunsaturated fatty acids. Furthermore, when fish are processed as a foodstuff, the unsaturated fatty acids will, if unprotected, readily undergo lipid peroxidation.

In the presence of proteins, enzymes, nucleotides and other classes of biological materials, lipid peroxidation is radiomimetic, that is, it produces similar if not the same effects as ionizing radiation—a damage mechanism which is known to be mostly free radical in nature. Accordingly, such materials as freeze-dried fish tissue, dried fish meals, and protein concentrates incompletely freed of residual unsaturation, are all prone to undergo various deteriorative changes as a consequence of lipid peroxidation.

Although much emphasis has been placed in the past, and continues to be at present, on the participation of radicals in events leading to damage, radicals of the oxy or peroxy type have not been directly characterized by electron paramagnetic resonance (EPR) spectroscopy in living systems or in emulsions for in these the steady state concentration of radicals is universally low. Nevertheless, the EPR method remains as the one method best suited for characterizing radicals.

This paper discusses recent research employing systems which, for the first time, are favorable for the detection and study of EPR signals which arise with the onset of lipid oxidation. Mechanisms for the formation of radicals as well as reactions of radicals themselves are discussed.

MATERIALS AND METHODS

Freeze-dried and isopropyl-extracted rockfish myofibrillar protein, and freeze-dried rockfish sacroplasmic protein were provided by the NMFS Technological Laboratory in Seattle. In addition, a polyunsaturated fatty acid (PUFA) concentrate (75% C22:6 + 25% C22:5) was also provided by this laboratory. Freeze-dried human serum albumin (Grade III) and bovine serum albumin (BSA; crude powder) were obtained from Sigma. Freeze-dried silver salmon light flesh was prepared from a slurry of fresh fillet. All freeze-dried materials were stored at —60° C in the dark under nitrogen prior to exposure to air. Lipid-protein mixtures were prepared merely by thoroughly mixing the C22:6 concentrate with protein, usually in a ratio of 2:1 (protein to lipid) by weight. All oxidations were conducted in air at room temperature. All EPR studies were conducted at room temperature according to the procedures of Roubal (1970).

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RESULTS AND DISCUSSION

ELECTRON MIGRATION IN PROTEINS

As a base for comparing EPR signals in processed food materials, it will be instructive at this point to consider briefly the mechanism(s) which may be operative (disregarding for the moment, lipid oxidation) in the production of the observed signals in *carefully* freeze-dried tissue. In recent years, considerable attention has been given to the idea of the migration of energy over comparatively long distances in cells; such transfer of electrons is implicated in the process of mutation by ionizing radiation, in the process of nerve conduction, and in certain biochemical photo dissociations. There are problems of a theoretical nature associated with such hypotheses, and these are reviewed in the discussions of Blyumenfel'd (1957).

Terenin (1947) and Terenin and Krasnovskii (1949) have criticized Szent-Györgyi's hypothesis that electrons move along the protein polypeptide backbone in conductivity bands analoguous to the movement of electrons in conductivity bands of semiconducting metals. Although polypeptide chains exhibit properties reminiscent of conjugated unsaturation, Terenin poses the interesting observation that some 70 kcal/mole, a large amount of energy, would be required to mobilize electrons; evidently at usual temperatures, conductivity bands are empty.

Notwithstanding the fundamental objections to energy migration in native proteins, Blyumenfel'd (1957) considered the participation of triplet states in energy transfer, a possibility that is partially confirmed by the fact that the phosphorescence spectrum of proteins lies in the region of 4000 Å and corresponds closely to the calculated excitation energy which would be sufficient for energy transfer across molecular orbitals originating within the framework of hydrogen bonds. However, in the absence of prosthetic groups, it seems reasonable that few, if any, electrons would be free for mobilization. When prosthetic groups are present, however, and when they lie close to those of the protein (energetically speaking), electrons could be transferred into low lying orbitals of protein. Once in these molecular orbitals, the forbidden triplet transition to the singlet ground state would tend to maintain electrons in the triplet state energy levels. Under these circumstances, the electron would move along a chain of peptide hydrogen bonds until disposed of into some other favorably bound group.

RADICAL CONTENT IN PROCESSED FISHERY MATERIALS

For conventionally processed foodstuffs, tissues, and meals (that is, solvent-extracted proteins or proteins processed at room temperature), the situation is quite different. No longer do we have a protein substance identical in character to the native material; bonds have been broken, and the original geometrical arrangement of the protein chains has been completely disrupted. In many instances, certain compounds or classes of compounds have been selectively removed while at other times other substances are purposely added back to the protein.

Interestingly, the present study has shown that such materials as protein concentrates containing some residual lipid, fish meal, and freeze-dried tissue samples not processed with utmost caution in the freezing and freeze-drying steps, all exhibit characteristic EPR signals when exposed to air. Indeed, it is generally true that the radical content of haphazardly handled materials is usually higher than for an equal weight of similar material which has been processed by careful handling, freezing in liquid nitrogen, and careful removal of water. Unable to migrate along conductive pathways, effective charge transfer to radicals from donor molecules or reactants is apparently reduced (thus the radicals act as though caged or matrixed) and no longer do radicals interact freely with one another nor do they react at once with other cellular constituents. However, the efficiency in the reduction of charge transfer undoubtedly depends on the nature of the sample and its treatment. Just how immobilized such radicals are is open to conjecture. They are im-

mobilized sufficiently, however, so as to be detectable for fairly long periods of time.

CHARACTERISTIC FORM AND APPEARANCE OF EPR SIGNALS OBSERVED IN DRIED PRODUCTS

Although a high resolution EPR analysis can usually be performed with dilute solutions of soluble, low molecular weight organic radicals, the same is seldom true for powdered samples, and especially for powdered samples of complex molecules. The requirements for resolution are: magnetically dilute systems (in order to prevent spin-spin interaction), long relaxation times, and a low rf field. In the solid state, relaxation times are shortened because of the more effective coupling between spin states and the surrounding lattice—cooling the sample (perhaps to the temperature of liquid nitrogen or below) will often increase the relaxation time to acceptable values. Related to relaxation is the line broadening arising with molecular dipole interactions. The notable example is the so-called "oxygen effect"—some radicals will be far removed from the magnetic influences of the molecular oxygen di-radical while other free radicals in the sample will be near oxygen molecules. Consequently, free radicals of the sample will experience a variety of magnetic fields, producing a collective band of resonances resulting from the distribution of collective magnetic fields superimposed on the external instrument magnetic field. Therefore, in solid state studies, radicals, because of their random alignment, exhibit anisotropic coupling which broadens the lines and makes interpretation difficult. Spectral features which can be used to characterize the radicals are the measurement of the g-value, line shape, and changes in these parameters upon chemical or physical treatment of samples.

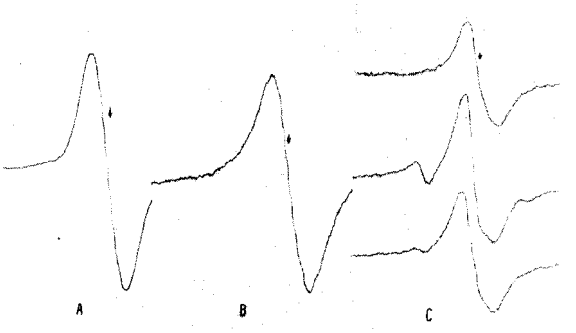


FIGURE 1.—EPR spectra for protein essentially free of lipid and for a lipid-treated protein, all exposed to air. A. Freeze-dried and solvent extracted rockfish myofibrillar protein. B. Freeze-dried human serum albumin. C. Crude bovine serum albumin (BSA) (upper trace). Crude BSA + C22:6 fatty acid (2:1 by wt) oxidized in air at room temperature for 2 hr (middle trace). Same material in air at room temperature at the end of 4 hr (lower trace). The arrows denote the g = 2 or free-spin value.

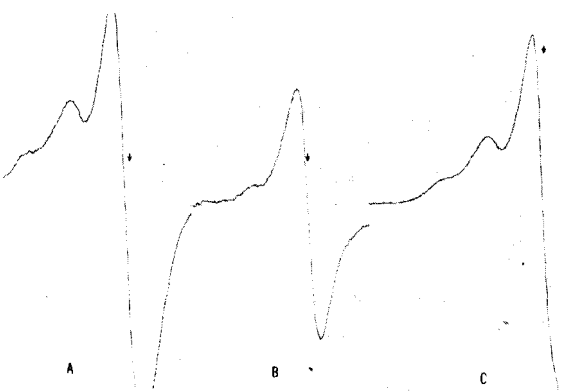


FIGURE 2.—EPR lipid signals in marine protein concentrates exposed to air. A. Freeze-dried rockfish flesh exposed to air for 20 hr at room temperature. B. Commercially available FPC, now 2 years old, low lipid initially, which still exhibits a weak lipid signal. C. Freeze-dried silver salmon light flesh exposed to air for 10 hr at room temperature. Arrows denote g = 2.

EPR SPECTRA OF FISHERY PRODUCTS

Shown in Figures 1, 2, and 3 are EPR signals which are observed in lipid-protein models and in dry ice-frozen, freeze-dried fish tissue, all of which are under investigation in this laboratory. As with carefully freeze-dried samples of the type discussed above (liquid nitrogen frozen and freeze-dried), the resonances are devoid of hyperfine structure of the type normally

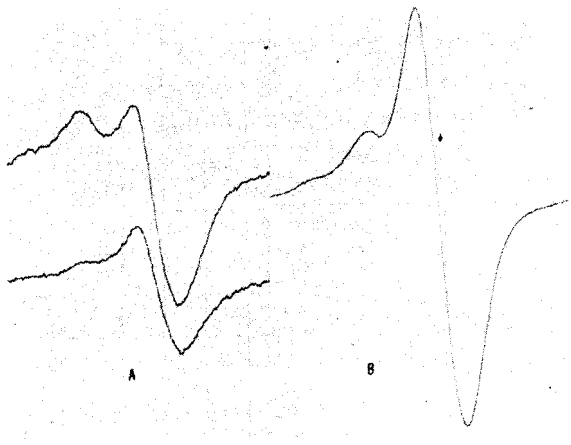


FIGURE 3.—EPR lipid signals in a hydroperoxide-treated protein and in a sacroplasmic protein. A. Rockfish myofibrillar protein upon removal from the freeze-drier (lower trace). The same material 40 min after incorporating a small amount of a hydroperoxide mixture prepared from a marine oil (upper trace). B. Freeze-dried rockfish sacroplasmic protein after storage in air at room temperature for 1 day. Arrows denote $g = 2$.

observed for dilute solutions of low molecular weight radicals. Without exception, all powdered materials of proteinaceous nature, which exhibited any type of signal at all, gave a single absorption line in the "free-spin" or $g = 2$ region. The $g = 2$ signal is exemplified in Figure 1a (for solvent-extracted myofibrillar protein), or in Figure 1b for human serum albumin. I will have more to say about the $g = 2$ signal; however, let us concern ourselves for the moment with other resonances which are seen in those samples containing oxidizable lipid in addition to protein.

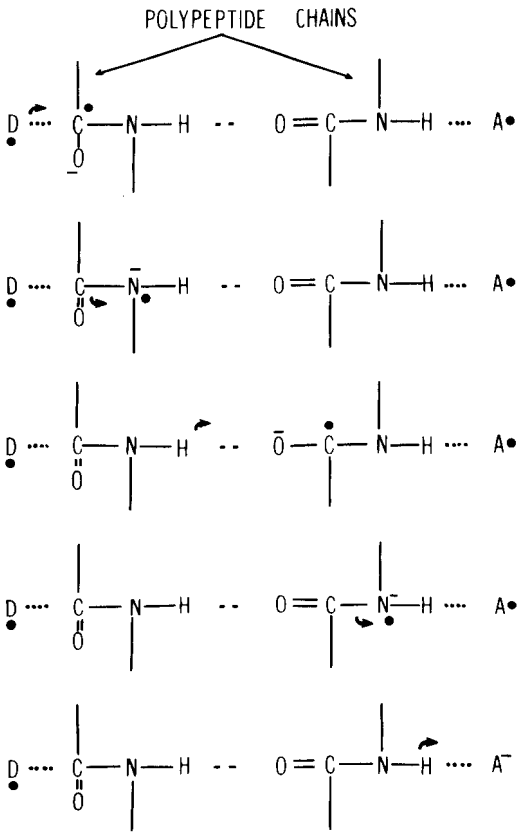
Freeze-dried Pacific cod, silver salmon, rockfish, and other marine fish, though devoid of the $g = 2$ signal initially (before lipid oxidation has taken place) soon give rise to two resonances when samples are exposed to air—the central $g = 2$ resonance and, downfield (to the left) from the central resonance, an area of EPR activity which I have designated as the "lipid signal" region (Figures 2 and 3). Unlike tissue samples, many single proteins considered to be quite pure exhibit a $g = 2$ resonance only. When, however, a thin film of oxidizable lipid is deposited on such materials and the mixture is exposed to air, in addition to the central resonance, a lipid signal is also observed (Figure 1). A preliminary study of lipid signals in various models is to be found in the recent work of the author (1970). Although it has not been possible to measure the g-value with the necessary precision needed to fingerprint the radical completely, the available data suggest a radical of the peroxy type. This is further illustrated by the two traces of Figure 3a. No indications of hyperfine splitting (hfs) are also consistent with a radical of this nature.

CHARGE TRANSFER IN TISSUES

In the present study, it is of particular interest to find that in carefully handled freeze-dried tissue samples, there often occurs after the lipid signal reaches a maximum, an abrupt increase in the $g = 2$ region. In those "native" samples containing a complement of cellular lipid, this may indicate a charge migration (a strong D→A—donor-to-acceptor interaction; strong charge-transfer process) between a cellular constituent acting as a donor and a peroxy radical acceptor. Such a process is also consistent with the observation that it is at this point in time that the lipid signal begins to decay. This draws our attention to the likelihood that once radical content has increased to some critical concentration, overlap of wave functions between a radical acceptor and a donor is sufficient to allow reactions to proceed. The abrupt change in the $g = 2$ region is illustrated by the spectra of Figure 3. Figure 3b for sacroplasmic protein under air for 1 day is to be compared with the lower trace of Figure 3a for the same material immediately on removal from the freeze-dryer.

Another point in favor of a mechanism of this type is the fact that only proteins are really effective as matrices for the formation as well as for the decay of radicals. Powdered glass, quartz wool, and amino acids are essentially without effect when used as substrates for thin films of reactants. Although there are many unanswered questions concerning the mechanism

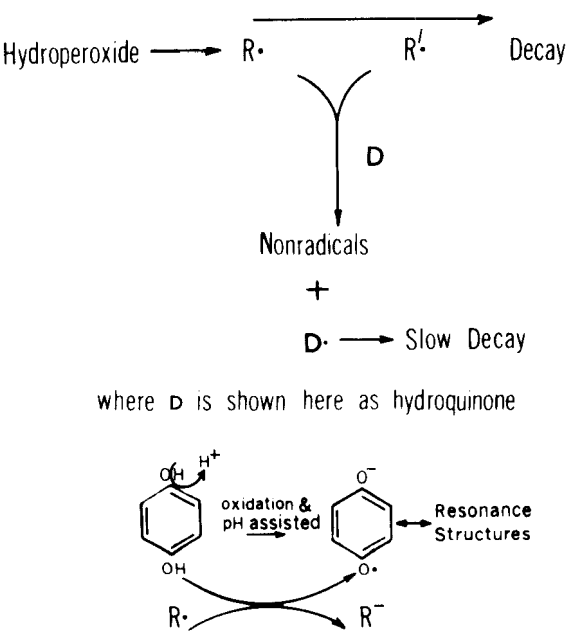
of trapping and charge migration, the data are consistent with the scheme shown below:



SCHEME 1.—Charge-transfer between donor (D) and a free radical acceptor (A) in biological systems. Although the actual pathway is not known with certainty, available data are in accord with the idea that hydrogen bonding of the type shown may play a role in the transfer of charge.

where D is a cellular electron donor material acting in the role of an antioxidant and A is a peroxy radical acceptor. To completely substantiate that such a mechanism does exist would be very exciting indeed for this would be the first instance in which the role of an antioxidant at the molecular level could be designated as a strong D→A interaction.

The data are also in accord with the idea put forth sometime ago by some Russian investigators, that the g = 2 signal in tissue is semiquinone in nature (Chetverikov, Blyumenfel'd, and Fomin, 1965). In the present work, for instance, when hydroquinone or various hydroquinone derivatives with free hydroxyl groups are incorporated into proteins coated with thin films of unsaturated lipid, an enhanced central resonance is obtained which is identical to that obtained for oxidizing tissue alone. For the protein-lipid hydroquinone systems, the author has shown that central resonance consists chiefly of trapped semiquinone radical ions. The g = 2 resonance in "lipid-free" proteins may very well indicate prior lipid oxidation but now at a point in time at which lipid signal has decayed. The g = 2 retention could then be explained because of resonance stability or other stabilizing factors for this species. For instance, some commercial preparations of bovine serum albumin exhibit an EPR signal somewhat displaced from the usual g = 2 signal while others do not. Likewise some of the samples that are EPR-active exhibit fluorescence quite characteristic of malonaldehyde-amino acid interaction (formation of iminopropene derivatives). The various data taken collectively suggest the following mechanism:



SCHEME 2.—Interaction between radicals (R·, R·') and cellular antioxidants (D).

Other radicals, however, may make some small contribution to the central resonance pattern. A recent investigation by Wekell and Roubal[2] has shown that free radicals arise during carbonyl amine browning. What is more, although hfs is seen in early stages of the browning, the signal changes to a single line in the $g = 2$ region as polymerization progresses. The browning reaction, without the implication of free radicals in lipid-protein systems, though not a dominant pathway to pigments, was first discussed by Venolia and Tappel (1958) as a possible cause of color formation during the oxidation of such systems [but because the more recent work, polymeric masses are, for the most part, attributed to lipid peroxy induced protein-protein polymerization together with malonaldehyde cross-linked proteins (conjugated Schiff base)].

Thus, although recent studies in this laboratory have uncovered new facts concerning radicals in lipid-protein systems, the exact nature of the resonances observed in freeze-dried tissue and in dry model mixtures remains to be fully characterized. Nonetheless, this pioneering piece of research has paved the way for use of EPR studies in systems of oxidizing lipids together with other cellular constituents. Concurrent studies have shown that transition metal ion impurities, if present, play only a minor role in radical production. Other studies of this laboratory have shown that protein-free fish bone does not give EPR signals. Freshly prepared freeze-dried tissue samples give no lipid signal resonances, but signal amplitudes grow on exposure to oxygen. Depending on the type of protein, type and amount of lipid, or added material, lipid signals exhibit various lifetimes ranging from hours to years. (For instance, compare Figure 1c for BSA with Figure 2b for 2-year-old FPC.) Polysaccharides are only partially effective as radical matrices.

For living systems, or for any system containing residual and unprotected oxidizable lipid, the implications of the various interactions discussed are significant. It is known that products of lipid oxidation in lipid-protein systems interact with proteins, enzymes, and nucleotides. Not only are these native biopolymers further polymerized by such interactions, constituent building blocks are destroyed (Roubal and Tappel, 1966b, 1967); notable are the sulfur amino acids which have been shown to be easily destroyed by free radicals (Roubal and Tappel, 1966a). In this presentation I have not discussed consequences of unwanted lipid peroxidation in nutritional deficiency states or in other pathologies in living systems. Nevertheless, such lipid-protein interaction is quite significant. The geronotological implications of these reactions leading to the formation of age pigments, based on studies of Roubal and Tappel (1966b) and others, have been reviewed by Bjorksten (1965), Packer, Deamer, and Heath (1967), and Tappel (1968).

[2] Unpublished data; to be published.

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