Trapped Radicals in Dry Lipid-Protein Systems Undergoing Oxidation

WILLIAM T. ROUBAL, U.S. Bureau of Commercial Fisheries,
Food Science Pioneer Research Laboratory, Seattle, Washington 98102

Abstract

Free radicals derived from peroxidizing lipids in close association with proteins and other biomolecules have been implicated as being the principal reactants leading to polymerization of proteins and destruction of amino acids. The present study, which deals with lipid-protein systems low in moisture, shows that radicals derived from oxidizing lipid are readily trapped. Partial characterization of these radicals has been possible. The possibility that these radicals are the ones which participate in events leading to biological damage is explored.

Introduction

In vitro studies have proved that unsaturated lipids in close association with proteins, enzymes, nucleotides and amino acids induce polymerization of proteins, enzymes and nucleotides (1,2) and cause destruction of amino acids (3,4). A substantial body of evidence suggests that free radicals derived from oxidizing lipids are the dominant cause of such reactions but, because of the low steady state concentration of radicals, no definitive electron paramagnetic resonance (EPR) study has been performed which describes such interactions. Harmon and Piette observed faint EPR signals above background noise in human serum at room temperature, but these investigators were unable to assign the signals specifically to lipid oxidation (5). Nevertheless, Desai and Tappel's earlier hypothesis that oxidizing lipids act as cross-linking agents in the polymerization and formation of insoluble masses (6) has been modified by more sophisticated studies in vitro, which have shown that in solution, protein polymerization leading to insolubility is most likely a free radical-induced process not actively incorporating lipid (1). However, to just what extent nonradical reactants participate in cross-linking or other events leading to damage remains unanswered. Product fluorescence and product analyses in studies of protein polymerization by other investigators point to the participation of malonaldehyde or other aldehydes as cross-linkers (7; also, Choi and Tappel, personal communication).

This paper reports evidence for the formation of radicals in oxidizing unsaturated lipid-protein systems, specifically in systems low in moisture. Although the solid state system was required by necessity if radicals were to be observed, it is argued that the relatively high concentration of radicals observed gives insight into radical participation in lipid-protein emulsions and biological systems.

Experimental Procedures

Hemoglobin (Hb; bovine, 2x crystallized) and ovalbumin (2x crystallized) were obtained from Nutritional Biochemicals. Fish protein concentrate (FPC) of commercial quality, and solvent extracted, was obtained from the Bureau of Commercial Fisheries Technological Laboratory, College Park, Maryland. Freeze-dried salmon (containing 20% lipid on a dry basis) and freeze-dried cod (containing 5% lipid on a dry basis) were prepared from dry ice-cooled, and frozen slurries of pink flesh of silver salmon and light flesh of true cod, respectively.

Lipid-protein models for EPR investigation were prepared by incorporating 5–10% (by weight) of the $C_{22:6}$ concentrate with the dry protein materials; the usual length of time allowed for oxidation in air at room temperature for models as well as for freeze-dried tissue was 12 hr. All samples were analyzed at room temperature in thin-walled 3 mm o.d. quartz sample tubes using a Varian X-band spectrometer equipped with Fieldial control and a TE_{102} universal cavity. Typically, 8 milliwatts of incident rf power and a modulation amplitude of 5 gauss at 100 kHz were used. In all studies, 100 mg samples and identical instrument settings were used for both control and test samples so that direct comparisons could be readily made.

Results and Discussion

Oxidation of Samples With and Without Added Antioxidant

All powdered samples that exhibited any EPR signal at all, gave a dominant central resonance in the "free-spin" or $g = 2$ position. Line widths were of the order of 10 ± 1 gauss. When oxidized samples were scanned, other resonances not previously observed appeared to the left or downfield from the $g = 2$ resonance. One and often two shoulders or distinct peaks were observed and accounted for much of the interest in the present study. Figure 1a shows the spectrum of hake FPC. An unresolved peak is seen to the left of the central resonance; this region of the spectrum has been designated as the "lipid signal" region.

Figure 1b (upper trace) shows the spectrum of freeze-dried salmon that was allowed to oxidize at room temperature overnight. Figure 1b (lower trace) shows the spectrum of freeze-dried salmon to which hydroquinone was incorporated before the onset of oxidation (1–2 mg hydroquinone stirred into the powdered protein). Figure 1c (upper trace) shows the spectrum of freeze-dried and solvent extracted ($CHCl_3$-MeOH) salmon flesh to which was added 10% of the $C_{22:6}$ concentrate and which was then allowed to oxidize. The data show that the effect of oxidation, as shown in Figure 1a or 1b, can be duplicated by readdition of oxidizable lipid to essentially lipid-free protein. The lipid signal can be greatly reduced or even eliminated by addition of antioxidant, as indicated in Figure 1b (lower trace) or 1c (lower trace). Not only was the lipid signal of Figure 1b completely eliminated by antioxidant, the $g = 2$ resonance for materials with a natural lipid complement was also reduced. Also, the individual spectra for lipid-protein reaction products of Figure 2a or 2b for ovalbumin and myofibrillar protein, respectively, were similar, as were those for addition of antioxidant to the sample (compare the middle trace with the lower trace of Figure 2b). The spectra of unoxidized and oxidized freeze-dried cod flesh are shown in Figure 2c.

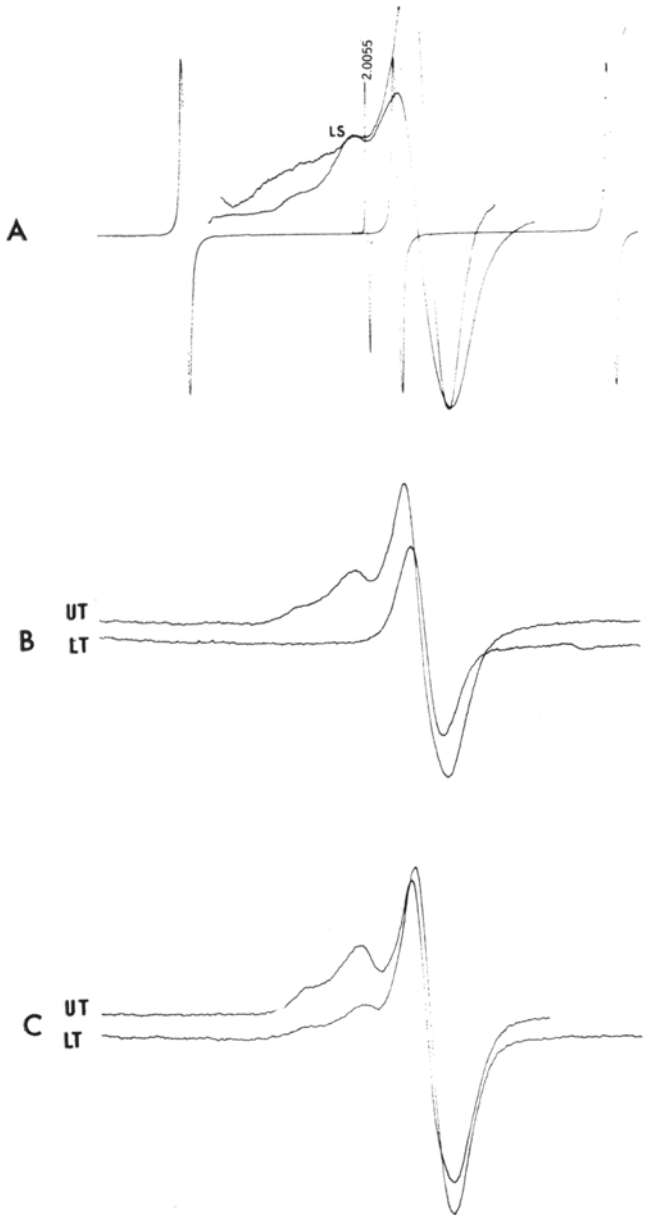

FIG. 1. EPR spectra of FPC, whole fish tissue, and reconstituted tissue. A: FPC at normal and high receiver gain. The narrow lines are peroxylamine disulfonate markers; the g-value of the marker nearest the lipid signal (LS) is 2.0055. B: Freeze-dried whole light flesh of silver salmon, oxidized overnight in air (upper trace), and the same material into which hydroquinone was incorporated before onset of oxidation (lower trace). C: Freeze-dried and solvent-extracted whole light flesh of silver salmon to which was added $C_{22:6}$ and which was allowed to oxidize (upper trace), and the same material into which hydroquinone was incorporated before onset of oxidation (lower trace). Air-oxidized, freeze-dried and solvent-extracted tissue gave a weak $g = 2$ signal only. Abbreviations: UT, upper trace; LT, lower trace.

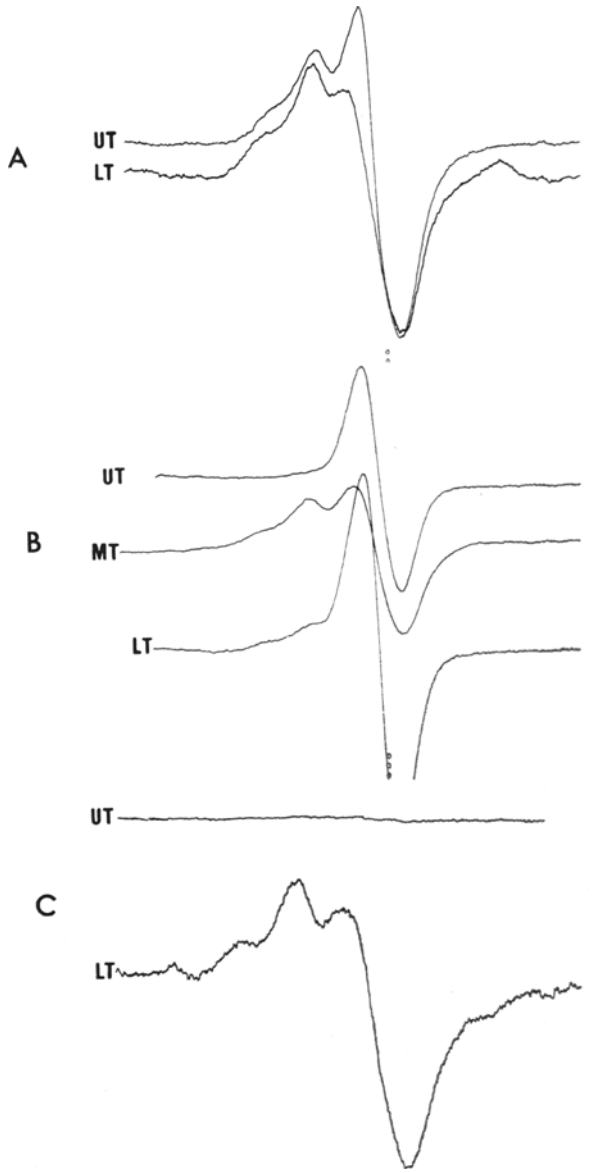

FIG. 2. EPR spectra of lipid-protein models and of freeze-dried light flesh of true cod. A: Oxidized whole salmon tissue (upper trace) is compared with air oxidized albumin containing $C_{22:6}$ (lower trace). B: Myofibrillar protein (upper trace) is compared with the same material + $C_{22:6}$ and oxidized (middle trace). The lower trace is for oxidized myofibrillar protein containing $C_{22:6}$ to which hydroquinone was added before onset of oxidation. C: Unoxidized freeze-dried true cod (upper trace) and the same material, air oxidized (lower trace). Abbreviations: UT, upper trace; MT, middle trace; LT, lower trace.

The spectra clearly indicate that the new peaks in the lipid-treated or lipid-containing samples are related to lipid oxidation. Added antioxidant greatly suppresses the lipid signal, whereas the usual increases noted for the $g = 2$ resonance for antioxidant-treated samples fortified with added lipid are attributed to trapped semiquinone radicals produced in the highly oxidizing environment. An explanation for the reduction of the $g = 2$ amplitude in antioxidant-treated, freeze-dried fish samples is that antioxidant also inhibits signals that arise from constituents other than lipid.

Nature of Trapped Radicals

From our point of view, the work of Commoner et al., who described radicals in freeze-dried animal and plant tissue and fluids, is of particular interest (8). These workers concluded that the resonances are associated with protein radicals. Blyumenfel'd, in a repeat of Commoner's work comments on the unique EPR signals that were obtained (9). Blyumenfel'd points out that the narrow line width (about three gauss) is especially remarkable. The sharp narrowing is attributed to metabolic interaction and delocalization of the unpaired electron. Furthermore, there is a lack of hyperfine structure.

Contrasted with native proteins, the situation for processed proteins is not the same; bonds have been

broken, the native geometry of polypeptide chains has been disrupted, and in many cases certain compounds or classes of compounds have been selectively removed during processing. Yet, as we have seen, such materials as FPC, freeze-dried tissue not quickly frozen in liquid nitrogen, and lipid-protein models, all exhibited relatively strong EPR resonances. Indeed, the radical content of slowly frozen, freeze-dried tissue was usually higher than for an equal weight of fresh tissue from which the water was removed after instant freezing of sample in liquid nitrogen. This observation is indicative of cellular damage which arises from growing ice crystals unless quenched by quick freezing of sample in liquid nitrogen. Unable to migrate along the conductive pathways discussed by Blyumenfel'd, some of the radicals in slowly frozen samples derived from oxidizing lipid undoubtedly are caged or trapped. Just how immobilized such radicals are is a matter of conjecture. More than one type of radical may be produced, and, of these, some possibly more reactive than others may be influential in further reducing protein quality, possibly by free radical attack on amino acids.

Munday states that most of the central resonance in liquid nitrogen-frozen and freeze-dried foods is due to physically absorbed oxygen (10) (with one exception he observed a $g = 2$ signal only). In the present study, however, when samples were evacuated to 10^{-6} torr for two days, or when air in samples was displaced with benzene, the signal decreased by only 20%. Munday concludes that moisture has a marked effect on signal intensity (presumably by displacing oxygen), but he makes no mention as to sample presentation other than that the spectra were obtained from samples studied at room temperature. Unless he took into account the dipolar absorption of microwave energy by water at room temperature, he would observe a decrease in signal on addition of water to dry samples. In any event, the present investigation has shown that samples can be saturated with water, the water can be removed under vacuum, and the spectra can be obtained under nitrogen with only a 25% loss in $g = 2$ signal. Munday assumes that a second peak in the single instance of accelerated freeze-dried herring is due to melanin; however, this assumption was based on studies by Munday of melanins isolated from plants and insects. Here Munday showed that a strong EPR resonance was associated with black pigments in seed coats and insect cuticle; he did not investigate fish. Munday gives no g-values for his melanin studies but his recorded spectra suggest that the g-value is in the free-spin region and is not the same as lipid signal observed in the present study. Evidently, the lipid signals observed in the present study are unique and reflect the trapping ability of the denatured materials.

Ovalbumin, shown in previous studies to be an efficient nonmetalloprotein prooxidant for unsaturated lipid (3), produced an easily detected lipid EPR signal, which decayed with a half life of two days. However Hb, which gave a strong, odd-shaped $g = 2$ resonance for the native protein, gave the identical resonance devoid of lipid signal for the Hb-lipid reactant in the dry state. These observations substantiate the already known catalytic effect of these proteins. Trapped radicals on ovalbumin undergo a moderately fast destruction. However, radicals in Hb, even in the dry state, evidently are destroyed almost as quickly as they are produced.

Lipid Signal g-Value

Longwave ultraviolet (UV) irradiation of freeze-dried fish or of models that exhibited the lipid signal resulted first in an initial increase in signal followed by a gradual loss. Concomitant with the change in the lipid signal, the dominant resonance slowly increased in amplitude and maintained this growth. This observation does not support a transfer reaction in which an unpaired electron is transferred to sulfur as in the case of X-irradiated sulfur-containing proteins; that the lipid signal might be the result of an anisotropy in the g-value for sulfur does not appear to be valid. Various sulfur amino acids were reacted with $C_{22:6}$, but no EPR signals could be detected. Although longwave UV-irradiated sulfur amino acids in air did produce strong resonances, the various spectral parameters did not match those of the lipid-protein reaction products.

It is noteworthy that the uncharacterized g-value of 2.004 for the faint signals in human serum observed by Harmon and Piette (5) is close to the g-value of 2.005 observed for the lipid signal in the present study and may indicate a possible radical relationship. Although Zirlin and Karel, in a recent study of gelatin-linoleate interaction, suggest that in the dry state reaction of protein radicals with oxygen may result in the formation of POO· radicals (11), it is not certain whether the g-value of 2.005 observed in the present study is of the lipid peroxy type and similar to LO· but shifted from the known value of 2.015 (in solution) by the solid-state system or whether solid state POO· radicals (if, indeed these are what are being observed), in contrast to LO· radicals in solution, exhibit an intrinsically different value, namely 2.005.

Radical Lifetime

Contrasted with emulsion systems, dry, unsaturated lipid-protein systems undergoing oxidation produce easily detected EPR resonances. Freshly prepared samples give weak resonances, if any at all, but signals increase on exposure of samples to air, reaching a maximum in 12–20 hr. Once formed, the lipid signals slowly decay. After the samples stood in air at room temperature for a month, lipid signals generally fell to about 40% of those for materials oxidized for 12 hr.

Lipid Peroxidation Damage in Biological Systems

Although the observation of free radicals in the present study is no guarantee that these same radicals are the ones which participate in emulsion systems, this study does show, for the first time, that radicals derived from oxidizing lipid can be trapped in biochemical systems.

In dried biologicals containing oxidizable lipids, regardless of the fact that trapping in a matrix reduces radical reactivity, radicals may be expected to react eventually. In so doing, it is reasonable that they will participate in events leading to a variety of damaging alterations similar to the way they do in emulsion systems.

For living systems, the implications of the various interactions discussed is significant. Increasing evidence indicates that free radical reactions are the cause of the gradual rise in polymeric, hydrolytic enzyme-resistant lipofuscin age pigments observed to accumulate in organs such as heart and brain in both man and animals. The gerontological implications of these reactions leading to the formation of

polymer-like materials, based on the studies of Tappel, Roubal and Tappel, and others, has been reviewed by Tappel (12), Packer et al. (13), and Bjorksten (14).

It seems quite reasonable that since free radicals precede aldehydes in the oxidation of lipid, radicals are the principal causes of protein polymarization and amino acid destruction. Hassinsky indicated some time ago that lipid peroxidation is similar to ionizing radiation, a known free radical mechanism, and that lipid peroxidation may have a similar mechanism of damage at the molecular level (15).

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