Interaction of Peroxidizing Methyl Linoleate with Some Proteins and Amino Acids

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Peroxidizing methyl linoleate reactions with protein lysozyme and with other selected proteins and amino acids were studied in model systems. Electron spin resonance established that reactions in the dry state between peroxidizing linoleate and lysozyme result in production of protein free radicals. Ionizing radiation and reaction with peroxides of methyl linoleate produced similar free radicals of carbon atoms in various proteins, and similar sulfur radicals in proteins having free sulfhydryl groups. Peroxides did not, however, break disulfide bonds in proteins. Studies of amino acids concentrated on the isolation and characterization of products of amino acid reactions with linoleate peroxides. Histidine gave several reaction products, one of which was identified as histamine. Methionine was oxidized to methionine sulfoxide, but under some conditions sulfone was formed. Several lysine reaction products were also separated and tentatively identified.

A great deal of evidence indicates that lipid peroxidation in lipid–protein systems results in reactions with the proteins. These reactions often lead to the formation of colored, insoluble, or sparsely soluble complexes.

Peroxidation of unsaturated fatty acids is a free-radical reaction, the course of which is shown schematically in Figure 1, and its major initial products consist of hydroperoxides. As oxidation progresses, hydroperoxide breakdown products accumulate, and eventually the hydroperoxide concentration decreases. The reaction is very sensitive to the environment, especially to temperature, electromagnetic radiation, state of dispersion, as well as to the presence of catalysts and inhibitors.

The reactions of peroxidizing lipids may be important biologically, either because they result in toxic or otherwise biologically active compounds in foods, or because there is actual peroxidation *in vivo*. *In vivo* peroxidation may be involved in: (a) damage to lipoprotein membranes of subcellular particles; (b) formation of the so-called "aging pigment;" (c) cross-linking of various polymers in aging animals. The possibility of *in vivo* peroxidation is still a controversial subject, but is supported by the following observations. (a) Extracted lipids show signs of peroxidation in some tissues even when they are extracted under conditions which minimize the danger of oxidation. (b) Free-radical signals in tissues are increased in some disease states, including some malignancies. (c) Some studies have shown life-prolonging effects among antioxidants, and some diseases and toxic effects are alleviated by antioxidant intake. (d) Studies on isolated subcellular particles, including liver and brain microsomes, and liver mitochondria show that peroxidation of membrane lipids can occur and cause severe functional damage. (e) Studies on isolated proteins show that reactions with peroxides damage the biological functions of such proteins.

Among the effects of lipid peroxides on proteins *in vitro* are: loss of enzyme activity (Chio and Tappel, 1969), loss of solubility due to aggregation or complex formation (Pokorny and Janicek, 1968; Andrews *et al.*, 1965), chain scission (Zirlin and Karel, 1969), as well as loss of specific amino acids. Cysteine, lysine, histidine, and methionine are the most susceptible (Roubal and Tappel, 1966a; Karel and Tannenbaum, 1965; Roy and Karel, 1973; Tannenbaum *et al.*, 1969).

As shown schematically in Figure 2, reaction products of lipid peroxidation react with proteins in a number of ways. One reactive oxidation intermediate, malonaldehyde (MA), is a product of hydroperoxides of linolenic, arachidonic, and other oxidized fatty acids, and forms the basis for the use of the TBA test. The Kummerow group (Kummerow, 1966; Nishida and Kummerow, 1960; Narayan *et al.*, 1964) studied the formation of lipid–protein complexes in model systems, and attributed the complex formation to secondary bonds, presumably including H bonds. Pokorny (1963) and Pokorny and Janicek (1968) studied casein reactions with peroxidized lipids. They also found that complex formation, especially the polarity of the solution, depended strongly on the environment. They attributed the complex formation to hydroperoxides. Tappel's group in California (Roubal and Tappel, 1966a,b) found a distinct similarity between the effects of reactions with oxidizing lipids on proteins, and the effects of ionizing radiations. They found damage to several proteins including cytochrome *c*, hemoglobin, and ovalbumin. Cytochrome *c* damage in reaction with hydroperoxides was also reported by O'Brien and Frazer (1966). The work of Roubal and Tappel led to their suggestion that protein insolubilization can result from protein–protein interactions initiated by lipid-free radicals. Zirlin and Karel (1969) studied gelatin–linoleate interaction in dry state, and concluded that the reactions of gelatin with lipids can lead to scission of the protein as well as to cross-linking.

There is a strong similarity between radiation effects and the reactions of proteins with peroxides. Some of the similarities were pointed out by Tappel (1973). It has been shown in radiation-induced protein reactions that high water activities and the presence of sulfur in the protein tend to promote cross-linking, but that proteins having no sulfur (*e.g.*, collagen) will tend to undergo scission in the dry state (Friedberg, 1969; Bailey *et al.*, 1964). Our results with proteins reacted with peroxidizing lipids showed a similar pattern (Zirlin and Karel, 1969; Takahashi, 1970), and it seemed reasonable to assume that the similarity is due to the presence of similar free-radical reaction patterns. Accordingly, we decided to study the formation of free radicals in proteins using electron spin resonance (esr).

Esr has been used extensively to study free radicals in irradiated proteins, but only very few reports are available on esr of oxidizing lipid–protein systems. These reports are mainly the work of Roubal (1970, 1971). Roubal's approach was to use freeze-dried fish with various lipid contents, extracted fish flesh with added unsaturated lipid, and various pure proteins, with and without added lipid. In some cases hydroquinone was incorporated as an antioxidant before oxidation. The characteristics of the esr signals he obtained are as follows: in most cases esr resonance with *g* values of 2.0 developed after freeze-drying, whether with or without lipid. After oxidation in air in the presence of lipid, an additional downfield "shoulder" signal appeared, increased with time to some maximum,

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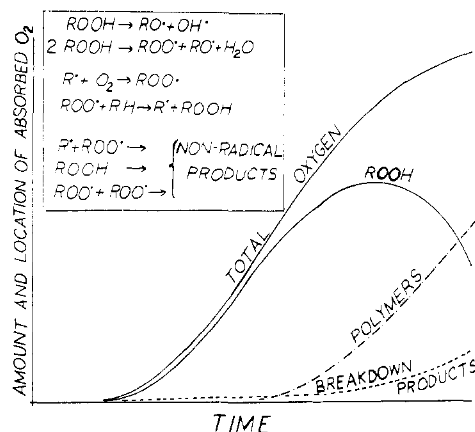

Figure 1. Course of oxygen absorption during peroxidation of lipids.

SOME POTENTIAL INTERACTIONS OF PROTEINS WITH LIPID OXIDATION

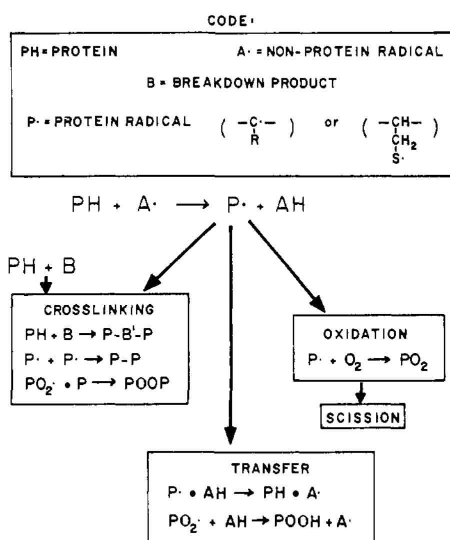

Figure 2. Schematic representation of reactions of proteins with peroxidizing lipids.

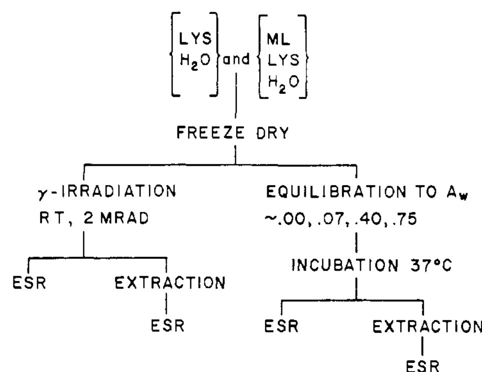

Figure 3. Experimental design for comparison of effects of irradiation and of oxidation on lysozyme.

then decreased. Simultaneously with this so-called "lipid" signal decay, the $g = 2$ resonance increased. Roubal explained this reaction as being caused by charge transfer from some cellular constituents, perhaps proteins, to lipid peroxy radicals.

Roubal postulated that denatured protein uniquely formed a matrix which could trap and stabilize lipid radicals. Powdered glass, quartz wool, and amino acids were ineffective, and polysaccharides were partially effective as traps.

Roubal's results leave doubt concerning the identities of the radicals in the systems. The $g = 2$ signals are most likely protein radicals, since the "pure proteins" exhibited signals without the presence of lipid. The g values reported were only estimated and may have been subject to some error. Roubal excluded the possibility that the "shoulder" signal was caused by sulfur radical and not peroxy radicals by two observations: (1) free sulfur amino acids reacted with oxidizing lipid did not form free radicals; (2) spectral parameters of irradiated sulfur amino acids did not match those of the experimental systems.

However, he only obtained the shoulder signal with proteins containing sulfur amino acids; bovine hemoglobin, for example, contains no sulfur, and exhibited no shoulder signal. Further, irradiation studies have shown that spectra of sulfur radicals in proteins are not identical with those of amino acid radicals, because of orbital immobilization and superposition of spectra from all affected amino acids. Therefore, there is some reason to suspect that the shoulder signals may have been caused by sulfur radicals, rather than by "bound" peroxy radicals. There is thus no conclusive proof in Roubal's results inconsistent with radical transfer from the lipid to protein, where it becomes energetically, rather than physically, stabilized.

Roubal's pioneer results do indicate that electron spin resonance may be a potentially powerful technique for studying radical production in oxidizing lipid–protein systems. Further investigations are needed to clarify the role of lipid radicals in protein damage mechanisms.

This paper reports results obtained in studies aimed at the following objectives: (1) to show whether free radicals are induced in proteins by reaction with peroxidizing lipids, and to study the nature of these radicals; (2) to obtain further information on the nature of products formed in reactions between amino acids or amino acid residues in proteins and peroxidizing lipids.

MATERIALS AND METHODS

Studies on Free-Radical Production in Proteins. Most of the studies were conducted on a model system using lysozyme. The procedure used is described below.

Model System. *Preparation.* A model system consisting of methyl linoleate (ML) (Hormel Institute) and lysozyme (Nutritional Biochemical, 3× crystallized) (10:1 molar ratio) was emulsified with distilled water by mixing for 5 min in a Sorvall Omni-Mixer, quick-freezing in liquid nitrogen, and then freeze-drying for 24 hr in a Virtis laboratory freeze-drier. Control systems of lysozyme and water, but no ML, were prepared in the same manner.

Treatment. As shown in Figure 3, after freeze-drying, both experimental and control systems were either oxidized in dry air following equilibration at 37° over $CaSO_4$, or over salt solutions in desiccators to water activities of approximately 0, 0.07, 0.04, and 0.75; or the systems were exposed to 2 Mrads γ irradiation. Irradiation was accomplished in sealed ampoules at ambient temperature in the ^{60}Co source of the U.S. Army Radiation Laboratory, Natick, Mass., with a dose rate of 3.92×10^4 rads/min, or in a Gammacell ^{60}Co irradiator at MIT (dose rate of 9500 rads/min).

Esr studies were conducted on systems without further treatment as well as on some from which lipid was extracted. Lipid was extracted with a benzene–ethanol azeotrope (32.4:67.6, v/v) solvent by shaking 30 min under N_2 and then filtering through a Büchner funnel.

Esr Measurements. All spectra were recorded for powder samples in 3.5-mm i.d. quartz tubes. To avoid interference from dielectric energy absorption by water, all samples were dried *in vacuo* over $CaSO_4$ before analyses.

The esr spectrometer was a Varian 4502 apparatus operating in X band with 100-kHz field modulation frequency.

The sweep rate of the external magnetic field was 10 G/min for radical concentration and principal g-value determinations, and 50 G/min for general spectra recording. Microwave frequency was 9433 MHz; microwave power was usually 3 mW, although higher power levels were used to study saturation effects. First derivatives of the absorption spectra were recorded with identical instrumental settings for all samples. The magnetic field was measured with a proton resonance field meter. The oscillation frequency for proton resonance was measured with a Heath-Schlumberger SM 100B electronic counter. Radical concentrations were determined by double integration of the spectra and comparison with diphenylpicrylhydrazyl standard samples measured under identical conditions.

Oxidation Measurements. The course of lipid oxidation was followed by iodimetric determination of peroxide values for the lipid extracted from incubated samples (American Oil Chemists' Society, Method AOCS-Cd-8:53).

Proteins and Amino Acids Other than Lysozyme. Several proteins and amino acids in addition to lysozyme were allowed to react with peroxidizing methyl linoleate. [Lysozyme (hen's egg white), bovine serum albumin, catalose, α-lactalbumin, myoglobin, l-asparagine, D,L-aspartic acid, L-cysteine, L-cystine, L-glutamine, L-glutamic acid, reduced glutathione, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine were obtained from Nutritional Biochemicals Corp. Casein, gliadin, and ovalbumin were obtained from Mann Research Laboratories; thiolated gelatin was from Schwarz BioResearch, Inc.; urease was from Sigma Chemical Co.; L-alanine, L-arginine, L-histidine, and D,L-tryptophan were from Eastman Kodak Co.; glycine was from Fisher Scientific Co.; and L-methionine was from Calbiochem.]

Reactions with methyl linoleate or its peroxides prepared by the method of Banks *et al.* (1959) were conducted in the following systems. Histidine (39–156 mg) and methyl linoleate (308 mg) were oxidized in air at 37° while dispersed on filter paper. Histidine (155 mg) and ML (308 mg) were oxidized in air at 37° while stirred in a 25-ml beaker in the absence of water.

Histidine (200 mg), ML (400 mg), and 1 ml of water were stirred in air at room temperature. In some experiments the water contained 10^{-7} M ferrous sulfate, or cobalt nitrate. Details of the procedure are reported by Roy and Karel (1973).

Methionine. L-Methionine was used without further purification. Experiments were conducted on methionine by exposing it to oxidation at 37° in air-stirred emulsions consisting of an aqueous solution of 1 mmol of the amino acid (pH 4) and 300 mg of methyl linoleate. Experiments were also conducted on the reactants dispersed on filter paper as in the case of histidine.

Lysine. An aqueous solution of lysine (146 mg in 0.5 ml) was dispersed on filter paper and dried in a vacuum desiccator overnight. Methyl linoleate (308 mg) in hexane (1 ml) was dispersed on the filter paper and the paper was redried, and then incubated for 14 days at 37°.

The paper was then cut in small pieces, extracted with chloroform, and then extracted with dilute hydrochloric acid, and filtered. The filtrate was neutralized with pyridine, and evaporated to dryness under reduced pressure. The dry residue was redissolved in water or in dilute HCl and examined on tlc plates. Ninhydrin and iodine vapor were used to visualize the spots. Additional analytical tests were conducted on some fractions, including ir and nmr.

RESULTS AND DISCUSSION

Free Radicals in Proteins and Amino Acids. Esr signals were observed in lysozyme exposed to peroxidizing methyl linoleate. The signals were singlets characterized by line widths of 11 ± 3 G, signal widths of 50 ± 15 G, and g values of 2.0051 ± 0.0005. As shown in Table I, the signal was observed only in lysozyme which was exposed to peroxidizing lipids, irradiation, or to very high temperatures. Peroxidizing lipids in the absence of protein gave no signals, probably because the half-life of the radicals present was too short to allow the buildup of detectable concentrations.

Table I. Esr Signals in Irradiated and Peroxidized Reaction Systems

System	Presence or absence of esr signal	Presence or absence of principal g value of 2.0051 ± 0.0005
Lysozyme alone		
Lyophilized	−	−
Lypohilized incubated	−	−
Irradiated	++	+
Heated in air or *in vacuo*, 160°	+	+
Lipid alone		
ML[a] irradiated	−	−
Vegetable oils,[b] irradiated	−	−
ML oxidized in bulk	−	−
ML oxidized on avicel	−	−
ML + lysozyme		
Lyophilized	−	−
Lyophilized incubated	+	+
Irradiated, lyophilized, or direct mix	++	+

[a] Methyl linoleate. [b] Peanut oil, safflower oil, and Wesson oil.

Table II. Reduction in Esr Signal Intensity Due to Treatment with Specified Solvents

	% reduction in esr signal	
	Lysozyme plus ML peroxides	Lysozyme irrad. with 1 Mrad
Chloroform–methanol (3:1)	80	89
Ethanol	72	73
Benzene–ethanol (32.4:67.6)	61	70
Hexane	58	52
Benzene	38	49

The signals were present in the lysozyme extracted with lipid-removing solvents, and in those which were not extracted. The signal intensity, however, was reduced in samples extracted with these solvents. This reduction was apparently due to radical quenching by the solvent, and not to the removal of the lipid. Table II shows that the reduction in intensity caused by the solvents was similar in lysozyme exposed to peroxidized methyl linoleate, and in lipid-free lysozyme exposed to γ irradiation.

A major difference between the signals from irradiated and oxidized lysozyme is the presence of signals caused by

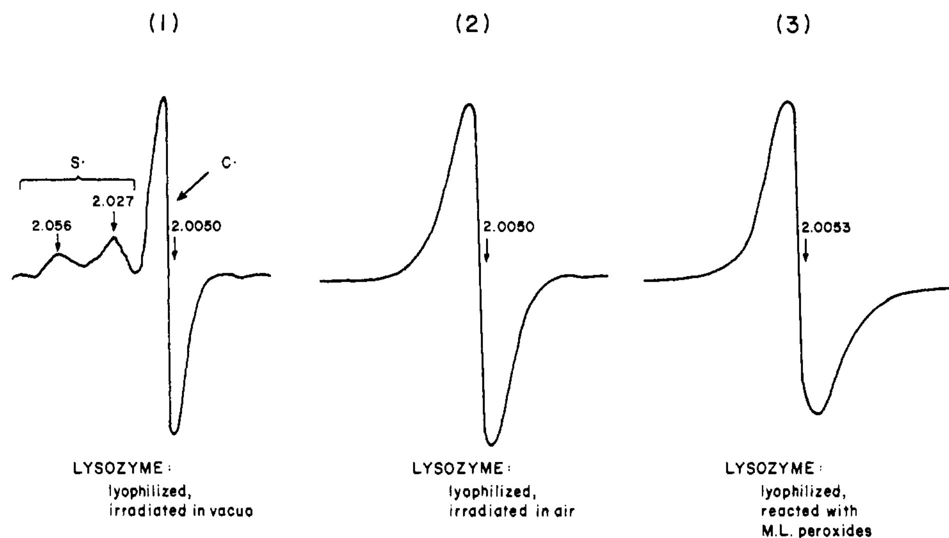

Figure 4. Typical esr signals of lyophilized lysozyme irradiated with γ-rays or exposed to methyl linoleate peroxides.

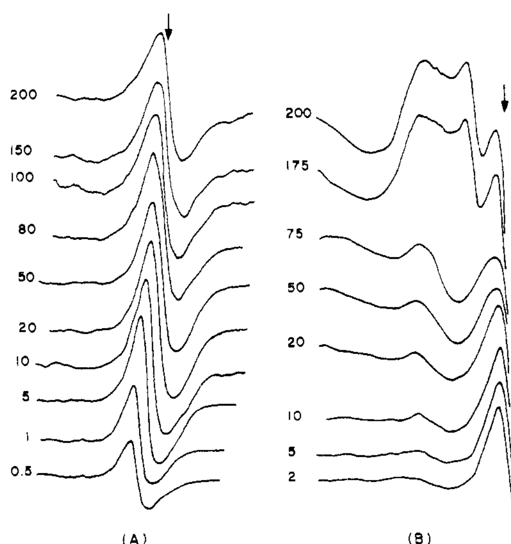

Figure 5. Comparison of saturation characteristics of lysozyme exposed to: (A) oxidizing methyl linoleate; (B) 1 Mrad of γ-radiation *in vacuo*. Microwave power levels are indicated on the traces.

Table III. Relative Intensity of Esr Signals of Proteins and Amino Acids Exposed to Radiation or to Incubation with Methyl Linoleate Peroxides

	Rel intensity	
	C radicals	S radicals
Lyophilized lysozyme		
Irradiation (vacuum)	++++	++
Irradiation (air)	++++	−
ML peroxides (air)	+++	−
Crystalline lysozyme		
Irradiation (air)	++++	+
ML peroxides (air)	+++	−
ML peroxides (vacuum)	++	−
Cysteine		
ML peroxides (air)	+	++++
Cystine		
ML peroxides (air)	+	++
Reduced glutathione		
ML peroxides (air)	++	++++

the sulfur radicals in lysozyme irradiated *in vacuo*. Lysozyme irradiated in air shows a great diminution in signal intensity due to sulfur, presumably because of sulfur oxidation. In lysozyme exposed to oxidizing methyl linoleate, sulfur radicals were not detected. Typical esr signals are shown in Figure 4. The esr signals from lysozyme exposed to oxidizing lipid (either linoleate or arachidonate) in either direct-mix systems or lyophilized emulsions exhibited α-carbon resonance essentially identical with that of irradiated lysozyme, but sulfur resonance was absent.

Composite spectra with contributions from a number of different radicals may often be at least partially resolved by saturation techniques, since each radical species saturates at a different power level. By sequentially increasing the power, resonance from some radical may be reduced or eliminated, and previously obscured weak resonances may then become detectable. The technique is particularly useful in detecting any obscured sulfur radicals, since carbon radicals easily saturate while sulfur resonance is progressively amplified as the power level increases. Typical saturation characteristics of lysozyme signals are shown in Figure 5. For convenience, only the downfield portion of the spectra is presented, since the sulfur radicals would be contributed by that portion of the spectrum. In both systems, the α-carbon resonance (the major central line with g = 2.0050 denoted by arrows) was easily saturated, as indicated by the decrease in line intensity with increasing power above about 15 mW. In lysozyme irradiated with γ-rays, the sulfur resonance increasingly predominated as the power increased, but analogous changes did not occur in lysozyme exposed to oxidizing lipid, suggesting that sulfur resonance is not present at all. Further attempts to detect presence of weak sulfur signals by using high gain and lower microwave power were similarly unsuccessful.

There are four disulfide bonds in lysozyme and no free sulfhydryl groups. Two of the four disulfide bonds are buried in the interior, and thus are definitely inaccessible to lipid peroxides. Consideration of the crystal structure of lysozyme suggests that of the remaining two bonds, Cys-6–Cys-30 would seem to be most accessible, particularly if the molecule was partially denatured. To determine whether the lack of sulfur resonance was caused by the inability of lipid peroxides to break the disulfide bonds, experiments were conducted in which lysozyme was denatured with guanidine hydrochloride and reduced with mercaptoethanol and then incubated with methyl linoleate. After 3 days incubation sulfur radicals were read-

Table IV. Esr Spectral Characteristics of Various Proteins Allowed to React with Oxidized Methyl Linoleate

Protein	*g* values	
	Carbon	Sulfur
Lysozyme	2.0053	
Reduced, denatured lysozyme	2.0061	2.015, 2.023
Cysteine	2.0087	2.010, 2.024, 2.055
Thiolated gelatin	2.0050	2.017, 2.023
Bovine serum albumin	2.0053	2.017, 2.023[b]
Gliadin	2.0052	2.016, 2.026
Ovalbumin	2.0052	2.015, 2.023
Catalase[a]	2.0058	2.015, 2.042
Lactalbumin	2.0057	
Trypsin	2.0057	

[a] Has also signals with *g* values of 1.863, 1.922, 2.094, and 2.154. [b] Only after long incubation.

ily detected. Apparently lipid peroxides and/or free radicals can readily abstract hydrogen from sulfhydryl groups in proteins, but do not readily break disulfide bonds. This hypothesis was confirmed by exposing a number of proteins and amino acids to oxidizing methyl linoleate. The results shown in Tables III and IV indicate that, of the proteins containing no free sulfhydryl groups, only gliadin showed signals caused by sulfur radicals. This protein, however, is known to undergo -S-S to -SH exchange very readily. Exposure of crystalline amino acids to linoleate peroxides produced both carbon and sulfur radicals in cysteine as well as in cystine, but no signals in methionine. Free-radical signals were observed also in tryptophan, arginine, lysine, and histidine, but not in any of the other amino acids present in lysozyme. More details on the relative intensity of signals in different proteins are presented by Schaich (1974). The spectra of the above four amino acids indicate that the signals were primarily caused by carbon radicals, but there was some evidence of esr signals in nitrogen. We believe that the process of free-radical transfer to the proteins and amino acids occurs *via* complex formation as shown

$$PH + LOOH \longrightarrow [PH\cdots LOOH] \longrightarrow P\cdot + LOO\cdot + H_2O$$

where PH refers to protein and LOOH refers to lipid hydroperoxides.

Products of Reactions of Amino Acids with Peroxidizing Methyl Linoleate. Preliminary work on the separation and tentative identification of reaction products of histidine, lysine, and methionine with peroxides of methyl linoleate has been completed.

In the case of histidine we have published results indicating the formation of histamine, aspartic acid, and several other reaction products (Roy and Karel, 1973). Additional studies on histidine are in progress.

From methionine exposed to peroxidizing methyl linoleate we isolated methionine sulfoxide. Purified methionine sulfoxide was then readily oxidizable to the sulfone, but in a methionine–methionine sulfoxide mixture this conversion did not occur.

A number of products derived from lysine were isolated after this amino acid was allowed to react with peroxidizing methyl linoleate. Major reaction products which were tentatively identified included: diaminopentane; aspartic acid; glycine; alanine; 1,10-diamino-1,10-dicarboxydecane; α-aminoadipic acid; and pipecolinic acid. Details of the identification will be presented in a subsequent paper.

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