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Lipid Hydroperoxide Reactivity with Proteins and Amino Acids: A Review

H. W. Gardner

Lipoxygenase is responsible for the production of lipid hydroperoxides in inadequately processed foods. The hydroperoxides as well as their products of decomposition are potentially reactive substances that can cause deterioration of food proteins or amino acids. Among the many consequences of protein exposure to peroxidized lipids is the formation of lipid–protein complexes that are bound through purely physical forces. Chemical changes caused by interaction of lipid hydroperoxide and protein are protein–protein cross-links, protein scission, protein–lipid adducts, and amino acid damage. The secondary products arising from hydroperoxide decomposition also readily damage protein and amino acids through formation of covalent bonds. Among the secondary products, aldehydes have received the most attention because of their propensity to form Schiff base adducts with amino groups, and, in particular, the bifunctional malondialdehyde can cross-link protein via Schiff base formation.

It is remarkable that living tissue is relatively immune to lipid peroxidation although all the necessary ingredients are present, i.e., polyunsaturated lipids, O_2, and catalysts. The resistance to lipid oxidation attests to the precise cellular compartmentalization by which a high degree of organization protects the cell from self-destruction. When tissue is disrupted as encountered in the isolation of cellular organelles, lipid oxidation is initiated, and this eventually leads to damage of membrane structure (Mead, 1976; McKnight et al., 1965; Robinson, 1965) and inactivation of enzymes (Bernheim et al., 1952). In particular, protein in biomembranes exists in close molecular proximity to polyunsaturated lipids, thus membrane protein would be readily damaged by lipid oxidation. In many foodstuffs the cellular integrity has been disrupted in varying degrees. As a result, care must be exercised to prevent lipid oxidation and attendant loss of quality and flavor. If large quantities of lipid peroxides accumulate in food, they can be toxic when ingested (Barber and Bernheim, 1967). Protein and amino acids are only one class of biochemicals in foods that is susceptible to the damaging effects of lipid hydroperoxides.

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The terminologies, lipid peroxides, peroxidized lipid, lipid hydroperoxides, are used interchangeably in the text. The term "peroxide" strictly is inaccurate nomenclature, but it is one that has gained wide usage. For the most part, hydroperoxides are the initial products of lipid oxidation and usually account for the majority of bound oxygen measured by peroxide value. Various endoperoxides and peroxides are usually secondary products and probably amount to a relatively small part of the total. In the text the term "peroxidizing" is distinguished from "peroxidized" since a lipid actively in the process of oxidation may affect protein or amino acids differently than a lipid that already had been peroxidized. Also, the term "peroxidized" should be distinguished from a specific isolated lipid hydroperoxide, such as linoleic acid hydroperoxide, because a "peroxidized" lipid implies an impure mixture containing hydroperoxides, unoxidized lipid, and secondary products.

The interaction between lipid hydroperoxide and protein/amino acids is complex indeed, a complexity that is contributed to by both lipid hydroperoxide and its secondary products of decomposition. In one such interaction, the lipid hydroperoxide and/or secondary products will physically complex with protein. In another, various types of covalent bonds are the result. These chemical reactions are categorized as to type in the text below. Other reviews that cover different aspects of the

topic are available (Tappel, 1973; Karel et al., 1975; Schauenstein, 1967; Kaunitz, 1967; Pokorny and Janicek, 1975).

NONCOVALENT COMPLEXES

When proteins are exposed to peroxidized lipid, a large proportion of the lipid complexes with the protein through hydrophobic association and/or hydrogen bonds. This phenomenon was recognized by Narayan and Kummerow (1958, 1963) and Narayan et al. (1964), who suggested that egg albumin physically complexed with oxidized lipids largely through hydrogen bonds. They did not obtain evidence for covalent bonding, but their methods would tend to discriminate between gross differences in complexed and native protein rather than small differences due to a comparatively few lipid–protein covalent bonds that may have been present.

The strength of the lipid–protein complex can be defined by a series of extraction steps (Pokorny, 1963; Pokorny et al., 1975a, 1976b; Kanazawa et al., 1975). A stepwise increase in solvent polarity extracted weakly bound lipids first, and the hydrogen-bonded lipids, which contained the largest amount of oxygenated functionality, were extracted last (Pokorny et al., 1976b). Kanner and Karel (1976) used 8 M urea and sodium dodecyl sulfate at low pH to break the complexes. Although most of the peroxidized lipid usually can be removed from protein by methods that disrupt hydrogen bonds, the lipid resistive to these methods can be extracted only after chemical treatment, e.g., by hydrolysis. Such lipid is bound mainly by covalent bonds.

If ignored, this physical complexing phenomenon could lead researchers to make false interpretations of their results. For example, Kanazawa et al. (1975) noted that losses in amino acid residues from lipid hydroperoxide treated protein were dependent upon the extraction procedure prior to protein hydrolysis. Ether-extracted protein suffered severe amino acid losses, but exhaustive extraction with polar solvents resulted in minimal losses. Additionally, the oft-noted inactivation of enzymes by lipid hydroperoxides may be misinterpreted. According to Matsushita et al. (1970), linoleic acid was sometimes as effective as linoleic acid hydroperoxide in its ability to inhibit a number of enzymes over a range of conditions. Binding of the fatty acids to the enzyme's hydrophobic regions, rather than a chemical reaction, could have been responsible for modification of activity. However, Matsushita and Kobayashi (1970) observed a unique activation of pepsin by linoleic acid hydroperoxide that could not be duplicated by linoleic acid.

Secondary products derived from the degradation of lipid hydroperoxide readily complex with protein. In this regard, aldehydes are one category of secondary products that have received much attention because of their low flavor threshold (Sessa and Rackis, 1977; Eriksson et al., 1976). The binding of a homologous series of volatile aldehydes and methyl ketones to a variety of proteins was determined by Franzen and Kinsella (1974). They demonstrated the participation of hydrophobic sites by a decrease in binding capacity of leaf protein concentrate after removal of residual lipid. Beyeler and Solms (1974) concluded that the binding affinity of aldehydes, ketones, and alcohols to either soy protein or bovine serum albumin was characterized by weak unspecific forces with nearly unlimited binding capacity. They suggested binding may involve both electrostatic and hydrophobic attractions. Similarly, the binding of hexanal and hexanol to partially denatured soy protein was attributed to hydrophobic association with binding energies (ΔF) of −3.007 and −2.558 kcal for hexanal and hexanol, respectively (Arai et al., 1970). Although hexanal and hexanol could not be removed by either hexane extraction or vacuum distillation, they were readily removed after enzymic proteolysis. It should be emphasized here that eventually aldehydes chemically react to some extent with the amino groups of proteins through formation of Schiff base adducts (see Reactions with Secondary Products section).

RADICAL REACTIONS

As initiators of radical chains, lipid hydroperoxides in contact with protein cause formation of protein-centered radicals which ultimately result in various types of damage. The process is greatly accelerated by radical initiators, such as transition metal ions or metalloproteins. The following depicts such a chain with ionic iron acting as an example of an initiator:

$$\mathrm{ROOH} + \mathrm{Fe^{3+}} \rightarrow \mathrm{ROO\cdot} + \mathrm{H^+} + \mathrm{Fe^{2+}}$$

$$\mathrm{ROOH} + \mathrm{Fe^{2+}} \rightarrow \mathrm{RO\cdot} + \mathrm{OH^-} + \mathrm{Fe^{3+}}$$

$$\mathrm{PH} + \mathrm{ROO\cdot} \rightarrow \mathrm{P\cdot} + \mathrm{ROOH}$$

$$\mathrm{PH} + \mathrm{RO\cdot} \rightarrow \mathrm{P\cdot} + \mathrm{ROH}$$

$$\mathrm{P\cdot} \rightarrow \text{products (cross-links, scission, oxidation)}$$

Recent research has indicated that radical damage to protein by lipid hydroperoxides may be of greater importance than damage caused by secondary products of hydroperoxide decomposition.

Evidence for Protein Radicals. Roubal (1970) reported electron paramagnetic resonance (EPR) signals from dehydrated protein-peroxidized lipid mixtures, thereby showing the existence of protein radicals. As assessed by destruction of amino acid residues from protein, the major damage occurred concomitant with maximum EPR signal intensity, whereas most damage had subsided by the time fluorescence (malondialdehyde damage) reached its maximum value at a later time (Roubal, 1971). Schaich and Karel (1975) and Karel et al. (1975) confirmed and extended Roubal's EPR investigations with a lysozyme-peroxidized methyl linoleate mixture. The main signal at $g = 2.0051 \pm 0.0005$, assigned to a carbon-centered radical, increased in intensity in proportion to the peroxide value of the extracted lipid. Moreover, the signal intensity increased further after the lipid peroxides started to decompose, indicating that protein radicals were induced by lipid peroxides.

To gain information on the origin of the EPR signals in protein, Schaich and Karel (1976) obtained EPR spectra of amino acid peroxidized methyl linoleate mixtures. Only lysine, arginine, histidine, tryptophan, cysteine, and cystine yielded signals intense enough for detection. It is of interest that in protein most of these amino acids are the most sensitive to damage by peroxidized lipid (cf. Protein Scission and Amino Acid Damage section). Much like the protein EPR spectra, the central feature of the amino acid EPR signal was at $g = 2.004$, which was assigned to α carbon or side-chain carbon radicals. Particularly with arginine, there was partial delocalization of spin density presumably on nitrogen. The remarkable similarity between tryptophan and nonsulfhydryl protein signals led Schaich and Karel to surmise that tryptophan was the major contributor to protein EPR spectra as can be seen in Figure 1.

Henriksen et al. (1976) pointed out that in irradiated protein, sulfur tends to accumulate unpaired spin density by intra- and intermolecular free radical migration. Such free radical migrations are important processes in localizing unpaired spin density in specific regions of the protein. Sulfur radicals were readily induced by peroxidizing

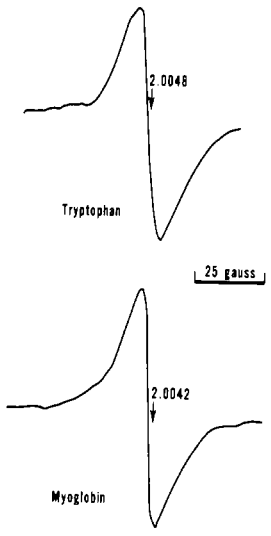


Figure 1. Electron paramagnetic resonance spectra of tryptophan and a nonsulfhydryl protein, myoglobin, reacted with peroxidizing methyl linoleate. Reprinted by permission from the authors and the American Oil Chemists' Society; from Schaich and Karel (1976).

methyl linoleate in both sulfhydryl proteins and cysteine (Schaich and Karel, 1975, 1976; Karel et al., 1975). The EPR spectrum of a cysteine-peroxidizing methyl linoleate mixture initially displayed an α-carbon resonance at g = 2.0065, as well as resonance shoulders at g = 2.00, 2.015, and 2.022 which were assigned to sulfur radicals (Schaich and Karel, 1976). After 8 days of incubation, the spectrum changed slightly (Figure 2) but still retained the sulfur signals at g = 2.015 and 2.020. As can be seen in Figure 2, the sulfhydryl protein, ovalbumin, also displayed similar α-carbon and sulfur signals after exposure to peroxidizing methyl linoleate. According to Schaich and Karel, the sulfur resonances probably were due to sulfur–oxygen radicals rather than thiyl radicals. The facile formation of radicals from the sulfhydryl group is probably related to the observed inactivation of sulfhydryl enzymes by peroxidizing lipids (Wills, 1961; Lewis and Wills, 1962; Little and O'Brien, 1966) and the observed oxidation of protein thiols by linoleic acid hydroperoxide (Little and O'Brien, 1968).

Although cystine resisted formation of sulfur resonances, longer incubation times did result in sulfur signals (Schaich and Karel, 1976). On the other hand, the sulfur signals were absent in disulfide proteins treated with peroxidizing lipid. Lysozyme, which contains four disulfide bonds and no sulfhydryl groups, did not display sulfur resonances in the presence of peroxidized lipid unless the enzyme was denatured with guanidine hydrochloride and the disulfide bonds were reduced with mercaptoethanol (Karel et al., 1975).

In order to effect radical transfer from lipid hydroperoxide to protein, contact must be established between them. Such intimate contact is achieved through noncovalent complexes (see prior section), and it is known that such occluded lipid can be high in peroxide content (Roubal and Tappel, 1966b). Schaich and Karel (1976) postulated that radical transfer takes place through complexes between lipid hydroperoxide (ROOH) and the nitrogen or sulfur centers of reactive amino acid residues of the protein (PH) as shown:

$$\mathrm{ROOH + PH \rightarrow [ROOH\cdots HP] \begin{cases} \nearrow \mathrm{RO\cdot + P\cdot + H_2O} \\ \searrow \mathrm{RO\cdot + \cdot OH + PH} \end{cases}}$$

$$\mathrm{PH + RO\cdot \rightarrow P\cdot + ROH} \qquad (6)$$

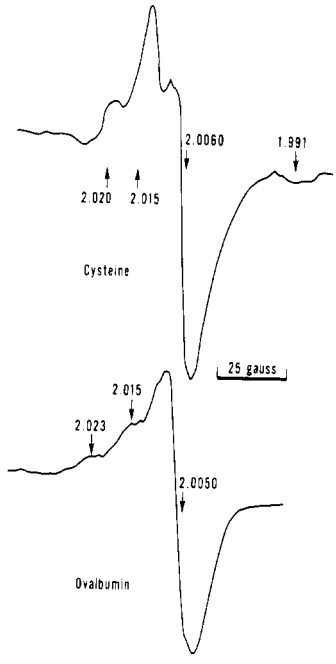


Figure 2. Electron paramagnetic resonance spectra of cysteine and a sulfhydryl protein, ovalbumin, after incubation with peroxidizing methyl linoleate. Reprinted by permission from the authors and the American Oil Chemists' Society; from Schaich and Karel (1976).

Protein to Protein Cross-Links. In aqueous solution or at high water activities, proteins commonly form protein–protein cross-links in the presence of peroxidizing lipids. Roubal and Tappel (1966b) discovered that peroxidized protein had cross-linked into a range of oligomers with concomitant protein insolubility. Likewise, a mixture of histidine and peroxidizing ethyl arachidonate resulted in histidine oligomers as demonstrated by gel permeation chromatography. In studies with dehydrated protein–lipid mixtures that were adjusted to various water activities, more cross-linking occurred at higher water activities, e.g., a_w = 0.75 (Kanner and Karel, 1976). Additionally, greater fluorescence was observed in samples incubated at higher water activities. Since fluorescence is an indicator of malondialdehyde cross-linking of protein (see Secondary Products section), it is not now possible to assess the relative importance of malondialdehyde vs. radical cross-linking reactions.

A few radical mechanisms for protein cross-linking have been proposed. Roubal and Tappel (1966b) suggested a bimolecular homolytic substitution or radical displacement reaction as follows (P = protein):

$$\mathrm{P\cdot + P \rightarrow P\text{–}P\cdot}$$

$$\mathrm{P\text{–}P\cdot + P \rightarrow P\text{–}P\text{–}P\cdot}$$

Because of a high activation energy, bimolecular homolytic substitution is unlikely to occur at sp 3-hybridized 4-coordinate carbon, but such substitution possibly could occur on aromatic amino acid residues. Schaich and Karel (1976) postulated that cross-linking occurred through termination reactions, which limits the reaction to collision of two protein radicals:

$$\mathrm{P\cdot + P\cdot \rightarrow P\text{–}P}$$

Lipid to Protein Cross-Links. As noted above, large amounts of peroxidizing lipids are simply occluded by protein; however, some lipid appears to bind covalently to protein (Zirlin and Karel, 1969; Roubal and Tappel, 1966b; Desai and Tappel, 1963; Matsushita, 1975; Nielsen, 1978). In most investigations, the covalent bonding of lipid

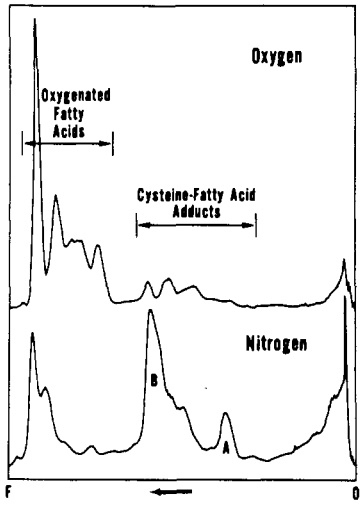


Figure 3. Thin-layer densitogram of products obtained from incubation of 13-hydroperoxylinoleic acid (3.2 mM), cysteine (12.8 mM), and $FeCl_3$ (10^{-5} M) in ethanol–H_2O (8:2) under either O_2 or N_2 atmosphere. Identity of spot A, 9-*S*-cysteine-10,13-dihydroxy-*trans*-11-octadecenoic acid; B, 9-*S*-cysteine-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid. Solvent system, $CHCl_3$–CH_3OH–H_2O–acetic acid (65:25:4:1) (filter paper liner). From Gardner, H. W., unpublished.

to protein has been studied per se rather than the characterization of the lipid–protein bond, and it is difficult to assess whether the lipid hydroperoxide or a secondary product is involved.

In a model system, 13-hydroperoxylinoleic acid was bound covalently to the sulfhydryl amino acids, cysteine or *N*-acetylcysteine (Gardner et al., 1976; Gardner et al., 1977). When catalyzed by 10^{-5} M $FeCl_3$ in aqueous ethanol solvent, *N*-acetylcysteine or cysteine was added by thiyl bond to the cis-9 olefinic carbon of 13-hydroperoxylinoleic acid; the 13-hydroperoxy group was reduced to a 13-hydroxy group; and the olefinic carbon-10 became substituted with an ethoxy or hydroxy group. Thus, with cysteine, the products were 9-*S*-cysteine-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid and 9-*S*-cysteine-10,13-dihydroxy-*trans*-11-octadecenoic acid. As shown in Figure 3, the absence of oxygen is required for production of high yields of the fatty acid adducts with cysteine. Under nitrogen, the only other product of significance was 13-oxooctadecadienoic acid which migrated close to the solvent front on thin-layer chromatography (TLC) plates. As seen in Figure 3, the approximate 4:1 ratio of the quantity of B to A (10-ethoxy adduct vs. 10-hydroxy adduct) reflects the 4:1 ethanol/water solvent used in the experiment. Gardner et al. (1976) proposed a mechanism of adduct formation that was consistent with the experimental findings (Figure 4).

When cysteine was reacted with 13-hydroperoxylinoleic acid in the presence of oxygen, the products shifted from fatty acid–cysteine adducts to a number of oxygenated fatty acids (Figure 3). Gardner et al. (1974) identified nine oxygenated fatty acids from such a reaction. The absence of cysteine–fatty acid adducts and the production of oxygenated fatty acids can be explained only by the active participation of oxygen in the reaction. Oxygen is an effective competitor for radicals and possibly would overwhelm any other radicals present by reacting with them as proposed in Figure 5.

In a reaction similar to the cysteine–fatty acid addition discussed above, Ham et al. (1975) reported addition of either cysteine, glutathione, or protein to prostaglandin A_1. Cagen et al. (1976) characterized the glutathione–

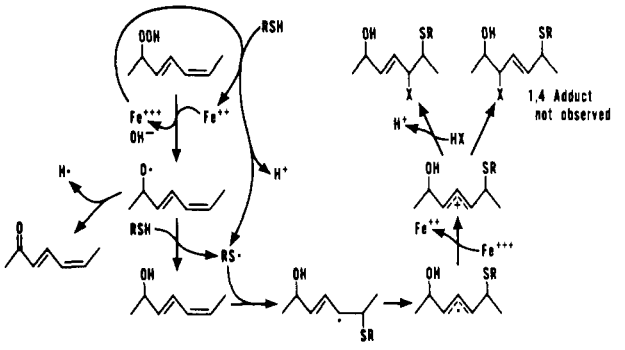


Figure 4. Pathway proposed for formation of adducts between 13-hydroperoxylinoleic acid and cysteine under N_2 atmosphere. HX, solvent; RSH, cysteine; fatty acid structures are abbreviated. Reprinted by permission from the American Oil Chemists' Society; from Gardner et al. (1976).

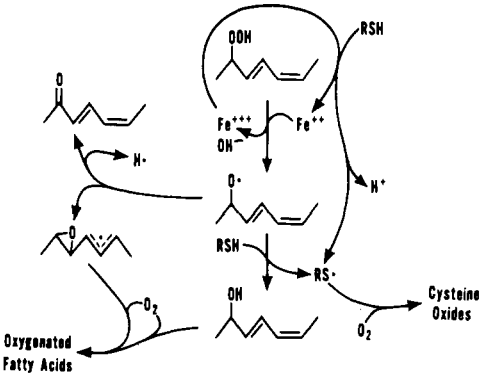


Figure 5. Pathway proposed for formation of oxygenated products from a reaction of 13-hydroperoxylinoleic acid and cysteine under an O_2 atmosphere. RSH, cysteine; fatty acid structures are abbreviated.

prostaglandin A_1 product from red blood cells as a thiyl addition to the double bond of the cyclopentenone. This product was typical of anti-Markownikoff thiyl-radical adducts (Griesbaum, 1970). It is not known if the reaction was radical initiated, but ample opportunity for radical initiation would be expected in red blood cells.

Protein Scission and Amino Acid Damage. Protein scission is favored over protein–protein cross-linking when peroxidized lipid is mixed with protein in the dehydrated state. Freeze-dried gelatin suffered up to a fourfold decrease in molecular weight after exposure to peroxidizing methyl linoleate, but scission was inhibited after increasing the water activity of the mixture (Zirlin and Karel, 1969). An increased content of protein amide groups after peroxidation indicated that cleavage occurred at the α carbon. Thus, these workers proposed that protein peroxides are formed through oxygen attack on α-carbon-centered radicals. Subsequent cleavage at the peroxide-bearing α carbon would result in protein scission and an increased amide content.

Amino acid residues in protein are damaged from exposure to lipid hydroperoxide regardless of whether the mixture is incubated in an aqueous system or dehydrated state. Table I lists the amino acid residues in proteins most labile to damage by lipid hydroperoxides. In general, histidine, cysteine/cystine, methionine, lysine, and tyrosine are among the most sensitive residues. Although tryptophan has not been measured frequently enough to assess its susceptibility to damage, this amino acid probably is among the most labile (O'Brien, 1966; Kanazawa et al., 1975). Except for methionine and tyrosine, most of these very sensitive amino acids are exceptional among the other

Table I. Damage to Amino Acid Residues in Proteins Exposed to Peroxidized Lipids[a]

protein	damage to amino acid residues (% loss)	type of lipid and (conditions)	reference
cytochrome c	(A) His (59), Ser (55), Pro (53), Val (49), Arg (42), Met (38), Cys (35)	peroxidizing linolenic acid (aqueous, 37 °C, 5 h)	Desai and Tappel (1963)[b]
	(B) Tyr (60-75), Try (48), Cys (37), His (31-35), Met (18-37)	linoleic acid hydroperoxide	O'Brien (1966)
γ-globulin	Lys (59), His (52), Tyr (51), Met (38), Cys (33)	peroxidizing ethyl arachidonate (aqueous, 37 °C)	Roubal and Tappel (1966a)[b]
bovine serum albumin	Gly (83), Cys (64), His (54), Ala (50), Val (48), Met (48)	peroxidizing ethyl arachidonate (aqueous, 37 °C)	Roubal and Tappel (1966a)[b]
hemoglobin	Tyr (91), Met (59), Lys (59), His (58)	peroxidizing ethyl arachidonate (aqueous, 37 °C)	Roubal and Tappel (1966a)[b,c]
ovalbumin	Met (80), His (38), Thr (28), Pro (28), Gly (28)	peroxidizing ethyl arachidonate (aqueous, 37 °C)	Roubal and Tappel (1966a)[b,c]
catalase	Lys (42), Ser (22), Val (21), Met (20), His (18)	peroxidizing ethyl arachidonate (aqueous, 37 °C)	Roubal and Tappel (1966a)[b,c]
ribonuclease A	Lys (35), His (35), Tyr (16)	peroxidizing ethyl arachidonate (aqueous, 37 °C, 22 h)	Chio and Tappel (1969b)[b]
ribonuclease	Met (99), Tyr (62), His (54), Lys (51), Cys (40)	linoleic acid hydroperoxide (aqueous, 37 °C, 40 min)	Gamage and Matsushita (1973)[b]
trypsin	Met (83), His (12)	linoleic acid hydroperoxide (aqueous, 37 °C, 40 min)	Gamage and Matsushita (1973)[b]
pepsin	Met (99), Arg (13), Glu (13)	linoleic acid hydroperoxide (aqueous, 37 °C, 40 min)	Gamage and Matsushita (1973)[b]
lysozyme	Try (56), His (42), Lys (17), Met (14), Arg (9)	linoleic acid hydroperoxide (aqueous, 37 °C, 8 days)	Kanazawa et al. (1975)
ovalbumin	Lys (50), Met (42), Leu (22), His (21), Val (21)	peroxidized ethyl linoleate (80% relative humidity, 60 °C, 4 days)	Horigome and Miura (1974)[b,c]
casein	Lys (50), Met (47), Ile (30), Phe (30), Arg (29), Asp (29), Gly (29), His (28), Thr (27), Ala (27), Tyr (27)	peroxidized ethyl linoleate (80% relative humidity, 60 °C, 4 days)	Horigome and Miura (1974)[b,c]
ovalbumin	Met (17), Ser (10), Lys (9), Ala (8), Leu (8)	peroxidized ethyl linoleate (aqueous, 55 °C, 24 h)	Horigome et al. (1974)[b,c]
casein	Lys (10), Thr (10), Val (10), Ala (9), Tyr (8), Phe (8), Ser (8), Arg (8), Asp (8)	peroxidized ethyl linoleate (aqueous, 55 °C, 24 h)	Horigome et al. (1974)[b,c]

[a] Only the most labile amino acids are tabulated by arbitrary selection. For a complete listing of damaged amino acids, refer to the original publications. [b] Tryptophan not analyzed. [c] Cystine not analyzed.

Table II. Products Obtained after Exposing Amino Acids to Peroxidized Lipids

amino acid	products	type of lipid	reference
histidine	(A) imidazole lactic acid; imidazole acetic acid; two histidine-aldehyde Schiff base adducts	peroxidizing methyl linoleate or methyl linoleate hydroperoxide	Yong and Karel (1978a)
	(B) histamine; valine; aspartic acid; ethylamine	peroxidizing methyl linoleate	Roy and Karel (1973)
cysteine	(A) cystine; H_2S; alanine	peroxidizing ethyl arachidonate	Roubal and Tappel (1966a)
	(B) cystine; cysteic acid; cystine disulfoxide	peroxidizing linoleic acid	Lewis and Wills (1962)
methionine	methionine sulfoxide	peroxidizing methyl linoleate	Karel et al. (1975)
lysine	diaminopentane; aspartic acid; glycine; alanine; 1,10-diamino-1,10-dicarboxydecane; α-aminoadipic acid; pipecolic acid	peroxidizing methyl linoleate	Karel et al. (1975)[a]

[a] Tentative identification.

amino acids in that they yielded EPR signals in the presence of peroxidizing lipid (Schaich and Karel, 1976). It is reasonable to conclude that amino acids susceptible to formation of stable radicals also are vulnerable to radical attack.

For the reason that the radical chemistry of these destructive reactions might be learned, there is considerable interest in identifying the degradation products of the labile amino acids. After exposure to lipid hydroperoxide, the amino acids, histidine, cysteine/cystine, lysine, or methionine, yielded the products summarized in Table II. The oxidation of methionine to methionine sulfoxide probably can be accounted for by the ease of delocalization of electrons on sulfur. Presumptive evidence for the oxidation of methionine residues in casein to methionine sulfoxide after exposure to autoxidizing methyl linoleate has been presented by Tannenbaum et al. (1969). The formation of the thiyl radical from cysteine is facile and accounts for the products derived from cysteine. Products from the nonsulfur amino acids can be explained on the basis of radical formation at the α carbon and to some extent on the side chains. In the degradation of histidine, Yong and Karel (1978a) theorized the following sequence of events: (1) formation of an α-carbon radical by deamination, (2) hydroperoxidation of the α carbon, and (3) hydroperoxide homolysis which led to observed products, imidazole lactic acid, and imidazole acetic acid. Additionally, the imidazole side chain was susceptible to

attack as determined with the model compound, 4-methylimidazole (Yong and Karel, 1978b), or with α-amino derivatives of histidine, such as *N*-acetylhistidine (Yong, 1978). Lysine products also can be explained by radical attack at the α carbon and side-chain carbons (Table II). Among the side-chain carbons the ϵ carbon seems to be the most labile. Schiff base formation is a particularly important degradative reaction for lysine, but discussion of this is deferred to a later section.

Activated Oxygen and Oxidation. Until recently, reactions of activated oxygen, i.e., singlet oxygen, superoxide, and hydroxyl radical, were unsuspected in biochemical transformations. The role of activated oxygen in interactions between lipid hydroperoxide and protein can not be assessed fully at this time, but it does seem certain that some active oxygen originates from lipid hydroperoxide or peroxidizing lipid.

The involvement of superoxide (oxygen radical anion) in peroxidizing lipids or decomposing hydroperoxides is incompletely known; for a recent review of superoxide biochemistry, see Fridovich (1976). Although superoxide itself is reactive, its deleterious action probably is the result of production of the more reactive hydroxyl radical through the following:

$$O_2^{-}\cdot + H_2O_2 \rightarrow OH^- + \cdot OH + O_2$$

Gutteridge (1977) observed superoxide formation during metal-ion catalyzed lipid oxidation. Gutteridge's observation may explain partly why actively peroxidizing lipids are commonly more destructive than pure lipid hydroperoxides. Superoxide also has been implicated in the oxidation of thiols, including cysteine (Asada and Kanematsu, 1976). The reverse reaction occurred as well; i.e., autoxidation of thiols generated superoxide (Misra, 1974). At this time an assessment of the effect of superoxide on other amino acids or protein can be only conjecture.

Singlet oxygen (spin-paired oxygen) recently has been identified during the decomposition of lipid hydroperoxide by either ceric ions, methemoglobin, or hematin (Hawco et al., 1977). Singlet oxygen produced by light-sensitized dyes readily decomposed tryptophan, methionine, histidine, and cysteine (Foote, 1976), and these amino acids are among the most labile to damage by lipid hydroperoxide. Whether there is a real or casual relationship between singlet oxygen damage and the lipid hydroperoxide destruction of protein and amino acids is unknown.

The γ irradiation of proteins produced EPR signals somewhat like those seen with lipid peroxidized protein (Karel et al., 1975). Irradiation is known to produce hydrated electrons, hydroxyl radicals, and hydrogen peroxide. Largely because of the reactivity of the hydroxyl radical, irradiation of protein or amino acids destroyed aromatic, heterocyclic, and sulfur amino acids (Simic, 1978; Henriksen et al., 1976). Hydroxyl radicals are commonly proposed as one pathway to homolytic scission of hydroperoxy groups, but their relative importance in lipid hydroperoxide decomposition has not been assessed.

The coordinate metal of metalloproteins usually is oxidized to a higher oxidation state by lipid hydroperoxide. This type of reaction affects the red color of meat through oxidation of oxymyoglobin to brown-colored metmyoglobin (Lin and Hultin, 1977). O'Brien and Frazer (1966) reported the facile oxidation of reduced cytochrome *c* by linoleic acid hydroperoxide.

REACTIONS WITH SECONDARY PRODUCTS

"Secondary product" is a generic term used to describe a mixture of aldehydes, epoxides, ketones, and other products obtained from the decomposition of lipid hydroperoxides (Gardner, 1975). Some of the secondary products react with protein or amino acids, but in peroxidizing mixtures it is difficult to assess the relative importance of secondary products vs. the primary lipid hydroperoxide. A few such comparative studies tested isolated lipid hydroperoxide and secondary products for their effect on enzymes; the results were not predictable. For example, pepsin was activated by secondary products but was inhibited by linoleic acid hydroperoxide; whereas trypsin was inactivated by secondary products but linoleic acid hydroperoxide had no effect (Gamage and Matsushita, 1973). Both secondary products and linoleic acid hydroperoxide damaged amino acid residues of protein (Kanazawa et al., 1975; Gamage and Matsushita, 1973), and both were bound to protein (Matsushita, 1975).

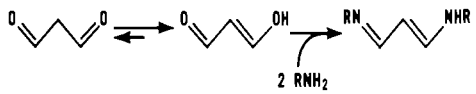


Figure 6. Formation of a Schiff base adduct between malondialdehyde and two amino groups.

Malondialdehyde. Malondialdehyde (MDA) has long been recognized as a secondary product of lipid peroxidation, and its detection with 2-thiobarbituric acid is the basis of a much utilized test for lipid rancidity. Pryor et al. (1976) proposed that MDA originates from prostaglandin-like endoperoxides formed via autoxidation of polyunsaturated fatty acids containing three or more double bonds. Fatty acids with less than three double bonds also appear to give rise to smaller amounts of MDA (Tarladgis and Watts, 1960).

Early investigations indicated that MDA was forming an acid-labile bond with either food proteins (Kwon et al., 1965) or bovine serum albumin (Kwon and Brown, 1965). When exposed to bovine plasma albumin, MDA blocked the ϵ amino of lysine and the N-terminal amino group of aspartic acid (Crawford et al., 1967). Similarly, ninhydrin-sensitive amino groups were lost after trout myosin was reacted with MDA (Buttkus, 1967); ninhydrin only reacts with free amino groups, the most notable being the ϵ amino of lysine. During the storage of frozen herring Kuusi et al. (1975) noted that free ϵ-amino groups decreased as the 2-thiobarbituric acid value increased.

Because of the bifunctionality of MDA, cross-linking between free amino groups of proteins is possible. Andrews et al. (1965) demonstrated that mixtures of gelatin and peroxidized methyl linoleate led to cross-linked gelatin. Bisulfite, which forms adducts with aldehydes, prevented the cross-links. In a model system composed of ribonuclease and MDA, dimers, trimers (Menzel, 1967), and tetramers (Shin et al., 1972) were observed. Davidkova et al. (1975) found that the lysine ϵ-amino groups of collagen were important to cross-linking by MDA. This cross-link was defined by Chio and Tappel (1969a) as a Schiff base conjugate between MDA and two amino groups, i.e., an N,N′-disubstituted 1-amino-3-iminopropene (Figure 6). The MDA Schiff base is a conjugated fluorochrome with an excitation maximum at ~370 nm and an emission maximum at ~450 nm. Fluorescence was demonstrated in MDA-induced dimer and trimer of ribonuclease A (Chio and Tappel, 1969b). Intramolecular cross-linking also was evident because fluorescence was observed in the ribonuclease A monomer.

Since the discovery of MDA-induced fluorescence, there has been considerable effort in this area of research, particularly by Tappel's group (Tappel, 1973), who often have correlated MDA-induced fluorescence with a similar fluorescence observed in age pigments (ceroid or lipofuscin

pigments). According to Tappel, lipofuscin pigments could accumulate in an aging organism as a result of in vivo lipid peroxidation, and the observed fluorescence of lipofuscin or ceroid is due to the MDA Schiff base. Using this approach, Trombly et al. (1975) examined fluorescent products extracted from aging human donors, and Seligman et al. (1977) used fluorescence to determine in vivo trauma to cat spinal cord. Desai et al. (1975) demonstrated that rats maintained on a tocopherol-deficient diet developed highly fluorescent ceroid pigments in their tissue presumably because of increased in vivo lipid peroxidation. Ceroid pigment accumulation in rats was inversely proportional to dietary tocopherol concentration and directly proportional to levels of polyunsaturation in dietary fats (Reddy et al., 1973). An analysis of lipofuscin pigment from human brain proved lipofuscin to be a lipid-soluble polymer having a composition suggesting lipid and phospholipid bound to amino acids or protein (Taubold et al., 1975).

Additionally, the fluorescence measurement is a convenient assay for peroxidation damage in vitro as demonstrated with rat liver microsomes (Bidlack and Tappel, 1973a) and other subcellular organelles (Chio et al., 1969). The rate of fluorescence development in vitro in rat liver microsomes and mitochondria as well as heart sarcosomes was dependent on the dietary status of the rat from which the organelles were isolated (Dillard and Tappel, 1971). Rats fed increased levels of α-tocopherol yielded organelle preparations that resisted production of fluorescence, whereas increased polyunsaturation in the diet caused the organelles to be more susceptible to fluorescence development.

Fluorescence measurement has been developed into a useful analytical method for quantitating peroxidation damage of biological materials (Fletcher et al., 1973). The method was extended by Trombly and Tappel (1975) who found another excitation maximum at 260–280 nm in addition to the commonly observed excitation at 350–390 nm. The 1-amino-3-iminopropene fluorescence could be distinguished from other fluorochromes by the application of various tests used to perturb the fluorophore, such as adjustment to basic pH or addition of a metal coordinator (Malshet et al., 1974). Measurement of fluorescence polarization and decay times were additional characteristic tests utilized. In the analytical method described above $CHCl_3$–CH_3OH extractable material was analyzed for fluorescence rather than water-soluble material mainly because the $CHCl_3$–CH_3OH solubles yielded more reproducible data. Undoubtedly, these lipid-soluble fluorochromes are Schiff base adducts of MDA with amino phospholipids. MDA adducts with phosphatidyl ethanolamine and phosphatidyl serine gave excitation (365–370 nm) and emission (435–440 nm) maxima that were essentially identical with MDA adducts with protein or amino acids (Bidlack and Tappel, 1973b). Dillard and Tappel (1973) noted that phosphatidyl ethanolamine (a lipid-soluble adduct) competed more effectively for MDA than did phenylalanine (water-soluble adduct).

Malshet and Tappel (1973) proposed that the fluorochrome was produced from MDA and not from other carbonyls derived from lipid hydroperoxides. Specifically, the structure necessary for the fluorochrome is an electron-donating group in conjugation with an imine. However, a similar fluorescence was produced simply by air oxidation of polyunsaturated lipids adsorbed on silica gel particles (Shimasaki et al., 1977). Buttkus (1975) found that the enolic salt of MDA self-condensed into fluorescent compounds, but the fluorescence spectrum (ex. max. 390 and 468 nm, em. max. 550 nm) was not comparable to MDA-amine conjugates. When amines were added to the MDA self-condensation product, the spectrum shifted to ex. max. 385 nm and em. max. 460 nm. Extracts of rancid herring yielded fluorescence spectra characteristic of both MDA-amine and MDA self-condensation products. A glucose–glycine browning reaction also produced MDA-amine type of fluorochrome with ex. max. 350 nm and em. max. 430 nm (Adhikari and Tappel, 1973). They proposed the following partial structures:

$$HOOCCH_2N{=}CHCH{=}\overset{|}{C}NH-$$

$$HOOCCH_2N{=}CHCH{=}C(OH)-$$

While it is expected that MDA will react with free amino groups of protein, particularly the ϵ-amino group of lysine, certain other amino acid residues also are sensitive to MDA. When myosin was incubated with MDA at −20 °C for 6 days, methionine, lysine, tyrosine, and arginine residues were partially lost; at 100 °C for 1 min, histidine, tyrosine, arginine, and methionine were damaged (Buttkus, 1967). With ribonuclease A, MDA damaged tyrosine, methionine, and lysine residues (Chio and Tappel, 1969b). Free methionine reacted with MDA to form an MDA-amine Schiff base, which hardly explained its lability while incorporated in protein. On the other hand, both the amino and sulfhydryl functions of cysteine reacted with MDA (Buttkus, 1969). It is interesting to note that the structures proposed by Buttkus for both the cysteine- and methionine-MDA products did not include the 1-amino-3-iminopropene cross-link. Shin et al. (1972) found that the sulfhydryl group of *N*-acetylcysteine was insensitive to MDA, in contrast to its lability in the presence of peroxidizing linolenic acid.

Aldehydes and Browning. Various aldehydes are the scission products of lipid hydroperoxides (Grosch, 1976; Galliard and Matthew, 1977; Eskin et al., 1977; Gardner, 1975). Since aldehydes both physically bind (see Noncovalent Complexes section) and react covalently (Maier, 1973) with protein, their presence in foods is important to both flavor and quality.

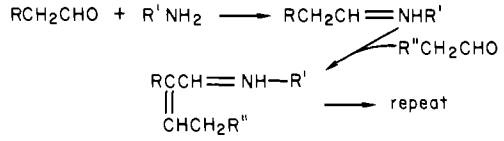
Like MDA discussed above, alkanals, alkenals, and alkdienals form Schiff bases with amino groups. Acetaldehyde (Mohammad et al., 1949), acrolein, propanal (Bowes and Cater, 1968), and a homologous series of aldehydes (Pokorny et al., 1973b) alkylated the free amino groups of protein. Aldehydes were cited as the cause of blocking the free amino groups of insulin that had been exposed to autoxidizing methyl linoleate (Andrews et al., 1965). They noted that the ϵ-amino group of lysine and the N-terminal amino groups were inaccessible for reaction with Sanger's reagent, and these amino acids were recovered after acid hydrolysis.

Unlike MDA, very few of the other aldehydes encountered in lipid rancidity appear to cause protein cross-linking. According to Milch (1964), aldehydes in aqueous solution had to exist primarily in the hydrated or *gem*-dihydroxy configuration before gelatin cross-linking occurred. With few exceptions, straight-chain alkanals and alkenals are in the unhydrated or free carbonyl state and, as a consequence, do not cross-link.

Nonenzymic browning long has been recognized as a consequence of peroxidizing lipids in the presence of protein. For example, the highly unsaturated nature of fish lipids results in the browning or rusting of fish. According to Pokorny et al., browning is affected by parameters, such as free amino groups (1975b), the degree of lipid peroxidation (1973a, 1974), and lipid unsaturation (1976a). Ever since Mohammad et al. (1949) discovered

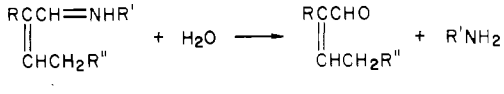
that acetaldehyde caused protein browning, it has been recognized that Schiff base adducts lead to brown pigments. Reportedly, even more rapid browning occurred with α,β-unsaturated alkenals than with saturated alkanals (Burton et al., 1963).

Most investigators theorize that nonenzymic browning during lipid autoxidation arises from aldol polycondensations of aldehydes with aldehyde–amine Schiff bases (Montgomery and Day, 1965; Janicek and Pokorny, 1971; Tai et al., 1974; Davidek and Jirousova, 1975). Such condensations were reported by Patrick (1952) as follows:



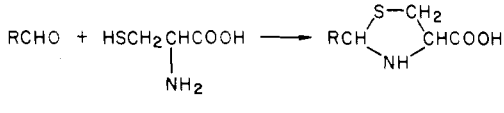
Subsequent aldol condensations extend the conjugated chromophore, and eventually the amino compound even may cleave from the polymer. For these reasons the N content of brown pigments can vary over a range of values. Low N contents have been observed in brown pigments by Pokorny et al. (1975b) and Davidek and Jirousova (1975). In studies with heptanal–tyrosine ethyl ester mixtures, Montgomery and Day (1965) concluded that the brown pigments did not contain nitrogen. When tetradecanal was permitted to react with either benzidine or aniline, the condensation products were composed of two or more aldehyde molecules for each amino group (Janicek and Pokorny, 1971). Likewise, Mohammad et al. (1949) observed that more aldehyde could be accommodated than there were available amino groups in the protein. An increase in the conjugated chromophore during storage time has been indicated by a bathochromic shift of the brown pigment toward longer wavelengths (Pokorny et al., 1973a). The browning of sugar–amine mixtures, while more complex, undergoes some of the same Schiff base polycondensations as lipid-induced browning described above (Hodge, 1953; Burton and McWeeny, 1964).

Often volatiles are synthesized in conjunction with browning reactions, such as those occurring in sugar–amine browning (Hodge, 1967). The aldehydes commonly found as a result of lipid oxidation are usually volatile and often emanate potent odors. In browning reactions, these aldehydes produce gluey odors (Pokorny et al., 1976d) and fish aromas (Pokorny et al., 1976c) that were uncharacteristic of the aldehyde odors. From a mixture of heptanal and tyrosine ethyl ester, 2-alkenals were produced (Montgomery and Day, 1965). They suggested that 2-alkenal formation is consistent with regeneration of the amino acid from the aldol condensation product as follows:



Similarly, a hexanal–glycine browning reaction yielded volatiles that could be explained by the above sequence of events (Davidek and Jirousova, 1975); however, the authors pointed out that hexanal boiled in water produced the same volatiles, demonstrating that glycine was not a prerequisite for aldol condensation to occur.

For many years it has been known that free cysteine reacts with aldehydes to yield a 2-substituted thiazolidine-4-carboxylate derivative (Schubert, 1936; Schmolka and Spoerri, 1957) as follows:

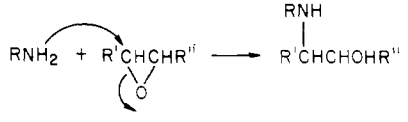


The thiazolidine-4-carboxylate derivative of 9-oxononanoic acid has been isolated from a reaction of linoleic acid hydroperoxide and cysteine catalyzed by $FeCl_3$ (Gardner et al., 1977). Presumably 9-oxononanoic acid was derived from scission of the fatty acid hydroperoxide. Similar thiazolidine derivatives are synthesized from cysteine and α,β-unsaturated aldehydes, but additionally another cysteine molecule was added by a thiyl bond to the β-unsaturated carbon (Esterbauer et al., 1976).

Other. Among the products of lipid hydroperoxide decomposition are nonvolatile fatty acids with oxygenated substituent groups, such as hydroxyl, ketone, and epoxide (Gardner, 1975). Several studies indicated that these products reacted with protein or amino acids as discussed below.

A mixture of 10-oxo-9-hydroxystearic and 9-oxo-10-hydroxystearic acid formed a brown color when heated with albumin (Pokorny et al., 1967). Janicek and Pokorny (1971) demonstrated that oxohydroxystearic acid could bind with two amino groups, but the specific structure was not characterized.

Although Pokorny et al. (1966) observed the binding of 9,10-epoxystearic acid or its methyl ester to albumin, the structure was not identified. One would assume this reaction would be a typical nucleophilic substitution:



Such a substitution recently has generated considerable interest, i.e., the reaction between the 2-amino group of polyguanylic acid and the mutagen, 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (Koreeda et al., 1976). Fatty epoxides very often are major products of the degradation of lipid hydroperoxides. For example, 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid is a significant product of homolytic degradation of linoleic acid hydroperoxide (Gardner et al., 1974) and accumulates in soybean homogenates during the oxidation of linoleic acid (Gardner and Kleiman, 1977). In our laboratory we currently are investigating the reactions between 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid and glycine. Preliminary results indicate that a number of products from this reaction are fatty acids bound covalently to glycine.

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Role of Lipoxygenase and Lipid Oxidation in Quality of Oilseeds

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Lipoxygenase is a prime suspect for catalyzing lipid oxidation in raw peanuts but it is destroyed in the roasting process. After roasting, however, lipid oxidation is catalyzed primarily by nonenzymic catalysts. Examination of several fresh samples of commercial peanut butters showed that the initial peroxide contents differed. This suggested that the samples were already in different stages of peroxidation and demonstrated the need for controlling oxidation in the peanuts before roasting and processing. We found that minor constituents such as metals, metalloproteins, and salts are possible catalysts of lipid oxidation. When ascorbyl palmitate, citric acid, or ethylenediaminetetraacetic acid were added to the peanut butter before storing, oxidation was decreased or completely controlled. Some natural compounds were also examined as possible inhibitors of lipoxygenase activity in raw peanuts.

Today, one of the major concerns of consumers is a growing awareness of the nutritional composition of foods. This concern has focused on the peanut because of its potential value as an economic source of high protein. In addition to containing 25–30% protein, peanuts are high in phosphorus and B vitamins, low in carbohydrates, and exceptionally high in oil (ca. 50%). Furthermore, the oil is an excellent source of unsaturated fatty acids (80%), of which 20–30% is polyunsaturated.

While peanuts are used in many food products, including candies, cookies, ice cream, and breakfast cereals, more than half of the peanuts produced in the United States are consumed as peanut butter. The protein in peanut butter makes it a good supplementary food in menu planning. Also, it ranks very high on the list of food energy sources. In the United States, peanut butter is second only to dry beans as the most economical source of protein (USDA, 1977). Addition of peanut butter can also improve the flavor of baked goods.

The causes of lipid oxidation in vegetable oils are numerous and involve such factors as light, air, high temperature, enzymes, microorganisms, trace metals, and the presence of free fatty acids. Because of the high percentage of unsaturated fatty acids, peanuts are susceptible to oxidative rancidity, staling, and loss of desirable flavors or odors. Therefore, they present a storage problem if high quality in the final products is to be retained. Many of these reactions are catalyzed by metals or enzymes, particularly lipoxygenase. This enzyme has been isolated and characterized in raw peanuts by several workers (Siddiqi and Tappel, 1957; St. Angelo and Ory, 1972; Sanders et al., 1975). The hydroperoxide products formed by the action of lipoxygenase on polyunsaturated fatty acids or triglycerides can be decomposed into acids, ketones, aldehydes, or other substances that form during processing or storage. These degradative products can then react with amino acids and proteins to impair flavor and/or lower nutritive value of peanuts or food products in which they are incorporated. Recently, Kuck et al. (1978) has shown that oxidized linoleic acid can react with lysine and threonine to form new reaction products.

In our laboratory, we have been concerned with preserving the desirable characteristics and high quality of peanuts and peanut products. This paper will discuss the functions of lipoxygenase and other constituents as related to quality and storage of oilseeds, particularly raw peanuts, and the ultimate effect of these functions on roasted products.

MATERIALS AND METHODS

Peanuts and peanut butter samples were obtained from commercial suppliers. Rapeseeds were a gift of Dr. R. Ohlson, A/B Karlshamns Oljefabriker, Sweden. Soybean lipoxygenase was purchased from Sigma Chemical Co., St. Louis, MO. Erucic acid was purchased from Eastman Chemical Co., Rochester, NY.

Peanuts were water blanched or spin blanched commercially and sent to us immediately after blanching, along with comparable unblanched peanuts. Procedures for fractionation of oil and proteins from raw or roasted peanuts or peanut butter were similar to those used previously (St. Angelo and Ory, 1975b; St. Angelo et al., 1977b).

Lipoxygenase activity was calculated either from initial rates of oxygen uptake, assuming an initial dissolved oxygen concentration of 240 nmol/L at 25 °C, or from formation of conjugated diene hydroperoxide (CDHP) as

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