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Damage to proteins by peroxidized lipids

I. D. Desai and A. L. Tappel

Department of Food Science and Technology,
University of California,
Davis, California

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SUMMARY

The purpose of this research was to determine the mechanism of damage to protein by the free radical intermediates formed during the peroxidation of lipids. The reaction system consisted of linolenic acid, cytochrome c, and oxygen. The addition reaction of peroxidizing linolenic acid with cytochrome c was measured using linolenic acid-C^{14}. There was considerable damage to cytochrome c as measured by its decreased solubility. Paper chromatographic studies of the amino acids of the linolenic peroxy–cytochrome c product showed losses of amino acids that are labile to oxidation. Linkages between linolenic acid and cytochrome c were identified as either peroxy or ether by infrared spectra. Hydrolysis revealed that 70% of the linolenic acid was bound to cytochrome c through peroxy bonds, and the rest through ether or possibly carbon-to-carbon bonds. Measurements of thiobarbituric acid value and peroxide number substantiated the chemical nature of peroxidative damage to cytochrome c.

Studies of damage to vitamin E or antioxidant-deficient animals have focused attention on the mechanism of damage to proteins, enzymes, and other important compounds by peroxidation of lipids (1). Damage resulting from ionizing radiation may also include that from induced lipid peroxidation (2). Damage to enzymes by lipid peroxidation has been studied in subcellular particles, especially mitochondria (3), and in reactions of pure lipids and enzymes (4). The chemical mechanism of damage has not been determined, although free radicals are implicated.

Evidence of peroxidation *in vivo* includes the formation of "Ceroid" type pigments that have been identified as peroxidized lipid–protein products (5). Peroxidized lipid–protein interactions result in brown polymer-like products (6); the mechanism of the reaction is still open to question.

This paper reports chemical evidence for protein damage and for addition reactions between peroxidizing linolenate and cytochrome c.

METHODS

The reaction system consisted of 1 g linolenic acid[1] and 0.5 g cytochrome c[2] dissolved in 2.5 ml of 0.1 M phosphate buffer pH 7.0. In the labeling experiment, 25 μc of linolenic acid-1-C^{14} were included. Peroxidation of linolenic acid was followed by manometric measurement of oxygen consumption at 37°.

Aliquots were taken out at intervals equivalent to 0.01, 0.10, 0.50, and 1.00 mole of oxygen absorption per mole of linolenic acid. The rate of reaction was 0.01 mole oxygen/mole linolenic acid/hr during the initial period and reached a maximum of 0.1 mole oxygen/mole linolenic acid/hr after about 5 hr. Aliquots of the reaction mixture were extracted four times with cold butanol followed by four extractions with methanol–chloroform 1:1 to assure complete removal of free lipids. The solubility in water and the C^{14} content of linolenate peroxy–cytochrome c product were then determined. Solubility was determined by measuring the concentration of dissolved material spectrophotometrically. Incorporation of C^{14} was measured in a Geiger-Müller gas-flow counter.

Infrared spectra were recorded on 0.5% KBr pellets of finely powdered samples. As a precaution against further peroxidation, samples were stored below 0° and under nitrogen.

Amino acids were determined by hydrolyzing the samples in 6 N HCl for 18 hr in an autoclave and analyzing the hydrolysate by two-dimensional paper chromatography. The solvent systems used were sec-butanol–tert-butanol–butanone–water 4:4:8:5, and *n*-butanol–acetic acid–water 4:1:5 (7).

[1] Linolenic acid was purchased from Hormel Foundation, Austin, Minnesota.

[2] Horse heart cytochrome c from Sigma Chemical Co., St. Louis, Missouri.

TABLE 1. LINOLENATE PEROXIDATION DAMAGE TO CYTOCHROME C AND LINOLENIC ACID-C^{14} ADDITION

Oxidation (Mole O_2/Mole Linolenate)	Linolenic Acid-C^{14} Addition (Mole Linolenate/Mole Cytochrome C)	Solubility (% Original)	Mole Cytochrome C Damaged/ Mole Peroxy Radical
0.01	0.03	62	0.400
0.10	0.04	34	0.070
0.50	0.22	21	0.017
1.00	0.99	3	0.010

More specific hydrolysis was carried out by refluxing the polymer with 2.5 N HCl in 80% aqueous ethanol at 85° for 2 hr (8). The hydrolysis mixture was either diluted with distilled water and thiobarbituric acid (TBA) reactants distilled for determining TBA value, or re-extracted 4 times with an equal volume of anhydrous diethyl ether to estimate the amount of lipid released. Lipids were determined gravimetrically after drying with sodium sulfate and evaporation of solvent.

TBA reactants were determined by reacting the distillate from the hydrolyzed product with 0.67% TBA in 0.1 N HCl for 10 min at 100° and measuring the color at 535 mμ.

Peroxide number was determined using conventional iodometric titration with thiosulfate. The sample was suspended in a mixture of acetic acid–isopropanol 3:2 previously flushed with nitrogen.

RESULTS AND DISCUSSION

Peroxidative Damage to Cytochrome C. The results in Table 1 show a concomitant reduction in solubility of cytochrome c, an increase in the peroxidation of linolenic acid, and an increase in the incorporation of peroxidized linolenate into cytochrome c as measured by radioactive tracer. The ratio of mole cytochrome c damaged per mole peroxy radical was calculated as follows. Damaged cytochrome c was measured by loss of solubility. The amount of peroxy radicals formed was approximated from the amount of oxidation since each mole of oxygen consumed produces a mole of peroxide by the well-known mechanism involving peroxy radicals. This allows comparison of the effect of peroxidation with damage to proteins by free radicals produced by ionizing radiation, where the analogous term expressing damaged protein per free radical pair is ionic yield. Ionic yield varies with the protein and conditions but some typical examples may be cited. Values for invertase, catalase, cytochrome c, and ribonuclease are 0.05, 0.03, 0.10, and 0.48, respectively (9). Damage to protein by peroxidation is of the same magnitude as damage by ionizing radiation, indicating how damaging lipid peroxidation might be *in vivo*. Other comparisons of the effects of lipid peroxidation *in vivo* with ionizing radiation are available (1). In similar studies involving reactions of other unsaturated fatty acids (arachidonic, eicosapentaenoic, and docosahexaenoic) with other pure proteins (hemoglobin, catalase, ovalbumin, pepsin, trypsin, and chymotrypsin), similar ratios of protein damaged per peroxy radical were found.

TABLE 2. LINOLENATE PEROXIDATION DAMAGE TO AMINO ACIDS OF CYTOCHROME C

Amino Acid	Amino Acid Analysis: Pure Cytochrome C	Amino Acid Analysis: Lipid-Free Linolenate Peroxy–Cytochrome C	Loss
	mmoles/100 mg		%
Phenylalanine	19.8	14.9	24.8
Leucine	30.1	21.9	27.2
Isoleucine	29.5	22.5	23.7
Methionine	21.6	13.3	38.3
Valine	21.2	10.8	49.3
Tyrosine	11.7	8.1	31.0
Threonine	54.5	38.0	30.3
Alanine	23.7	16.5	30.3
Serine	12.6	5.7	54.6
Glycine	22.7	16.8	26.0
Histidine	19.8	8.2	58.8
Glutamic acid	37.4	28.6	23.5
Aspartic acid	25.0	18.2	27.2
Arginine	16.5	9.6	41.9
Lysine	23.9	19.8	17.2
Cystine	22.5	14.7	34.7
Proline	31.4	14.7	53.2

Comparison of cytochrome c damaged per peroxy radical with linolenic acid-C^{14} incorporation shows that, especially at the lower oxidation levels, most of the damage must be ascribed to free radical oxidation of cytochrome c without a concurrent addition reaction. Thus at 0.01 mole O_2 per mole linolenate, 2.5 peroxy radicals cause insolubility of a cytochrome c molecule but only 1 out of 83 linolenate peroxy radicals undergoes an addition reaction resulting in C^{14} incorporation.

Linolenate Peroxidation Damage to Amino Acids of Cytochrome C. Damage to the protein of cytochrome c was studied by analyzing its amino acids. In Table 2 is presented the amino acid analysis of pure cytochrome c and the linolenate peroxy–cytochrome c on a lipid-free basis. The fact that all amino acids show losses of 17% or greater and the observation of some hydrolysis

residue suggests that a loss of about 20% of all amino acids could have resulted from the resistance of the linolenate peroxy–cytochrome c product to hydrolysis. Since valine peptides are difficult to hydrolyze, an additional loss of valine may have resulted from this cause.

Thus, the evidence indicates that histidine, serine, proline, and arginine were most labile to peroxidative damage while sulfur amino acids, methionine, and cystine were next in order. It is interesting that this overall pattern of amino acid susceptibility to peroxidative damage is similar to that of free radical damage to irradiated proteins and amino acids. Irradiation studies of cytochrome c show the most radiolabile amino acids to be: methionine, histidine, cystine, phenylalanine, and serine in that order (10). Mechanisms for damage to individual amino acids probably include hydrogen abstraction and addition of peroxy radicals to amino acids.

Infrared Spectra of Peroxidized Cytochrome C. Typical infrared spectra of linolenate peroxy–cytochrome c and pure cytochrome c are presented in Fig. 1. Absorption between 2 and 7 μ is similar in both samples. The characteristic differences arise mainly between 7 and 12.5 μ where a number of new bands are present that can be related to the addition of linolenate peroxy radicals to cytochrome c.

Absorption in the region of 7.9–8.0 μ has been assigned to C—O stretching of acid groups and, since this peak is not seen in pure cytochrome c, it can be interpreted as absorption of the linolenic acid residue attached to cytochrome c.

Distinct new bands at 8.8 and 8.9 μ can be due to

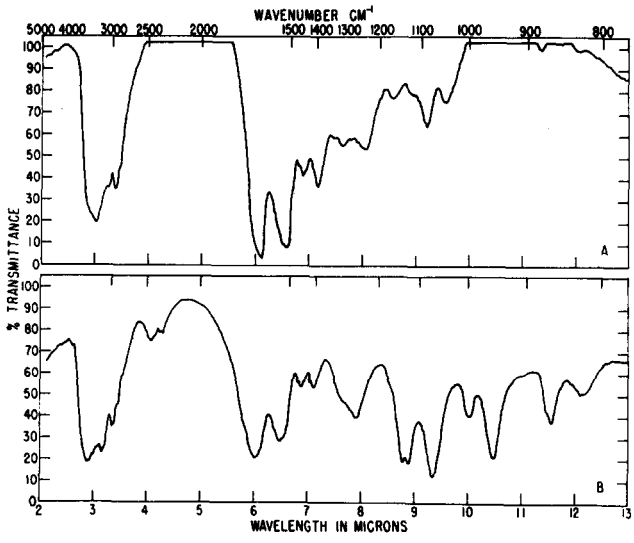

FIG. 1. Infrared spectra of (A) pure cytochrome c and (B) linolenate peroxy–cytochrome c.

TABLE 3. CHARACTERIZATION OF LINOLENATE PEROXY–CYTOCHROME C PRODUCT

	Before Hydrolysis		After Hydrolysis	
	Extent of peroxidation during reaction (mole O_2 per mole linolenate)			
	0.5	1.0	0.5	1.0
TBA reactants in steam distillate, mg malonaldehyde per 1000 g sample	0.0	0.0	0.072	0.165
Peroxide number, mEq peroxide per kg sample	143.8	268.8	0.0	12.5
Extractable lipid				
Weight % of total polymer	0.0	0.0	9.8	13.4
Weight % of linolenate in polymer			70	70

aliphatic ether (—C—O—C—) bonds (11). Ether could result by addition reactions of oxy radicals to cytochrome c. Oxy radicals are produced in high yield by the cytochrome c-catalyzed homolytic scission of linolenate peroxides (12).

Other unique absorptions are in the region of 10.0–10.5 μ, indicating the presence of conjugated double bond systems. According to O'Connor (13), absorption between 10.0 and 10.1 μ can be due to *trans-trans-trans* conjugation; *cis-trans-trans* conjugation, or *cis-cis-trans* conjugation. Absorption between 10.47 and 10.50 μ can be mainly ascribed to *cis-trans* conjugation. Since the 10.47-μ peak is much stronger than the 10.03-μ peak, there seems to be a predominance of *cis-trans* conjugation. This conjugation is a measure of the peroxidized linolenate, which has reacted with the cytochrome c.

Absorptions at 11.6 μ and between 12.1 and 12.2 μ indicate the presence of alkyl hydroperoxides. Williams and Mosher (14) assign absorption between 11.4 and 11.8 μ to the —O—O— stretching of hydroperoxides. Primary and secondary peroxides show bands between 12.0 and 12.5 μ. Peroxides are later shown to be the major link between linolenate and cytochrome c. Other peroxides, particularly hydroperoxides, can be present in the linolenate since 1 molecule of linolenate can peroxidize in several places along the chain.

Analysis and Hydrolysis of the Linolenate Peroxy–Cytochrome C. Results in Table 3 show that the linolenate peroxy–cytochrome c showed no TBA reactants and a high peroxide number. There were 2 to 3 peroxides formed per mole of linolenate attached to the cytochrome c. After hydrolysis, 9.8–13.4% of the bound lipid was re-extractable by lipid solvents. Since

this type of acid hydrolysis (2.5 N HCl) specifically breaks peroxide linkages in lipid polymers (8), it is estimated that 70% of the total linolenate bound to cytochrome c is linked through peroxy bonds and 30% by more stable ether or carbon-to-carbon bonds. The products of hydrolysis had a high TBA value and practically no peroxides. This indicates that peroxy bonds hydrolyze on acid treatment giving TBA-reacting aldehydic components.

From the overall results presented here, some of the generalities can be derived for the mechanisms of reactions of peroxidizing linolenate with proteins and damage to protein. The main reactants are the peroxy radicals formed in high yield from linolenate (L),

$$L\cdot + O_2 \rightarrow LOO$$

and the alkoxy radical from linolenate peroxide decomposition,

$$LOOH \xrightarrow{\text{cytochrome c}} LO\cdot + \cdot OH$$

Mechanisms by which these free radicals can damage proteins are largely unknown. Studies of ionizing radiation damage to proteins and amino acids show the variety of oxidative reactions that can be ascribed to HO· and HOO·. Similar oxidative reactions probably result from RO· and ROO· and require investigation. At present these reactions can be designated as H abstraction from protein (P),

$$LOO\cdot + P \rightarrow LOOH + P(-H)$$

$$LO\cdot + P \rightarrow LOH + P(-H)$$

Addition reactions have been measured here giving qualitative and quantitative data. They are of the general type,

$$LOO\cdot + P \rightarrow \cdot LOOP$$

$$LO\cdot + P \rightarrow \cdot LOP$$

Oxidative reactions and addition reactions can account for the loss of solubility in cytochrome c with little increase in molecular weight. At high levels of linolenate oxidation, however, the product from cytochrome c shows characteristics of polymerization through crosslinking by the multifunctional linolenate peroxy radicals,

$$\cdot LOOP + O_2 \rightarrow \cdot OOLOOP$$

$$\cdot OOLOOP + P \rightarrow POOLOOP$$

Characteristics observed include complete insolubility in water, difficulties in HCl hydrolysis, hardness in grinding, and formation of brown color. Similar observations have been made for reactions of other unsaturated fatty acids and pure proteins.

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