Free Rad. Res. Comms., Vol. 3, No. 1–5, pp. 213–225
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Printed in Great Britain

BIOLOGICAL SIGNIFICANCE OF SECONDARY LIPID OXIDATION PRODUCTS

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(Received July 22nd 1986)

KEY WORDS: Autoxidation; singlet oxygen; hydroperoxides; photosensitized oxidation; cyclic peroxides; malonaldehyde; decomposition; fluorescence; mutagenicity.

INTRODUCTION

In biological systems, lipid peroxidation involving free radicals and other reactive species such as singlet oxygen has been regarded as a rather harmful reaction, implicated in a variety of diseases, including cancer, strokes, atheriosclerosis, inflammation, and the aging process.[1–4] Biological aging is now believed to be due to random attack of hydroxyl radicals that can arise from undesirable radical-generating chemicals in foods, or from environmental sources, such as ultraviolet and energy radiation.[5] This paper reviews recent structural studies of complex volatile and non-volatile decomposition products of lipid oxidation. We investigated the biochemical effect of these oxidation products by determining the fluorescence formed from their interaction with DNA in the presence of metals and ascorbic acid,[6] and by Ames tests for mutagenicity.[7] More recent work includes fluorescence formation with DNA nucleosides, with keto fatty esters and polyunsaturated aldehydes from oxidized linolenate, and the effect of singlet oxygen quenchers and antioxidants. We have been interested in volatile oxidation products because they have a profound impact on the flavor of lipid-containing foods, and because they are associated with cellular damage, and with a decrease in the safety of food lipids.

THERMAL DECOMPOSITION OF LIPID OXIDATION PRODUCTS

Malonaldehyde (MA) is one of the volatile lipid oxidation products that has been implicated in many biological reactions causing cell damage by interactions with DNA, enzymes, proteins and phospholipids.[8–11] In previous papers[12–16] we reported that the thermal decomposition of monohydroperoxides and cyclic peroxides produced complex mixtures of volatile compounds identified by gas chromatography-mass spectrometry. Although cyclic peroxides have been considered to be important sources of MA, under our conditions for thermal decomposition, we found no evidence for MA formation from various cyclic peroxides that we isolated from oxidized linoleate and linolenate. More recently, however, we obtained evidence for

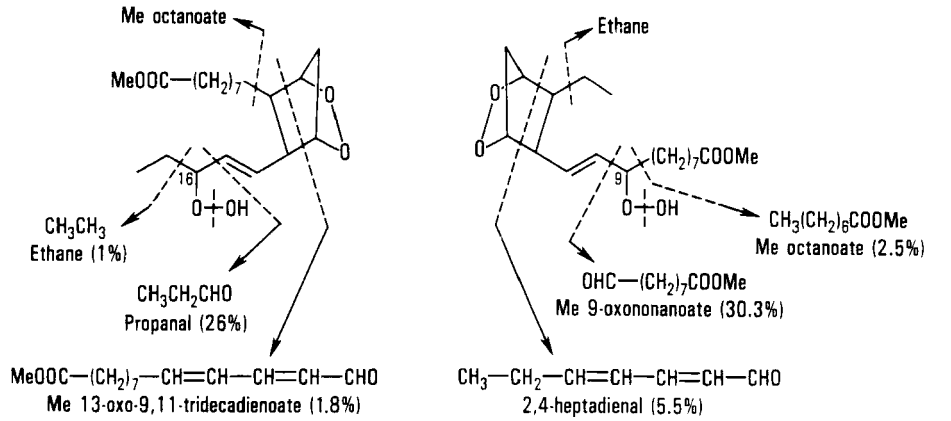


FIGURE 1 Thermal decomposition of a mixture of 9-hydroperoxy- and 16-hydorperoxy-bicycloendoperoxides from oxidized methyl linolenate (based on reference[16]).

MA formation when various lipid oxidation products were decomposed with a dilute solution of HCl in methanol at room temperature.[16,17]

Thermal fragmentation of hydroperoxy bicycloendoperoxides showed a dominant cleavage between the hydroperoxide group and the allylic double bond to form 26% propanal from the 16-isomer, and 30% methyl 9-oxononanoate from the 9-isomer (Figure 1). Cleavage on the other side of the hydroperoxide group produced small amounts of ethane from the 16-isomer and methyl octanoate from the 9-isomer. Cleavage across the endoperoxide ring explains the formation of methyl 13-oxo-9,11-tridecadienoate (16-isomer), and 2,4-heptadienal (9-isomer). This same cleavage would also be expected to produce MA, as suggested by Pryor *et al.*[18] However, this dialdehyde was not detected under our conditions of thermal decomposition.[16]

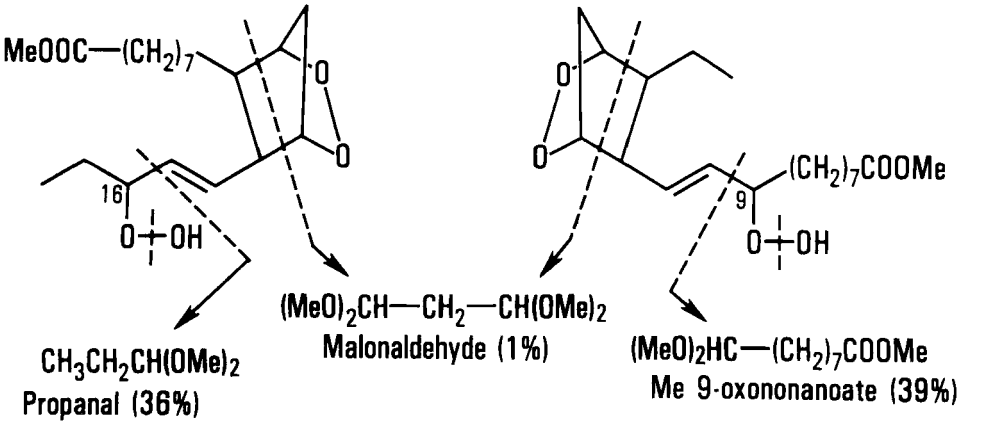
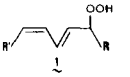
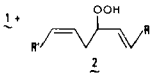
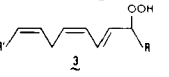
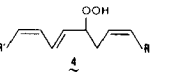
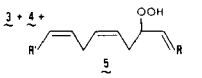
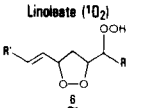
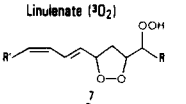
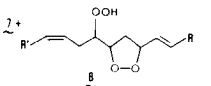
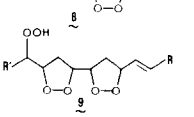
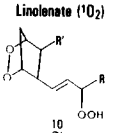
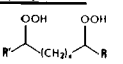


FIGURE 2 Acid decomposition of a mixture of 9-hydroperoxy- and 16-hydorperoxy-bicycloendoperoxides from oxidized methyl linolenate (based on reference[16]).

TABLE I

Formation of malonaldehyde and TBA reactants by acid decomposition of autoxidation ($^3O_2$) and singlet ($^1O_2$) oxidation products of methyl linoleate and linolenate (based on reference[15])

	Malonaldehyde (% by wt.)	TBA ("MA", %)
Monohydroperoxides		
Linoleate ($^3O_2$)		
 1	0.04	0.12
Linoleate ($^1O_2$)		
 1 + 2	0.06	0.04
Linolenate ($^3O_2$)		
 3	0.05	0.42
 4		
Linolenate ($^1O_2$)		
 3 + 4 + 5	0.10	0.32
Hydroperoxy Epidioxides		
Linoleate ($^1O_2$)		
 6	5.04	0.34
Linulenate ($^3O_2$)		
 7	0.49	0.84
Linolenate ($^1O_2$)		
 7 + 8	0.46	0.52
 9	1.08	2.00
Hydroperoxy Bicycloendoperoxides		
Linolenate ($^1O_2$)		
 10	0.79	0.65
Dihydroperoxides		
		
Linolenate ($^1O_2$)		
10,12	1.06	0.84
13,15	0.96	2.00
9,12 + 13,16	0.18	1.40
9,16	0.0	1.70
10,16	0.0	0.61

ACID DECOMPOSITION OF LIPID OXIDATION PRODUCTS

Mild acid decomposition at room temperature was investigated to determine if MA can be detected among the decomposition products of different oxidation products. Aldehyde fragmentation products were identified by MS as the dimethyl acetals.[16,17] Under these acid conditions, heterolytic cleavage of bicycloendoperoxides (from oxidized methyl linolenate) produced a simple mixture consisting of 36% propanal (from the 16-isomer), 39% C-9 aldehyde ester (from the 9-isomer), and 1% MA (from both isomers) (Figure 2). The first two aldehydes are formed by selective heterolytic rupture of the bond between the hydroperoxide group and the allylic double bond. MA is formed by cleavage across the two rings. Minor products included 1% 3-hexenal (from the 9-isomer) and 2% methyl 12-oxo-9-dodecenoate (from the 16-isomer), which correspond to the central fragments remaining after formation of MA.

The concentration of MA formed from different lipid oxidation products was estimated by GC as the tetramethyl acetal derivative under our acid decomposition conditions. The results of this procedure were compared with those of the thiobarbituric acid (TBA) test because this method is extensively used, especially in biological systems, on the assumption that it measures MA. As expected, monohydroperoxides produced very little MA by the GC procedure. On the other hand, significantly higher TBA values were obtained with the monohydroperoxides of methyl linolenate than with those of methyl linoleate (Table I). Higher levels of MA were obtained by the GC method with the isomeric hydroperoxides from linolenate oxidized with singlet oxygen than with the corresponding hydroperoxides from free radical autoxidation, but the reverse trend was shown by the TBA test. The five-membered hydroperoxy epidioxides, from linoleate and linolenate, the bis-epidioxides and the bicycloendoperoxides from linolenate, were rich sources of MA. The 1,3- and 1,4-dihydroperoxides from linolenate were also important precursors of MA and, as expected, the 1,7- and 1,8-dihydroperoxides were completely inert in forming MA as determined by the GC procedure. On the other hand, the TBA test gave high values with the bicycloendoperoxides and *all* the dihydroperoxides. There was no correlation between the TBA values and the MA analyses by the GC method (Table I).

FORMATION OF FLUORESCENCE AND TBA REACTANTS

MA can react bifunctionally with the amino group of enzymes, proteins and DNA to form intra- and inter-molecular crosslinks.[8–11] This mechanism has been generally accepted for the biological damage assumed for MA. However, there is now a large body of evidence that other bifunctional lipid oxidation products such as the hydroxyalkenals have considerable biological reactivity.[19–22] Our recent evidence also suggests that lipid oxidation decomposition products *other than MA* are important in their interactions with DNA in the presence of traces of metals and reducing agents such as ascorbic acid.

The biochemical effect of a series of lipid oxidation products was investigated by determining the fluorescence formed from their interactions with DNA, metals and reducing agents.[6] The fluorescence spectra of DNA reacted with several mono- and di-hydroperoxides in the presence of $FeCl_3$ and ascorbic acid showed an excitation maximum at 320 nm and emission maximum at 420 nm (Figure 3), corresponding to

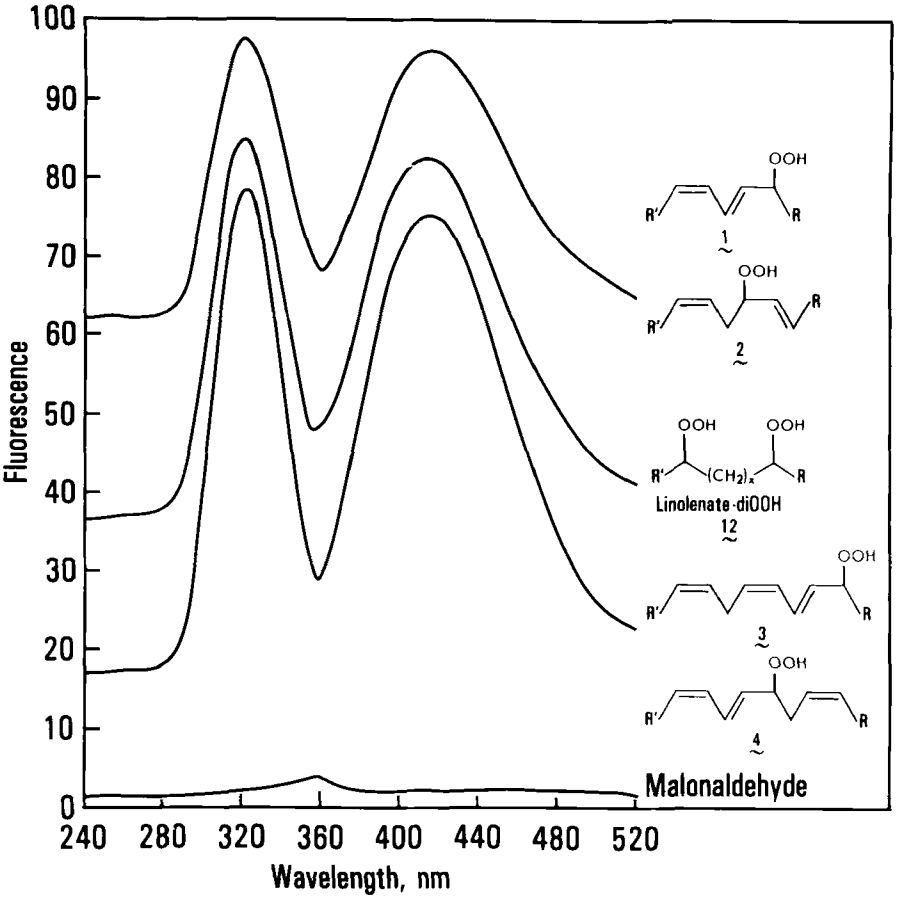
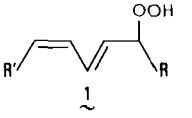
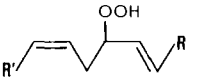
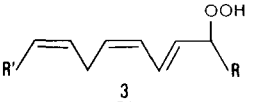
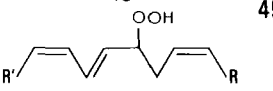
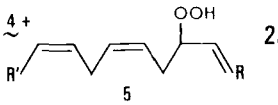


FIGURE 3 Fluorescence spectra of reaction products between DNA and lipid oxidation products and malonaldehyde in the presence of $FeCl_3$ and ascorbic acid (based on reference[6]).

the conjugated Schiff base reported from the reaction of DNA with oxidized arachidonic acid.[22] In contrast, MA showed very little fluorescence under our conditions. We found that at low concentrations of oxidation products (1 mM), fluorescence formation required the presence of metals and ascorbic acid. In contrast, a positive TBA reaction was obtained with many lipid oxidation products without metals or ascorbic acid (Table II). Monohydroperoxides from both autoxidized methyl linoleate and linolenate produced the highest levels of fluorescence. With Fe(III) and ascorbic acid, linolenate hydroperoxides gave higher levels of fluorescence than linoleate hydroperoxides. Very little fluorescence was obtained with either lipid oxidation products alone or with only Fe(III).

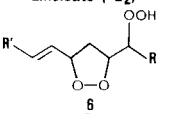
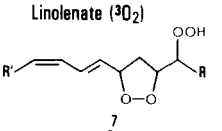
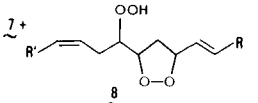
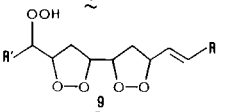
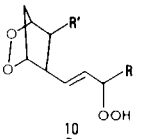
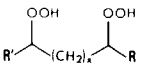
The TBA values showed no correlation with fluorescence formation under our conditions. Unoxidized linoleate and linolenate gave high TBA values in either the absence or presence of iron and ascorbic acid. The hydroperoxides of linolenate gave higher TBA values than the hydroperoxides of linoleate in the presence of iron and ascorbate. However, in the absence of iron and ascorbate, the TBA values were quite

TABLE II
Formation of fluorescence and TBA reactants from oxidation products and DNA. Effect of $FeCl_3$ and ascorbic acid (based on reference 6)

Me Esters	Fluorescence			TBA	
	Alone	+ Fe^{3+}	+ Fe^{3+} + AA	Alone	+ Fe^{3+} + AA
Linoleate	8	7	2	2	4.0
Linolenate	5	8	5	4.3	4.9
Monohydroperoxides					
Linoleate ($^3O_2$)  1	28	43	472	0.4	0.5
Linoleate ($^1O_2$) 1 +  2	18	16	335	0.4	0.8
Linolenate ($^3O_2$)  3  4	45	40	725	2.2	3.6
Linolenate ($^1O_2$) 3 + 4 +  5	23	24	545	2.7	4.2

Fluorescence after 72 hr. incubation (32°C)
TBA (relative to malonaldehyde x 10^{-3}) Maximum values 3-72 hr.
AA = Ascorbic Acid

TABLE III
Formation of fluorescence and TBA reactants from oxidation products and DNA in the presence of $FeCl_3$ and ascorbic acid (based on reference[6])

	Fluorescence[a] + Fe^{3+} + AA[c]	TBA[b] + Fe^{3+} + AA
Hydroperoxy Epidioxides		
Linoleate ($^1O_2$)  6	180	0.7×10^{-3}
Linolenate ($^3O_2$)  7	365	8.1×10^{-3}
Linolenate ($^1O_2$)  7 + 8	190	2.2×10^{-3}
 9	326	15.6×10^{-3}
Hydroperoxy Bicycloendoperoxides		
Linolenate ($^1O_2$)  10	246	6.0×10^{-3}
Dihydroperoxides		
		
Linoleate (9-12),(10-13)-diOOH 11 ($^1O_2$)	360	1.0×10^{-3}
Linolenate (9-12)-diOOH (13,16) (9,16) 12 ($^3O_2$)	245	13.1×10^{-3}
Linolenate (9,12)-diOOH (10,12) + (13,15) (9,15) + (9,16) (10-16) + (13,16) 13 ($^1O_2$)	450	5.3×10^{-3}
Malonaldehyde		
	8	1.0

[a] Fluorescence after 72 hr. incubation (32°C)
[b] TBA (relative to malonaldehyde) Maximum values 3-72 hr.
[c] AA = Ascorbic Acid

low for the linoleate hydroperoxides but relatively high for the linolenate hydroperoxides.

These results indicate that the TBA test cannot be relied upon to measure either the degree of peroxidation or hydroperoxide decomposition. The lack of specificity of the TBA reaction for MA has been shown by many previous investigators.[23-33].

Hydroperoxy epidioxides with conjugated diene systems gave higher levels of fluorescence than the corresponding cyclic peroxides from linoleate with only one allylic double bond (from singlet oxidation), or those from linolenate with two isolated double bonds. The bis-cyclic peroxide from linolenate treated with singlet oxygen was also a rich source of fluorescence (Table III).

As previously shown with the monohydroperoxides, we found no correlation between fluorescence and TBA values. The bis-cyclic peroxide gave the highest TBA value (twice as much as the monocyclic peroxide with conjugated double bonds from autoxidized linolenate). The monocyclic peroxide from linoleate gave the lowest TBA value, in contrast to our previous study which showed that this compound gave the highest amount of MA by acid decomposition.

Hydroperoxy bicycloendoperoxides and dihydroperoxides were also active secondary oxidation products forming fluorescence in the presence of iron and ascorbic acid. On the other hand, under our experimental conditions, MA produced very little fluorescence, which was in the same order of magnitude as the unoxidized linoleate and linolenate. In contrast, with a base value of 1 for MA used as the references compound for the TBA test, the other oxidation products gave TBA values in the 10^{-3} range. As with the other products tested, no correlation was found between fluorescence and TBA values. We conclude, therefore, that the importance of MA in its crosslinking properties with DNA, may have been greatly exaggerated in the literature.

To elucidate the nature of the secondary oxidation products responsible for the fluorescence, methyl linoleate hydroperoxides were interacted with DNA, nucleosides, and bases contained in DNA in the presence of ascorbic acid and Fe(III).[34] Adenosine was the only nucleoside that produced significant fluorescence between pH 4 and 9. Adenine derived from it also produced similar fluorescence. To clarify the mechanism of fluorescence formation between DNA and lipid degradation products, we recently also examined a number of carbonyl compounds and volatile aldehydes formed from the metal-catalyzed decomposition of linolenate hydroperoxides. Methyl keto ricinoleate (12-oxo-9-octadecenoate) and keto linoleate (mixture of 9-oxo-10,12- and 13-oxo-9,11-octadecadienoate) produced only little fluorescence compared to linolenate hydroperoxides. Keto oleate (mixture of allylic 8-, 9-, 10-, and 11-oxooctadecenoate) produced no fluorescence with DNA in the presence of iron and ascorbate. Alkanals and 2-alkenals produced little or no fluorescence; 2,4-alkadienals were more active and 2,4,7-decatrienal was the most active. A mixture of volatile aldehydes prepared from linolenate hydroperoxides decomposed with iron and ascorbate had the same activity as 2,4,7-decatrienal. Higher molecular-weight products from the decomposition of linolenate hydroperoxides[35] also showed low activity.

The relatively low fluorescence obtained with the decomposition products of linolenate hydroperoxides suggested that other active species may be formed in the presence of iron and ascorbate. We then examined the effect of singlet oxygen quenchers and antioxidants. We found that beta-carotene and alpha-tocopherol

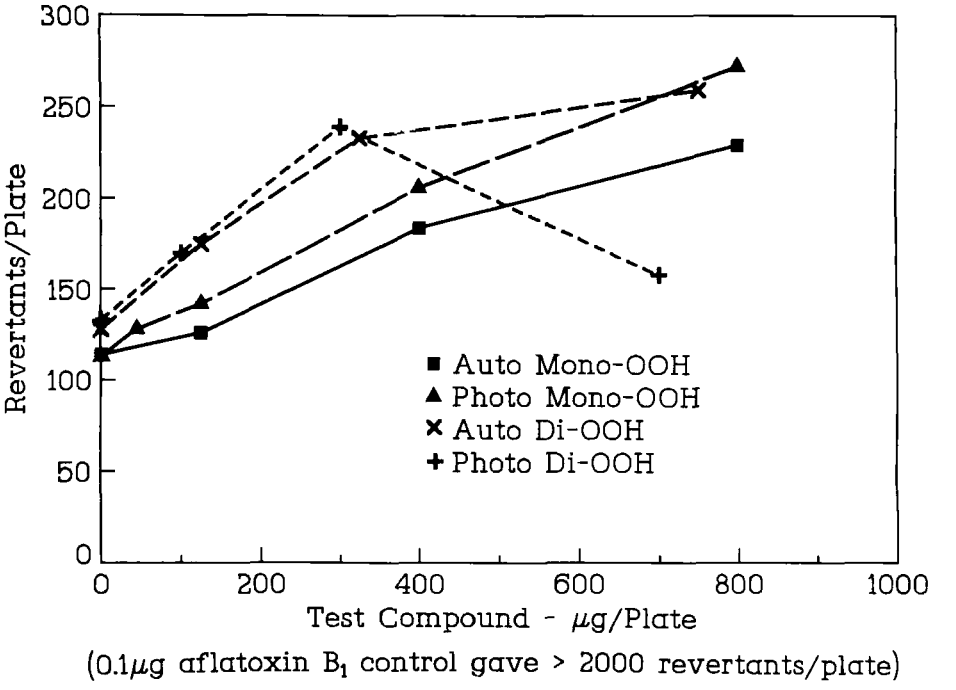


FIGURE 4 Effect of linolenate hydroperoxides on reversion of Salmonella TA-100 with S9 (based on reference[7]).

effectively reduced the amount of fluorescence formed by linolenate hydroperoxides under our test conditions. Therefore, our results suggest that singlet oxygen and perhaps free radical species may significantly contribute to the fluorescence formed from the interaction of linolenate hydroperoxides with DNA in the presence of iron and ascorbate.

BACTERIAL MUTAGENICITY OF LIPID OXIDATION PRODUCTS

Because we have shown that lipid oxidation products react with DNA in the presence of metals and ascorbic acid, it was of interest to investigate the relationship of this activity with their mutagenicity. With the collaboration of toxicologists at the Western Regional Research Center in Berkeley, CA. we tested a series of lipid oxidation products for bacterial mutagenicity by the Ames test.[7]

Generally, all of our oxidation products were weakly mutagenic toward *SALMONELLA TYPHIMURIUM* strains TA97 and TA100. In TA100, all of the oxidation products tested had similar activity. Rat liver microsomes S-9 reduced the toxicity of all oxidation products to the tester strains. The greatest mutant yields were usually obtained in the presence of S-9, but mutagenic potency was sometimes greater without S-9. We found no significant difference between the monohydroperoxides from autoxidation and from photosensitized oxidation of methyl linolenate (Figure 4). With the dihydroperoxides, the activity was slightly higher than with the mon-

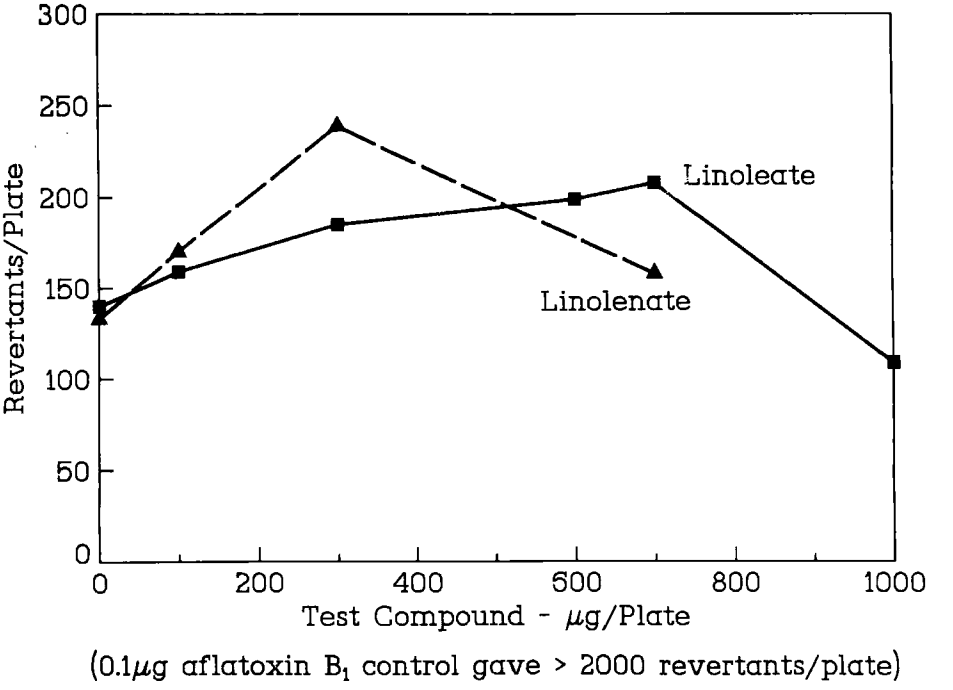


FIGURE 5 Effect of di-hydroperoxides on reversion of Salmonella TA-100 with S9 (based on reference[7]).

ohydroperoxides (Figure 5). The activities observed ranged between 150 and 250 revertants per plate, which is weak compared to the positive control for aflatoxin B1 of more than 2000 revertants per plate.

The cyclic peroxides behaved the same way as the mono- and dihydroperoxides in showing only weak mutagenic activity, with values of 150 to 250 revertants per plate (Figure 6). We also found that these cyclic peroxides exhibited significantly higher activity in strain TA97 than did the monohydroperoxides or the dihydroperoxides. The dihydroperoxides of linolenate were slightly more active than those of linoleate.

The structural feature common to all of the mutagenic oxidation products was the presence of a hydroperoxide group, suggesting that this characteristic is responsible for the observed mutagenicity, either directly or through a common degradative pathway to reactive products of lower molecular weight.

SAFETY OF OXIDIZED FATS

Ames[36] reviewed an increasing body of evidence suggesting that a large number of potent carcinogens arise from natural processes, and that carcinogens are formed in cooking by reactions involving proteins and fats. He suggested that since unsaturated fats are easily oxidized on standing and in cooking (to form hydroperoxides and cholesterol epoxides), they form mutagens, promoters and carcinogens. These rancid fats are, therefore, according to Ames, possible causative agents of colon and breast

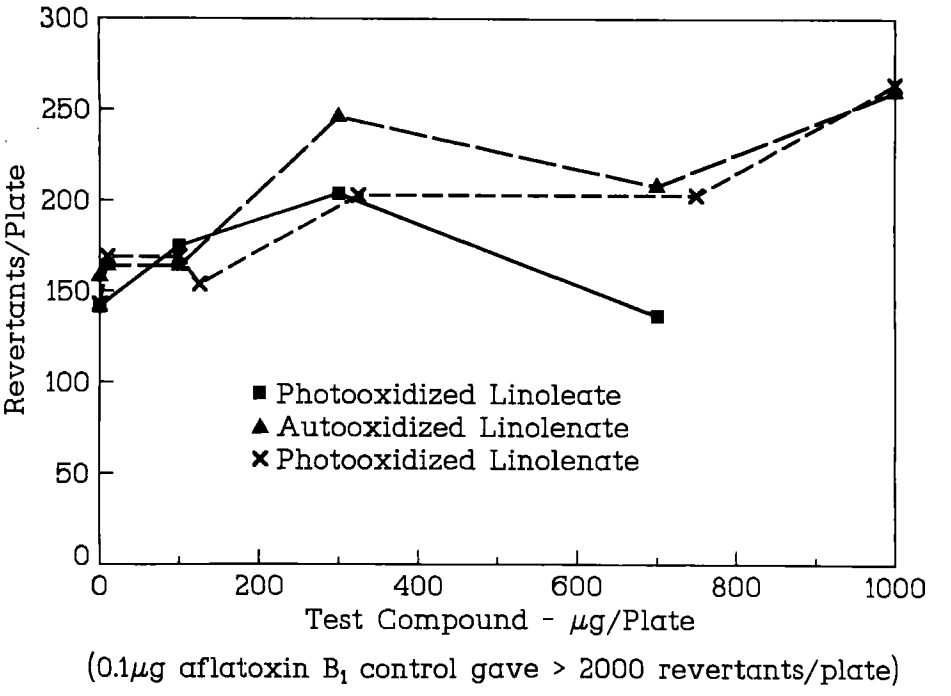


FIGURE 6 Effect of hydroperoxy epidioxides on reversion of Salmonella TA-100 with S9 (based on reference[7]).

cancer in humans. Colon and digestive tract are exposed to many fat-derived carcinogens (fatty acid hydroperoxides, cholesterol epoxides). Human breast fluid can contain high levels of cholesterol epoxides, which could originate from either ingested oxidized fat or in vivo oxidative processes. Ames further indicated that colon and breast cancer account for a major protion of all the cancer deaths in the U.S. He also identified many antioxidants as anticarcinogens. He concluded, that dietary intake of natural antioxidants could be an important aspect of the body's defense mechanism against these carcinogenic agents.

Our results presented above with pure oxidation products isolated from highly oxidized lipids (peroxide values of 800 to 3000) showed only weak mutagenic activity (Figures 4–6). Under conditions of actual food use, oxidation is associated with much lower peroxide values, usually lower than 10. We, therefore, concluded that our results do not suggest a marked genotoxic potential from the occurence of these compounds in dietary fats.

Because of their unpalatable nature, one would not expect rancid fats to be ingested to any significant extent. The situation is different, however, with fats thermally oxidized during frying. Although fats heated to frying temperatures (180–200°C) contain no hydroperoxides, we found by GC that cyclic acids in commercial frying fats range between 0.1 and 0.5%.[37] These ranges are considered below the toxic levels in rat feeding studies.[38] In addition to cyclic acids, we found 0.6 to 8% of other polar oxidation products,[37] and 3 to 6% of dimers.[39] The nature of these materials is very

complex and has not been completely elucidated. The possible biological effects of these materials are unknown.

In conclusion, a better understanding of the biological effects of complex products of hydroperoxide decomposition is needed to improve our basis for controlling the course of potentially harmful biological reactions and the deterioration of the nutritional value of lipid-containing foods.

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Accepted by Prof. H. Sies