Hydrocarbon Gases Produced During In Vitro Peroxidation of Polyunsaturated Fatty Acids and Decomposition of Preformed Hydroperoxides

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ABSTRACT

Hydrocarbon gases have been used previously as an index of lipid peroxidation in vivo and in vitro. In vitro experiments are reported on the formation of hydrocarbon gases from peroxidizing ω-3 and ω-6 fatty acids. Hydrocarbon gases were not released during a 20-hr peroxidation phase but were released following the decomposition of hydroperoxides by addition of excess ascorbic acid. The major hydrocarbon gas products in iron, copper, or hematin catalyzed peroxidation systems were ethane or ethylene from linolenic acid, and pentane from linoleic acid and arachidonic acid. Calculations of the ratios of hydrocarbon gases formed were based on fatty acid decrease and/or change in diene conjugation and peroxide values. Depending on the fatty acid, catalyst, and calculation basis used, pentane formation was as high as 1.3 mol %, ethane 4.3 mol %, and ethylene 10.6 mol %.

INTRODUCTION

There are a number of methods used for the measurement of lipid peroxidation in vivo and in vitro; e.g., determination of peroxide value, diene conjugation, malonaldehyde and fluorescent products. Since the appearance of hydrocarbon gases during the autoxidation of fats was first noted (1), several papers have described the measurement of these products to follow lipid peroxidation. Saturated hydrocarbon gases were shown to arise early during autoxidation of soybean oil and methyl linoleate (2,3). Pentane was the predominant short chain hydrocarbon gas product noted to arise through thermal decomposition of lipoxidase derived products of linoleic acid (4), and several low molecular weight hydrocarbon gases were found in oxidized butterfat (5). Correlations of flavor scores and pentane formation were used to determine rancidity of oils (6-8). In dehydrated food systems, oxidation processes were measured by thermal release of hydrocarbons, and several predicted hydrocarbon gases were found (9).

Plaa and Witschi (10) concluded that the concept of lipid peroxidation in vivo is one of the important concepts of current experimental pathology and toxicology, in spite of the fact that some of the most convincing evidence for the role of lipid peroxidation is indirect and that many conclusions have been drawn by inference. Most conclusions have been based on the finding of increased malonaldehyde levels in tissues (11) and on the presence of fluorescent products that arise in part by malonaldehyde reaction with other biological compounds (12). Ethylene formation was shown to be coupled with the peroxidation of lipids in rat liver microsomes in a cuprous-generating system (13). It was suggested (14) that "activated" linolenic acid was the possible source of ethylene. Ethane production was shown to be characteristic of lipid peroxidation in vivo (15-17). Recently, pentane was shown to be an even more sensitive index of lipid peroxidation in rats fed a vitamin E-deficient diet (18) and additionally stressed by exposure to ozone (19).

Earlier studies (18,19) showed that the measurement of hydrocarbon gases is advantageous in that it is a nondestructive method that is applicable to experiments in vivo and that it is an easy and direct measurement of lipid peroxidation products. It was the purpose of this study to quantitate the hydrocarbon gases produced from iron and copper catalyzed decomposition of hydroperoxides.

EXPERIMENTAL PROCEDURES

Materials

Methyl linoleate and methyl arachidonate were obtained from The Hormel Institute, Austin, MN, and methyl linolenate from Analabs, North Haven, CT; all were >99% pure and were used without further purification. Hydroperoxides of the three fatty acid methyl esters were prepared by the procedure of O'Brien (20). The peroxidation time was 4-7 days. The last purification step by preparative thin layer chromatography was omitted.

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Peroxidation of Fatty Acid Methyl Esters and Decomposition of Hydroperoxides

Erlenmeyer flasks (25 ml) with rubber stoppers were used as reaction vessels. The 3-ml reaction mixture contained: 0.2 ml of 170-180 mM methyl linoleate, methyl linolenate, or methyl arachidonate in methanol that contained methyl pentadecanoate as an internal standard; 0.2 ml of 10 mM $FeCl_3$, $CuSO_4$ or hematin; 0.2 ml of 20 mM ascorbic acid; and 2.4 ml of 0.1 M potassium phosphate buffer, pH 7.0, in 1% Brij 35. After incubation with shaking at 37 C for 20 hr, an additional 0.2 ml of 360 mM ascorbic acid was added to reduce the cations that mediate decomposition of hydroperoxides. The blanks contained methanol without fatty acids. Samples of 0.25 ml were removed prior to incubation, after 20 hr and 20 min following the addition of the second aliquot of ascorbic acid. The samples were immediately added to 1.25 ml of butanol and stored at -20 C for subsequent analysis of fatty acids, diene conjugation, and peroxide value. Parallel headspace gas samples taken with a gastight syringe were analyzed immediately via gas chromatography.

Decomposition of Preformed Hydroperoxides

A 0.2-ml sample that contained a known concentration (60-100 mM) of the different fatty acid methyl ester hydroperoxides in methanol was mixed with 2.4 ml 0.1 M potassium phosphate buffer, pH 7.0, 0.2 ml of 10 mM $FeCl_3$, $CuSO_4$, or hematin, and 0.2 ml of 360 mM ascorbic acid added via a syringe through the rubber stopper. Headspace gases were analyzed before and after addition of catalyst and ascorbic acid.

Analytical Methods

Fatty acid methyl esters were measured in a Packard model 427 gas chromatograph equipped with a flame ionization detector and a glass column (6 ft x 2 mm) packed with 3% OV-1 on 80/100 Supelcoport. A nitrogen carrier gas flow rate of 20 ml/min was used. The injector and detector temperatures were 260 C and 300 C, respectively. The column temperature was programmed as follows: 190 C for 1 min, followed by a 39 C rise/min to a temperature of 240 C, which was held for 1 min. The fatty acid methyl esters were quantitated on a basis of the methyl pentadecanoate internal standard.

Conjugated diene measurements of peroxidized fatty acids were made after dilution of the samples in hexane. Further dilution of the samples was required to avoid interference by ascorbic acid. The conjugated dienes of preformed hydroperoxides were determined on a methanolic solution. Calculations were based on an extinction coefficient of $\epsilon_{232} = 25000$ $M^{-1}cm^{-1}$ (21).

Fatty acid hydroperoxides were determined by a photometric method (22). Sample aliquots of 0.5 ml were added to 6 ml chloroform-acetic acid (1:2) at 37 C and were constantly deaerated by bubbling a stream of nitrogen through the solution. A 0.1-ml aliquot of half-saturated KI solution was added. After 10 min, the volume was adjusted to 10 ml with methanol and mixed well. Following dilution of the sample 1:10 with methanol, the absorption was measured immediately at 370 nm. The conversion factor used for calculation of the peroxide content was verified by comparison with the results of an enzymatic measurement of linoleic acid hydroperoxide (23). One microequivalent of iodine, equal to 1 microequivalent of peroxide, gave an absorption of 0.385.

For hydrocarbon gas analysis, a Varian-Aerograph model 1520 gas chromatograph with a flame ionization detector was used. A stainless steel column (1/8 in. x 5 ft) filled with activated alumina (80-100 mesh) was used with a nitrogen carrier gas flow rate of 25 ml/min. The injector temperature was 165 C, and the detector temperautre was 265 C. The column temperature was programmed at 70 C for 1 min followed by a 30 C rise/min to 250 C. The total program was for 8 min. Headspace gas samples of 0.25-0.5 ml were injected, and the relative peak areas of the hydrocarbon gases were calculated from pentane and ethane standards (Matheson Gas Products, Newark, CA). A mixed gas standard (C1 to C6, 15-20 ppm, Scott Environmental Technology, Inc., Plumsteadville, PA) was used to quantitate the other hydrocarbon gases.

RESULTS

Hydrocarbon Gas Formation after Peroxidation of Fatty Acid Methyl Esters and after Decomposition of Hydroperoxides

Peroxidation of methyl linoleate, methyl linolenate, and methyl arachidonate was catalyzed with iron, copper, or hematin. Using the decrease in fatty acid as a measure of peroxidation, the data presented in Table I show that hematin was the most effective catalyst for methyl linoleate and that copper was most effective as a catalyst for peroxidation of methyl linolenate and methyl arachidonate. The overall decrease of fatty acids varied from 53 to 98% in 20 hr.

Diene conjugation and peroxide values in the

TABLE I

Fatty Acid Decrease and Products of Iron, Copper, and Hematin Catalyzed Peroxidation of Methyl Linoleate, Linolenate, and Arachidonate[a]

Fatty acid methyl ester	Catalyst	Fatty acid decrease (μmoles)	Diene conjugation (μmoles)	Peroxide value (μmoles)	Major hydrocarbon gas	
					Ethane (nmoles)	Pentane (nmoles)
Linoleate	Fe	23.3	12.6	16.5		121.7
	Cu	17.6	11.4	11.7		105.2
	Hematin	25.4	6.4	4.2		5.0
Linolenate	Fe	19.2	7.2	10.4	210.9	
	Cu	27.5	7.0	9.1	301.6	
	Hematin	20.9	2.2	6.6	3.2	
Arachidonate	Fe	22.9	8.0	11.0		91.2
	Cu	35.7	5.7	5.8		19.2
	Hematin	23.6	4.8	2.0		1.9
	Hematin[b]	27.4	4.1	3.0		9.3

[a]The reaction mixtures, containing 35-36 μmoles of fatty acid, and conditions are described in Experimental Procedures. Fatty acids, diene conjugation, and peroxide values were measured prior to decomposition and hydrocarbon gases following decomposition of hydroperoxides initiated by excess ascorbic acid. Each value is the average of data obtained from five different reaction mixtures.

[b]Excess ascorbic acid was added at the beginning of the incubation period.

TABLE II

Ethane and Pentane Evolved Following Peroxidation of Methyl Linoleate, Linolenate, and Arachidonate[a]

Fatty acid methyl ester	Catalyst	Major hydrocarbon gas	Major hydrocarbon gas relative to:		
			Fatty acid decrease (mol %)	Diene conjugation (mol %)	Peroxide value (mol %)
Linoleate	Fe	Pentane	0.5	1.0	0.7
	Cu	Pentane	0.6	0.9	0.9
	Hematin	Pentane	<0.1	0.1	0.1
Linolenate	Fe	Ethane	1.1	2.9	2.0
	Cu	Ethane	1.1	4.3	3.3
	Hematin	Ethane	<0.1	0.1	<0.1
Arachidonate	Fe	Pentane	0.4	1.1	0.8
	Cu	Pentane	0.1	0.3	0.3
	Hematin	Pentane	<0.1	<0.1	0.1
	Hematin[b]	Pentane	<0.1	0.2	0.3

[a]The reaction mixtures are described in Experimental Procedures. Except where indicated, hydrocarbon gases were measured in headspace gases following 20 hr of incubation and addition of excess ascorbic acid.

iron and copper catalyzed systems show that hydroperoxides are the major products after 20 hr of peroxidation. The larger decreases in fatty acid compared with increases in diene conjugation and peroxides indicate that some decomposition of the hydroperoxides occurred during the 20 hr incubation. The extent of decomposition was greater in the fatty acids with the greatest degree of unsaturation, indicating that the hydroperoxides with less unsaturation were more stable. In the hematin catalyzed systems, diene conjugation and peroxide values were much lower than in the iron and copper catalyzed systems even though the decreases in fatty acids were in a similar range.

During the 20 hr of incubation to peroxidize the fatty acids, the production of hydrocarbon gases was negligible. The hydrocarbon gases analyzed were methane, ethane, ethylene, propane, butane, pentane, and hexane. However, the methane peak could not be quantitated because it was too close to the air pressure peak following sample injection. By estimation, methane was noted to be in the range of other minor hydrocarbon gases. Hexane was not detected in the samples because it was either not released or not volatile enough at 37 C. The amounts of hydrocarbon gases shown in Table I are those measured after the addition of excess ascorbic acid which resulted in rapid evolution of the gases. Since it is known that hydroperoxide decomposition by Fe^{3+} or Cu^{2+} is

TABLE III

Minor Hydrocarbon Gases Evolved Following Peroxidation of Methyl Linoleate, Linolenate, and Arachidonate[a]

Fatty acid methyl ester	Catalyst	Major hydrocarbon gas	Butane (mol %)	Propane (mol %)	Ethylene (mol %)	Ethane (mol %)
Linoleate	Fe	Pentane	16.3	6.4	<1.0	5.0
	Cu	Pentane	14.5	2.4	<1.0	2.1
Linolenate	Fe	Ethane	0	0	<1.0	
	Cu	Ethane	0	0	46.3	
Arachidonate	Fe	Pentane	13.5	1.7	<1.0	5.2
	Cu	Pentane	27.6	8.4	7.2	15.9

[a]The reaction mixtures are described in Experimental Procedures. Hydrocarbon gases were measured following addition of excess ascorbic acid after 20 hr of incubation. Calculations of mol % are expressed relative to the major hydrocarbon gas evolved.

TABLE IV

Hydrocarbon Gases Evolved During Iron, Copper, and Hematin Catalyzed Decomposition of Preformed Hydroperoxides of Methyl Linoleate, Linolenate, and Arachidonate[a]

Fatty acid methyl ester hydroperoxide	Catalyst	Major hydrocarbon gas	Major hydrocarbon gas relative to: Diene conjugation (mol %)	Peroxide value (mol %)
Linoleate	Fe	Pentane	1.3	1.3
	Cu	Pentane	0.7	0.6
	Hematin	Pentane	0.3	0.3
Linolenate	Fe	Ethane	3.0	2.3
	Cu	Ethylene	10.6	8.3
	Hematin	Ethane	0.4	0.3
Arachidonate	Fe	Pentane	0.9	0.7
	Cu	Pentane	0.5	0.4
	Hematin	Pentane	0.3	0.2

[a]The reaction system for decomposition of preformed hydroperoxides is described in Experimental Procedures. Data shown represent the average of values obtained for analysis of three separate reaction systems.

markedly stimulated by a hydrogen donor like ascorbic acid (20), it is assumed that the formation of hydrocarbon gases occurs during the decomposition of hydroperoxides only. The amount of hydrocarbon gases formed in the hematin catalyzed reactions was much smaller than that formed in the iron and copper catalyzed systems. As shown in Table I, the amount of pentane formed from methyl arachidonate in the hematin catalyzed system was greater when higher amounts of ascorbic acid were added at the beginning of the peroxidation. In this system, pentane was measured at intervals during the 20-hr incubation and was found to be evolved continuously. The increased formation of pentane was probably dependent upon maintenance of the reduced state of the iron in the hematin.

The major hydrocarbon gases formed were ethane from methyl linolenate and pentane from methyl linoleate and methyl arachidonate. The ratios of these gases formed following decomposition of the hydroperoxides are shown in Table II. Depending on the fatty acid, catalyst, and calculation basis, pentane formation was as high as 1.1 mol % and ethane, 4.3 mol %. Since hydroperoxides are not the only peroxidation products, as shown in Table I, the ratios of hydrocarbon gases produced based on the decrease of fatty acid were always smaller than those based on diene conjugation or peroxide value.

The amounts of other low molecular weight hydrocarbon gases that were detected, relative to the major hydrocarbon gas products, are shown in Table III. In the copper catalyzed methyl linolenate systems, about 46% as much ethylene was detected as ethane, and in the methyl linoleate and methyl arachidonate systems, between 13.5 and 27.6% as much butane as pentane was produced. Other hydrocarbon gases were only of minor importance. Measurement of the peroxidation reaction by following the decrease in fatty acid showed that continuation of peroxidation during the decomposition of hydroperoxides by excess ascorbic acid for

20 min was negligible. Small amounts of ethane and ethylene formed by decomposition of Brij 35, a polyethylene ether of lauryl alcohol, were corrected for by the use of appropriate blanks.

Hydrocarbon Gas Formation Following Decomposition of Preformed Hydroperoxides of Fatty Acid Methyl Esters

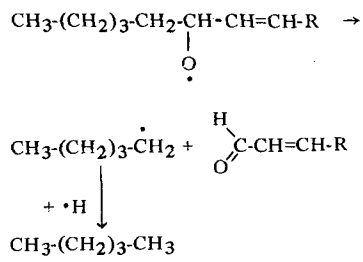
After it was found that hydrocarbon gases were formed only during the decomposition of the fatty acid hydroperoxides, similar assays were done with known amounts of preformed hydroperoxides. Table IV shows that with one exception the ratios of hydrocarbon gases in the total system are similar to those shown in Table II. The ratio of ethane:ethylene formed in the copper catalyzed methyl linolenate hydroperoxide decomposition systems was 2:1 in the total peroxidizing system and 1:12 in the preformed hydroperoxide system.

DISCUSSION

This investigation of the use of the low molecular weight hydrocarbon gases as indices of lipid peroxidation was initiated following the reports that ethane production in vivo was related to treatment of mice with carbon tetrachloride (15), and that measurement of ethane and pentane was used to show protection by vitamin E against lipid peroxidation in rats (18). Further literature references show that many volatile compounds have been measured from peroxidizing food systems. Pentane evolved from peroxides during their decomposition has been correlated with sensory tests for rancidity (6-8), and it also has been measured as a decomposition product of 13-hydroperoxy-octadeca-9,11-dienoic acid, a lipoxidase oxidized product from linoleic acid (4,24). In dehydrated food systems where oxidation was measured by thermal release of hydrocarbons, the following gas products were found: ethane from linolenic acid; octane from oleic acid; pentane from linoleic acid; pentane and butane from myristoleic acid; and heptene and hexane from vaccenic acid (9).

This report shows that the hydrocarbon gases are evolved only during the decomposition of fatty acid hydroperoxides and not during the peroxidation phase. The major hydrocarbon gas products are pentane, which arises from ω-6 fatty acids, and ethane or ethylene, which arise from ω-3 fatty acids.

The general route for formation of ethane and pentane from free radical products of hydroperoxide decomposition has been visualized by Evans et al. (4). The main reaction of β-scission is well-known for oxy radicals from decomposing peroxides (25-27). β-Scission is the production of a stable molecule by unpairing of the electrons in the bond which is β to the free radical. The most likely precursor for pentane formation is an alkoxy radical that undergoes β-scission very readily. Cleavage of an ω-6 fatty acid chain on the side of the peroxy group closest to the methyl end results in the formation of a pentane free radical as shown in reaction (I). Hydrogen abstraction by the resulting free radical forms pentane.



(I)

This study demonstrates that pathway (I) is even more likely once the route of transition metal-catalyzed decomposition of hydroperoxides is considered:

$$ROOH + M^{+n} \rightarrow RO\cdot + OH^- + M^{+n+1} \quad \text{(II)}$$

$$ROOH + M^{+n+1} \rightarrow ROO\cdot + H^+ + M^{+n} \quad \text{(III)}$$

In their lower but not in their higher oxidation states, the iron and copper catalysts used in these experiments are effective reducers of ROOH (28). Iron, hematin, and copper are the metals that decompose hydroperoxides and are present in higher concentrations in vivo when compared to other heavy metals. Therefore, it can be assumed that alkoxy radicals are major decomposition intermediates that eventually lead to release of pentane or ethane by β-scission.

The amount of hydrocarbon gases formed in the hematin catalyzed reactions was much smaller than that formed in the iron and copper catalyzed systems. This was probably dependent upon the fact that the reduced state of iron in hematin is harder to achieve than it is in free iron ions. Hematin is known to be a very effective inducer of lipid peroxidation, and contrary to free ions, it is a potent decomposer of hydroperoxides (20,29). For these reasons, it is assumed that the decomposition reaction mainly follows route (III).

It would be interesting to account for the origin of the minor hydrocarbons, butane, propane, ethane, and ethylene, that derive from

the ω-6 fatty acid hydroperoxides. Considering that β-scission is a predominant route of scission reactions (25-27), the origin of these minor hydrocarbons might be visualized as follows. β-Scission of the 13-hydroperoxyl radical (III) would yield the 13-alkyl radical, and β-scission of this radical would yield butane radical. A β-scission of butane radical gives ethylene and ethane radical. In a similar manner, β-scission of pentane radical of (I) would give ethylene and propane radical. Hydrogen abstraction by these alkyl radicals gives the alkanes, butane, ethane, and propane.

It is assumed that the general decomposition mechanism in the ω-3 fatty acid systems also follows reaction (I), but gives rise to ethane. Copper is known to react with alkyl radicals to give the corresponding a alkene (25). This reaction (IV) can account for the formation of ethylene.

$$C_2H_5^{\circ} + Cu^{++} \rightarrow CH_2{=}CH_2 + H^+ + Cu^+ \qquad (IV)$$

Ethylene was produced in larger amounts from preformed hydroperoxides, but the conditions favoring results of this are not apparent.

Only a qualitative statement about the relationships among conjugated dienes, peroxide values, and hydrocarbon gases can be given. Hydrocarbon gases can be produced upon the addition of ascorbic acid only after the appearance of conjugated dienes and peroxides, and they are not detectable when conjugated dienes and peroxides are negligible. Earlier studies by Jarvi et al. (7) showed a close correlation between peroxide values and pentane released after thermal decomposition of soybean oil hydroperoxides.

The calculated molar ratios show that hydrocarbon gases are only minor lipid peroxidation products compared to those obtained in studies by Chan et al. (30), where on a molar basis a 67-80% yield of volatile cleavage products of thermally decomposed methyl linoleate hydroperoxide was found in the form of hexanal, methyl octanoate, 2,4-decadienals, and methyl 9-oxononanoate. A major usefulness of the molar ratios determined is that they can be applied to measurements of pentane and ethane produced during lipid peroxidation in vivo. These ratios allow a calculation of the approximate amounts of fatty acid hydroperoxides formed and decomposed in vivo. The advantages of the measurement of hydrocarbon gases as products of lipid peroxidation are that they are volatile at ambient temperature, that they are stable and chemically inert, and that they are easily detectable in minimal amounts both in vivo and in vitro.

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