Formation of Reactive Aldehydes from Fatty Acids in a Fe^{2+}/H_2O_2 Oxidation System

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The lipid peroxidation products formed from arachidonic acid, linolenic acid, linoleic acid, oleic acid, and their esters upon Fe^{2+}/H_2O_2 oxidation were analyzed by two derivatization methods. α,β-Unsaturated aldehydes, such as acolein and 4-hydroxy-2-nonenal, and β-dicarbonyl compounds, such as malonaldehyde, were derivatized with *N*-methylhydrazine. Saturated normal aldehydes, such as formaldehyde and hexanal, were derivatized with cysteamine. Subsequently, a specific quantity of each derivative 1-methylpyrazoline, 1-methylpyrazole, and thiazoline was analyzed by gas chromatography. Formaldehyde, which has never been quantified satisfactorily in a lipid peroxidation system, was the major product, and its quantities ranged from 142 nmol/mg in ethyl linolenate to 50 nmol/mg in ethyl arachidonate. Malonaldehyde was found in all samples, in levels between trace in oleic acid and 97 nmol/mg in ethyl arachidonate. 4-Hydroxy-2-nonenal was found in arachidonic and linoleic acids and their ethyl esters, whereas 4-hydroxy-2-hexenal was found only in linolenic acid and its ethyl ester. Hexanal was one of the main aldehydes formed from arachidonic acid (43 nmol/mg) and linoleic acid (141 nmol/mg) and from their ethyl esters, but propanal was produced in significant amounts only from linolenic acid and its ethyl ester (35 and 23 nmol/mg, respectively).

Fatty acids in foods or microsomes prepared from a mammalian liver are known to produce many different carbonyl compounds, such as malonaldehyde (MA) and 4-hydroxy-2-nonenal (4-HN), via peroxidation reactions (Esterbauer, 1982). Consequently, formation of certain carbonyl products has been monitored to determine the degree of peroxidation in lipids. The most widely used assay for lipid peroxidation is a thiobarbituric acid (TBA) method that measures total products that react with TBA. Therefore, the results obtained by this method are not always specific to the product of interest.

Recently, we developed a gas chromatographic (GC) method to analyze MA as the *N*-methylhydrazine (NMH) derivative 1-methylpyrazole (Umano et al., 1988). In this method, monocarbonyl compounds such as formaldehyde, α,β-unsaturated aldehydes such as acrolein and 4-hydroxynonenal, and β-dicarbonyl compounds such as MA can be analyzed simultaneously as hydrazones, 1-methyl-2-pyrazolines, and 1-methylpyrazoles, respectively. Free and bound MA formed in liver microsomes prepared from a rat administered CCl_4 were measured by this method (Ichinose et al., 1989). Acrolein formed from heated pork fat was also analyzed as the NMH derivative 1-methyl-2-pyrazoline (Yasuhara and Shibamoto, 1989). Among the drawbacks of this NMH derivatization method are that hydrazones formed from aldehydes were not stable and that those with high molecular weights are difficult to analyze by GC.

On the other hand, aldehydes including C_1–C_{10} normal aldehydes were analyzed as cysteamine derivative thiazolidines more satisfactorily (Hayashi et al., 1985; Hayashi and Shibamoto, 1985; Umano and Shibamoto, 1986). In contrast to NMH, cysteamine does not react readily with α,β-unsaturated aldehydes or β-dicarbonyl compounds. Therefore, this derivatization method is useful to analyze monocarbonyl compounds selectively. For example, Hayashi et al. (1986) satisfactorily measured trace formaldehyde in various foods and beverages as thiazolidine with GC using a nitrogen–phosphorus specific detector (NPD).

In the present study, carbonyl compounds formed from various fatty acids and their ethyl esters upon Fe^{2+}/H_2O_2 oxidation were analyzed as either NMH or cysteamine derivatives by GC with a NPD.

EXPERIMENTAL PROCEDURES

Materials. Acrolein, purchased from Aldrich Chemical Co. (Milwaukee, WI), was dried over anhydrous sodium sulfate and purified by distillation immediately before use. All other aldehydes, fatty acids, and ethyl esters were purchased from Aldrich and used without further purification. *trans*-4-Hydroxy-2-nonenal was synthesized according to the methods of Tamura and Shibamoto (1991). All other authentic chemicals were obtained from reliable commercial sources at the highest grade and used without further treatment.

Oxidation of Fatty Acids and Their Ethyl Esters with Fe^{2+}/H_2O_2. An aqueous solution (5 mL) containing a fatty acid or its ethyl ester (1.5 mg/mL), 0.25 mmol of Trizma buffer (pH 7.4), 0.75 mmol of potassium chloride, and surfactant sodium dodecyl sulfate (0.2%) was stirred with or without 1 μmol of ferrous chloride and 0.5 μmol of hydrogen peroxide at 37 °C for 16 h. The oxidation reaction was stopped by adding 4 ppm of butyrated hydroxyl toluene (BHT).

Effect of Temperature on the Lipid Peroxidation. Ethyl linoleate in the above oxidation system was heated at 25, 37, 50, or 90 °C for 16 h to investigate the effect of temperature on the lipid peroxidation. The amount of MA and 4-HN formed under these temperatures was measured.

Preparation of NMH Derivatives of the Lipid Peroxidation Products. NMH (40 μL) was added to the above oxidized solutions of fatty acids and their ethyl esters and then stirred for 1 h at 25 °C. The reaction solution was extracted with 8 mL of dichloromethane by using a liquid–liquid continuous extractor for 3 h. The solution was saturated with NaCl prior to extraction to prevent emulsion formation. The extract was adjusted to 10 mL by adding dichloromethane. After 100 μL of 2-methylpyrazine was added as a GC internal standard, the solution was analyzed by GC with a NPD.

Preparation of Cysteamine Derivatives of the Lipid Peroxidation Products. Cysteamine (100 mM) was added to the above oxidized solution of fatty acids and their ethyl esters, and the pH of the solution was adjusted to 8.5 with 6 N sodium

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Table I. Lipid Peroxidation Products Recovered as NMH Derivatives from Fatty Acids and Their Ethyl Esters

	arachidonic acid		linolenic acid		linoleic acid		oleic acid	
compd	free acid	ethyl ester	free acid	ethyl ester	free acid	ethyl ester	free acid	ethyl ester
acrolein	7.7 ± 1.9[a]	13.3 ± 8.9	4.0 ± 3.6	8.2 ± 3.4	9.3 ± 7.3	0.8 ± 0.5	*b*	*c*
crotonaldehyde	*b*	*b*	1.1 ± 0.7	2.0 ± 0.9	0.8[d]	0.4[d]	1.1[d]	*c*
2-pentenal	*b*	*b*	1.1 ± 0.1	3.3 ± 1.8	*c*	*b*	*c*	*c*
2-hexenal	*b*	0.7[d]	*b*	*c*	0.8 ± 0.3	1.0 ± 0.1	*c*	*c*
2-heptenal	0.4 ± 0.1	0.4 ± 0.0	*c*	*c*	1.6 ± 0.1	1.2 ± 0.1	*b*	*c*
2-octenal	1.0 ± 0.4	2.2 ± 0.3	*c*	*b*	1.7 ± 0.2	1.3[d]	*c*	*b*
malonaldehyde	27.5 ± 3.8	97.2 ± 15.7	20.0 ± 2.5	63.1 ± 10.3	57.9 ± 7.0	43.1 ± 6.1	*b*	0.3[d]
4-hydroxy-2-hexenal	*c*	*c*	5.1 ± 0.8	15.4 ± 3.4	*c*	*c*	*c*	*c*
4-HN	10.3 ± 2.2	26.1 ± 12.6	*c*	*c*	15.1 ± 1.5	9.1 ± 2.8	*c*	*c*

[a] Values are mean ± standard deviation in nanomoles per milligram (n = 3). [b] Trace. [c] Not detected. [d] n = 2.

Table II. Lipid Peroxidation Products Recovered as Cysteamine Derivatives from Fatty Acids and Their Ethyl Esters

	arachidonic acid		linolenic acid		linoleic acid		oleic acid	
compd	free acid	ethyl ester	free acid	ethyl ester	free acid	ethyl ester	free acid	ethyl ester
formaldehyde	115.7 ± 31.3[a]	49.9 ± 35.3	106.6 ± 63.2	141.5 ± 92.2	59.3 ± 62.8	104.1 ± 6.3	100.9 ± 31.6	119.7 ± 59.8
acetaldehyde	40.2 ± 28.6	23.0 ± 18.9	22.7 ± 10.7	20.9 ± 6.3	67.3 ± 47.8	58.9 ± 49.5	45.4 ± 27.3	17.4 ± 7.8
propanal	*b*	*b*	35.0 ± 7.5	22.9 ± 0.8	*b*	*b*	*b*	*b*
pentanal	3.5 ± 1.9	*b*	*b*	*b*	13.3 ± 4.1	7.3 ± 3.9	*b*	*b*
hexanal	43.2 ± 24.6	57.1 ± 50.6	*b*	*b*	141.0 ± 93.6	64.4 ± 35.5	*b*	*b*

[a] Values are mean ± standard deviation in nanomoles per milligram (n = 3). [b] Not detected.

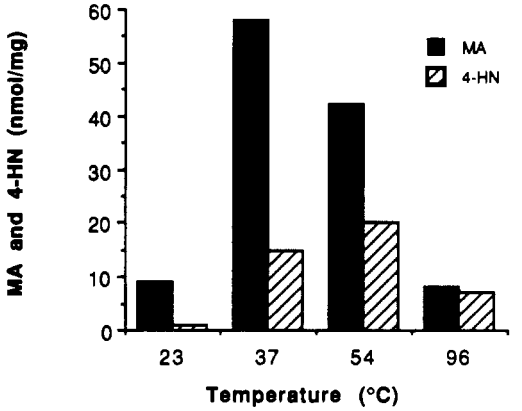


Figure 1. Formation of MA and 4-HN from ethyl linoleate over different temperatures.

hydroxide solution. The solution was stirred for 1 h at 25 °C by use of a magnetic stirrer. The reaction solution was extracted with 8 mL of chloroform by using a liquid-liquid continuous extractor for 3 h. The extract was adjusted to 10 mL by adding chloroform. After the addition of 100 μL of *N*-methylacetamide as a GC internal standard, the extract was analyzed by GC with a NPD.

Instrumentation. A Hewlett-Packard (HP) Model 5880 gas chromatograph equipped with a nitrogen–phosphorus detector and a 30 m × 0.25 mm i.d. DB Wax bonded-phase fused silica capillary column (J&W Scientific, Folsom, CA) was used for routine analysis. GC peak areas were integrated by using an HP-3390A integrator. The oven temperature was held at 35 °C for 1 min, programmed to 190 °C at 4 °C/min, and held until all components were eluted. The helium carrier gas flow was 30 cm/s. The injector and detector temperatures were 270 and 300 °C, respectively.

A gas chromatograph (HP 5890A) interfaced to a VG Trio-2 mass spectrometer was used to obtain mass spectra of gas chromatographic components of the samples. The ionization voltage was 70 eV, and the ion source temperature was 140 °C.

Infrared (IR) spectra were obtained on an HP 5965 IR detector interfaced to an HP 59970 IRD Chem Station and an HP 5890 GC. GC conditions for both instruments were the same as those described previously.

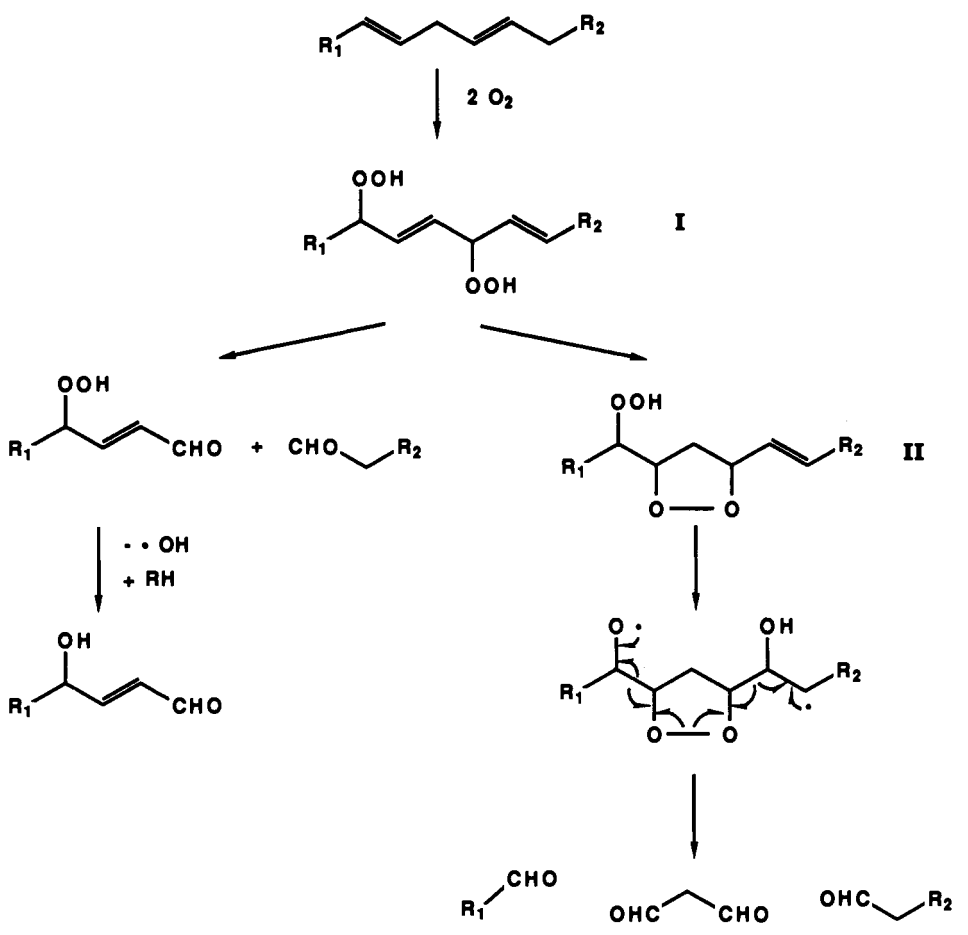
Qualitative and Quantitative Analyses of NMH or Cysteamine Derivatives. Qualitative analyses of the derivatives were done by comparing the GC Kovats retention index (I) and MS fragmentation pattern of each derivative to those of the authentic compounds. GC calibration curves used for quantitative analysis of each derivative were prepared by using a corresponding authentic compound. 2-Methylpyrazine and *N*-methylacetamide were used as an internal standard for analysis of NMH and cysteamine derivatives, respectively. Gas chromatographic peak area ratios of derivatives and standard against quantity of derivatives were plotted (Ettre, 1967). The authentic compounds of NMH and cysteamine derivatives were synthesized according to the methods reported by Dennis and Shibamoto (1990) and Yasuhara and Shibamoto (1989), respectively.

RESULTS AND DISCUSSION

Figure 1 shows the amounts of MA and 4-HN produced from ethyl linoleate at different temperatures. Production of MA and 4-HN reached a maximum at 37 °C. When the temperature was raised to over 50 °C, formation of these compounds was reduced, suggesting that these reactive carbonyl compounds underwent secondary reactions. It is interesting that 37 °C is the temperature used to incubate most biological samples. Therefore, 37 °C was used to induce lipid peroxidation for further experiments in the present study.

Table I shows carbonyl products formed from various fatty acids and their esters in a Fe^{2+}/H_2O_2 oxidation system. The products were analyzed as NMH derivatives. 2-Heptenal and 2-octenal formed only from arachidonic acid and linoleic acid and their ethyl esters. On the other hand, relatively large amounts of crotonaldehyde and 2-pentenal were produced from linolenic acid and its ethyl ester. These results are consistent with those reported previously (Esterbauer, 1982; Yasuhara and Shibamoto, 1989) except in the case of 2-hexenal. 2-Hexenal was a characteristic product of linoleic acid and its ethyl ester in the present study, although it was found in the largest amount in linolenic acid in previous studies (Esterbauer, 1982; Frankel et al., 1981).

Acrolein was recovered from all samples except oleic acid and its ethyl ester. There are virtually no reports of the formation of acrolein in lipid peroxidation systems prior to the present study. This may be due to a lack of an appropriate analytical method for acrolein. The determination of acrolein is a difficult analytical procedure because the compound is highly volatile and polymerizes readily (Hess et al., 1978). Recently we developed and validated a new analytical method for trace acrolein using



	R_1	R_2
Arachidonic acid	$CH_3(CH_2)_3CH_2$ -	- $CH{=}CHCH_2CH{=}CH(CH_2)_2CH_2COOH$
Ethyl arachidonate	$CH_3(CH_2)_3CH_2$ -	- $CH{=}CHCH_2CH{=}CH(CH_2)_2CH_2COOC_2H_5$
Linoleic acid	$CH_3(CH_2)_3CH_2$ -	- $CH_2(CH_2)_4CH_2COOH$
Ethyl linoleate	$CH_3(CH_2)_3CH_2$ -	- $CH_2(CH_2)_4CH_2COOC_2H_5$
Linolenic acid	CH_3CH_2 -	- $CH{=}CH(CH_2)_6CH_2COOH$
Ethyl linolenate	CH_3CH_2 -	- $CH{=}CH(CH_2)_6CH_2COOC_2H_5$

Figure 2. Proposed formation mechanisms of MA, 4-HN, 4-hydroxy-2-hexenal, propanal, and hexanal from arachidonic acid, linolenic acids, linoleic acids, and their ethyl esters.

NMH (Yasuhara and Shibamoto, 1989). In this method, analysis of 56 pg of acrolein is possible. Also, acrolein is reportedly formed in large amounts by high-temperature treatment of lipid-rich foods such as cooking oils (Umano and Shibamoto, 1987). One of the major advantages of the NMH method is that free malonaldehyde can be analyzed (Umano et al., 1988). In the present study, all samples produced malonaldehyde in amounts ranging from trace in oleic acid to 97 nmol/mg in ethyl arachidonate. By use of the same method, 0.17–0.22 mg/g of liver of free malonaldehyde was previously found in the liver microsome prepared from a rat administered CCl_4 (Ichinose et al., 1989).

In the present study, 4-HN was found in arachidonic acid, linoleic acid, and their ethyl esters, whereas 4-hydroxy-2-hexenal was found only in linolenic acid and its ethyl ester. 4-HN is reportedly one of the major lipid peroxidation products of fatty acids, including linoleic, γ-linolenic, and arachidonic acid, and of liver microsomes (Esterbauer, 1982). Because 4-HN has received much attention as a lipid peroxidation product that possesses cytotoxicity (Esterbauer, 1982), further study on toxicity of 4-hydroxy-2-hexenal is in order.

Table II shows aldehydes identified as cysteamine derivatives. Formaldehyde was recovered from all samples in relatively large amounts. Among them, ethyl linolenate produced the highest level of formaldehyde (142 nmol/mg). Formaldehyde has never been reported as a lipid peroxidation product prior to the present study. Formaldehyde is difficult to extract from an aqueous solution with an organic solvent because it is highly soluble in water, and in an aqueous medium it exists as a polymer. Moreover, formaldehyde is difficult to separate from common organic solvents because of its high volatility (bp 19.5 °C). Therefore, formaldehyde may have never been recovered from an aqueous medium used for lipid peroxidation studies prior to the present study. Acetalde-

hyde was also found in all samples but in smaller amounts than formaldehyde.

Only linolenic acid and its ethyl ester produced propanal in significant quantities. Hexanal was one of the main aldehydes formed from arachidonic acid and linoleic acid and their ethyl esters, although it was produced from linolenic acid and oleic acid and their ethyl esters only in trace amounts. Different production of various aldehydes may clarify the lipid peroxidation mechanisms.

Pryor et al. (1976) proposed the presence of a hydroperoxy cyclic peroxide intermediate on the basis of the mechanisms suggested by Dahle et al. (1962). They also proposed the formation of malonaldehyde with a prostaglandin-like endoperoxide mechanism. Later, Frankel et al. (1982) summarized the formation mechanisms of various aldehydes from thermal decomposition cleavage of hydroperoxy cyclic peroxides. Formation mechamisms of 4-hydroxy-2-alkenals were not included in their proposed mechanisms. We modified the mechanisms proposed by Pryor et al. (1976) to include 4-HN and 4-hydroxy-2-hexenal.

The proposed formation mechanisms of MA, 4-HN, propanal, and hexanal from the fatty acids and their esters used in the present study are shown in Figure 2. A proposed dihydroperoxide intermediate (I) explains the formation of 4-HN from linoleic acid and arachidonic acid and of 4-hydroxy-2-hexenal from linolenic acid. Linoleic acid and arachidonic acid form 13-hydroperoxy-10,12-cyclic peroxide intermediate (II) and 15-hydroperoxy-13,14-cyclic peroxide intermediate (II), respectively, and then produce hexanal predominantly. Linolenic acid forms 16-hydroperoxy-14,15-cyclic peroxide intermediate and then produces propanal predominantly. MA is also formed as a result of hexanal or propanal formation from the cyclic peroxide intermediates in addition to the pathway proposed by Pryor et al. (1976).

In the present study, reactive aldehydes were satisfactorily determined by using derivatization methods. Aldehydes formed from lipids upon oxidation play an important role in various biological and chemical phenomena. Formaldehyde is a suspected carcinogen (Karns, 1980). Acrolein is reportedly toxic to animals, plants, and unicellular organisms (Izard and Libermann, 1978), and malonaldehyde has been implicated in aging, mutagenesis, and carcinogenesis (Shamberger et al., 1979). Therefore, further investigation of aldehyde formation in foods and environment is in order.

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Registry No. MA, 542-78-9; 4-HH, 17427-08-6; 4-HN, 29343-52-0; H_2O_2, 7722-84-1; Fe, 7439-89-6; acrolein, 107-02-8; crotonaldehyde, 4170-30-3; 2-pentenal, 764-39-6; 2-hexenal, 505-57-7; 2-heptenal, 2463-63-0; 2-octenal, 2363-89-5; formaldehyde, 50-00-0; acetaldehyde, 75-07-0; propanal, 123-38-6; pentanal, 110-62-3; hexanal, 66-25-1; arachidonic acid, 506-32-1; ethyl arachidonate, 1808-26-0; linolenic acid, 463-40-1; ethyl linolenate, 1191-41-9; linoleic acid, 60-33-3; ethyl linoleate, 544-35-4; oleic acid, 112-80-1; ethyl oleate, 111-62-6.