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Characterization of 4-Oxo-2-nonenal as a Novel Product of Lipid Peroxidation

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Fe^{II}-mediated decomposition of 13-[*S*-(*Z*,*E*)]-9,11-hydroperoxyoctadecadienoic (hydroperoxylinoleic) acid resulted in the formation of three α,*β*-unsaturated aldehydes. At low Fe^{II} concentrations or at early time points after the addition of Fe^{II}, two major products were observed. The least polar product had chromatographic properties that were identical with those of 4-oxo-2-nonenal. Conversion of this product to its bis-oxime derivative with hydroxylamine hydrochloride resulted in two *syn*- and two *anti*-oxime isomers that had chromatographic and mass spectral properties identical with the properties of products derived from an authentic standard of 4-oxo-2-nonenal. This confirmed for the first time that 4-oxo-2-nonenal is a major product of the Fe^{II}-mediated breakdown of lipid hydroperoxides. The more polar product had chromatographic properties that were similar to those of 4-hydroperoxy-2-nonenal. LC/MS analysis of its *syn*- and *anti*-oxime isomers confirmed this structural assignment. Thus, 4-hydroperoxy-2-nonenal is a previously unrecognized major product of lipid hydroperoxide decomposition. At high Fe^{II} concentrations and at longer incubation times, a third more polar product was observed with chromatographic properties that were identical to those of 4-hydroxy-2-nonenal. The *syn*- and *anti*-oxime isomers had chromatographic and mass spectral properties identical with the properties of products derived from an authentic standard of 4-hydroxy-2-nonenal. It appears that 4-hydroperoxy-2-nonenal is formed initially and that it is then converted to 4-hydroxy-2-nonenal in the presence of high Fe^{II} concentrations or by extended incubations in the presence of low Fe^{II} concentrations. It is conceivable that some of the 4-hydroperoxy-2-nonenal is also converted to 4-oxo-2-nonenal. However, we cannot rule out the possibility that it is also formed by a concerted mechanism from a rearrangement product of 13-[*S*-(*Z*,*E*)]-9,11-hydroperoxyoctadecadienoic acid.

Introduction

The role that endogenously produced chemicals may play in the etiology of cancer has become increasingly important over the past decade (*1*). This is largely due to epidemiological data, which show that apart from tobacco smoke and sunlight, exposure to genotoxic environmental carcinogens accounts for only a minority of human cancers (*2*). Lipid peroxidation has long been thought to produce endogenous genotoxins that are derived from lipid hydroperoxides (*3*). The formation of lipid hydroperoxides is a complex process, which involves a number of different radical intermediates (*4*, *5*). However, lipid hydroperoxides are also readily formed as a consequence of LOX-mediated oxidation of endogenous polyunsaturated fatty acids (PUFAs)[1] (*6*). Therefore, there are both enzymatic and nonenzymatic pathways by which lipid hydroperoxides can be formed. In view of their potential role as genotoxins, it is important to understand how structural modifications to DNA can occur from reactions with lipid hydroperoxides. Their genotoxic properties are thought to result from the generation of bifunctional electrophiles such as malondialdehyde (*7*) and 4-hydroxy-2-nonenal (*8*). These bifunctional electrophiles then react at electron rich sites of the DNA bases to form DNA adducts (*1*). Evidence has been obtained for the presence of both malondialdehyde- (*9*, *10*) and 4-hydroxynonenal-derived DNA adducts (*11*) in vivo. Transition metal ions are known to enhance the formation of bifunctional electrophiles through a homolytic process (*12*). The presence of transition metal cations bound to the polar sugar residues of DNA can potentially enhance the breakdown of lipid hydroperoxides to genotoxic bifunctional electrophiles (*13*). We have recently studied the decomposition of 13-HPODE (a prototypic $\omega - 6$ PUFA) in the presence of the DNA bases dGuo and dAdo. From the structures of the resulting DNA adducts, we proposed that the covalent modifications arose through the generation of 4-oxo-2-nonenal from 13-HPODE (*14*, *15*). We have also demonstrated that the same adducts were formed when the DNA bases were treated with synthetic 4-oxo-2-nonenal (*14*, *16*). Surprisingly, we were unable to detect covalent modifications resulting from 4-hydroxy-2-nonenal. This led us to speculate that 4-oxo-

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[1]Abbreviations: ESI, electrospray ionization; 4-HNE, 4-hydroxy-2-nonenal; 4-HPNE, 4-hydroperoxy-2-nonenal; 13-HPODE, 13-hydroperoxy-[*S*-(*Z*,*E*)]-9,11-octadecadienoic acid; 13-(*E*,*E*)-HPODE, 13-hydroperoxy-(*E*,*E*)-9,11-octadecadienoic acid; 9-(*E*,*Z*)-HPODE, 9-hydroperoxy-(*E*,*Z*)-10,12-octadecadienoic acid; 9-(*E*,*E*)-HPODE, 9-hydroperoxy-(*E*,*E*)-10,12-octadecadienoic acid; LC/MS, liquid chromatography/mass spectrometry; LOX, lipoxygenase; MS^n, multiple tandem mass spectrometry; 4-ONE, 4-oxo-2-nonenal; PUFA, polyunsaturated fatty acid; TIC, total ion current.

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2-nonenal was the major breakdown product of lipid hydroperoxides rather than 4-hydroxy-2-nonenal. However, it is possible that the DNA adducts could have arisen from reaction of the DNA base with an unstable intermediate during lipid hydroperoxide decomposition. We now report definitive evidence that 4-oxo-2-nonenal is indeed a major breakdown product of lipid hydroperoxides. In addition, we provide evidence for the unexpected formation of 4-hydroperoxy-2-nonenal as an additional lipid peroxidation breakdown product.

Materials and Methods

Materials. 4-Hydroxy-2-nonenal was purchased from Cayman Chemical Co. (Ann Arbor, MI). Soybean lipoxidase (type V), tetrahydrofuran, and hydroxylamine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Ammonium iron(II) sulfate hexahydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Chelex-100 chelating ion-exchange resin (100−200 mesh size) was obtained from Bio-Red Laboratories (Hercules, CA). HPLC grade water was obtained from Fisher Scientific Co. (Fair Lawn, NJ). HPLC grade methanol was purchased from Burdick and Jackson (Muskegon, MI). Gases were supplied by BOC Gases (Lebanon, NJ). Normal phosphate buffer was prepared from potassium hydrogen phosphate (KH_2PO_4, ACS reagent grade, Aldrich) and HPLC grade water (Optima, Fisher Scientific Co.).

Mass Spectrometry. The data were acquired on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with a Finnigan electrospray source. The mass spectrometer was operated in the positive ion mode with a potential of 4.25 kV applied to the electrospray needle. Nitrogen was used as the sheath (60 units) and auxiliary (5 units) gas to assist with nebulization. The capillary temperature was held at 200 °C. Full scanning analyses were performed in the range of *m*/*z* 100−800. Collision-induced dissociation (CID) experiments coupled with multiple tandem mass spectrometry (MS^n) were carried out with helium as the collision gas. The relative collision energy was set at 20% of the maximum (1 V).

Liquid Chromatography. Chromatography was performed using a Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA). Gradient elutions were all performed in the linear mode. Gradient systems 1 and 4 included a Hi-Chrom silica column (250 mm × 4.6 mm i.d., 5 μm; Regis, Morton Grove, IL) at a flow rate of 1 mL/min. A YMC C_{18} ODS-AQ column (250 mm × 4.6 mm i.d., 5 μm; YMC, Inc., Wilmington, NC) was used in systems 2 and 3. The LC/UV/MS experiments were performed using a Hitachi L-4200 UV detector at 234 nm for detection of conjugated dienes (systems 2 and 4) and at 226 nm (system 1) and 265 nm (system 3). A postcolumn split was employed such that an 800 μL/min flow was delivered to the UV detector and a 200 μL/min flow to the mass spectrometer. For normal phase HPLC, all of the mobile phase was diverted through the UV detector. For system 1, solvent A was hexane and 2-propanol (197:3, v/v) and solvent B was hexane and 2-propanol (70:30, v/v). The gradient conditions were as follows: 3% B at 0 min, 3% B at 15 min, 85% B at 25 min, 85% B at 28 min, and 3% B at 30 min, followed by a 5 min equilibration time. For system 2, solvent A was tetrahydrofuran, methanol, water, and acetic acid (25:30:44.9:0.1, v/v) and solvent B was methanol and water (9:1, v/v). Both solvents A and B contained 5 mM ammonium acetate. The gradient conditions were as follows: 70% B at 0 min, 70% B at 3 min, 100% B at 10 min, 100% B at 20 min, and 70% B at 23 min, followed by a 7 min equilibration time. For system 3, solvent A was 5 mM ammonium acetate in water containing 0.01% trifluoroacetic acid and solvent B was 5 mM ammonium acetate in methanol containing 0.01% trifluoroacetic acid. The gradient conditions were as follows: 30% B at 0 min, 30% B at 5 min, 84.5% B at 40 min, 100% B at 42 min, 100% B at 49 min, and 30% B at 51 min, followed by a 5 min equilibration time. For system 4, isocratic elution was performed with hexane and 2-propanol (197:3, v/v) containing 0.1% acetic acid at a flow rate of 1 mL/min.

Synthesis of 4-Oxo-2-nonenal. 4-Hydroxy-2-nonenal diethyl acetal was oxidized with activated MnO_2 as described by Esterbauer and Weger (*17*). The resulting 4-oxo-2-nonenal diethyl acetal was then hydrolyzed by citric acid/HCl as described previously (*14*).

Preparation of Pure 13-HPODE. 13-HPODE was prepared using linoleic acid (5.0 mg, 17.9 μmol) and soybean lipoxidase (20 000 units, type V) in 3 mL of 0.2 M borate buffer (pH 9.0). The solution was mixed by magnetic stirring while the temperature was maintained at 0 °C. After 5 h, the reaction mixture was acidified to pH 3 with 1 N HCl and the 13-HPODE was extracted with diethyl ether (2 × 5 mL). The combined extracts were washed with water, dried over sodium sulfate, and evaporated under a stream of nitrogen. The 13-[*S*-(*Z*,*E*)]-HPODE was then purified using normal phase system 4. 13-(*E*,*E*)-HPODE had a retention time of 11.9 min under these conditions, and 13-(*Z*,*E*)-HPODE had a retention time of 13.2 min. The pure 13-[*S*-(*Z*,*E*)]-HPODE dissolved in ethanol, and its concentration was determined by UV spectroscopy ($\lambda_{max} = 234$ nm, $\epsilon = 25\,600$). It was stored in ethanol at −70 °C, and the solution was reanalyzed by reversed phase LC/MS using gradient system 2 before it was used. A single chromatographic peak was observed. The mass spectrum contained a dominant ammoniated molecular ion at *m*/*z* 330 ($M + NH_4$) together with the expected fragment ion at *m*/*z* 195 ($M - C_6H_{12}OOH$). No fragment ion was detected at *m*/*z* 155 ($M - C_9H_{16}OOH$) from potentially coeluting 9-(*E*,*Z*)-HPODE. It would have been possible to detect <0.5% contamination under the conditions used in the analysis. Both 13-(*E*,*E*)-HPODE and 9-(*E*,*E*)-HPODE are readily separated from 13-[*S*-(*Z*,*E*)]-HPODE, and neither of these compounds was observed in the chromatogram. When the pure 13-[*S*-(*Z*,*E*)]-HPODE was analyzed by LC/UV under normal phase conditions using system 1, no early eluting peaks corresponding to α,β-unsaturated aldehydes were detected.

Preparation of Metal Ion Free Phosphate Buffer. A slurry of 3.0 mL of hydrated Chelex-100 resin (approximately 200 mg dry weight) in the sodium form was packed into a Pasteur pipet. The resin was washed with 15 mL of 2.5 M HNO_3 to elute any trace metal contamination. Excess acid was removed by washing the resin with 10 mL of water. The resin was transformed to the NH_4^+ form by the addition of 2.0 M NH_4OH (10 mL). The pH of the last few drops eluted was checked with pH paper. Residual NH_4OH was removed from resin with 15 mL of water. The 0.5 M KH_2PO_4 buffer (10 mL) was then allowed to pass through the column, and the effluent was collected.

Decomposition of 13-HPODE in Normal and Metal Ion Free Phosphate Buffer. A solution of 13-HPODE (42 μg, 0.13 μmol) in ethanol (10 μL) was added to phosphate buffer (0.5 M KH_2PO_4, pH 7.0, 190 μL), and incubations were carried out for 24 h with continuous shaking at 37 °C after vortex mixing for 1 min. At the end of the incubation, the samples were cooled in an ice bath and extracted with diethyl ether (200 μL). The reaction was monitored by LC/UV (gradient system 1) and LC/MS with concomitant UV monitoring (gradient system 2) for 24 h. The experiment was repeated in metal ion free phosphate buffer.

Decomposition of 13-HPODE in the Presence of Fe^{II}. An ethanol solution of 13-HPODE (42 μg, 0.13 μmol, 0.65 mM) in ethanol (10 μL) was added together with $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ in water (10 μL) to 180 μL of phosphate buffer (0.5 M KH_2PO_4, pH 7.0). Final Fe^{II} concentrations were in the range of 0−17 mM. The reaction mixture was vortex mixed for 1 min, followed by incubations with continuous shaking at 37 °C. After 30 min, each sample was cooled in an ice bath and extracted with diethyl ether (200 μL). Aliquots (20 μL) were analyzed directly by normal phase LC/UV (226 nm) using gradient system 1.

Preparation of Oxime Derivatives. A solution of 13-HPODE (210 μg, 0.67 μmol, 3.35 mM final concentration) in

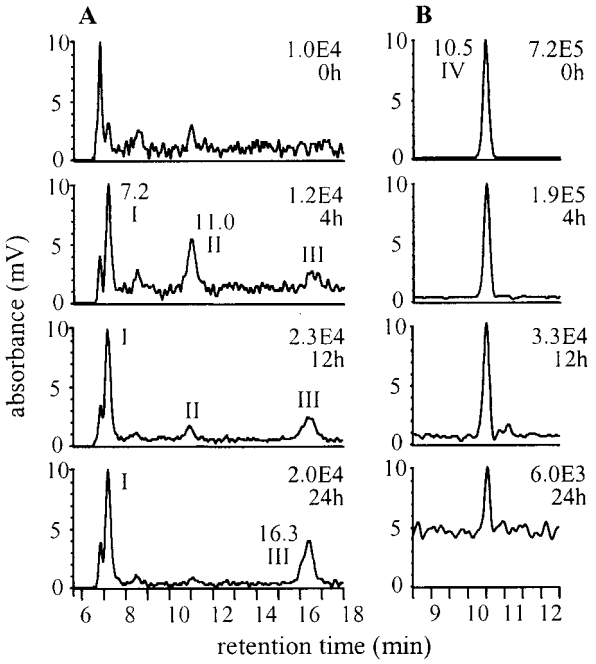


Figure 1. LC/UV analysis of the decomposition of 13-HPODE in normal buffer solution when heated for 24 h at 37 °C. (A) Normal phase analysis of α,β-unsaturated aldehydes using system 1 at 226 nm. (B) Reversed phase analysis of 13-HPODE using system 2 at 234 nm. Peaks: I, 4-oxo-2-nonenal; II, 4-hydroperoxy-2-nonenal; III, 4-hydroxy-2-nonenal; IV, 13-HPODE.

ethanol (10 μL) was added together with $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ (40.1 μg, 0.68 μmol, 3.40 mM final concentration) in water (10 μL) to 180 μL of phosphate buffer (0.5 M KH_2PO_4, pH 7.0). The reaction mixture was allowed to stir for 30 min at 37 °C. Hydroxylamine hydrochloride (2 mg, 28.8 μmol) was added, and the sample was sonicated for 15 min, followed by incubation for an additional 1 h at 37 °C. After cooling to room temperature, the aqueous solution was extracted with diethyl ether (2 × 200 μL). The combined extracts were dried under nitrogen and reconstituted in ethanol (100 μL). LC/MS with concomitant UV monitoring (265 nm) was conducted on a 10 μL aliquot of this solution using gradient system 3 at a flow rate of 1 mL/min.

Results

Decomposition of 13-HPODE in Normal and Metal Ion Free Phosphate Buffer. After incubation of 13-HPODE in normal phosphate buffer (pH 7.0) for 4 h at 37 °C, two major products were observed in the chromatogram at 7.2 and 11.0 min (Figure 1A). Product I with a retention time of 7.2 min coeluted with authentic 4-oxo-2-nonenal. Product II with a retention time 11.0 min had chromatographic properties that resembled those of 4-hydroperoxy-2-nonenal, so it was tentatively identified as such. A minor product (III) was observed at 16.3 min. After heating for 12 h, the signal for 4-hydroperoxy-2-nonenal was reduced in intensity, whereas the signals for 4-oxo-2-nonenal and product III had increased in intensity by a substantial amount (Figure 1A). By 24 h, only 4-oxo-2-nonenal and product III were detected. Product III was subsequently shown to have a retention time identical to that of 4-hydroxy-2-nonenal. Over the 24 h of the experiment, the intensity of the signal derived from 13-HPODE was reduced by more than 99% (Figure 1B). When a similar experiment was performed using Chelex-100-treated phosphate buffer, no α,β-unsaturated aldehydes were formed and the signal from the 13-HPODE remained essentially constant.

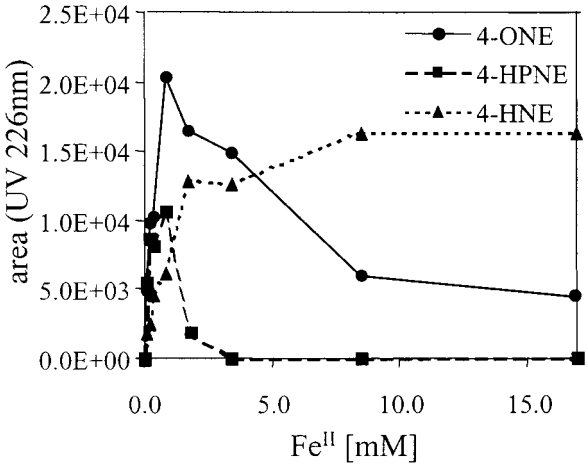


Figure 2. Effect of Fe^{II} concentration on the conversion of 13-HPODE (650 μM) to α,β-unsaturated aldehydes after 30 min at 37 °C.

Decomposition of 13-HPODE in the Presence of Fe^{II}. As the concentration of Fe^{II} was increased with the 13-HPODE held constant, increases in the amounts of 4-oxo-2-nonenal and 4-hydroperoxy-2-nonenal were observed. The amount of these two products was maximal when the Fe^{II} concentration (850 μM) was in slight excess over the 13-HPODE concentration (650 μM). As the concentration of Fe^{II} increased, there was a dramatic decrease in the amount of 4-hydroperoxy-2-nonenal with a concomitant increase in the amount of 4-hydroxy-2-nonenal. At an approximate 2-fold excess, the 4-hydroperoxy-2-nonenal was almost undetectable. There was a much more modest decline in the amount of 4-oxo-2-nonenal until the concentration of Fe^{II} reached a 10-fold excess (8.5 mM). At this point, the amount of 4-oxo-2-nonenal had fallen by approximately 75% compared to that from the reaction conducted with 850 μM Fe^{II}. There was no concomitant increase in the amount of 4-hydroxy-2-nonenal (Figure 2). In separate experiments, 4-oxo-2-nonenal and 4-hydroxy-2-nonenal were shown to be stable at Fe^{II} concentrations of >10 mM at 37 °C.

Characterization of Oxime Derivatives from Fe^{II}-Mediated Decomposition of 13-HPODE. LC/MS analysis of the ether extract from the oxime derivatization suggested that several derivatives had been formed (Figure 3A). Examination of the mass spectra revealed that there were in fact three different oxime derivatives present. The first had an MH^+ ion at *m*/*z* 185, which corresponded to 4-oxo-2-nonenal-bis-oxime; the second had an MH^+ ion at *m*/*z* 188, which corresponded to 4-hydroperoxy-2-nonenal-oxime, and the third had an MH^+ ion at *m*/*z* 172, which corresponded to 4-hydroxy-2-nonenal-oxime.

The selected ion chromatogram of the ion at *m*/*z* 185 revealed the presence of the expected two *syn*- and two *anti*-isomers of 4-oxo-2-nonenal-bis-oxime with retention times of 31.9, 32.3, 33.4 (major isomer), and 34.3 min, respectively (Figure 3B). The relative intensities, retention times, mass spectra, and MS^2 spectra were identical with those of the *syn*- and *anti*-isomers of an authentic standard of 4-oxo-2-nonenal-bis-oxime. The selected ion chromatogram of the ion at *m*/*z* 188 revealed the presence of *syn*- and *anti*-isomers of 4-hydroperoxy-2-nonenal-oxime with retention times of 34.1 and 34.3 min, respectively (Figure 3C). The MS^2 characteristics of these *syn*- and *anti*-oxime derivatives were identical with those

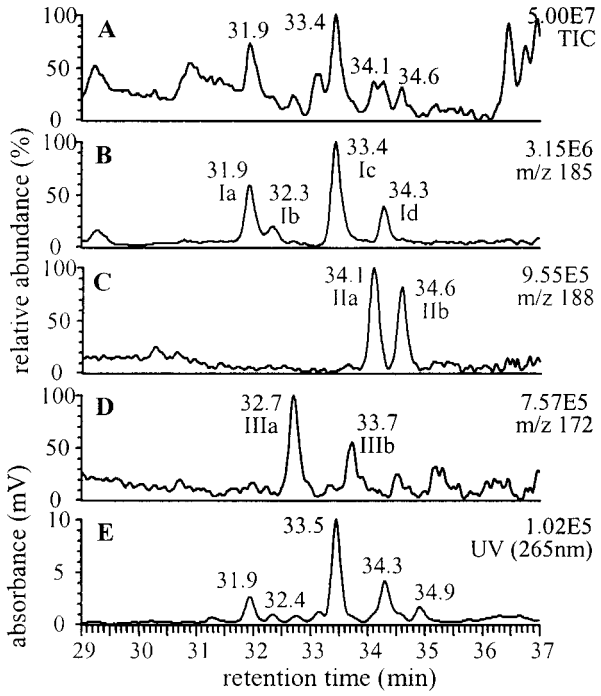


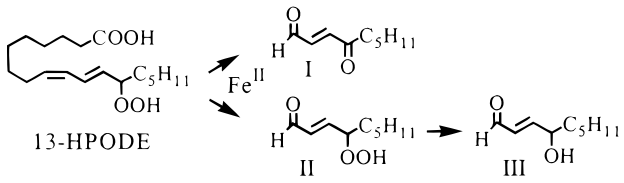
Figure 3. Reversed phase LC analysis of oxime derivatives of α,*β*-unsaturated aldehydes derived from Fe^{II}-mediated decomposition of 13-HPODE using system 3. (A) TIC chromatogram. (B) Selected ion chromatogram for MH^+ (*m*/*z* 185) of *syn*- and *anti*-isomers of 4-oxo-2-nonenal-bis-oxime. (C) Selected ion chromatogram for MH^+ (*m*/*z* 188) of *syn*- and *anti*-oxime derivatives of 4-hydroperoxy-2-nonenal. (D) Selected ion chromatogram for MH^+ (*m*/*z* 172) of *syn*- and *anti*-oxime derivatives of 4-hydroxy-2-nonenal. (E) UV chromatogram (265 nm).

expected for a hydroperoxy compound. Thus, significant product ions were observed at *m*/*z* 171 ($MH^+ - OH$) and 154 ($MH^+ - H_2O_2$) for each isomer. The selected ion chromatogram of the ion at *m*/*z* 172 revealed the presence of the expected *syn*- and *anti*-isomers of 4-hydroxy-2-nonenal-oxime with retention times of 32.7 (major) and 34.3 min (minor), respectively (Figure 3D). The relative intensities, retention times, mass spectra, and MS^2 spectra were identical with those of the *syn*- and *anti*-isomers of an authentic standard of 4-hydroxy-2-nonenal-oxime. The UV chromatogram did not reveal the presence of any other α,*β*-unsaturated aldehydes (Figure 3E).

Discussion

To ensure that 4-oxo-2-nonenal was formed specifically from 13-HPODE, great care was taken to ensure that 9-HPODE and minor contaminants were completely absent. Initial decomposition experiments with pure 13-HPODE were conducted with buffers made from HPLC grade water, which we had previously shown to contain low concentrations of metal ions. When 13-HPODE was heated at 37 °C and pH 7.0, a gradual loss of the starting material was observed by reversed phase HPLC until more than 99% of it had been consumed after 24 h (Figure 1B). There was a concomitant increase in the level of formation of α,*β*-unsaturated aldehydes (Figure 1A). After incubation for 4 h, the least polar α,*β*-unsaturated aldehyde (I) was tentatively identified as 4-oxo-2-nonenal on the basis of its coelution with an authentic standard. Product II was clearly not 4-hydroxy-2-nonenal. However, on the basis of a previous study by Gardener and Grove (*18*), it was tentatively identified

Scheme 1. Formation of 4-Oxo-2-nonenal and 4-Hydroperoxy-2-nonenal from 13-HPODE



as 4-hydroperoxy-2-nonenal. As the incubation progressed, more 4-oxo-2-nonenal was formed and the level of the tentatively identified 4-hydroperoxy-2-nonenal declined. There was a concomitant increase in the level of formation of a compound which had chromatographic properties that were identical with those of 4-hydroxy-2-nonenal (III). To provide unequivocal proof of structure for the α,*β*-unsaturated aldehydes, we required a more facile system for their generation. Therefore, the effect of Fe^{II} concentrations on the decomposition of 13-HPODE was examined. The reaction was much faster as the Fe^{II} concentration was increased. At equimolar concentrations of Fe^{II} and 13-HPODE, a substantial amount of the 13-HPODE was decomposed after only 30 min. Analysis of the product profile at this 30 min time point revealed that a slight excess of Fe^{II} gave optimal yields of all three α,*β*-unsaturated aldehydes (Figure 2). The reaction was then performed on a slightly larger scale, and the products in the aqueous solution were converted to oxime derivatives with hydroxylamine hydrochloride. We reasoned that the more polar oxime derivatives would have improved ESI/MS characteristics when compared with the parent aldehydes and that this would make structural identification easier. The oximes were extracted from the aqueous solution with ether and analyzed by reversed phase LC/ESI/MS with on-line UV monitoring (Figure 3).

Unequivocal LC/MS evidence was obtained for the structures of all three α,*β*-unsaturated aldehydes. Unlike with GC/MS (*18*), it was possible (for the first time) to obtain molecular ion information for the 4-hydroperoxy-2-nonenal-oxime. Both 4-oxo-2-nonenal-bis-oxime and 4-hydroxy-2-nonenal-oxime had identical LC/MS properties when compared with authentic standards. Therefore, we have shown for the first time that both 4-oxo-2-nonenal and 4-hydroperoxy-2-nonenal can be formed from lipid hydroperoxides (Scheme 1). From our data, it is clear that the 4-hydroxy-2-nonenal was formed from 4-hydroperoxy-2-nonenal (Figure 2). This probably explains why we have been unable to detect any DNA adducts derived from 4-hydroxy-2-nonenal when DNA bases were incubated with lipid hydroperoxides (*14*, *15*). The formation of 4-hydroxy-2-nonenal from 4-hydroperoxy-2-nonenal is clearly incompatible with the mechanism that has been proposed previously (*8*). Therefore, additional studies will be required to elucidate the mechanisms by which 4-hydroperoxy-2-nonenal is formed from 13-HPODE. It is possible that 4-hydroperoxy-2-nonenal is also the precursor for 4-oxo-2-nonenal. However, the presence of several oxidation states of Fe that were present in our incubations makes it difficult to delineate possible mechanisms that would account for the formation of 4-oxo-2-nonenal.

In summary, we have provided unequivocal evidence that 13-HPODE, a prototypic $\omega - 6$ lipid hydroperoxide, undergoes Fe-mediated decomposition to form 4-oxo-2-

nonenal. Initially, 4-hydroperoxy-2-nonal is also formed. However, this is converted to 4-hydroxy-2-nonenal in the presence of high concentrations of Fe^{II} or by extended incubations in the presence of low concentrations of Fe^{II}. It is conceivable that some of the 4-hydroperoxy-2-nonenal is also converted to 4-oxo-2-nonenal by a mechanism similar to that proposed by Gardener and Grove (*18*). However, we cannot rule out the possibility that it is also formed by a concerted mechanism from a rearrangement product of 13-HPODE as we have proposed previously (*14*). Studies directed toward resolving this issue are currently in progress.

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