Gamma-Irradiation of 7-Ketocholesterol in Aqueous Dispersions and Liposomes

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7-Ketocholesterol in aqueous sodium stearate dispersions or incorporated into liposomes was exposed to gamma radiation at 0–4°C to a dose of 50 kGy. Substrate degradation was noted in all systems, but occurred to a much greater extent in dispersions (~90%) than in liposomes (0–25%). The difference in degree of degradation is attributed to dissimilarities in the microenvironment of 7-ketocholesterol in the two systems. The principal decomposition product was identified as 7-ketocholestanol, a compound that can arise from hydrogenation of the double bond of the starting material. Also detected, but in lower concentration, were the epimeric 7-hydroxycholesterols resulting from the reduction of the original carbonyl function. Both 7-ketocholestanol and the epimeric 7-hydroxycholesterols are believed to be secondary species generated by the interaction of the starting material with the primary radiolysis products of water. In addition, many minor degradation products were noted but not identified.

KEY WORDS: **Gamma irradiation, 7-ketocholestanol, 7-ketocholesterol, liposomes, radiation-induced degradation, sodium stearate dispersions.**

The susceptibility of cholesterol to oxidative change has been studied in different media for fifty years (1), and prevailing knowledge of cholesterol autoxidation has recently been reviewed (2,3). The principal product of the autoxidation of cholesterol in aqueous sodium stearate dispersion (4), in solvent (5), and in liposomes (6,7) is 7-ketocholesterol (3β-hydroxycholest-5-en-7-one). Additional products include 7α-and 7β-hydroxycholesterol (cholest-5-ene-3 β,7α-diol and cholest-5-ene-3β,7diol, respectively) and the α and β-epoxides (cholesterol 5α, 6α-epoxide and cholesterol 5β, 6β-epoxide), with the total epoxides being formed at about one-tenth of the amount of 7-ketocholesterol.

Additionally, investigators have examined the effect of ionizing radiation on cholesterol in various systems, and have demonstrated that some of the chemical changes induced by irradiation are similar to those occurring in the autoxidation process (8-10).

The work reported here, the irradiation of 7-ketocholesterol, is part of a study of the effect of ionizing radiation on cholesterol. The goal of this study is to determine whether the product distribution of irradiated cholesterol is substantially different from that of autoxidized cholesterol and whether this difference can be employed to identify animal-derived foods that have been irradiated.

Earlier experiments performed in our laboratory (11,12) indicated that the ratio of 7-ketocholesterol to the sum of the two cholesterol epoxides was less than unity after irradiation and between six and ten after autoxidation when both processes were performed on sodium stearate dispersions of cholesterol. Other investigators also reported data indicating that the ratio 7-ketocholesterol/total epoxides in autoxidized cholesterol dispersions is 6-10 (4). Studies of irradiated cholesterol dispersions saturated with N_2O (8) and of irradiated cholesterol-containing liposomes (10) indicated 7-ketocholesterol/total epoxide ratios of approximately 1. Lakritz and Maerker (11) hypothesized that the low 7-keto/total epoxide ratios encountered may be due to an instability of 7-ketocholesterol to irradiation. This work was undertaken to test this hypothesis. We chose to investigate this phenomenon by exposing 7-ketocholesterol to irradiation, both in sodium stearate dispersion and as a component of liposomes at 0-4°C with a dose of 50 kGy. As autoxidation has been shown not to occur to any appreciable extent at this temperature over a 2-week period (11), the data presented here are representative of the effects of irradiation, and other effects are negligible.

EXPERIMENTAL

Reagents. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Sephadex G50-80, Trizma Base, Hepes buffer, and 7-ketocholesterol (5-cholesten-3β-ol-7-one) were purchased from Sigma Chemical Co. (St. Louis, MO), and 7β-hydroxycholesterol (cholest-5-ene-3β, 7β-diol) was acquired from Steraloids, Inc. (Wilton, NH). Research Plus, Inc. (Bayonne, NJ) was the source of 7α-hydroxycholesterol (cholest-5-ene-3β, 7α-diol) and 7-ketocholestanol (5α-cholestan-3β-ol-7-one). Purification of technical grade stearic acid was accomplished by double recrystallization from acetone. Thin-layer chromatography (TLC) plates, silica gel GHL (250 μm) were obtained from Analtech, (Newark, DE). Other chemicals utilized were of reagent grade quality, and solvents were "distilled in glass" grade. Water used was double-deionized, glass distilled.

Cholesterol dispersions. Stearic acid (21.2 mg) which had previously been dissolved in 2 mL ethanol was added dropwise to 22 mL of 2 μM Na_2HPO_4 at 80°C with vigorous stirring. The solution was allowed to cool at room temperature after it had been clarified by the addition of 1 N NaOH (12). The pH was adjusted to 8.0 with 10% H_3PO_4 after which the solution was reheated to 80°C. Then 7-ketocholesterol (2.2 mg in 1 mL ethanol) was added dropwise with stirring. When the dispersion had cooled to room temperature, 2.0 mL aliquots were put into 5 mL glass vials, capped with a Teflon-lined screw cap, and refrigerated at 4°C overnight prior to irradiation.

Liposome preparation and characterization. Large unilamellar vesicles (LUVs) were prepared by the method of Deamer and Bangham (13) and Deamer (14). Phospholipids and 7-ketocholesterol were dispensed from organic stock solutions into a 15 mL test tube with a Teflon-lined screw cap in the proper molar ratio (DPPC:DPPG:7-ketocholesterol, 60:20:20), mixed, and the solvent was evaporated under a stream of N_2. The resulting thin film of lipid on the test tube's inner wall was redissolved in pentane:isopropanol

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(8:2) and vortexed vigorously, yielding a 4 mM solution of lipids. This was injected through a 24 gauge needle from a 10 mL syringe into 5 mL buffer (50 mM Hepes, 145 mM KCl, and 24.8 mM Trizma Base) in the internal chamber of a Liebig condenser. Water, warmed at 65°C, was passed through the jacket of the condenser by a circulating pump. The injection rate was maintained at 0.15 mL/min by use of a syringe pump. Nitrogen was introduced through the top into the internal chamber of the condenser during lipid infusion to prevent oxidation and to enhance evaporation of the organic solvent.

Multilamellar vesicles (MLVs) were prepared using the same proportions and amounts of lipids used above (12 μmoles DPPC, 4 μmoles DPPG, and 4 μmoles 7-ketocholesterol). The stock solutions were dispensed into a 15 mL test tube provided with a Teflon-lined screw cap and mixed thoroughly. Solvent was removed under nitrogen while the tube was swirled to distribute the dried lipids evenly over the inner surface. The lipids were hydrated with 10 mL Hepes buffer (prepared as above) and allowed to swell for 2 hr in a water bath at 50°C with vortexing every 30 min.

Following preparation, vesicles (LUVs and MLVs) were filtered through 0.4 μm pore polycarbonate membranes to ensure a rather uniform size distribution (15). Subsequently, each batch was chromatographed on a 25 cm × 1.6 cm column of Sephadex G50-80 using Hepes buffer. Vesicles were collected as they eluted, the batch was mixed to ensure homogeneity, and samples (2 or 3 mL) were put into 5 mL glass vials capped with Teflon-lined screw caps and refrigerated overnight prior to irradiation.

Phospholipid content of vesicles was determined by measuring inorganic phosphorus according to the procedure of Eng and Noble (16). Quantification of liposome 7-ketocholesterol to determine initial lipid composition of vesicles was accomplished by gas chromatographic analysis of control (unirradiated) samples.

Gamma radiation. Dispersions or vesicles containing 7-ketocholesterol were irradiated using a ^{137}Cs source (0.12 kGy/min) at 0-4°C to a dose level of 50 kGy. Controls consisted of unirradiated refrigerated (0-4°C) samples. Samples and controls were extracted immediately after irradiation or frozen overnight and extracted the following day.

Extraction. Samples were extracted three times with equal volumes of ethyl acetate. Combined extracts were dried over anhydrous sodium sulfate, transferred to 2 mL vials, and evaporated to dryness under nitrogen. Aqueous layers were saved for subsequent inorganic phosphorus analysis.

Samples were reconstituted in 200 μL ethyl acetate, and an aliquot (5-10 μL) of each was applied to one-quarter of a scored 20 × 20 cm silica gel GHL TLC plate (prewashed with chloroform: methanol, 2:1, and activated at 110°C) for visualization of the oxidation products of cholesterol. Another portion (20-30 μL) was spotted onto an adjacent quarter of the plate. The TLC plates were developed with benzene:ethyl acetate:acetic acid (60:40:1), and the scored sections intended for visualization were sprayed with 50% sulfuric acid to locate the cholesterol oxides present. The area between the origin and 5 mm above 7-ketocholesterol was scraped from the corresponding section for analysis and extracted three times with 5 mL ethyl acetate. After each extraction, samples were centrifuged to pellet the silica gel, and the three extracts were combined. The samples were evaporated under nitrogen and reconstituted in 200-400 μL ethyl acetate prior to gas chromatography.

Gas chromatography (GC). Samples were analyzed by GC as described previously (17) with the following modifications. All work was performed on a Varian 3600 gas chromatograph equipped with an on-column injector and a flame ionization detector held at 325°C. The injector was programmed from 60°C (hold 30 sec) to 250°C (30°C/min) (hold at 250°C for 10 min). The column used was a Hewlett-Packard Ultra 2 performance column of crosslinked 5% phenyl methyl silicone (25 m × 0.2 mm × 0.33 μm film thickness). The column was programmed from 100°C (hold 3 min) to 265°C (30°C/min) to 285°C (0.6°C/min) to 300°C (30°C/min) (hold for 25 min). Quantitation was accomplished by the use of 7-ketocholesterol as an external standard.

Gas chromatography/mass spectrometry (GC/MS). Mass spectrometry was accomplished by use of a Varian model 3400 gas chromatograph interfaced directly with a Finnigan MAT 8230 high resolution magnetic sector mass spectrometer. Data were processed with a Finnigan MAT SS300 data system. Chromatography was performed using a J&W 30 m × 0.32 mm i.d. DB-5 column with 0.25 μm film thickness. Injection temperature was 260°C and the column was programmed from 100°C (hold 3 min) to 250°C (20°C/min), then to 320°C (2°C/min), holding there for 15 min. The mass spectral library used was that of the National Bureau of Standards. GC/MS was performed by the Center for Advanced Food Technology, Cook College, Rutgers University, New Brunswick, NJ.

RESULTS AND DISCUSSION

7-Ketocholesterol in aqueous sodium stearate dispersions or incorporated in liposomes (LUVs or MLVs) was irradiated at 0-4°C to a dose of 50 kGy. The extent to which irradiation caused substrate degradation clearly depended on the environment of the 7-ketone. In dispersions about 90% of the starting material was converted to other products, presumably by the radiolysis products of water (Table 1). However, in liposomes 7-ketocholesterol appeared considerably more stable, so that the difference in 7-ketocholesterol content of irradiated and unirradiated liposomes was statistically not significant (except in one experiment) (Table 2). Minor amounts of degradation products indicated, however, that even in liposomes some destruction of 7-ketocholesterol occurred during irradiation.

TABLE 1

Irradiation of 7-Ketocholesterol in Aqueous Dispersions

	Irradiated (n = 3)	Control (n = 3)
μg 7-Ketocholesterol ($\bar{x}$ ± SD)	12.4 ± 1.9	147.9 ± 18.8
μg 7-Ketocholestanol ($\bar{x}$ ± SD)	20.6 ± 4.2	N.D.[a]
μg 7β-Hydroxycholesterol($\bar{x}$ ± SD)	8.6 ± 1.7	1.0 ± 1.0
μg 7α-Hydroxycholesterol ($\bar{x}$ ± SD)	3.1 ± 0.6	N.D.

[a]N.D. = not detected.

The degradation of 7-ketocholesterol as a result of exposure to γ-irradiation in aqueous media was recognizable by the appearance of new peaks on the GC trace as well as by the disappearance of starting material when irradiated samples

TABLE 2

Irradiation of 7-Ketocholesterol in Liposomes

	LUVs[a]		LUVs[b]		MLVs[c]	
	Irrad.	Ctrl.	Irrad.	Ctrl. (n = 3)	Irrad.	Ctrl. (n = 3)
μg 7-Ketocholesterol ($\bar{x} \pm$ SD)	24.8 ± 0.3	27.7 ± 1.8	106.6 ± 4.1	141.4 ± 4.0	139.9 ± 1.7	137.6 ± 15.1
μg 7-Ketocholestanol ($\bar{x} \pm$ SD)	1.0 ± 0.5	N.D.[d]	2.3 ± 0.5	N.D.	1.2 ± 0.4	N.D.
μg 7β-Hydroxycholesterol ($\bar{x} \pm$ SD)	N.D.	N.D.	0.1 ± 0.2	0.3 ± 0.3	0.4 ± 0.3	0.2 ± 0.2
μg 7α-Hydroxycholesterol ($\bar{x} \pm$ SD)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a]7.5 Mol% 7-ketocholesterol.
[b]25 Mol% 7-ketocholesterol.
[c]22 Mol% 7-ketocholesterol.
[d]N.D.—not detected.

and unirradiated controls were compared. No effort was made to isolate and identify the majority of the degradation products. Some of these were eliminated from consideration by the TLC procedure employed in this study, which only isolated 7-ketocholesterol and those products that were as polar or more polar than the 7-ketone.

Among the degradation products observed on the GC traces of irradiated 7-ketocholesterol, one was particularly prominent. Its retention time was slightly less than that of 7-ketocholesterol and roughly the same as 6-ketocholestanol, a compound that is sometimes used as internal standard in analysis of cholesterol oxides (17). Analysis by GC/MS and by cochromatography with an authentic standard established this compound to be 7-ketocholestanol. The amount of 7-ketocholestanol formed from 7-ketocholesterol was about 14% in dispersions (Table 1) and 1-4% in liposomes (Table 2).

Other degradation products of 7-ketocholesterol found, though in lesser amounts, were the isomeric 7-hydroxycholesterols. One of the isomers, 7β-hydroxycholesterol, was present as an impurity in the starting material and was detected in some instances at low levels (<1%) in the unirradiated controls. Both 7α- and 7β-hydrocholesterol were generated in irradiated dispersions of 7-ketocholesterol (Table 1). The β-isomer was found at 2-3 times the concentration of the α-isomer, a fact that is satisfactorily explained by the greater thermodynamic stability of the quasiequatorial β-isomer (8). Conceivably, the two 7-hydroxycholesterol epimers were also formed in the irradiation of liposomes containing 7-ketocholesterol, but because of the low level of degradation of the starting material the 7-hydroxy derivatives were not detected by the current methodology.

Kučera *et al.* (9) irradiated water/ethyl acetate emulsions of cholesterol at dose levels of 13-120 kGy. Among the irradiation products that they identified were 7-ketocholesterol, 7-ketocholestanol and 7β-hydroxycholesterol, but strangely 7α-hydroxycholesterol was not reported. These authors plotted concentration of products versus dose and reported that 7-ketocholesterol concentration increased rapidly between 0 and 5 kGy and then decreased gradually between 5 and 12 kGy. Meanwhile, 7-ketocholestanol increased gradually over the dose range. These data are consistent with the formation of 7-ketocholestanol from 7-ketocholesterol found in their work as well as in ours.

Among the primary radiolysis products of water are the hydrated electron (e^-_{aq}), and both the hydrogen radical (H ·) and molecular hydrogen (H_2) (18). In the current work, which was carried out in an aqueous medium, the formation of 7-ketocholestanol from 7-ketocholesterol appears to occur by hydrogenation of the double bond, while 7-hydroxycholesterol is formed from the same precursor by reduction of the 7-ketone. Radiation-induced hydrogenation of lecithin has been discussed by Coleby (19) and has been proposed for 7-ketocholesterol by Kučera *et al.* (9). The hydrated electron is an extremely strong reducing agent (20) and may well be the active species in the reduction of the 7-ketone.

Previous studies of the effects of ionizing radiation on cholesterol in dispersions or liposomes indicated yields of cholesterol oxides on the order of 1% or less of the cholesterol treated (8,10,11). The current work on the irradiation of 7-ketocholesterol gives evidence that in aqueous dispersions about 90% of the original material is degraded while in liposomes less than 10% is destroyed. These data suggest that 7-ketocholesterol is more susceptible than cholesterol to the (mostly secondary) effect of ionizing radiation. This may be due to the presence in 7-ketocholesterol of an α, β-unsaturated carbonyl functionality which increases its reactivity to at least some of the energetic species produced in the radiolysis of water (18). The data also suggest that 7-ketocholesterol, and perhaps to a lesser extent also cholesterol, finds itself in a distinctly different microenvironment in the predominantly micellar structure of dispersions than in the lipid bilayer surroundings of liposomes.

It is generally assumed that relatively nonpolar molecules such as cholesterol or 7-ketocholesterol insert into fatty acid micelles and into bilayer vesicles in a similar manner. In the stearate micelles utilized in the current work, the expectation is that the hydrophobic side chain of the sterols, and perhaps their C and D rings, are buried in the hydrophobic interior of the micelle, while the A and B rings tend to be located to some extent in the interfacial region. Likewise, in liposomes it is expected that the sterol molecules are packed between the phospholipid molecules in a chain-extended conformation. This causes the A and B rings to be near the polar head groups of the lamellae while the side chains of the sterols are buried in the hydrocarbon-like interior of the bilayers. Recently, however, Shobha *et al.* (21) have carried out experiments that demonstrate that certain probe molecules are packed differently in micelles than they are in bilayer vesicles. The authors, and other investigators cited by them, present convincing evidence that in small micelles having large surface-area-to-volume ratios these probes loop and curl along the micelle/water interface, making them more accessible to reagents in the aqueous phase. With increasing micellar size (lower surface-area-to-volume ratios),

and in lamellar vesicles, the probe molecules unbend, become inserted in the expected straight conformation and become less accessible to bulk-phase reagents. An example of this phenomenon quoted by Shobha *et al.* (21) was the reduction of testosterone with sodium borohydride contained in the bulk aqueous phase. With testosterone in small spherical micelles, the reaction rate was 0.0167 $M^{-1}sec^{-1}$, in larger, rod-like micelles it was 0.0067 $M^{-1}sec^{-1}$, and solubilized in liposomes, it did not react. A similar surface effect may be responsible for the difference in the reactivity of 7-ketocholesterol to the radiolysis products of water in the current work. In the small stearate micelles used here in dispersions, the 7-ketocholesterol is apparently rather accessible to the bulk phase reagents, i.e., the radiolysis products of water. On the other hand, the greatly reduced reactivity of 7-ketocholesterol in either LUVs or MLVs is then explainable by a decreased exposure to aqueous radiolysis products.

In earlier work in this laboratory (11) it was determined qualitatively that in sodium stearate dispersions 7-ketocholesterol, as well as the two epoxides, were degraded by radiation. The order of susceptibility to radiolysis was 7-ketocholesterol > β-epoxideα > α-epoxide, the 7-ketone/total epoxide ratio of the isolated product was less than one, and the apparent conversion of cholesterol to these three products was about 0.2%. The current studies show that 7-ketocholesterol, and by implication the two epoxides, are more shielded from radiolysis reactions in liposomes than they are in dispersions. This should result in higher apparent conversions to these three products when cholesterol in liposomes is irradiated. Preliminary work in our laboratory supports this projection.

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