Gamma-Irradiation of Individual Cholesterol Oxidation Products

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Cholesterol and seven of its oxidation products in aqueous suspensions of multilamellar vesicles or sonicated aqueous suspensions were subjected individually to γ-radiation (10 KGy) at 0–4°C in air, N_2 or N_2O. All compounds underwent some changes under the influence of radiation. β-Epoxide (cholesterol 5β,6β-epoxide) and, to a much lesser extent, α-epoxide (cholesterol 5α,6α-epoxide) were converted in low yield to 6-ketocholestanol (5α-cholestan-3β-ol-6-one). 7β-Hydroxycholesterol (cholest-5-ene-3β,7β-diol) and, to a lesser extent, 7α-hydroxycholesterol (cholest-5-ene-3β,7α-diol) gave low yields of 7-ketocholestanol (5α-cholestan-3β-ol-7-one). The latter compound also was obtained by irradiation of 7-ketocholesterol (cholest-5-ene-3β-ol-7-one). 6-Ketocholestanol and 7-ketocholestanol are potential biomarkers for irradiated meat and poultry.

KEY WORDS: Cholesterol, cholesterol oxides, irradiation, 6-ketocholestanol, 7-ketocholestanol, vesicles.

Under the influence of oxidizing conditions, cholesterol gives rise to a substantial number of products collectively known as "cholesterol oxides" (1). The principal product of the autoxidation of cholesterol in aqueous sodium stearate dispersions (2), in organic solvents (3) and in liposomes (4,5) is 7-ketocholesterol. (For the correct chemical names of the cholesterol oxides discussed in this paper see Table 1). Other prominent products of this autoxidation reaction include the anchimeric 7α- and 7β-hydroxycholesterols and the α- and β-epoxides.

Cholesterol oxides also are formed when cholesterol in various aqueous systems is exposed to ionizing radiation (6–9). The nature of these products is similar to those formed by autoxidation (10,11), but the relative amounts formed by the two processes are substantially different (12). Specifically, the ratio of 7-ketocholesterol to total epoxides generated by irradiation is generally less than unity, whereas the ratio of the same products formed in autoxidation is greater than 10.

An explanation for this difference in ratios may be that the same products are apparently formed by different reaction paths in the two processes. It is generally acknowledged that in solutions or dispersions the first relatively stable derivatives of cholesterol under autoxidizing conditions are the anchimeric 7-hydroperoxycholesterols (11), and that the 7-keto and 7-hydroxy products are derived from the 7-hydroperoxycholesterols intramolecularly and the 5,6-epoxides intermolecularly. By contrast, 7-hydroperoxycholesterol intermediates do not seem to be involved in the formation of cholesterol oxides from irradiated cholesterol in aqueous systems, at least not in the absence of oxygen (6). Radiolysis of water gives rise to three primary radicals, OH radical, hydrated electron e^-_{aq} and H-atom (13), which presumably may interact further with cholesterol in the aqueous phase to give the observed products.

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An alternative reason, or perhaps a supplementary one, for the observed difference in product ratios in autoxidized cholesterol *vs.* irradiated cholesterol may be differences in radiolytic stability of the various products formed. The susceptibility of 7-ketocholesterol to further radiolysis has been examined (14), and its radiolytic degradation has been found to be dependent on its microenvironment. To our knowledge, the radiolytic stability of other individual cholesterol oxides has not been tested.

In our continuing efforts to find methods that distinguish meat and poultry that have been irradiated from those that have not, this work was undertaken to determine whether the unusual radiolytic product ratios of cholesterol may be due to the lability of individual products to the effects of γ-radiation. An additional goal was to discover whether the radiolysis products of cholesterol react further to generate compounds that might be useful as biomarkers in irradiated foods.

EXPERIMENTAL PROCEDURES

Materials and reagents. Cholesterol, 6-ketocholestanol, α-epoxide and Sephadex G50-80 were purchased from Sigma Chemical Co. (St. Louis, MO), β-epoxide and 7α-hydroxycholesterol from Research Plus, Inc. (Bayonne, NJ), and 7β-hydroxycholesterol, triol and desmosterol from Steraloids, Inc. (Wilton, NH). Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Celite 545 was purchased from Fisher Scientific (Malvern, PA). All solvents used were "distilled in glass grade", and chemicals were of reagent grade. Nitrous oxide (N_2O) was purchased from Scott Specialty Gases (Plumbsteadville, PA). Water was double-deionized, glass-distilled (ddI).

Lipid film preparation. A lipid film was prepared from 44.0 mg (60 μmol) DPPC, 14.5 mg (20 μmol) DPPG and 40 μmol of cholesterol or a cholesterol oxide. The phospholipids and cholesterol oxide were dissolved, with vigorous agitation in chloroform (1 mL) in a 15-mL screw top test tube. Solvent was removed under N_2 while the tube was rotated at an angle so as to evenly coat the lipids on the lower half of the tube wall. Nitrogen (N_2) purge was continued until the odor of chloroform was no longer detectable.

Preparation of N_2- and N_2/N_2O-sparged water. Water was allowed to boil for 30 min with vigorous N_2 sparging. The water was allowed to cool to room temperature with continued N_2 sparging. N_2-sparged water was sparged vigorously with N_2O at room temperature for 30 min.

Preparation of liposomes. Water (5.0 mL) saturated with air, N_2 or N_2/N_2O was added to the lipid film prepared as described above. The capped tube was placed in a water bath held at 55°C for 3 hr to permit formation of multilamellar vesicles (MLV). The tube was removed for vigorous agitation every 15 min during swelling. Additional water (5 mL) was added to the liposome preparation, and the suspension was agitated to insure homogeneity. Suspensions prepared with N_2/N_2O-sparged

water were further sparged with N_2O for 10 min at room temperature. Aliquots (1 mL) of all suspensions were placed into glass vials (1.8-mL) provided with Teflon-lined screw caps, and those prepared with N_2 or N_2/N_2O were blanketed with N_2 or N_2O. Samples were refrigerated (0–4°C) overnight prior to irradiation.

Preparation of chromatographed liposomes. Lipid films were hydrated with air-saturated water by swelling at 55°C as described above. After swelling, the liposome preparation was allowed to cool to room temperature and was filtered through a 0.4-μm-pore polycarbonate membrane to ensure rather uniform size distribution (15). The suspension was chromatographed on a 26 cm × 1.2 cm column of Sephadex G50-80 with double-deionized water as eluent. A 10-mL fraction containing the vesicles was collected, agitated, and divided into 1-mL aliquots as described above.

Preparation of epoxide dispersions. The method of liposome preparation described above was not suitable for cholesterol epoxides. Instead, water (10 mL) saturated with air, N_2 or N_2/N_2O was added to the lipid film preparation. The tube containing this suspension, with screw cap on, was sonicated at room temperature for two 15-min periods. The suspension was agitated before, between and after the sonication periods. Before division into aliquots, N_2O was bubbled through the suspension prepared with N_2/N_2O saturated water. Aliquots (1 mL) of the dispersions were placed into glass vials (1.8 mL) provided with Teflon-lined screw caps. Individual samples prepared with N_2 and N_2/N_2O were blanketed with the respective gases before the vials were closed.

Extraction and isolation of cholesterol oxides. Cholesterol oxides were extracted from 1-mL liposome preparations or from 1-mL dispersions by the dry column method of Maerker and Jones (12). Each eluate was evaporated to dryness with N_2, and the residue was reconstituted in 10.0 mL methylene chloride. The internal standard desmosterol (20 μL, 1 μg/μL in ethyl acetate) was added to each aliquot (1.0 mL) and the solvent was removed with N_2. The sample was reconstituted in 500 μL ethyl acetate for gas chromatographic (GC) analysis.

Irradiation of cholesterol oxides in liposome suspensions or in dispersions. Aliquots (1 mL) of MLV suspensions or of sonicated dispersions were irradiated in a ^{137}Cs source (0.118 KGy/min) at 0–4°C to a dose of 10 KGy. Controls consisted of unirradiated, refrigerated (0–4°C) samples. Controls and irradiated samples were extracted on the day of irradiation.

Gas chromatography (GC). Samples were analyzed by GC without prior derivatization as described previously for cholesterol oxides (12). Response factors of cholesterol and of seven cholesterol oxides relative to the internal standard desmosterol were determined by the method of Lee *et al.* (16). Gas chromatography/mass spectrometry (GC/MS) analyses were conducted with a Varian 3400 gas chromatograph (Varian Associates, Palo Alto, CA) directly interfaced to a Finnigan MAT (San Jose, CA) 8230 high resolution magnetic sector mass spectrometer. Data were acquired and processed with a Finnigan MAT SS300 data system. Computerized library searches of mass spectra were conducted with the NIST mass spectra data base.

Aliquots (0.5 μL) of the sterol radiolysis products and reference standards were injected into the GC/MS under the following conditions. The GC was equipped with a 30 meter × 0.32 mm i.d. DB-5 capillary column containing a 0.25 μm film thickness (J&W Scientific, Folsom, CA). The injector temperature was 250°C and on-column injection technique was utilized. The column was temperature programmed from 100°C (hold 2 min) to 265°C (hold 0 min) at a rate of 20°C per min; then, it was programmed to 275°C (hold 5 min) at a rate of 0.5°C per min; finally, it was programmed to 300°C (hold 15 min) at a rate of 5°C per min. The mass spectrometer was operated in electron ionization mode scanning masses 50–550 once each second. GC/MS was performed by the Center for Advanced Food Technology, Cook College, Rutgers University (New Brunswick, NJ).

RESULTS AND DISCUSSION

The seven cholesterol oxides that were examined in this study along with the cholesterol are listed in Table 1. Initial exploratory experiments indicated that irradiation of the β-epoxide caused the formation of 6-ketocholestanol as one of the products. The 6-keto derivative, which is not a cholesterol autoxidation product (1), previously had been used as an internal standard by us and by others (17–19), but in this study it presented analytical difficulties. In its place, a plant sterol that can be purchased in suitable purity, desmosterol, was chosen as an appropriate

TABLE 1

Sterols Examined

Chemical name	Trivial name	Purity	Relative[a] retention time	Response[a] factor
Cholest-5-ene-3β-ol	Cholesterol	93.2	0.95	0.97
Cholesta-5,24-dien-3β-ol	Desmosterol	99.5+	1.0	1.0
Cholesterol 5β,6β-epoxide	β-Epoxide	97.5	1.12	1.09
Cholesterol 5α,6α-epoxide	α-Epoxide	99.4	1.15	1.05
Cholest-5-ene-3β,7α-diol	7α-Hydroxycholesterol	97.5	1.22	1.17
Cholest-5-ene-3β,7β-diol	7β-Hydroxycholesterol	98.6	1.24	1.14
5α-Cholestan-3β-ol-7-one	7-Ketocholestanol	99.5+	1.29	1.08
5α-Cholestan-3β-ol-6-one	6-Ketocholestanol	98.9	1.34	1.10
Cholest-5-ene-3β-ol-7-one	7-Ketocholesterol	99.5+	1.38	1.11
5α-Cholestan-3β,5,6β-triol	Triol	97.3	1.64	1.36

[a]Relative to desmosterol.

alternative internal standard. Retention times and response factors of the various sterols relative to desmosterol are shown in Table 1.

To provide an experimental environment approaching that of biological membranes, the test compounds were incorporated in liposomes (multilamellar vesicles) with dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG). The molar ratio of DPPC/DPPG/cholesterol oxide was 3:1:2. Molecular sieve chromatography (20,21), in which liposomes are eluted and nonliposomal particles are retained on the column, was not used, because it was desired to examine all sterols in the suspensions. Comparison of a liposomal suspension, prepared with 7β-hydroxycholesterol and tested for 7β-hydroxycholesterol content before and after molecular sieve chromatography, indicated that about two-thirds of that cholesterol had been incorporated in the MLV.

Formation of MLV involved swelling for three hours above the transition temperature (T_c), which is 41°C for both phospholipids (22). Both epoxides proved to be unstable to the swelling conditions and formed triol by hydrolysis. The β-epoxide was affected more severely than the α-epoxide, as was expected from previous studies (18). To minimize this problem, each of the two epoxides and the two phospholipids were sonicated in the presence of water at room temperature twice for 15 min to generate suspensions, the nature of which was not determined. The epoxides did not hydrolyze substantially under these conditions and the suspensions appeared stable during the irradiation procedure.

Cholesterol and seven of its oxides were irradiated in aqueous suspensions saturated with air under a headspace filled with air. The results are shown in Table 2. All of the irradiated samples showed loss of starting material compared to the unirradiated controls, but in most instances the losses were only partly accountable by identifiable products. The fate of the balance of the starting material is not known. The isolation/quantitation procedure was rather uncomplicated and essentially involved only two steps—dry column extraction of the aqueous suspension and GC analysis of an aliquot of the dry column eluate. The missing materials must be retained in either or both of these steps, because exhaustive elution of the dry column gave no evidence of additional products and GC traces, in most cases, contained few minor peaks.

Previous studies (14) demonstrated that 7-ketocholesterol, in aqueous sodium stearate suspensions as well as in liposomes, was subject to radiolytic degradation. For purposes of comparison that compound was included in the current study, and the formation of 7-ketocholestanol (5α-cholestan-7-one) as the principal radiolytic product of 7-ketocholesterol was confirmed.

The two epoxides gave rise to substantial amounts of triol when they were irradiated, the β-epoxide forming more triol than the α-isomer (Table 2). The degradation of the epoxides to triol is attributed to a decrease in the pH of the irradiated suspensions rather than to a direct radiolytic effect. Comparison of the pH of the unirradiated controls and the irradiated samples showed that the pH decreased from 6.5–7.0 for the controls to 2.5–3.0 for the irradiated samples. This decrease in pH may be caused by the radiolysis of phospholipids to phosphatidic acids and other products (P.W. Tinsley, private communication) and is being investigated further. The lability of the epoxides to acid aqueous systems is well known (18,23).

6-Ketocholestanol was found to be a direct radiolysis product of the β-epoxide and, to a much lesser extent, the α-epoxide (Table 2). 6-Ketocholestanol has been reported once to be present in pigs' spleen (24), and its presence in trace amounts in some processed meats has been mentioned (25), but neither of these reports has been confirmed by other workers. The compound is not otherwise known to occur in cholesterol-containing foods. The identity of the 6-ketocholestanol found in this study has been confirmed by GC/MS and by GC retention time (spiking). A plausible reaction path from the β-epoxide to 6-ketocholestanol is shown in Scheme 1.

Irradiation of the isomeric 7-hydroxycholesterols causes the formation of 7-ketocholesterol, as well as 7-ketocholestanol (Table 2). The generation of the latter by irradiation of 7-ketocholesterol (14) was mentioned earlier. Its production from irradiated 7-hydroxycholesterols, as shown here, thus constitutes a second source for this compound. To our knowledge, the presence of 7-ketocholestanol in

TABLE 2

Irradiation[a] of Cholesterol Oxides in MLV Under Air

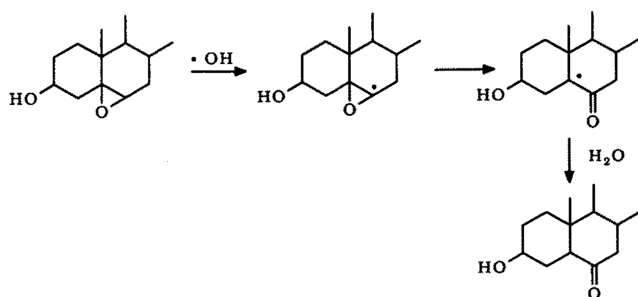
Compound irradiated	Loss of starting material (percent)	Radiolysis product 1: Name	Radiolysis product 1: Conversion of starting material (percent)	Radiolysis product 2: Name	Radiolysis product 2: Conversion of starting material (percent)
Cholesterol	14.1[b]	6-Ketocholestanol	1.8	7-Ketocholestanol	0.95
β-Epoxide	26.4	Triol[c]	16.0	6-Ketocholestanol	8.1
α-Epoxide	26.8	Triol[c]	7.5	6-Ketocholestanol[d]	trace
7β-Hydroxycholesterol	35.4	7-Ketocholesterol	14.2	7-Ketocholestanol	1.5
7α-Hydroxycholesterol	17.7	7-Ketocholesterol	3.0	7-Ketocholestanol	trace
6-Ketocholestanol	14.8	Unknown RRT (1.40)	3.2		
7-Ketocholesterol	26.4	7-Ketocholestanol	3.9		
Triol	19.1	Unknown RRT (1.35)	3.1		

[a]Dose, 10 KGy; dose rate, 0.118 KGy/min; and temperature, 0–4°C.

[b]Principal radiolysis products include 7-ketocholesterol, 7α- and 7β-hydroxycholesterl, and α- and β-epoxide.

[c]This product is believed to be formed by acid-catalyzed hydrolysis rather than by radiolysis (see Discussion).

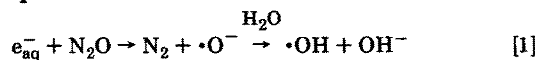
[d]Tentative identification.



SCHEME 1

foods has not been reported, and it is not formed in the autoxidation of cholesterol.

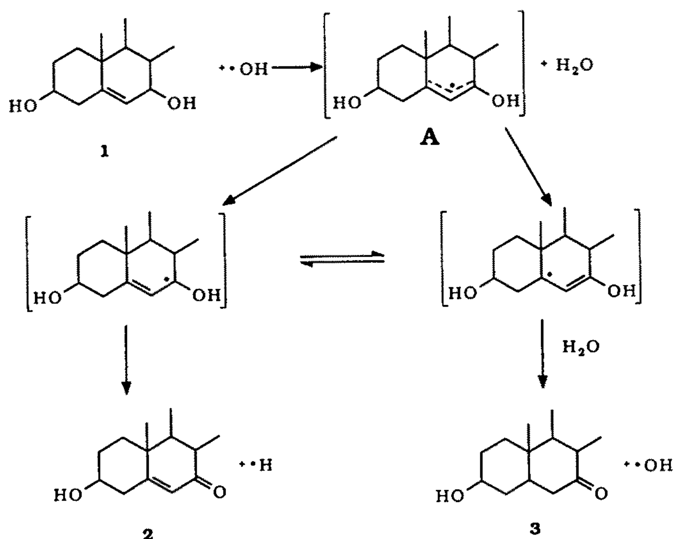
In the predominantly aqueous system under study here, the primary effect of impacting γ-radiation is the radiolysis of water. The radiation chemical yields (G-values) of the three principal species are OH radical, 2.7; e^-_{aq}, 2.7; and H atom, 0.5 (13). These yields are essentially independent of pH above 3 (26). Hydroxy radical is a strongly oxidizing species that has a strong affinity for carbon-bound hydrogen atoms and a much lower affinity for oxygen-bound hydrogen (13). The hydrated electron e^-_{aq} reacts with N_2O to form hydroxy radical (26) according to the following equation:



$$e^-_{aq} + N_2O \rightarrow N_2 + \cdot O^- \xrightarrow{H_2O} \cdot OH + OH^- \quad [1]$$

Accordingly, saturation of aqueous liposome preparations with N_2O leads to an increase in the amount of OH radical formed during irradiation, as well as an increase in pH.

Scheme 2 depicts a plausible pathway for the formation of 7-ketocholesterol and 7-ketocholestanol in the radiolysis of 7β-hydroxycholesterol (2, 3 and 1, respectively, in Scheme 2). In this view, abstraction of allylic hydrogen at the 7α-position of 1 leads to the intermediate radical A from which 2 and 3 are generated. Apparently, the



SCHEME 2

7β-hydrogen of 7α-hydroxycholesterol is much less susceptible to abstraction than the corresponding 7α-hydrogen of 7β-hydroxycholesterol because 7α-hydroxycholesterol forms much less 7-ketocholesterol and only trace amounts of 7-ketocholestanol.

The radiolysis of 6-ketocholestanol and triol also has been examined. Both compounds are secondary products formed from primary products (epoxides) but have been included here for completeness. On exposure to γ-radiation both secondary products form small amounts of products that have not yet been identified.

The irradiation of cholesterol under the conditions of these experiments gave rise to the same principal products, 5,6-epoxides, 7-hydroxycholesterols and 7-ketocholesterol, reported previously (12). In addition, as shown in Table 2, 6-ketocholestanol and 7-ketocholestanol were identified among the products.

Four of the cholesterol oxides also were irradiated in aqueous suspensions that had been saturated with nitrogen or with nitrous oxide. The results are shown in Table 3, where experiments carried out under air have been included for comparison. Use of a nitrogen atmosphere did not affect the results greatly as compared to air, demonstrating that air oxidation does not play a significant role in determining the composition of the irradiated samples. Use of an N_2O atmosphere reduced radiolysis of the compounds tested and minimized the formation of triol from epoxides. This is in accordance with expectations, because the hydroxide anion formed in the reaction of e^-_{aq} with N_2O (see Eq. [1]) is likely to neutralize (at least partly) the acid radiolysis products. Indeed, irradiated samples saturated with N_2O had a higher pH than those saturated with air or N_2. For β-epoxide, the pH of the irradiated samples saturated with N_2O was 5.5, while the corresponding samples saturated with N_2 had a pH of 3.0.

The results of this study show that the product ratios observed in the radiolysis of cholesterol (12) are unlikely to be caused by selective reactivity of individual members. The principal radiolysis products of cholesterol, 7-ketocholesterol and α- and β-epoxide, react to about the same extent to further radiation exposure and, hence, their ratio is not affected.

On the other hand, examination of the effect of γ-radiation on individual cholesterol oxides has revealed the formation of two compounds, 6-ketocholestanol and 7-ketocholestanol, that are not now known to be present in foods. If one or both of these compounds could be demonstrated to be present in irradiated meat and poultry and absent in unirradiated foods, their presence might serve as evidence of past irradiation. We have begun to address this problem in our laboratory. Meanwhile, we are attempting to establish the identity of additional secondary radiolysis products of cholesterol.

REFERENCES

1. Smith, L.L., in *Cholesterol Autoxidation*, Plenum Press, New York, 1981.
2. Kimura, M., Y. Jin and T. Sawaya, *Chem. Pharm. Bull. (Tokyo) 27*:710 (1979).
3. Muto, T., K. Sugino and S. Matsushita, *Ibid. 30*:3172 (1982).
4. Terao, J., K. Sugino and S. Matsushita, *J. Nutr. Sci. Vitaminol. 31*:499 (1985).
5. Lijana, R.C., M.S. McCracken and C.J. Rudolph, *Biochim. Biophys. Acta 879*:247 (1986).

TABLE 3

Irradiation[a] of Selected Cholesterol Oxides Under Air, N_2 and N_2O

Compound irradiated	Air			Nitrogen			Nitrous oxide		
	Loss of starting material	Principal product	Conversion	Loss of starting material	Principal product	Conversion	Loss of starting material	Principal product	Conversion
7-Ketocholesterol	26.4	7-Ketocholestanol	3.9	19.1	7-Ketocholestanol	3.9	14.4	7-Ketocholestanol	2.3
7-β-Hydroxycholesterol	35.4	7-Ketocholesterol	14.2	35.3	7-Ketocholesterol	17.0	27.8	7-Ketocholesterol	10.2
α-Epoxide	26.8	Triol	5.2	21.5	Triol	1.7	15.4	Triol	1.5
β-Epoxide	26.4	Triol	16.0	32.9	Triol	2.9	16.0	6-Ketocholesterol	6.8
		6-Ketocholestanol	8.1		6-Ketocholestanol	3.1			

[a]As in Table 2.

6. Ansari, G.A.S., and L.L. Smith, *Photochem. Photobiol. 30*:147 (1979).
7. Kŭcera, J., V. Schwarz and M. Sykora, *J. Radioanal. Nucl. Chem. 87*:219 (1984).
8. Sevanian, A., and L.L. McLeod, *Lipids 22*:627 (1987).
9. Lakritz, L., and G. Maerker, *J. Food Sci. 54*:1569 (1989).
10. Smith, L.L., *Chem. Phys. Lipids 44*:87 (1987).
11. Maerker, G., *J. Am. Oil Chem. Soc. 64*:388 (1987).
12. Maerker, G., and K.C. Jones, *Lipids 26*:139 (1991).
13. von Sonntag, C., in *The Chemical Basis of Radiation Biology*, Taylor & Francis, Inc., Philadelphia, 1987, pp. 32–34, 37.
14. Moriarity, N.J., and G. Maerker, *J. Am. Oil Chem. Soc. 67*:923 (1990).
15. Olson, F., C.A. Hurt, F.C. Szoka, W.J. Vail and D. Papahadjopoulos, *Biochim. Biophys. Acta 557*:9 (1979).
16. Lee, M.L., F.Y. Yang and K.D. Bartle, in *Open Tubular Column Gas Chromatography. Theory and Practice*, John Wiley & Sons, New York, 1984, p. 222.
17. Maerker, G., and J. Unruh, Jr., *J. Am. Oil Chem. Soc. 63*:767 (1986).
18. Maerker, G., and F.J. Bunick, *Ibid. 63*:771 (1986).
19. Missler, S.R., B.A. Wasilchuck and C. Merritt, Jr., *J. Food Sci. 50*:595 (1985).
20. Chatterjee, S.N., and S. Agarwal, *Free Radical Biol. Med. 4*:51 (1988).
21. Chapman, D., in *Liposome Technology, Vol. I, Preparation of Liposomes*, edited by G. Gregoriadis, CRC Press, Inc., Boca Raton, 1984, pp. 1–8.
22. Szoka, F.C., and D. Papahadjopoulos, *Ann. Rev. Biophys. Bioeng. 9*:467 (1980).
23. Maerker, G., E.H. Nungesser and F.J. Bunick, *Lipids 23*:761 (1988).
24. Churý, J., *Chem. Abstr. 42*:7405i (1948).
25. Higley, N.A., S.L. Taylor, A.M. Herian and K. Lee, *Meat Sci. 16*:175 (1986).
26. Buxton, G.V., in *Radiation Chemistry, Principles and Applications*, edited by Farhataziz and M.A.J. Rogers, VCH Publishers, Inc., New York, 1987, pp. 332–342.

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