Effect of Low-Dose γ-Radiation on Individual Phospholipids in Aqueous Suspension

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A series of individual phospholipids (phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines and phosphatidylglycerols) containing either saturated or unsaturated fatty acid chains was irradiated at 9.66 kGy and 0–4°C in aqueous suspension. The phospholipids were analyzed by normal-phase high-performance liquid chromatography on a silica column with an evaporative light scattering detector. Phospholipid disappearance and production of two radiolytic products, phosphatidic acid and the lysophospholipid, after irradiation were quantitated from calibration curves of synthetic standards. Dipalmitoylphosphatidic acid and monopalmitoylphosphatidylcholine from irradiated dipalmitoylphosphatidylcholine were identified by liquid secondary-ion mass spectrometry.

KEY WORDS: High-performance liquid chromatography, liquid secondary-ion mass spectrometry, lysophospholipid, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, γ-radiation.

Acceptance and utilization of irradiation in food preservation varies greatly worldwide. Thus, fast and efficient analytical methods are needed to determine whether food has been irradiated. Because phospholipids play an important structural and functional role in all cellular membranes, a thorough examination of phospholipid radiolysis could yield an effective means for the detection of irradiated food. Because regulation of irradiated meat is a primary concern, the two major meat phospholipid classes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were of greatest interest in this study. Two minor phospholipid classes, phosphatidylserine (PS) and phosphatidylglycerol (PG), were also examined. Due to the chemical complexity of meat, a model system is essential in developing an understanding of the effect of γ-radiation on phospholipids. Phospholipids in nature are usually in close proximity with water, thus an appropriate model should involve phospholipids in an aqueous environment.

Phospholipids are routinely detected by thin-layer chromatography (TLC) (1,2), and recently methods have been developed for their quantitation by high-performance liquid chromatography (HPLC) (3–11). TLC, while an excellent screen for phospholipids, lacks the sensitivity required for this study. Because phospholipids contain no strong chromophors, most HPLC analyses are based on normal-phase methods monitored at lower ultraviolet (UV) wavelengths (200–210 nm) (2–5). With the recent introduction of evaporative light-scattering detectors (ELSD), the quantitation of phospholipids by HPLC has become easier and more sensitive (8–11). Thus, in this study, phospholipid quantitation was accomplished by normal-phase HPLC monitored by an ELSD.

In 1959, Coleby (12) exposed lecithin and hydrogenated lecithin to high-energy electrons (2 MeV) and reported the formation of lysophosphatidylcholine, fatty acids and phosphorylcholine. Nawar (13–15) reported that dipalmitoylphosphatidylethanolamine (PPPE) or palmitic acid irradiated at 500 kGy formed similar volatile hydrocarbons and demonstrated by TLC the formation of lysophosphatidylethanolamine and phosphorylethanolamine from irradiated PPPE. Sevilla (16) detected several radicals, including the phosphorylethanolamine radical, by electron spin resonance (ESR) when polycrystalline PPPE was irradiated at 500 kGy.

The effect of γ-radiation on phospholipids incorporated in model and in natural membranes also has been examined. Ianzini (17) showed by TLC the formation of lysophosphatidylcholine and palmitic acid upon irradiation of PC incorporated into multilayer liposomes. Cantafora (18) reported a decrease in polyunsaturated fatty acid content of PE, but not of PC, after erythrocyte ghosts were irradiated with increasing doses from 0.5 to 4.6 kGy.

Several biological samples have been analyzed for the effect of γ-radiation on the endogenous phospholipids. Bancher (19) found phosphatidic acid to be a radiolytic product of both peanut oil irradiated at 100 kGy and walnut oil irradiated at 5 and 100 kGy. Lysophosphatidylcholine was also formed during the radiolysis of walnut oil. Hafez (20) showed by TLC that both phosphatidic acid and lysophosphatidylcholine are formed from soybean seeds irradiated at 40–100 kGy, but not at lower doses.

The above studies indicate that phosphatidic acid, lysophospholipids, fatty acids, the phosphoryl-base and volatile hydrocarbons are phospholipid radiolytic products. However, no attempt has been made to measure either the disappearance of the irradiated phospholipid or the amount of radiolytic products generated. Likewise, the studies did not positively identify the radiolytic products.

This manuscript examines the effect of low-dose γ-radiation on individual phospholipids in aqueous suspension. Normal-phase HPLC methods, monitored by an ELSD, were developed to determine the effect of γ-radiation on the individual phospholipids and to quantitate and identify several phospholipid radiolysis products.

MATERIALS AND METHODS

Chemicals. All HPLC-grade solvents (chloroform, methanol and water) were obtained from Burdick and Jackson (Muskegon, MI). ULTREX ultrapure reagent-grade ammonium hydroxide (22.9%) was obtained from J.T. Baker (Phillipsburg, NJ). Synthetic dipalmitoylphosphatidylcholine (PPPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), dioleoylphosphatidylcholine (OOPC), dipalmitoylphosphatidylethanolamine (PPPE), 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE), dioleolylphosphatidylethanolamine (OOPE), dipalmitoylphosphatidylserine (PPPS) sodium salt, 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) sodium salt, dioleoylphosphatidylserine (OOPS) sodium salt, dipalmitoylphosphatidylglycerol (PPPG) sodium salt, 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) sodium salt, dioleoylphosphatidylglycerol (OOPG) sodium salt, monopalmitoylphospha-

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tidylethanolamine (LPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Synthetic dipalmitoylphosphatidic acid (PPPA) free acid and PPPS free acid were obtained from Sigma Chemical (St. Louis, MO).

Phospholipid suspensions. Aliquots (250 μL) of a stock solution (20 μmol/mL) for each phospholipid dissolved in chloroform or 2:1 chloroform/methanol (PPPE and non-salt PPPS) were evaporated to dryness with a nitrogen stream, and 1.0 mL of HPLC-grade water was added to give a final suspension of 5.0 μmol/mL. The samples were suspended by vortexing for 1 min and sonicating for 5 min, twice in sequence. The samples were kept at 0–4°C and were vortexed just prior to irradiation.

γ-Radiation. Phospholipid aqueous suspensions (5 μmol/mL) were irradiated at 0–4°C with a ^{137}Cs source (0.114 kGy/min) to a final dose of 9.66 kGy. The dose rate was established by using National Physical Laboratory (Middlesex, United Kingdom) dosimeters. Variations in dose were minimized by the use of small samples placed in a uniform portion of the radiation field. Samples were maintained at the desired temperature during irradiation by the injection of liquid nitrogen into the irradiation chamber. Control samples consisted of nonirradiated suspensions kept at 0–4°C. Both irradiated and control samples were frozen at −80°C, lyophilized to dryness (~12 h) and stored at −80°C until analyzed by HPLC.

HPLC instrumentation. Phospholipid HPLC analyses were performed with a Waters (Millipore, Milford, MA) 510 pump operated with an Autochrom (Milford, MA) Model 300 static gradient controller, followed by a Spectra-Physics (San Jose, CA) SP8500 dynamic mixer. Chromatography was performed on a Chrompack (Raritan, NY) ChromSep 7-micron LiChrosorb Si 60 silica glass column (10 cm × 3.0 mm) preceded by a silica guard column (10 × 2.1 mm) in a metal cartridge system. Samples were reconstituted in 250 μL of 2:1 chloroform/methanol prior to injection on a Rheodyne (Cotati, CA) Model 7125 injector fitted with a 50 μL loop. A Varex (Rockville, MD) Model IIA ELSD was operated at 80°C (exhaust at 54.7°C) with nitrogen as the nebulizing gas (43 psi).

Phospholipid chromatography. Becart's HPLC method (8) was modified. A binary gradient HPLC method (Table 1) was developed for the chromatography of the individual phospholipids of the PC, PE and PG classes. The gradient HPLC method was used in determining the disappearance of the phospholipids after irradiation. The individual PS phospholipids, used for the disappearance study, were determined by isocratic HPLC with a mobile phase of chloroform/methanol/water/ammonium hydroxide (70.25:27:2:0.75) at a flow rate of 0.6 mL/min.

Radiolytic product chromatography. Two binary-gradient HPLC methods (Table 1) were developed for the chromatography of PPPA and the lysophospholipids, the radiolytic products of PPPC and PPPE. The PPPA gen-

TABLE 1

Mobile-Phase Gradients for the Determination of Phospholipids and the Radiolytic Products Formed from PPPC and PPPE[a]

Gradient for determination of phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol

Time (min)	Mobile phase percent (vol/vol)		Flow rate (mL/min)
	Eluant A[b]	Eluant B[c]	
0	78	22	0.6
4	78	22	
17	65	35	
20	40	60	
22	40	60	
27	78	22	

Gradient for the determination of the radiolytic products of PPPC

Time (min)	Mobile phase percent (vol/vol)		Flow rate (mL/min)
	Eluant C[d]	Eluant B	
0	80	20	0.4
20	20	80	
23	80	20	

Gradient for the determination of the radiolytic products of PPPE

Time (min)	Mobile phase percent (vol/vol)		Flow rate (mL/min)
	Eluant C	Eluant B	
0	100	0	0.8
2	100	0	
20	30	70	
25	100	0	

[a]All injections were made every 37 min. PPPC, dipalmitoylphosphatidylcholine; PPPE, dipalmitoyl phosphatidylethanolamine.
[b]Eluant A: chloroform/methanol/ammonium hydroxide (85:14:3:0.7).
[c]Eluant B: chloroform/methanol/water/ammonium hydroxide (60:34:5.3:0.7).
[d]Eluant C: chloroform/methanol/ammonium hydroxide (80:19.3:0.7).

erated from irradiated PPPG was determined by isocratic HPLC in chloroform/methanol/water/ammonium hydroxide (76.5:21:1.75:0.75) at a flow rate of 0.4 mL/min.

Quantitation. For each phospholipid analyzed, a calibration curve was constructed from the phospholipid stock solution (20 μmol/mL) and two or three stock solution dilutions. For each radiolytic product, the calibration curve was constructed by dilutions of stock synthetic lipid solutions. The detector response (peak area) was plotted against the phospholipid mass (mol). Standards, control samples and irradiated samples were analyzed in duplicate, except the PPPC and PPPE calibration curve standards for the radiolytic quantitation studies, which were analyzed by single determinations. All calibration curves were fitted to a nonlinear exponential model by means of the curve-fitting software "Table Curve" from Jandel Scientific (Corte Madera, CA). An average of the coefficients of determination for the calibration curves for each phospholipid is given in Table 2 and 3.

Mass spectrometry. PPPA and LPC were collected from several HPLC injections of irradiated PPPC and evaporated to dryness with a stream of nitrogen. Liquid secondary-ion mass spectrometry (LSIMS) in both the negative (−) ion and positive (+) ion mode was performed on a VG Analytical (Manchester, United Kingdom) ZAB-T mass spectrometer. LSIMS ionization was effected by the

TABLE 2

Disappearance of Phospholipids After Irradiation[a]

Phospholipid irradiated	Recovery of control[b]	Disappearance after irradiation[c]			
	mol percent	mol percent (range)	N[d]	r^{2e}	t_R[f]
PPPC[g]	98.5	3.1 (2.7–3.5)	3	0.988	11.7
POPC	97.5	2.7 (1.5–3.5)	3	0.999	10.7
OOPC	95.8	7.5 (7.3–7.9)	3	0.988	10.2
PPPE	96.5	2.0 (0.4–3.1)	4	0.973	6.8
POPE	92.5	1.9 (−2.2–5.9)	2	1.000	6.3
OOPE	98.5	−0.3 (−0.5–0.4)	2	1.000	6.3
PPPG (Na^+ Salt)	91.0	28.6 (26.4–30.8)	2	1.000	5.7
POPG (Na^+ Salt)	94.5	22.9 (22.3–23.4)	2	0.996	5.3
OOPG (Na^+ Salt)	95.0	21.1 (20.5–21.6)	2	0.993	4.8
PPPS	97.0	3.4 (0.5–7.3)	4	0.991	12.0
PPPS (Na^+ Salt)	95.1	10.7 (9.4–12.0)	2	0.993	12.2
POPS (Na^+ Salt)	99.0	19.0 (17.9–19.8)	4	0.994	11.6
OOPS (Na^+ Salt)	100.3	26.9 (13.9–40.0)	4	0.983	9.2

[a]Five μmol/mL phospholipid aqueous suspension irradiated at 9.66 kGy (0–4°C).
[b]The recovery of the phospholipid in the unirradiated sample after lyophilization as determined from a calibration curve.
[c]The phospholipid in the control sample minus the phospholipid in the irradiated sample.
[d]The number of determinations.
[e]The mean of the coefficient of determination for all the calibration curves used.
[f]The mean of the retention times (t_R) for four injections of phospholipid (1 μmol).
[g]PPPC and PPPE as in Table 1. POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; OOPC, dioleoylphosphatidylcholine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; OOPE, dioleoylphosphatidylethanolamine; PPPG, dipalmitoylphosphatidylglycerol; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; OOPG, dioleoylphosphatidylglycerol; PPPS, dipalmitoylphosphatidylserine; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine; OOPS, dioleoylphosphatidylserine.

TABLE 3

Quantitation of Radiolysis Products from Phospholipids[a]

PL[d]	Disappearance after irradiation[b]			Phosphatidic acid formed after irradiation[c]			Lysophospholipid formed after irradiation[c]		
	mol percent	r^{2e}	t_R[f]	mol percent	r^{2e}	t_R[f]	mol percent	r^{2e}	t_R[f]
PPPC[g]	2.4	0.975	10.6	0.41	1.000	23.6	0.59	1.000	28.5
PPPE	1.3	0.945	9.6	0.17	1.000	23.4	0.063	1.000	19.9
PPPG	25.8	0.997	4.7	19.6	0.995	23.9	—	—	—

[a]Five μmol/mL phospholipid aqueous suspension irradiated at 9.66 kGy (0–4°C).
[b]The phospholipid in the control sample minus the phospholipid in the irradiated sample. PL, phospholipids.
[c]Based on the total phospholipid in the irradiated sample.
[d]Phospholipid irradiated.
[e]The coefficient of determination for the calibration curves used.
[f]The mean of the retention times (t_R) for three injections. Note that for each phospholipid a different high-performance liquid chromatographic method was used (see text).
[g]Abbreviations as in Table 2.

impact of high-energy cesium ions on the sample dispersed in a matrix of triethanolamine. The LSIMS sample ions were accelerated to a potential of 8 kV. The mass range of 50–2000 amu for (+) ion LSIMS and 100–1000 amu for (−) ion LSIMS was calibrated against cesium iodide. The mass spectrometry was performed by the Center for Advanced Food Technology, Cook College, Rutgers University (New Brunswick, NJ).

RESULTS AND DISCUSSION

The HPLC gradient system was developed to monitor the disappearance of each phospholipid after irradiation and the production of the expected radiolytic products. The same binary gradient (Table 1) was suitable for all the phospholipid classes studied (PC, PE and PG), except for the PS class. Because PS co-elutes with the radiolytic products, an isocratic method was required for the PS phospholipids. The HPLC gradients for the quantitation of the radiolytic products (Table 1) effectively separated those products, thus improving detection sensitivity. Because the ELSD response is nonlinear, standard calibration curves were constructed for each quantitated phospholipid.

Most of the reported HPLC methods for phospholipids were developed to separate the phospholipid classes from biological samples (3–6, 8–11). Due to the complexity of biological samples, the published methods usually involve lengthy gradients. The current study deals with the chromatography of individual synthetic phospholipids, thus the analysis time was reduced by using faster HPLC gradients. The retention time (t_R) for each phospholipid is reported in Table 2. The following details were crucial for the development of the HPLC gradients: (i) Changing the mobile-phase composition of chloroform or methanol markedly affected phospholipid separation, but the presence of water at a low percentage was required for the elution of the lysophospholipids; (ii) the addition of ammonium hydroxide to the mobile phase (8) improved both peak shape and standardized elution times, especially for phosphatidic acid; (iii) due to the gradual water gradient on the silica column, consistent retention times were observed only by making consistently timed injections (8,21); (iv) an initial blank gradient was required for equilibration of the silica column prior to an experimental sequence of injections.

The disappearance of each phospholipid after radiolysis of the aqueous phospholipid suspension is presented in Table 2. Of the primary meat phospholipids, all three PC phospholipids and PPPE are significantly affected by γ-radiation. All the PG and the PS sodium salt phospholipids are considerably more sensitive to the effects of γ-radiation. Among the phospholipid classes, there is no apparent correlation in the disappearance of the saturated as opposed to the unsaturated phospholipids.

All of the non-salt phospholipids have a lower percent disappearance (2.9 ± 2.4%) after irradiation than do the phospholipids in sodium salt form (21.5 ± 6.4%). The sodium salt phospholipids dispersed more completely in water than any of the non-salt phospholipids. The effect of γ-radiation on phospholipids in aqueous suspension may be due to the reaction of the phospholipid with reactive water radiolysis products (*i.e.*, hydroxyl radicals, hydrated electrons and hydrogen atoms) (22) or to a direct effect of the γ-radiation on the phospholipid (23). Thus, the increased hydration of the sodium salt phospholipids could lead to more direct contact with reactive radiolytic water products and help explain the higher disappearance of the sodium salt phospholipids as compared to the non-salt phospholipids.

Phosphatidic acid and the lysophospholipids were the only major radiolytic products observed for the phospholipids studied. Representative chromatograms for the control and the irradiated PPPC are shown in Figure 1. PPPA formed by the irradiation of all the dipalmitoyl phospholipids co-eluted with synthetic PPPA. Likewise, LPC and LPE, the radiolytic products of PPPC and PPPE, respectively, co-eluted with the synthetic monopalmitoylphospholipid. Attempts were made to identify the peak labelled "Artifact" in Figure 1. The results, though not completely conclusive, strongly suggested that the peak is a chromatographic artifact and not a radiolysis product.

The amounts of PPPA and the lysophospholipid formed by radiolysis of PPPC and PPPE are reported in Table 3. Because different chromatographic conditions are used for both analyses, t_R is reported for each lipid. A representative calibration curve for the radiolytic products of PPPE is shown in Figure 2. Phosphatidic acid (19,20) and the lysophospholipids (12–15,17,19,20) from irradiated phospholipids have been reported in the literature, but never quantitated. PPPA and the lysophospholipid generated by radiolysis represents, at most, 45% of the total disappearance of PPPC or PPPE. Thus, a significant portion of the radiolysis products could be compounds not determined by this methodology, such as the volatile hydrocarbons and related residues (13–15), fatty acids (12,18), phosphoryl-bases (12–17) and diglycerides.

Phospholipids are prone to acid or alkaline hydrolysis, especially at higher temperatures. Sample hydrolysis was minimized by the low temperatures (0–4°C) during irradiation and a low storage temperature (−80°C). However, a small amount of lysophospholipid formation could be seen in several of the control samples (Fig. 1) and was most likely due to hydrolysis that occurred during sample preparation.

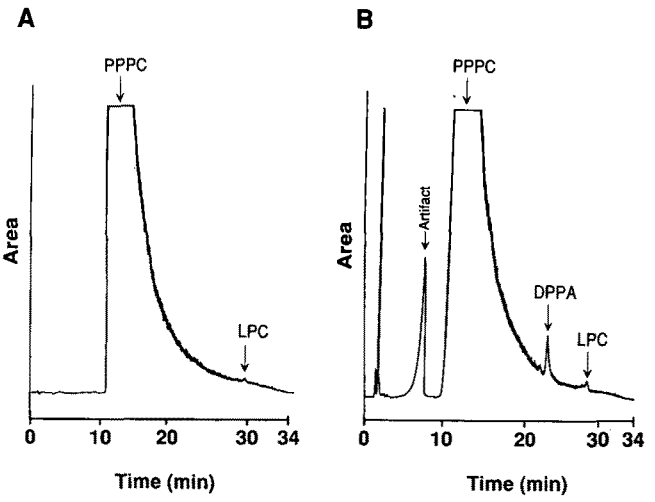

FIG. 1. Gradient high-performance liquid chromatography of unirradiated PPPC (A) and irradiated PPPC (B). PPPC, dipalmitoylphosphatidylcholine; LPC, monopalmitoylphosphatidylcholine; DPPA, dipalmitoylphosphatidic acid.

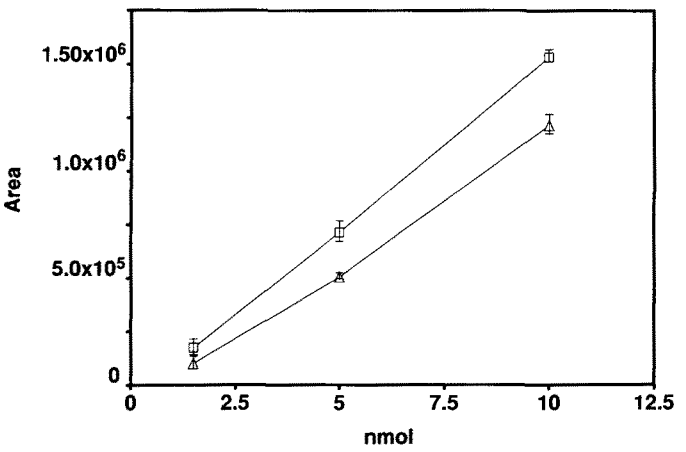

FIG. 2. PPA (□) and LPE (△) calibration curves used for quantitation of the radiolytic products from PPPE. PPA, phosphatidic acid; LPE, monopalmitoylphosphatidylethanolamine; PPPE, dipalmitoylphosphatidylethanolamine.

Of the phospholipids studied, PPPG was the most labile to the effect of γ-radiation at 28.6% disappearance. Most of the PPPG disappearance (76%) was accounted for by the formation of PPPA (Table 3).

PPPA and LPC, formed during γ-radiation of PPPC, were positively identified by LSIMS of collected and pooled HPLC peaks (data not shown). The LSIMS was operated in both the (+) ion and (−) ion modes, thus forming unique fingerprint cations and anions, respectively. The (+) ion LSIMS spectrum of LPC from irradiated PPPC identically matched the spectrum of synthetic LPC, and all the major peaks corresponded to published spectra (24). The (+) ion LSIMS spectrum of PPA from irradiated PPPC showed the identical spectrum as synthetic PPPA, but the PPPA spectrum from irradiated PPPC also showed two extra peaks. The (+) ion LSIMS spectrum of PPPA is difficult to interpret, and no published data are available for comparison. Therefore, an identical match of the (−) ion LSIMS of PPPA (25) was used to further confirm the identity of the radiolytically generated PPPA. The two extra peaks seen in the (+) ion LSIMS spectrum of radiolytically generated PPPA appear to be a molecular ion and its dimer, thus representing a second compound collected with PPPA. The chromatogram of the collected PPPA sample shows two small peaks eluting just after PPPA. The significance of these peaks is under further investigation.

Phospholipid radiolysis in aqueous suspension is a complex process. No clear correlation was shown for the susceptibility of the different phospholipid classes to γ-radiation or the effect of γ-radiation on the saturated as opposed to the unsaturated phospholipids. However, phospholipid radiolysis is likely to be complicated even more by the introduction of the phospholipid into a biological environment. Phospholipids in food are usually incorporated in the bilayers of cellular membranes. Cellular membranes contain other components, such as proteins and cholesterol. Because the phospholipid aqueous suspensions contained no interfering components, the resulting effect of radiation on phospholipids in cellular membranes may be considerably different from the results seen in aqueous suspension. Two predicted radiolysis products, phosphatidic acid and the lysophospholipids, were quantitated and positively identified in aqueous suspension. However, both are endogenous lipids in food; thus, neither lipid can be used effectively to develop an analytical method for detection of irradiated food.

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