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The Redistribution of Bulk Aqueous Phase Phospholipids During Thermal Stressing of Phospholipid-stabilized Emulsions

M. J. GROVES AND C. J. HERMAN*

Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago (M/C 964), 850 W. Taylor (2014 SEL), Chicago, IL 60607, USA

Abstract—The mechanism of the stabilization of triglyceride emulsions by phospholipids has been studied using an HPLC-FID method to determine phosphatidylcholine, lyso-phosphatidylcholine and phosphatidylethanolamine in the oil and aqueous phases of a model emulsion consisting of soybean oil 20 g, egg phospholipid 1·2 g, glycerol 2·25 g and water to 100 mL. It was shown that, on heat sterilization of the emulsions, the phospholipids rapidly relocate from the aqueous phase to the oil phase. It is suggested that the phospholipids concentrate in the oil/water meso phase, forming a cubic liquid crystalline phase, the bulk of which is converted to a lamellar phase on cooling, and that this organization of interfacial material accounts for the enhanced stability of phospholipid emulsions after heat sterilization.

Phospholipid-stabilized emulsions are injected parenterally to provide calories for debilitated patients. Development of these systems can be traced back to the initial suggestion by Yamakawa et al (1929) but within a decade Narat (1937) had demonstrated that injected lecithin-stabilized olive oil emulsions could keep starved dogs alive. Systematic comparison of emulsifiers for the delivery of intravenous fat was undertaken by McKibbon et al (1945) and within another decade Schuberth & Wretlind (1961) had started on the studies that led to the commercial introduction of Intralipid by Vitrum (now Kabi-Pharmacia). Use of these terminally heat-stabilized emulsions for the safe delivery of calories has been established throughout the world over the past thirty years. More recently, these emulsions have shown some promise as delivery systems for sparingly soluble drugs (Prankerd et al 1988) and a diazepam-containing emulsion is commercially available based on the pioneering investigations of Jeppsson & Ljungberg (1975).

The mechanism involved in stabilization remains somewhat obscure despite extensive investigations by Hansrani (1980) and others (Rydhag & Wilton 1981; Washington & Davis 1987; Washington et al 1989; Rubino 1990a, b). In part, this may have been because individual purified phospholipids do not stabilize emulsions on their own (Hansrani 1980), and mixtures of naturally-occurring phospholipids such as those available from avian eggs or soybeans are, by their very nature, complex and variable in composition.

The main components of commercial mixtures (usually described as "lecithin") used as emulsifiers are long chain phosphatidylcholines (PC) and phosphatidylethanolamines (PE), together with small amounts of phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SPM) and cholesterol (Rydhag & Wilton 1981). The acyl side chains vary in length from C_{12} to C_{20}, and may contain different centres of unsaturation. Each phospholipid itself is unstable so that associated degradation products may also be present.

Two main degradation mechanisms are involved. In the first, oxidation of the acyl chains occurs at unsaturated sites (Kemps & Crommelin 1988a). In the second, especially in the presence of water, hydrolysis occurs at the polar head, splitting off one or both of the acyl chains to form the corresponding monoacyl (lyso) derivative or, ultimately, the corresponding glycerophosphoryl compound (Marriott 1969; Kensil & Dennis 1981; Kemps & Crommelin 1988b; Grit et al 1989; Grit 1991; Herman & Groves 1992).

Although the phospholipids and, to a lesser degree, the constituent triglycerides are readily hydrolysed, the large-volume parenteral emulsions are terminally heat-sterilized in their final container by autoclaving. Inevitably, therefore, the constitution of the final, sterilized emulsion is somewhat different from the unsterilized starting material considered from a chemical standpoint. For example, the amounts of the lyso-PC and lyso-PE compounds are significantly increased, as are the free fatty acids present in the system (Kemps & Crommelin 1988b). From a physical standpoint, however, the particle size of the disperse phase droplets remains unchanged or decreases after sterilization (Lee & Groves 1981), and there is some evidence that the stability of these emulsions is enhanced on sterilization (Hansrani 1980; Hansrani et al 1983).

Grit (1991) measured stability kinetics of phospholipid breakdown in aqueous liposomal dispersions and we have substantially confirmed his results in emulsion systems. However, there is little information about the behaviour of phospholipids at interfaces and we evaluated the effect of heat exposure on the composition of the phospholipids distributed between the aqueous and oil phases.

Materials and Methods

Materials

Asahi, injectable grade, purified egg phospholipid was

* Present address: Mallinckrodt Specialty Chemicals Company, PO Box 5439, St Louis, MO 63147, USA.

Correspondence: M. J. Groves, Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago (M/C 964), 840 W. Taylor (2014 SEL), Chicago, IL 60607, USA.

received as a gift from Austin Chemical, Chicago, IL, USA. Egg phosphatidylcholine, egg lysophosphatidylcholine, egg phosphatidylethanolamine and egg lysophatidylethanolamine were from Matreya Inc., Pleasant Gap, PA, USA, and were used as received. Soybean oil USP, both pharmaceutical grade and "super refined", were gifts from Croda (US) Inc., Edison, NJ, USA. Glycerol USP, all other reagents and chromatographic reagents were from Fisher Scientific, Itasca, IL, USA.

Model emulsion

This consisted of soybean oil 20 g, egg phospholipid 1·2 g, glycerol 2·25 g, and water to 100 mL. The emulsion was prepared by dissolving the glycerol in about 95% of the water at 70°C and stirring the egg phospholipid into this system until dispersed. The soybean oil was then added and mixed to a crude emulsion before passing through a Microfluidics Model 110T homogenizer for a total of 10 cycles (Washington & Davis 1988; Lidgate et al 1989). The pH was adjusted to 8·0 with 0·01 M sodium hydroxide before making up to volume. The final product was then packed in heat-sealed 2 mL all-glass ampoules, with, if required, a nitrogen overlay. Ampoules were thermally stressed in cabinets at 37 and 50°C or, for higher temperatures, in heating blocks (Fisher 'Dry Baths') filled with oil for uniform temperature exposure. Controls were stored in the refrigerator at ~4°C.

Development of an analytical procedure

The development of a mass-based high-performance liquid chromatographic procedure has been described elsewhere (Herman & Groves 1991; Herman 1992). Initial evaluation of UV adsorption methods demonstrated that the method basically detected phospholipids with multiple unsaturated sites on the molecule and had little sensitivity to even disaturated acylphospholipids. The loss of an unsaturated fatty acid residue from a C_2 position on a phospholipid would therefore be misinterpreted by UV adsorption methods as a greater degradation than had actually occurred. Oxidation of unsaturated sites on the acyl chain also reduced UV detection levels and sensitivity. Accordingly mass detection procedures based on refractive index measurement or flame ionization detection were evaluated. Refractive index measurement, using a Waters model 401 detector, was significantly better than refractive index methodologies which could not detect, for example, dipalmitoyl PC at the levels of 10 μg ($1{\cdot}4 \times 10^{-8}$ M) readily measured by the former method. However, the baseline drift and fluctuation experienced with the refractive index method made it somewhat less than valuable. Flame-ionization detection (FID), using a Tremetrics (formerly Tracor) 945 LC Detector, was sensitive to dipalmitoyl PC at a level of 0·5 μg ($17{\cdot}1 \times 10^{-10}$ M) and the baseline drift was significantly reduced. Overall, the method was difficult to set and operate but, once functional, performed satisfactorily.

The system consists of a rotating belt swept under two flames, one of hydrogen and oxygen, for cleaning and the other, of hydrogen and air, for detection. This detector flame requires careful optimization for successful operation and is critically affected by some solvents and impurities. Water-based systems were found to volatilize too slowly and previously undetected impurities in the methanol used interfered with the FID sensitivity. These impurities were later found to be readily removed by adsorption onto a silica column (Herman 1992). Electrolytes also interfere in the flame ionization process, ruling out certain buffer salts. Acetonitrile is reported (Tracor FID manual) to be thermally degraded to hydrogen cyanide which has the potential for a significant laboratory hazard. A mobile phase described by Hax & Van Kessel (1977), consisting of 2-propanol:hexane:water, was successfully applied to a silica column and proved to be effective as a general solvent. PC, PE and lyso-PE could all be readily separated and measured using a 2-propanol:hexane:water mobile phase of 5·5:4:1. To separate the lyso-PC, the constitution was changed to 3:1:0·9. Glycerophosphatidylcholine (GPC) could be separated with a methanol:water (3:2) mixture but the analogous ethanolamine compound (GPE) could not be separated under these conditions.

One other advantage of FID was that triglycerides could be measured, although a hexane:2-propanol (95:5) system was required as mobile phase using a Lichrosorb Si60 column for separation. Lyophilization of the emulsion samples gave a residue, consisting of triglycerides and phospholipids with their degradation products, together with glycerol and residual water, that could be dissolved in a small volume of chloroform. However, the phospholipids could be separated from most of the lipids by extraction with methanol and adsorbed onto a Baker 1°/2° NH_2-NH SPE column after evaporation of the methanol extract and resolution in chloroform. The adsorbed phospholipids are then eluted with a small volume of fresh methanol and injected into the HPLC-FID system. Calibration was achieved using standards as listed. Validation was carried out by analysing samples of aged commercial emulsions (Intralipid 20%; Liposyn 20% (Herman 1992)).

Emulsion centrifugation

The separation and investigation of phospholipid stabilized emulsions was described by Groves et al (1985). This method requires prolonged ultracentrifugation and, from an analytical point of view, may produce structural damage to the interface. Others have used less drastic methods to produce substantial but not total separation of the two phases (Sherman 1968; Lutz et al 1990). Accordingly, 10 mL samples of a model 20% emulsion were spun at 13000 rev min^{-1} for 40 min at 4°C in a Sorvall (Dupont, Wilmington, DE, USA) refrigerated centrifuge. Two distinct layers could

Table 1. Phospholipid concentration of the aqueous and oil droplet compartments of a 20% model emulsion stored at 50°C for 6 and 9 weeks.

	Concn (mol × 10^{-5}/100 mL emulsion)[a]			
	6-week sample[b]		9-week sample[b]	
Phospholipid	Oil layer	Aqueous layer	Oil layer	Aqueous layer
PC	53·0	23·2	35·3	11·9
PE	14·2	6·8	10·5	3·7
PC:PE ratio	3·7	3·4	3·4	3·2
Lyso-PC	27·5	10·4	45·1	7·6

[a] Molar concentrations of PC and PE were calculated using the molecular weights of dipalmitoyl-PC and dipalmitoyl-PE. [b] A single sample from each storage condition was analysed.

Table 2. Phospholipid concentrations of the aqueous and oil droplet compartments in 20% model emulsions[a] after exposure to 120°C and refrigeration for 9 weeks.

Time at 120°C (min)	Oil compartment				Aqueous compartment			
	PC[b]	% of control	PE[b]	% of control	PC[b]	% of control	PE[b]	% of control
Control	59·0	—	16·2	—	48·2	—	19·2	—
20	79·0	+34	24·3	+50	26·9	−44	10·0	−48
40	81·2	+38	24·7	+52	25·4	−47	11·0	−43
60	95·3	+62	25·4	+57	17·5	−64	7·9	−59

[a] Duplicates. [b] PC and PE, mol/100 mL ($\times 10^5$).

be seen; a heavy white supernatant over a cloudy aqueous infranatant. The infranatant was readily removed for analysis by perforating the end of the plastic centrifuge tube with a 25 gauge hypodermic needle and gently squeezing the liquid into a collecting tube.

Results and Discussion

An initial experiment was carried out on a model emulsion system stressed at 50°C for 6 and 9 weeks. The system was centrifuged and both oil and water phase analysed for the major phospholipid components (Table 1). From this data it appeared that the lyso-PC partitioned into the oil compartment, depending to some extent on the degree of phospholipid degradation. The PC and PE both degraded more completely by the time the 9 week sample had been taken. At this earlier time the lyso-PC had separated into roughly equal molar fractions in the oil and water compartments. However, after an additional four weeks, significantly more lyso-PC had formed and was to be found substantially in the oil compartment. It was also evident from Table 1 that the phospholipids themselves were able to relocate from one compartment to another. This feature was therefore evaluated in a further series of experiments.

Model emulsion samples, stressed at 120°C for various periods and subsequently refrigerated for 9 weeks were compared with unstressed material (Table 2). Phospholipids moved from the aqueous phase to the oil during the application of thermal stress without returning on cooling. Autoclaving at different temperatures showed that the process had essentially gone to completion, even after 10 min at 100°C (Table 3).

Accordingly, a systematic evaluation of PC and PE movement was undertaken by subjecting unsterilized emulsions to temperatures of up to 90°C, and sampling at time intervals of 5 min. The results shown in Table 4 suggest that the process is rapid and occurs within 10 min of elevated heat exposure during the thermal sterilization process. The kinetics of this process were determined between 70 and 120°C and results for PC are shown in Fig. 1, PE being similar.

Both PC and PE were rapidly depleted from the aqueous compartment, taking less than 5 min at 100°C to go to equilibrium. In addition, a greater quantity of PC left the aqueous phase than PE so that, eventually, a higher proportion of PC accumulated in the oil compartment. The rates at which PE and PC redistributed from the aqueous compart-

Table 3. Phospholipid concentration of the aqueous compartment of a 20% model emulsion after 10-min autoclave temperature exposure[a].

Temperature (°C)	Phospholipid concn (mol × 10^{-5}/100 mL emulsion)			
	PC	% of control	PE	% of control
Control	61·3	—	23·9	—
100	37·6	−39	16·4	−31
110	37·3	−39	16·4	−31
120	38·9	−37	16·2	−32

[a] Duplicate samples.

Table 4. Phospholipid concentration of the aqueous compartment of model emulsion sampled at 90°C[a].

Time at 90°C (min)	Phospholipid concn (mol × 10^{-5}/100 mL emulsion)			
	PC	% of control	PE	% of control
Control	61·3	—	23·9	—
5	44·7	−27	21·1	−12
10	40·2	−33	19·1	−20
15	38·0	−38	17·5	−27

[a] Duplicate samples.

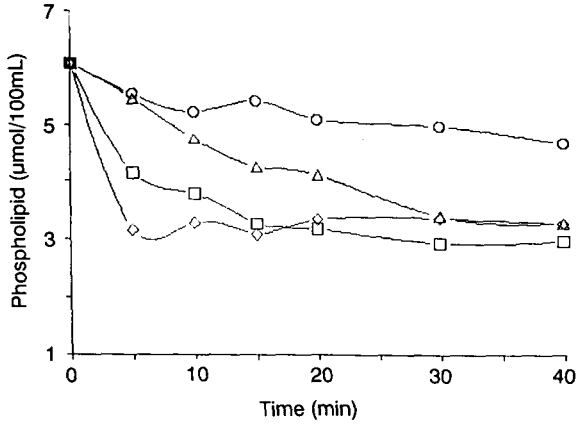

FIG 1. The rate of PC disappearance from the aqueous phase of a 20% model emulsion after stressing at temperatures of 70–100°C. ○ 70, △ 80, □ 90 and ◇ 100°C.

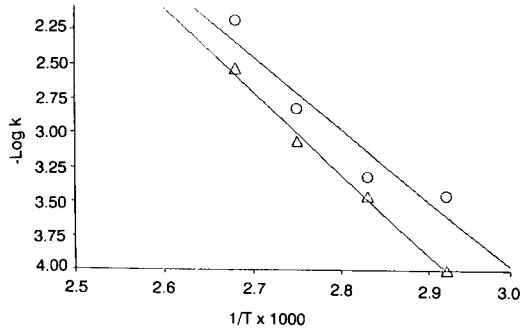

FIG. 2. The rates of PC and PE transfer from a model 20% emulsion plotted according to Arrhenius. ○ E_APE = 285, △ E_APC = 226 J M^{-1}.

ment approximated a second order process (Connors et al 1986) but are also described by an Arrhenius relationship (Fig. 2).

Other emulsion systems were evaluated by this methodology. A system prepared with hydrogenated phospholipids (Herman 1992) and stressed at 100°C for 15 min showed no change in composition. An emulsion system, similar in composition to the model emulsion described here but made by a reverse method in which the phospholipid was dispersed in the oil phase before adding the water/glycerol and homogenizing, showed identical behaviour (Fig. 3). In addition, as noted by Herman (1992), there was no significant change of particle size or of aqueous compartment triglyceride during the heat stressing processes.

It should be pointed out here that the method of separation is not total since some water is incorporated in the oil compartment and the aqueous compartment contains oil as is evidenced by the appearance and the measured triglyceride composition. However, it seems reasonable to speculate that what is seen analytically is a direct reflection of phospholipid behaviour at the oil/water interface. This interface is by no means a sharp boundary and would probably be more accurately described as a 'mesophase' since it is evidently composed of triglycerides, water and phospholipids. In addition, phospholipids are dispersed in the aqueous compartment and liposomes have been reported in commercial emulsions of similar composition (Groves et al 1985). What appears to be occurring is a movement of

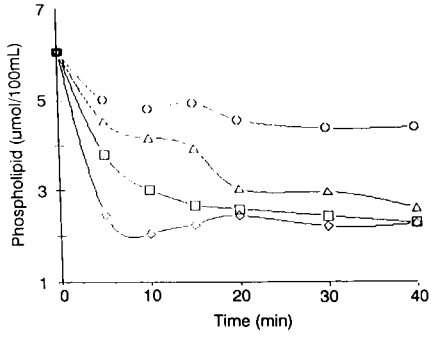

FIG. 3. The rate of PC disappearance from the aqueous compartment of a 20% emulsion made by dispersing the egg phosphatide in the oil phase (reverse process to the model system). ○ 70, △ 80, □ 90 and ◇ 100°C.

phospholipids from the aqueous phase into the oil/water mesophase and it is the composition of this mesophase that is of interest. Handa et al (1990) reported that PC alone formed a stable monolayer at an oil/water interface and was able to stabilize oil droplets in water whereas PE was not. Hansrani (1980) and Rydhag & Wilton (1981) found that neither phospholipid formed satisfactory emulsions in practice. Nevertheless, the preference of PC for an interface may account for the preferential movement of PC into the oil compartment.

The ability of mixed phospholipids to form liquid crystalline phases is well documented (Eriksson et al 1985; Das & Rand 1986; Seddon 1990; Siegel et al 1989), but formation of these systems has not been reported in emulsions. Direct observation of liquid crystalline phases in stable oil/water emulsions proved to be difficult in our hands and their presence must be regarded as possible but unproven. Heat-induced cubic phase formation by phospholipids has been reported for isolated systems (Eriksson et al 1985; Rilfors et al 1986; Lindblom & Rilfors 1989; Luzzati et al 1992) and components encouraging this process are certainly present in the heat-degraded emulsions, including lyso-PC, lyso-PE, free fatty acids and both mono- and diglycerides (Seddon 1990).

Accordingly, we suggest that a cubic liquid crystalline phase is formed at the oil/water interface during heat sterilization of these emulsions and that the bulk of this cubic material is then converted to a lamellar phase on cooling. The two phases, of differing composition, may exist in equilibrium at ambient room temperature (Eriksson et al 1985), but this organization of interfacial material would certainly account for the enhanced stability of phospholipid emulsions observed by others after heat sterilization (Hansrani 1980; Herman 1992).

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