Phosphatidylglycerol in Rat Lung. I. Identification as a Metabolically Active Phospholipid in Isolated Perfused Rat Lung†

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ABSTRACT: Isolated rat lungs, when perfused for 2 hr with [U-^{14}C]glucose, [2-^{14}C]lactate, or [1-^{14}C]acetate, were found to contain a phospholipid which represented a small percentage of the total phospholipid (3.9%), and possessed the highest specific activity of any phospholipid. Using chromatographic, chemical, and mass spectral analysis, the phospholipid has been identified as phosphatidylglycerol. When [2-^{14}C]lactate was present in the perfusion medium, 15.3% of the lactate incorporated into phospholipids was incorporated into phosphatidylglycerol with a relative specific activity of 5.1 compared to phosphatidylcholine, 1.0, and phosphatidylethanolamine, 0.5. Phosphatidylglycerol also had the highest specific activity when lungs were perfused with [1-^{14}C]acetate and [U-^{14}C]glucose. While the significance of the content and apparent metabolic activity of phosphatidylglycerol are unknown, its possible role in stabilizing the surfactant complex of lung is discussed.

Phosphatidylglycerol was first identified as a minor component of rat lung phospholipids by chromatographic methods in 1967 (Gray, 1967a,b). Since that time comparable concentrations of phosphatidylglycerol have been reported for rabbit, monkey, and dog lung and greater concentrations have been reported as a component of surfactant lipids isolated from dog, rabbit, and human lung (Baxter *et al.*, 1969; Body, 1971; Pfleger and Thomas, 1971; Rooney *et al.*, 1974).

As has been pointed out (Body, 1971; Pfleger *et al.*, 1972, Rooney *et al.*, 1974) confusion exists in the literature as to the identification of several of the less polar of the phospholipids isolated from lung, particularly in reference to discrimination between phosphatidylglycerol and phosphatidyl-*N,N*-dimethylethanolamine (Morgan *et al.*, 1965; Wolfe *et al.*, 1970; DiAugustine, 1971). As a result, in some cases phosphatidylglycerol may have been mistakenly identified as phosphatidyl-*N,N*-dimethylethanolamine. An unknown lung phospholipid possessing similar chromatographic properties as those reported for phosphatidyl-*N,N*-dimethylethanolamine (Morgan *et al.*, 1965) was found to incorporate $H^{32}PO_4$ at a rate higher than any other phospholipid except phosphatidylcholine but was present at the lowest concentration (Weinhold and Villee, 1965).

A high specific activity phospholipid present as a minor component in isolated rat lung perfused with medium containing radioactively labeled glucose, lactate, and acetate was found to be present during the course of our investigations. Studies have been carried out which establish the identity of this compound as phosphatidylglycerol and a comparison of the incorporation of radioactive substrates into phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol has been made.

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Experimental Procedures

Authentic phosphatidylglycerol was obtained from Applied Science Lab. Inc., State College, Pa. Lungs from fed adult male Wistar strain rats (National Laboratory Animal Co., O'Fallon, Mo.) were used in all experiments. All ^{14}C-labeled compounds were purchased from New England Nuclear Corp., Boston, Mass. Procedures related to the isolated lung perfusions including conditions during perfusion, perfusion media, tissue sampling, lipid extraction and washing, and separation of the phospholipid fraction from other lipids by silicic acid column chromatography were as previously reported (Godinez and Longmore, 1973). Fresh rat lungs used for determination of phospholipid composition were perfused free of blood with 0.9% saline *in situ*. The phospholipids (20–30 μmol) were further separated using (method 1) a 2-g silicic acid column (Unisil, Clarkson Chemical Co., Williamsport, Pa.) from which the phospholipids were eluted with a discontinuous gradient system as follows: (v/v) $CHCl_3$–CH_3OH (19:1), 48 ml; $CHCl_3$–CH_3OH (13:1), 48 ml; $CHCl_3$–CH_3OH–H_2O (11:1:0.06), 80 ml; $CHCl_3$–CH_3OH–H_2O (5:1:0.06), 48 ml; $CHCl_3$–CH_3OH–H_2O (3:1:0.08), 48 ml. A flow rate of 2 ml/min was obtained using a syringe pump (Harvard Apparatus Co., Boston, Mass.). Alternatively (method 2) the phospholipids (20–30μmol) were separated using the method of Gray (1967a) modified in that only three solvent mixtures were used for elution as follows on a 2-g silicic acid column: $CHCl_3$, 50 ml; tetrahydrofuran–dimethoxymethane–CH_3OH–H_2O (10:6:4:1, v/v), 25 ml; CH_3OH, 75 ml. Further purification of the acidic phospholipids was carried out using thin-layer chromatography on silica gel H as described (Gray, 1967b; Skipski *et al.*, 1967).

Phospholipid phosphorus was determined by a modification (Dittmer and Wells, 1969) of the Bartlett procedure and specific radioactivities were obtained by counting appropriate aliquots by liquid scintillation counting methods. Phospholipase A (from *Crotalus adamanteus*) hydrolysis of phosphatidylglycerol was carried out (Wells and Hanahan, 1969) and the hydrolysis products were separated by thin-layer chromatography on silica gel H using tetrahydrofu-

Table I: Comparison of the Chemical Composition of Authentic Phosphatidylglycerol with that of a Phospholipid Isolated from Lung.[a]

Sample	Composition (μmol)				Ratios of Compositions		
	Phosphorus	Acyl Ester	Glycerol	Nitrogen	Acyl Ester/Phosphorus	Glycerol/Phosphorus	Glycerol/Acyl Ester
Phosphatidylglycerol standard	0.068	0.13	0.16	0.00	1.9	2.3	1.2
Isolated lung phospholipid; 1	0.016	0.04	0.04	0.00	2.4	2.5	0.9
2	0.15	0.29	0.33	0.00	2.0	2.3	1.1

[a] All assays were carried out in duplicate and mean values are presented.

ran-dimethoxymethane-CH_3OH-2 M NH_4OH (10:6:4:1, v/v).

Phospholipase C (from *Bacillus cereus*) hydrolysis of phospholipids was performed (Haverkate and Van Deenen, 1965) and the resulting products were separated by thin-layer chromatography on silica gel H using hexane–ethyl ether (70:30, v/v) against standards of 1,2 and 1,3 diglycerides.

Assay of nitrogen, glycerol, and acyl esters and the mild alkaline hydrolysis (deacylation) of phospholipds was carried out as described (Dittmer and Wells, 1969). Mass spectral analysis of deacylated isolated and authentic phosphatidylglycerol was performed on a LKB-9000 gas chromatograph mass spectrometer (LKB, Stockholm, Sweden) as previously reported (Duncan *et al.*, 1971). Separation was carried out on a 6 ft × 3 mm column of OV-17, 1% on Gas Chrom Q at 180°. Helium flow was 25 cm^3/min. The flash heater was maintained at 222°, the molecular separator at 241°, and the ion source of the mass spectrometer at 290°.

Results

During the course of studies to determine the distribution of incorporated [U-^{14}C]glucose into the phospholipids of isolated perfused rat lungs a phospholipid with a high specific activity and which constituted approximately 4% of the total lung phospholipid phosphorus was found to be consistently present. This phospholipid was first observed upon silicic acid column chromatography (method 1, see Experimental Procedures) and was eluted in the first 20 ml of the $CHCl_3$-CH_3OH-H_2O (5:1:0.06, v/v) fraction. It was further purified by thin-layer chromatography (Skipski *et al.*, 1967). Further studies suggested that the compound was phosphatidylglycerol and could be isolated more readily by discontinuous gradient elution from a silicic acid column (method 2, see Experimental Procedure) in which phosphatidylglycerol was isolated in the tetrahydrofuran–dimethoxymethane-CH_3OH-H_2O (10:6:7:1, v/v) fraction. This fraction containing for the most part phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylserine was then separated by thin-layer chromatography on silica gel H (0.5 mm) developed in a system of tetrahydrofuran–dimethoxymethane-CH_3OH-2 M NH_4OH (10:6:4:1 v/v) (Gray, 1967b). The high specific activity material, which cochromatographed with authentic phosphatidylglycerol, was isolated by scraping the silicic acid from the plate and extracting the silicic acid two times with 5 ml of $CHCl_3$-CH_3OH (1:1, v/v) and once with 5 ml of methanol. The phosphatidylglycerol isolated has been found to migrate with authentic phosphatidylglycerol in two other thin-layer chromatography systems on silica gel H, $CHCl_3$-CH_3OH-H_2O (65:25:4, v/v) (Gray, 1967b) and $CHCl_3$-CH_3OH-1 M NH_4OH (80:36:2) on basic plates (Skipski *et al.*, 1967) and has been chromatographed to constant specific activity. The compound isolated from lung in this manner was then used in studies to establish its further identity as phosphatidylglycerol.

The isolated compound was analyzed for its content of phosphorus, acyl ester, glycerol, and nitrogen. The results of these analyses are given in Table I and are compared to a similar analysis of authentic phosphatidylglycerol. The result indicates a composition compatible with phosphatidylglycerol, but not defining the arrangement of the components to form phosphatidylglycerol.

Enzymatic hydrolysis of the compound and authentic phosphatidylglycerol with phospholipase A and separation of the products by thin-layer chromatography yielded compounds identified as lysophosphatidylglycerol and free fatty acid. Similarly phospholipase C hydrolysis of both compounds yielded 1,2 diglyceride. These results suggested the compound contained adjacent fatty acids on the same glycerol molecule while the other glycerol moiety was free of acyl groups. The compound reacted with Schiff's reagent denoting the presence of adjacent free hydroxyl groups indicating that the unacylated glycerol was esterified to phosphate at the 1 position.

To establish as carefully as possible the identity of the compound as phosphatidylglycerol, the trimethylsilyl derivatives of deacylated authentic posphatidylglycerol and the deacylated compound thought to be phosphatidylglycerol were prepared for glc–mass spectral analysis essentially as described (Duncan *et al.*, 1971). Figure 1 compares the mass spectrum of the two compounds. Major peaks for both the standard and unknown are seen at *m/e* 591, 516, 503, 461, 445, 347, 299, and 73. The theoretical molecular ion (M*) at *m/e* 606 was not consistently observed in either the unknown or the standard; *m/e* 357 (M* − ·CH_2OTMS) was taken as the base peak. A calculated difference spectrum indicated the compounds were identical.

The phospholipid composition of fresh rat lung which had been perfused free of blood (see Experimental Section) is given in Table II. Phosphatidylglycerol was found to constitute 3.9% and phosphatidylcholine 54% of the total phospholipid pool. However, when isolated rat lung was perfused with medium containing [2-^{14}C]lactate (Table III), phosphatidylglycerol contained 15.3% of the total radioac-

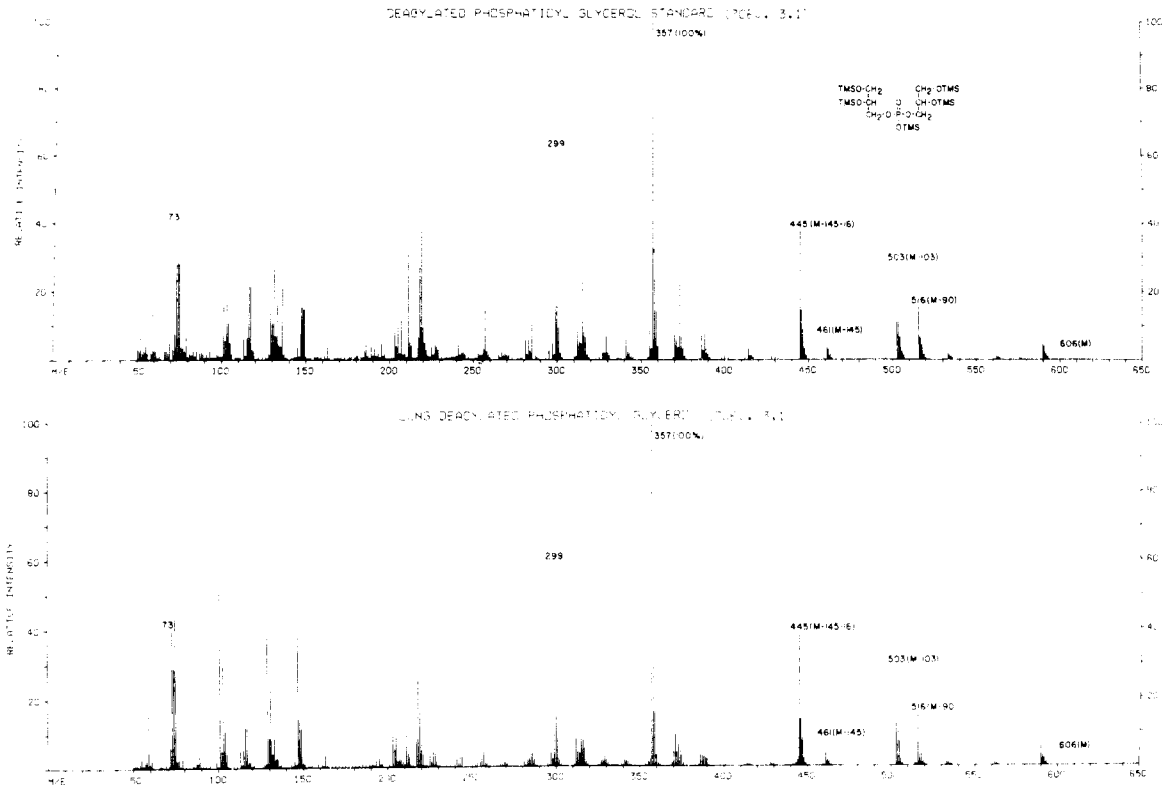

FIGURE 1: The 70-eV mass spectrum of pentakis(trimethylsilyl)-α-glycerophophorylglycerol. Spectra were obtained on the trimethylsilyl derivative of the deacylated phospholipids (upper standard, lower obtained from lung) using LKB 9000 gas chromatograph–mass spectrograph.

Table II: Composition of Rat Lung Phospholipids.[a]

Phospholipid	Per Cent
Phosphatidylcholine	54.3 ± 1.35[b]
Phosphatidylethanolamine	20.2 ± 1.14
Phosphatidylserine + phosphatidylinositol	5.5 ± 0.5
Sphingomyelin	12.6 ± 0.6
Phosphatidic acid and cardiolipin	3.9 ± 1.0
Phosphatidylglycerol	3.9 ± 0.3

[a] Total phospholipid content: 1.64 μmol/mg of protein. [b] Mean ±S.E. of 28 determinations.

Table III: Incorporation of [2-^{14}C]Lactate into Phospholipids of the Isolated Perfused Lung.[a]

Phospholipid	Distribution of Incorporated Radioactivity, % Total dpm	Incorporation of Radioactivity: Specific Activity (dpm pmol)	Incorporation of Radioactivity: Specific Activity Relative to Phosphatidylcholine
Phosphatidylcholine	75.0	13.8	1.0
Phosphatidylglycerol	15.3	70	5.1
Phosphatidylethanolamine	5.6	7.2	0.5
Phosphatidylserine and phosphatidylinositol	2.3	4.7	0.3
Sphingomyelin	1.8	1.5	0.1

[a] Perfusion medium substrate concentrations: L-Lactic acid, 2.0 mM (20 μCi), glucose, 5.7 mM. Perfusions were of 2 hr duration. All assays were conducted in duplicate and mean values are presented.

tivity incorporated into lung phospholipids, second only to incorporation into phosphatidylcholine (75.0%). The relative specific activity of phosphatidylglycerol was 5.1 compared to phosphatidylcholine (1.0). Mild alkaline hydrolysis indicated approximately 97% of the incorporated radioactivity was present in the fatty acid moieties of phosphatidylcholine and phosphatidylglycerol. All other phospholipids present had significantly lower specific activities compared to phosphatidylcholine although present at concentrations similar to or lower than phosphatidylglycerol.

Studies of the incorporation of [U-^{14}C]glucose and [1-^{14}C]acetate into lung phospholipids were carried out to permit further comparison of the rate of labeling of phosphatidylcholine, phosphatidylglycerol, and phosphatidylethanolamine. The results (Table IV) indicate that the incorporation of [U-^{14}C]glucose into phosphatidylglycerol was similar to that of [2-^{14}C]lactate. Phosphatidylglycerol was found to have a relative specific activity of 4.5 when compared to incorporation into phosphatidylcholine (relative specific activity 1.0). Phosphatidylglycerol was also found to have a greater specific activity than phosphatidylcholine (relative specific activities of 1.9 and 1.0, respectively) fol-

Table IV: Relative Specific Activities of the Incorporation of [U-^{14}C]Glucose, [2-^{14}C]Lactate, and [1-^{14}C]Acetate into Phosphatidylcholine, Phosphatidylglycerol, and Phosphatidylethanolamine in the Isolated Perfused Lung.[a]

Phospholipid	Incorporation of (Relative Specific Activity)		
	[U-^{14}C]-Glucose[b]	[2-^{14}C]-Lactate[c]	[1-^{14}C]-Acetate[d]
Phosphatidylcholine	1.0	1.0	1.0
Phosphatidylglycerol	4.5	5.1	1.9
Phosphatidylethanolamine	0.2	0.5	0.3

[a] Perfusions were of 2 hr duration. All assays were conducted in duplicate and mean values were used to calculate relative specific activities. [b] Perfusion medium contained 6 mM (50 μCi) [U-^{14}C]glucose. [c] Perfusion medium contained 2.0 mM (20 μCi) [2-^{14}C]lactate and 5.7 mM glucose. [d] Perfusion medium contained 4 mM (20 μCi) [1-^{14}C]-acetate and 5.2 mM glucose.

lowing perfusion of the lungs with medium containing [1-^{14}C]acetate. The specific activity of phosphatidylethanolamine was found to be markedly less than either phosphatidylcholine or phosphatidylglycerol following perfusion with each of the three substrates, [U-^{14}C]glucose, [2-^{14}C]lactate, and [1-^{14}C]acetate. In each case the relative specific activity of phosphatidylethanolamine ranged from 0.2 to 0.5 compared to 1.0 for phosphatidylcholine. As glucose would be expected to be incorporated into the glycerol phosphoryl-x moiety of phospholipids, as well as into the fatty acid moieties, mild alkaline hydrolysis of the phospholipids was performed to determine the percentage incorporation of [U-^{14}C]glucose into the glycerol phosphoryl-x moiety and fatty acid moieties. The results given in Table V show that while the concentration of glucose present in the medium perfusing the lung (2.1 or 6.6 mM) did not significantly alter the incorporation into the two fractions, the glycerol phosphoryl-x moiety of phosphatidylglycerol contained nearly twice the percentage of total counts as the same moiety of phosphatidylcholine. The incorporation into the second glycerol moiety of phosphatidylglycerol accounts in part for the greater incorporation into phosphatidylglycerol as compared to incorporation into phosphatidylcholine (Table IV).

Table V: Percentage of [U-^{14}C]Glucose Incorporated into the Fatty Acid Moieties of Lung Phosphatidylcholine, Phosphatidylglycerol, and Phosphatidylethanolamine.[a]

Phospholipid	Glucose Concentration (mM) in Perfusion Medium	
	2.1	6.6
Phosphatidylcholine	61.6	64.0
Phosphatidylglycerol	31.0	36.2
Phosphatidylethanolamine	46.1	46.1

[a] Perfusions were of 2 hr duration. All assays were conducted in duplicate and mean values were used to calculate percentages.

Discussion

A series of observations pertinent to the occurrence and metabolism of lung phospholipids may be cited which bear upon one another. In 1965 an unidentified relatively nonpolar phospholipid present in low concentration in adult rat lung was found to incorporate [^{32}P]phosphoric acid at a rate higher than phosphatidylcholine (Weinhold and Villee, 1965). Beginning in 1967 chromatographic evidence for the presence of phosphatidyylglycerol in lung tissue of various species has been presented by several investigators (Baxter *et al.*, 1969; Body, 1971; Pfleger and Thomas, 1971; Rooney *et al.*, 1974). Other investigators have suggested that phosphatidyl-*N,N*-dimethylethanolamine was present in dog lung (Morgan *et al.*, 1965) and that a methylation pathway for the conversion of this intermediate to phosphatidylcholine was present (Morgan, 1969). This pathway has also been reported as present in newborn infants and adults (Gluck *et al.*, 1972). Attempts in our laboratory to identify phosphatidyl-*N,N*-dimethylethanolamine as a prominent phospholipid in rat lung capable of significant incorporation of phospholipid precursors have failed. Due to the similarity of migration of phosphatidyl-*N,N*-dimethylethanolamine and phosphatidylglycerol in thin-layer chromatography systems used by several investigators it now seems that the compound was in all cases phosphatidylglycerol. Pfleger and Thomas (1971), Body (1971), and Rooney *et al.* (1974) have also challenged the identity of the lung phospholipid in question as phosphatidyl-*N,N*-dimethylethanolamine.

It now appears that phosphatidylglycerol is distinctive in lung, both because of the quantity present and because of its ability to incorporate phospholipid precursors at a rate greater than other phospholipids present in lung. The concentration of phosphatidylglycerol in whole lung has been reported to be in the range of 2–5%. The variability is dependent both upon the species used and the laboratory reporting the results. The concentration of phosphatidylglycerol in the isolated extracellular surfactant of lung has been reported to be as high as 14% (Baxter *et al.*, 1969; Body, 1971; Pfleger and Thomas, 1971; Rooney *et al.*, 1974). Prior to these reports of relatively high concentrations of phosphatidylglycerol in lung, phosphatidylglycerol had been reported only as a minor component of animal tissues. Mitochondria of rat liver, kidney, and brain and beef and sheep heart had been shown to contain small quantities of glycerylphosphorylglycerol following deacylation of the lipid fraction (Strickland and Bensen, 1960) and phosphatidylglycerol had been isolated from rat liver mitochondria (Gray, 1964). Phosphatidylglycerol is a major lipid component of plants, primarily in chloroplasts (Benson and Maneo, 1958) and of bacteria (Kanfer and Kennedy, 1964). In *Escherichia* phosphatidylglycerol has been shown to be a precursor of cardiolipin (Hirschberg and Kennedy, 1972). In the present studies the observed high specific activity of phosphatidylglycerol following *ex vivo* perfusion of lung with [U-^{14}C]glucose, [2-^{14}]lactate, or [1-^{14}C]acetate is of particular interest. While the specific activity might be expected to be higher than phosphatidylcholine or phosphatidylethanolamine because of its smaller pool size, it should

be pointed out that other phospholipids present in similar or smaller amounts in lung do not possess similar high specific activities.

Both [2-^{14}C]lactate and [1-^{14}C]acetate label predominantly the fatty acid moieties of the phospholipids while [U-^{14}C]glucose is also incorporated into both glycerol moieties of phosphatidylglycerol (Table V). The observation that [2-^{14}C]lactate incorporation yields phosphatidylglycerol with a higher specific activity when compared to phosphatidylcholine than does incorporation of [1-^{14}C]acetate cannot be explained with available data. It should be noted, however, that acetate must undergo conversion to acetyl CoA by an acyl CoA synthetase which is not involved in the pathway leading to acetyl CoA from lactate or glucose. Acyl CoA synthetases are known to vary in activity in different organs of the same species and also to be specific for differences in chain length of the acyl compound (Aas, 1971). Should phosphatidylglycerol synthesis be restricted to a certain cell type in the lung and phosphatidylcholine synthesis be common to several cell types, the difference between lactate and acetate incorporation could be due to differing abilities of the cell types to synthesize acetyl CoA from acetate for use in fatty acid synthesis.

The significance of phosphatidylglycerol in the lung and its apparent metabolic activity are unknown. It has been determined that the surface tension lowering properties of phosphatidylglycerol are similar to those of dipalmitoylphosphatidylcholine (Henderson and Pfleger, 1972). Thus phosphatidylglycerol may function as a surfactant to some degree. Characteristically however, phosphatidylglycerol is associated with membrane structures displaying high metabolic activity such as the mitochondria of animal tissue, the chloroplasts of photosynthetic cells, and the protoplasts of bacteria. Among the possible roles assigned to phosphatidylglycerol in bacteria are transport of amino acids (Macfarlane, 1962) and transport of sugars (OpDen Kamp *et al.*, 1969). Surfactant phospholipids are thought to be stored in the lamellar body of the type II pneumocyte (Schaefer *et al.*, 1964; Redding *et al.*, 1972) prior to secretion onto the alveolar surface of the lung. Nuclear magnetic resonance studies have shown that phosphatidylglycerol possesses greater hydrogen bonding capacity than most other phospholipids including phosphatidylcholine (Henderson *et al.*, 1974). In another study (Michaelson *et al.*, 1973) it has been shown that phosphatidylglycerol when cosonicated with equal molar quantities of phosphatidylcholine forms inverted vesicles which contain on the outer surface twice as many phosphatidylglycerol as phosphatidylcholine molecules. These properties of phosphatidylglycerol may permit it to play a role in stabilizing the surfactant lipoprotein complex after secretion or in the storage of surfactant in the lamellar bodies of the type II pneumocyte.

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