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Transmembrane Movement of Phosphatidylglycerol and Diacylglycerol Sulfhydryl Analogues†

Barry R. Ganong and Robert M. Bell*

ABSTRACT: Transmembrane movement of phospholipids is a fundamental step in the process of biological membrane assembly and intracellular lipid sorting. To facilitate study of transmembrane movement, we have synthesized analogues of phosphatidylglycerol and diacylglycerol in which a sulfhydryl group replaces a hydroxyl group in the polar head group. A rapid, continuous assay for the movement of phospholipids across single-walled lipid vesicles was developed that exploits the reactivity of these analogues toward 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a nonpenetrating, colorimetric, sulfhydryl reagent. In the reaction of DTNB with vesicles containing phosphatidylthioglycerol, a phosphatidylglycerol analogue, two kinetic phases were seen, which represent the reaction of DTNB with phosphatidylthioglycerol in the outer and inner leaflets of the bilayer. Analysis of the slow second phase indicated that the half-time for phosphatidylthioglycerol transbilayer movement was in excess of 8 days. In a similar experiment using dioleoylthioglycerol, a diacylglycerol analogue, the reaction was complete within 15 s. The large difference in translocation rates between these two lipids indicates that the primary barrier to transmembrane movement is the polar head group and implies that phospholipid translocation events in biological membranes may not be unlike those for molecules similar to the polar head groups alone.

In mammalian cells, the endoplasmic reticulum is the primary site of phospholipid synthesis. Efforts are currently being made in a number of laboratories to elucidate the molecular mechanisms by which phospholipids synthesized in this membrane are distributed among the various membrane systems in the cell. Phospholipid assembly occurs on the cytoplasmic face of the endoplasmic reticulum (Bell et al., 1981). A fundamental step in the process of intracellular lipid sorting is the movement of phospholipids across the membrane to the lumenal surface.

The rate of phospholipid movement across biological membranes varies over several orders of magnitude depending on the membrane under investigation. Values of half-times range

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received March 16, 1984.* Supported by U.S. Public Health Service Grants AM20205, GM20015, and AM07020.

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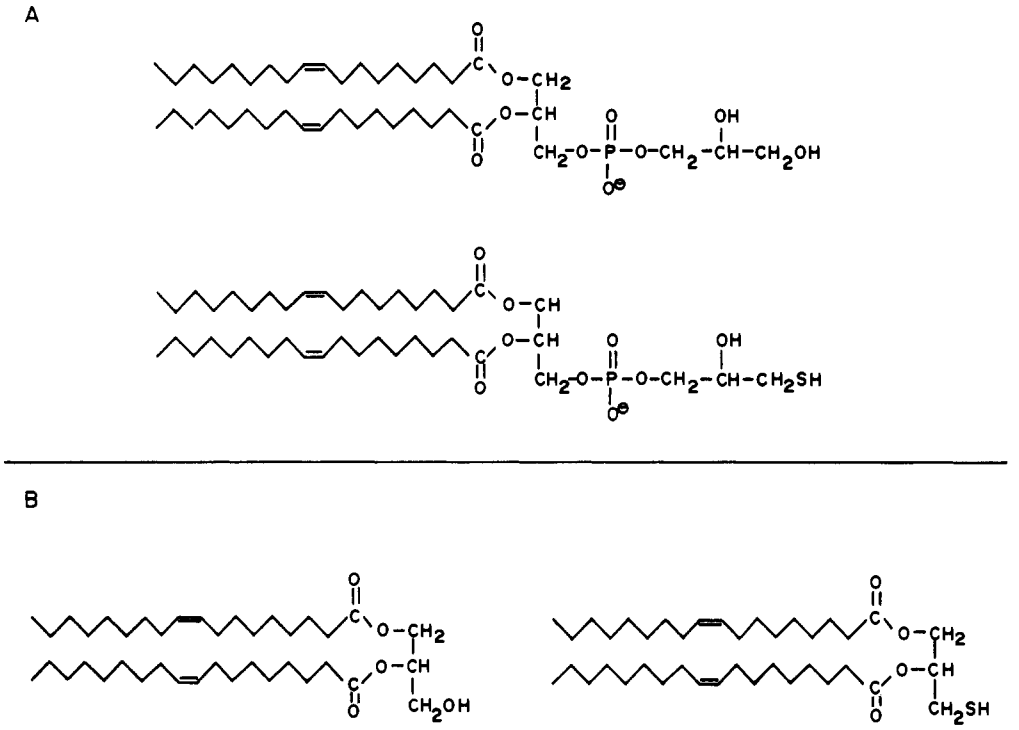

FIGURE 1: Structures of lipids and their sulfhydryl analogues: (A) phosphatidylglycerol and phosphatidylthioglycerol; (B) dioleoylglycerol and dioleoylthioglycerol.

from less than 5 min for rat liver endoplasmic reticulum (van den Besselaar et al., 1978; Hutson & Higgins, 1982) and the membrane of *Bacillus megaterium* (Rothman & Kennedy, 1977) to more than 10 days for influenza virus membrane (Rothman et al., 1976). Some of these data have been reviewed by Thompson (1978). The factors that influence the rate of phospholipid translocation in biological membranes are not known. In general, membranes in which transbilayer movement has been shown to be rapid are those involved in phospholipid biosynthesis, such as the endoplasmic reticulum of rat liver and the cytoplastic membrane of the bacterium *B. megaterium*. Bretscher (1974) suggested that specific proteins may be present in these membranes that stimulate transmembrane movement.

The search for, and study of, such proteins would be greatly facilitated by a simple, rapid, continuous assay for transbilayer phospholipid movement. Methods that have been used to date involve complicated procedures for generating transbilayer asymmetry and/or for labeling, separating, and quantifying lipid products. In this paper we report a spectrophotometric assay for transmembrane lipid movement that is rapid and requires few manipulations. The assay was employed to investigate the rate of translocation of phosphatidylglycerol and diacylglycerol sulfhydryl analogues. The novel sulfhydryl analogue of phosphatidylglycerol developed for this assay should also prove useful in other studies of phospholipid metabolism.

Experimental Procedures

Materials. Oleic anhydride was purchased from Serdary, Inc., London, Ontario, Canada, and octyl glucoside (octyl β-D-glucopyranoside) and α-thioglycerol were obtained from Calbiochem. DTNB,[1] phosphatidylethanolamine (*Escherichia coli*), phosphatidylcholine (egg), phosphatidylglycerol (egg phosphatidylcholine), dioleoylphosphatidylcholine, methyl methanethiosulfonate, reduced glutathione, silica gel for column chromatography, and cabbage phospholipase D, Type I, were products of Sigma. DEAE-Sephacel and Sephadex G-50 were obtained from Pharmacia, Inc. Phospholipase C was purified from *Bacillus cereus* (Zwaal et al., 1971). Thin-layer chromatography was performed on silica gel 60 plates (E. Merck, Darmstadt, West Germany) obtained from American Scientific Products. For analytical work a layer thickness of 0.25 mm was used, and for preparative use the thickness was 2 mm.

Analytical Methods. Phosphorus was determined by the method of Ames & Dubin (1960), and acyl ester was quantified by the procedure of Stern & Shapiro (1953). Absorbance of DTNB was measured at 412 nm by using $\epsilon = 13\,600$ M^{-1} cm^{-1} (Ellman, 1959). Mass spectra were obtained with a VG Model 7070HS/11-250 spectrometer/data system operated in electron impact ionization mode.

Synthesis and Purification of Phosphatidylthioglycerol. Phosphatidylthioglycerol, a structural analogue of phosphatidylglycerol (Figure 1A), was synthesized from phosphatidylethanolamine by the transphosphatidylation reaction catalyzed by cabbage phospholipase D (Yang et al., 1967). A solution of phosphatidylethanolamine (30 μmol) in chloroform was dried under nitrogen and dissolved in 6 mL of diethyl ether. To this solution was added 12 mL of reaction mixture containing 0.2 M sodium acetate, pH 5.6, 10 mM $CaCl_2$, 2000 units of phospholipase D, and 5% (v/v) thioglycerol. The two-phase mixture was vortexed for 15–20 min at room temperature and acidified to pH 3 with HCl to stop the reaction. The amount of phospholipase D and the time employed were determined for each batch of phospholipase D. The ether was removed under a stream of nitrogen and lipids were extracted from the buffer by the procedure of Bligh & Dyer (1959).

The chloroform phase was dried under a stream of nitrogen, and the lipid film was dissolved in 3.8 mL of chloroform–methanol–water (1:2:0.8 v/v) and loaded onto a small column (5-mL bed volume) of DEAE-Sephacel in the chloride form, equilibrated with chloroform–methanol–water (1:2:0.8 v/v).

[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Me_3Si, trimethylsilyl; BHA, butylated hydroxyanisole.

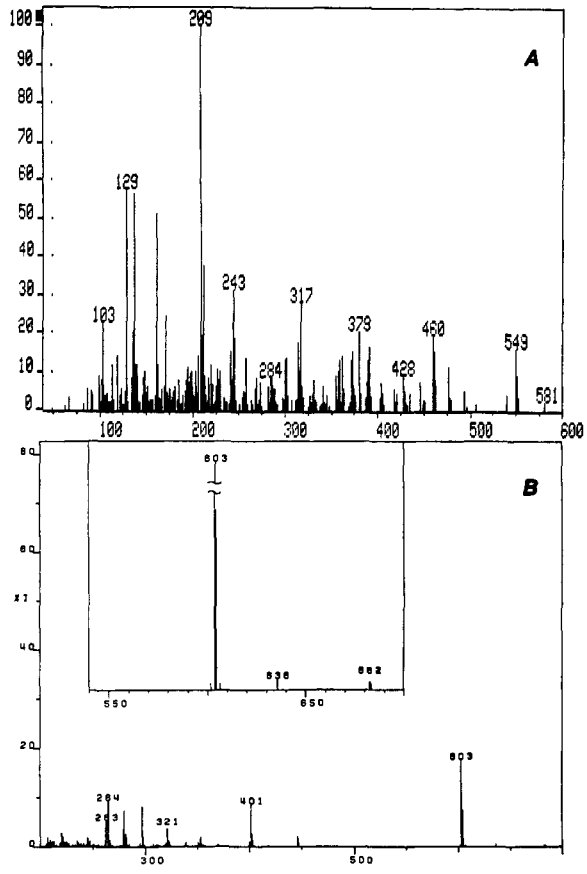

FIGURE 2: Mass spectra of sulfhydryl lipid analogues. (A) Dioleoylphosphatidylthioglycerol was deacylated and trimethylsilylated prior to analysis. (B) Dioleoylthiomethylthioglycerol; insert shows enlargement of region from *m/e* 550 to 700.

The lipids were then eluted with a step gradient of Tris-HCl, pH 8, in the same solvent mixture, with the aqueous component made from 0 to 100 mM Tris-HCl in steps of 10 mM. Phosphatidylethanolamine (R_f = 0.16), phosphatidylthioglycerol (R_f = 0.60), and phosphatidic acid (R_f = 0.43) were identified in fractions by thin-layer chromatography in the solvent system chloroform–methanol–acetic acid (65:25:10 v/v). The lipids were recovered from the eluates by addition of 1 volume each of chloroform and 0.1 N HCl to extract the lipids. A typical yield of phosphatidylthioglycerol was 40% based on phosphatidylethanolamine.

The ratio of free sulfhydryl/phosphate/ester was found to be 0.97/0.94/2.00. For mass spectrometric analysis of dioleoylphosphatidylthioglycerol, the sulfhydryl was first protected by reacting 5 μmol of phosphatidylthioglycerol in 1 mL of chloroform with 1 μL of methyl methanethiosulfonate and 10 μL of pyridine at room temperature for 1 h to form the methyl disulfide derivative. When this compound was trimethylsilylated and analyzed, peaks were seen at *m/z* 603 and 677, corresponding to the diacylglycerol and trimethylsilylated diacylglycerol, respectively (Horning et al., 1969). Deacylation followed by trimethylsilylation as described by Duncan et al. (1971) confirmed the expected structure (Figure 2A). No signal was seen corresponding to M^+ at 596, but diagnostic fragments were found at *m/z* 209 (loss of glycerylphosphoryl moiety), 379 (loss of glyceryl moiety with back-transfer of Me_3Si and H), 549 (loss of thiomethyl group), and 581 (loss of methyl groups). Minor diagnostic fragments were seen at *m/z* 476 (loss of Me_3Si and thiomethyl group), 493 (loss of Me_3Si–O–CH_2), and 506 (loss of Me_3Si–OH).

Synthesis and Purification of Dioleoylglycerol and Dioleoylthioglycerol. The structures of these compounds are shown in Figure 1B. *sn*-1,2-Dioleoylglycerol was prepared by digestion of dioleoylphosphatidylcholine with phospholipase C (Marvis et al., 1972). Dioleoylphosphatidylcholine (100 mg) in chloroform was dried under nitrogen and dissolved in 5 mL of diethyl ether. To this was added 1 mL of 50 mM potassium phosphate, pH 7, containing 5 units of phospholipase C. The two-phase mixture was vortexed intermittently at room temperature for 2 h. Another 5 units of enzyme was added and vortexing was continued for 2 h. The ether was evaporated under nitrogen, and the lipids were extracted from the aqueous phase and dissolved in chloroform. Traces of residual phosphatidylcholine were removed by passing the solution over a small bed of silica gel in chloroform. Dioleoylglycerol in chloroform was stored at −20 °C.

Thioglycerol (5 mmol), methyl methanethiosulfonate (5.5 mmol), and pyridine (6 mmol) were dissolved in 10 mL of chloroform. The reaction was complete within 5 min. Thiomethylthioglycerol was purified by chromatography on silica gel in chloroform, eluting with a gradient of 1–15% methanol. Fractions containing thiomethylthioglycerol were identified by thin-layer chromatography developed with chloroform–methanol–water (65:25:4 v/v). Spots were visualized by staining with iodine vapors. The R_f of thioglycerol was 0.46, of pyridine 0.69, of methyl methanethiosulfonate 0.81, and of thiomethylthioglycerol 0.58.

Thiomethylthioglycerol (30 μmol) was dried under nitrogen and vacuum and dissolved in 1 mL of heptane. Oleic anhydride (65 μmol) and pyridine (125 μmol) were added, and after mixing, the solution was left in the dark at room temperature for 24 h. The solvents were removed under nitrogen, and the residue was extracted by the method of Bligh & Dyer (1959) using 0.1 N HCl as the aqueous component. The lower phase was washed 3 times with preequilibrated acidic upper phase to remove pyridine, concentrated under nitrogen, and applied to a preparative thin-layer chromatography plate (2-mm thickness) that had been prerun twice in acetone and dried at 70 °C. The chromatogram was developed in heptane–diethyl ether–acetic acid (25:75:1 v/v). The material of greatest R_f (0.76) was visualized by ultraviolet light and scraped off the plate. Dioleoylthiomethylthioglycerol was eluted with chloroform and stored at −20 °C. Prior to use the protecting thiomethyl group was removed by reacting dioleoylthiomethylthioglycerol in chloroform containing 1% pyridine, with a 30-fold excess of dithiothreitol at 45 °C for several hours. After the chloroform was removed under a stream of nitrogen, the products were partitioned between heptane and water. The heptane layer, containing the dioleoylthioglycerol, was washed once with water.

Both the dioleoylglycerol and the dioleoylthiomethylthioglycerol gave one spot on thin-layer chromatograms run in heptane–diethyl ether–acetic acid (25:75:1 v/v). Analysis of dioleoylglycerol by mass spectrometry gave an M^+ peak at *m/z* 620, as expected. Other prominent peaks were seen at 602 (loss of water), 339 (loss of oleate), and 264 (oleoyl cation).

Dioleoylthiomethylthioglycerol (Figure 2B) gave a small M^+ peak at *m/z* 682 (insert), and more prominent peaks at 603 (loss of –S–S–CH_3), 401 (loss of oleate), 321 (loss of oleate and –S–S–CH_3), and 264 (oleoyl cation), confirming the predicted structure. The ratio of free sulfhydryl to ester was found to be 1.05/2.00.

Preparation of Vesicles. Phospholipid vesicles were prepared according to Mimms et al. (1981). Lipids (5 μmol) were dried from chloroform to give a thin film in a test tube. Buffer (0.5 mL of 25 mM Tris, 25 mM potassium phosphate, 5 mM EDTA) was added to the lipid film, and a 25-fold molar excess

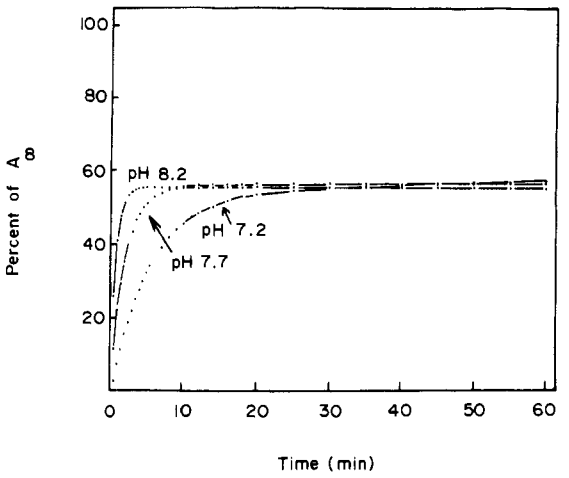

FIGURE 3: Titration of phosphatidylthioglycerol, incorporated into unilamellar phospholipid vesicles, with DTNB. Absorbance at 412 nm was read directly from a digital spectrophotometer at various times after addition of DTNB and corrected for light scattering. Total A_{412} was determined in the presence of detergent.

Table I: Kinetic Analysis of DTNB Titration and Leakage

		half-times		
pH	outer/inner[a]	first phase (min)[b]	second phase (h)[b]	leakage (h)[c]
7.2	56/44	4.0	25	30
7.7	57/43	1.43	90	81
8.2	54/46	0.50	190	230

[a] Inferred distribution of phosphatidylthioglycerol between monolayers. [b] Reaction of DTNB with phosphatidylthioglycerol in outer and inner monolayers. [c] Leakage of DTNB trapped in vesicles.

of octyl glucoside was added. A clear solution resulted. This mixed-micellar solution was applied to a 1 × 16.5 cm column of Sephadex G-50 at room temperature and eluted at a flow rate of about 0.5 mL/min. Vesicle-containing fractions were identified by light scattering and pooled.

Results

Transmembrane Movement of Phosphatidylthioglycerol. Vesicles were prepared from phosphatidylcholine, phosphatidylglycerol, and phosphatidylthioglycerol with a mole ratio of 8:1:1. A sample of these vesicles was suspended in buffer containing DTNB, 1 mM final concentration, and the absorbance at 412 nm was followed for 1 h at room temperature. The data were corrected for light scattering by subtracting the absorbance of vesicles in the absence of DTNB and expressed as percent of infinite absorbance, which was determined by measuring A_{412} with 0.2% Triton X-100 included. Time courses for this reaction at pH 7.2, 7.7, and 8.2 are shown in Figure 3. In each case the vesicles were prepared at the indicated pH so the pH was the same inside and outside the vesicles.

After an initial rapid reaction, the absorbance reached a plateau value of about 55%, after which the absorbance increased very slowly. These kinetics were analyzed by fitting the data to mathematical models of the sum of two exponential reactions. The results of such an analysis are presented in Table I.

The pool of phosphatidylthioglycerol that reacts rapidly is presumed to be that in the outer leaflet of the bilayer, whose sulfhydryl groups are accessible to the external, nonpenetrating DTNB. The pool that reacts slowly is presumed to be that in the inner leaflet. The distribution of this analogue between these two pools is shown in the second column and is seen to

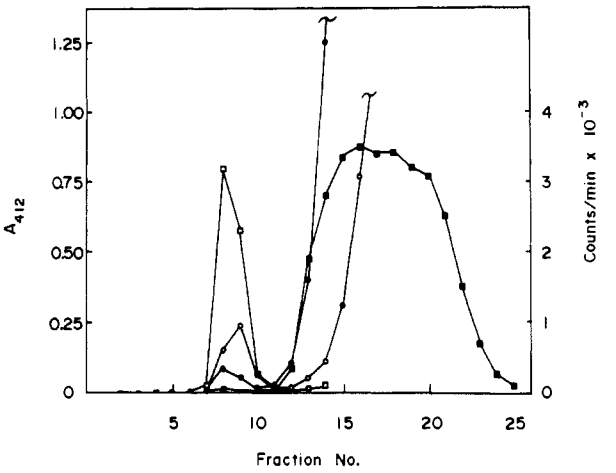

FIGURE 4: Trapping of DTNB in vesicles. Vesicles were prepared by octyl glucoside removal on a Sephadex G-50 column as described in the text. Open circles show A_{412} of fractions directly. This represents light scattering for fractions 8–10 and absorbance due to DTNB after fraction 12. The closed circles present absorbance of external DTNB, that which is accessible to 1 mM glutathione. Open squares show the increase in absorbance resulting from addition of 10 mM β-mercaptoethanol, which reacts with trapped DTNB. Closed squares present the distribution of [^{14}C]octyl glucoside.

be independent of pH, with about 55% present in the outer monolayer.

The third column shows the half-times, expressed in minutes, of the first phase of the reaction. These values represent the averages from two determinations that differed from one another by no more than 10%. This parameter is clearly pH dependent, and the pH dependence of the rates is approximately what would be expected for a disulfide exchange reaction that requires ionization of the sulfhydryl group (Fava et al., 1957). These rates are considerably slower than those seen in the presence of detergent. We attribute this to an electrostatic interaction with the negatively charged vesicle surface, since the rate of reaction of DTNB with vesicles is considerably enhanced by molar concentrations of salt (data not shown).

The half-times, in hours, of the slow second phase are presented in the fourth column. These values are the averages of two determinations that differed by as much as 50%.[2] This parameter is also pH dependent but in the opposite direction from the first phase, suggesting that the rate of the second phase is limited by a step that requires the protonation of an ionizable group. The reaction of external DTNB with phosphatidylthioglycerol of the inner leaflet indicates either flipping of the lipid head group across the bilayer or leakage of DTNB into the lumen of the vesicles. The latter possibility was tested by measuring the rate of leakage of DTNB out of vesicles in which it had been trapped.

Leakage of DTNB Trapped in Vesicles. Vesicles were prepared from phosphatidylcholine and phosphatidylglycerol with a mole ratio of 8:2. The buffer used to resuspend the lipid film contained 20 mM DTNB, and the column was equilibrated to one-fourth of its length with 20 mM DTNB in buffer. The vesicles, which contained trapped DTNB, emerged from the column ahead of the major band of DTNB-containing buffer.

Figure 4 illustrates the trapping procedure. Light scattering was measured at 412 nm in the absence of a sulfhydryl compound (open circles). The absorbance in fractions 8–10 is due

[2] Although the error was in some cases quite large, due to the relatively short assay time, the difference in magnitudes at different pHs was clearly greater.

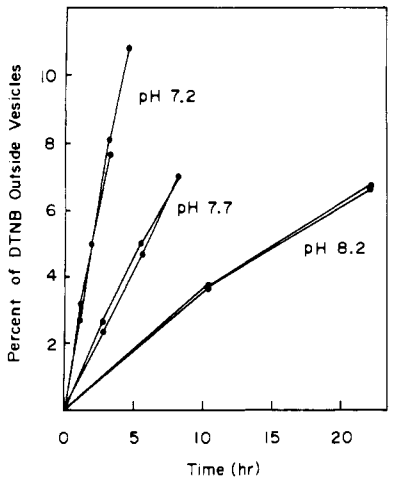

FIGURE 5: Leakage of DTNB trapped in phospholipid vesicles. DTNB (20 mM) was trapped in vesicles, and the amount that had leaked out at room temperature was determined at various times by reaction with 1 mM glutathione.

chiefly to light scattering as these fractions were visibly turbid. After fraction 13, the background color of DTNB increased noticeably. Adding 1 mM glutathione, a nonpenetrating sulfhydryl compound, to a portion of the vesicle suspension and subtracting absorbance due to scattering reveals external DTNB (solid circles). The open squares show the increase in A_{412} occurring upon addition of 10 mM β-mercaptoethanol, which permeates the vesicles and reacts with trapped DTNB. Beyond fraction 14 this increase becomes a very small difference between two very large numbers and is therefore not presented. A trace of [^{14}C]octyl glucoside was included in the solubilization mixture (about 1600 cpm/μmol), and the solid squares present the distribution of radioactivity. The small amount of ^{14}C in the vesicle-containing fractions (8–10) indicates an octyl glucoside:phospholipid mole ratio of about 1:100.

Leakage of DTNB out of vesicles was measured by determining the amount of DTNB outside of the vesicles at various times. A portion of the vesicles was incubated at room temperature, and at various intervals the A_{412} of a sample was measured in the presence of 1 mM glutathione. The data were corrected for light scattering and expressed as percent of total absorbance, which, as above, was determined in the presence of 0.2% Triton X-100.

Time courses of the leakage of up to 10% of the trapped DTNB at pH 7.2, 7.7, and 8.2 are shown in Figure 5. From the initial rates, half-times for the process were calculated and are presented in the last column of Table I. The close correlation between these values and those for the slow second phase of the reaction between DTNB and phosphatidylthioglycerol leads us to conclude that this second phase represents the leakage of DTNB into the vesicles and that transmembrane movement of phosphatidylthioglycerol is extremely slow, with a half-time in excess of 8 days.

Permeability Coefficient of DTNB. Figure 6 presents the size distribution of lipid vesicles containing trapped DTNB, determined from an electron micrograph. From these data, a number-average vesicle volume was calculated and found to be 1.3×10^{-21} cm^3. The average vesicle volume was independently calculated from the ratio of included volume to moles of phospholipid as described by Mimms et al. (1981). Assuming efficient trapping by the above procedure, the volume inside vesicles was 5.1 μL/mL of vesicle suspension, determined by measuring included DTNB. The phospholipid concentration of the suspension was found to be 2.8 μmol/mL.

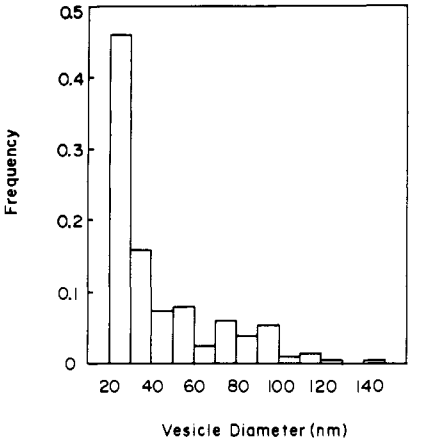

FIGURE 6: Size distribution of vesicles containing trapped DTNB.

The ratio of these numbers indicates an average volume of 1.6×10^{-21} cm^3, in good agreement with the above value.

The rate constant for DTNB leakage from vesicles at pH 8.2, calculated from the half-time, was 3.0×10^{-3} h^{-1}, or 8.4×10^{-7} s^{-1}. The permeability coefficient (P) of DTNB was calculated by using the relationship P(membrane area/vesicle) = k (internal volume/vesicle) and was found to be about 1×10^{-12} cm/s. This value is comparable to permeability coefficients determined for Na^+ (Mimms et al., 1981) and indicates that these vesicles are tightly sealed.

Transmembrane Movement of Dioleoylthioglycerol. Vesicles containing phosphatidylcholine, *sn*-1,2-dioleoylglycerol, and dioleoylthioglycerol with mole ratio 38:1:1 were prepared. Titration of the sulfhydryl groups in these vesicles with DTNB at pH 8.2 revealed a single fast-reacting component with a half-time of less than 15 s. However, the color yield ranged from 30 to 50% of what was expected, and thin-layer chromatographic analysis of lipids extracted from vesicles prior to DTNB treatment indicated that the dioleoylthioglycerol had oxidized to the disulfide form. The half-time for leakage of DTNB from vesicles composed of phosphatidylcholine and *sn*-1,2-dioleoylglycerol (19:1 mole ratio) was measured as above and found to be 22 h. This is about an order of magnitude faster than that seen with phosphatidylcholine–phosphatidylglycerol vesicles (Table I).

Discussion

Since the primary function of biological membranes is to separate functionally distinct aqueous compartments, it is to be expected that membrane-associated functions are asymmetrically disposed across the plane of the membrane bilayer. Results from this laboratory and others have shown that phospholipid synthesis in biogenic membranes such as endoplasmic reticulum and the bacterial cytoplasmic membrane is asymmetric (Vance et al., 1977; Bell et al., 1981; Rothman & Kennedy, 1977; Hutson & Higgins, 1982). In the case of endoplasmic reticulum, it has been demonstrated that the active sites of all the glycerolipid biosynthetic enzymes face the cytoplasmic, rather than the lumenal, side of the membrane (Bell et al., 1981). Therefore, some mechanism must exist for translocation of phospholipids made on one side of the bilayer to the other side.

The transmembrane movement of phospholipids would be expected to be an energetically unfavorable process, and measurement of the rates of transbilayer movement of phosphatidylcholine and phosphatidylethanolamine in unilamellar lipid vesicles gives half-times from 4 days to greater than 80 days (Rothman & Davidowicz, 1975; Johnson et al., 1975; Roseman et al., 1975; DiCorleto & Zilversmit, 1979). Rates

of this magnitude clearly could not account for the doubling of membrane mass every 24 h in the case of mammalian cells in culture or every 30–60 min in the case of exponentially growing bacteria. In fact, half-times for transmembrane movement of phosphatidylethanolamine and phosphatidylcholine in rat liver endoplasmic reticulum, and phosphatidylethanolamine in bacterial cytoplasmic membranes, have been estimated to be less than 5 min (Hutson & Higgins, 1982; van den Besselaar et al., 1978; Rothman & Kennedy, 1977). This rapid rate is not characteristic of all biological membranes, however.

In rat erythrocyte membranes, transmembrane movement of phosphatidylcholine has been reported to have a half-time of 2.3–11 h [Bloj & Zilversmit, 1976; Renooij et al., 1976; Crain & Zilversmit, 1980; reviewed by Thompson (1978)]. A similar rate has been observed in spikeless vesicular stomatitis virions (Shaw et al., 1979), whereas in influenza virus membranes the half-time has been shown to be greater than 10 days (Rothman et al., 1976). Slow rates of transmembrane movement of phosphatidylcholine, on the order of 1–4 days, have also been reported in the inner mitochondrial membrane of rat liver and in the plasma membrane of LM cells (Rousselet et al., 1976; Sandra & Pagano, 1978).

Since all biological membranes do not show the very high rate of transmembrane phospholipid movement seen in endoplasmic reticulum and bacterial cytoplasmic membranes, it may be concluded that the presence of substantial amounts of membrane protein is not in itself sufficient to promote this movement. A study of the energetics of transmembrane movement of phosphatidylethanolamine in *B. megaterium* membranes suggests that this movement is not dependent on metabolic energy (Langley & Kennedy, 1979), and the authors concluded that there must be processes or structures unique to these membranes that facilitate transbilayer movement. What might be the nature of such a process? Bretscher (1974) has proposed that specific membrane proteins may exist whose function is to stimulate translocation of lipids. From the difference in translocation rates between dioleoylthioglycerol and phosphatidylthioglycerol, it appears that there is no great hindrance to movement of the diacylglyceryl moiety across the membrane and that the primary barrier is to the charged head group. Proteins that promote the transport of phosphate, glycerol, and glycerol phosphate across membranes are known (Lin et al., 1962; Sanno et al., 1968; Willsky et al., 1973; Sprague et al., 1975). A phospholipid "translocase" protein might function in an analogous manner, by providing a hydrophilic channel through which the head group could traverse the hydrophobic core of the bilayer.

The study of this process has been impeded by the lack of a convenient assay for transmembrane movement. Presently used methods suffer from several drawbacks. The use of impermeant probes, such as trinitrobenzenesulfonic acid and various phospholipases, to chemically modify bulk lipid requires time-consuming extractions, separations, and quantitations to measure products. Furthermore, these methods result in significant structural perturbation of the bilayer. Assay methods that produce a minimal perturbation of the bilayer structure, such as those employing phospholipid transfer proteins or nuclear magnetic resonance, have their own handicaps. The use of phospholipid transfer proteins involves long incubations, making kinetic analysis on short time scales difficult. To study transmembrane movement by nuclear magnetic resonance, it is necessary first to create an asymmetric bilayer by using a method such as transfer protein-mediated exchange.

The assay we have described herein circumvents most of these problems. Once vesicles containing a sulfhydryl analogue have been prepared, spectrophotometer traces may be easily obtained under various conditions with very few manipulations. Kinetic analysis is as easy as that for any spectrophotometric assay. The analogue need comprise only 2–3% of the total phospholipid to generate a significant signal. The product of the reaction between DTNB and phosphatidylthioglycerol is a mixed disulfide of 5-thio-2-nitrobenzoic acid with the lipid, which remains associated with the bilayer. Incorporation of this lipid product into vesicles in place of phosphatidylthioglycerol does not affect their permeability to DTNB (data not shown), so this assay does not appear to perturb the bilayer structure significantly. Finally, the leakage of trapped DTNB provides a very convenient control for the integrity of the vesicles.

Other researchers have used a similar method for measuring the rate of transbilayer movement of thiocholesterol by titration with DTNB (Huang et al., 1970; Dawidowicz & Backer, 1981). Dawidowicz and Backer reported that, for unknown reasons, their color yield was about 70% of that predicted. When we measured the rate of transmembrane movement of dioleoylthioglycerol, the color yield was 30–50% of what we expected. Thin-layer chromatographic analysis of the neutral lipids extracted prior to DTNB treatment, from vesicles prepared with phosphatidylcholine, dioleoylglycerol, and dioleoylthioglycerol, showed the presence of four compounds: 1,2-dioleoylglycerol, 1,3-dioleoylglycerol, dioleoylthioglycerol, and an unknown lipid migrating just ahead of the latter. Treatment of this neutral lipid fraction with dithiothreitol converted the unidentified lipid to dioleoylthioglycerol, confirming our suspensions that the dioleoylthioglycerol had oxidized during vesicle preparation. Neither careful degassing of buffers nor inclusion of β-mercaptoethanol or BHA in the mixed-micellar solution inhibited this oxidation. Interestingly, despite the sensitivity of dioleoylthioglycerol and, possibly, thiocholesterol to oxidation, we have never observed the formation of oxidized phosphatidylthioglycerol in vesicle preparations. Perhaps the local environment of the sulfhydryl group, whether at the hydrophobic/water interface or extending well into the water phase, affects its susceptibility to oxidation.

Phosphatidylthioglycerol provides a means for conveniently determining the dynamics of phosphatidylglycerol in bilayer systems. The localization of phosphatidylglycerol has been investigated in the membrane of *Micrococcus lysodeikticus* and *Acholeplasma laidlawii* by using combinations of lipid transfer proteins and phospholipases (Barsukov et al., 1976; Bevers et al., 1978) and in bacteriophage PM2 by reaction with sulfanilic acid diazonium salt (Schafer et al., 1974). Studies of the distribution of this lipid in unilamellar liposomes have been briefly reviewed by Lentz et al. (1980), who used periodate oxidation followed by chemical determination of released formaldehyde to quantify accessible phosphatidylglycerol. Using this method these workers were able to measure a minor redistribution of phosphatidylglycerol in vesicles induced by divalent cations (Lentz et al., 1982). However, the unique chemistry afforded by a sulfhydryl analogue greatly facilitates such measurements. Our results, which represent the first attempt to measure spontaneous transbilayer translocation of phosphatidylglycerol, are consistent with previous conclusions that phospholipid movement across lipid bilayers is extremely slow.

We plan to extend the application of this assay, using a larger selection of sulfhydryl analogues, to test the hypothesis that specific membrane proteins facilitate the transbilayer

translocation of phospholipids in biogenic membranes. Sulfhydryl lipid analogues may be useful in other areas of lipid metabolism. For example, they might be useful as readily traceable probes in studies of intracellular lipid movement. Alternatively, phospholipid analogues with sulfhydryl groups in the polar head group could be convenient substrates for phospholipases C and D, whose water-soluble products could be titrated by DTNB. Lipid analogues with esterified sulfhydryl groups in the hydrophobic portion have already proved useful for this purpose (Aarsman & van den Bosch, 1981).

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Registry No. DTNB, 69-78-3; dioleoylphosphatidylthioglycerol, 91860-73-0; dioleoylthioglycerol, 91860-74-1; dioleoylphosphatidylthiomethylthioglycerol trimethylsilyl derivative, 91860-75-2; thioglycerol, 96-27-5; methyl methanethiosulfonate, 2949-92-0; thiomethylthioglycerol, 60033-22-9; oleic anhydride, 24909-72-6; dioleoylthiomethylthioglycerol, 91860-76-3; *sn*-1,2-dioleoylglycerol, 24529-88-2.

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