Phospholipid Asymmetry in LM Cell Plasma Membrane Derivatives: Polar Head Group and Acyl Chain Distributions†

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ABSTRACT: The transbilayer distribution of the major phospholipids in mouse LM cell plasma membrane derivatives was studied. Cells were grown on various radiolabeled phospholipid precursors and subsequently allowed to phagocytose latex spheres (0.5–2 μm diameter). The resulting phagosomes, assumed to be inside-out plasma membrane derivatives, were isolated and purified from cell homogenates, and subsequently characterized with respect to lipid composition and plasma membrane markers. The distribution of phosphatidylcholine (PC) in the isolated phagosomes was determined by use of purified beef liver PC-specific exchange protein. The exchange protein catalyzed PC exchange from the radiolabeled latex phagosomes to either unilamellar PC vesicles or unlabeled phagosomes, present in excess. The kinetics of this process are consistent with two exchangeable PC pools, with ~52% being readily exchangeable. Phosphatidylethanolamine (PE) distribution was determined by trinitrobenzenesulfonic acid (TNBS) labeling. TNBS labeling of isolated phagosomes rapidly converted approximately 70% of PE to the Tnp derivative. In contrast, when TNBS labeling of intact cells was carried out, followed by feeding of latex beads and isolation of the phagosomes, only about 24% of the PE was derivatized. These data are consistent with a transbilayer distribution in which PE is enriched on the cytoplasmic face of the plasma membrane, while PC is approximately equally distributed. From indirect evidence, it is concluded that the remaining major phospholipid, sphingomyelin, is enriched on the external half of the membrane. The transbilayer acyl chain composition of these phospholipids was determined using phagosomes from cells grown on [^{3}H]acetate. PC (and PE) from the cytoplasmic face of the bilayer was hydrolyzed and its acyl chain composition determined by radio gas–liquid chromatographic analysis. These data were compared with the acyl chain composition of total PC (and PE) extracted from phagosomes. No major differences could be detected in the transbilayer distribution of acyl chains within a phospholipid class, but differences in acyl chain composition among lipid classes result in an apparent enrichment of unsaturated fatty acids on the cytoplasmic face of the phagosome bilayer.

The transbilayer distribution of phospholipids within the plasma membrane has been determined in the red blood cell by the use of specific phospholipases (Renooij et al., 1976), covalent coupling reagents (Gordesky et al., 1975), and phospholipid exchange proteins (PLEP)[1] (Bloj and Zilversmit, 1976). In addition, these methods have been applied to the determination of phospholipid asymmetry in enveloped viruses which obtain a portion of the host cell plasma membrane as a result of a budding process (Rothman et al., 1976). All of these studies have concluded that the lipid bilayer is asymmetric with respect to the distribution of at least some phospholipid species. The fact that such studies have been limited to relatively simple membrane systems emphasizes the formidable problems of obtaining plasma membrane preparations which are free of contaminating lipids, sealed, and of uniform sidedness (Rothman and Lenard, 1977).

In order to extend the study of membrane asymmetry to more complex eukaryotic cells, we have taken advantage of a general membrane mediated event characteristic of many metabolically active cells. When such cells are suspended in the presence of inert latex particles of defined size, the particles are rapidly ingested as membrane bound phagosomes. In some systems, these isolated latex phagosomes have been shown to have a lipid (Ulsamer et al., 1971) and protein (Hubbard and Cohn, 1975) composition similar to that of isolated plasma membranes and, from the topography of the phagocytotic process, presumably preserve an inside-out membrane sidedness.

In the present study we have used the latex phagosome system to obtain plasma membrane derivatives in cultured mouse LM cells. These cells can be grown without serum and are capable of readily incorporating exogenous fatty acids into cellular phospholipids (Wisnieski et al., 1973; Williams et al., 1974; Ferguson et al., 1975). In addition, a great deal of information is available on the chemical and physical properties of normal LM cell membranes and those having an altered phospholipid composition (Schroeder et al., 1976; Esko et al., 1977; Wisnieski and Iwata, 1977). Using these phagosomes as membrane derivatives, we have determined both polar head group and acyl chain transbilayer distribution of the major LM cell membrane phospholipids.

Materials and Methods

Cell Cultures and Labeling. Mouse LM cells were obtained from the American Type Culture Collection (Rockville, Md.) and grown in complete medium consisting of Eagle's minimal essential medium (GIBCO, Grand Island, N.Y.) supplemented with 0.5% Bactopeptone (DIFCO, Detroit, Mich.), 50 mg/L gentamycin (Schering, Kenilworth, N.J.), and 2.2 g/L $NaHCO_3$, pH 7.4. Monolayer cultures were grown at 37 °C in a water-saturated atmosphere of 5% CO_2 in air.

Cells were radiolabeled as follows. (i) Methyl[^{3}H]choline chloride (4.2 Ci/mmol) was added to cultures in complete

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[1] Abbreviations used: DOPC, dioleoylphosphatidylcholine; GLC, gas–liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLEP, phospholipid exchange protein; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin-layer chromatography; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TnpPE, *N*-2,4,6-trinitrophenylphosphatidylethanolamine; Tris, tris(hydroxymethyl)aminomethane.

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medium minus choline at a final concentration of 2 μCi/mL. (ii) Carrier-free [^{32}P]orthophosphate was added to cultures at 20 μCi/mL in medium containing 0.1× the normal phosphate concentration. (iii) Sodium [^{3}H]acetate (686 mCi/mmol) was added to cultures at 20 μCi/mL. (iv) [^{3}H]Palmitic acid (340 μCi/mmol) was coupled to defatted bovine serum albumin (Chen, 1967) by the method of Spector and Hoak (1969). This complex was added to complete medium at 1 μCi/mL. Cells were generally grown on radiolabeled precursors for times up to 96 h, except for [^{3}H]acetate (up to 72 h). Our results on PC and PE asymmetry were not significantly affected by varying the labeling time from 48 to 96 h, suggesting that asymmetry of labeling is unlikely. All radioisotopes were obtained from New England Nuclear Corp. (Boston, Mass.).

Lipid Extraction and Identification. Lipids were extracted from isolated latex phagosomes with 95% ethanol (Ulsamer et al., 1969) followed by evaporation under argon and extraction of the residue with chloroform–methanol (Bligh and Dyer, 1959). The latter method was also used for cell homogenates. Lipid extraction with ethanol was complete as judged by the release of ~99% of the radioactivity from [^{3}H]choline or [^{3}H]palmitate labeled phagosomes.

Phospholipids were separated by two-dimensional TLC on 20 × 20 cm silica gel plates (EM Laboratories, Elmsford, N.Y.) using (i) chloroform–methanol–28% ammonium hydroxide (65:25:5) and (ii) acetone–chloroform–methanol–acetic acid–water (40:30:10:10:5) as the solvent systems. In the case of TNBS labeled material, chloroform–methanol–water (65:25:4) was substituted for the first dimension. For ^{3}H-labeled extracts, appropriate phospholipid standards were included so that spots could be visualized by brief exposure to iodine vapor. For ^{32}P-labeled phospholipids, spots were detected by autoradiography using Kodak RPX-Omat film. Radioactive lipid spots were scraped and counted in Triton–toluene scintillation fluid.

Extracted [^{3}H]acetate-labeled phospholipids to be analyzed for acyl chain composition were separated by TLC after the addition of ^{32}P-labeled phospholipid standards. The TLC plates were covered with x-ray film to localize the desired phospholipids, which were then processed for GLC. Fatty acids were transmethylated by incubation with methanol–3% HCl for 24 h at 40 °C in an argon atmosphere. SM was hydrolyzed and transmethylated as described (Shinitzky and Barenholtz, 1974). Fatty acid methyl esters were extracted with hexane (Litman, 1975). Following hydrolysis, ^{32}P from the lipid standards quantitatively partitioned into the aqueous phase, and ^{3}H into the organic phase. GLC analysis of the methyl esters and appropriate standards was carried out using a Hewlett-Packard Model 5711A instrument (Avondale, Pa.) equipped with an effluent splitter and gas fraction collector (Packard Instruments, Downer's Grove, Ill.).

Preparation and Characterization of Ingested Latex Beads. Latex beads were obtained from Dow Chemical Co., Indianapolis, Ind. Both polystyrene beads (diam. = 0.500, 0.760, and 0.945 μm; density = 1.05 g/mL) and polyvinyltoluene beads (diam. = 2.02 μm; density = 1.027 g/mL) were used. Beads were sonicated for 60 s and washed three times with phosphate buffered saline to remove any contaminating material (Weisman and Korn, 1967).

Radiolabeled LM cells were washed six times with ice-cold phosphate-buffered saline in situ, removed from the culture dish using a Teflon policeman, centrifuged, and resuspended in LM medium. The cells were incubated with ~3000 beads/cell at 37 °C from 15 to 60 min with continuous shaking. Bead uptake was terminated by placing the cell suspension on ice and centrifuging them through fetal calf serum or 8% bovine serum albumin in phosphate-buffered saline (10 min, 250*g*). The cell pellet was twice resuspended in phosphate-buffered saline and centrifuged for 5 min at 1000*g*. As assessed by phase-contrast microscopy, virtually no free beads were found in this pellet. Electron microscopy revealed that these beads were either within the cell or in the process of being phagocytosed. The beads were isolated from the cells by a modification of the method of Hubbard and Cohn (1975). Briefly, washed cells were suspended in a minimal volume of 0.01 M Tris-HCl, pH 7.4, and 1 mM $MgCl_2$ for 3 min. The suspension was brought to 40% sucrose (w/v) and homogenized with 5–10 strokes in 10 mM Tris-HCl, pH 7.4, using a tight Dounce homogenizer. One milliliter of the homogenate was overlayered with 1.5 mL each of 27% and 10% sucrose, 1 mL of 5% sucrose, and 0.5 mL of 10 mM Tris-HCl, pH 7.4, and centrifuged at 100 000*g* for 90 min in a SW 50.1 rotor. The latex beads were carefully removed from the 27–10% sucrose interface in the case of the polystyrene beads and the 10–15% interface for the polyvinyltoluene material. The beads were diluted with 10 mM Tris-HCl, pH 7.4, 100 mM KCl, pelleted by centrifugation for 2 min in an Eppendorf microfuge, and washed twice with the same buffer.

The following enzyme assays were performed on isolated latex phagosomes as well as cell homogenates using commercially available (Sigma, St. Louis, Mo.) enzyme assay kits: 5′-nucleotidase, lactic dehydrogenase, and acid phosphatase.

Phosphatidylcholine Exchange Protein Assays. PC-specific exchange protein was isolated from beef liver and purified according to the procedure for Kamp et al. (1973). The preparation had a specific activity of 7000 units/mg protein, determined by the red blood cell ghost-vesicle assay (Rothman et al., 1976). The exchange protein was stored at −20 °C in 50% glycerol and dialyzed against 100 mM KCl, 10 mM 2-mercaptoethanol, and 10 mM Tris-HCl, pH 7.4, in the presence of 0.1 mg of bovine serum albumin/mL.

PC exchange reactions between vesicles and latex beads were carried out at 37 °C with continuous shaking in 1 mL of 100 mM KCl, 5 mM mercaptoethanol, 0.2% NaN_3, 10 mM Tris-HCl, pH 7.4, 0.1 mg of bovine serum albumin mL, and 20 units/mL PLEP. [^{3}H]Acetate, [^{3}H]choline, or ^{32}P-labeled latex phagosomes were incubated with sonicated DOPC vesicles which were present in approximately a 30-fold excess lipid phosphorus. The reaction was stopped by chilling to 20 °C and then centrifuged for 2 min on an Eppendorf microfuge to pellet the latex beads. In control incubations, PLEP was omitted. Aliquots of the supernatant or pellet were removed for phospholipid analysis and radioactivity determination. The fraction of radiolabeled PC transferred from beads to vesicles was calculated from the loss of PC radioactivity in the beads after incubation in the presence of PLEP minus the loss after incubation without PLEP. Alternatively, transfer was determined by the increase in PC radioactivity in the supernatant containing the vesicles.

PC exchange reactions between radiolabeled (0.945 μm; 1.05 g/mL) and unlabeled (2.02 μm; 1.027 g/mL) latex beads were performed as above. Control experiments were carried out either with noningested (2.02 μm) beads in excess, or without PLEP. After incubation, the beads were pelleted, suspended in 40% sucrose, and separated on a discontinuous sucrose gradient. The gradient was fractionated and analyzed for radioactivity, and the fraction of PC transferred from donor to acceptor beads calculated.

TNBS Labeling. Isolated ^{32}P-labeled latex phagosomes were reacted with TNBS by incubating the beads for various

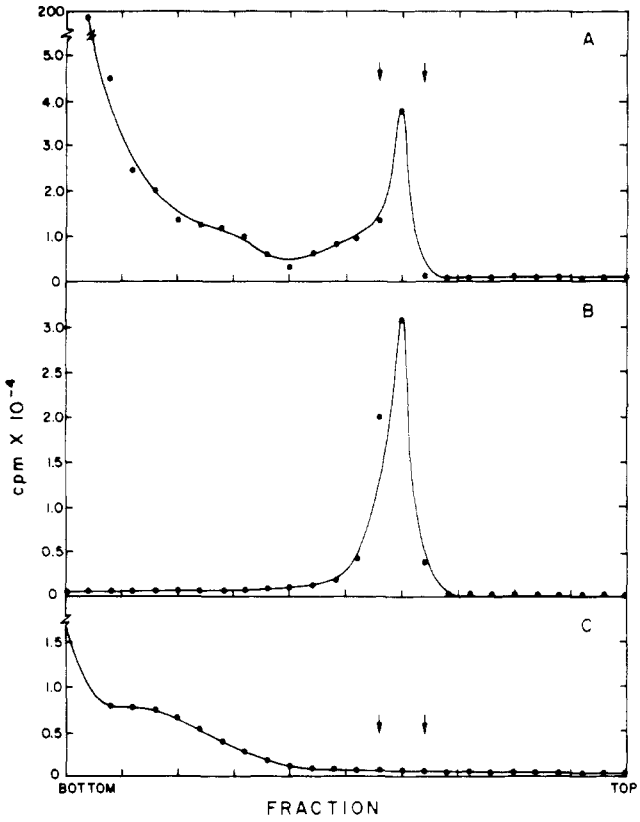

FIGURE 1: Isolation of latex phagosomes from [^{3}H]choline-labeled LM cells on sucrose step gradients. (A) Bead-containing cell homogenate; arrows represent position of latex beads; (B) isolated latex phagosomes rerun under conditions identical with A; (C) homogenate of [^{3}H]choline-labeled cells containing no ingested beads.

times at either 4, 20, or 37 °C in Hanks' balanced salt solution buffered with 1.8% $NaHCO_3$, pH 8.5. The final TNBS concentration was 5 mM, diluted from a freshly made aqueous stock solution. The reaction was stopped by either the addition of 0.5 N HCl or complete LM medium. Beads were centrifuged for 2 min and washed once with the same solution. Phospholipids were extracted and analyzed by two-dimensional TLC. The plates were autoradiographed to determine the positions of radioactive PE and TnpPE, which were scraped and counted. Control plates containing authentic [^{32}P]PE and Tnp[^{32}P]PE were run in each experiment.

Intact cells were reacted with TNBS by suspending washed ^{32}P-labeled LM cells in Hanks' balanced salt solution containing 1.8% $NaHCO_3$ at pH 8.5, and 5 mM TNBS. The reaction was stopped by addition of a large volume of LM medium. The cells were washed and incubated 30 min at 37 °C with 0.945 μm latex beads, and the phagocytosed beads isolated and analyzed for radioactive PE and TnpPE.

Other Materials and Analytical Methods. Cholesterol was purchased from Applied Science Laboratories, State College, Pa. TNBS was a gift from Dr. B. Litman. DOPC was synthesized and unilamellar vesicles prepared by sonication as previously described (Huang and Pagano, 1975). Inorganic phosphate was determined by the method of Bartlett (1959). Protein was determined by the method of Lowry et al. (1951).

Results

Characterization of Ingested Beads. Ingested latex beads from [^{3}H]choline labeled cells were isolated on sucrose density gradients. Fractionation of the gradients revealed a peak of radioactivity banding in the same position as the beads (arrows, Figure 1A). When the fractions comprising this peak were pooled and rerun under the same conditions, the beads and radioactivity were again found to comigrate (Figure 1B). The radioactivity in the peak was more than 99% chloroform–methanol extractable and accounted for approximately 1% of the total chloroform–methanol extractable radioactivity and about 1% of the lipid phosphorus in the cell homogenate. When labeled cell homogenates were run in the absence of beads, no radioactivity was detected at the position ordinarily occupied by beads (Figure 1C). When phagocytosis of beads was inhibited by incubating cells and beads at 2 °C, only negligible amounts of radioactivity became associated with the isolated beads. Approximately 80% of the radioactivity associated with the phagocytosed beads could be released by first subjecting the isolated phagosomes to three 1 min bursts of sonication followed by a low-speed centrifugation (2 min, 8000g) to pellet the beads.

The ingested phagosomes were further characterized by examination of their radiolabeled phospholipids (Table I). Ingested beads from cells radiolabeled with choline, palmitate, orthophosphate, or acetate were enriched in both radiolabeled SM and PE relative to PC when compared with whole cell homogenates. Furthermore, the amount of radiolabeled lipid present on the isolated latex phagosomes derived from [^{3}H]-choline or [^{3}H]palmitate labeled cells was found to be directly proportional to the surface area of the ingested latex beads. This suggests that each latex bead is enveloped by a portion of the LM cell plasma membrane during phagocytosis.

Biochemical analyses of enzyme activities associated with the beads and cell homogenates were also carried out. No lactic dehydrogenase activity was detected in the phagosome preparation whereas 5′-nucleotidase was enriched sixfold in the phagosomes compared to the cell homogenate (3 vs. 0.5 μM h^{-1} (mg of cell protein)$^{-1}$). Acid phosphatase activity was detected in both cells and phagosomes, with the phagosome activity enriched fourfold over that found in the homogenate (10.3 vs. 2.5 μM h^{-1} (mg of cell protein)$^{-1}$).

PC Asymmetry: Phagosome–Vesicle Exchange. When radiolabeled phagosomes were incubated with PLEP and an excess of sonicated DOPC acceptor vesicles, bead PC was transferred to the vesicles. The specificity for PC in this PLEP catalyzed exchange reaction is shown in Figure 2A, in which the amount of ^{32}P-labeled phagosome lipids transferred to vesicles in the presence and absence of PLEP is given. It is apparent that PC is the only phospholipid transferred to the acceptor vesicles to any appreciable degree. Furthermore, the background levels of lipid transfer found in the absence of PLEP were never more than 10–15% of the total exchange. While PC is the only phospholipid that was exchanged by PLEP, the presence of other ^{32}P-labeled phospholipids and other minor radiolabeled components could be detected in both PLEP containing and control incubations. This is seen in Figure 2B, which shows an autoradiogram of the ^{32}P-labeled lipids transferred to acceptor vesicles in an exchange reaction. We wish to emphasize that the lipid extractable portion of this background material comprised a complex mixture of lipids which are not well separated from PC by TLC in one dimension. Therefore to minimize the possibility of comigration of unidentified minor components with exchanged PC, two-dimensional TLC was used for all analyses.

Figure 3 shows the kinetics of PLEP-catalyzed exchange between DOPC vesicles and phagosomes isolated from cells grown in [^{3}H]choline. When the fraction of PC remaining on the phagosomes was plotted on a logarithmic scale vs. incubation time with PLEP, two kinetic components were observed.

TABLE I: Percent Radioactivity in Phospholipid of LM Cell Homogenates and Ingested Latex Beads.[a]

	[^{3}H]Choline		[^{3}H]Palmitate		[^{32}P]Orthophosphate		[^{3}H]Acetate	
	Cells	Beads	Cells	Beads	Cells[b]	Beads	Cells	Beads
PC	85.5 ± 2.9	78.2 ± 2.8	72.5 ± 6.1	42.1 ± 4.2	55.2 ± 4.1	40.4 ± 2.1	53.2 ± 0.9	44.6 ± 0.4
PE			14.6 ± 1.4	34.3 ± 2.0	25.9 ± 0.9	35.0 ± 1.0	22.2 ± 0.3	31.5 ± 0.7
SM	14.5 ± 0.5	21.8 ± 0.8	8.0 ± 1.6	15.7 ± 7.3	8.4 ± 0.6	13.1 ± 0.7	17.1 ± 0.2	16.8 ± 0.5
PS + PI			4.9 ± 0.2	7.9 ± 0.5	10.5 ± 0.8	11.5 ± 0.6	7.5 ± 1.5	7.1 ± 0.3

[a] LM cells were labeled with the indicated precursor as described in Materials and Methods. The cells were allowed to ingest latex beads (0.945 μm diam.) for 30 min. Cells were then homogenized and beads isolated on sucrose gradients. Phospholipids were extracted from the beads, separated by TLC, and analyzed for radioactivity. Phospholipids from cells which had not ingested latex beads were identically extracted and analyzed. Values are ± standard error of the mean. [b] These values are in good agreement with those of Schroeder et al. (1976).

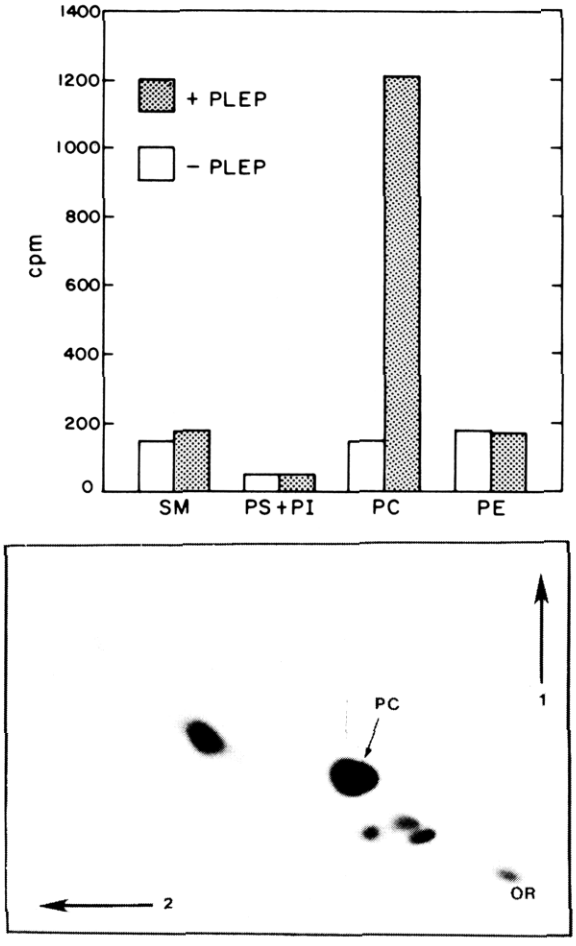

FIGURE 2: PC specificity of beef liver PLEP. ^{32}P-labeled phagosomes were incubated (9 h, 37 °C) with DOPC vesicles and 20 units/mL PLEP. Donor and acceptor membranes were separated by centrifugation and the latter extracted and analyzed by TLC for radiolabeled lipids. (A, top) Exchange of major phospholipids in the presence and absence of PLEP. (B, bottom) Two-dimensional TLC autoradiogram of ^{32}P-labeled lipids present in acceptor membranes after exchange with PLEP. OR = origin; chromatograms were developed in solvent systems: (1) chloroform–methanol–28% ammonia (65:25:5, v/v); and (2) acetone–chloroform–methanol–acetic acid–water (40:30:10:10:5, v/v).

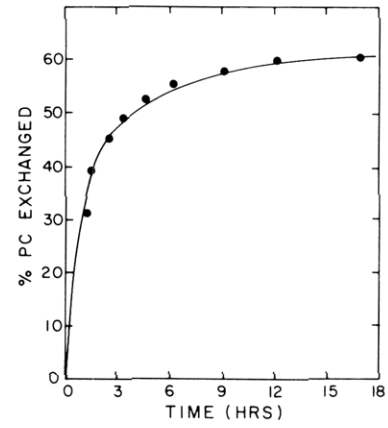

FIGURE 3: Kinetics of PLEP catalyzed PC exchange between [^{3}H]choline-labeled latex phagosomes and DOPC vesicles. Incubation conditions were as in Materials and Methods.

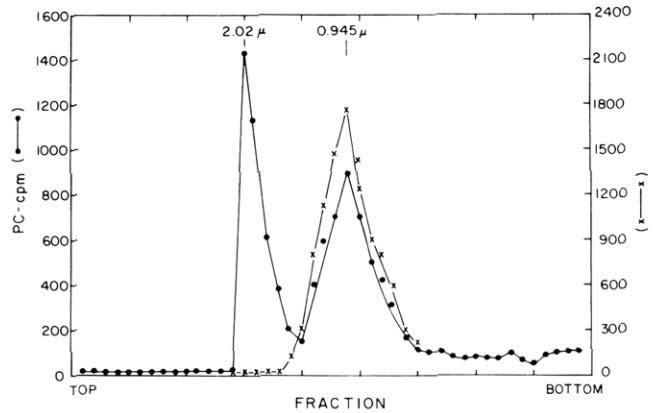

FIGURE 4: Exchange of PC between phagosomes. [^{3}H]Choline-labeled latex phagosomes (diam. = 0.945 μm; density = 1.05 g/mL) were incubated (37 °C, 9 h) with unlabeled latex phagosomes (diam. = 2.02 μm; density = 1.027 g/mL) in the presence of PLEP. The phagosomes were then separated on discontinuous sucrose gradients which were analyzed for PC radioactivity. (×—×) Before exchange; (●—●) after exchange.

From least-squares fit of the data, the fast ($t_{1/2} \simeq 0.9$ h) and slow ($t_{1/2} \simeq 88$ h) components were found to comprise respectively 52 and 48% of the phagosomal PC. Regression coefficients were 0.99 and 0.97, respectively. The addition of more PLEP or DOPC acceptor vesicles during the incubation did not affect the relative sizes of the PC pools. Furthermore, these were not changed when the times for phagocytosis of beads were varied from 15 to 60 min. Qualitatively similar results to those in Figure 3 were also obtained using phagosomes from cells grown in the presence of [^{32}P]- or [^{3}H]palmitate.

PC Asymmetry: Phagosome–Phagosome Exchange. PLEP could also mediate exchange of PC between donor and acceptor populations of latex phagosomes. This result was obtained using an excess of unlabeled latex phagosomes (2.02 μm; 1.027 g/mL) as acceptor and [^{3}H]choline-lipid labeled beads (0.945 μm; 1.05 g/mL) as donor membranes. Following incubation with PLEP, the two populations of phagosomes were separated on sucrose density gradients (Figure 4) and analyzed for radioactivity. As is seen, coincubation of labeled and unlabeled

TABLE II: Percent Fatty Acid Composition of Latex Phagosome Phospholipids.[a]

	PC		PE			
Fatty acid	Total	+PLEP	Total	+TNBS	SM	PS + PI[b]
16:0	21.1 ± 1.8	26.7 ± 0.6	6.4 ± 0.9	5.2 ± 1.0	91.7 ± 0.8	3.5 ± 0.7
16:1	11.3 ± 0.5	14.3 ± 1.3	5.2 ± 0.3	3.2 ± 0.4	1.0 ± 0.2	1.5 ± 0.4
18:0	5.6 ± 1.3	7.1 ± 2.0	17.2 ± 0.5	18.1 ± 0.4	1.8 ± 0.8	43.6 ± 0.6
18:1	62.0 ± 2.3	57.1 ± 0.1	71.2 ± 0.7	74.2 ± 1.4	5.4 ± 0.9	51.5 ± 0.9
% unsaturated	73.3	71.4	76.4	77.4	6.4	53.0

[a] Phospholipids on latex phagosomes were extracted from [^{3}H]acetate-labeled LM cells and separated as described in Materials and Methods. Radio GLC was carried out on the methyl ester fatty acid derivatives. Results are expressed in terms of percent relative abundance within the four major fatty acids ± standard error of the mean. [b] PS and PI are shown together because the TLC system did not completely separate these two phospholipids.

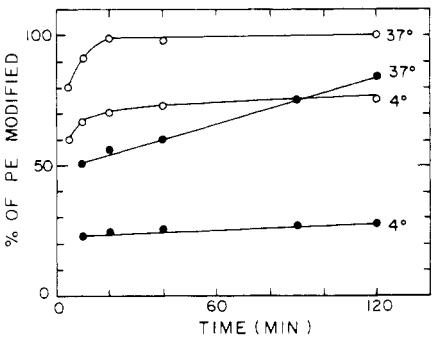

FIGURE 5: Appearance of Tnp-[^{32}PE by labeling isolated latex phagosomes (O—O), and phagosomes derived from TNBS-labeled cells (●—●) (see Materials and Methods) at 4 and 37 °C.

phagosomes results in a transfer of radioactive PC between the phagosome populations. No exchange takes place when PLEP was omitted from the incubation, or when uningested latex beads were used as acceptor "membranes". The quantitative aspects of this experiment are also in good agreement with the exchange data presented in Figure 3.

PE Asymmetry. Isolated ^{32}P-labeled latex phagosomes were incubated with 5 mM TNBS in Hanks' balanced salt solution–bicarbonate buffer, pH 8.5, for various times and the [^{32}P]PE and [^{32}P]TnpPe subsequently separated by TLC, detected by autoradiography, and assayed by radioactivity measurements. At 4 °C (Figure 5, open circles) or 20 °C (not shown), a rapid labeling of PE was observed. In 20 min, 70% of the PE was converted to the Tnp derivative. At 4 °C this value increased to 75% over the next 100 min, whereas at 20 °C, 90% of the PE reacted with TNBS during this time period. At 37 °C, the initial rate of the reaction was even faster, with >95% of the PE converted to TnpPE within 20 min.

TNBS labeling was also carried out on intact LM cells. Cells were incubated with 5 mM TNBS for various times at 4, 20, or 37 °C. The cells were then washed and allowed to ingest latex beads. Bead uptake was not adversely affected by the prelabeling of cells with TNBS. After isolation of the ingested beads, the proportion of PE and TnpPE in the phagosomes was determined. In cells preincubated with TNBS for 20 min at 4, 20, or 37 °C, 24, 30, or 55%, respectively, of the phagosomal PE was present as TnpPE. The proportion of phagosomal TnpPE did not significantly increase when cells were exposed to TNBS for times up to 120 min at 4 °C (Figure 5, closed circles) or 20 °C (not shown). However, when these incubations were carried out at 37 °C, the proportion of phagosomal TnpPE increased linearly with time (Figure 5), possibly suggesting an active cellular uptake of the probe.

Additionally, the procedures of TNBS labeling of intact cells and of isolated phagosomes were carried out sequentially in a single experiment. Intact cells were labeled with TNBS (20 min, 4 °C), and fed latex beads. The resulting phagosomes were isolated, and also labeled with TNBS (20 min, 4 °C). Virtually all (~99%) of the phagosomal PE in this experiment was found as the Tnp derivative.

Phospholipid Acyl Chain Disposition. Cells labeled with [^{3}H]acetate were used to determine the transbilayer acyl chain distribution of phagosomal PC and PE. ^{3}H-labeled latex phagosomes were incubated with PLEP and DOPC vesicles for 6 h at 37 °C, or with TNBS for 20 min at 4 °C. The exchanged PC and derivatized PE were isolated, hydrolyzed, and analyzed by radio GLC. The four major fatty acids (16:0, 16:1, 18:0, 18:1) which comprise ~95% of total LM cell phospholipid fatty acids were quantified relative to their total amount. As seen in Table II the % unsaturated fatty acids found in the PLEP exchangeable PC pool is not significantly different from that of the total phagosomal PC. Similarly, the % unsaturated fatty acids found in TnpPE were virtually identical with total phagosomal PE. The fatty acid compositions of phagosomal PS + PI and SM were also determined (Table II). PS and PE are predominately composed of the 18:0 and 18:1 fatty acids (~95%). Palmitic acid was the predominant fatty acid found in the phagosomal SM.

Discussion

Phagosomes as Plasma Membrane Derivatives. In this study, the transbilayer distribution in ingested latex phagosomes, of PE and PC, the major phospholipids of the LM cell (Table I; Glaser et al., 1974; Ferguson et al., 1975) was determined. Latex phagosomes can be easily isolated and several lines of evidence support their plasma membrane origin. Firstly they are characterized by an enrichment of radiolabeled SM and PE relative to PC (Table I). LM cell plasma membranes isolated by conventional sucrose gradient techniques show a similar enrichment (Schroeder et al., 1976). Secondly, isolated phagosomes showed an enrichment in activity of a commonly accepted plasma membrane marker, 5′-nucleotidase, as well as absence of the mitochondrial marker, lactic dehydrogenase. The direct relationship between the radioactivity associated with different sizes of latex beads ingested by radiolabeled cells and bead surface area suggests that each isolated bead was enveloped by the plasma membrane during phagocytosis. Furthermore, as a result of the topology of their ingestion, isolated latex phagosomes presumably have a uniform sidedness, opposite to that of the plasma membrane.

While the properties cited above make the latex phagosome system well suited for studies of plasma membrane lipid asymmetry, we recognize certain complicating factors which arise as a result of the phagocytotic process. It has been sug-

gested that the plasma membrane lipids in specialized phagocytotic cells such as the macrophage, polymorph, and granulocyte undergo compositional changes during phagocytosis (Mason et al., 1972; Smolen and Shohet, 1974; Berlin and Fera, 1977) and that the phagosome may not be completely representative of the plasma membrane. The plasma membrane origin of the phagosome membrane is also complicated by the possible changes in membrane composition that occur after fusion with lysosomes (van Vliet et al., 1976). Indeed, the presence of acid phosphatase activity in our isolated phagosome preparations suggests formation of phagolysosomes. To control for these factors, we have varied both bead size (0.5 to 2 μm) and time of phagocytosis (15 to 60 min) and determined transbilayer PE and PC distribution in the resulting phagosomes. The distribution of PE and PC was independent of both these variables.

PC Asymmetry in Latex Phagosomes. By the addition of PLEP to sonicated vesicles and red blood cell ghost acceptor membranes, it has been demonstrated that only the outer leaflet of the vesicle lipids is readily available for exchange (Johnson et al., 1975; Rothman and Dawidowicz, 1975). This finding makes PLEP a useful, nonperturbing probe for studying membrane lipid asymmetry. In the present study we have shown (Figure 3) that PC in the latex phagosome membrane exists in two exchangeable pools. Based on the action of PLEP in model systems, we equate the more rapidly exchanging PC pool to that which occupies the outer leaflet (cytoplasmic face) of the phagosome membrane bilayer. Alternatively, masking of PC by proteins or other membrane components could result in an exchangeable pool which is not necessarily related to PC asymmetry. While such a possibility is considered unlikely (Rothman and Lenard, 1977), this question could be studied by comparing the size of the exchangeable pools in membrane preparations having uniform and opposite sidedness to the latex phagosome. However, we were unsuccessful in numerous attempts to obtain such plasma membrane derivatives. Furthermore, attempts to exchange PC by PLEP from the outer leaflet of the plasma membrane of intact LM cells using sonicated vesicles as acceptor membranes were found to be impractical due to high background levels of vesicle-cell fusion (Pagano and Huang, 1975) and adhesion (Pagano and Takeichi, 1977).

Previous studies utilizing phospholipid exchange proteins to determine membrane asymmetry have generally been limited to acceptor and donor membranes with sufficiently different properties to facilitate their physical separation following exchange. This requirement usually means that the donor and acceptor membranes differ chemically. For example, sonicated lipid vesicles are often used as acceptor membranes during incubation with PLEP and natural membrane donors. Recently, however, it has been shown that the composition and size of these acceptor vesicle membranes can strongly influence the exchange reaction in some systems (DiCorleto and Zilversmit, 1977). Thus, adsorption or fusion of liposomes with donor membranes could result in an underestimation of the size of the exchangeable pool. This possibility can be ruled out in the present study since exchange of phagosome PC to unilamellar DOPC vesicles or to nonradioactive latex phagosomes worked equally well (Figures 3 and 4). Furthermore, these data demonstrate that, for this system, the composition and physical properties of the acceptor membrane do not markedly affect the PLEP-catalyzed exchange reaction.

Equating the fast and slowly exchanging PC pools with the PC residing on the cytoplasmic and external leaflets of the phagosome membrane bilayer, we calculated a half time of transbilayer movement (flip-flop) from the data shown in Figure 3 of ~88 h. This value is in good agreement with values of PC flip-flop obtained in viruses ($t_{1/2} \simeq 10$ days; Lenard and Rothman, 1976) and in vesicles ($t_{1/2} \simeq$ several days; Johnson et al., 1975; Rothman and Dawidowicz, 1975; Roseman et al., 1975), but it is considerably slower than that seen in intact erythrocytes and their ghosts (Bloj and Zilversmit, 1976; Renooij et al., 1976) and in microsomes (Zilversmit and Hughes, 1977) where half-times for flip-flop of only a few hours have been observed. Our present finding of a slow rate of transbilayer movement of PC in phagosome membranes, a plasma membrane derivative, suggests that, once formed, the two halves of the membrane bilayer are compositionally independent of one another. Although we have not as yet explored the rates of PC flip-flop in intracellular membranes of the LM cell, it seems likely, based on the studies cited above, that flip-flop times will be strongly dependent upon the composition, structure, and function of the intracellular membrane chosen for study. Such a variability in rates of lipid transposition across the varied membranes of a single cell type raises many intriguing questions on the origin and maintenance of lipid asymmetry in eukaryotic cells which will require further study.

PE Asymmetry in Latex Phagosomes. TNBS has been used under nonpenetrating conditions as a probe for the sidedness of amino-containing lipids in erythrocyte and bacterial cell membranes (Gordesky et al., 1975; Rothman and Kennedy, 1977). Since at low temperatures, a discrete pool of phagosomal PE is accessible to TNBS, whereas at elevated temperature, virtually all of the PE is labeled by this probe, we suggest that TNBS does not penetrate the phagosome membrane bilayer at low temperatures (4 or 20 °C). Under these conditions, approximately 70% of the phagosomal PE is converted to the Tnp derivative, suggesting a preponderance of this lipid on the outer surface of the phagosome. From the topology of the latex bead ingestion, a similar enrichment of PE would be expected on the cytoplasmic face of the plasma membrane. The finding of PE enrichment on the cytoplasmic face of the phagosome membrane bilayer is in agreement with studies of lipid asymmetry in other simpler membrane systems which show an enrichment of amino lipids on the inner half of the membrane bilayer (see Bergelson and Barsukov, 1977).

Because TNBS reacts with intact cells without affecting their viability, the subsequent isolation and analysis of latex beads ingested by such cells gave an independent estimate of the transbilayer distribution of PE. In this experiment, approximately 24% of the phagosome PE was isolated as the Tnp derivative when cell labeling was carried out at 4 °C. This value of 24% is nearly complementary to the value of 70% obtained when TNBS labeling is carried out directly on isolated phagosomes. Thus the PE pools were determined from both sides of the phagosome membrane and their size accounted for nearly all of the phagosomal PE. This result suggests that the transbilayer distribution of PE, determined by TNBS labeling, is not complicated by inaccessibility of the probe due to masking of the lipids.

Model for Polar Head Group and Acyl Chain Asymmetry in Latex Phagosomes. In the present study, the transbilayer distribution of PE and PC, which together comprise about 75% of the phagosomal phospholipids (Table I), has been determined directly. Our results suggest that 52% of the phagosome PC and 70% of its PE are on the cytoplasmic half of this plasma membrane derivative. The distribution requires that the remaining phospholipids (mostly SM) are localized predominantly on the outer leaflet of the membrane (Figure 6A), assuming an equal transbilayer distribution of total phospholipid. The present model for phagosomes is in good agreement with

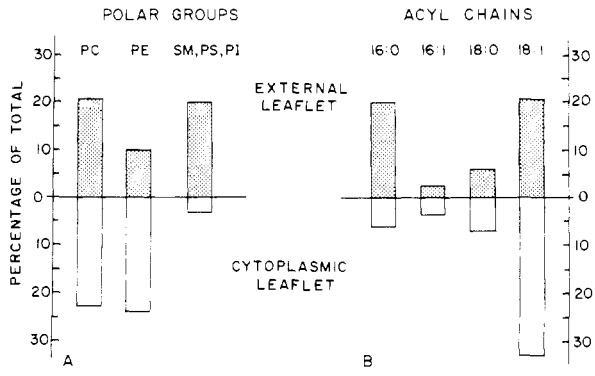

FIGURE 6: Model for phospholipid asymmetry in LM cell plasma membrane derivatives (latex phagosomes). (A) Polar head groups; (B) acyl chains.

the PE asymmetry seen in erythrocytes (see Rothman and Lenard, 1977; Bergelson and Barsukov, 1977), but differs from this system in that no PC asymmetry was seen. In budding viruses, the only other eukaryotic membrane derivative to be examined for lipid asymmetry, varying degrees of PE and PC asymmetry have been found depending on the virus used, host cell type, and assumptions made about the distribution of total phospholipids across the viral envelope (Fong et al., 1976; Rothman et al., 1976). Thus it is more difficult to make a direct comparison between these virus systems and the phagosome membranes used in the present study.

Our results on the transbilayer acyl chain distribution in PC and PE (Table II) show that, within each of these phospholipid classes, there is no significant acyl chain asymmetry. However, the polar head group distribution (Figure 6A), taken together with the marked differences in acyl chain composition of each of the major lipid classes, results in the overall acyl chain distribution shown in Figure 6B, in which the cytoplasmic face of the bilayer contains a higher fraction of unsaturated fatty acids than the external leaflet of the membrane (37% vs. 23% of total). Thus, we have found both polar head group and, as a consequence, acyl chain asymmetry in the LM cell phagosome membrane.

It has been postulated that as a result of phospholipid asymmetry, the bilayer halves may respond differentially to chemical and physical stimuli (Sheetz and Singer, 1974), leading to changes in membrane associated cellular functions (e.g., motility, morphology, and fusion). By documenting the transbilayer distribution of acyl chains and polar head groups, the present study in LM cells provides a starting point for testing some of these ideas.

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