Biochimica et Biophysica Acta, 649 (1981) 162–174
Elsevier/North-Holland Biomedical Press

BBA 79457

ALTERED PHOSPHOLIPID COMPOSITION AFFECTS ENDOCYTOSIS IN CULTURED LM FIBROBLASTS

FRIEDHELM SCHROEDER

Department of Pharmacology, University of Missouri School of Medicine, Columbia, MO 65212 (U.S.A.)

(Received March 26th, 1981)

Key words: Phospholipid, Polar head group, Endocytosis, (Cultured fibroblast)

The phospholipid polar head group composition of LM fibroblast membranes was altered by growing the cells in a chemically defined, serum-free medium containing choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine. The cells incorporated these bases into their membrane phospholipid such that 29–40% of the total plasma membrane phospholipids contained these polar head groups. Alteration of the phospholipid composition correlated with a depression of polystyrene bead phagocytosis by 36, 55 and 85% when the cells had been supplemented with *N,N'*-dimethylethanolamine, *N*-monoethylethanolamine, or ethanolamine, respectively. Pinocytotic uptake of horseradish peroxidase was depressed 44, 39, and 32%, respectively. The phagosomal membrane phospholipid composition qualitatively resembled that of the primary plasma membrane from which it was derived. However, enrichment of phosphatidylcholine, and other quantitative differences were noted in the phagosomal membranes as compared to the parent primary plasma membrane. Approx. 50% of the phagosomal membrane's phosphatidylethanolamine was accessible to the chemical labelling reagent trinitrobenzenesulfonate at 4°C. The asymmetric distribution of phosphatidylethanolamine across the phagosomal membrane did not appear to be altered by base analogues except in the case of phagosomes from cells supplemented with ethanolamine. The data were consistend with a nonrandom site for endocytosis with regard to phospholipid composition.

Introduction

Endocytotic processes in eucaryotic cells have recently been reviewed [1–3]. The process of endocytosis involves internalization of a significant portion of the cell's surface membrane. Each minute macrophages and L cells in culture pinocytose 3.1 and 0.8% of the cell surface area, thereby internalizing an equivalent of their surface membrane every 0.5–2.9 h [4]. The role of membrane lipid components in endocytosis is not well understood, although the plasma membrane lipid components appear to be largely retained in the internalized phagosomal membrane. In contrast, the content of cholesterol, the sterol/phospholipid, and the microviscosity of the remaining parent membrane all seem to be reduced [5–8]. In actively phagocytosing polymorphonuclear leucocytes, phosphatidylcholine newly formed from lysophosphatidylcholine was all associated with the phagosomal membrane [9–11]. These data lend support to the possibility that endocytosis may occur at specialized areas of the surface membrane or that remodeling of the cell membrane must take place [12]. It is well known that blood lipids freely exchange between lipoproteins and blood cell membranes and that plasma lipid contents may be genetically, nutritionally, and hormonally regulated [13–19]. In addition, macrophages and L cell phagocytosis and pinocytosis appear to respond to the level of unsaturated fatty acids and sterol in the parent surface membrane [20–22]. Thus, membrane lipid composition may, in part, regulate endocytic processes. However, the response of endocytosis to alterations in the polar head groups of phospholipids,

Abbreviation: SDS, sodium dodecyl sulfate.

the major lipid component of membrane bilayers, has not been determined. A potential role for phospholipid composition in endocytic cells may be proposed on the basis of several blood cell pathologies Abnormal functioning of the immune system is closely related to leukocyte and lymphocyte phospholipid composition [23–25]. In addition, specific alterations in lymphocyte phospholipid composition have been noted during chronic renal failure [27]. The LM fibroblast, a choline-requiring cell line, can be cultured in serum-free medium and provides an excellent model system for alterations in membrane phospholipid polar head groups [28], diethyl ether-linked lipids [29], fatty acids [30], and sterols [31]. The response of phagocytosis and pinocytosis to altered phospholipid composition in LM fibroblast membranes is detailed herein.

Materials and Methods

Cell culture. LM cells, a strain of mouse fibroblasts, were obtained from the American Type Culture Collection (CCL 1 2). The cells were grown in suspension in a chemically defined, serum-free medium as described earlier [28] The medium contained either choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine (Eastman Organic Chemicals, Rochester, NY) at a concentration of 40 μg/ml. Cells ($1 \cdot 10^6$ cells/ml) were cultured for 3 days and harvested in exponential growth phase. The analogues of choline did not affect the growth characteristics of the LM fibroblasts during this time period. For phospholipid compositional investigations, the cells were first cultured for 5 days in choline-containing medium with 2 μCi $^{32}P_i$/ml (New England Nuclear Inc , Boston, MA). The cells were then placed on choline- or choline analogue-containing medium with the same specific activity of $^{32}P_i$.

Plasma membrane isolation. Plasma membranes were isolated as described by Schroeder et al [28], with minor modifications. The cells were first washed two times with 10 vol phosphate-buffered saline made without calcium and magnesium. The cells were homogenized with a tight-fitting Dounce (7.5 ml vol.) homogenizer and breakage was assessed by phase contrast microscopy Unless otherwise stated, the discontinuous sucrose gradient contained 0.5 ml 55% (w/w) sucrose, 2 ml 40% sucrose, 1 ml 35% sucrose, 2 ml 32% sucrose, 2 ml 29% sucrose, 2 ml 27% sucrose, and 1 ml 20% sucrose, unless otherwise specified. After centrifugation in an SW 41 or SW 40 rotor at 39 000 rev./min ($1.96 \cdot 10^5 \times g$) for 16 h, the bands of turbid material at the interfaces were removed and designated as follows 20–27%, I; 27–29%, II, 29–32%, III; 32–35%, IV; 35–40%, V; and 40–55%, VI. Each fraction was collected as previously described [28]. Fractions were taken from the sucrose gradient and placed on a dextran gradient [28]. Fractions I, II, and V from the sucrose gradient were pelleted and resuspended in 1 ml of 0.25 M sucrose/1 mM triethanolamine, pH 7.4, and layered on a discontinuous dextran 170 gradient (average molecular weight 1.7 10^5, Sigma Chemical Co , St. Louis, MO) of the following composition: 3 ml 25% (w/w) dextran; 3 ml 16% dextran; and 3 ml 10% dextran Fractions I, II, and V from the discontinuous sucrose gradient were centrifuged for 4, 4, and 2 h, respectively, on the discontinuous dextran gradient in a SW 41 rotor at 39 000 rev./min ($1.95 \cdot 10^5 \times g$). The interfaces on the dextran gradient were collected and labelled as follows: 8.3% sucrose/10% dextran, I'; 10–16% dextran, II', 16–25% dextran, III', and the pellet at the bottom of the tube, IV'. Material designated as Fractions I and II from the sucrose gradient was treated similarly, except that 4 h of centrifugation at 39 000 rev./min was used. Samples were taken at each step of preparation and from each gradient fraction and centrifuged at $1\,05 \cdot 10^5 \times g$ for 60 min for assay of particulate protein and enzyme activity [28] The relative distribution of marker activities on the discontinuous sucrose or dextran 170 gradients was determined as before [28] Relative specific activity was calculated as the per cent in each fraction of the total enzymatic activity recovered from the gradient divided by the per cent in that fraction of the total protein recovered from the gradient. The specific activity and purification relative to crude homogenate of ouabain-sensitive ($Na^+ + K^+$)-ATPase and 5'-nucleotidase in the purified plasma membrane fraction were the same as the fold purification and specific activity of these enzymes in the phagosomal membranes described below.

Phagocytosis and phagosome isolation. Phagocytosis was carried out by the procedure of Sandra and Pagano [32] modified as follows: suspension cultured cells were washed two times with 20 vol.

phosphate buffered saline (Ca^{2+}- and Mg^{2+}-free) [28]. Unless otherwise specified, 5 10^8 cells were exposed to latex beads (2000 beads/cell) in 30 ml of fresh medium. Unless otherwise specified, the cell/bead mixture was then gently shaken at 37°C for 30 min, on a gyrotory shaker (model G-10, New Brunswick Scientific, New Brunswick, NJ) Phagocytosis was terminated by placing the incubation mixture on ice at 4°C. Unphagocytized beads were separated from cells by overlaying the incubation mixture over 10 ml 8% bovine serum albumin (Pentex Fraction V, Miles Research Labs, Kankakee, IL) in phosphate-buffered saline at 4°C in 50-ml centrifuge tubes. The tubes were centrifuged for 15 min at 244 × *g* and the supernatant was decanted. The pellet was resuspended in phosphate-buffered saline at 4°C and the process was repeated. The pellet was then resuspended in 1 ml 0.25 M sucrose/1 mM Tris-HCl (pH 7 2)/1 mM $MgCl_2$ and incubated at 4°C for 3 min. Then 2 ml 60% sucrose (w/w)/1 mM $MgCl_2$ was added, the mixture was vortexed gently for 1 min and placed on ice at 4°C. The phagosomes were released from the cells by Dounce homogenization (7 ml with tight-fitting pestle; Kontes Glass Co , Vineland, NJ) using 30 up-and-down strokes at 4°C. The homogenate was placed in the bottom of a polyethylene centrifuge tube and overlayered with 4 ml 27% sucrose (w/w) followed by 3 ml 10% sucrose (w/w) and centrifuged in a Sw 40 or SW 41 swinging bucket rotor on a Model L5-65 ultracentrifuge (Beckman Instr , Fullerton, CA) at 39 000 rev./min for 90 min at 4°C. Phagocytized beads were removed from the 10–27% interface and stored at 4°C for treatment with trinitrobenzenesulfonic acid or frozen at −70°C pending lipid analysis. Any unphagocytosed beads not removed in the washing procedure appeared at the top of the gradient. Phagocytosed polyvinyltoluene beads were isolated from a 10–15% (w/w) sucrose interface as described earlier [32]. The degree of phagocytosis was determined by extracting the phagosomal lipids and either quantitating membrane phospholipid phosphate as by [^{32}P]-phospholipid counting Since all phospholipid species were uniformly labelled to the same specific activity after 5 days in culture, dpm [^{32}P]phospholipid and nmol phospholipid were directly proportional Similar results were obtained if phagosomal membrane protein was measured. In addition, the [^{32}P]phospholipid extracted from the phagosomes was not due to nonspecific adherence of membrane particles during homogenization for the following reasons (1) less than 5% as much ^{32}P was found in the phagosomal fraction when beads were added to a LM cell homogenate, and (2) the lipid composition and sterol/phospholipid ratio did not resemble that of intracellular membranes such as microsomes or mitochondria

Trinitrobenzenesulfonate labelling. The asymmetric distribution of aminophospholipids across phagosome membranes may be determined by use of the chemical labelling reagent, trinitrobenzenesulfonic acid, which covalently trinitrophenylates amino groups on phosphatidylethanolamine, phosphatidylserine, or proteins. The details of the whole cell labelling procedure are provided elsewhere [32–36]. Similarly, isolated phagosomes were trinitrophenylated by resuspending the phagosomal pellet obtained in the previous section in 8 ml of 4 mM trinitrobenzenesulfonate labelling reagent [33], shaking on a gyrotary shaker for 80 min at 4 or 37°C; terminating the reaction by adding 40 ml 0.15 M Tris-HCl, pH 7.0, and centrifuging at 33 000 × *g* for 20 min at 4°C This washing procedure was repeated one more time with Tris-HCl buffer and one time with phosphate-buffered saline.

Lipid determinations. All organic solvents were glass-distilled and all glassware was washed with sulfuric acid/dichromate before use. Phagosomes were suspended in 1 0 ml of the phosphate-buffered saline solution mentioned above A 0 2-ml aliquot was removed for protein determination. The remaining 0.8 ml aliquot was extracted as described earlier [28] Neutral and phospholipids were separated by silicic acid chromatography. Unisil (100–200 mesh, Clarkson Chemical Co., Williamsport, PA) was washed with methanol and activated at 100°C overnight. Just prior to use, each column was eluted with 5 ml chloroform/4 ml methanol, and finally with 5 ml chloroform. The phospholipids were separated by two-dimensional thin-layer chromatography on Silica Gel G plates (250 μm thick, Analtech Inc , Newark, DE) that had been preactivated with acetone. The phospholipid components were visualized by autoradiography [28], scraped, and counted in 10 ml of a 3A70 scintillation mixture (Research Products International Corp., Elk Grove Village, IL) to which 0.5

ml distilled water had been added and mixed. The relative mobility values and identity of the phospholipids were determined by two-dimensional chromatography in two solvent systems as described previously [28]: (A) chloroform/methanol/water (65:25.4); (B) *n*-butanol/glacial acetic acid/water (6:2:2). The determination of the polar head groups of phospholipids separated by this system was accomplished as described earlier [28]. The following phospholipid standards were also used for co-chromatography and identification of unknown phospholipids. phosphatidylserine, lysophosphatidylethanolamine, cardiolipin, and phosphatidic acid standards obtained from Serdary Research Labs, London, Ontario Canada; phosphatidylglycerol and sphingomyelin from Applied Science Labs, State College, PA; lysophosphatidylcholine from Supelco, Bellefonte, PA; phosphatidyl-*N,N'*-dimethylethanolamine, phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-2-amino-1-butanol, and phosphatidyl-3-aminopropanol generously provided by Dr. M. Glaser, Department of Biochemstry, University of Illinois, Urbana, IL, 61801.

Pinocytosis assay. Pinocytosis was measured by the horseradish peroxidase uptake assay of Steinman and Cohn [37] and Heininger and Marshall [22] as described below. Reagents: Horseradish peroxidase was stored at −20°C. Stock solutions of 10 mg/ml and 1.0 ng/μl in distilled water were prepared fresh daily Phosphate buffer was prepared by mixing 97.3 ml KH_2PO_4 (9.073 g/l) and 0 7 ml Na_2HPO_4 (1.187 g/100 ml) and adjusting pH to 5.5. A 1.0% *o*-dianisidine (Sigma Chemical Co., St. Louis, MO) solution in absolute methanol was kept in the dark at −20°C. The *o*-dianisidine was light-sensitive. A 0.3% H_2O_2 solution was prepared fresh daily. A 0.1% SDS solution was prepared. Procedures: 2 ml of cells ($3 \cdot 10^6$ cells) in fresh medium were placed in an acid-washed test tube. The 0.7 ml medium and 0.3 ml horseradish peroxidase (10 mg/ml) were added to give a final concentration of 1 mg/ml horseradish peroxidase. The sample tube was capped with a Teflon-lined cap and inverted to mix, followed by incubation at 37°C for 1 h on a reciprocating shaker water bath. Pinocytosis was stopped by placing the sample on ice at 4°C. The cells were pelleted at $244 \times g$ in a swinging bucket IEC centrifuge at 4°C. The cells were washed five times with phosphate-buffered saline [28] to remove extracellular horseradish peroxidase. The final cell pellet was then placed on ice. The substrate for the enzymatic assay was prepared at 4°C by mixing 6 ml of the above phosphate buffer, 0.1 ml 0.3% H_2O_2, and 0 05 ml 1% *o*-dianisidine and placed in a brown bottle. A horseradish peroxidase standard curve from 0–10 ng (0 ng horseradish peroxidase as a reference) was constructed with a Cary 14 Spectrometer (Varian Instr., Park Ridge, IL) by mixing 0.1 ml horseradish peroxidase with phosphate buffer and 0.1 ml 0.1% SDS in the cuvette. Then 0.9 ml substrate was added, the cuvette was mixed and color development was followed at 460 nm for 3–5 min. Pinocytosed horseradish peroxidase was measured after lysing the cell pellet with 1 ml SDS, vortexing for 1 min, taking a 0.1-ml aliquot (immediately placing the aliquot in the cuvette) and adding 0.1 ml phosphate buffer and 0.9 ml substrate. The cuvettes were mixed and color development was followed immediately. The amount of horseradish peroxidase uptake was quantitated by comparison with the standard curve.

Analytical procedures. Protein was determined by the method of Lowry et al. [38].

Results

Effect of choline analogue supplementation on polystyrene bead uptake by LM fibroblasts

LM fibroblasts cultured in the presence of choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine incorporated these bases into the cellular membrane phospholipids such that 40 6, 28.7, 31 and 39% of the surface membrane phospholipids were phosphatidylcholine, phosphatidyl-*N,N'*-dimethylethanolamine, phosphatidyl-*N*-monomethylethanolamine, and phosphatidylethanolamine, respectively (Table I). These results are similar to those reported earlier from this laboratory [28]. The effect of the altered phospholipid composition on the rate of uptake of polystyrene beads is detailed below.

The rate of membrane internalization by LM fibroblasts grown with choline analogues may be dependent on several variables, including time, bead/cell ratio, and bead size. As shown in Fig. 1, uptake appears to be maximal by 5–10 min. However, compared to growth in the presence of choline, the maximal uptake was reduced by 36, 55, and 85% when the

TABLE I

PHOSPHOLIPID COMPOSITION OF PLASMA MEMBRANES FROM LM FIBROBLASTS SUPPLEMENTED WITH ANALOGUES OF CHOLINE

LM fibroblasts were cultured in suspension with choline, *N,N'*-dimethylethanolamine (DME), *N*-monomethylethanolamine (MME) or ethanolamine (E) and plasma membranes were isolated as described in Materials and Methods. Abbreviations PC, phosphatidylcholine; PDME, phosphatidyl-*N,N'*-dimethylethanolamine, PMME, phosphatidyl-*N*-monomethylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol, PS, phosphatidylserine; LPC, lysophosphatidylcholine, PG, phosphatidylglycerol. Values are phospholipid composition expressed as percentages.

Phospholipid species	Supplement			
	Choline	DME	MME	E
PC	40 6	11.8	12.9	17 0
PDME	0.1	28 7	0.1	0.1
PMME	1 3	2.1	31.0	0.1
PE	24.9	22.2	17.7	37.0
PI + PS	11.1	13.3	14.4	15.6
Sphingomyelin + LPC	9.7	13.6	14.7	19.4
PG	10.0	3.5	2.3	3 6
Others	2.3	4.8	6.9	5.2

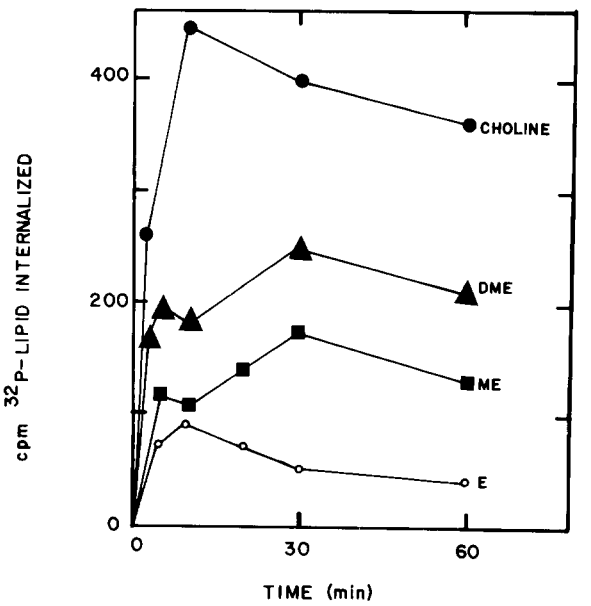

Fig. 1. Uptake of polystyrene beads by LM fibroblasts cultured with analogues of choline. LM fibroblasts were cultured in the presence of choline, *N,N'*-dimethylethanolamine (DME), *N*-monomethylethanolamine (ME), or ethanolamine (E) as described in Materials and Methods. The cells were exposed to 0.760 μm diameter polystyrene beads (800 beads/cell) for the time periods indicated and the quantity of surface membrane internalized was determined as described in Materials and Methods.

LM cells were grown in the presence of *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, and ethanolamine, respectively The effect of bead/cell ratio on internalization of the polystyrene heads is shown in Table II. The internalization of the polystyrene beads appears to be proportional to the bead/cell ratio. This finding is in agreement with the results of others investigating latex bead uptake by polymorphonuclear leukocytes and Ehrlich ascites carcinoma cells [39] The inhibition of bead internalization in choline analogue-supplemented LM cells was greatest at the higher bead/cell ratios (2000 and 800 beads/cell). Fig. 2 indicates that at different bead sizes (0 497 μm vs. 0.760 μm diameter) the rate of bead uptake was maximal at 5–10 min exposure of the cells to beads. Supplementation of the LM cells with *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, and ethanolamine reduced the internalization of 0.497 μm beads by 40, 46 and 36%, respectively (Table III). In contrast, uptake of the larger 0.760 μm beads was reduced 21, 52, and 69% when compared to the choline-grown controls. Internalization of polystyrene beads was much higher for 0.760 μm polystyrene beads than for the 0.497 μm beads The membrane internalization may also be calculated on the basis of bead diameter, surface area, or volume (mass). Bead uptake by control choline-supplemented LM cells was not proportional to the bead diameter or bead surface area. In contrast, compared to the uptake of 0.760 μm beads, equal volumes or masses of 0.497 μm beads were internalized (567 ± 35 vs 538 ± 16 cpm [^{31}P]phospholipid/10^6 cells). When the same volume-correction calculations were performed for the cells supplemented with choline analogues, the 0 497 μm beads were taken up at rates of 339 ± 32, 335 ± 21, and 307 ± 28 cpm [^{32}]phospholipid internalized/10^6 cells for LM fibroblast supplemented with *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, and ethanolamine, respectively. These values illustrate that the control choline-grown LM fibroblasts take up equal volumes of different size beads, while choline

TABLE II

EFFECT OF BEAD TO CELL RATIO ON INTERNALIZATION OF POLYSTYRENE BEADS BY LM FIBROBLASTS SUPPLEMENTED WITH CHOLINE ANALOGUES

LM fibroblasts were cultured as described in the legend of Fig. 1 and exposed to 0.760 μm diameter polystyrene beads for 30 min at varying bead/cell ratios, as described in Materials and Methods. Abbreviations as in Table I.

Choline analogue	Bead/cell ratio	cpm [^{32}P] labelled lipid internalized/30 min
Choline	2000	600 ± 30
	800	390 ± 25
	200	210 ± 10
DME	2000	410
	800	250 ± 20
	200	190 ± 18
MME	2000	230
	800	175 ± 15
	200	125 ± 13
E	2000	170
	800	60 ± 8
	200	55 ± 6

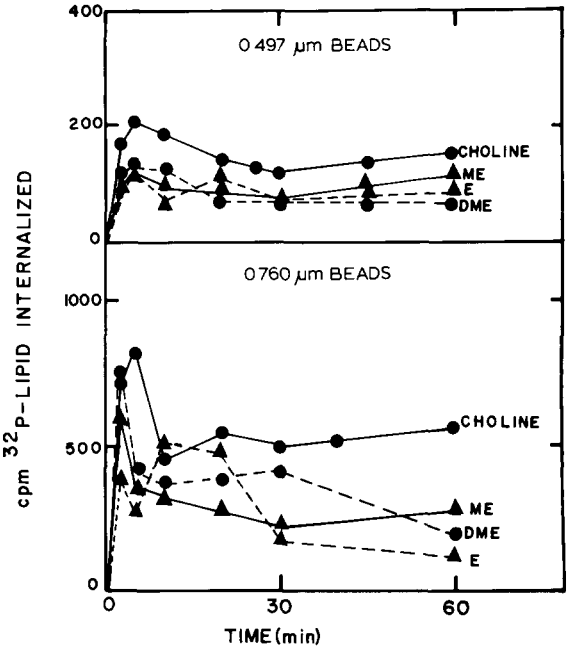

Fig. 2. Effect of bead size on uptake rates of LM fibroblasts cultured with analogues of choline. All conditions were described in legend of Fig 1, except that either 0.497 μm or 0.760 μm diameter beads were used at a bead/cell ratio of 2000 beads per cell. Abbreviations as in Fig. 1.

analogue-grown cells did not Choline analogue-supplemented cells also internalized fewer polystyrene beads. Choline analogues had no effect on phagocytosis if the analogues were not incorporated into membrane phospholipids (data not shown).

Effect of choline analogue supplementation on pincytosis of horseradish peroxidase by LM fibroblasts

Pinocytosis represents the endocytic uptake of fluid, solutes, and small proteins or particles as opposed to uptake of large particulates during phagocytosis [1]. Fluid-phase pinocytosis can be measured by the uptake of horseradish peroxidase [1,37]. LM fibroblasts do not contain this enzyme. Therefore, this enzyme is a useful marker for pinocytic rate. Fig. 3 shows the effect of increasing content of

TABLE III

EFFECT OF SIZE ON LM CELL SURFACE MEMBRANE INTERNALIZATION BY PHAGOCYTOSIS

LM cells grown on choline or choline analogues for 3 days were exposed to beads (2000 beads/cell) for 30 min as described in Materials and Methods. Phagosomes were isolated and the phospholipid was quantitated by counting [^{32}P]phospholipid in the phagosomes contained in $1 \cdot 10^6$ cells. Values represent the mean ± S.E. ($n = 6$) Abbreviations as in Table I.

Bead size (μm)	Surface membrane internalized (cpm [^{32}P]phospholipid/10^6 cells)			
	Choline	DME	MME	E
0.497	159 ± 10 [a]	95 ± 98 [a]	94 ± 6 [a]	86 ± 8 [a]
0.760	538 ± 16 [a,b]	423 ± 8 [a,b]	256 ± 10 [a,b]	167 ± 9 [a,b]

[a] $P \leqslant 0.01$ as compared to choline-grown controls.
[b] $P \leqslant 0.01$ comparing phagosomes from 0.497 μm and 0.760 μm beads.

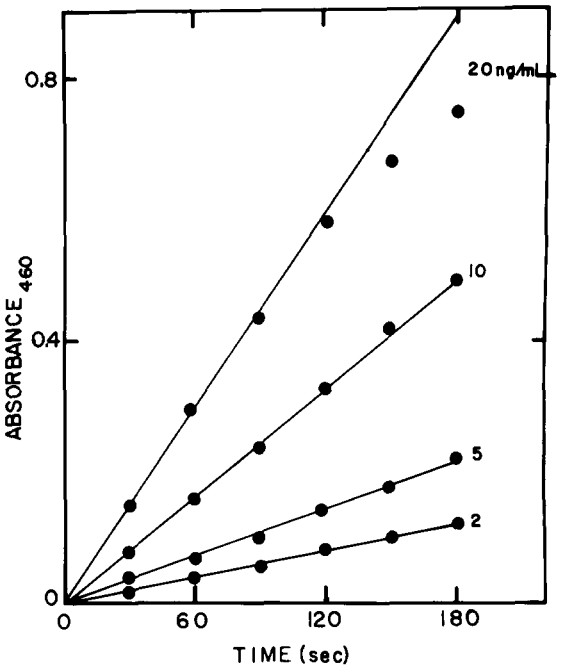

Fig. 3. Linearity of the horseradish peroxidase enzymatic assay. The assay was performed as described in Materials and Methods.

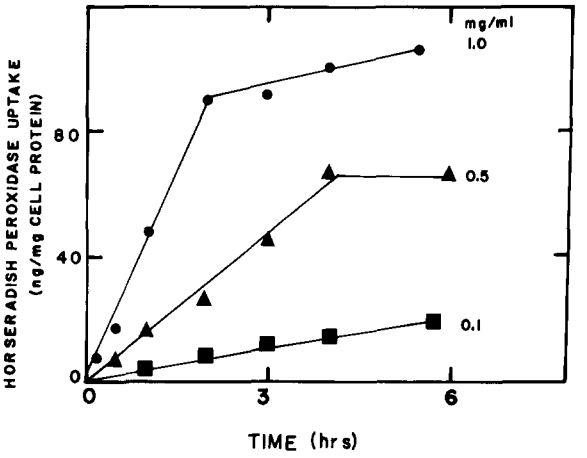

Fig 4 Uptake of horseradish peroxidase by LM fibroblasts as a function of time and extracellular concentration. 20 ml of LM fibroblasts ($1 \cdot 10^6$ cells/ml) were incubated with 1.0, 0.5, or 0 1 mg horseradish peroxidase/ml at 37°C for up to 6 h. At the indicated time points, 2-ml aliquots were removed, placed on ice, and the amount of horseradish peroxidase pinocytosed was determined as described in Materials and Methods.

horseradish peroxidase in the assay on color development. The assay was linear for 180 s at concentrations of horseradish peroxidase ranging from 2–10 ng/ml. At 20 ng/ml, the assay was linear only for 120 s. These data are in agreement with those of Steinman and Cohn [37]. The uptake of horseradish peroxidase by LM cells as a function of time and concentration is shown in Fig. 4. Uptake was followed for up to 6 h. At each time point, samples of cells were taken, washed, and internalized horseradish peroxidase was determined. At low concentrations (0.1 mg horseradish peroxidase/ml) the uptake was linear up to 6 h, while at 1.0 ng/ml and 0.5 mg/ml, the uptake was linear for 2 and 4 h, respectively. Therefore, all horseradish peroxidase uptake rates were followed for 2 h. At 2 h exposure of the LM fibroblasts to horseradish peroxidase, uptake was directly related to the amount of cell protein present incubation (Fig. 5). During exponential growth phase, 200 μg cell protein corresponds to approx 1 10^6 LM fibroblast suspension cells The slope in Fig 5 is 0 2 ng horseradish peroxidase/μg protein per 1 h. Thus, $1 \cdot 10^6$ LM fibroblasts pinocytose horseradish peroxidase at a rate of 40 ng/h. This represents 0.0040% of the administered horseradish peroxidase The y intercept in the Fig. 5 may indicate

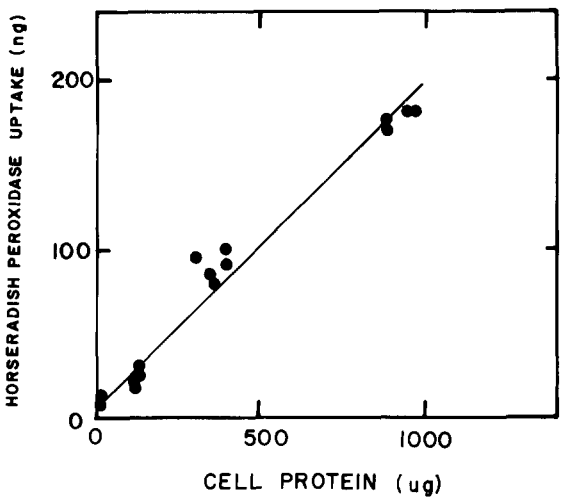

Fig. 5. Effect of LM cell concentration on horseradish peroxidase uptake. All conditions were as described in legend of Fig. 4, except that different amounts of LM cells (0–6(· 10^6) cells/ml) were incubated with 1 mg/ml horseradish peroxidase for 1 h.

the residual small amount of horseradish peroxidase bound to the cell surface and not removed by washing. Fig. 6 illustrates the linear dependence of horseradish peroxidase uptake with the concentration of horseradish peroxidase in the medium.

LM fibroblasts were cultured in the presence of choline or choline analogues for 3 days, and then the uptake of horseradish peroxidase was measured as shown in Table IV. Supplementation of the cells with *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, and ethanolamine reduced the horseradish peroxidase uptake by 44, 39, and 32%, respectively. Thus, alterations in surface membrane phospholipid polar head groups may decrease the pinocytic as well as phagocytic rate of LM fibroblasts. Such decreases were not noted if the choline analogues were simply added to the LM cell medium, but not incorporated into membrane lipids (data not shown). The degree of phagocytosis and pinocytosis inhibition was directly correlated with the alteration in phospholipid composition.

Effect of choline analogue supplementation on the phospholipid composition of internalized phagosomes

It is possible that phagocytosis in LM fibroblasts occurs only at nonrandom sites within the surface membranes. Such sites could have a particular type of lipid composition. Thus, when the lipid composition of the surface membrane changes (Table I), there may be fewer endocytic sites. Such a possibility may

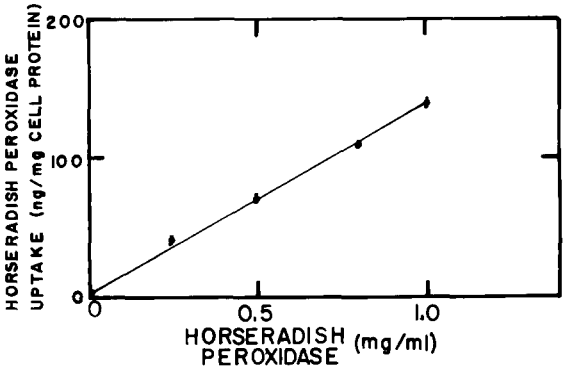

Fig. 6. Effect of horseradish peroxidase concentration on pinocytic rate. All conditions were as described in legend of Fig. 5, except that $1 \cdot 10^6$ cells/ml were used and exposed to varying concentrations of horseradish peroxidase (0–1 mg/ml) for 1 h.

TABLE IV

EFFECT OF CHOLINE ANALOGUES ON HORSERADISH PEROXIDASE PINOCYTOSIS BY LM FIBROBLASTS

LM cells were cultured on choline- or choline analogue-containing medium for 3 days. The cells were washed and incubated with horseradish peroxidase (1 mg/ml medium) at 37°C for 1 h. Cells were then washed five times with phosphate-buffered saline at 4°C; the pellets were lysed with 0.1% SDS (1 ml) and the horseradish peroxidase assay was performed as described in Materials and Methods. Values represent the mean ± S.E. with the number of experiments given in parenthesis. Abbreviations as in Table I.

Analogue	Horseradish peroxidase uptake (ng/mg per h)
Choline	183 ± 9 (5)
DME	103 ± 13 [a] (6)
MME	113 ± 17 [a] (4)
E	126 ± 19 [a] (5)

[a] $P \leq 0.025$ as compared to choline-fed cells.

explain the reduced phagocytic and pinocytic rates noted above in choline analogue-supplemented cells. The data in Table V indicate that the phagosomes from LM cells supplemented with choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, and ethanolamine contained 48.2 ± 1.3% phosphatidylcholine, 43.9 ± 2.3% phosphatidyl-*N,N'*-dimethylethanolamine, and 35.8 ± 1.4% phosphatidylethanolamine, respectively. Thus, the new analogue-containing phospholipids are not excluded from the phagosomal membranes. In contrast, as compared to the primary plasma membrane phospholipid composition (Table I in the present work and Table V in Ref. 28), the phagosomes from LM cells supplemented with choline or *N,N'*-dimethylethanolamine were enriched in phosphatidylcholine (48.2 ± 1.3% vs. 40.6–37.8%) and phosphatidyl-*N,N'*-dimethylethanolamine (43.9 ± 2.3% vs. 28.7–33.8%), respectively. Phagosomes from LM cells supplemented with *N*-monomethylethanolamine or ethanolamine had slightly less phosphatidyl-*N*-monomethylethanolamine (27 1 ± 2.6% vs. 3.10–32.9%) and phosphatidylethanolamine (35.8 ± 1.4% vs 39.9–39.5%), respectively, than did the primary plasma membrane. In addition, the choline analogue-containing lipids largely displaced the phosphatidylcholine content in the surface membrane (Table I in the present work; Table V in Ref. 28) such that the

TABLE V

EFFECT OF CHOLINE ANALOGUES ON PHOSPHOLIPID COMPOSITION AND ON DISTRIBUTION OF PHOSPHATIDYLETHANOLAMINE IN PHAGOSOMAL MEMBRANES

LM cells were cultured on choline analogues for 72 h, fed 0.497-μm beads (2 000 beads/cell) for 30 min, and phospholipid composition was determined as described in Materials and Methods. Values, expressed as percentage phospholipid composition, represent the mean ± S.E (n = 3) TNBS, trinitrobenzenesulfonic acid Other abbreviations as in Table I

Phospholipid species	Choline	DME	MME	E
PC	48 2 ± 1.3	16 6 ± 1.1 [a]	21.3 ± 3 3 [a]	27 8 ± 1 7 [a]
PDME	–	43 9 ± 2 3	4.4 ± 1 6	–
PMME	–	–	27 1 ± 2.6	–
PE	28.3 ± 1 7	15.9 ± 1 8 [a]	16.6 ± 2.8 [a]	35.8 ± 1.4 [a]
PI + PS	7.4 ± 1.0	7.2 ± 1.0	8 4 ± 1.3	8 1 ± 0.4
Sphingomyelin + LPC	5 1 ± 0.8	7.6 ± 1.6	10 9 ± 1.2 [a]	14 3 ± 0.8 [a]
PG	6.1 ± 2.6	5.0 ± 1.0	9 3 ± 3.1	8 9 ± 2.6
PC/PE	1 70	1 04 [a]	1.28 [a]	0 78 [a]
Anionic/zwitterionic	1.14	3.13 [a]	2 11 [a]	1 38 [a]
% PE labelled with TNBS	52.3 ± 1.2	51.1 ± 0.5	51 ± 1.2	42 ± 2 1 [a]

[a] $P \leq 0$ 025 as compared to choline control.

phosphatidylcholine content of the primary plasma membrane decreased 72 ± 2%, 67 ± 1%, and 58 ± 1%, respectively, in *N,N'*-dimethylethanolamine-, *N*-monomethylethanolamine- and ethanolamine-supplemented cells. In the corresponding phagosomal membranes (Table V), the phosphatidylcholine control decreased only 66 ± 2%; 44 ± 7%, 42 ± 4%, respectively Thus, the phagosomal membranes from choline analogue-supplemented cells also appear to be selectively enriched in phosphatidylcholine as compared to phagosomal membranes from choline-supplemented cells. The phagosomal membranes seem to have less phosphatidylinositol + phosphatidylserine and less sphingomyelin + lysophosphatidylcholine than the corresponding primary plasma membrane. Lastly, as shown in Table V, the ratios of phosphatidylcholine/phosphatidylethanolamine and anionic/zwitterionic phospholipids were lower and higher, respectively, in the phagosomes from analogue-supplemented cells than choline-supplemented cells. These data argue against the existence of a single highly specialized area of the surface membrane that has a single type of phospholipid composition or being the 'site' of endocytosis However, preference does seem to be shown for phosphatidylcholine-enriched areas.

TABLE VI

ASYMMETRIC DISTRIBUTION OF PHOSPHATIDYLETHANOLAMINE ACROSS PHAGOSOMAL MEMBRANES

LM cells were cultured on choline analogues for 72 h, treated with trinitrobenzenesulfonic acid under nonpenetrating conditions, fed 0.497-μm beads (2000 beads/cell) for 30 min, and phagosomes were isolated as described in Materials and Methods Phospholipid composition was determined as described in Materials and Methods. Values represent the mean ± S E (n = 3) Alternately isolated phagosomes instead of the whole cells were treated with trinitrobenzenesulfonic acid TNBS, trinitrobenzenesulfonic acid. Other abbreviations as in Table I

Supplement	Treatment with TNBS	% PE trinitrophenylated
Choline	Cells	8 1 ± 0.9
DME	Cells	6.7 ± 1.3
MME	Cells	9 6 ± 0.9
E	Cells	8 7 ± 0 8
Choline	Phagosome	52.3 ± 1 2
DME	Phagosome	51.1 ± 0.5
MME	Phagosome	51 0 ± 1.2
E	Phagosome	42 0 ± 1 9 [a]

[a] $P \leq 0.01$ as compared to choline-supplemented cells

Effect of choline analogue supplementation on the reactivity of phosphatidylethanolamine in the phagosomal membrane with trinitrobenzenesulfonic acid

The asymmetric distribution of phosphatidyl-

ethanolamine across LM cell membrane bilayers can be determined using trinitrobenzenesulfonic acid [32–36]. At 4°C and pH 8.5, the reagent does not penetrate the LM fibroblast surface membrane. Thus, only phosphatidylethanolamine in the outer monolayer of the plasma membrane is trinitrophenylated. Only 4.3 ± 0.3% of the plasma membrane phosphatidylethanolamine was trinitrophenylated. The data in Table V shows that the phagosomes internalized about 8–10% of the trinitrophenylphosphatidylethanolamine, thereby indicating a 2-fold enrichment of this phospholipid in the membrane domain at which the latex beads were internalized by the cell. No significant effect of choline analogues on this enrichment were noted. However, when phagosomes isolated from untreated LM cells were exposed to trinitrobenzenesulfonic acid, approx. 50% of the phosphatidylethanolamine was trinitrophenylated in phagosomes from choline-, *N,N'*-dimethylethanolamine-, and *N*-monomethylethanolamine-supplemented cells. In contrast, significantly less phosphatidylethanolamine (42.0 ± 1.9%) was trinitrophenylated in phagosomes from ethanolamine-supplemented cells. If the phagosomes were treated with trinitrobenzenesulfonic acid under penetrating conditions (37°C), then greater than 95% of the phosphatidylethanolamine was trinitrophenylated. Similar results were obtained when phagosomes were treated at 4°C with isethionyl acetimidate, a nonpenetrating probe [33] which labelled 55% of the phagosomal phosphatidylethanolamine, or with methyl acetimidate, a penetrating probe [33] which labelled greater than 92% of the phagosomal phosphatidylethanolamine. These results regarding the impermeability of phagosomal membranes to trinitrobenzenesulfonic acid at 4°C were also confirmed by other investigators [32].

Discussion

Role of lipids in the immune system

Serum lipid components [13–19,40], disease state [23–27,40], and drugs [41,42] can alter the lipid composition of cells in the immune system. Activation of lymphocytes involves stimulation of phospholipid metabolism [41,42]; transformation of lymphocytes to neoplastic cells alters membrane fluidity and cholesterol content [43,44], while lowered membrane cholesterol content and lowered unsaturated fatty acid contents decrease the phagocytic and pinocytic activity of macrophages and LM cells [20–22]. Alterations in phospholipid metabolism have also been shown to occur during endocytosis [9–11]. Thus, it seems entirely possible that the endocytic processes of the immune system and of other cells (capillary endothelial cells, thyroid enthathelial cells, yolk sac, oocytes, carcinoma cells, sarcoma cells, L cells, LM cells, etc.) known to actively endocytose, may be regulated at least in part by the lipid composition of the surface membranes [1,9–11, 20–22,32,37,39,47,48]. In addition, recent methods for drug delivery include encapsulation in liposomes [49]. The liposomes are actively phagocytosed by tissue macrophages. Thus, cellular lipid composition may also affect the rate of drug delivery by this means in rheumatoid arthritis, cancer, and respiratory distress syndrome [49].

Effect of alterations in phospholipid composition on endocytic processes

The data presented herein indicate that alterations in the phospholipid composition of LM fibroblasts dramatically affect endocytosis. Enrichment of the surface membranes with phosphatidyl-*N,N'*-dimethylethanolamine, phosphatidyl-*N*-monomethylethanolamine, or phosphatidylethanolamine resulted in decreased uptake of polystyrene beads (phagocytosis) and horseradish peroxidase (pinocytosis). This is in agreement with other investigators who have shown that virus budding from LM cells and chick embryo fibroblasts supplemented with certain analogues of choline was severely reduced [45,46]. It is possible that endocytic rates may be correlated with the degree of cell surface 'ruffling'. Confluent LM cells differed considerably in morphology from cells in exponential growth phase [47]. Confluent cells had many more microvilli, blebs, and ruffles than growing cells. These confluent cells had 2–4-fold increased pinocytic activity Choline analogue supplementation with the exception of *N*-monomethylethanolamine and L-2-amino-1-butanol did not, however, alter the morphology of chick embryo fibroblasts [46]. In both of the latter cases, the cells became shorter, broader, round and refractile. The cells supplemented with L-2-amino-1-butanol also had ruffles and more microvilli. Thus, simply on the basis of morphological alterations, one would have expected increased endo-

cytosis with increased ruffling and microvilli formation when LM cells were grown in the presence of choline analogues. However, the results presented here were found to be exactly opposite: phagocytosis and pinocytosis both decreased rather than increased when LM cells were grown in the presence of choline analogues.

Phospholipids of the phagosomal membranes

Other investigators have shown increased formation of phosphatidylcholine in lymphocytes on stimulation with tumor promoters [42], decreased content of phosphatidylcholine in leukemic lymphocytes [44], and increased incorporation of phosphatidylcholine from lysophosphatidylcholine into phagosomal membranes from leukocytes [9–11]. The results presented herein also indicate an enrichment or a preferential incorporation of phosphatidylcholine into phagosomal membranes from LM cells supplemented with choline or choline analogues. Thus, although the phospholipid composition of phagosomes reflects the same qualitative pattern as the primary plasma membrane, the quantitative pattern was significantly different. In addition, trinitrophenylphosphatidylethanolamine was preferentially incorporated into phagosomal membranes. These data are in agreement with those of Charalampous [12], indicating that endocytosis occurs either at nonrandom sites on the surface of the cell or that rearrangement of the lipids occurs.

Asymmetric distribution of phosphatidylethanolamine across the phagosomal membrane

The asymmetric distribution of lipids in membranes has recently been reviewed [50]. The only eucaryotic surface membrane that has been investigated thoroughly is the red blood cell membrane. However, very little has been done regarding investigations of surface membrane lipid asymmetry in other eucaryotes, except for the tumorigenic LM fibroblasts plasma membrane [33–36,51,52] and the synaptosomal plasma membrane [34,53]. In contrast, model membrane systems, virus membranes, and bacterial membranes have been investigated in detail [50]. Membrane derivatives such as eveloped viruses were once believed to reflect random areas of cell surface membrane and could therefore reflect surface membrane properties as a whole [54]. However, this is not the case [50]. Similarly, a recent report indicates that phagosomal membranes from LM cells may simply represent inside-out surface membranes [32]. Again as shown above, the phagosomal lipids do not resemble in composition the plasma membrane of LM cells. It was also shown that if LM fibroblasts are cultured in monolayer, released by scraping, exposed to trinitrobenzenesulfonic acid under nonpenetrating conditions, and exposed to polystyrene beads, then approx. 25% of the phosphatidylethanolamine was trinitrophenylated. The two numbers convincingly add up to 100% and were therefore interpreted as representing the inverted asymmetric distribution of phosphatidylethanolamine in the LM surface membrane. The same experiment performed with suspension-grown LM cells herein provided 8 1 and 52.3% trinitrophenylphosphatidylethanolamine. These numbers add up to only 60.4%. The differences between these two investigations could be the medium used, the type of culture (monolayer vs. suspension), as well as other factors. In the present investigation, suspension cultures were used while in previous work [32], monolayer cells were used. Plasma membrane lipids from suspension-cultured cells versus cells in monolayer are not identical in composition. We have found that scraping LM fibroblasts from monolayers results in greater penetration of the trinitrobenzenesulfonic acid labelling reagent into the cell. This could account for the high trinitrophenylation (25%) noted by others [32]. When the experiment with monolayer cells was performed in our laboratory, the monolayer culture itself was exposed to the reagent prior to scraping or exposure to polystyrene beads. In this case, the percent trinitrophenylphosphatidylethanolamine was 13.3 and 63.0%, respectively (unpublished data). These values add up to 76.3%, and also fall short of 100%. Lower than 100% trinitrophenylation could be explained by either lack of complete reactivity of phosphatidylethanolamine with the trinitrobenzenesulfonic acid [55], a structural reorganization of the surface membrane in which selective patches are internalized, or altered asymmetric distribution due to transbilayer redistribution ('flip-flop') during endocytosis. These possibilities are presently under investigation.

In summary, alterations in the surface membrane phospholipid composition correlated with a reduction in the phagocytosis of polystyrene beads and in the

pinocytosis of horseradish peroxidase. The data are consistent with a nonrandom composition of endocytic sites in the LM fibroblast membrane. These considerations may be of importance in endocytic processes such as the immune surveilance system internalization of plasmodium (malarial parasites), tumor cell nutrition, and endocytic uptake of drug containing liposomes targeted against neoplastic cells.

Acknowledgements

The author wishes that thanks be extended to Ms. Hsiao-Mei Wiedemeyer for her excellent technical assistance. This investigation was supported in part by grants from the National Cancer Institute (USPH CA 24339) and by the American Heart Association (AHA 78734).

References

1 Silverstein, S.C., Steinman, R.M. and Cohn, A.Z. (1977) Annu. Rev. Biochem. 46, 669–722
2 Sherman, I.W. (1979) Microbiol. Rev. 43, 453–495
3 Kavet, R.I. and Brain, J.D. (1980) J. Reticuloendothel. Soc. 27, 201–221.
4 Steinman, R.M., Brodie, S.E. and Cohn, Z.A. (1976) J Cell Biol. 60, 665–687
5 Seed, T.M. and Kreir, J.P. (1972) Proc. Helminthol. Soc. Wash. 39 (Spec. Issue), 387–411
6 Beach, D.H., Sherman, I.W. and Holz, G.G., Jr. (1977) J. Parasitol. 63, 62–75
7 Howard, R.J. and Sawyer, W.H. (1980) Parasitology 80, 331–342
8 Berlin, R.D. and Fera, J.P. (1977) Proc Natl. Acad. Sci. U.S.A. 74, 1972–1976
9 Elsbach, P. (1968) J. Clin. Invest. 51, 1910–1914
10 Elsbach, P., Patriarca, P., Pettri, P., Stossel, T.P , Mason, R.J. and Vaughan, M. (1972) J. Clin. Invest. 51, 1910–1914
11 Sastry, P.S. and Hokin, L.E. (1966) J. Biol Chem 241, 3354–3361
12 Charalampous, F. (1979) Biochim. Biophys. Acta 556, 38–51
13 Eisenberg, S. and Levy, R.I. (1976) Annu. Rev Med. 1–89
14 Smith, L.C., Pownall, H.J and Gotto, A.M. (1978) Annu. Rev. Biochem. 47, 751–777
15 Bruckdorfer, K.R., Demel, R A., De Gier, J. and Van Deenen, L.L.M (1969) Biochim Biophys. Acta 183, 334–345
16 Schroeder, F., Goh, E.J. and Heimberg, M. (1976) J. Biol. Chem. 254, 2456–2463
17 Schroeder, F. and Goh, E.H. (1976) J. Biol. Chem. 254, 2464–2470
18 Keyes, W.G. and Heimberg, M. (1979) J. Clin Invest. 64, 182–190
19 Yamamoto, R.S., Crittenden, L.B., Sokoloff, L and Jay, G.E., Jr. (1963) J Lipid Res. 4, 413–418
20 Mahoney, E.M., Hamill, A.L., Scott, W.A. and Cohn, Z.A. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4895–4899
21 Schroit, A.J. and Gallily, R. (1979) Immunology 36, 199–205
22 Heminger, J -J. and Marshall, J D. (1979) Cell Biol. Int. Rep. 3, 409–420
23 Gottfried, E L. (1972) Semin. Hematol. 9, 241–250
24 Kalofoutis, A and Stratakis, N. (1978) I.R.C.S. Med. Sci. 6, 45
25 Kalofoutis, A., Koutselinis, A., Dionissiou-Asteriou, A. and Miras, C. (1978) Acta Pharmacol Toxicol. 43, 81–85
26 Capetanakis, J., Tsamboos, D. and Kalofoutis, A. (1974) Dermatologica 154, 85–89
27 Kalofoutis, A. (1980) Clin. Chem. 26, 247–249
28 Schroeder, F., Perlmutter, J.F., Glaser, M. and Vagelos, P R. (1976) J. Biol Chem. 251, 5015–5026
29 Schroeder, F. and Vagelos, P.R. (1976) Biochim. Biophys. Acta 441, 239–254
30 Schroeder, F., Holland, J F. and Doi, O. (1979) Arch. Biochem. Biophys. 194, 431–438
31 Saito, Y. and Silbert, D.F. (1979) J. Biol. Chem. 254, 1102–1107
32 Sandra, A. and Pagano, R.E (1978) Biochemistry 17, 332–338
33 Fontaine, R.N. and Schroeder, F. (1979) Biochim. Biophys. Acta 558, 1–12
34 Fontaine, R.N., Harris, R A and Schroeder, F (1980) J. Neurochem. 34, 269–277
35 Schroeder, F. (1980) Biochim. Biophys Acta 599, 254–270
36 Schroeder, F., Fontaine, R.N., Feller, D. and Weston, K. (1980) Biochim. Biophys Acta 643, 76–88
37 Steinman, R M. and Cohn, Z.A (1972) J. Cell Biol. 55, 186–204
38 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1954) J. Biol. Chem 193, 265–275
39 Roberts, J. and Quastel, J.H. (1963) Biochem. J 89, 150–156
40 Inbar, M., Goldman, R., Inbar, L., Bursuker, I., Goldman, B., Akstein, E., Segal, P., Ipp, E. and Ben-Bassat, I. (1977) Cancer Res. 37, 3037–3041
41 Florin, C.H., Nordgren, H. and Bilsson, A. (1977) Biochemistry 8, 331–340
42 Wertz, P.W. and Mueller, G C. (1978) Cancer Res 38, 2900–2904
43 Van Blitterswijk, W.J., Emmelot, P., Hilkmann, H.A.M., Oomenmeulemans, E.P.M. and Inbar, M. (1977) Biochim. Biophys Acta 467, 309–320
44 Hildebrand, J , Marique, D and Vanhouche, J (1975) J. Lipid Res. 16, 195–199
45 Maeda, M , Doi, O. and Akamatsu, Y. (1980) Biochim Biophys. Acta 597, 552–563
46 Hale, A.H., Pessin, J.E., Palmer, F., Weber, M.J. and Glaser, M. (1977) J. Biol. Chem 252, 6190–6200

47 Steinman, R.M., Silver, J.M. and Cohn, Z.A (1974) J. Cell. Biol. 63, 949–969
48 Trouet, A., Compeneere, D.D. and De Duve, C. (1972) Nat New Biol. 239, 110–112
49 Tom, B.H. and Dix, H.R. (1980) Liposomes and Immunology, pp. 255–315, Elsevier, New York
50 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
51 Schroeder, F. (1978) Nature 275, 528–530
52 Schroeder, F. (1980) Eur. J Biochem. 112, 293–307
53 Fontaine, R.N., Harris, R.A. and Schroeder, F. (1979) Life Sci. 24, 395–400
54 Wisnieski, B.J. and Iwata, K.K. (1977) Biochemistry 16, 1321–1328
55 Bishop, D.G , Bevers, E.M., Van Meer, G., Op den Damp, J.A F. and Van Deenen, L.L.M. (1979) Biochim Biophys. Acta 551, 122–128