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Generation of pH-Sensitive Liposomes: Use of Large Unilamellar Vesicles Containing *N*-Succinyldioleoylphosphatidylethanolamine†

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ABSTRACT: By use of a carboxylated derivative of phosphatidylethanolamine, *N*-succinyldioleoylphosphatidylethanolamine (COPE), pH-sensitive liposomes have been designed that have a wide range of leakage properties. The leakage rate of the vesicle contents, as determined by the release kinetics of the water-soluble fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid complexed with the quencher *p*-xylenebis(pyridinium) dibromide [Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532], was found to be dependent on the lipid composition and the pH of the incubation medium. Pure COPE vesicles released their encapsulated contents at pH 7.4 but not at pH 4.0. Leakage of these vesicles appears to be due to the electrostatic interactions between the COPE molecules. A dramatic reversal of the leakage properties was observed in mixed-lipid vesicles composed of COPE containing increasing amounts of dioleoylphosphatidylethanolamine (DOPE). Unlike pure COPE vesicles, COPE/DOPE (3:7) vesicles were more leaky under acidic conditions (pH 4.0) than they were at neutral pH. Studies employing a fluorescent COPE analogue, *N*-succinyl-1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylethanolamine, suggested that the mechanism of leakage might be a result of lipid-packing defects due to the nonbilayer properties of DOPE and protonation of the COPE molecules. Hence, the mechanism of release is different from that of other recently described pH-sensitive liposomes where either fusion or aggregation of the vesicles results in the release of vesicle contents.

Liposomes have recently attracted considerable interest as a potential modality for selective drug delivery in vivo (Gregoriadis & Allison, 1980). Many concerns have been raised, however, about their effectiveness in delivering drugs to extravascular sites because of anatomical barriers imposed by the peripheral circulation (Poste, 1983). In an effort to circumvent these problems, strategies have been suggested that take advantage of the reduced local pH environments of pathologic tissues (Yatvin et al., 1980) by employing pH-sensitive liposomes. Indeed, it has been shown that sites of primary tumors, metastasis, inflammation, and infection have an ambient pH considerably lower than that of normal tissues (Kahler & Robertson, 1943; Gullino et al., 1965; Meyer et al., 1948; Naeslund & Swenson, 1953). Conversely, the passive localization of liposomes to cells of the reticuloendothelial system has been exploited to deliver immunomodulators to these cells (Alving, 1983). Since the primary mechanism of liposome uptake by these cells is via endocytosis followed by sequestration of the vesicles into acidic intracellular vacuoles (Straubinger et al., 1983), utilization of this "pH-sensitive pathway" has been suggested as a means of improving the delivery of liposome-encapsulated compounds to macrophages (Poste & Kirsh, 1983; Straubinger et al., 1983; Straubinger et al., 1985).

The general strategy employed in the construction of pH-sensitive liposomes has been to include lipids containing pH-sensitive groups such as *N*-palmitoyl-L-homocysteine (Yatvin et al., 1980) and cholesteryl hemisuccinate (Ellens et al., 1984). These lipids have the ability to destabilize the lipid bilayer when exposed to acidic environments, which results in liposome–liposome fusion (Conner et al., 1984) and liposome–liposome aggregation (Ellens et al., 1984), respectively, followed by concomitant release of the entrapped compounds. The acid-induced leakage from liposomes that contain *N*-palmitoyl-L-homocysteine required the presence of lipids near their phase transition (Yatvin et al., 1980), and the release of contents from cholesteryl hemisuccinate containing liposomes has been shown to depend on the presence of auxiliary lipids, in particular, on the inclusion of phosphatidylethanolamine (Ellens et al., 1984).

In this paper, we describe the preparation and properties of COPE,[1] which, when incorporated into liposomes, conveys

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upon them a wide range of leakage properties that are dependent on the pH of the medium and the lipid composition of the vesicles. The stability of the liposomes was monitored by a quantitative assay that utilizes the fluorophore ANTS, which has been shown to be relatively insensitive to any pH above 4.0 and which can be quenched by DPX (Ellens et al., 1984; Smolarsky et al., 1977). This assay has been recently characterized in lipid systems and found to satisfy the requirements for reliable and convenient monitoring of pH-induced leakage of encapsulated contents. Our results indicate that the mechanism of leakage from the COPE-containing liposomes is not a result of vesicle aggregation and/or fusion, as has been reported in other pH-sensitive liposome systems (Ellens et al., 1984; Connor et al., 1984), but results from bilayer destabilization induced by pH-dependent lipid-packing defects and electrostatic interactions between the negatively charged COPE molecules.

Experimental Procedures

Materials. EPC, EPE (transphosphatidylated EPC), DOPC, DOPE, NBD*-PE, *N*-NBD-PE, and *N*-Rh-PE were obtained from Avanti Polar Lipids (Birmingham, AL). The ANTS and DPX were obtained from Molecular Probes, Inc. (Junction City, OR). All lipids were chromatographically pure, as determined by two-dimensional thin-layer chromatography using activated silica gel 60 thin-layer plates (Merck). Steady-state fluorescence and light scattering were quantified at 20 °C with a dual-beam Farrand MK II spectrophotofluorometer. Polycarbonate filters (0.1-μm pore size) were obtained from Nucleopore (Pleasanton, CA).

Preparation of COPE. DOPE was carboxylated by a modification of the procedure used by Kinsky et al. (1984). DOPE (270 μmol) was dried under nitrogen in a 100-mL round-bottom flask and placed under high vacuum for 2 h to remove residual solvent. The dry lipid was dissolved in freshly distilled pyridine (10 mL) containing succinic anhydride (540 μmol) and sealed under nitrogen. The reaction mixture was then stirred for 2 h under nitrogen at 55 °C. Thin-layer chromatography in $CHCl_3$/MEOH/H_2O (65:25:4) revealed complete conversion of DOPE to COPE, as shown by ninhydrin (no staining) and phosphate reagent sprays. Following removal of the pyridine by evaporation under reduced pressure, the excess succinic anhydride was hydrolyzed by resuspending the residue in $CHCl_3$/MeOH/0.58% NaCl (1:2:0.8 v/v). COPE was then extracted by partitioning after the addition of 1 volume of $CHCl_3$ and 1 volume of 0.58% NaCl. The lower phase was washed 3 times with $CHCl_3$/MEOH/0.58% NaCl (3:48:47), and the lower organic phase containing pure COPE was subsequently dried by rotary evaporation and resuspended in $CHCl_3$. Typical yields of chromatographically pure COPE averaged 90% with respect to the initial amount of DOPE used.

Preparation of LUVETs. All leakage studies were carried out with LUVETs prepared by the extrusion technique of Hope et al. (1985), which has been shown to yield homogeneous vesicle preparations. Briefly, LUVETs were prepared by extruding a multilamellar preparation through two stacked polycarbonate filters (0.1-μm pore size) under moderate nitrogen pressure (150 psi) a total of 10 times. The vesicles were then separated from unencapsulated material by passing the LUVETs through a small Sephadex G-50 column (1-mL bed volume) equilibrated in the appropriate buffer. Typically, vesicles were prepared in buffer that contained 50 mM ANTS/DPX and 10 mM HEPES, pH 7.4, and the unencapsulated solution (the external medium) was exchanged with an equiosmotic solution of pH 7.4 or 4.0 of 150 mM NaCl containing 10 mM HEPES or 20 mM sodium acetate buffer, respectively.

The ANTS/DPX leakage assay was done as described by Ellens et al. (1984), with excitation and emission wavelengths of 358 and 520 nm, respectively. The residual fluorescence of the liposomes containing 50 mM DPX and 50 mM ANTS was measured immediately after elution from the Sephadex column and taken as 0% release. Maximum fluorescence after solubilization of the liposomes with 1% Triton X-100 (final concentration) was taken as 100% release. Since there is no significant binding of ANTS to the liposomes (Ellens et al., 1984; R. Nayar and A. J. Schroit, personal observations), relative fluorescence measured at any time represents leakage of the liposome contents. All experiments were done at room temperature in buffers of different pH (10 mM HEPES, pH 7.4; 20 mM sodium acetate/acetic acid, pH 4.0, 4.5, 5.0, and 6.0, containing 150 mM NaCl), which were equiosmotic with the encapsulated ANTS/DPX buffer. The experiment was started by mixing 20 μL of concentrated liposomes (1 mM lipid) into 1.5 mL of the buffer. Relative fluorescence was then measured immediately and at various time intervals, with the excitation beam shutter kept closed between measurements to prevent bleaching of the fluorophore.

Fluorescence Energy Transfer and Quenching Experiments. To determine the possible mechanism of leakage in the COPE and COPE/DOPE systems, membrane fusion was monitored by fluorescence energy transfer (Struck et al., 1981), and alterations in lipid packing were monitored by differences in the quantum yield of NBD-labeled lipid analogues (Hoekstra, 1982).

Briefly, membrane fusion was monitored according to the procedure described by Struck et al. (1981); *N*-NBD-PE was used as the energy donor and *N*-Rh-PE as the energy acceptor. The LUVETs contained 1% *N*-NBD-PE and 1% *N*-Rh-PE, and the efficiency of energy transfer was measured over 2 h in the presence of a 10-fold excess of unlabeled vesicles at pH 7.4 and 4.0. The quenching experiments were done by employing a NBD-COPE analogue synthesized from C_6-NBD-PE as described above. The quantum yield of pure NBD-COPE vesicles was determined in the absence and presence of detergent in buffers of pH 7.4 and 5.0.

Results

Leakage Characteristics of Pure COPE LUVETs. To determine the pH effect on liposomes composed of pure COPE, LUVETs containing 50 mM ANTS/50 mM DPX were suspended in pH 7.4 and pH 4.0 buffers, and the development of fluorescence was monitored. Figure 1 shows that the rate of leakage is relatively fast at pH 7.4, with about 65% of the entrapped contents released within 15 min and almost all of the contents released after 1 h. In contrast, the leakage of ANTS/DPX from the vesicles was significantly slower at pH 4.0, with about 4% release occurring in 15 min and only 40% after 4 h of incubation.

Leakage Characteristics of COPE/DOPE and COPE/EPC LUVETs. Figure 2a shows the release characteristics of LU-

[1] Abbreviations: COPE, *N*-succinyldioleoylphosphatidylethanolamine; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; DPX, *p*-xylylenebis(pyridinium) dibromide; EPC, egg phosphatidylcholine; EPE, egg phosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; LUVETs, large unilamellar vesicles prepared by extrusion technique; NBD*-PE, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine.

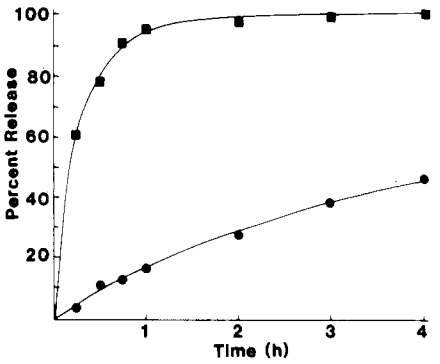

FIGURE 1: Effect of pH-induced leakage on pure COPE LUVETs. Twenty microliters of COPE LUVETs (1 mM) in pH 7.4 buffer containing 50 mM ANTS/DPX and 10 mM HEPES was injected into 1.5 mL of buffers (final lipid concentration 15 μM) of pH 7.4 (■) (150 mM NaCl/10 mM HEPES) or pH 4.0 (●) (150 mM NaCl/20 mM sodium acetate/acetic acid), and the development of fluorescence was measured as a function of time at room temperature. Leakage of ANTS/DPX was expressed as the percent release, where 0% was the residual fluorescence and 100% was the fluorescence measured after the addition of Triton-X-100 (1% final concentration) minus the initial background fluorescence.

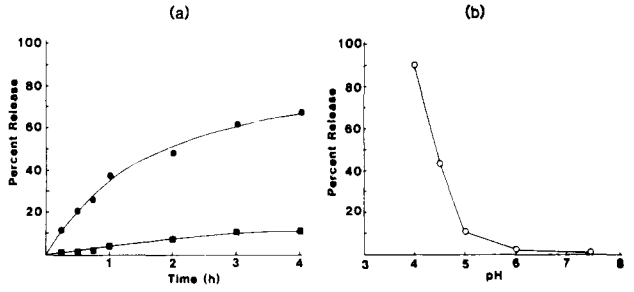

FIGURE 2: Effect of pH-induced leakage on COPE/DOPE (3:7) LUVETs (panel a) and the amount of leakage at various pHs (panel b). Twenty microliters of COPE/DOPE LUVETs (1 mM) was injected into 1.5 mL of buffers (final lipid concentration 15 μM) of pH 7.4 (■) and pH 4.0 (●), and fluorescence was measured as a function of time (see legend to Figure 1). Panel b illustrates the amount of ANTS/DPX released from COPE/DOPE LUVETs after a 2-h incubation in buffers of pH 7.4 (150 mM NaCl/10 mM HEPES) or pH 4.0, 4.5, 5.0, or 6.0 (150 mM NaCl/20 mM sodium acetate/acetic acid buffers).

VETs composed of COPE/DOPE (3:7) at pH 7.4 and 4.0. Incorporation of DOPE results in a dramatic reversal of the release characteristics, with more leakage occurring at pH 4.0 than at pH 7.4. It can be seen that almost 50% of the entrapped contents were released from the vesicles after 2 h at pH 4.0, whereas only approximately 7% was released at pH 7.4. To investigate the pH dependence of leakage, liposomes were injected into buffers ranging from pH 4.0 to pH 7.4, and the percentage of the entrapped ANTS/DPX released in 2 h was determined. The results presented in Figure 2b indicate that very little leakage occurred above pH 6.0. However, as the pH decreased, there was a concomitant enhancement in the amount of ANTS/DPX released from the liposomes with half-maximal release occurring at approximately pH 4.5.

The reversal in the release characteristics of COPE/DOPE liposomes could possibly be due to the known bilayer-stabilizing properties of negatively charged phospholipids in lipid systems containing nonbilayer lipids such as DOPE (Cullis & Hope, 1980; Cullis et al., 1983). To investigate this possibility, the release of ANTS/DPX from COPE vesicles containing decreasing amounts of DOPE was determined. The results presented in Figure 3 show that LUVETs containing less DOPE (COPE/DOPE 3:7, 1:1, 7:3) released proportionally less ANTS/DPX at pH 4.0. In the case of equimolar

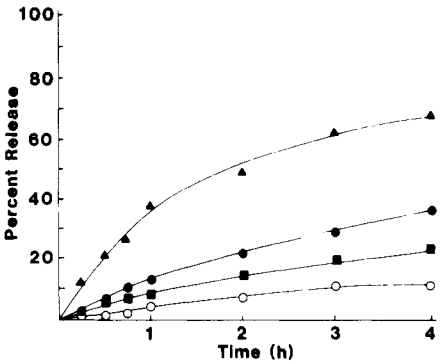

FIGURE 3: Effect of different COPE/DOPE ratios on the leakage of ANTS/DPX at pH 4.0. LUVETs containing different COPE/DOPE ratios [3:7 (▲), 1:1 (●), 7:3 (■)] were monitored for release of encapsulated ANTS/DPX at pH 4.0 (150 mM NaCl/20 mM sodium acetate/acetic acid). The final lipid concentration was 15 μM. For all COPE/DOPE ratios, the leakage of ANTS/DPX was identical at pH 7.4 (○).

Table I: Release of ANTS/DPX from Vesicles of Different Phospholipid Compositions

	percent release			
	1 h		4 h	
LUVET lipid compn	pH 4.0	pH 7.4	pH 4.0	pH 7.4
COPE/DOPE (3:7)	38	4	70	10
DOPC	6	4	22	13
EPC	8	5	12	10
COPE	16	100	40	100
COPE/DOPC (3:7)	7	0	19	4
COPE/EPC (3:7)	8	10	25	21
COPE/EPE (3:7)	6	1	16	5

COPE/DOPE, 35% of the entrapped contents were released after 4 h, whereas vesicles containing 30 mol % DOPE released only about 20% of their contents. Interestingly, there were no significant differences in leakage at pH 7.4 with any of the DOPE concentrations used, unlike the effects observed when pure COPE vesicles were used (see Figure 1).

To assess the possible role of acyl-chain unsaturation and nonbilayer lipids on the pH-induced release of the mixed COPE vesicles, EPC, DOPC, and EPE were substituted for DOPE. As shown in Table I, the dramatic differences in leakage rates observed with the COPE vesicles at acidic and neutral pH significantly decreased when EPE, EPC, or DOPC was substituted for DOPE.

Fluorescent Studies on the Mechanism of pH-Induced Release of COPE and COPE/DOPE LUVETs. In two other recently described pH-sensitive liposome systems, release of the encapsulated vesicle contents has been shown to be due to either liposome–liposome fusion or liposome–liposome aggregation when palmitoylhomocysteine (Conner et al., 1984) and cholesteryl hemisuccinate (Ellens et al., 1984), respectively, have been used. To determine if these or other mechanisms of release were responsible for our results with COPE/DOPE vesicles, membrane fusion was monitored by fluorescence energy transfer (Struck et al., 1981), and aggregation was monitored by light scattering and analysis of concentration-dependent leakage rates (Bentz & Nir, 1981).

The possibility of acid-induced liposome fusion was investigated by determining the efficiency of resonance energy transfer with COPE/DOPE LUVETs containing 1 mol % of *N*-NBD-PE and *N*-Rh-PE. In this assay any decrease in the surface density of the energy acceptor (*N*-Rh-PE) results in a proportional decrease in the measured transfer efficiency (E); hence, a measure of lipid dilution as would occur during vesicle–vesicle fusion. The results of these experiments in-

Table II: Effect of pH on Fluorescence of NBD-Labeled Phospholipid[a]

	pH 7.4	pH 5.0
NBD-PC		
−Triton	0.048	0.047
+Triton	2.16	2.19
Δ	45.0	46.6
NBD-COPE		
−Triton	0.174	0.082
+Triton	2.98	2.98
Δ	17.1	36.3

[a] Pure NBD-PC and NBD-COPE vesicles were monitored for efficiency of NBD fluorescence (λ_{ex} 469 nm; λ_{em} 520 nm) in the absence and presence of 1% Triton X-100 at pH 5.0 and 7.4. Addition of detergent relieves self-quenching of the NBD molecules to identical fluorescence valves, implying no pH-induced decrease in fluoroescence efficiency of the NBD probe per se. The detergent enhancement is expressed as a ratio of fluorescence in the presence and absence of detergent, Δ.

dicated essentially no significant alterations in E over a period of 2 h with up to a 10-fold excess of unlabeled COPE/DOPE vesicles at either pH 4.0 (E = 85%) or pH 7 (E = 90%). It should be noted that this assay was indeed indicative of fusion since the addition of excess calcium (approximately 10 mM) resulted in an immediate decrease in E to approximately 36% similar to that of a positive control with PE/PS (3:1) vesicles, which readily fuse in the presence of calcium (results not shown here).

Aggregation-dependent mechanisms of liposome leakage that have been shown to exhibit concentration-dependent leakage rates (Conners et al., 1984) were also investigated. ANTS/DPX release profiles from COPE/DOPE LUVETs at pH 4.0 were found to be concentration-independent (for up to 4 h, from 10 to 50 μM), and no alterations in light scattering by vesicles were detected, strongly suggesting that the mechanism of leakage is independent of liposome–liposome contact/aggregation.

The results presented above suggest that a pH-dependent rearrangement of lipids in the COPE/DOPE system might be responsible for the enhanced leakage at pH 4.0, whereas our results showing more leakage at pH 7.4 with the pure COPE could be explained by electrostatic repulsion of the unprotonated molecules. Since the fluorescent efficiency of NBD-labeled lipid analogues is extremely sensitive to the probes' density in bilayer membranes (Hoekstra, 1982), we reasoned that any alterations in lipid packing at either acidic or neutral pH should be detectable by a concomitant alteration in the fluorescence efficiency. To investigate this possibility, vesicles were made from NBD-COPE and the fluorescent efficiency at both pH 5.0 and pH 7.4 was determined by comparing the relative fluorescent intensities of the vesicles in the presence and absence of detergent. The results presented in Table II clearly show a concomitant decrease in vesicle fluorescence and higher detergent-mediated enhancement with decreasing pH, suggesting a higher effective lipid density (closer packing) of NBD-COPE at the lower pH values. It should be noted that the decrease in fluorescence was not due to a pH-induced decrease in fluorescence efficiency of the NBD probe per se, since the relative fluorescence of the lipid was essentially identical at both pHs in the presence of detergent. Furthermore, no pH-induced alterations in the fluorescence of NBD-PC were observed in the presence or absence of detergent.

Discussion

By use of COPE alone and in mixed-phospholipid systems, pH-sensitive liposomes have been designed that have a wide range of leakage properties. For example, pure COPE LUVETs were leaky at pH 7.4 but not at pH 4.0. However, in the presence of 70 mol % DOPE, the leakage properties of COPE/DOPE LUVETs were reversed, with more leakage under acidic conditions. When EPE, DOPC, and EPC were substituted for DOPE, the pH-induced leakage of the vesicles at pH 4.0 was completely abolished.

The molecular mechanism of leakage in the lipid systems described here seems to be unlike two other recently described pH-sensitive liposome systems, where either liposome–liposome aggregation (Ellens et al., 1984) or liposome–liposome fusion (Conner et al., 1984) induced vesicle leakage. Leakage of the pure COPE liposomes appears to be a result of electrostatic repulsion and lipid-packing defects induced by the unprotonated carboxyl groups of the COPE molecules at pH 7.4 (net charge 2−). Conversely, protonation of the carboxyl moiety at acidic pH (net charge 1−) results in tighter packing and less leakage of ANTS/DPX. This is supported by fluorescent-quenching experiments employing a COPE analogue (Table II) in which enhanced quenching of NBD-COPE was observed at pH 5.0, implying tighter packing of the COPE molecules. In this system, the possibility of membrane fusion was also ruled out by fluorescent energy-transfer experiments that used nonexchangeable fluorescent phospholipid analogues as described by Struck et al. (1981).

The leakage characteristics of the COPE/DOPE system are similar to those described by Ellens et al. (1984) for DOPE/cholesteryl hemisuccinate, although the mechanism of leakage appears to be different. Our results suggest that the greater negative charge of COPE at pH 7.4 increases the bilayer-stabilizing properties of COPE in DOPE-containing systems similar to those observed in other mixed acidic phospholipid systems (Tilcock & Cullis, 1981; Cullis et al., 1983). This property is most clearly demonstrated in the COPE titration experiment, in which increasing the COPE content from 30 to 70 mol % led to the reduction of ANTS/DPX leakage from the COPE/DOPE vesicles from 70% to 23% after 4 h at pH 4.0. In addition, the rate of leakage from the COPE/DOPE vesicles was independent of lipid concentration, strongly implying that the release process was not due to liposome–liposome aggregation (Bentz & Nir, 1981). Several lines of evidence point to this mechanism of release. First, the specificity for the pH-induced release is dependent on the presence of DOPE and cannot be substituted with DOPC, EPC, or EPE, suggesting that lipid shape might be involved in the release process. In addition, the ability to form relatively stable COPE liposomes with DOPC, EPC, and EPE at pH 7.4 and 4.0 implies that acyl-chain saturation and the PE head group per se are not contributing factors. Since DOPE can undergo H_{II} phase transitions at room temperature and is stabilized into a bilayer conformation in the presence of acidic phospholipids (Cullis et al., 1983), it is conceivable that a similar mechanism of nonbilayer structures destabilizing the membrane in the COPE/DOPE vesicles is also operative here. Indeed, it is quite likely that protonation of COPE at pH 4.0 would disturb the molecular arrangement of the bilayer and result in analogous packing defects (pores), such as has been observed in dimyristoylphosphatidylcholine bilayers at the phase-transition temperature (Van Hoogevest et al., 1984).

In conclusion, pH-sensitive liposomes have been designed in which the leakage properties of the trapped contents can be easily manipulated by altering the lipid composition. Whether these liposomes are potentially useful for selectively delivering their contents to cells of the reticuloendothelial system is currently under investigation. Indeed, taking into

consideration that phagocytosed liposomes encounter acidic pH compartments after their internalization (Straubinger et al., 1983, 1985), it is conceivable that such a system would be therapeutically advantageous by remaining stable (i.e., nonleaky) until acidified intracellularly.

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Registry No. COPE, 97782-02-0; DOPC, 10015-85-7; DOPE, 2462-63-7.

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