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Permeability behavior of liposomes prepared from fatty acids and fatty acid methyl esters

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The permeability properties of liposomes prepared at pH 8.7 from a fatty acid and either methyl oleate or methyl elaidate, with or without cholesterol, were investigated. The fatty acids used were oleic acid, elaidic acid, and the selenium-containing fatty acids 9-selenaheptadecanoic acid and 13-selenaheneicosanoic acid. The liposomes trapped sucrose and carboxyfluorescein. Their volume change resulting from osmotic shock was directly proportional to the change in absorbance (light scattering). Liposomes prepared from oleic acid and either methyl oleate or methyl elaidate underwent osmotic swelling much more slowly than liposomes prepared from elaidic acid and either methyl oleate or methyl elaidate. Incorporation of cholesterol decreased the initial rate of erythritol permeation, especially in liposomes containing methyl oleate. The swelling rates of liposomes prepared with the selenium-containing fatty acids indicated that incorporation of methyl elaidate gave more tightly packed bilayers than did incorporation of methyl oleate. The effect of cholesterol on the initial rate of erythritol influx was greater in oleic acid and elaidic acid liposomes than in selenium-containing fatty acid liposomes, indicating that the large bulk of the selenium heteroatom suppresses the ability of cholesterol to interact with the hydrocarbon chain.

The self-assembly of surfactant molecules in water into micelles and vesicles has been considered in detail because both the practical and theoretical aspects are of interest (for reviews of the applications of surfactant aggregation, see Refs. 1 and 2; for reviews of the theory, see Refs. 3 and 4). Although single-chain amphiphiles generally form micelles, Gebicki and Hicks [5] showed that closed multilamellar vesicles are formed by vortex mixing of oleic or linoleic acid in an alkaline medium (pH 8–9); furthermore, recent biophysical measurements showed that equimolar mixtures of fatty acids and fatty acid soaps in water consist of crystalline and liquid-crystalline aggregates at pH 7.4 [6]. These model lipid bilayers trap glucose and shrink and swell in response to osmotic gradients, in a manner analogous to that of multilamellar vesicles from phospholipids bearing two long hydrocarbon chains. When single-chain amphiphiles containing an anionic head group (such as carboxylate, sulfate, or phosphate) are mixed with approximately equimolar amounts of long-chain alcohols [7,8] or lysophospholipid [9], lamellar structures are formed at alkaline pH. Hargreaves and Deamer [7] concluded that long-chain alcohols serve as spacer molecules, maintaining a separation of charge between the head groups of the anionic surfactants and allowing the close packing of the hydrocarbon chains required for formation of bilayers.

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The existence of stable membranes in organisms such as the alga *Ochromonas danica*, whose lipids consist mainly of single-chain amphiphiles (alkyl sulfates and free fatty acids) [10], makes investigation of model membranes containing anionic lipids especially interesting. The properties of vesicles from single-chain amphiphiles are also of interest because these structures entrap a variety of substances, and thus represent prototypes of the simple cells that arose spontaneously under prebiotic conditions [5,7]. In addition to their use in very simple model membrane mimetic systems, single-chain amphiphiles have been incorporated into vesicles with lysophospholipids [9] and diacylphospholipids [11], and their effects on vesicle stability have been analyzed at low and high pH. In this report, the permeability behavior of membranes containing fatty acids is examined. Previous findings with fatty acid liposomes attributed a decrease in absorbance (light scattering) to the swelling of liposomes exposed to osmotic shocks and showed an increase in leakiness when cholesterol was incorporated [5]. In contrast, we show here that the absorbance change is directly proportional to the volume change and that cholesterol incorporation decreases the initial rate of nonelectrolyte permeation into fatty acid liposomes.

In addition, we report the permeability properties of multilamellar vesicles from selenium-containing fatty acids and methyl esters of fatty acids. Lipid-associated selenium has been observed in marine invertebrates, fish, and higher plants [12]. The possibility of covalent bonding between selenium and carbon in algae lipids has been considered. However, it is not known exactly how selenium is incorporated into the lipids of marine animals and plants. Organoselenium compounds have also been used as radiopharmaceuticals, in which ^{75}Se is substituted for sulfur or carbon. Long-chain fatty acid analogs in which ^{75}Se is inserted between carbon-carbon bonds are attractive as myocardial imaging agents because they are taken up into the heart after administration to rats [13,14]. In this paper we examine the effect of placing the large selenium heteroatom into two positions along the fatty acyl chain on the permeability behavior of the vesicles. The steric effect of this heteroatom may be analogous to introducing an ethylene or cyclopropyl group into the hydrocarbon chain.

Materials. Oleic and elaidic acids, methyl oleate, and methyl elaidate were purchased from Sigma Chemical Co. and used without further purification. Cholesterol (Sigma) was recrystallized twice from absolute ethanol. The selenium-containing fatty acids, 9-selenaheptadecanoic acid (referred to as 9-Se-C_{16}), and 13-selenaheneicosanoic acid (referred to as 13-Se-C_{20}), were synthesized and purified by Dr. F.F. Knapp, Jr., Oak Ridge National Laboratory [14]. These lipids were dissolved in chloroform and stored under argon at $-20°C$. Carboxyfluorescein was purchased from Eastman Kodak; after recrystallization from methanol, it gave a single spot on cellulose MN thin-layer plates (elution with chloroform/methanol/water, 65 : 25 : 4, by vol.).

Preparation of liposomes. Liposomes were prepared by withdrawing the desired volumes of stock solutions of the lipids in chloroform. After the solutions were mixed, the solvent was evaporated under a stream of nitrogen, the lipid film was dried under vacuum, and then suspended in a solution of 0.20 M sucrose or 0.10 M choline chloride in 50 mM Tris (pH 8.7). The lipids were dispersed by vigorous agitation on a vortex mixer for 1 min in the presence of three glass beads. Aqueous dispersions from 13-Se-C_{20} were prepared by vortex mixing of the suspension at 70°C for 2.5 min. The suspensions obtained after vortex mixing of the various lipids in sucrose/Tris buffer were sonicated for 10 min under nitrogen at $38 \pm 2°C$ in a bath-type sonicator (Lab Supply Co., Hicksville, NY), whereas those in choline chloride-Tris buffer were sonicated in a Heat Systems Model W375A sonicator equipped with a microtip at power level 3 and 40% pulse for 6 min. Cholesterol-containing liposomes were sonicated an additional 3–5 min. Since no pellet formed on centrifugation of the suspensions for 20 min in a Sorvall centrifuge in a SS34 rotor at 10000 rpm, the lipid concentration of the liposomes was calculated from the concentrations of the stock solutions. The liposomes were allowed to stand overnight at 37°C before the permeability measurements were carried out.

Entrapment of carboxyfluorescein. All of the liposomes trapped the fluorescent dye carboxyfluorescein. For example, the procedure for trapping carboxyfluorescein in vesicles containing 9-

Se-C_{16}, methyl oleate, and cholesterol was as follows. A dry film of 25 μmol total lipid (equimolar amounts of 9-Se-C_{16} and methyl oleate, and 28 mol% cholesterol) was vortexed in the presence of 1 ml of 50 mM Tris (pH 8.7) containing 0.20 M sucrose and 0.16 M carboxyfluorescein. After sonication for 1.5 min using a microtip, the liposomes were passed through a Sephadex G-50 column (1×35 cm) and eluted with sucrose-Tris buffer, removing untrapped carboxyfluorescein. The trapped volume was calculated from the absorbance at 490 nm, assuming an extinction coefficient of $6.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$. The cholesterol concentration was determined as cited previously [15]. The volumes trapped in liposomes made from 36 mol% fatty acid (oleic acid, elaidic acid, 9-Se-C_{16} or 13-Se-C_{20}), 36 mol% methyl elaidate or methyl oleate, and 28 mol% cholesterol were between 0.4 and 0.5 liter per mol of lipid. Phase-contrast microscopy of suspensions from 9-Se-C_{16} and methyl oleate showed multilamellar liposomal structures and a small number of crystalline structures; therefore, the molar ratio of Se-fatty acid : methyl ester in the liposomes may not be exactly 1 : 1.

Permeability measurements. The absorbance changes at 400 nm as a result of light scattering of liposomes exposed to osmotic shrinking and swelling conditions were measured at equilibrium at 37°C in a Cary 14 spectrophotometer. An aliquot of liposomes (30 to 50 μl) was added rapidly to an aqueous solution of sucrose (0.1 to 0.5 M) in 50 mM Tris (pH 8.7). The resulting suspension was mixed thoroughly, and the absorbance was recorded after equilibrium was reached.

Initial rates of penetration of erythritol were measured as follows. A solution (0.75 ml) of erythritol (0.1 to 1.0 M) in 50 mM Tris (pH 8.7), containing 0.10 M choline chloride was placed in a thermostatted cell holder of a Perkin Elmer Model 320 spectrophotometer. The solution was maintained at 37°C. A 50-μl aliquot of liposomes (25 mM initial total lipid concentration) in 0.10 M choline chloride/50 mM Tris (pH 8.7) was then injected into the erythritol solution, and the contents were mixed rapidly. An initial phase of decreasing absorbance (shrinking) was observed, which arises from the loss of water from the spaces between the lamellae in order to equalize the osmolarity gradient. This phase was followed by a slower phase of increasing absorbance, which corresponds to swelling because of erythritol influx. The initial rate of permeation of erythritol into the liposomes was estimated from the slope of the straight line that followed the initial phase of water movement. No absorbance change was observed when liposomes were injected into isosmolar solution.

Results and Discussion. The dispersions prepared from oleic and elaidic acids in the presence of equimolar amounts of their methyl esters formed closed liposomes, as shown by their ability to trap carboxyfluorescein and their response to osmotic shock. All of the liposomes were impermeable to sucrose. When hypo- or hyperosmotic shocks of sucrose were applied, the liposomes underwent volume changes due to water permeability. A decrease in the absorbance (light scattering) at 400 nm was observed at equilibrium on application of osmotic shrinking pulses, and an increase after swelling. Fig. 1 shows the linear relationships between the absorbance of the liposomes suspension and the reciprocal of the final osmolarity. Since the latter parameter is proportional to liposome

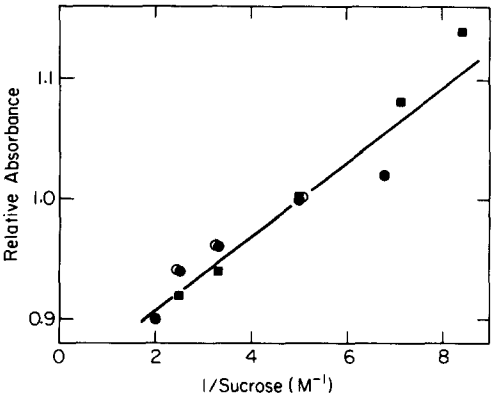

Fig. 1. Plot of the absorbance at 400 nm at equilibrium vs. the reciprocal of the final osmolarity of liposomes prepared from equimolar mixtures of (●) oleic acid and methyl oleate and (○) elaidic acid and methyl oleate. Osmotic swelling and shrinking experiments were also performed using liposomes prepared from (■) elaidic acid, methyl oleate, and cholesterol (1 : 1 : 0.4, molar ratio). The final lipid concentration was 6.25 mM. One volume of liposomes in 50 mM Tris (pH 8.7) containing 0.2 M sucrose was diluted with 7 volumes of sucrose solutions (0.1–0.5 M) in 50 mM Tris (pH 8.7). Absorbance values are relative to the following A_{400} values in isosmolar sucrose solution: ●, 0.46; ○, 0.40; □, 0.36.

volume, the data in Fig. 1 suggest that the change in absorbance arising from scattered light is directly proportional to the change in volume. Thus our results differ from the report that liposomes from oleic and linoleic acids display the usual relationship between volume change and the reciprocal of the absorbance (in the limited range of about 0.07–0.20 M sucrose) [5]. It seems unlikely that the presence of fatty acid methyl esters in the liposomes we used can account for this difference. When a suspension of oleic acid (8 mg) in 0.15 M KCl (3 ml), adjusted with KOH to pH 7.0, was subjected to osmotic shocks at 37°C with 0.1–0.7 M sucrose solutions, we observed increases in the absorbance at 400 nm under osmotic swelling conditions and decreases under osmotic shrinking conditions. There are several other examples of biological membranes and isolated subcellular particles that display a linear dependence between absorbance and volume changes (Ref. 16, and references cited therein), although the more commonly observed relationship is that absorbance is inversely proportional to the two-thirds power of the total particle volume (see, for example, Ref. 17).

The initial rates of erythritol permeation into liposomes of elaidic acid/methyl elaidate and elaidic acid/methyl oleate are plotted as a function of the osmolarity change in Fig. 2. The initial rates of swelling were similar when the liposomes contained methyl elaidate or methyl oleate. The initial rates of swelling of the liposomes were reduced dramatically when cholesterol was incorporated at 28 mol%. Cholesterol incorporation into liposomes of oleic acid and either methyl oleate or methyl elaidate also decreased the initial rates of erythritol permeation (data not shown). Fig. 2 shows that the cholesterol-induced reduction in the rate of swelling was greater in liposomes containing methyl oleate than in liposomes containing methyl elaidate. This effect of cholesterol on fatty acid/fatty acid methyl ester liposomes is similar to that found with phospholipid liposomes above the gel to liquid-crystalline phase transition (see for example, Ref. 18). However, our results differ from those of Gebicki and Hicks [5], who showed that liposomes from oleic or linoleic acid, at pH 8, became leaky to glucose at 17 mol% cholesterol, and highly permeable when more than

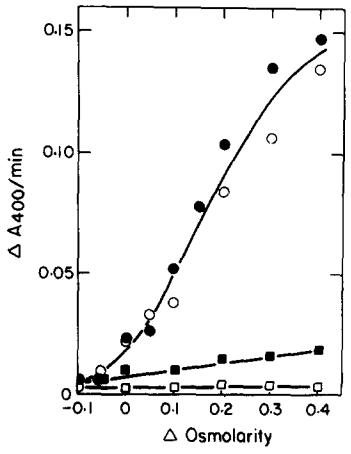

Fig. 2. Plot of the initial rate of erythritol permeability, ΔA/min, vs. the change in osmolarity for swelling of liposomes prepared from an equimolar mixture of elaidic acid and either methyl oleate or methyl elaidate, with and without cholesterol. ○, Elaidic acid and methyl oleate; ●, elaidic acid and methyl elaidate; □, elaidic acid, methyl oleate, and cholesterol; ■, elaidic acid, methyl elaidate, and cholesterol. Cholesterol, when present, was incorporated at 28 mol%. The liposomes were prepared in 50 mM Tris (pH 8.7) containing 0.10 M choline chloride. The final total lipid concentration was 1.6 mM.

30 mol% cholesterol was incorporated.

The initial rates of swelling (erythritol permeation) into liposomes from oleic acid and methyl oleate or methyl elaidate are strikingly different from the initial rates of swelling of liposomes from elaidic acid and either of these esters. The initial rate of swelling of liposomes containing oleic acid is about fifty times slower than that of liposomes containing elaidic acid. For example, at a ΔosM of 0.2 the initial rates of swelling were $8.0 \cdot 10^{-2}$ and $1.7 \cdot 10^{-3}$ min^{-1} for liposomes of elaidic acid/methyl oleate and oleic acid/methyl oleate, respectively. The molecular basis for this large difference is not known. No significant difference in initial rates of swelling was found in liposomes prepared with elaidic acid and methyl elaidate compared with those prepared with elaidic acid and methyl oleate; the effects of these methyl esters on liposomes of oleic acid were also similar.

Fig. 3 shows that the relative absorbance of liposomes from 9-Se-C_{16} and methyl oleate (with or without cholesterol) is linearly dependent on the reciprocal of the osmolarity at equilibrium. The inset shows the same type of behavior with

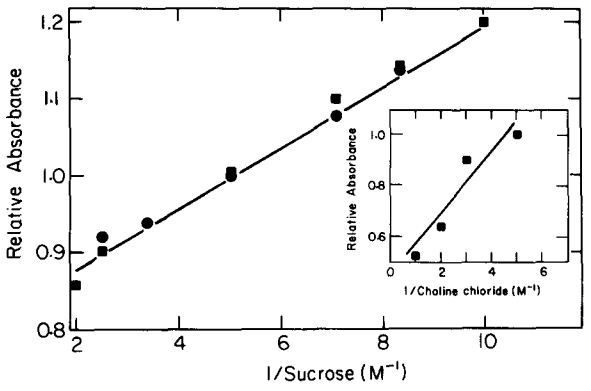

Fig. 3. Plot of the absorbance at 400 nm at equilibrium vs. the reciprocal of the final osmolarity of liposomes prepared from (●) an equimolar mixture of 9-Se-C_{16} and methyl oleate and from (■) 9-Se-C_{16}, methyl oleate, and cholesterol (1 : 1 : 0.8, molar ratio). The final lipid concentration was 3.1 mM. Liposomes were prepared in 50 mM Tris (pH 8.7) containing 0.2 M sucrose, and subjected to osmotic shock with buffered sucrose solutions. Absorbance values are relative to the following A_{400} values in isosmolar sucrose solution: (○), 0.36; (■), 0.29. Inset: Plot of the absorbance at 400 nm at equilibrium vs. the reciprocal of the final osmolarity of liposomes subjected to osmotic shock with choline chloride (0.1–1.0 M) in 50 mM Tris (pH 8.7). Liposomes were prepared from 13-Se-C_{20}, methyl oleate, and cholesterol (1 : 1 : 0.74, molar ratio) in 50 mM Tris (pH 8.7) containing 0.1 M choline chloride. The final lipid concentration was 3.2 mM. The absorbance in isosmolar choline chloride solution was 0.38.

liposomes containing 13-Se-C_{20}, methyl oleate, and cholesterol; here, the osmotic shocks were achieved using choline chloride instead of sucrose. This behavior is similar to that observed with liposomes from oleic and elaidic acids and their methyl esters (Fig. 1). As noted for the liposomes from fatty acids lacking selenium, we also observed that an absorbance increase results from swelling and an absorbance decrease results from shrinking of these liposomes. The initial rate of swelling of liposomes prepared with selenium-containing fatty acids on erythritol influx is similar to that of the liposomes containing elaidic acid. The effects of methyl oleate and methyl elaidate on the initial rates of swelling of liposomes containing 9-Se-C_{16} and 13-Se-C_{20} are compared in Table I. In contrast to the lack of a significant difference in the erythritol permeability of liposomes from oleic and elaidic acids in the presence of methyl elaidate compared with methyl oleate, liposomes from the selenium-containing fatty acids display markedly different initial swelling rates in the presence of these two methyl esters. Higher rates of swelling were found in the presence of methyl oleate than in the presence of methyl elaidate. The *cis* double bond of methyl oleate causes a kinked structure in the membrane, which is expected to decrease the degree of lipid order and promote erythritol permeation.

Finally, the effect of cholesterol on the initial rate of swelling was examined. Incorporation of cholesterol at 28 mol% caused a reduction in the initial rate of swelling of liposomes from 9-Se-C_{16} or 13-Se-C_{20} and methyl oleate at a difference in

TABLE I

EFFECT OF CHOLESTEROL AND FATTY ESTERS ON THE INITIAL RATES OF ERYTHRITOL PERMEATION INTO LIPOSOMES PREPARED WITH 9-Se-C_{16} OR 13-Se-C_{20}

Aliquots (50 μl) of liposomes suspended in 0.10 choline chloride were mixed rapidly with 0.75 ml of erythritol solutions in 0.10 M choline chloride at 37°C. The desired osmolarity changes were obtained by varying the erythritol concentration. The initial rate of absorbance change was measured at 400 nm. The final concentration of fatty ester was 12.5 mM. The total lipid concentration was 25 mM.

ΔosM	Initial rate of swelling (ΔA/min)						
	9-Se-C_{16}				13-Se-C_{20}		
	methyl oleate	methyl elaidate	methyl oleate and cholesterol	methyl elaidate and cholesterol	methyl oleate	methyl elaidate	methyl oleate and cholesterol
0.10	0.032	0.025	0.025	0.023	0.072		0.067
0.15					0.083	0.066	0.063
0.20	0.092	0.050	0.055	0.051	0.120		0.080
0.30	0.288	0.079	0.072	0.078		0.084	
0.40	0.260	0.096	0.068	0.096			

osmolarity greater than 0.2. However, as shown in Table I, in the more tightly packed liposomes prepared from selenium-containing fatty acids and methyl elaidate, cholesterol incorporation did not have a large effect on the swelling rate. A comparison of the data in Fig. 2 and Table I indicates that cholesterol inhibits the initial rates of erythritol permeation less in the Se-containing fatty acid liposomes than in liposomes from oleic or elaidic acids. The large bulk of the selenium heteroatom may partially suppress the ability of cholesterol to increase the order of the hydrocarbon matrix of the membrane. Similarly, liposomes from polyunsaturated phospholipids undergo smaller increase in lipid order on cholesterol incorporation than liposomes from the corresponding saturated phospholipids.

Our permeability studies indicate that solutes can be trapped in vesicles from oleic acid and elaidic acids in the presence and absence of cholesterol, and that the resulting vesicles are sealed and stable at pH 8.7. There is considerable interest in acid-sensitive liposomes as carriers to deliver drugs to target organs when the liposomes encounter an acidic environment (see, for example, Refs. 11 and 19). Since fatty acids are available in high purity and at low cost, liposomes formed from fatty acids may be useful in cytoplasmic drug delivery and may offer an alternative to the pH-sensitive phospholipid-containing liposomes. Recently, the pharmacological efficacy of other non-phospholipid liposome systems for drug delivery has been explored. For example, cholesterol derivatives [20] and a mixture of single and double long-chain nonionic surfactants [21] have been shown to form closed, stable liposomes; these lipids, however, are not susceptible to pH-induced liposome fusion. We have found from experiments in which leakage of carboxyfluorescein was measured as a function of pH that liposomes of oleic acid/methyl oleate and oleic acid/methyl elaidate display marked enhancement in membrane permeability on acidification. For example, the efflux of carboxyfluorescein from liposomes of oleic acid and methyl oleate was 2–3-fold greater at pH 7.4 than at pH 8.7, and 4–6-fold greater at pH 6.5 than at pH 8.7. Future experiments are required to determine whether liposomes containing fatty acids can be prepared so that their stability is significantly higher at pH 7.4 than at pH 7.0 or lower.

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