

## Liposomes from Ionic, Single-Chain Amphiphiles†

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**ABSTRACT:** In studies of the minimum physicochemical requirements for lipid membrane formation, we have made liposomes from dilute, aqueous dispersions of C(8)–C(18) single-chain amphiphiles. In general, membrane formation from ionic soaps and detergents requires the presence of uncharged amphiphiles. Vesicles were characterized by phase-contrast microscopy, by trapping of ionic dyes, as well as by negative-stain and freeze-fracture electron microscopy. They were typically heterogeneous in size, but the average diameter could be experimentally varied in some cases over the range of 1 to 100  $\mu\text{m}$ . Uni-, oligo-, and multilamellar vesicles were observed. Membrane permeability to various solutes was determined in

part by a new technique which utilized phase-contrast microscopy; when impermeable vesicles exclude added solutes such as sucrose, refractive index differences are created between vesicle contents and surrounding medium, so that the vesicles appear bright in the phase microscope. Permeant solutes do not produce this effect. Spectrophotometric permeability determinations confirmed the results of this technique and provided quantitative measures of permeability. Monoalkyl liposomes have potential uses as models of biomembranes and in drug delivery. They are also relevant to the prebiotic origin of biomembranes.

Lipid bilayer membranes form spontaneously when membrane lipids isolated from most living organisms are dispersed in water. These bilayer-forming compounds almost universally consist of various glycerolipids or sphingolipids containing two long hydrocarbon chains. The few exceptions include flagellar membrane lipids, found in a protozoan. These are composed of single-chain hydrocarbons containing sulfate and chlorine, together with sterols and free fatty acids (Haines, 1973; Chen et al., 1976). While some single-chain lipids (fatty acids and lysophospholipids) are normally found in biomembranes in low concentrations, these compounds have been considered incapable of forming stable lipid bilayers in water at low lipid concentrations (Tanford, 1973). However, Gebicke and Hicks have published accounts of lipid vesicles forming spontaneously from *cis*- $\Delta^9$ -octadecenoic acid (oleic acid) in dilute aqueous dispersions (Gebicke and Hicks, 1973, 1976). We have been interested in the minimum physicochemical requirements for lipid membrane formation and became aware that single-chain lipids can form stable liposomes during experiments employing dodecanoate, a 12-carbon saturated fatty acid. Subsequently, we have determined that a wide variety of saturated single-chain amphiphiles varying in length from 8 to 18 carbons are capable of forming lipid vesicles.

The purpose of this paper is to define the conditions under which liposomes can be prepared from monoalkyl<sup>1</sup> compounds and to describe some characteristics of these vesicles.

Before proceeding, we must define our use of some terms which might otherwise be ambiguous. We have used the word "liposome" to describe certain spherical or tubular structures formed by amphiphiles dispersed in an aqueous medium. These structures contain lipid bilayers and enclose aqueous compartments and are thereby differentiated from micelles. We

have operationally defined liposomes by their microscopic appearance and by their ability to entrap small, polar molecules. We have coined the term "oligolamellar" to describe liposomes which, by phase-contrast and electron microscopy, appear to contain an aqueous compartment surrounded by one or several concentric lipid bilayers. We feel that the term "unilamellar" should be used only when chemical or ultrastructural evidence is presented which can differentiate between these two vesicle types. Using our operational criteria, "multilamellar" vesicles are those which appear dark by phase-contrast microscopy and which fail to become bright on dilution of vesicle dispersions with an impermeant nonelectrolyte such as sucrose (see Materials and Methods for details of this technique).

### Materials and Methods

**Sources and Purity of Materials.** C(8:0)–C(16:0) and C(18:1 $\Delta^9$ ) (oleic) acids, decanol, and NaDodSO<sub>4</sub><sup>2</sup> were purchased from Sigma Chemical Co., St. Louis, Mo.; dodecanol was obtained from Aldrich, Milwaukee, Wis. All were used as received. Thin-layer chromatography of these compounds was performed on silica gel F-254 plates (EM Laboratories, Elmsford, N.Y.) in hexane–ethyl ether–acetic acid (80:20:1) and in chloroform–methanol–water (65:25:4). The separated components were visualized with a Rhodamine 6G spray and illuminated at 366 nm. Each compound revealed only a single spot when 1 and 10  $\mu\text{g}$  of lipid were analyzed. Gas-liquid chromatography of saturated fatty acids was performed using the boron trifluoride–methanol reagent (Metcalf and Schmitz, 1961) and a 10% DEGS column. Oleic acid was received sealed under nitrogen, then diluted with chloroform, purged with argon, and stored at  $-20^\circ\text{C}$ . Except for C(8) fatty acid [ $>97.4\%$  pure, containing small amounts of C(10) and C(12) fatty acids], all saturated fatty acids were greater than 99.7% pure. The dye 6-carboxyfluorescein (6CF) was pur-

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<sup>1</sup> "Monoalkyl" and "single-chain" will be used interchangeably to describe amphiphiles with a single hydrocarbon chain derived from either fatty acids or fatty alcohols.

<sup>2</sup> Abbreviations used: CMC, critical micelle concentration; EDTA, ethylenediaminetetraacetic acid, disodium salt; HTMAB, hexadecyltrimethylammonium bromide; PC, phosphatidylcholine; PS, phosphatidylserine; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; TBS, 10 mM triethylaminoethanesulfonic acid (TES), 130 mM NaCl, 10 mM KCl; 6CF, 6-carboxyfluorescein; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

chased from Eastman Kodak and was used as received.

**Formation of Fatty Acid Vesicles.** (1) *Vesicle Formation by Titration.* Liposomes could be prepared by titrating alkaline micellar solutions of fatty acids with dilute acid. Weighed amounts of free fatty acid were dispersed to form clear solutions by stirring the lipid in a slight molar excess of NaOH or KOH, at a temperature above the bulk melting point. The initial volume of lipid dispersions was 10 mL. HCl (0.2 or 1.0 N) was added in 5- $\mu$ L aliquots while the sample was stirred in a thermostat-equipped water bath. The pH was monitored using a Radiometer 26 meter with a Thomas combination electrode. Standardization was performed at the titration temperature with pH 7.0 and 9.2 or 7.0 and 4.0 buffers, depending on the buffering range of the dispersion being titrated.

Translucence appeared at a precise pH which varied with temperature and with hydrocarbon chain length and was caused by either microscopic crystals or by liposomes, depending on temperature. Phase-contrast microscopy on a temperature-controlling slide (see Figure 1 and "Light Microscopy" below) disclosed whether vesicles or crystals were present in a translucent dispersion. At a specific temperature ( $T_m$ ) the crystals were observed to melt and form spherical liposomes. (The significance of  $T_m$  will be presented under Results and Discussion.)

(2) *Incorporation of Fatty Alcohol.* In addition to titrating pure soap solutions, vesicles were prepared by mixing equimolar quantities of the homologous fatty alcohol with pH 10–12 fatty acid solutions. For vesicles which were fluid at 22 °C (decanoate–decanol), simple vortex mixing of the two lipids resulted in the appearance of liposomes. In some experiments, cholesterol was added to oleic acid in the same manner with similar results (mole ratio 1:1).

To form vesicles from dodecanoate–dodecanol, it was necessary to heat the dispersion above the melting temperature of the resultant liposomes. Heating was accompanied either by vortex mixing or by probe sonication (40 W nominal power for 0.5–2 min, 55 °C).

(3) *Dilution from Ethanol.* Vesicles also formed when equimolar mixtures of pure fatty acid and fatty alcohol were dissolved in a small volume of 95% ethanol and then diluted with alkali to pH 8–12. As with methods 1 and 2, the temperature had to exceed the  $T_m$  either during or subsequent to the dilution step. The rate and extent of dilution, as well as the precise sequence of dilution and warming steps, probably affect the average diameter and average internal aqueous volume of these liposomes. Typically, the dissolved lipid was diluted rapidly with 10 or 20 volumes of 0.05 N KOH with concomitant vortex mixing. With C(12) mixtures, dilution resulted in the formation of a turbid dispersion which was subsequently warmed to 55 °C for 30 s to form vesicles.

**Sodium Dodecyl Sulfate–Dodecanol Vesicles.** Vesicles were made from various molar ratios of sodium dodecyl sulfate and dodecanol by adding melted dodecanol (>22 °C) dropwise to a stirred micellar solution of NaDodSO<sub>4</sub>. The final total concentration of the two amphiphiles was 50 mM. Below 27–30 mol % dodecanol, the solution remained clear. When the dodecanol content exceeded 27 mol %, turbidity appeared. Phase microscopic examination showed that the turbidity was due to microscopic crystals at temperatures below  $T_m$  (see Results). If the slide was heated to temperatures above  $T_m$ , the crystals melted and expanded into liposomes. Neither dodecanol nor NaDodSO<sub>4</sub> by themselves exhibited this effect.

The most stable vesicles formed when dodecanol was present at 70 mol %. These were prepared by weighing appropriate amounts of dodecanol and NaDodSO<sub>4</sub> into test tubes. Enough

buffer was added to produce a final concentration of 50 mM amphiphile, and the suspension was vortexed briefly at 55 °C and then sonicated by probe (40 W, nominal power) at the same temperature for 2 min. Microscopic examination of the relatively clear dispersion revealed large numbers of 0.1–1- $\mu$ m vesicles. Incubation of dispersions at room temperature or at 38–40 °C produced a larger size distribution of vesicles; these preparations were used for the volume-trapping experiments described below.

**Volume-Trapping Measurements.** Experiments were performed to determine trapping volumes of a monoalkyl vesicle preparation. Sodium dodecyl sulfate and dodecanol were weighed into 20-mL glass-stoppered tubes to give 30–70 mol % dodecanol (ROH). Ten milliliters of buffer-containing dye (120 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM K<sub>2</sub>CrO<sub>4</sub>, 2 mM Bromophenol blue, 0.1% NaN<sub>3</sub>, pH 7.2) was added to give a total lipid concentration of 50 mM, and the tubes were heated to 55 °C with vortex mixing. Probe sonication was performed as described above. The dispersion was then incubated at the appropriate temperature before an experiment was performed. Initial results indicated that turbidity and trapped volumes increased during incubation following sonication. After 24 h (48 h for 70 mol % ROH) such changes were no longer detectable; thus, all dispersions were incubated for 24–48 h or more before use.

Trapping volumes of vesicles were determined by Sephadex G-50 gel filtration. Coarse beads (100–300 mesh) in a 1 × 23 cm water-jacketed column were equilibrated with 120 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM K<sub>2</sub>SO<sub>4</sub>, 0.1% NaN<sub>3</sub>, pH 7.2. For experiments utilizing fluid vesicles (max  $T_m$  = 39–41 °C), the temperatures of the column and the buffer reservoir were maintained at 42 °C with water baths. Aliquots of a vesicle dispersion (0.1–0.2 mL) were applied to the column and eluted with column buffer. The excluded fraction was easily visible due to trapped Bromophenol blue dye and was collected as one fraction (4–8 mL) within 1–3 min from initiation of the run. In order to monitor separation of trapped from free dye, a subsequent small fraction (ca. 2 mL) was collected prior to elution of the free dye. Triton X-100 was added to the vesicle fraction to form a clear solution (2.5 mM final concentration), and the total amount of dye in each fraction was determined spectrophotometrically using  $A_{376}$  for chromate and  $A_{590}$  for Bromophenol blue.

**Long-Term Stability of Vesicles.** We determined the long-term stability and permeability of NaDodSO<sub>4</sub>–dodecanol vesicles in the gel state. Liposomes containing 70 mol % dodecanol (50 mM lipid) were formed in the presence of a saturated solution of 6CF (ca. 50 mM) in phosphate-buffered saline (50 mM sodium phosphate, 100 mM NaCl, pH 7.2) containing 0.1% sodium azide as an antibacterial agent. The dispersion was probe sonicated as described above and then incubated at room temperature for 24 h. Gel filtration on Sephadex G-50 in the same buffer was monitored with a handheld, long-wave UV light, and the vesicle fraction was collected. Fluorescence microscopy showed that the dye was present inside liposomes (see Figure 11 for details). Incubation was continued at room temperature for 7 weeks, at which time fluorescence micrographs were obtained.

#### Electron Microscopy

**Negative Staining.** Samples for negative-stain electron microscopy were suspended in water or in TBS and then diluted 1:1 with 1% aqueous uranyl acetate on carbon-formvar-coated grids. After 1 min, grids were drained on absorbent tissue and allowed to air dry. Oleate vesicles (8 mM lipid, pH 9) were fixed prior to staining by dilution with an equal volume of

glycine-buffered osmium (0.5%  $\text{OsO}_4$  and 50 mM glycine, pH 9). After 30 min at 22 °C, vesicles were mixed with stain. Phase-contrast microscopy was used to ascertain that intact vesicles were present at this step. Staining on grids was then carried out as described above.

**Freeze-Fracture.** Freeze-fracture electron microscopy was performed after mixing samples with equal volumes of 50% glycerol, using standard techniques (Deamer, 1974).

### Light Microscopy

**Identification of Vesicles by Phase-Contrast Microscopy.** Phase-contrast microscopy was routinely used to monitor lipid dispersions. Differences in the refractive index of lipid and water allowed discrimination between emulsions (phase-bright droplets), oligolamellar vesicles (low phase-contrast spheres), and multilamellar vesicles (phase-dark spheres). Confirmation of the efficacy of this technique was obtained in several ways. First, oligolamellar vesicles frequently enclosed smaller vesicles which moved freely within the apparent aqueous compartment of the larger liposome. In addition, we have compared monoalkyl vesicles with red blood cell ghosts and with oligolamellar phospholipid vesicles prepared by the ether-injection technique of Deamer and Bangham (1976). All of these exhibited similar low-contrast appearance in the phase-contrast microscope.

**Microscopic Determinations of Thermotropic Phase Transitions.** Crystalline or amorphous structures, which formed during titration of fatty acids or after addition of dodecanol to  $\text{NaDodSO}_4$  solutions, could be reversibly transformed into fluid vesicles by heating. This transition occurred over a reproducible, narrow temperature range. We assume that these visible transitions correspond to the major gel to liquid-crystalline phase transitions reported for phospholipid dispersions and for biomembranes (see Melchior and Steim 1976a). Determinations of melting points of lipid dispersions were made using a thermistor-equipped hollow microscope slide (Figure 1). This device was attached to a circulating water bath and permitted phase-contrast microscopy over a temperature range of 10–60 °C. Bulk melting point determinations were made with a Fisher-Johns melting point apparatus.

**Microscopic Determinations of Vesicle Permeability.** Differences in the refractive index between vesicle contents and surrounding medium allowed us to use phase-contrast microscopy to qualitatively determine vesicle permeability to glycerol, sucrose, inulin, or dextran-T-40 (Pharmacia). These compounds are all highly polar, but are of increasing molecular weight (92, 342, ~5000, and ~40 000, respectively). We expected to find some vesicles which were permeable to lower molecular weight sugars but impermeable to larger molecules. We have determined that 0.4 M glycerol, 0.1 M sucrose, 3.5% (w/v, ~5 mM) inulin, and 3.4% (w/v, ~0.1 mM) dextran provided refractive index differences which were easily distinguished when diluted 1:1 with impermeable vesicles. The solutions contained 5 mM sodium azide, 1 mM TES, and 0.1 mM EDTA (pH 7.9). Inulin and dextran solutions were prepared by heating briefly in a boiling water bath, the former remaining clear for weeks at 22 °C, while the latter remained soluble indefinitely at this temperature.

As an example of phase-contrast permeability measurement, dilution of a suspension of impermeable vesicles with isotonic sucrose resulted in a change in the refractive index of the medium but not of the liposomal contents. This caused the vesicles to appear bright by phase-contrast microscopy. If vesicles were made permeable to sucrose by the addition of dilute Triton X-100 to the sample, phase brightness disappeared leaving a membrane "ghost". This phenomenon was observed for phospholipid vesicles mixed with sucrose, as well as with res-

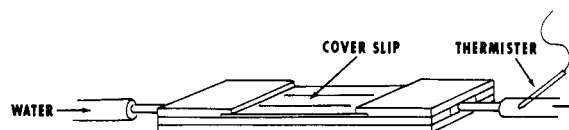


FIGURE 1: Microscopic observation of thermotropic phase transitions. In order to observe the melting of microscopic crystals to form fluid liposomes, a water-filled slide was constructed which could maintain a lipid dispersion at various temperatures, yet was suitable for observation with a Zeiss standard microscope. The device was assembled from standard glass slides and a 22 × 50 mm cover slip, using cyanocrylate adhesive (Super Glue-3, Woodhill Sales Corp., Cleveland, Ohio). Two glass strips were cut to form a channel in the center through which water could flow. After assembly, silicon adhesive (Dow Corning) was used to mount the aluminum nipples and to seal the assembly externally. Water was driven through the slide by a recirculating, variable-temperature water bath (Bronwill Scientific, Rochester, N.Y.). In order to vary the flow rate, the pump was driven by a rheostat. For additional control of water flow through the fragile glass chamber of the slide, a bypass valve allowed some water to return to the bath reservoir without passing through the slide. Temperature was monitored with a small thermistor positioned at the outlet of the slide.

ealed erythrocyte ghosts mixed with inulin. Triton X-100, in the amounts used to eliminate sucrose-induced brightness, had no effect on the phase brightness of lipid droplets in a dodecanol emulsion.

**Spectrophotometric Determinations of Permeability.** While we did not perform extensive quantitative comparisons of vesicle permeability, we ascertained that this was possible using a spectrophotometric technique modified from that of Cohen and Bangham (1972). The initial absorbance of 2 mL of a vesicle dispersion was adjusted to 0.6–0.8 by varying the measured wavelength between 400 and 760 nm. Then, 0.2 mL of a 2 M solute was added with rapid mixing. Time-dependent changes in absorbance following mixing with solute or with a water-control aliquot were recorded. Cohen and Bangham (1972) have shown that the slope immediately following the absorbance peak is proportional to the permeability of the vesicles to added solute.

Vesicles tested included potassium decanoate-decanol (1:1 molar ratio, 50 mM lipid concentration), potassium dodecanoate-dodecanol (1:1 molar ratio, 12.5 mM lipid), and potassium oleate-cholesterol (1:1 molar ratio, 25 mM lipid, pH 10). The C(10) and C(12) vesicle preparations were made by dissolving both lipids in ethanol and then diluting in 40 volumes of 0.05 M KOH. Dispersions were then warmed to 55 °C and probe sonicated (50 W nominal power) for 1 min to reduce turbidity. Oleate-cholesterol vesicles were prepared by method 2. Crystalline cholesterol was added to an alkaline potassium oleate solution (50 mM lipid), which was then vortexed briefly, diluted 1:1 with water, and incubated at 23 °C for 48 h prior to assay. Sodium azide (5 mM) was included to prevent bacterial growth, and an argon atmosphere was maintained to preclude lipid oxidation. For comparison, phospholipid vesicles (egg PC/PS, 90:10 molar ratio, 0.5 mM lipid, in 100 mM phosphate buffer) were prepared by the ether-injection method (Deamer and Bangham, 1976) and assayed for permeability.

### Results

Initial experiments indicated that a 50 mM sodium dodecanoate dispersion equilibrated at 50 °C is a clear solution above approximately pH 8.5, a translucent dispersion of liposomes between approximately pH 7 and 8.5, and a milky emulsion below pH 7. These results suggested that vesicles might be formed at a precise pH during titration, and more detailed experiments were undertaken.

**The Formation of Vesicles by Titration.** We were interested

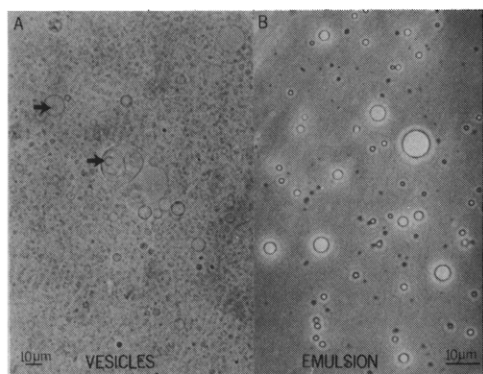


FIGURE 2: Phase-contrast microscopy of dodecanoate vesicles and dodecanol emulsion. (A) C(12) fatty acid (50 mM) equilibrated at 50 °C, pH 7.5, forms liposomes up to 25  $\mu\text{m}$  in diameter. The presence of an aqueous compartment is shown by the low contrast of spheres and by the presence of trapped vesicles (arrows). (B) This emulsion, formed by sonicating dodecanol briefly in water, demonstrates that refractive index differences between lipid and water cause lipid droplets to appear bright by phase contrast.

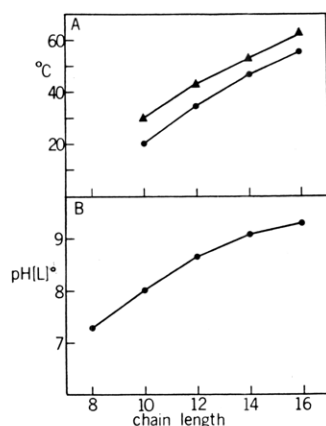


FIGURE 3: Vesicle characteristics vary with chain length. (A) Melting points of anhydrous and dispersed lipid. Each point represents a single determination. Bulk fatty acid (anhydrous) melting points ( $\blacktriangle$ ) were determined as described in the text. Micellar, aqueous lipid dispersions titrated with HCl formed vesicles or crystals, depending on the temperature. When crystals were slowly warmed on the temperature-controlled microscope slide (see text), a reversible transition to vesicles occurred; the  $T_m$  ( $\bullet$ ) was 7–10 °C lower than the corresponding bulk  $T_m$ . (B) pH at which lamellar dispersions are formed [ $\text{pH(L)}^*$ ] vs. chain length. Fatty acids were titrated above their CMC [C(8) at 500 mM; C(10) at 200 mM; C(12)–C(16) at 50 mM] at  $T_m + 5$  °C.  $\text{pH(L)}^*$  was detected as the appearance of translucence, and confirmed by phase-contrast microscopy.

in determining the precise effect of pH on fatty acid vesicle formation. Since either liposomes or lipid droplets could have been present, it was important that vesicles were easily distinguished from lipid droplets by phase-contrast microscopy (Figure 2). Titrations of C(12) and longer chains at 22 °C resulted in the formation of needle-shaped crystals. At higher temperatures vesicles were present. It was thus necessary to determine the melting points ( $T_m$ ) of crystals formed during titration of fatty acids (Figure 3A). As determined by phase-contrast microscopy (see Materials and Methods), this occurred 7–10 °C below the melting points of bulk (anhydrous) lipid.

When titrated with HCl above the  $T_m$ , the micellar soaps of all fatty acids tested formed liposomes. Pure fatty acid vesicles were metastable when cooled below this temperature, undergoing gradual crystallization. For example, dispersions of dodecanoate equilibrated at length above the  $T_m$  remained uncrystallized for hours after cooling to room temperature.

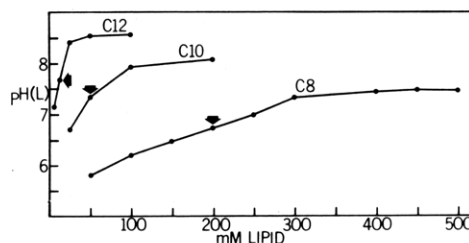


FIGURE 4: Dependence of  $\text{pH(L)}$  on lipid concentration. Potassium soaps of C(8)–C(12) fatty acids were titrated at  $T_m + 5$  °C over a concentration range spanning their respective reported CMC's [ca. 400 mM for C(8), 100 mM for C(10), and 25 mM for C(12); Mysels and Mukerjee, 1971]. Vesicles in all cases were present (arrows) at about  $\geq$  half the CMC.

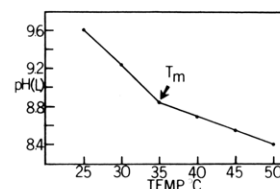


FIGURE 5: Temperature dependence of  $\text{pH(L)}$ . Potassium dodecanoate (50 mM) was titrated from pH 10 at various temperatures. A sharp change in temperature sensitivity of  $\text{pH(L)}$  occurs at the  $T_m$  (the crystal-liquid crystal transition).

Crystals which appeared slowly were flat and angular. Brief reheating of these crystals resulted in the formation of vesicles which rapidly degenerated into feathery crystals during cooling and only subsequently into the platelike form.

Alkaline, micellar dispersions titrated with HCl at 5 °C above their respective  $T_m$  values showed distinct pH values at which vesicles appeared. These values varied with chain length (Figure 3B) and ranged from approximately 7.45 for octanoic acid to 9.25 for palmitic acid. Oleic acid formed vesicles at pH 9.8 at 22 °C. It appears that this pH is less sensitive to chain length for hydrocarbon chains longer than C(12). The average slope in Figure 3B is approximately 0.31 for C(8)–C(12), while that from C(12)–C(16) is 0.14, a reduction of 55%.

The pH at which translucence appeared decreased with lipid concentration below the critical micellar concentration (CMC; see Figure 4). The minimum lipid concentration required for the formation of liposomes visible in the phase-contrast microscope (0.1  $\mu\text{M}$  or larger) was not rigorously determined. However, this value was approximately 200 and 50 mM for C(8) and C(10) fatty acid dispersions, respectively (Figure 4). Thus, vesicles were present in translucent dispersions at approximately half the CMC (cf. Mysels and Mukerjee, 1971). Below the concentrations at which vesicles formed (indicated in Figure 4), translucence appeared during titration but was apparently caused by lipid droplets which were visible by phase-contrast microscopy.

The effect of temperature on the appearance of microscopic crystals or vesicles was striking. This was apparent in the pH at which translucence appeared during titration. For a given dispersion [C(12), Figure 5], translucence appeared at a lower pH as temperature was increased. Surprisingly, an abrupt change in this temperature sensitivity occurred at the transition temperature. Below  $T_m$ , the change in pH units was approximately 0.75 per degree celsius. Above  $T_m$ , it was only 0.035 pH unit degree celsius. The shape of dodecanoate titration curves also differed above and below the transition temperature (Figure 6). At all temperatures, however, translucence appeared at the beginning of the upper plateau. Below 35 °C, translucence was apparently caused by microscopic, needle-shaped crystals, while at 35 °C or above, only fluid liposomes

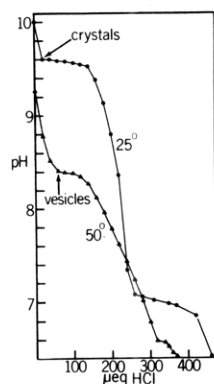


FIGURE 6: Temperature effect on C(12) titration curves. Below 35 °C ( $T_m$ ), potassium dodecanoate solutions (50 mM) form crystals during titration with HCl, while above this temperature liposomes are formed. To the right of the lower inflection of each curve, macroscopic lipid aggregates appeared.

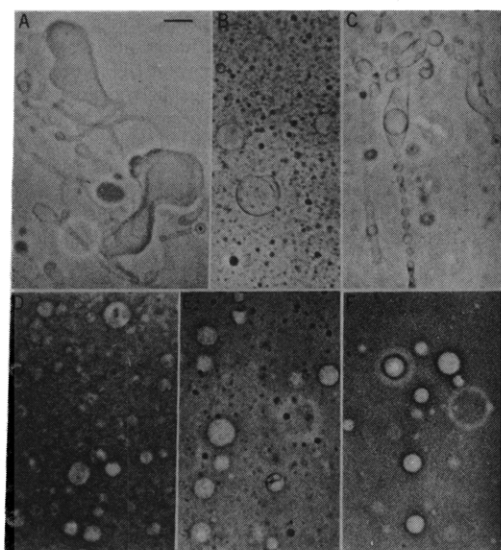


FIGURE 7: Phase-contrast micrographs of fatty acid vesicles. These observations were made at 25 °C; all frames are the same magnification (bar = 10  $\mu$ m). (A) Vesicles and tubules are present in 200 mM octanoate, pH 6.2. At higher lipid concentrations, dark spherical vesicles are formed. (B) Decanoate-decanol (1:1, 100 mM lipid), pH 8. (C) Potassium oleate (5 mM lipid), pH 9.0. (D) Same preparation as B, diluted 1:1 with 3.4% inulin to show impermeable (bright) vesicles. (E) Dodecanoate-dodecanol (1:1, 20 mM lipid) diluted from ethanol (5%, v/v, final concentration) with 0.05 M KOH, shown after dilution 1:1 with 100 mM sucrose to reveal impermeable (bright) vesicles. (F) For comparison, egg PC-beef brain PS (90:10) liposomes, prepared by the technique of Deamer and Bangham (1976) and diluted 1:1 in 100 mM sucrose.

were visible and the plateaus were much shorter. At the end of the lower plateau, all of the lipid aggregated as an amorphous mass.

**Effect of Fatty Alcohol.** The titration curves described above seem to result from anionic sites with two distinct  $pK_a$  values, located at the plateaus or inflections for each temperature. When we dispersed dodecanoate with dodecanol (both 25 mM) in 0.1 N NaOH (using method 2 to form vesicles), the titration curve was that of a monoprotic compound with a  $pK_a$  of about 7.0 at 55 °C (data not shown). Vesicles were present even at pH 11.6 and were stable at room temperature for at least 24 h.

**Microscopic Determinations of Permeability.** In order to quickly obtain qualitative information about liposome permeability to polar solutes, we developed the phase-contrast technique (described under Materials and Methods under

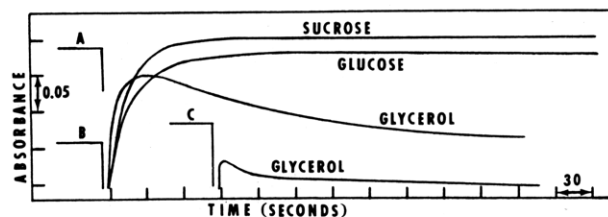


FIGURE 8: Spectrophotometric determination of vesicle permeability. Oleate-cholesterol vesicles underwent absorbance changes following dilution with hypertonic nonelectrolyte solutions (see text for details of methods). The slope immediately following the absorbance peak is proportional to permeability of the vesicles to the added sugar (Cohen and Bangham, 1972). (A and B) The absorbance baselines prior to the addition of sucrose (A) and of glucose or of glycerol (B) to oleate-cholesterol vesicles. These vesicles were moderately permeable to glycerol, very slightly permeable to glucose, and apparently totally impermeable to sucrose. (C) For comparison, phospholipid vesicles (no cholesterol) were assayed for permeability (see text for details). These liposomes appeared more permeable to glycerol than did oleate-cholesterol vesicles. Results of similar experiments using C(10) and C(12) fatty acid vesicles are described in the text.

“Light Microscopy”). A problem exists in the application of this technique to monoalkyl dispersions, especially those containing only fatty acid. Vesicles in such dispersions are relatively unstable in the presence of high concentrations of ionic and nonionic solutes. For example, octanoate vesicles, which were fluid at room temperature (Figure 7A), formed lipid droplets on the addition of sucrose, inulin, or dextran, making permeability determinations by the phase-contrast method impossible. Decanoate vesicles (Figure 7B) were also fluid at room temperature (23 °C) but were moderately stable in 100 mM sucrose and quite stable in inulin and dextran. Only dextran was excluded from these vesicles (not shown). Dodecanoate vesicles, which are in the gel state at room temperature, appeared impermeable to sucrose and to glycerol (not shown). These vesicles were rapidly disrupted by added solute, however, making permeability determinations difficult.

The formation of stable vesicles was readily achieved by the incorporation of equimolar fatty alcohol as described under Materials and Methods (methods 2 and 3). The addition of an equimolar amount of decanol to a pH 12 potassium decanoate solution (100 mM final lipid concentration) resulted in the formation of vesicles which were impermeable to sucrose (Figure 7D). Using method 3 (with a 20-fold dilution of the ethanol-lipid solution), oligoamellar dodecanoate-dodecanol liposomes formed which were 1–15- $\mu$ m in diameter and appeared highly impermeable to glycerol or sucrose (Figure 7E). For comparison, Figure 7F shows impermeable phospholipid vesicles mixed with isotonic sucrose. Figure 7C depicts oleic acid vesicles (formed by method 1). These were fluid at room temperature and appeared impermeable to sucrose using this assay (not shown).

**Spectrophotometric Determination of Permeability.** In order to estimate vesicle permeability more quantitatively, we used the spectrophotometric assay devised by Bangham and colleagues (e.g., Cohen and Bangham, 1972), modified as described under Materials and Methods. Oleate-cholesterol liposomes exhibited absorbance changes on dilution with hypertonic nonelectrolytes; the response indicated low permeability, especially to glucose and to sucrose (Figure 8A,B). Phospholipid vesicles (Figure 8C) showed a steeper slope for the decline of the glycerol absorbance peak, indicating greater permeability to this compound than that shown by the monoalkyl vesicles. We also determined that C(10) vesicles (soap/fatty alcohol, 1:1) were moderately permeable to glycerol, less permeable to glucose, and essentially impermeable to sucrose



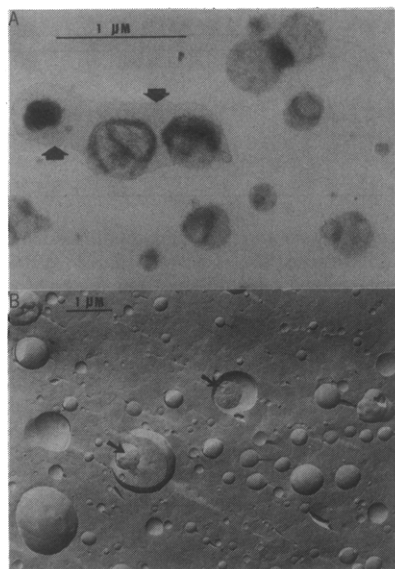


FIGURE 9: Electron microscopy of oleate vesicles. (A) After fixation with osmium tetroxide and staining on a formvar-carbon-coated grid with uranyl acetate (see text), wrinkled vesicles were present. Broken membrane fragments (arrows) seemed to result from the tearing of large, unilamellar vesicles during drying of the sample on the grid. (B) Freeze-fracture of oleic acid vesicles using standard techniques (see text). Vesicles trapped in aqueous compartments of larger liposomes are noted (arrows).

(data not shown). Absorbance curves were thus similar to those of oleate-cholesterol vesicles. By the same criteria, similar preparations of C(12) vesicles were totally impermeable to glycerol and larger sugars, as well as to urea and to KCl (data not shown).

**Electron Microscopy of Fatty Acid Vesicles.** Negative-stain electron microscopy (Bangham and Horne, 1964; Castle and Hubble, 1976) and the freeze-fracture method (Deamer et al., 1970) were employed to confirm the presence of aqueous compartments and lipid bilayers, as well as to detect vesicles too small to be visualized in the light microscope ( $<0.1 \mu\text{m}$ ).

Uranyl acetate was used as a negative stain because it exhibited superior spreading characteristics on carbon-formvar-coated grids, compared to stains such as ammonium molybdate and sodium phosphotungstate. However, negative-stain electron microscopy of dodecanoate dispersions did not reveal intact vesicles. Monitoring of lipid dispersions by phase-contrast microscopy during the addition of uranyl acetate revealed breakdown of vesicles into amorphous aggregates. Oleic acid vesicles fixed with osmium tetroxide before staining were stabilized against disruption by the divalent uranyl cation. Fixed vesicles were agglutinated but not crystallized by 10 mM  $\text{CaCl}_2$ , which disrupted unfixed liposomes at much lower concentrations. Electron micrographs showed small, wrinkled vesicles as well as membrane fragments from larger, apparently unilamellar vesicles (Figure 9A).

Freeze-fracture electron microscopy preserves membrane structure without drying artifacts which are possible in negative-stain preparations. However, problems were encountered similar to those described in microscopic permeability determinations. High concentrations of glycerol ( $\sim 4 \text{ M}$ ) are commonly used to reduce ice crystal formation during freezing of samples. Addition of glycerol caused crystallization of lipid in dispersions of saturated pure fatty acid. The use of fatty alcohol stabilized vesicles but did not entirely prevent changes in the microscopic appearance of short-chain [C(10)] dispersions. However, pure oleate dispersions were relatively stable in 25% glycerol. The freeze-fracture electron micrograph of

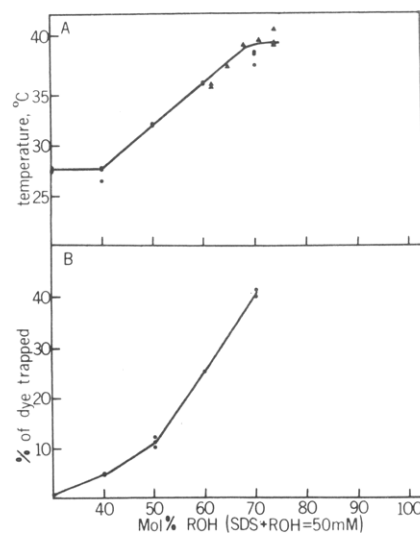


FIGURE 10: Characteristics of dodecyl sulfate-dodecanol vesicles. (A) Melting points of crystals, membrane fragments, or gel-state vesicles were determined microscopically (see text) at different molar ratios of dodecyl sulfate (SDS) to dodecanol (ROH). Values plotted represent individual determinations of the initiation of melting; the transition was usually complete within 1–1.5 °C: (● and ▲) two different preparations of vesicles. (B) Trapping volumes of vesicles were calculated as the percentage of total chromate absorbance ( $A_{372}$ ) which was recovered in vesicles, after gel filtration at 39–42 °C on Sephadex G-50. Points represent individual determinations for a typical vesicle preparation (● in A). Other data suggest that chromate trapping does not increase beyond ca. 70 mol % ROH.

such a preparation disclosed oleate vesicles up to  $1.2\text{-}\mu\text{m}$  in diameter (Figure 9B). Liposomes which appear unilamellar are visible, and these have entrapped smaller vesicles.

**Dodecyl Sulfate-Dodecanol Vesicles.** We tested the trapping capability and stability of vesicles made from various ratios of  $\text{NaDodSO}_4$  and dodecanol. Liposomes were formed from 50 mM dispersions containing 30–74 mol % dodecanol. The melting points of vesicles (Figure 10A) were determined as for fatty acids. Liposomes containing 30 and 40% dodecanol were unstable below their melting points, seeming to open and simultaneously roll up into 1–2- $\mu\text{m}$  long, spindle-shaped crystals. Reheating the sample caused the immediate re-formation of large, oligolamellar vesicles. At 50% dodecanol or greater, vesicles were stable for hours or days at room temperature but precipitated if stored at 0–4 °C overnight. Increased dodecanol up to approximately 70 mol % resulted in higher transition temperatures (Figure 10A) and an apparent increase in vesicle size.

**Trapping Volumes of Vesicles.** Trapping volumes of vesicles containing 2 mM Bromophenol blue dye (to allow visual detection of vesicles during gel filtration) and 25 mM potassium chromate (as a volume marker) were determined after prolonged equilibration of vesicles following sonication. Trapping volume is defined here as the percentage of the total dye eluted from the column which was recovered in the fraction excluded by Sephadex G-50 (mol wt ca.  $>15\,000$ ). Initial measurements indicated that trapping by 40–60 mol % dodecanol vesicles increased during postsonication incubation for up to about 24 h, while trapping by 70 mol % dodecanol vesicles increased for 48 h.

Trapping volumes of vesicles equilibrated at 39–42 °C are shown in Figure 10B. It was necessary to add Triton X-100 to the vesicle fraction prior to spectrophotometry to eliminate light scattering. We found that Bromophenol blue absorbance was affected rather unpredictably by the presence of Triton. For this reason, chromate absorbance was used to calculate

trapped volumes. Below 33% dodecanol, essentially no trapping occurred, although light microscopy of lipid eluting in the 30 mol % dodecanol "vesicle fraction" revealed many 0.1–1.0- $\mu\text{m}$  diameter vesicles. Trapping increased approximately linearly between 50 and 70 mol % dodecanol.

If gel filtration was performed below the melting point of vesicles containing less than 50% dodecanol, trapping was negligible. For vesicles with 50 mol % dodecanol, trapping was reduced but still substantial if gel filtration was performed below the  $T_m$ , while those containing 60 mol % or more dodecanol showed similar trapping volumes above and below their melting points (data not shown). Maximal trapping volumes (up to 42% of the volume of the dispersion) were obtained after overnight incubation of 70 mol % dodecanol vesicles at their  $T_m$  (39 °C). This dispersion, which was very viscous, contained oligolamellar vesicles as large as 100  $\mu\text{m}$  in diameter. As the Sephadex beads were 100–300  $\mu\text{m}$  in diameter, some of the largest vesicles were trapped at the top of the column and were not eluted with the vesicle fraction; thus, the actual trapped volumes probably exceeded the 42% measured.

**Microscopic Determination of Vesicle Permeability and Stability.** As measured by phase-contrast microscopy, gel-state vesicles which contained trapped 6CF were impermeable to added glycerol or sucrose. After 7 weeks at room temperature, Sephadex-filtered vesicles were largely oligolamellar and were still impermeable to added sucrose (Figure 11A). The dye 6CF could still be seen by fluorescence microscopy to be concentrated inside vesicles (Figure 11B).

## Discussion

**Comparison of Monoalkyl and Phospholipid Vesicles.** Vesicular model membranes made from phospholipids are finding increasing use in biomembrane research (Poste et al., 1976; Tyrell et al., 1976). While monoalkyl compounds are not found in substantial quantities in membranes of most organisms, the results presented here demonstrate that model membranes formed from these amphiphiles in dilute dispersions share several characteristics with phospholipid membranes. For example, if aqueous solutions of fatty acids or  $\text{NaDodSO}_4$  are combined with uncharged amphiphiles ( $\text{RCOOH}$  or  $\text{ROH}$ ), they exhibit self-assembly into vesicles. Like phospholipid vesicles, these are impermeable to small polar solutes, undergo gel to liquid-crystalline thermotropic phase transitions, and reveal smooth fracture planes by freeze-fracture electron microscopy.

The ways in which monoalkyl liposomes differ from phospholipid vesicles are also significant, because they point to specific characteristics of phospholipids that are important to biological function. Unique characteristics of monoalkyl liposomes include the tendency for gel-state vesicles to undergo a transformation to lamellar crystals, the requirements of a pH greater than 7 (for fatty acid dispersions), and the relatively high concentration of lipid required for membrane formation. It is striking that the same minimum chain length [C(10)] is required for the formation of "normal" vesicles from diacylphosphatidylcholine (see Grover et al., 1975) and from monoalkyl dispersions. Yet the CMC of didecanoylphosphatidylcholine is substantially below 0.19 mM (Bonsen et al., 1972), while vesicles do not form in C(10) single-chain dispersions below about 50 mM lipid.

**Previous Reports of Monoalkyl Model Membranes.** Recent theoretical and experimental considerations of the behavior of mono and dialkyl polar lipids (Tanford, 1973; Israelachvili et al., 1976, 1977) resulted in the conclusion that single-chain amphiphiles only form bilayer membranes at high lipid concentrations (>50% lipid by weight).

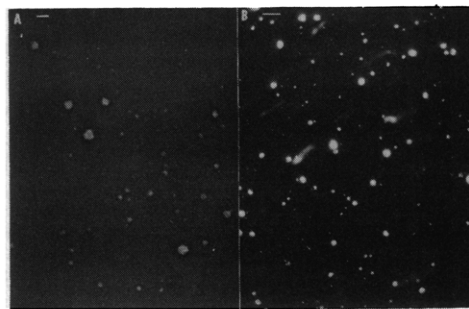


FIGURE 11: Long-term trapping by dodecyl sulfate-dodecanol vesicles. 70 mol % dodecanol vesicles (50 mM lipid) made in the presence of 6CF (ca. 50 mM in phosphate-buffered saline) and 0.1% sodium azide were incubated at 22–25 °C for 7 weeks after removal of free dye on Sephadex G-50. Bars = 10  $\mu\text{m}$ . (A) Phase-contrast micrograph of vesicles diluted with hypertonic sucrose to show impermeable (bright), partially collapsed vesicles. (B) Fluorescence micrograph of same vesicle preparation showing 6CF trapped inside of vesicles. Fluorescence microscopy was performed using the Zeiss standard fluorescence microscope with dark field optics, BG3 excitation filter and 530-nm barrier filter. Tri-X film was exposed for 30 s and developed in diluted Microdol-X.

However, numerous older studies of the liquid-crystalline phases exhibited by amphiphiles (recently reviewed by Ekwall, 1975) include descriptions of lamellar mesophases (e.g., mesophase B, Ekwall, 1975) which can exist at lower lipid concentration (70–90% water). This phase was described by Ekwall as "probably a coherent, double lipid layer alternating with layers of water" and was reported for several ternary systems. These included (1) C(8)–C(10) soaps and C(6)–C(10) *n*-alcohols, (2) potassium oleate plus C(10) alcohol, (3) octyl sulfate plus C(10) alcohol, (4) octylamine-HCl or octyldimethylamine-HCl plus C(10) alcohol, and (5) either of two nonionic detergents plus oleic acid.

It appears likely to us that Ekwall's mesophase B is a dispersion of fluid vesicles. The fact that it has been reported only for short chain or unsaturated lipids is probably the result of observations being carried out near 22 °C. This temperature is below the  $T_m$  of lamellar phases from C(12) and longer lipids. These early studies were largely performed without knowledge of the self-assembly of membrane vesicles by phospholipids (Bangham and Horne, 1964; Bangham, 1968) or of the importance of fluid lamellar phases (Singer and Nicolson, 1972) and thermotropic phase transitions (Melchior and Steim, 1976a,b) to biomembrane function.

Such considerations were first applied to research with single-chain amphiphiles by Gebicki and Hicks (1973, 1976), who varied pH, degree of unsaturation, and hydrocarbon chain length in attempts to form liposomes from carboxylic acids. They found, using freeze-fracture electron microscopy, that moderately alkaline dispersions of oleic acid contained heterogeneous populations of multilamellar vesicles. Light-scattering changes were induced in such dispersions by dilution with hypo- or hypertonic solutions of sucrose. These results suggested that oleate and linoleate vesicles were moderately impermeable to sucrose. Gebicki and Hicks concluded that only unsaturated, long-chain fatty acids could form vesicles and, thus, called these structures *ufasomes* (for unsaturated fatty acid liposomes).

In the work reported here, we have confirmed the formation of vesicles in alkaline dispersions of oleic acid. We have also found that such vesicles are moderately permeable to sucrose. However, in the presence of cholesterol at high pH, oleate forms vesicles which appear quite impermeable to sucrose, as determined by the spectrophotometric technique described under Materials and Methods.

We have shown that short- and long-chain saturated am-

phiphiles can form liposomes which behave similarly in many respects to those formed from biological phospholipids. The requirements for membrane formation have been investigated by varying pH, temperature, and lipid concentration, as well as chain length and polar head group. We have found that membrane formation in dilute dispersions of monoalkyl amphiphiles is readily and predictably attained. Therefore, in our opinion, the term *ufasomes* is misleading, since saturated fatty acids as well as detergents can form similar vesicles. Since these structures are similar in many respects to phospholipid vesicles and since the components fall under the definition of lipids, we propose that *liposome* is sufficiently descriptive.

**Effect of pH on Vesicle Properties.** The titration of alkaline soaps with HCl at 22–23 °C has been described by several groups investigating the effects of surface charge on lipid–water interfaces (Rosano et al., 1969; Lucassen, 1966). In these experiments, the appearance of a plateau (buffering capacity) at a precise pH during titration was coincident with the formation of lipid crystals. This phenomenon results from the formation of insoluble, lamellar  $(\text{RCOOH}-\text{RCOO}^-)_x$  complexes from  $(\text{RCOO}^-)_x$  micelles (Rosano et al., 1969). Since these “acid–soap” complexes form at alkaline pH, lipid carboxyls can clearly be protonated above their  $\text{pK}_a$  of 4.76 (Smith and Tanford 1973). This apparent shift in the  $\text{pK}_a$  of lipid anions has been attributed to the local decrease in pH at highly charged surfaces (see Gebicki and Hicks, 1976).

In our experiments, acid titration of alkaline, micellar soap also resulted in the appearance of a plateau at a precise pH, which corresponded to the formation of vesicles or crystals. Freeze–fracture and negative-stain electron microscopy of vesicles (Figure 9) and crystals (not shown) indicate that both these structures are lamellar. Because of the central role of micellar to lamellar transitions in the formation of liposomes from soaps, we have called this pH the  $\text{pH(L)}$ . Because  $\text{pH(L)}$  varies with temperature, with lipid concentration below the CMC, and probably with ionic strength, we have designated a standard value,  $\text{pH(L)}^\circ$ . This is measured at a temperature equal to  $T_m + 5^\circ\text{C}$ , a lipid concentration exceeding the CMC, and an ionic strength equivalent to that achieved by titrating with HCl from pH 10.0.

We have found that  $\text{pH(L)}^\circ$ , like  $T_m$ , varies with hydrocarbon chain length (Figure 3B). In a previous study reporting the titration of micellar C(12) and C(14) fatty acids,  $\text{pH(L)}$  was determined at 23 °C (Rosano et al., 1969). Differences in  $\text{pH(L)}$  for the two chain lengths were attributed to a higher packing density of the C(14) amphiphiles, which created a higher surface charge density and thus a greater shift in the apparent  $\text{pK}_a$ . This interpretation is supported by the work of Cornwell and colleagues (Heikkilä et al., 1970; Patil et al., 1972, 1975) which emphasizes the dependence of fatty acid ionization on packing density, which in turn is affected by hydrocarbon chain length.

Our results are consistent with the concept that associative forces decrease with decreasing chain lengths. It appears that these forces become so tenuous at the C(8) chain length that they are barely adequate to maintain bilayers in water. Grover et al. (1975) have observed a similar phenomenon with synthetic PC of varying chain length. While didecanoylphosphatidylcholine forms vesicles similar to those from longer chain analogues, the dioctanoylphosphatidylcholine disperses in water as “very elongated insoluble micelles”. This description suggests similarities between dispersions of dioctanoylphosphatidylcholine and those of C(8) monoalkyl vesicles. PC containing two C(6) chains is apparently monomeric in water at the concentrations tested by Grover et al. (1975).

**Effect of Fatty Alcohol Inclusion.** We have considered what

forces might contribute to the formation of membranes from ionic monoalkyl amphiphiles. These are interesting in that they may also contribute to the stability of dialkyl membranes. While both hydrophilic and hydrophobic forces are probably involved, hydrogen bonding between polar heads groups could promote parallel chain packing in single-chain dispersions. Rosano et al. (1969) concluded that acid–soap crystals which form during titration of micellar soaps are stabilized by hydrogen bonding between a protonated fatty acid and the carbonyl of an ionized soap molecule. This suggested to us that a supplementary proton donor, ROH, could participate in the formation of lamellar vesicular mesophases at pH values where no protonated carboxyls could exist. These inferences were supported by the formation of vesicles from C(10) and C(12) soaps mixed 1:1 (molar ratio) with the homologous fatty alcohol. Such liposomes were sensitive to low pH as were vesicles formed by acid titration but did not dissolve at very high pH (greater than pH 12).

Addition of dodecanol to clear solutions of NaDodSO<sub>4</sub> also caused the formation of lamellar mesophases. Lawrence (1958, 1968) detected birefringence in such structures but apparently did not heat them above the  $T_m$ . The progressive change in both trapped volume and  $T_m$  of liposomes formed from increasing mol % ROH is quite interesting but not easily interpreted. However, a general conclusion which can be drawn from these results is that increased hydrophobic interactions or decreased charge repulsion between adjacent membrane lipids (either of which would serve to raise the  $T_m$ ), can serve to increase membrane stability and thus vesicle size.

**Temperature Effect.** Saturated fatty acids form vesicles if the pH and temperature are appropriately adjusted; very short chain compounds [C(8), C(10)] can form fluid liposomes at 22 °C, as reported for long-chain, unsaturated lipids (Gebicki and Hicks, 1973, 1976). Vesicles from fatty acids are fluid and stable above a temperature ( $T_m$ ) which is characteristic for each chain length and degree of unsaturation.

The transition to fluid vesicles can be detected macroscopically in a vial of acid–soap crystals which is gradually heated. As the  $T_m$  is reached, the turbid dispersion becomes translucent. This phenomenon can be monitored more precisely using a hollow microscope slide (Figure 1) connected to a variable-temperature, circulating water bath. At the  $T_m$ , microscopic crystals or angular, gel-state vesicles form spherical or tubular liposomes which are visibly fluid.

The  $T_m$  for acid–soap crystals occurs consistently 7–10 °C below the melting temperature of bulk (anhydrous) lipid (Figure 3A). Thus, the hydrated lipid behaves as if it contains 1.2–1.8 fewer carbon atoms per chain. This result is generally in agreement with Tanford’s suggestion (Tanford, 1973, p 12) that the CH<sub>2</sub> group proximal to the head group in an amphiphile should not contribute to the hydrophobic effect.

The  $\text{pH(L)}$  of a micellar C(12) soap solution is greatly affected by changes in temperature, especially if the lamellar structures which form during titration are crystals. We were surprised to see a dramatic change in this temperature sensitivity at the  $T_m$  of dodecanoate (Figure 4). Below this temperature (35 °C),  $\text{pH(L)}$  changes 0.075 pH unit per degree celsius, while above the  $T_m$  this value is reduced to 0.035 pH unit per degree celsius. We propose that  $\text{pH(L)}$  is related to the  $\text{pK}_a$  of lipid anions present in micelles. This  $\text{pK}_a$  appears very sensitive to temperature changes. For comparison, the  $\text{pK}_a$  of Tris buffer, which is also very sensitive to temperature compared to other common buffers (Good et al., 1966), changes only 0.03 pH unit per degree celsius. Thus, below the  $T_m$ , the  $\text{pK}_a$  of dodecanoate is more than twice as sensitive to temperature changes as Tris buffer. This phenomenon may be



related to temperature-dependent changes in hydrocarbon packing in micelles.

**Permeability.** Previous studies of liposome permeability have used dialysis (Papahadjopoulos et al., 1977; Gebicki and Hicks, 1973, 1976) or gel filtration (Gebicki and Hicks, 1976) to remove external solutes or spectrophotometry to monitor changes in light scattering which result from osmotic effects on vesicles (Cohen and Bangham, 1972). While all of these approaches can provide quantitative information about the rate of leakage of a given solute, they cannot report on the permeability of individual vesicles within a large population.

The phase-contrast microscopic technique which we have introduced allows qualitative measurement of the permeability of individual liposomes. Information can be obtained immediately after mixing a potentially impermeant solute with vesicles. As an example of how this technique may prove useful in membrane research, we mixed large-volume vesicles made from egg phospholipids in phosphate-buffered saline (Deamer and Bangham 1976) with isotonic sucrose to a final sucrose concentration of 150 mM. Liposomes appeared as bright, 1–10- $\mu$ m spheres. After incubation for more than 10 months at room temperature (under air in a sealed vial, with 5 mM sodium azide), some vesicles were no longer bright but appeared as oligolamellar "ghosts". Vesicles which were still bright presumably remained impermeable to sucrose for the duration of the incubation period. Thus, it seems that vesicles are impermeant to sucrose while they retain their structural integrity. Averaging techniques such as dialysis, however, would not distinguish between the gradual accumulation of lysed vesicles and the gradual leakage of sucrose from all vesicles.

**Other Monoalkyl Vesicles.** Two other classes of monoalkyl vesicles have been observed in preliminary experiments (Hargreaves and Deamer, unpublished observations). First, equimolar mixtures of NaDodSO<sub>4</sub> with cationic HTMAB (50 mM lipid) form crystals at 22 °C. On warming to 47 °C or higher, fluid vesicles appear which are impermeable to sucrose. On cooling, these vesicles rapidly degenerate into angular membrane fragments.

Second, the long-chain "detergents" octadecyl phosphate and octadecyl sulfate (see Morse and Deamer, 1973, for synthesis) can be dissolved in water or buffer by heating briefly above 70 °C (lipid concentration 1–5 mM). On slow cooling, solutions become translucent and large oligolamellar vesicles are visible by phase-contrast microscopy. In the case of octadecyl sulfate, planar disks approximately 10  $\mu$ m in diameter are formed when lipid is dissolved in distilled water and slowly cooled. Subsequent addition of NaCl to 75 mM results in saltatory transition of disks to large (5–10  $\mu$ m diameter) oligolamellar vesicles.

Negative-stain electron microscopy of octadecyl phosphate vesicles revealed both large (1–5- $\mu$ m diameter) and small (0.05- $\mu$ m diameter and smaller) liposomes. These all appear unilamellar and are very similar to osmium-fixed, oligolamellar PC vesicles which have been stained with uranyl acetate (Hargreaves and Deamer, unpublished).

Vesicles obtained from both of these long-chain detergents enclose large aqueous compartments. In order to assess their potential use in drug delivery to human cells (discussed below), we observed mixtures of octadecyl phosphate or octadecyl sulfate liposomes and washed human erythrocytes (Hargreaves and Deamer, unpublished). It is significant that no lysis or other detectable change occurred in these cells. In contrast, incubation of NaDodSO<sub>4</sub>-HTMAB vesicles with erythrocytes resulted in agglutination and gradual lysis of the cells.

**Biological Significance of Monoalkyl Vesicles.** Liposomes

have potential uses as carriers of drugs and molecular probes in cell cultures and in organisms (Poste and Papahadjopoulos, 1976; Tyrrell et al., 1976). It may be possible to use single-chain lipids to tailor liposome characteristics (charge, fluidity, permeability, affinity for protein components, vesicle size, and stability) to maximize their utility for a particular function. Some monoalkyl ionic lipids have already been employed to modify the characteristics of phospholipid vesicles (see Tyrrell et al., 1976). However, with a greater understanding of the membranogenic characteristics of single-chain amphiphiles, other applications may be possible. The use of monoalkyl vesicles with living cells would require that the lytic effects of any micellar or monomer lipid be minimal. Our preliminary experiments involving the incubation of erythrocytes with octadecyl sulfate and octadecyl phosphate vesicles (see above) demonstrate that such deleterious effects can be avoided.

Monoalkyl liposomes are relevant as models of some biomembranes. The phytoflagellate alga, *Ochromonas danica*, utilizes for its flagellar membrane single-chain chlorosulfolipids, as well as free fatty acids (Haines, 1973; Chen et al., 1976). That fatty acids do not arise from hydrolysis of more complex lipids during isolation has been demonstrated (T. Haines, personal communication). Cells were prefixed in glutaraldehyde to immobilize and inactivate potential phospholipases prior to lipid extraction, with no effect on the yields of free fatty acid.

Monoalkyl chlorosulfolipids have been conclusively identified in five other species of algae and in *Nostoc*, a blue-green alga (Mercer and Davies, 1974, 1975). These lipids were extracted from whole cells rather than from isolated membranes and are not necessarily membrane components. However, these results imply that the utilization of monoalkyl amphiphiles as the major lipid components in plasma membranes may not be limited to *Ochromonas*.

Our results show that interactions of NaDodSO<sub>4</sub> and dodecanol result in the formation of stable membranes. Similar interactions could stabilize the organization of the naturally occurring sulfolipid membranes.

One final consequence of the formation of liposomes from monoalkyl amphiphiles is the likelihood that such compounds, believed to have been present in the primeval ocean, contributed to the formation of semipermeable membranes on the prebiotic Earth (Hargreaves et al., 1977; Hargreaves and Deamer, in press).

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## Lipid-Protein Associations in Chromatophores from the Photosynthetic Bacterium *Rhodospseudomonas sphaeroides*<sup>†</sup>

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**ABSTRACT:** Lipid-protein interactions were examined in chromatophores isolated from the photosynthetic bacterium *Rhodospseudomonas sphaeroides* using lipid spin-labels. The chromatophores contain fluid bilayer and a significant amount of lipid immobilized by membrane proteins. For a typical preparation of cells grown under 600 ft-c illumination, 59% of the spin-labeled fatty acids were bound. Essentially the entire length of the 18-carbon fatty acid chain was immobilized, judging from results obtained with the spin-label at the 7, 12, and 16 positions. The amount immobilized varies directly

with the bacteriochlorophyll content of the chromatophore material, suggesting that a significant fraction of the lipid spin-labels is immobilized on the hydrophobic surfaces of the chlorophyll-binding proteins. Changing the lipid spin-label head group from a negatively charged carboxyl group to a positively charged quarternary amine greatly decreased the amount of immobilized lipid. The changes in immobilized lipid with light level and polar head group suggest that the antenna bacteriochlorophyll-binding proteins preferentially associate with negatively charged lipids.

**B**iological membranes consist largely of protein and lipid. The lipid forms a two-dimensional fluid bilayer in which integral membrane proteins are embedded. It is known that lipid-protein interactions are important to membrane function (Griffith & Jost, 1978; Gennis & Jonas, 1977). These can be

viewed as consisting of a hydrophobic interaction between the lipid chains and nonpolar protein residues and an ionic interaction between lipid polar head groups and the protein at the aqueous interface. The available evidence suggests that the hydrophobic lipid tails are immobilized by binding to hydrophobic surfaces of integral membrane proteins and that the immobilized lipid forms a boundary layer or annulus around the protein (Jost et al., 1973a). This layer is apparently required for activity. The boundary layer lipid does not exhibit the usual segmental motion seen in fluid bilayer (Jost et al.,

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