Uniform Preparations of Large Unilamellar Vesicles Containing Anionic Lipids†

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ABSTRACT: A general procedure for the preparation of large unilamellar vesicles of selected sizes has been developed. The procedure consists of dissolving the lipid in organic solvent, washing with mild acid, removing the solvent, adding salt (0.15 M KCl) solution, and adjusting the pH (raising it to about pH 10 and lowering it immediately to pH 7.55). The procedure takes less than 30 min. The resulting unilamellar vesicles are of a single size with a rather low standard deviation. The sizes of these preparations range between 150 and 1000 nm in diameter. Sizes and polydispersities were measured to within 1–2% by photon correlation spectroscopy. Vesicle size varies with (1) the phospholipid structure, (2) the composition of the phospholipid mixture, (3) the ionic strength of the medium, (4) the alkyl chain composition, (5) the cholesterol content of the phospholipid mixture, and (6) the timing of the pH adjustment procedure. Uniform preparations of vesicles have been obtained from the dioleoyl esters of phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylserine, from diphytanyl ethers of glycolipid sulfate, phosphatidylglycerol, phosphatidylglycerol phosphate, and phosphatidylglycerol sulfate, from bovine liver phosphatidylinositol, from *Escherichia coli* phosphatidylethanolamine, from membrane lipid extracts from *E. coli* and *Holabacterium cutirubrum*, and from dodecanesulfonate–alkanol mixtures and free oleic acid. The preparation of unilamellar vesicles from oleic acid is novel, and the size range is 600–3000 nm; the preparations are relatively uniform. Vesicles of phospholipids in which sucrose and trehalose replace salt as the impermeant do not differ significantly from those prepared in pentaerythritol.

Procedures for rapid preparation of unilamellar vesicles have been developed in recent years to obtain model membranes for studying transport proteins, membrane fusion, and delivery of drugs (and possibly proteins and/or genes) into living cells (Gregoriadis & Allison, 1980; Poste, 1983). Many methods are now available for synthesizing unilamellar vesicles (Batzri & Korn, 1973; Barenholz et al., 1979; Saunders et al., 1962; Papahadjopolous & Miller, 1967; Deamer & Bangham, 1976; Parente & Lentz, 1984; Mimms et al., 1981; Zumbuehl & Weder, 1981, Petri et al., 1980; Enoch & Strittmater, 1979). Most of these methods produce a wide range of sizes of vesicles in a single preparation. A method has been available that yields uniform vesicles in the 30–50 nm diameter size range (Huang, 1969; Chrzeszczyk et al., 1977; Huang & Mason, 1978) or vesicle that are mostly larger in size but also contain the smaller sized vesicles (Hope et al., 1985; Mayer et al., 1986). Special mention should be made of the latter method developed by P. R. Cullis. It is an ingenious method that uses filtration extrusion with a variety of pore sizes for making vesicles with selected diameters. This method has an advantage over the procedure reported herein, which requires anionic lipids, in that any lipid can be used to make the vesicles. However, the formation of a large number of smaller vesicles by the extrusion procedure can be a problem.

The work reported herein describes a simple, quick procedure for making uniform preparations of unilamellar vesicles by a pH adjustment method described earlier (Aurora et al., 1985). Its origins can be traced to the method developed by Hauser and Gains (Hauser & Gains, 1982; Hauser et al., 1983; Gains & Hauser, 1983) for obtaining small unilamellar vesicles. The procedure requires that anionic lipids be at least one component of the vesicle bilayer. In addition to producting a narrow standard deviation in size (Aurora et al., 1985), the method allows the size of the vesicles to be selected by varying the composition, the structure of the component lipids, the ionic strength of the medium, and other conditions used for making the vesicles.

Our previous work showed that (1) vesicle preparations made by the pH-adjustment method were unilamellar and uniform in size, as established by PCS,[1] EM, internal volume, and osmotic swelling studies (Aurora et al., 1985), (2) vesicle size depended on maximum pH of the pH-adjustment method, cholesterol content in DOPA, and different osmolal media, and (3) osmotic swelling could be used on these preparations to establish the membrane modulus, the surface expansion, and the transbilayer pressure difference at bursting during swelling of the vesicles (Li et al., 1986).

This paper shows that uniform preparations can be made of virtually any anionic membrane lipids including natural lipid mixtures and that the size of the resulting vesicles depends upon the ionic strength of the medium, although other factors have important effects on the vesicle size.

Materials and Methods

Materials. DOPA, DOPG, DOPE, DOPS, DOPC, bovine liver PI, *Escherichia coli*. PE, and *E.* coli lipids were procured from Avanti Polar Lipids, Inc. (Birmingham, AL). They were checked for purity by TLC. Ultrapure sucrose was purchased from Schwarz/Mann (Orangeburg, NY). Pentaerythritol and trehalose were purchased from Sigman (St. Louis, MO). DΦGLS, DΦPGP, DΦPG, and DΦPGS were isolated from

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[1] Abbreviations: DOPA, 1,2-dioleoyl-3-*sn*-phosphatidic acid; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; DOPE, 1,2-dioleoly-3-*sn*-phosphatidylethanolamine; DOPG, *rac*-1,2-dioleoylphosphatidylglycerol; DOPS, 1,2-dioleoyl-3-*sn*-phosphatidylserine; DΦGLS, diphytanylglyceroglycolipid sulfate; DΦPGP, diphytanylphosphatidylglycerol phosphate; DΦPG, diphytanylphosphatidylglycerol; DΦPGS, diphytanylphosphatidylglycerol sulfate; EM, electron microscope; LUV, large unilamellar vesicle; PCS, photon correlation spectroscopy; PI, (bovine liver) phosphatidylinositol; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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cells of *Halobacterium cutirubrum* by Laura Stewart in Dr. Kates' laboratory, Ottawa, Canada, using the procedure described previously (Kates, 1978). Dodecanesulfonic acid (sodium salt) was purchased from Aldrich (Milwaukee, WI). All chemicals were reagent-grade unless otherwise indicated. All solvents were redistilled before use.

Preparation of Vesicles. The modified pH-adjustment procedure for making unilamellar and uniform vesicles was described in the previous work (Aurora et al., 1985). The lipid (5–10 mg) was dissolved in 2 mL of chloroform. In order to remove sodium (or ammonium) ions, this solution was washed up to 4 times with 2 mL of chloroform/methanol/0.2 M HCl (3:48:47 v/v/v). The upper phase was discarded each time. The resulting lower phase was washed with a 2-mL mixture of chloroform/methanol/water (3:48:47 v/v/v). In the vesicle preparations of lipid mixtures, the lipids were mixed in solvent at this point. The lower phase was then rotary evaporated in a round-bottom flask to form a phospholipid film. The phospholipid film was dried under a water aspirator at room temperature for about 10 min. The dried film (total sample of 5–10 mg) was then dispersed in 3.0 mL of the desired medium for vesicle formation. The mixture was stirred with a stirring bar for up to 30 min at room temperature in order to suspend the lipid film in the solution. This solution was titrated with 0.1 N NaOH to a pH of 7.55–11.0, and the pH then was immediately adjusted to 7.55 with 0.1 N HCl.

Negative-Staining Electron Microscopy. Fatty acid vesicles were studied with a Philips 300 EM at 60 kV. Formvar-coated grids were prepared with a 0.5% formvar solution on uncoated grids. A drop of solution containing phospholipid vesicles was placed on the Formvar grid. The excess solution was adsorbed with No. 1 filter paper after 15 s. A drop of uranyl acetate solution (5%) was placed on the grid. After 5 s, the excess was absorbed with filter paper. The grid was then allowed to dry in air for 10 min prior to examination with the EM. Phospholipid vesicles were earlier examined with EM (Aurora et al., 1985).

Photon Correlation Spectroscopy (PCS). Dynamic light scattering was used to determine the vesicle size (Aurora et al., 1985). The apparatus used is described by Hwang and Cummins (1982). Light from an argon ion laser at 488 nm was focused onto the vesicle sample in a glass cuvette maintained at constant temperature (20 °C) by a Lauda water circulator. The intensity of the scattered light was detected at 90° to the incident beam with a Hamamatsu (Middlesex, NJ) photomultiplier tube (PMT).

The radius of the vesicles in a sample is calcualted from the correlation function. In PCS, one measures the second-order correlation function $g^{(2)}(\tau)$, where τ is the delay time. For particles in Brownian motion, the correlation function decays exponentially with the delay time according to the following expression:

$$g^{(2)}(\tau) = B(1 + Ae^{-\Gamma\tau})$$

where B is the base line, Γ is the inverse correlation time, and A is a constant between 0 and 1 determined by the optical system. Γ is given by $2Dq^2$, where D is the translational diffusion coefficient and q is the scattering vector. D is related to a spherical particle's radius (R) by the Einstein–Stokes expression:

$$D = kT/(6\pi\eta R)$$

k = Boltzmann's constant, η = viscosity of the solution, and T = absolute temperature.

A typical set of data showing autocorrelation is displayed in Figure 1. The data for the standard polystyrene spheres

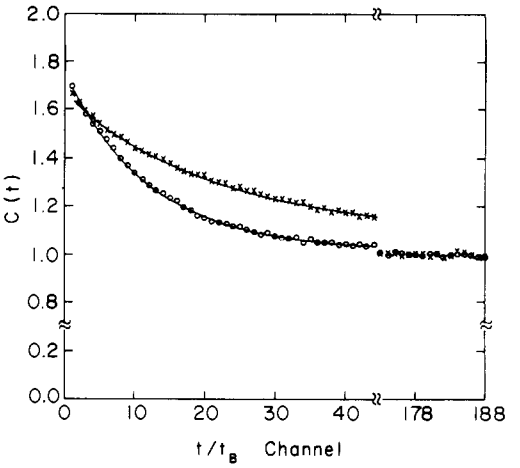

FIGURE 1: Light scattering intensity autocorrelation data for polystyrene spheres (diameter = 126.8 nm) in water (O) and for a DOPG vesicle preparation (diameter = 456.3 nm) in 150 mM KCl(+). The solid lines are single-exponential fits of the data to $C(t) = B(1 + ae^{-t/t_c})$. The correlation time, t_c, found from the fits is related to the translational diffusion constant, D, by $t_c = 1/(2Dq^2)$, where q is the scattering vector. The background, B, was found from the delayed channels. The bin times, t_b, for the two experiments were both 20 μs.

are shown. Comparisons were earlier made on the standard deviation of the particle sizes of both the spheres and the unilamellar vesicles by electron microscopy (Aurora et al., 1985).

By fitting a single exponential through the correlation data, one can determine the diffusion coefficient (D) and from this the particle radius (R). The light scattering apparatus was calibrated with a standard, monodisperse sample of polystyrene latex spheres (with diameter 126 nm).

Correlation data obtained in the measurement of vesicle size (Figure 1) were analyzed by the standard cumulants method from which the z-average radius $\langle R\rangle_z$ and the polydispersity index μ_2/Γ_0^2 were determined. For vesicles in the size range examined, the standard deviation σ of the size distribution is given approximately by $\sigma/R_0 = x\mu_2/\Gamma_0^2$ with $x \sim 3$ (Aurora et al., 1985).

Osmotic Swelling of Oleic Acid Vesicle Suspensions in KCl. Vesicle suspension (1.0 mL) was placed in a cuvette, and different amounts of the same diluted buffer (0.086 M KCl solution) were added to it. The dilution was carried out very slowly in order to prevent the vesicles from experiencing osmotic shocks. A syringe pump (Razel, Stamford, CT) was employed for conducting slow dilution 0.025 mL/min) of vesicle suspensions. The vesicle solution was continuously stirred with a magnetic stir bar during dilution and was kept in a closed cuvette (with a small hole in the Teflon stopper for the syringe needle) to prevent evaporation of water, which might give rise to size change by changing the solution concentration. The vesicles were suspended in buffer solutions that had been filtered through 0.3 μm pore size filter paper (Nuclepore, Pleasanton, CA) prior to dilution.

RESULTS

Synthesis of Uniform Vesicles with Anionic Lipids. A variety of lipid structures were examined for their capacity to form uniform, unilamellar vesicles. A summary of the results of these experiments is shown in Tables I–III. Attempts to make vesicles with DOPC alone were unsuccessful.

Table I illustrates that monounsaturated (or in the case of *E. coli* lipids, a mixture of monounsaturated and cyclopropane lipids) lipids that contain anionic head groups may be used

Table I: Variation of Vesicle Size and Polydispersity with Lipid Head Group for Vesicles Made of Monounsaturated Glycerol Lipids[a]

lipids	initial pH	maximum pH	vesicle size diameter (nm)	index of polydispersity
PI	5.17	10.0	226.3 ± 0.9	0.09
DOPS	4.41	8.50	277.8 ± 1.2	0.07
DOPA	4.52	10.0	318.1 ± 2.0	0.07
E. coli lipids/DOPA	4.49	9.03	445.5 ± 2.6	0.10
DOPG	4.10	10.0	456.0 ± 2.3	0.11
E. coli lipids	6.94	9.50	517.1 ± 3.1	0.09
DOPE	5.94	10.65	544.3 ± 2.3	0.06
E. coli PE	5.36	10.05	716.0 ± 4.5	0.12

[a] All preparations are in 0.15 M KCl solution. Vesicle size was measured by PCS. In the preparation of *E. coli*/DOPA, the mole ratio of the two components was 1:1. PI refers to bovine liver phosphatidylinositol. All vesicle preparations were restored to pH 7.55 after the maximum, except DOPE and *E. coli* PE which were adjusted to pH 8.60. The error represents error in measurement and not the standard deviation on the vesicle size.

to make vesicles in the 200–700 nm diameter size range.

In contrast, lipids containing the bulkier phytanyl chains (Table II) yield sizes in the 400–1000-nm range. The finding that increased bulk in the hydrocarbon domain of the bilayer results in larger sized vesicles is consistent with the earlier finding (Aurora et al., 1985) that vesicle size is directly proportional to the mole fraction of cholesterol in DOPA vesicles. These two observations suggest that very small changes in the average distance between the head groups relates to the curvature of the vesicles synthesized by this procedure.

Table II shows several features of our procedure that should be pointed out. First, the polydispersity of these vesicles is significantly higher than those of the straight-chain lipids. This means that the vesicle preparations are not uniform. Second, a comparison of the initial pH of DΦPGP vesicles made in 0.15 M KCl to that of those made in H_2O illustrates the extent to which acidic protons are sequestered at the polyanionic membrane surface in water. This does not occur in salt solution. When the lipids are suspended in water (as compared to saline), the surface pH is lower; consequently, the bulk-phase pH (initial pH) is higher. Because the head group has two phosphates, the protonated form would be expected to yield an unusually low initial pH. A similar statement might be made about DΦPGS, but this substance was available in limited supply. The possibility of solvolysis of the sulfate esters (Mayers et al., 1969) discouraged a thorough acid wash of this sample.

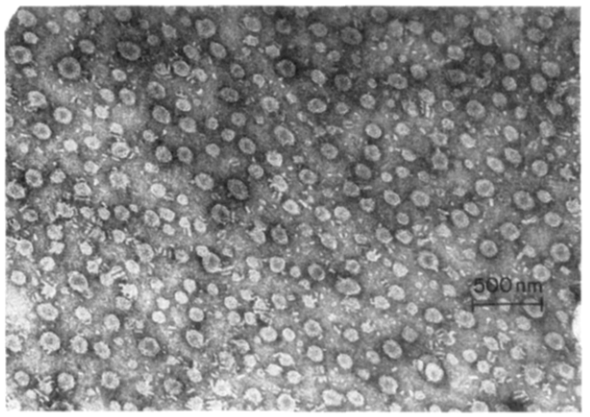

FIGURE 2: Negatively stained electron micrographs of vesicles made by pH adjustment with oleic acid in 0.15 M KCl. The maximum pH was 10.0. Grids were examined on a Philips 300 EM at the magnification 25000×. Uranyl acetate solution (5%) was used for negative staining. The vesicle size obtained from EM is smaller than those measured by using PCS; that is because the negative staining procedure shrunk the vesicles.

Table III shows that vesicles can be made with free fatty acids and with dodecanesulfonic acid/dodecanol (1:1) mixtures. The latter results show a sharp size dependence on the ionic strength and on the maximum pH. The formation of fatty acid vesicles is unique since, until this report, only fatty acid liposomes (oligolamellar) and not unilamellar vesicles have been obtained from free fatty acids (Gebicki & Hicks, 1976; Hargreaves & Deamer, 1978; Haines, 1983). The formation of unilamellar vesicles from free fatty acids in seawater may have its implications to the origin of the life on prebiotic earth. The vesicles of oleic acid are surprising because they are significantly larger than those of phospholipids and because they are uniform in size. Figure 2 shows a negatively stained electron micrograph of oleic acid unilamellar vesicles made in 0.15 M KCl. The size of the vesicles in the electron micrograph are much smaller than those obtained by PCS (Table III). This difference is presumably due to shrinkage during

Table II: Variation of Vesicle Size and Polydispersity with Lipid Head Group for Vesicles Made of Diphytanyl Ether Lipids[a]

lipids	suspension	initial pH	maximum pH	vesicle size diameter (nm)	index of polydispersity
Halobacterium lipids	0.15 M KCl	2.73	9.70	440.1 ● 3.5	0.12
DΦPGP	0.15 M KCl	3.60	10.0	631.7 ± 3.6	0.13
DΦPGP	H_2O	6.85	6.92	632.6 ± 3.3	0.13
DΦPG	0.15 M KCl	6.55	9.51	749.5 ± 4.8	0.11
DΦGLS	0.15 M KCl	6.04	9.68	759.2 ± 4.8	0.17
DΦPGS	H_2O	6.85	6.90	1045 ± 6	0.15

[a] Vesicle size was measured by PCS. Φ = phytanyl; *Halobacterium* lipids was whole lipid extract from *H. cutirubrum*. The error represents error in measurement and not the standard deviation on the vesicle size.

Table III: Vesicle Preparations with Fatty Acids[a]

fatty acids	suspension	initial pH	maximum pH	vesicle size diameter (nm)	index of polydispersity
oleic acid	0.15 M KCl	5.84	10.0	383.2 ± 1.7	0.07
oleic acid	0.15 M KCl	5.30	7.00	601.7 ± 3.8	0.07
oleic acid	4.0 M KCl	4.50	7.00	2450 ± 20	0.12
oleic acid	seawater (30 ppt)	6.29	6.29	1690 ± 14	0.12
oleic acid	seawater (35 ppt)	6.28	6.28	2800 ± 26	0.12
oleic acid	seawater (36.5 ppt)	6.48	6.48	1356 ± 12	0.11
oleic acid/oleyl alcohol	0.15 M KCl	6.15	7.00	355.0 ± 1.5	0.08
$C_{12}SO_3/C_{12}OH$	0.15 M KCl	6.75	6.75	3000 ± 24	0.09

[a] Vesicle size was measured by PCS. In the preparation of oleic acid/oleyl alcohol and $C_{12}SO_3/C_{12}OH$, the mole ratio of the two components was 1:1. The error represents error in measurement and not the standard deviation on the vesicle size.

Table IV: DOPG Vesicle Preparations Made in Solutions of Different Salt Concentrations[a]

suspension	initial pH	maximum pH	vesicle size diameter (nm)	index of polydispersity
H_2O	3.07	7.20	162.4 ± 0.8	0.06
0.15 M KCl	4.10	10.0	419.7 ± 2.8	0.09
0.50 M KCl	2.91	9.95	560.0 ± 3.2	0.11
1.00 M KCl	2.92	9.93	1170 ± 7	0.12
1.50 M KCl	2.93	9.96	2240 ± 16	0.10
2.00 M KCl	2.93	9.98	2920 ± 19	0.18
3.00 M KCl	2.73	9.94	3100 ± 23	0.20
4.00 M KCl	2.73	10.15	2990 ± 23	0.16
0.15 M KCl	4.05	4.05	543.3 ± 2.8	0.11
0.15 M KCl	4.54	4.54	446.9 ± 2.7	0.11
0.15 M KCl	11.58	11.58	450.3 ± 3.1	0.10

[a] Vesicle size was measured by PCS. For the pH of last three DOPG vesicle preparations, the pH was adjusted by addition of an appropriate concentration of NaOH to the suspending medium of the phospholipid film. No pH adjustment was made after the film was suspended. The vesicle size was measured at that pH. The error represents error in measurement and not the standard deviation on the vesicle size.

negative staining. In order to establish that the vesicle preparations of oleic acid are unilamellar, the osmotic properties (membrane modulus, maximum surface area increase, and transbilayer pressure difference at vesicle bursting) were studied on the vesicle preparations. The data are shown in Figure 3. Details of the osmotic experiments and calculations are given elsewhere (Li et al., 1986).

It may be seen in Table III that restricting the maximum pH to 7 produced substantially larger vesicles than were obtained at pH 10. The large value of polydispersity in high salt seems to be a characteristic of the method of vesicle preparation.

Preparation of Vesicles in Media of High Ionic Strength. The size of DOPG vesicles was found to vary with the salt concentration in which vesicles were made as shown in Table IV. It can be seen that the size increases up to 2.0 M KCl and then remains relatively constant. The very large sizes seen in Table IV are accompanied by a substantial increase in the polydispersity. In the last three of these experiments, the protonated phospholipid films suspended in 0.15 M KCl with or without base (as an alternative to conducting a pH adjustment *after* suspension) are approximately the same size but display a higher polydispersity.

Figure 4 shows the variation of the sizes of the vesicles obtained when DOPA vesicles were prepared in mixtures of 0.25 M sucrose and 0.15 M KCl. It can be seen that the vesicle size increases linearly with salt concentration in the range 0–0.15 M KCl.

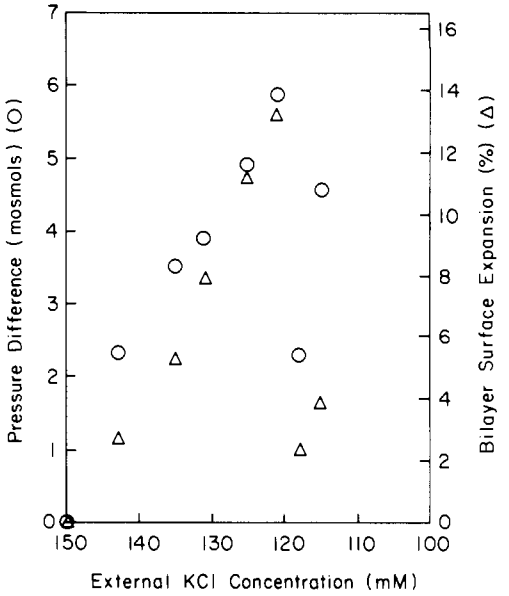

FIGURE 3: Transbilayer pressure difference (O) during the osmotic swelling of oleic acid vesicles (309 nm) in 0.15 M KCl. The KCl that was trapped inside the vesicles at the start of the swelling experiment was assumed to remain in the vesicle as it expanded. The concentration difference is used to calculate the transbilayer pressure difference (Li et al., 1986). The surface area expansion (Δ) suggests by the decrease size at 5.9 mosmol that the vesicles have burst.

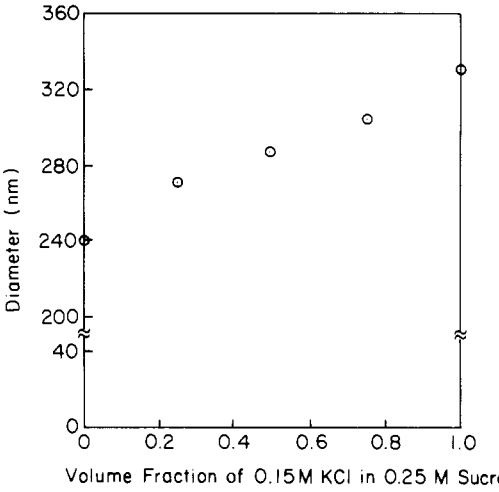

FIGURE 4: DOPA vesicle size varies with the volume fraction of 0.15 M KCl in 0.25 M sucrose; this mixture was used as a suspension medium during the synthesis of vesicles. Vesicles were prepared by the pH-adjustment method. Vesicle diameters were obtained by using PCS.

Table V: Vesicle Preparations in Low Ionic Strength Media[a]

lipids	suspension	initial pH	maximum pH	vesicle size diameter (nm)	index of polydispersity
DOPA	0.25 M pentaerythritol	5.10	10.1	177.7 ± 0.6	0.08
DOPA	0.25 M sucrose	4.80	10.0	231.3 ± 1.0	0.08
DOPA	0.25 M trehalose	5.52	10.1	290.7 ± 1.6	0.09
DOPA	KCl/sucrose	4.59	10.1	291.4 ± 2.0	0.09
DOPA	0.15 M KCl	4.82	9.85	325.5 ± 1.9	0.08
DOPA	0.05 M KP_i	7.37	7.55	535.8 ± 3.4	0.12
DOPG	H_2O	3.07	7.20	162.3 ± 0.8	0.06
DOPG	0.25 M pentaerythritol	4.90	10.02	202.4 ± 0.9	0.07
DOPG	0.25 M trehalose	5.32	9.96	268.2 ± 1.7	0.08
DOPG	0.25 M sucrose	4.94	10.0	335.6 ± 1.4	0.10
DOPG	KCl/sucrose	4.38	9.90	390.5 ± 2.5	0.11
DOPG	0.15 M KCl	4.10	10.0	419.6 ± 2.8	0.10
DOPG	0.05 M KP_i	7.30	7.50	536.9 ± 3.2	0.12

[a] Vesicle size was measured by PCS. The preparations in KCl/sucrose means the media used for making vesicles was a 1:1 volume ratio mixture of 0.15 M KCl and 0.25 M sucrose solution. The error represents error in measurement and not the standard deviation on the vesicle size.

Table VI: DOPG/DOPC Vesicle Preparations in KCl and Pentaerythritol[a]

DOPC in DOPG (molar %)	suspension	initial pH	maximum pH	vesicle size diameter (nm)	index of polydispersity
0	0.15 M KCl	4.10	10.0	456.4 ± 2.3	0.11
15	0.15 M KCl	4.80	10.05	500.7 ± 1.8	0.12
30	0.15 M KCl	4.84	10.05	560.3 ± 2.4	0.13
50	0.15 M KCl	5.15	10.03	624.7 ± 3.2	0.13
75	0.15 M KCl	6.15	10.06	808.2 ± 3.7	0.16
0	0.25 M pentaerythritol	4.90	10.02	199.5 ± 0.7	0.07
1.5	0.25 M pentaerythritol	5.00	10.0	218.9 ± 0.9	0.07
30	0.25 M pentaerythritol	5.02	10.25	245.6 ± 1.1	0.08
50	0.25 M pentaerythritol	6.10	10.0	280.4 ± 1.4	0.08
75	0.25 M pentaerythritol	6.15	10.08	454.7 ± 1.7	0.11

[a] Vesicle size was measured by PCS. The error represents error in measurement and not the standard deviation on the vesicle size.

Preparation of Vesicles in Media of Low Ionic Strength. Table V shows the preparation of vesicles of DOPA and DOPG in a variety of media with low ionic strength. The preparation of each in 0.15 M KCl is included for comparison. It may be seen that the size of the resultant DOPG vesicles increases in the following order of the suspending medium: water, pentaerythritol, trehalose, sucrose, KCl, and potassium phosphate. A similar comparison for DOPA vesicle sizes yields essentially the same progression except that trehalose and sucrose are reversed. These data show that there is no special relationship between the phospholipid head groups and the sugars in dilute solution in this system. Such a relationship has been found at high trehalose concentrations (Crowe & Crowe, 1984).

Vesicles Prepared with Lipid Mixtures Containing Anionic Lipid. Table VI shows the sizes of vesicles made of mixtures of DOPG and DOPC in 0.15 M KCl and in 0.25 M pentaerythritol. The studies show that the vesicle size increases as the mole fraction of DOPC increases. This statement applies to vesicles made in both media, although vesicles made in 0.15 M KCl were larger and displayed greater polydispersity.

Discussion

Vesicle Preparation of Anionic Phospholipids. A general procedure is outlined for making LUV in uniform preparations from a wide variety of anionic lipids. All biological membranes contain an excess of negative charges in their lipids. The technique is therefore useful for creating analogues of biological membranes. Using this pH-adjustment procedure, we were unable to make unilamellar vesicles from DOPC alone. Uniform vesicles of DOPA had been prepared earlier (Aurora et al., 1985) with this procedure. This paper reports that the method can be used to make uniform unilamellar vesicles of virtually any anionic lipid(s) including all of the common anionic phospholipids either alone or in combination with zwitterionic lipids as is found in natural membranes.

In our earlier work (Aurora et al., 1985), we were able to characterize the uniform preparation of DOPA vesicles by (1) electron microscopy, (2) Sepharose exclusion chromatography, (3) measurement of the entrapped volume and the phospholipid content of the vesicles, and (4) photon correlation spectroscopy. Subsequently, osmotic swelling of the vesicles by dilution of the salt in a stepwise manner permitted us (Li et al., 1986) to measure the membrane modulus, the maximum surface area expansion at bursting, and the transbilayer pressure difference at bursting on DOPA vesicles as well as several of the vesicles described in this work. Special emphasis in this study is directed toward the lipids of *H. cutirubrum* and *E. coli*. *Halobacteria*, extreme halophiles, lack zwitterionic lipids. Their charged lipids are solely anionic lipids. Additionally, their lipids contain exclusively phytanyl chains in ether linkage with the glycerol backbone (Kates, 1978). These phytanyl ethers, which contain methyl groups extending from the chain on every fourth carbon, are bulkier than the straight oleyl chains of the other lipids we have examined. Like cholesterol, these methyl groups increase the average distance between adjacent head groups and therefore yield larger vesicles as does cholesterol insertion into the bilayer (Aurora et al., 1985). It is possible that the increased average distance between the head groups decreases the hydrogen bonding (acid–anion formation), which represents putative attractive forces between the head groups (Haines, 1983). A decrease in attractive interactions necessarily (because the head groups are anionic) increases the repulsive interactions. We propose that the balance between attractive vs. repulsive interactions of the inner monolayer (of the forming vesicle) fixes the curvature of the vesicles. Increased repulsion between adjacent head groups results in larger vesicles; increased attraction (H bonding) results in smaller vesicles. Phytanyl chains with their bulky methyl groups provoke the formation of larger (750-nm diameter for DΦPG; Table II) vesicle formations than does the straight chain lipids (450-nm diameter for DOPG; Table I). It should be noted that the mixture of total lipids of *Halobacterium* yields smaller vesicles than vesicle preparations made from each of the anionic lipids in pure preparation. This is presumably due to the fact that the organism contains neutral, uncharged glycolipids (Kates, 1978) as well as anionic lipids. No attempts were made to make vesicles from the glycolipids of *Halobacteria*.

E. coli lipids were also investigated (Table I). In this study we examined analogues of the principal lipids of this organism, namely, DOPG, DOPE, and DOPS. Each of these yielded uniform vesicles preparations; however, DOPS formed remarkably small (30-nm diameter) vesicles above pH 9.0 (data not shown), so that it could not be used to make large unilamellar vesicles above pH 8.5. In contrast, DOPE formed a precipitate below pH 8.6, although an excellent preparation of vesicles was obtained at pH 10.6. We did not examine the PE vesicle preparation pattern in the intermediate pH range. The pH sensitivity of these two lipids is possibly due to the protonation of the amino group that is presumably involved in head group–head group interactions.

PE from *E. coli* contains largely cyclopropane fatty acids with some monounsaturated fatty acids esterified to the glycerol (Goldfine, 1982). In the context of the above discussion on the bulkiness of the phytanyl chains, we suspect that the larger vesicles of the *E. coli* PE result from a greater average distance between the head groups (compared to DOPE) caused by the slight bulk of the cyclopropane chains compared to that of the double bonds.

Unilamellar Vesicles Obtained from Single-Chain Lipids. Vesicles were also prepared from two single-chain amphiphiles: pure oleic acid and an equimolar mixture of dodecanesulfonate and dodecanol. Although these "lipids" are not natural

membrane lipids, there have been several attempts to make unilamellar vesicles from them. This work is largely motivated by the discovery of a class of chlorosulfolipids (alkyl disulfate lipids) that constitute the flagellar membrane of the phytoflagellate *Ochromonas danica* (Chen & Haines, 1976; Chen et al., 1976; Haines, 1973).

Liposomes (oligolamellar particles entrapping water) were first made of oleic acid by Gebicki and Hicks (1973, 1976). Hargreaves and Deamer (1978) elaborated and further characterized these liposomes and showed that similar liposomes could be made from alkyl sulfates and alcohols. The liposomes are generally made in this method by titrating from the micellar pH range (above pH 10) down to neutral pH. Haines (1983) explained the basis for stability of the liposomes on the basis of acid–anion formation and rapid exchange of the acid–anions. Acid–anion pairs are uncoupled, in this view, by protonation of the pair; each protonated carboxyl protonates a neighboring acid–anion. The time scale for these events would be faster than 10^{15}/s so that the statistical consequences of acid–anion formation is that acidic H atoms (not hydronium ions) provide attractive forces (via unique H bonding) between the negatively charged head groups. The attractive interactions are therefore due to the presence of nonhydrated protons in the head group domain (Haines, 1983). This principle has provided the framework for the development of the present method of synthesizing uniform preparations of unilamellar vesicles as described earlier (Aurora et al., 1985) and in this paper. The synthesis of unilamellar fatty acid vesicles is novel, as is the synthesis of unilamellar vesicles from the dodecanesulfonate–dodecanol mixture. We used a different approach to make the vesicles than that used by Hargreaves and Deamer (1978) in that we first made a film of protonated fatty acids and then suspended it in saline while adjusting the pH. In 0.15 M KCl, the result is a uniform preparation of large vesicles wherein the size depends upon the maximum pH and the salt concentration (Table III). It had earlier been shown (Gebicki & Hicks, 1976; Hargreaves & Deamer, 1978; Haines & Heller, 1982; Haines, 1983) that the liposomes are only stable between pH 7.15 and pH 9.45. We therefore sought vesicles only in this pH range.

The fact that vesicles could be made in the 4 M KCl (Table III) suggested that it might be possible to make vesicles in seawater. Earlier attempts to make liposomes of free fatty acids in seawater failed (D. Deamer, personal communication) presumably because the calcium in the seawater precipitates the fatty acids at high pH. Our attempts in three different seawaters each resulted in vesicles although these preparations, like that in 4 M KCl, displayed high polydispersity.

Verification of Unilamellar Fatty Acid Vesicles by Osmotic Swelling. In our study on osmotic swelling (Li et al., 1986) we established that the membrane modulus varies from 10^7 dyn/cm^2 in 0.25 M sucrose solutions to 10^9 dyn/cm^2 in 0.15 M KCl for DOPA, DOPG, and several other phospholipids we studied. For these lipid mixtures we found an 8–10% surface area expansion at bursting in 0.25 M sucrose solutions and a 3–5% maximum surface area expansion for vesicles in 0.15 M KCl solutions. Finally, the transbilayer pressure difference at bursting was found to be about 4–6 mosmol in sucrose while it is about 40 mosmol in 0.15 M KCl solutions. Osmotic swelling in oligolamellar liposomes (Gruner et al., 1985) has dramatically different properties and does not display a "bursting" pressure. The use of osmotic swelling is therefore a means of demonstrating that the vesicles are actually unilamellar. On the basis of such measurements we found the vesicles of oleic acid swell in 0.15 M KCl. The membrane modulus was found to be 1.0×10^7 dyn/cm^2, the maximum surface area expansion was 13%, and the transbilayer pressure difference at bursting was 6 mosmol. These data are consistent with the data found for phospholipid vesicles and submitochondrial particles as shown in the osmotic swelling studies (Li et al., 1986). It is interesting that the free fatty acid vesicles are made in KCl solution but they display properties that phospholipids display in sucrose.

The equimolar dodecanesulfonate–dodecanol vesicles represent analogues of dodecyl sulfate–dodecanol that are resistant to hydrolysis when converted to the protonated form. In conducting this experiment, many acid washes were necessary to completely convert the sodium sulfonate to the acid form. Oligolamellar liposomes of SDS–dodecanol were obtained by Hargreaves and Deamer (1978). On the basis of experiments with the *O. danica* alkyl sulfate lipids, we were concerned that preparing the protonated form of dodecyl sulfate would result in solvolysis (Mayers et al., 1969) of the dodecyl sulfate to yield dodecanol. The sulfonate is not susceptible to such solvolysis but is otherwise isosteric with the dodecyl sulfate. We assume therefore that beginning with the protonated form of the sulfate ester it will be possible to make unilamellar vesicles although the experimental details will need to be worked out.

Vesicle Preparations in Non-Saline Media. It can be seen from the data in Tables IV–VI, and displayed in Figure 4, that the salt concentration has a significant impact on the size of the vesicles prepared by the pH-adjustment method. Table IV shows that DOPG vesicles are more uniform when the pH adjusment is conducted on a preparation; i.e., the polydispersity is higher without the pH adjustment. This is seen by comparing the polydispersity of the first eight DOPG preparations in Table IV with that of the last three samples in the table that were not pH adjusted. The one sample that was adjusted up to pH 9.95 and then down to 7.55 had the lower polydispersity we generally obtain using our method (Table I). The concentrations of KCl in Table IV are much larger than those used in experimental work on membranes. The vesicle size increases but the polydispersity does as well. No investigations were conducted to see if there were two or more classes of sizes in these preparations as seems to be the case for extruded uniform vesicle preparations (Hope et al., 1985).

We have generally found smaller vesicles in preparations made in nonionic impermeants, and so we decided to compare the sizes of vesicles made in mixtures of sucrose and KCl solutions (Figure 4). It may be seen that the vesicle size increases in a linear manner with the KCl component of the KCl–sucrose mixtures. This does not, however, establish that it is salt alone that establishes the size difference. It is entirely possible that sucrose interacts in some specific way with the phospholipid head groups. Such interaction has been proposed for trehalose (Crowe & Crowe, 1984). For this reason, we used 0.25 M pentaerythritol to conduct experiments on DOPG vesicle synthesis. The shape of this molecule makes it unlikely that it interacts uniquely with phospholipid molecules. The results of these experiments (Table V) and of experiments using trehalose indicate that the use of any nonionic membrane impermeant for adjusting the osmolarity yields vesicles that are smaller than those produced in 0.15 M KCl. Indeed, vesicles synthesized in pentaerythritol are nearly as small as those produced in water.

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Registry No. DOPA, 61617-08-1; DOPC, 4235-95-4; DOPE, 4004-05-1; DOPG, 5487-64-9; DOPS, 70614-14-1; DΦGLS, 65941-02-8; DΦPGP, 2679-48-3; DΦPG, 42274-15-7; DΦPGS, 65845-51-4; (*Z*)-$Me(CH_2)_7CH{=}CH(CH_2)_7CO_2H$, 112-80-1; (*Z*)-$Me(CH_2)_7CH{=}CH(CH_2)_8OH$, 143-28-2; $C_{12}SO_3$, 1510-16-3; KCl, 7447-40-7; KP_i, 7778-77-0; pentaerythritol, 115-77-5; sucrose, 57-50-1; trehalose, 99-20-7; 1-dodecanol, 112-53-8.

REFERENCES

Aurora, T. S., Li, W., Cummins, H. Z., & Haines, T. H. (1985) *Biochim. Biophys. Acta 820*, 250–258.

Barenholz, Y., Amselem, S., & Lichtenberg, D. (1979) *FEBS Lett. 99*, 210–214.

Batzri, S., & Korn, E. D. (1973) *Biochim. Biophys. Acta 298*, 1015–1019.

Chen, L. L., & Haines, T. H. (1976) *J. Biol. Chem. 251*, 1828–1834.

Chen, L. L., Pousada, M., & Haines, T. H. (1976) *J. Biol. Chem. 251*, 1835–1842.

Chrzeszczyk, A., Wishnia, A., & Springer, C. S., Jr. (1977) *Biochim. Biophys. Acta 470*, 161–169.

Crowe, J. H., & Crowe, L. M. (1984) *Science (Washington, D.C.) 223*, 701–703.

Deamer, D., & Bangham, A. D. (1976) *Biochim. Biophys. Acta 443*, 629–634.

Enoch, H. G., & Strittmater, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 145–149.

Gains, N., & Hauser, H. (1983) *Biochim. Biophys. Acta 731*, 31–39.

Gebicki, J. M., & Hicks, M. (1973) *Nature (London) 243*, 232–234.

Gebicki, J. M., & Hicks, M. (1976) *Chem. Phys. Lipids 16*(2), 142–160.

Goldfine, H. (1982) *Cur. Top. Membr. Transp. 17*, 1–43.

Gregoriadis, G., & Allison, A. C., Eds, (1980) *Liposome in Biological Systems*, Wiley, New York.

Gruner, S. M., Lenk, R. P., Janoff, A. S., & Ostro, M. J. (1985) *Biochemistry 24*, 2833–2842.

Haines, T. H. (1973) *Annu. Rev. Microbiol. 27*, 403–411.

Haines, T. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 160–164.

Haines, T. H., & Heller, M. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 893 (Abstr.).

Hargreaves, W. R., & Deamer, D. W. (1978) *Biochemistry 17*, 3759–3768.

Hauser, H., & Gains, N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1683–1687.

Hauser, H., Gains, N., & Muller, M. (1983) *Biochemistry 22*, 4775–4781.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55–66.

Huang, C. H. (1969) *Biochemistry 8*, 344–351.

Huang, C., & Mason, J. T. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 308–310.

Hwang, J. S., & Cummins, H. Z. (1982) *J. Phys. Chem. 77*, 616–621.

Kates, M. (1978) *Prog. Chem. Fats Other Lipids 15*, 301–342.

Li, W., Aurora, T. S., Haines, T. H., & Cummins, H. Z. (1986) *Biochemistry* (in press).

Mayer, L. D., Hope, M. J., & Cullis, P. R. (1986) *Biochim. Biophys. Acta 858*, 161–168.

Mayers, G. L., Pousada, M., & Haines, T. H. (1969) *Biochemistry 8*, 2981–2986.

Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., & Reynolds, J. A. (1981) *Biochemistry 20*, 833–840.

Papahadjopolous, D., & Miller, N. (1967) *Biochim. Biophys. Acta 135*, 624–638.

Parente, R. A., & Lentz, B. R. (1984) *Biochemistry 23*, 2353–2362.

Petri, W. A., Jr., Estep, T. N., Pal, R., Thompson, T. E., Biltonen, R. L., & Wagner, R. R. (1980) *Biochemistry 19*, 3088–3091.

Poste, G. (1983) *Biol. Cell (1981) 47*, 19–38.

Saunders, L., Perrin, J., & Gammack, D. (1962) *J. Pharm. Pharmcol. 14*, 567–572.

Zumbuehl, O., & Weder, H. G. (1981) *Biochim. Biophys. Acta 640*, 252–262.