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Characterization of Surfactant Vesicles as Membrane Mimetic Agents. 1. Interactions and Reactions of Sodium 2,2,5,5-Tetramethyl-1-pyrrolidinyloxy-3-carboxylate Spin Probe in Sonicated Dioctadecyldimethylammonium Chloride

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Abstract: Hyperfine coupling constants, differential line widths, and line heights have been determined for the sodium 2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylate spin probe, $R^-{\cdot}Na^+$, in the presence of dioctadecyldimethylammonium chloride (DODAC) vesicles by electron paramagnetic resonance (EPR) spectroscopy. Distinct line broadening and asymmetry in the high-field line indicated $R^-{\cdot}Na^+$ to be in different environments on the EPR time scale. Line-shape analysis of the EPR spectra, obtained in solutions containing different concentrations of $R^-{\cdot}Na^+$ and DODAC, led to a value of 375 M^{-1} for the binding constant between the spin probe and the DODAC vesicle, $K_{R^-{\cdot}Na^+}$. These data also provided information on the amount of water trapped within and associated at the outside of the vesicle. Sodium ascorbate was found to scavenge $R^-{\cdot}Na^+$ associated with DODAC vesicles. Rate constants for the scavenging have been determined. Analysis of the kinetics led to a value of $K_{R^-{\cdot}Na^+}$ of 328 M^{-1} and to a value of 627 M^{-1} for the association constant of sodium ascorbate with the DODAC vesicle. Subsequent to scavenging by sodium ascorbate, a residual concentration of undestroyed $R^-{\cdot}Na^+$ remained in the presence of DODAC. Apparently on the longer time scale, some spin labels enter into the interior of the vesicle where sodium ascorbate cannot reach them. The good agreement of $K_{R^-{\cdot}Na^+}$ obtained by the two different methods substantiates the proposed model for single compartment DODAC vesicles. This involves spherical bilayers with an average diameter of 300 Å and a vesicle thickness of 50 Å.

Introduction

Attention is increasingly focused on obtaining an understanding of the intricate functions of biological membranes at the chemical level. Complexities of membranes have necessarily restricted detailed studies to membrane models. Liposomes, smectic mesophases of phospholipid bilayers, have been extensively investigated as membrane mimetic agents.[2-4] Stabilities, morphologies, permeabilities, fluidities, and temperature-dependent phase behaviors have been studied as functions of lipid composition and additives. Although highly significant insights have been gained, the relative complexities and chemical instabilities warrant the search for alternative and simpler membrane mimetic agents.

Under favorable conditions, closed stable vesicles may form from relatively simple synthetic surfactants. It is important to recognize the distinction between surfactant vesicles and micelles. Surfactant vesicles, like liposomes, are thermodynamically stable. They are likely to form if the surfactant phase diagram consists of a lamellar ($L\alpha$) and water two-phase region at an appropriate temperature.[5] Conversely, micelles are dynamically formed from amphiphatic molecules at a given concentration, the critical micelle concentration.[6] The first report of synthetic vesicle formation was published by Gebicki and Hicks in 1973.[7] Stable particles, ufasomes, were reported to form upon shaking thin films of oleic and linoleic acids in aqueous buffers.[7-9] Although ufasomes resemble membranes in some respects, their formation is inhibited by electrolytes, and they are unstable outside the pH 6-8 range, do not concentrate on centrifugation, and retain substrates poorly.[8] Additionally, both oleic and linoleic acids are unsaturated and are liable, therefore, to oxidative decomposition. These properties do not render ufasomes to be viable alternatives to liposomes as membrane models.[8] Vesicle formation from fatty acids, containing a single chain of 8-18 carbon atoms, have also been reported.[9b]

Surfactants having dialkyl chains can pack analogously to phospholipids and are likely to form functional membrane-like vesicles.[10] The observed phase behavior of surface active dialkyldimethylammonium salts are in accord with this expectation.[11] Indeed, the presence of lamellar and vesicle-like structures has been demonstrated by electron microscopy in ultrasonically dispersed long-chain dialkyldimethylammonium halides[12-16] and dihexadecyl phosphate.[17] Depending upon the surfactant and the sonication time, the diameter of these vesicles ranged between 300 and 1500 Å.[13-15] In a recent communication, we have characterized preliminarily predominantly single compartment dioctadecyldimethylammonium chloride (DODAC) vesicles.[15] In contrast to ufasomes,

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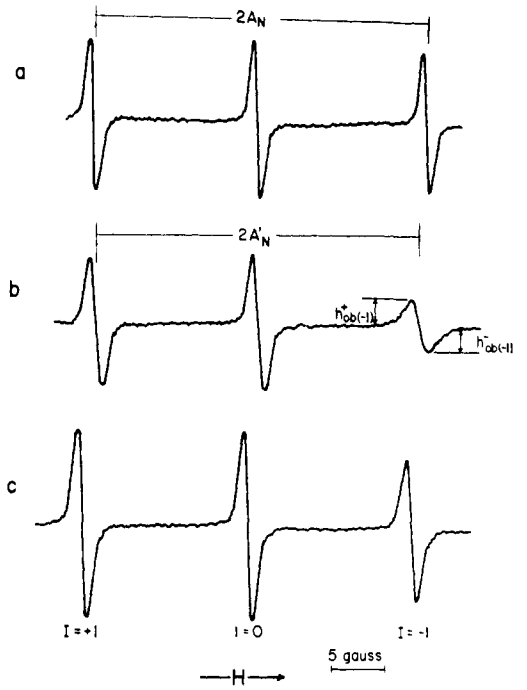

Figure 1. (a) EPR spectrum of 8.0×10^{-5} M $R^-{\cdot}Na^+$ in water. (b) EPR spectrum of 2.5×10^{-5} M $R^-{\cdot}Na^+$ in the presence of 3.37×10^{-2} M DODAC. (c) EPR spectrum of approximately 8.0×10^{-5} M $R^-{\cdot}Na^+$ in the presence of 5.0×10^{-3} M DODAC subsequent to lysing the surfactant vesicle by Triton-X.

DODAC vesicles could be prepared in the pH 1–13 range, remained stable for weeks, responded osmotically to electrolytes, and, most importantly, entrapped and retained substrates in substantial amounts.[16] The absence of unsaturated carbon atoms in DODAC obviates, of course, the possibility of oxidative degradation. These properties led us to suggest DODAC vesicles to be the simplest membrane mimetic agent utilized to date.[15]

Advantage is taken in the present work of the interaction of sodium 2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylate spin probe with DODAC. This nitroxide radical exhibits a three-line electron paramagnetic (EPR) spectrum due to the contact hyperfine interaction between the electron spin (S = 1/2) and ^{14}N nuclear spin (I = 1).[18] The observed EPR spectra in the presence of DODAC indicate the distribution of the spin probe among different environments. Substantial amounts of the radical are distributed between the bulk water and the outer charged surface of DODAC. Additionally, a small "residual" concentration of the spin probe is observed at the inner surface of DODAC vesicles. This behavior has been substantiated by determining the amounts and rates of radical scavenging by sodium ascorbate. Kinetic treatment of the data has been utilized to evaluate binding constants for the association of the spin probe and of ascorbate ion with DODAC as well as to rationalize the effects of DODAC on the rate of this reaction.

Experimental Section

Dimethyldioctadecylammonium bromide (Eastman) was recrystallized from acetone. The recrystallized sample was passed through an anion exchange column (chloride form, BioRad Lab, AG2-X, 20–50 mesh) using MeOH-$CHCl_3$ (70:30 v/v) as eluent. The solution was evaporated to dryness and was passed through once again a fresh anion exchange column. The solid, resulting from evaporation to dryness, was recrystallized three times from acetone–water (95:5 v/v). The product, dimethyldioctadecylammonium chloride (DODAC), was found to be free of amine hydrochloride by IR and NMR spectroscopy. No bromide ion was detected in DODAC by magenta test. Anal. Calcd for $C_{38}H_{80}N_1Cl_1{\cdot}H_2O$: C, 75.5; H, 13.7; N, 2.3; Cl, 5.9. Found: C, 75.69; H, 13.58; N, 2.28; Cl, 5.75 (Galbraith Laboratories, Knoxville, Tenn.). Thus DODAC preparations contained 1 mol of water crystallization which could not be removed by drying over P_2O_5 under vacuum for 3–4 days.

Sodium 2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylate, $R^-{\cdot}Na^+$, was prepared by mixing equivalent amounts of 2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid (Eastman) and sodium hydroxide in aqueous ethanol. The solvent was removed in a rotary evaporator and the crude product, $R^-{\cdot}Na^+$, was washed twice with ether. $R^-{\cdot}Na^+$ was recrystallized from ethanol–ether (30:70 v/v).

Vesicles were prepared by the sonic dispersal of the appropriate amounts of DODAC (typically 12 mg) in water (typically 2.0 mL) at 50 °C by means of the microprobe of a Braunsonic 1510 sonifier set at 70 W. Under this condition, optically clear solution appeared within 15 min subsequent to the beginning of sonication. All solutions were prepared in distilled, deionized water. Electron paramagnetic resonance spectra were taken on solutions which had been purged by purified (passing it through two bottles of alkaline pyrogallol solution) nitrogen for 15–20 min.

Absorption spectra were taken on a Cary 118C recording spectrophotometer. Electron paramagnetic spectra were obtained with a Varian E-6S spectrometer at room temperature (25 °C). In all line-width studies, the power was kept at or below 2 mW and the modulation amplitude at a level not exceeding 15% of the experimental peak-to-peak line width. The field was calibrated with Fremy's salt in saturated potassium carbonate solution where the distance between the two outermost lines is 26.182 G.[18] The hyperfine splitting constant (A_N) reported in this work is believed to have a maximum error of ±0.05 G. The line widths were carefully measured and the precision is ±0.01 G or even better in some cases. The pH of solutions was adjusted by means of a Radiometer pHM 26 instrument.

Kinetic experiments were carried out as follows. Appropriate concentrations of $R^-{\cdot}Na^+$ were added to the DODAC vesicles or were cosonicated with it. The resulting solution (0.8 mL) was degassed by purging with purified nitrogen (15–20 min) in a standard flask and stoppered with paraffin paper under positive N_2 atmosphere. Simultaneously, an appropriate concentration of aqueous sodium ascorbate (at pH ≃5.5–5.7) was degassed in another flask. Aqueous sodium ascorbate (0.2 mL) was injected into the vesicle solution and the two solutions were mixed quickly. The pH of this solution was 6.0. The first EPR monitoring of the middle-field line height was usually made 60–90 s subsequent to mixing. A positive nitrogen pressure was maintained above the solution throughout the measurements. All kinetic runs were carried out on freshly prepared DODAC vesicles. The temperature of the EPR cavity was maintained to ±0.2 °C throughout the period of a kinetic run by passing through it a steady stream of nitrogen. In all kinetic runs, the concentration of sodium ascorbate was at least 100-fold in excess of the concentration of $R^-{\cdot}Na^+$. Accordingly, good pseudo-first-order rate dependency was observed in all cases.

Results

In most of these studies, appropriate concentrations of the spin label were added to the already formed DODAC vesicles. EPR spectra of given samples were taken subsequent to a 20–30-min incubation. Alternatively, in some cases vesicles were formed by cosonicating DODAC and $R^-{\cdot}Na^+$. This latter preparation is referred to as cosonicated vesicles.

EPR spectra of $R^-{\cdot}Na^+$ in water, in the environment of intact DODAC vesicles, and that in surfactant destroyed DODAC vesicles are given in Figure 1. In water and in lysed DODAC vesicles, the spectral parameters are identical and agree well with those reported previously.[19] In the presence of DODAC vesicles, the spectral features are different, however. Distinct line broadening as well as asymmetry in the high-field line are observable. This behavior suggests that the spin label is in more than one environment and that the exchange frequency between these environments is slower than 10^6 s^{-1}. The distribution of the probe between different environments may be expressed by the symmetry parameter ($h^+_{ob(-1)}/h^-_{ob(-1)}$ in Figure 1). It is seen that in water or in surfactant lysed DODAC $h^+_{ob(-1)}/h^-_{ob(-1)}$ is equal to 1. Values for $h^+_{ob(-1)}/h^-_{ob(-1)}$, those for the differential line width (expressed as ω_{-1}

Table I. EPR Parameters of $R^-{\cdot}Na^+$ in Different Media

media	$10^5[R^-{\cdot}Na^+]$, M	symmetry parameter, $h^+_{ob(-1)}/h^-_{ob(-1)}$	$\omega_{-1} - \omega_{+1}$, G	A_N, G[a]	A_N', G[b]
water[c]				16.16	
benzene[c]				14.10	
0.10 M DAP aggregates in benzene[c]				14.25	
9.31 × 10^{-3} M sonicated DODAC in H_2O[d]	10.0	0.62			15.91
9.31 × 10^{-3} M sonicated DODAC in H_2O[d]	5.0	0.66			15.82
9.31 × 10^{-3} M sonicated DODAC in H_2O[d]	3.0	0.79	0.667		15.66
27.93 × 10^{-3} M sonicated DODAC in H_2O[d]	5.0	0.94	0.548		15.63
37.24 × 10^{-3} M sonicated DODAC in H_2O[d]	3.0	0.93	0.564		15.61
38.79 × 10^{-3} M sonicated DODAC in H_2O[d]	2.5	0.97	0.483		15.61
46.55 × 10^{-3} M sonicated DODAC in H_2O[d]	2.5	1.00	0.506		15.61
lysed DODAC		1.00			16.08
residual signal remaining subsequent to scavenging $R^-{\cdot}Na^+$ attached to DODAC by Asc^- [e]		~0.80			16.02
48.90 × 10^{-3} M DODAC cosonicated with radical	2.5	0.96	0.511		15.61
38.79 × 10^{-3} M DODAC cosonicated with cholesterol (mol/mol = 5/1) and probe added externally	2.5	0.99	0.572		15.66
27.93 × 10^{-3} M DODAC cosonicated with cholesterol (mol/mol = 5/1) and probe added externally	2.5	0.97	0.548		15.66

[a] Hyperfine coupling constant of $R^-{\cdot}Na^+$ in one environment. [b] One-half of the separation of the highest and lowest field lines of the composite spectra; $R^-{\cdot}Na^+$ being in more than one environment. [c] Taken from ref 19. [d] Radical added to the already formed DODAC vesicle. [e] Based on the average result of several samples from the kinetic experiment.

$- \omega_{+1}$, where ω_{-1} and ω_{+1} are the line widths of the −1 and +1 lines), and those for the hyperfine coupling constants A_N and A_N' (defined as one-half of the separation of +1 and −1 lines) in water, benzene, reversed micelles, DODAC vesicles, and surfactant destroyed DODAC vesicles are given in Table I.

It is constructive to consider the effects of increasing ratios of DODAC to $R^-{\cdot}Na^+$ concentrations on the EPR parameters. The trend in the symmetry parameter is informative. At the lowest surfactant to radical ratio (R = 93.1) the value for $h^+_{ob(-1)}/h^-_{ob(-1)}$ is 0.62. Increasing the amount of DODAC per radical results in increased ratios of $h^+_{ob(-1)}/h^-_{ob(-1)}$. At the highest DODAC to $R^-{\cdot}Na^+$ ratio (R = 1862), the high-field EPR line becomes symmetrical, $h^+_{ob(-1)}/h^-_{ob(-1)} \simeq 1$. There is a corresponding, although less pronounced, decrease in the A_N' value which approaches a limiting value of 15.61 G. These data are in accord with the equilibrium distribution of $R^-{\cdot}Na^+$ among different sites. As the concentration of surfactant per radical increases, more and more radicals are associated with the DODAC vesicles until essentially none remain in the bulk water phase. This interpretation is substantiated by the observed near-identical EPR parameters for $R^-{\cdot}Na^+$ in solutions prepared either by adding small amounts of the spin-label to high concentrations of the already formed DODAC vesicles ("limiting" condition) or by cosonicating $R^-{\cdot}Na^+$ with DODAC (Table I).

Distribution of the radical between different sites can be more quantitatively estimated from considering the amplitude of the resonance lines.[20] The following symbols are used to facilitate description of the method: $\omega_{b(i)}$ = line width of the i-line for radical bound to the vesicle surface, $\omega_{f(i)}$ = line width of the i-line in bulk water, $h_{b(i)}$ = line height of the i-line for radical bound to the vesicle surface, $h_{f(i)}$ = line height of the i-line for radical in bulk water, $h^-_{f(i)}$ = line height for the trough of the i-line for radical in bulk water (see Figure 2), $h^-_{ob(i)}$ = observed line height for the trough of the i-line for radical in both environments. $A_{N(f)}$ = hyperfine coupling constant of the radical in bulk water, $A_{N(b)}$ = hyperfine coupling constant of the radical bound to the vesicle, A_N' = observed hyperfine coupling for the radical being distributed in more than one environment. Values of $A_{N(f)}$ = 16.16, $A_{N(b)}$ = 15.61, $\omega_{b(0)}$ = 1.238, $\omega_{b(-1)}$ = 1.726, $\omega_{f(0)}$ = 1.12, and $\omega_{f(-1)}$ = 1.14 G are used. The following assumptions are required for treating the present data. Firstly, the spin probe is considered to reside mainly at two sites: that in bulk water and that associated (as yet in an unspecified manner) with the surfactant vesicles. Secondly, the EPR line shapes are assumed to be Lorentzian and to remain unchanged with population changes.

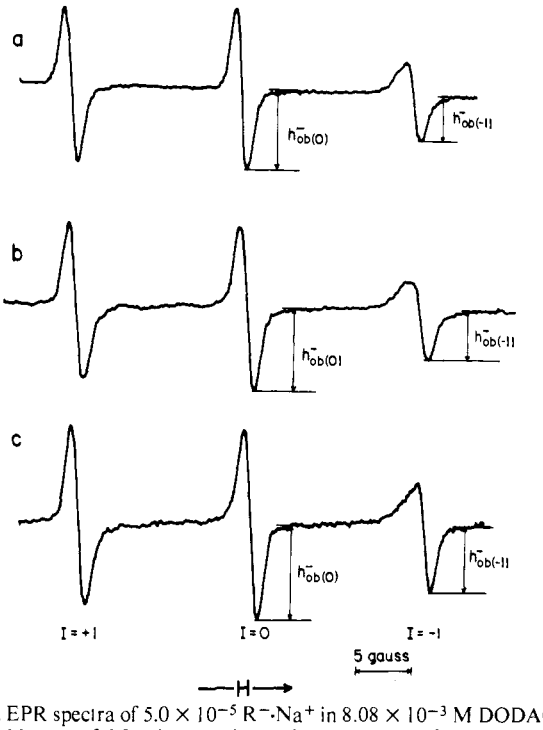

Figure 2. EPR spectra of 5.0 × 10^{-5} $R^-{\cdot}Na^+$ in 8.08 × 10^{-3} M DODAC (a), in 4.32 × 10^{-3} M DODAC (b), and in 2.13 × 10^{-3} M DODAC (c).

Thirdly, since the g value is known to change less than A_N, it is reasonable to assume that the 0-line, particularly the lower half of it, the trough, for the radical in bulk water is almost coincidental with that bound to the vesicle. Finally, remembering that $\Delta A_N \simeq 16.16 - 15.61 \simeq 0.55$ G and that the line-width difference for the -1 line is approximately 0.6 G, it is also reasonable to assume that the positions of the trough of the two (-1) lines are nearly coincident. This assumption is justifiable only when $\omega_{b(-1)}$ is much larger than $\omega_{f(-1)}$ so that fortuitously the positions of the minima of the two troughs are nearly identical. Under identical spectrometer settings, equal concentration of $R^-\cdot Na^+$ in two different environments would have the same intensity but the height of a given line and the square of its width could differ if the line widths are different. Since the intensity of a first derivative EPR line is proportional to the height of a given line and the square of its width, the ratio of the 0-th free-to-bound line heights can be calculated from the data by the equations

$$h_{f(0)}\omega_{f(0)}^2 = h_{b(0)}\omega_{b(0)}^2 \quad (1)$$

$$1.12^2 h_{f(0)} = 1.238^2 h_{b(0)} \quad (2)$$

$$h_{f(0)}/h_{b(0)} = 1.22\ (1) \quad (3)$$

The ratio of the -1 free-to-bound line heights can be similarly obtained by the equations

$$h_{f(-1)}\omega_{f(-1)}^2 = h_{b(-1)}\omega_{b(-1)}^2 \quad (4)$$

$$1.14^2 h_{f(-1)} = 1.726^2 h_{b(-1)} \quad (5)$$

$$h_{f(-1)}/h_{b(-1)} = 2.29\ (2) \quad (6)$$

Therefore, the relationship between free and bound line heights for the trough of the 0 and -1 lines is given by the equations

$$h^-_{f(0)} = 1.221 h^-_{b(0)} \quad (7)$$

$$h^-_{f(-1)} = 2.292 h^-_{b(-1)} \quad (8)$$

Letting x be the fraction of $R^-\cdot Na^+$ that is in the bulk water ($[R^-\cdot Na^+]_f = xC$, where $C = 5.0 \times 10^{-5}$ M), then $1 - x$ is the fraction of $R^-\cdot Na^+$ that is vesicle bound. Assuming that the line shape does not change with x, the observed line heights of the trough can be described by the equations

$$\begin{aligned} h^-_{ob(0)} &= xh^-_{f(0)} + (1-x)h^-_{b(0)} \\ &= 1.221xh^-_{b(0)} + (1-x)h^-_{b(0)} \\ &= (0.221x + 1)h^-_{b(0)} \end{aligned} \quad (9)$$

$$\begin{aligned} h^-_{ob(-1)} &= xh^-_{f(-1)} + (1-x)h^-_{b(-1)} \\ &= 2.292xh^-_{b(-1)} + (1-x)h^-_{b(-1)} \\ &= (1.292x + 1)h^-_{b(-1)} \end{aligned} \quad (10)$$

The ratio of eq 9 and 10 gives the equation

$$\frac{h^-_{ob(0)}}{h^-_{ob(-1)}} = \frac{(0.221x+1)h^-_{b(0)}}{(1.292x+1)h^-_{b(-1)}} = \frac{2.39(0.221x+1)}{1.292x+1} \quad (11)$$

which upon rearranging yields

$$x = \frac{2.39 - R}{1.292R - 0.53} \quad (12)$$

where

$$R = \frac{h^-_{ob(0)}}{h^-_{ob(-1)}}$$

The experimental data allowed the calculation of the right-hand side of eq 12 for each x value, which, in turn, was related to the number of radicals bound to DODAC, $n_b^{R^-\cdot Na^+}$, and those which are free in water, $n_f^{R^-\cdot Na^+}$, by the equation

$$\frac{x}{1-x} = \frac{n_f^{R^-\cdot Na^+}}{n_b^{R^-\cdot Na^+}} \quad (13)$$

An important consequence of localizing all the spin probes in the environment of surfactant vesicles is that their rotational correlation times, τ_r values, can be calculated. Equation 14[21,22] and the splitting tensors available for 2-doxylpropane[23] were used to calculate the rotational correlation times.

$$\tau_r = \frac{\sqrt{3}\pi(\Delta\nu_{+1} - \Delta\nu_{-1})}{b\Delta\gamma B_0\,(0.5333 + 0.4u)} \quad (14)$$

where

$$b = \frac{2}{3}\left[A_{zz} - \tfrac{1}{2}(A_{xx} + A_{yy})\right]\frac{g\beta}{\hbar}$$

$$\Delta\gamma = \frac{|\beta|}{\hbar}\left[g_{zz} - \tfrac{1}{2}(g_{xx} + g_{yy})\right]$$

$$u = 1/(1 + f_\omega^2\tau_r^2)$$

f_ω = resonance frequency

$\Delta\nu_{+1}$, $\Delta\nu_0$, and $\Delta\nu_{-1}$ are the peak-to-peak line widths (MHz) of the low-, middle-, and high-field lines, respectively, and B_0 is the laboratory magnetic field (3400 G). A_{xx}, A_{yy}, and A_{zz} are the respective principal hyperfine coupling constants; g_{xx}, g_{yy}, and g_{zz} are the respective g-tensor elements, β is the Bohr magneton, and $\hbar$ is Planck's constant. τ_r values of $R^-\cdot Na^+$ have been calculated to be 2.70×10^{-10} and 2.78×10^{-10} s for solutions prepared by adding $R^-\cdot Na^+$ to the already formed DODAC vesicles and for those containing the cosonicated constituents, respectively. These two correlation times are considered to be identical within the experimental error. A τ_r value of 3.08×10^{-10} s was obtained from the "limiting" spectra in cholesterol containing DODAC vesicles (DODAC:cholesterol = 5:1 (mol/mol)).

Sodium ascorbate quenches $R^-\cdot Na^+$.[19,24-27] Second-order rate constants for this reaction increase sigmoidally with increasing concentrations of DODAC (Table II). At pH $\geq$6, the rate of reaction is independent of the hydrogen ion concentration. At lower pH values, both $R^-\cdot Na^+$ and Asc^- protonate. Neither protonation equilibrium is assumed to be drastically altered at the surface of the DODAC vesicle. Significantly, in the presence of surfactant vesicles, a fraction of $R^-\cdot Na^+$ remains undestroyed for at least 30 half-lives subsequent to the completion of the kinetic run. Amounts of this remaining radical concentration, expressed as percent of residual $R^-\cdot Na^+$ (% of residual signal = 100% (residual signal height/(signal height at time zero)), are also given in Table II. Signal heights at time zero (time of mixing the constituents) were obtained by extrapolating the straight lines in the log (signal height) vs. time plots to time zero. On a somewhat longer time scale (>30 min) a small (10-30%) increase in the residual signal was observed following each kinetic run. After standing overnight, there was a considerable decrease in the signal height. The hyperfine coupling constant due to the residual $R^-\cdot Na^+$, 16.02 G, is indicative of a considerably polar environment. The observed asymmetry of the residual signal, $h^+_{ob(-1)}/h^-_{ob(-1)} \simeq 0.8$, is due to two kinds of overlapping spectra.

Discussion

Surfactant vesicles, formed upon sonic dispersal of DODAC, have been found to be stable for weeks. Addition of anionic spin probes to the already formed DODAC vesicles results in their distribution between the positively charged vesicle and bulk water. In the extreme, there are four different sites the $R^-\cdot Na^+$ radicals can occupy. They can freely move about in bulk water, they may be attached to the outer or inner charged surface of DODAC vesicles, and finally they may be entrapped in the interior of the vesicles. Figure 3 is a schematic representation

Table II. Rate Constants for the Destruction of $R^{-}\cdot Na^{+}$ at 25.0 °C[a]

media	k_2, M^{-1} s^{-1} [b]	% residual $R^{-}\cdot Na^{+}$
H_2O[c]	0.14	0
7.05×10^{-4} M sonicated DODAC in H_2O	1.94	6.0 (±0.4)
1.18×10^{-3} M sonicated DODAC in H_2O	2.35	4.0 (±0.4)
1.76×10^{-3} M sonicated DODAC in H_2O	2.50	2.5 (±0.7)
3.53×10^{-3} M sonicated DODAC in H_2O	2.96	1.2 (±0.7)
7.42×10^{-3} M sonicated DODAC in H_2O	3.30	2.5 (±0.7)
12 mg of DODAC consonicated with 4.8×10^{-5} M $R^{-}\cdot Na^{+}$ in 2 mL of H_2O	2.21	3.2 (±0.8)
12 mg of DODAC and 2.4 mg of cholesterol cosonicated with 4.8×10^{-5} M $R^{-}\cdot Na^{+}$ in 2 mL of H_2O	2.86	7.5 (±1.0)

[a] $[R^{-}\cdot Na^{+}] = 4.8 \times 10^{-5}$ M. $R^{-}\cdot Na^{+}$ added to the already formed DODAC vesicles, unless stated otherwise; $[Asc^{-}] = 5.0 \times 10^{-3}$ M. [b] ±5% error. [c] Determined in ref 19.

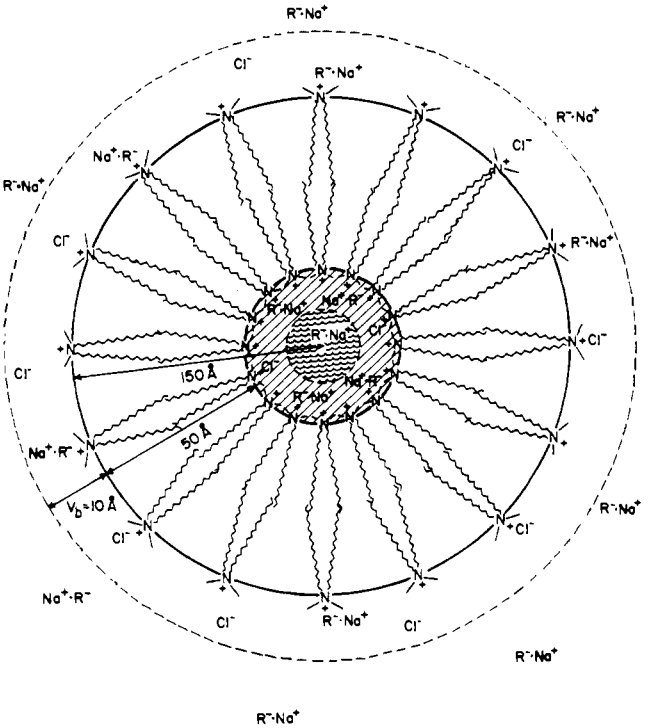

Figure 3. Schematic drawing of a DODAC vesicle indicating the likely sites of $R^{-}\cdot Na^{+}$. The radius of the vesicle is 150 Å and the thickness of the bilayer is 50 Å. Note that the radius of the vesicle interior is 100 Å and is not drawn to scale. Similarly, the region of the outer surface of the vesicle where immobilization of $R^{-}\cdot Na^{+}$ occurs, V_b, is not drawn to scale.

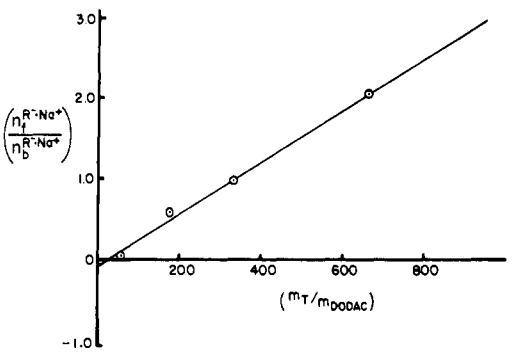

Figure 4. Ratio of free to vesicle bound $R^{-}\cdot Na^{+}$ as a function of the mass ratio of m_T/m_{DODAC}, according to eq 16.

of a single compartment bilayer DODAC vesicle and the different environments the spin probe may experience. If $R^{-}\cdot Na^{+}$ is added to the already formed cationic DODAC vesicles, electrostatic interactions are likely to hinder their penetration across the bilayer. Consequently, on a short time scale (~15–20 min) the radical is distributed predominantly between the bulk water and the outer surface of the surfactant vesicle. Indeed this was the a priori assumption in treating the experimentally determined EPR line heights at different DODAC and $R^{-}\cdot Na^{+}$ concentrations (see Results). Neglecting activity coefficients,[20] the equilibrium constant for the binding of $R^{-}\cdot Na^{+}$ to the outer vesicle surface, $K_{R^{-}\cdot Na^{+}}$, is given by

$$K_{R^{-}\cdot Na^{+}} \simeq \frac{n_b^{R^{-}\cdot Na^{+}} V_f}{n_f^{R^{-}\cdot Na^{+}} V_b} \qquad (15)$$

where $n_b^{R^{-}\cdot Na^{+}}$ and $n_f^{R^{-}\cdot Na^{+}}$ are the number of radicals bound to the outer phase of DODAC vesicles of volume V_b and those which are free in the bulk water of volume V_f. Since $n_b^{R^{-}\cdot Na^{+}}/n_f^{R^{-}\cdot Na^{+}}$ are related to the amplitude of the resonance lines (eq 13) and since $V_b = \bar{V}_b m_{DODAC}$ and $V_f = \bar{V}_f m_f$ (where $\bar{V}_b$ is the specific volume of the outer surface of DODAC vesicle per g of DODAC, m_{DODAC} is the mass of DODAC, $\bar{V}_f$ is the partial specific volume of water and is equal to 1, and m_f is the mass of the bulk solvent), eq 15 can be rearranged to

$$\frac{n_f^{R^{-}\cdot Na^{+}}}{n_b^{R^{-}\cdot Na^{+}}} = \left[\frac{1}{K_{R^{-}\cdot Na^{+}}} \frac{\bar{V}_f}{\bar{V}_b}\right]\left[\frac{m_T}{m_{DODAC}} - Q\right] \qquad (16)$$

where m_T represents the total mass of the solvent and Q is the sum of the solvents within the vesicle and those associated with it on the outside (expressed as mass of solvent per g of DODAC).[28] A plot of the left-hand side of eq 16 against m_T/m_{DODAC} gives a straight line (see Figure 4) from the slope and intercept of which $K_{R^{-}\cdot Na^{+}}\bar{V}_b = 311.5$ and $Q = 24.6$ are obtained. The value for $K_{R^{-}\cdot Na^{+}}$ can be evaluated if $\bar{V}_b$ is known. $\bar{V}_b$ is operationally defined as the volume, per g of lipid, occupied by the spin label, on the outer surface of a vesicle, giving rise to a bound EPR signal.[28] Taking 300 and 200 Å to be the diameters of a single compartment bilayer DODAC vesicle and that of its inner aqueous compartment, and further assuming 50 and 10 Å to be the thickness of the bilayer and that of the outer surface occupied by radicals (see Figure 3),[29] the internal (V_i) and vesicle (V_b) volumes are calculated to be 4.2×10^{-18} and 2.98×10^{-18} cm^3, respectively. Further, since each vesicle contains 6800 molecules of surfactant (of which there are 4000 at the outer surface), m_{DODAC} is 6.4×10^{-18} g. Using this value $\bar{V}_b = 0.83$ cm^3/g is obtained, which in turn yields $K_{R^{-}\cdot Na^{+}} = 375$ M^{-1}. Making the assumption that each DODAC molecule is hydrated by four molecules of water, Q is calculated to be 3.7. Considering the assumptions made and the uncertainty of the intercept in Figure 4, the agreement obtained between the experimentally obtained and calculated Q values is quite satisfactory.

Some useful information can also be derived from the scavenging experiments. The rate of $R^{-}\cdot Na^{+}$ scavenging by sodium ascorbate, Asc^{-}, R_t, is given by

$$R_t = k_2[R^{-}\cdot Na^{+}]_t[Asc^{-}]_t \qquad (17)$$

where k_2 is the observed second-order rate constant (values given in Table II) and $[R^{-}\cdot Na^{+}]_t$ and $[Asc^{-}]_t$ are the total concentrations of the reagents, related to the true concentrations at the surface of the DODAC vesicle, $[R^{-}\cdot Na^{+}]_b$ and

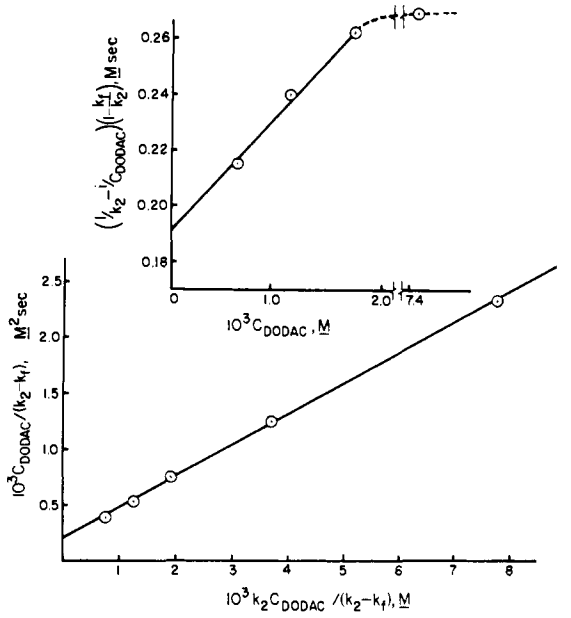

Figure 5. Kinetic treatment of $R^-\cdot Na^-$ scavenging by sodium ascorbate on the surface of DODAC vesicles, according to eq 25 and 29 (insert).

$[Asc^-]_b$, and in the bulk water, $[R^-\cdot Na^+]_f$ and $[Asc^-]_f$, by the equations

$$[R^-\cdot Na^+]_t = [R^-\cdot Na^+]_b C_{DODAC} V_b + [R^-\cdot Na^+]_f(1 - C_{DODAC}\bar{V}_b) \quad (18)$$

$$[Asc^-]_t = [Asc^-]_b C_{DODAC} V_b + [Asc^-]_f(1 - C_{DODAC}\bar{V}_b) \quad (19)$$

where C_{DODAC} is the stoichiometric DODAC concentration and $\bar{V}_b$ is the volume of the vesicle.[31] The total rate of scavenging, R_t, is the composite of reactions occurring at the outer surface of DODAC vesicles, whose rate, R_b, is governed by k_b:

$$[R^-\cdot Na^+]_b + [Asc^-]_b \xrightarrow{k_b} P \quad (20)$$

and that proceeding in bulk water, whose rate, R_f, is governed by k_f:

$$[R^-\cdot Na^+]_f + [Asc^-]_f \xrightarrow{k_f} P \quad (21)$$

and is described by the equation

$$R_t = R_b C_{DODAC}\bar{V}_b + R_f(1 - C_{DODAC}\bar{V}_b) \quad (22)$$

and hence by the equation

$$R_t = k_b[R^-\cdot Na^+]_b[Asc^-]_b C_{DODAC}\bar{V}_b + k_f[R^-\cdot Na^+]_f[Asc^-]_f(1 - C_{DODAC}\bar{V}_b) \quad (23)$$

Making the assumptions that partitioning of the reactants between the vesicle and bulk water is not altered by the consumption of the radical (i.e., the partitioning is rapid), that the volume fraction of the DODAC vesicles is small (i.e., $C_{DODAC}\bar{V}_b < 1$) and that both reagents appreciably bind to the vesicle k_2 can be expressed by the equation

$$k_2 = \frac{(k_b/\bar{V}_b)K_{R^-\cdot Na^+}K_{Asc^-}C_{DODAC} + k_f}{(1 + K_{R^-\cdot Na^+}C_{DODAC})(1 + K_{Asc^-}C_{DODAC})} \quad (24)$$

where $K_{R^-\cdot Na^+}$ and K_{Asc^-} are the binding constants for the association of the radical and sodium ascorbate to DODAC. Following the kinetic treatment derived for second-order reactions occurring in the presence of aqueous micelles[32] eq 24 can be transformed to the equation

$$\frac{C_{DODAC}}{k_2 - k_f} = (i) + (ii)C_{DODAC}\frac{k_2}{k_2 - k_f} + (iii)C_{DODAC}^2\frac{k_2}{k_2 - k_f} \quad (25)$$

where

$$(i) = \bar{V}_b(k_b K_{R^-\cdot Na^+} K_{Asc^-}) \quad (26)$$

$$(ii) = i(K_{R^-\cdot Na^+} + K_{Asc^-}) \quad (27)$$

$$(iii) = iK_{R^-\cdot Na^+}K_{Asc^-} \quad (28)$$

Figure 5 illustrates the plot of data according to eq 25. The intercept of this plot yields 2.00×10^{-4} M^2 s for (i). This value allows a further analysis of the data if eq 25 is rearranged to

$$\left(\frac{1}{k_2} - \frac{(i)}{C_{DODAC}}\right)\left(1 - \frac{k_f}{k_2}\right) = (ii) + (iii)C_{DODAC} \quad (29)$$

The insert of Figure 5 illustrates a plot of the left-hand side of eq 29 against DODAC concentration. From the intercept and slope of this plot, values of 0.191 M s and 41.14 s were obtained for (ii) and (iii), respectively. Substituting these values into eq 26-28 led to the estimation of $K_{R^-\cdot Na^+}$ and K_{Asc^-} to be 328 and 627 M^{-1}, respectively. In view of the approximations involved, these binding constants can only be considered to represent orders of magnitudes. Nevertheless, the agreement of $K_{R^-\cdot Na^+}$ values obtained by analyzing the EPR spectra of the spin probe and by the kinetic treatment of scavenging (i.e., by two independent methods) lends credence to the assumptions involved and indicate substantial binding of both $R^-\cdot Na^+$ and Asc^- to the DODAC vesicles. In addition to estimating binding constants, the rate constant for radical scavenging at the surface of DODAC vesicles, k_b, can be evaluated by combining eq 26 and 28 to

$$(iii) = \bar{V}_b/k_b \quad (30)$$

Substituting $\bar{V}_b = 0.83$ into eq 30 gives $k_b = 2.0 \times 10^{-2}$ M^{-1} s^{-1}.[32] This value indicates the radical scavenging at the surface of the surfactant vesicle to be some sevenfold slower than that in bulk water ($k_f = 1.4 \times 10^{-1}$ M^{-1} s^{-1}) when true concentrations of the reactants are used. Rate retardation, expressed as k_b, is not unexpected since two negatively charged reactants are less likely to encounter if bound to a surface at close proximity than when they are free to move about. An entirely similar situation has been observed in the scavenging of $R^-\cdot Na^+$ in reversed micelles.[19] The observed apparent rate enhancement, expressed in k_2 values, is the consequence of the effective binding of the reagents to the surface of the DODAC vesicles which, in turn, gives high local concentrations. Rate enhancements of second-order reactions in the presence of aqueous micelles have been rationalized analogously.[33-35]

The satisfactory kinetic treatment of the data for radical scavenging implies the predominant distribution of $R^-\cdot Na^+$ at sites which are accessible to Asc^-. These sites were shown to be the aqueous bulk phase and the outer surface of the DODAC vesicle. Under the experimental conditions given in Table II, the number of $R^-\cdot Na^+$ per vesicle ranged from 44 to 460. There remained, however, a residual concentration of $R^-\cdot Na^+$ which apparently is inaccessible to the attacking ascorbate ions. The site of this residual $R^-\cdot Na^+$ is proposed to be the inner surface of the DODAC vesicles. The dependence of the amounts of residual radicals on DODAC concentration is in accord with this postulate. At relatively low DODAC concentration, there are a greater number of $R^-\cdot Na^+$ per vesicle and therefore the probability of some of the spin probes "flipping inside" is greater. Accordingly, at 7.05×10^{-4} M

DODAC (each vesicle is associated with 460 $R^-{\cdot}Na^+$), 6% of the radicals are in a position inaccessible to Asc^-. As the stoichiometric DODAC concentration increases, the number of $R^-{\cdot}Na^+$ per vesicle decreases with the resultant increase of distribution of $R^-{\cdot}Na^+$ from the inside to the outside of the vesicle. Interestingly, the amount of residual radicals is not appreciably greater if $R^-{\cdot}Na^+$ is cosonicated with DODAC. This fact is indicative of the high concentration of bound and free chloride ions at the interior of the surfactant vesicles which cannot be as readily replaced as the outer counterions. Addition of cholesterol increases, however, the extent of residual $R^-{\cdot}Na^+$.

Conclusion

The binding constant for the association of $R^-{\cdot}Na^+$ has been assessed by two independent methods: analysis of EPR spectra and kinetic treatment of scavenging rates. Both methods relied on assumed geometries of the spherical bilayer DODAC vesicle. Agreement between the association constants obtained by the two methods may be taken as a substantiation of the proposed dimensions of the DODAC vesicle, schematically illustrated in Figure 3. It is significant that analogous EPR line analysis has been employed for association of spin probes with phospholipid vesicles and the kinetic treatment of radical scavenging followed that derived for reactions in the presence of aqueous micelles. Synthetic surfactant vesicles possess the relative chemical simplicity, found in micelles, and functionality observed in more complex lipid vesicles and membranes. These advantages merit extensive further studies.[36]

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(29) These diameters were derived from electron micrographs of DODAC vesicles.[15,30]
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(31) V_b used in the kinetic equations is, strictly speaking, not equal to that used in the EPR line analysis since the volumes occupied by the probe on the outer surface of DODAC vesicles may be different in the presence of sodium ascorbate. We shall, however, use a value of 0.83 cm^3/g for V_b for both calculations.
(32) Although we have no exact knowledge of the error in taking $\bar{V}_b$ to be 0.83, the close agreement between $K_{R^-{\cdot}Na^+}$ values (375 and 328) obtained by independent approaches suggests an experimental error of less than ±20%. The error introduced in V_b as a result of sodium ascorbate may not be serious enough to change it by an order of magnitude. The uncertainty of (iii) as can be seen from Figure 5 is very small (<1%). Thus, the comparison of k_b with k_l is meaningful.
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(36) NOTE ADDED IN PROOF. Using low angle laser light scattering and photon correlation spectroscopy, we have recently determined the molecular weight and hydrodynamic radius of 12 min sonicated DODAC (setting 3 on a Branson B-12 sonifier) vesicles to be 30 million and 309 Å. These values were best accommodated in terms of the formation of single compartment prolate DODAC vesicles (U. Herrmann and J. H. Fendler, to be published in *Chem. Phys. Lett.*, 1979).