On the Cleavage of Ellman's Reagent [5,5'-dithiobis(2-nitrobenzoic acid)] by Dithonite in the Presence of Dioctadecyldimethylammonium Chloride [Dodac] Surfactant Vesicles Laced with Cholesterol

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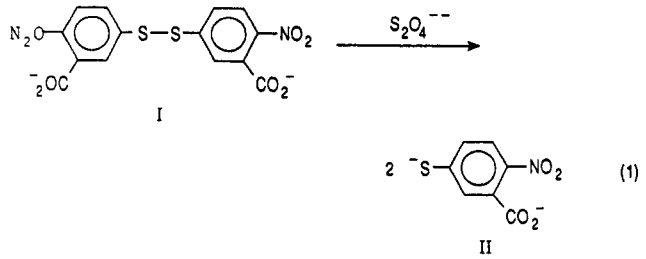
Received October 5, 1992®

Ellman's reagent (**I**) is distributed between the outer aqueous interface and the interior bilayer of DODAC vesicles upon contact when added to presonicated aqueous DODAC suspensions. This distribution also extends to the inner aqueous interface in about 10 min. This suspension reacts with aqueous alkaline sodium dithionite in two kinetic processes. The first process involves the bimolecular cleavage of **I** at the outer aqueous interface instantaneously. This is followed by a slow reaction of the same due to leakage of the bilayer-embedded substrate to the outer interface. This condition changes, however, when the DODAC vesicles are laced with cholesterol by cosonication of the aqueous solutions. Both Ellman's reagent and the reduced anion **II** product of cleavage by dithionite now are impermeable to this membrane but the protonated **III** does penetrate the bilayer. This result is indicated by loss of absorbance at 440 nm concurrent with gain in absorbance at 320 nm upon contact between **III** and cholesterol-laced vesicles. This result is interpreted in terms of the known permeability changes accompanying the incorporation of cholesterol into the vesicles. Thus Ellman's reagent (**I**) can be encapsulated by DODAC vesicles if they contain cholesterol in such a way that they are unreachable by dithionite and lyate species.

Stable multimolecular organized assemblies of dioctadecyldimethylammonium chloride (DODAC) molecules can be generated in aqueous solutions by sonication.[3] These so-called "DODAC vesicles" are globular in shape and range about 40 to 60 nm in diameter.[4] They are perhaps the simplest, most easily produced functional bilayer membrane models.

Fendler reported that the base-catalyzed hydrolysis of dianionic Ellman's reagent (**I**) was accelerated some 500-fold when **I** was first cosonicated with DODAC before placing in alkaline buffer.[4] This was interpreted to mean that the substrate **I** was concentrated at the inner and outer aqueous vesicular bilayer interfaces upon sonication due to the electrostatic effects of opposite surface charge. When these vesicles were combined with buffer, the hydroxide was also subject to these attractive forces and since the vesicular bilayer of DODAC is lyate-permeable, all substrate is destroyed in this reaction at an accelerated rate.[3]

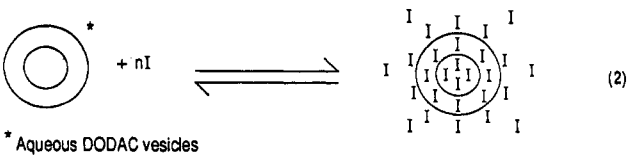
Moss and co-workers showed that the cleavage of **I** by dithionite, a bimolecular process as shown in eq 1, occurred



as two separate kinetic processes when Ellman's reagent was combined with dithionite after first sonicating with DODAC in pH 8 Tris buffer.[5] This was interpreted to mean that, as earlier reported by Fendler, some of the substrate **I** was encapsulated by the bilayer in its aqueous interior upon sonication. When it was combined with dithionite, substrate bound to the exterior vesicular surface reacted immediately, but the excess dithionite was unable to penetrate the bilayer surface due to its small size and large negative charge. The second slower kinetic process observed was due to the slow hydrolysis of encapsulated Ellman's reagent (**I**) as previously measured by Fendler.[4]

We were able to show that if this experiment were repeated, with the difference that the substrate is added after sonication of the DODAC and prior to combination with dithionite, two distinct kinetic processes, essentially identical to the first experiment reported by Moss, were again obtained. However, in our hands the rate of the slow step was about 20 times faster than that earlier reported regardless of the experiment.[6] Thus the interpretation of this data originally supplied by Moss cannot be completely correct.

Both experiments (see Scheme 1) are explained by the establishment of a favorable equilibrium to insert a portion of the substrate **I** into the vesicular bilayer with the majority bound at inner and outer aqueous interfaces as shown in eq 2. The slow kinetic process is due to an



unfavorable leakage of inserted substrate back into the

® Abstract published in *Advance ACS Abstracts*, March 1, 1994.

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(3) Fendler, J. H. *Acc. Chem. Res.* **1980**, *13*, 7-13.
(4) Fendler, J. H.; Hinze, W. L. *J. Am. Chem. Soc.* **1981**, *103*, 5439-47.
(5) Moss, R. A.; Swarup, S.; Zhang, H. *J. Am. Chem. Soc.* **1988**, *110*, 2914-19.
(6) Huffman, R. W.; Brown, D. M. *J. Org. Chem.* **1991**, *56*, 6477-79.

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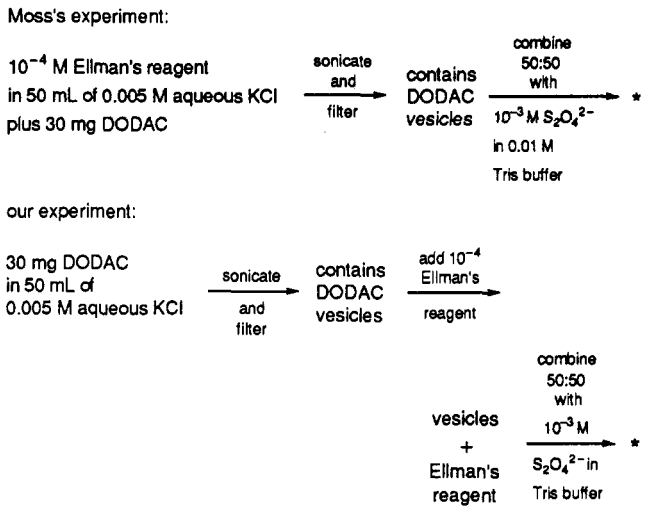
Scheme 1[a]



[a] (*) Follow rate of appearance (λ_{max} = 440 nm) in a spectrophotometer.

outer aqueous phase once the faster depletion of the outer surface bound substrate is complete. This explanation was offered by Moss and Schreck to explain similar behavior of Ellman's reagent in vesicles of the hexadecyldimethylammonium system.[7] Thus, as shown, Ellman's reagent is distributed between the two distinct phases upon mixing with the aqueous surfactant regardless of whether it is sonicated with surfactant or it is added after sonication. All cleavage of **I** occurs at the outer aqueous interface.

Fendler and co-workers studied the effects of added cholesterol prior to sonication of DODAC solutions.[8] Vesicles laced with cholesterol were found to be smaller, less viscous, and more rigid. Phase transitions, observed initially in pure DODAC vesicles, are maintained but occur at lower temperatures. These hybrid vesicles appear to be less permeable to small lyate ions but may be more permeable to larger ions especially above phase-transition temperatures.[3] It was of interest to investigate what effects cholesterol-laced DODAC bilayers would have on the cleavage of Ellman's reagent.

Results

The cleavage of Ellman's reagent (**I**) to produce Ellman's anion (**II**) by dithionite (eq 1) was studied at 25 °C in a thermostated Hewlett-Packard Model 8452A diode array UV-vis spectrophotometer. Kinetic measurements were made under pseudo-first-order conditions using the gain in absorbance of the 440-nm band due to the anion (**II**) (ϵ = 13 600).[5] Reactions were initiated in a quartz cuvette by the 50:50 combination of 0.005 M aqueous KCl containing 5×10^{-4} M DODAC and 5×10^{-5} M **I** with 7×10^{-4} M sodium dithionite in 0.01 M aqueous pH 8 Tris buffer (μ = 0.005). The KCL solution containing the DODAC and varying amounts of cholesterol was sonicated 15 min at 70 W at 60 °C prior to the addition of **I**. Sonication produced vesicles from the totally insoluble DODAC and cholesterol with mean particle diameters of 90–95 nm as measured by laser light scattering.[6] Solutions were prepared just prior to experimentation and sparged 15 min with nitrogen prior to mixing inside the spectrophotometer.

Figure 1 shows spectral data collected on the cleavage of **I** in the presence of DODAC vesicles containing no cholesterol. It clearly indicates two distinct kinetic steps involving a rapid initial burst of absorbance due to the anion **II** followed by a slower first-order absorbance increase (k = 0.648 ± 0.024 mins^{-1}).[6]

Figure 2 shows a series of curves of absorbance versus time at 440 nm for the reaction with dithionite involving experiments containing increasing amounts of cholesterol (see description of Figure 2 in title). Inspection of the curves indicates experiments containg little or no cholesterol give standard increasing absorbance across the entire time scale. Those with increasing amounts of added cholesterol involve a decreasing tendency to increase absorbance initially followed by greater absorbance decreases. Thus the anion **II** initially generated in all of these experiments is being converted more completely to something else as the amount of cholesterol in the vesicles increases.

Experiments involving added cholesterol are accompanied by an absorbance increase at λ_{max} = 320 nm (also first order). This absorbance is due to 5-mercapto-2-nitrobenzoic acid (**III**) (λ_{max} = 317 nm, ϵ = 15 292 in ethanol). A pK_a' value for the thiol group of **III** was determined to be 4.57 (λ_{max} = 328 nm, ϵ = 8840: λ_{max} = 412 nm, ϵ = 130) in aqueous solution.[9] The presence of cationic vesicles in these solutions would be expected to increase stability of the anionic conjugate base thus lowering this pK_a value. No obvious trends were apparent in values determined for the first-order rate constants in these experiments. These constants have multiple processes contained within them and thus are not reported.

Kinetic measurements were also conducted on the disappearance of the anion **II** in pH 8 aqueous Tris buffer containing DODAC vesicles. The difference in these experiments is that no dithionite was added. Experiments like those described above were initiated by combining 50:50 solutions containing 5×10^{-4} M presonicated DODAC and added cholesterol in 0.005 M KCl with 5×10^{-5} M **II** in 0.01 M pH 8 Tris buffer (μ = 0.005) in a cuvette inside the same spectrophotometer.

Figure 3 shows a series of plots of absorbance versus time at 440 nm for these latter experiments containing different concentrations of added cholesterol. Curve A shows the results of an experiment containing no added cholesterol indicating no absorbance change with time. Thus the concentration of the anion **II** remains constant over the duration of the experiment. However as the DODAC vesicles laced with increasing amounts of cholesterol are combined with **II** in curves B–D, increasing amounts of the anion are lost with time. The pseudo-first-order constant measured for this rate in curves B–D was k = 0.448 ± 0.011 min^{-1}.

These latter experiments are also accompanied by increasing absorbance at 320 nm. This also is due to the production of 5-mercapto-2-nitrobenzoic acid (**III**). Pseudo-first-order rate constants calculated for this rate increase are k = 0.480 ± 0.016 min^{-1}.

Experimental Section

Materials. Dioctadecyldimethylammonium chloride (Tokyo Kasei) [5,5'-dithiobis(2-nitrobenzoic acid)] (Aldrich), sodium

(7) Moss, R. A.; Schreck, R. P. *J. Am. Chem. Soc.* **1985**, *107*, 6634–39.
(8) Kano, K.; Romero, A.; Djermeuni, B.; Ache, H. J.; Fendler, J. H. *J. Am. Chem. Soc.* **1979**, *101*, 4030–36.
(9) Riddles, P. W.; Blakely, R. L.; Zerner, B. *Anal. Biochem.* **1979**, *94*, 75–81.

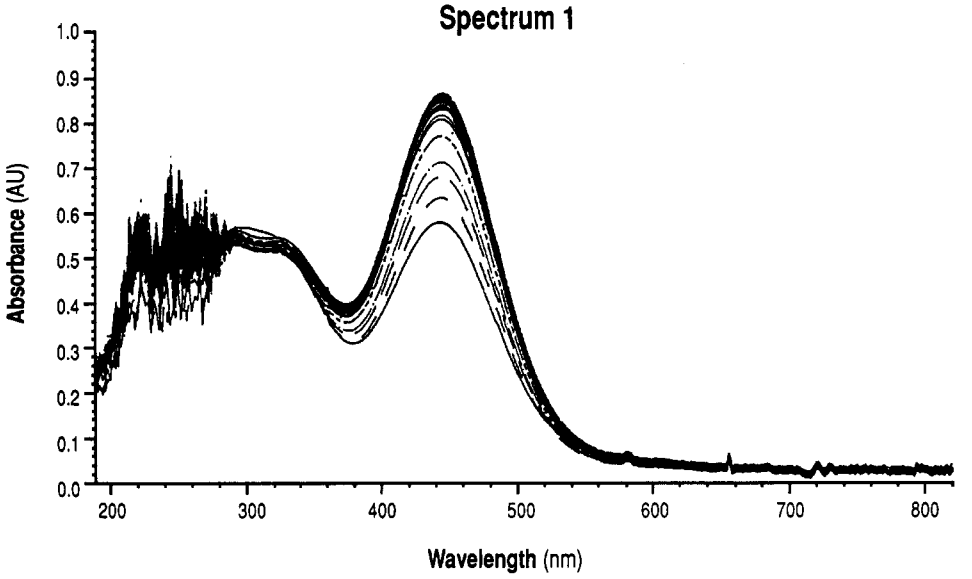


Figure 1. A series of spectral recordings at 0.5-min intervals for the cleavage of **I** by dithionite in the presence of DODAC vesicles in pH 8 aqueous Tris buffer, 25 °C. The lowest recording at 440 nm is the initial one and was taken about 5 s after mixing of the reagents. It represents the instantaneous liberation of **II**. Successive recordings climb in absorbance at regular intervals but at a much slower rate.

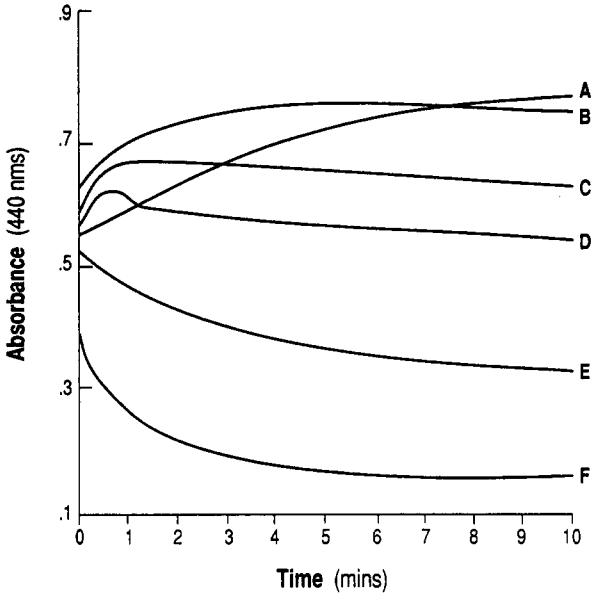


Figure 2. A series of curves showing changes in absorbance at 440 nm with time for solutions containing **I** and sonicated DODAC with cholesterol in aqueous KCl combined with dithionite in aqueous Tris buffer at pH 8. The cholesterol content varied as follows: curve A, 0 M; B, 1.3×10^{-4} M; C, 1.5×10^{-4} M; D, 2.6×10^{-4} M; E, 5.1×10^{-4} M; F, 7.7×10^{-4} M. Concentrations are as described in Results.

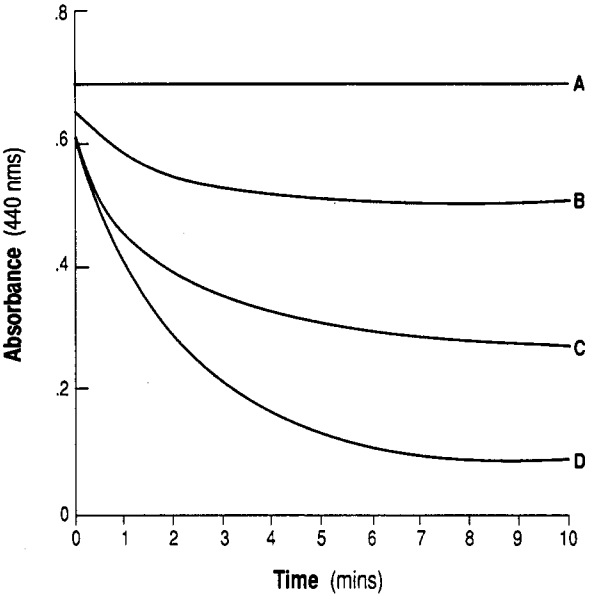


Figure 3. A series of curves showing changes in absorbance at 440 nm with time for solutions containing DODAC and cholesterol in aqueous KCl combined with the anion **II** in aqueous Tris buffer at pH 8 (no dithionite added). The cholesterol content varied as follows: curve A, 0 M; B, 1.3×10^{-4} M; C, 2.6×10^{-4} M; D, 5.1×10^{-4} M. Concentrations are as described in Results.

dithionite (Baker), potassium chloride (Baker), and tris(hydroxymethyl)aminomethane (Sigma) were used as purchased.[6] 5-Mercapto-2-nitrobenzoic acid was prepared according to Degani and Patchornik[10] as orange crystals: mp = 118–124 °C (lit. mp = 137–138 °C); λ_{max} = 412 nm for the dianion **II** (ϵ = 12 810)(lit. λ_{max} = 412 nm (ϵ = 13 600).

Solutions. Aqueous 0.01 M Tris buffer was prepared by dissolving Tris in the required amount of distilled water and adjusting the pH to 8 with dilute aqueous hydrochloric acid using a pH meter. Aqueous 0.005 M KCl was prepared by dissolving the required amount of KCl into distilled water. Ellman's reagent in alcohol was prepared by dissolving 50 mg in 25 mL of 95% ethanol.

Vesicle Preparation. DODAC and cholesterol were placed in 50 mL of buffer, sonicated, and filtered as previously reported.[6] Ellman's reagent was added by pipette to the resulting solution (0.5 mL in alcohol).

Light-Scattering Measurements. Vesicle size was determined on the vesicle preparations as previously reported.[6]

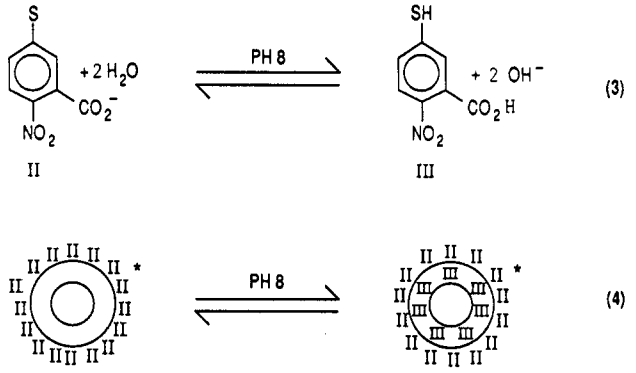
Data Collection and Handling. The light scattering and kinetic data were collected on instruments having computers and software packages to do plotting and error analysis as reported.

Discussion

The equilibria of eqs 3 and 4 comprise the explanation offered for the loss of absorbance at 440 nm observed in

(10) Degani, Y.; Patchornik, A. *J. Org. Chem.* **1971**, *36* (18), 2727–28.

Figures 2 and 3 due to the anion **II**. The pK_as of 5-mercaptobenzoic acid (**III**) are less than 4.57 for the thiol and about 3 for the carboxyl group. Little, if any of **III** could be present in the aqueous buffer used in these experiments. However, considerably greater stability might be expected for **III** within the (di-C18) bilayer of DODAC than for the anion **II** at the bilayer–aqueous interface due mainly to loss of charge. This would serve to pull the equilibria of eqs 3 and 4 in favor of colorless bilayer-inserted **III** (i.e. to the right side of eq 4).



Therefore the data of Figure 2 is explained by the rapid cleavage of **I** to **II** at the outer vesicular bilayer interface because with vesicles laced with increasingly larger amounts of cholesterol, **I** appears not to penetrate the bilayer even though it does so readily in pure DODAC. This is true because the initial absorbance increase observed in Figure 2 becomes more and more due only to the fast kinetic process, and the slow kinetic process, so prominent in vesicle solutions of pure DODAC, disappears as the reader progresses from curve A to B to C. The anion **II** also does not penetrate the cholesterol-laced bilayer even though it is reported to do so in solutions of pure DODAC vesicles.[5] Cholesterol-laced vesicles have been reported by Fendler to be less permeable in general to ions of high charge density and impermeable to aqueous lyate species.[3]

The loss of absorbance occurring in the experiments of curves D–F of Figure 2 and B–D of Figure 3 are the result of the displacement of the equilibria of eqs 3 and 4 toward the insertion of 5-mercaptobenzoic acid (**III**) into the vesicular bilayer. As the cholesterol content of the bilayer increases, the displacement of eq 4 to the right becomes more complete until the anion **II** is essentially removed from the solution after 10 min. Evidence for this is the accompanying increase in absorbance at 320 nm which is not shown. Also the rate of disappearance of absorbance at 440 nm in curves B–D of Figure 3 are within experimental error, the same as the appearance of absorbance at 320 nm.

The possibility that the loss of absorbance at 440 nm in Figures 2 and 3 is due to air oxidation of the anion **II** back to the substrate **I** was considered and rejected on the basis that all curves in Figure 3 are obtained in an identical manner with the exception of different cholesterol content. In addition, the anion **II** is stable in the buffer by itself. It is difficult to imagine a role for cholesterol in such a process.

The picture that emerges from this and the other studies is that pure DODAC vesicles in this system are permeable to both Ellman's reagent (**I**) and the dianion cleavage product **II**. Equilibria are established within a few seconds of contact in which both of these species are dispersed between the outer aqueous–bilayer interface and the internal part of the bilayer itself. It requires 10 to 15 min, however, to establish further dispersion to the inner aqueous–bilayer interface when **I** is added to preformed vesicles. A fast reaction with the small highly charged dithionite ion occurs completely only in the outer aqueous–bilayer in two stages, the second one being the slow migration of bilayer-embedded **I** to the reaction site. This model is similar to that used for an earlier study of this same reaction in the presence of (di-C16) vesicles by Moss and Schreck.[7] It can be represented by Scheme 2 in which **I** represents embedded substrate in equilibrium (K) with **I*** at the interface. **I*** is drawn from this arrangement rapidly by a reaction (k_r) with dithionite to form the dianion product **II**. Product **II** is also similarly dispersed as **I** but that is not represented here. Under these circumstances, the observed rate constant for the slow step of this reaction is given by

Scheme 2

$$\mathrm{I} \underset{}{\overset{K}{\rightleftharpoons}} \mathrm{I}^* \xrightarrow[k_r]{S_2O_4^{2-}} \mathrm{II}$$

Table 1: A Comparison of Rate and Equilibrium Constants Estimated from the Observed Data According to Scheme 2 for the Cleavage of I by Dithionite in Di-C16 and Di-C18 Vesicles

	K	k_r, s^{-1}
pure di-C16	1.1[a]	325
pure di-C18	2.0	50

[a] Data from ref 7.

$$k_{obs} = k_r K/(1 + K)$$

where the preequilibrium constant K is given by

$$K = \frac{\text{absorbance of the initial burst at 440 nm}}{\text{difference of final absorbance from that of the burst}}$$

Please see Figure 1 for clarification. Table 1 shows a comparison of values obtained for K and k_r as determined above for the (di-C18) surfactant in this study to that of the same study using the (di-C16) surfactant mentioned previously.[7]

Equilibrium constants vary by a factor of 2 in the two studies indicating that **I** is about half as stable in the interior binding sites of the (di-C18) bilayer compared to the surface electrostatic binding sites as it is in the (di-C16) bilayer. This is mainly due to the fact that the (di-C16) study was conducted with a bilayer at the gel–liquid phase-transition temperature, T_c = 25 °C in that system,[7] while the present study was conducted below this transition temperature, T_c = 36 °C in the (di-C18) bilayer.[3] The (di-C18) chains are therefore more tightly and firmly packed offering less room for and more resistance to entry and exit of substrate molecules. According to Scheme 2, the slow kinetic process of Figure 1 should be the rate of surface migration from the interior binding sites by **I**. These values are 2.4 s^{-1} for the (di-C16) study and 0.0103 s^{-1} in the present one. Thus the gel structure of the (di-C18) bilayer retards the migratory rate of **I** by a factor of 233 over the gel–liquid structure of the (di-C16) bilayer.

Values for the rate constants for the outer surface reaction between I and dithionite, which is k_r in Scheme 2 are also displayed in Table 1. The value for the shorter chained bromide vesicles is about 6.5 times that for the longer chained chloride vesicles of the present system. This is probably due to a more effective vesicular surface exchange of the harder dithionite ion with the softer bromide than with the smaller harder chloride ion.

When increasing amounts of cholesterol are included in the vesicular DODAC bilayer, the preequilibrium of Scheme 2 is increasingly displaced in favor of **I***. The k_r rate then becomes faster more completely producing the anion product **II** which also becomes increasingly less able to penetrate the hybrid bilayer. This leads to the insertion of the uncharged 5-mercaptobenzoic acid into the more tightly packed lyophobic hybrid bilayer with concurrent loss of the color due to **II**. The apparent driving force for the insertion mechanism is the loss of negative charge in a bilayer which is decidedly more nonpolar and lyophobic because of the addition of cholesterol. Structure **III** is also about half the size of the substrate **I**, an important fact when the interstitial bilayer space is packed with increasing amounts of added cholesterol. The interaction between **III** and the interior hybrid membrane is likely one of induced polarization strengthened by large surface area but even a reaction between **II** or **III** and cholesterol has not been ruled out.

Table 2 shows a comparison of K values from Scheme 2 calculated as shown above for experiments involving DODAC vesicles laced with 0–18.9 wt % cholesterol. The trend for these values is to increase from 2 to about 6 which is in support of the statements made in the previous paragraph. Although the data is meager in the (di-C16) study, also shown, the trend is similar. The effect of increasing amounts of added cholesterol might also be ascribed to the differences in T_c temperatures between the two bilayers. There would be more interstitial space available in the liquid bilayer compared to the gel. Thus the added cholesterol has less of an exclusion effect on **I** in the liquid bilayer than in the gel.

Table 2. A Comparison of Equilibrium Constants Estimated from the Observed Data According to Scheme 2 for the Cleavage of I by Dithionite in Di-C16 and Di-C18 Vesicles Laced with Varying Amounts of Cholesterol

wt % cholestrol	K $(C18)_2$	K $(C16)_2$[a]
0	2.06	1.1
3.2	2.05	
14.3	5.16	
16.7	5.60	
18.9	5.94	1.85

[a] Data from ref 7.

Thus, truly encapsulated Ellman's reagent, sought by Moss and co-workers, is achievable using DODAC vesicles substantially laced with cholesterol. Although it is encapsulated in pure DODAC vesicles also, it can cross the membrane and escape in about 10 min.

When an experiment identical to that of curve E of Figure 2 was repeated with the exception that the Ellman's substrate (**I**) was cosonicated with DODAC and cholesterol instead of its being added to preformed vesicles, the observed curve was very similar to that of E except that it occurred at about one-half the absorbance observed in Figure 2. Thus about one half of the substrate is now unreachable by the dithionite indicating prolonged entrapment.

Acknowledgment. Support for this work is gratefully acknowledged from the Research Corp. and the Flinn Foundation as well as the Northern Arizona University Organized Research Fund.