adduct was isolated in 42% yield as a yellow viscous oil whose proton NMR spectrum was identical with that reported previously.[17a]

Competitive Oxidation of Dibenzylamine and Dibenzyl Sulfide with Davis' Reagents. Dibenzylamine (0.197 g, 1.00 mmol) and dibenzyl sulfide (0.214 g, 1.00 mmol) were dissolved in $CDCl_3$ (3 mL). 2-(Phenylsulfonyl)-3-phenyloxaziridine (0.150 g, 0.57 mmol) was added in one portion, and the reaction mixture was stirred for 30 min. The ^1H NMR spectrum of the reaction mixture was obtained. The integral ratio of the methylene protons of dibenzyl sulfoxide (δ 3.91) and dibenzyl sulfide (δ 3.56) was 30.5 to 25.5, indicating that 54% of the 1 mmol of dibenzyl sulfide had been oxidized to dibenzyl sulfoxide. Thus 0.54 mmol (54% of 1 mmol) of the available 0.57 mmol of oxidant (95%) had reacted at sulfur. A similar experiment using a 1:1:1 ratio of reactants was carried out, and the reaction mixture was analyzed by ^{13}C NMR. Similar results were obtained as in the previous experiment.

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Transvesicular Reactions of Thiols with Ellman's Reagent

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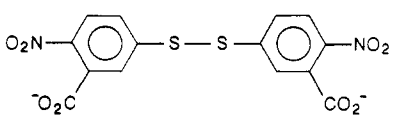
The cleavage of Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)], **1**, to chromophoric anion **2** by various thiols has been studied in pH 8 buffer, micellar cetyltrimethylammonium bromide (**4**), and vesicular dihexadecyldimethylammonium bromide (**5**) or dioctadecyldimethylammonium chloride (**6**) solutions. The thiols included thiocholesterol, thiophenol, 2-thionaphthol, DL-cysteine, glutathione, 1-butanethiol, and 1-octanethiol. Vesicles of **6** at 25 °C sequester **1** in distinct exovesicular and endovesicular binding sites, where reactions with added thiols are kinetically differentiated. Differences in thiol acidity and structure influence their rates of permeation and reaction with vesicle-bound **1**. Small quantities of covesicallized 1-hexanol (0.2 wt %) lower the gel to liquid crystalline transition temperature of vesicular **1** (from ~39 °C to 24 °C), enhance vesicular fluidity, accelerate the thiol/**1** reactions, and destroy the kinetic distinction between the exovesicular and endovesicular reactions.

Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)], **1**, is readily cleaved at its disulfide bond by a variety of nucleophiles to afford chromophoric anion **2**.[1] In the case of (excess) thiolate nucleophile, **1** is cleaved to 2 equiv of **2** via an intermediate "mixed disulfide",[2] whereas nucleophiles such as sulfite,[1b] or dithionite[3,4] give 1 equiv of **2**, together with a "Bunte" salt (e.g., $ArSSO_3^-$ from **1** + sulfite[1b]). In either case, conditions can be selected to make



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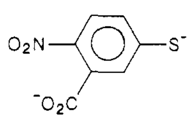

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the reductive cleavage of **1** rapid and quantitative, so that the reaction assumes analytical importance due to the intense (log ϵ 4.14) and conveniently located (λ_{max} 407 nm) absorption of Ellman's anion, **2**.[5]

Accordingly, Ellman's reagent has been used as a probe of reactions occurring on or within micelles, vesicles, and liposomes. Micelles are thermodynamically stable aggregates that form spontaneously from single chain surfactants, typically carrying 12–16 carbons in their alkyl chains. Vesicles or liposomes are usually composed of twin-tailed ionic surfactants or phospholipids. These form multilamellar or unilamellar vesicles depending upon the method of preparation. The unilamellar vesicles contain a central water core surrounded by a surfactant bilayer that has both inner and outer charged interfaces covering the hydrocarbon chain region. Micelles, on the other hand, contain a hydrophobic core composed of the alkyl chain hydrocarbons, surrounded by a single charged interface or Stern layer in contact with the aqueous solution.

Fendler and Hinze examined the hydroxide mediated cleavage of **1** in cetyltrimethylammonium (CTA) bromide micelles and in dioctadecyldimethylammonium chloride (18_2, DODAC) vesicles. In the latter case, reaction was slow, relative to OH^- permeation across the bilayer membrane, leading to a monophasic chemical process.[6] In contrast, the very rapid reactions of **1** with added sulfite or dithionite ions in dihexadecyldimethylammonium bromide vesicles (16_2) were kinetically biphasic, with dynamic behavior indicative of a rapid but kinetically resolvable equilibration (k_{equil} ~ 2–4 s^{-1}) of **1** between "subvesicular" (possibly intercalation) and exovesicular binding sites.[7] When **1** was *encapsulated* inside DODAC (18_2) vesicles, the more permeation-resistant 18_2 bilayers prevented the leakage of **1** and "shut off" exovesicular reactions with dithionite.[8]

Bizzigotti, analyzed reactions of **1** with thiol-functionalized 16_2, finding evidence that the reaction of 16_2S-SEll (the "mixed" disulfide) and 16_2S^- was unexpectedly slow.[9] This may have reflected a general phenomenon when the reactants were both integral parts of the bilayer. In the

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(5) These values were determined in N_2-purged, 0.01 M aqueous Tris buffer at pH 8.0, μ = 0.01 (KCl): Moss, R. A.; Swarup, S.; Schreck, R. P. *Tetrahedron Lett.* **1985**, *26*, 603. Note that λ_{max} is sensitive to its microenvironment; see below.
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same vein, Ganong and Bell interpreted the biphasic kinetics observed upon reaction of 1 with dioleoyl phosphatidylthioglycerol in terms of a slow ($\tau_{1/2} > 8$ days) transbilayer migration ("flip-flop") of the endovesicular thiolipid.[10]

Less clearcut were the reactions of 1 and thiocholesterol (3, TC) in egg lecithin liposomes.[11,12] When liposomes containing endovesicular *and* exovesicular TC were titrated with 1, biphasic kinetics were observed and attributed to rapid ($k \sim 2.5 \times 10^{-2}$ s^{-1}) and slow ($k \sim 2.7 \times 10^{-4}$ s^{-1}) reactions of 1 with exovesicular and endovesicular TC, respectively.[11] Dawidowicz and Bacher reported on the *inverse* experiment, where 1 was encapsulated in egg lecithin liposomes and then treated with TC.[12] They observed only a single, rapid reaction ($\tau_{1/2} \leq 1$ min at 20 °C) and concluded that TC rapidly crossed the lecithin bilayers. No comparison was offered with the experiments of Huang et al.,[11] which seemed at face value to indicate that endovesicular TC reequilibrated to exovesicular loci very slowly ($\tau_{1/2} \sim$40 min) before reacting with the exovesicular 1.

Our general interest in the modulation of chemical reactivity by vesicular membranes,[3,7,8,13-15] has now led us to a wider study of the reactions of 1 and thiols (including TC) in CTABr micelles and in 16_2 and 18_2 vesicular membranes. The observed responses of the reaction kinetics to surfactant and thiol structures help us to understand those factors that favor permeation-limited, "membrane-modulated" chemistry. Most importantly, we find that small quantities of added alcohols (e.g., 0.2 wt % of 1-hexanol) greatly enhance the fluidity of 18_2 membranes and eliminate permeation control. These results have implications for the more precise control of chemical reactions *inside* vesicles and liposomes.

Results and Discussion

Methodology. The surfactants used in this work were CTABr, 4, 16_2(Br), 5, and 18_2(Cl) (DODAC), 6. The thiol reactants included thiocholesterol (3, TC), thiophenol (7), 2-thionaphthol (8), DL-cysteine (9), glutathione (10), 1-butanethiol, and 1-octanethiol.

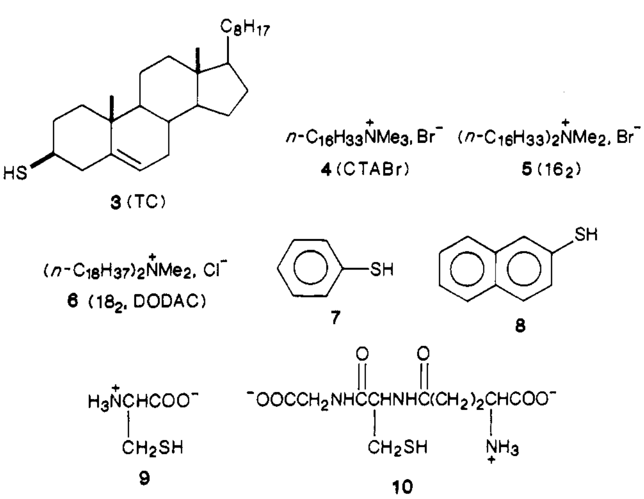

Table I. Rate Constants (k_ψ) for the Micellar Cleavage of 1 by Thiols[a]

thiol	pK_a[b]	k_ψ, s^{-1} buffer[c]	k_ψ, s^{-1} CTABr[d]	k_{CTABr}/k_{buffer}
7	5.9, 6.3	0.179	169	944
8	5.4, 6.2	0.198	312	1580
3	10.0[e]	*f*	0.0032	
9	8.5[g]	0.170	68.7	404
10	9.2[h]	0.037	141	3810

[a] Conditions: [1] = 1.25–2.5 × 10^{-5} M; [thiol] = 5 × 10^{-5} M; pH 8, Tris buffer, 25 °C. [b] See text for discussion. For 7 and 8, the first entry refers to 2.5 × 10^{-3} M CTABr micellar solution in Tris; the second entry refers to Tris alone. [c] Tris *buffer* refers to 0.01 M Tris and 0.01 M KCl in aqueous solution at pH 8. [d] Determined in 2.5 × 10^{-3} M CTABr in Tris buffer. [e] Determined in 2.5 × 10^{-3} M CTABr, see text. [f] Not measured due to the low solubility of TC in buffer. [g] In 0.02 M aqueous phosphate/borate buffer; ref 18. [h] In 2 × 10^{-4} M aqueous CTABr; ref 19.

Micelles were readily obtained from CTABr upon dissolution in aqueous buffers. Vesicles were generated from 16_2 by sonication[16] and from 18_2 either by sonication (small vesicles) or slow injection (large vesicles); details appear in the Experimental Section. The apparent hydrodynamic diameters of these vesicles, as determined by dynamic light scattering, were 300–400 Å (16_2), 700–800 Å (small 18_2 vesicles), and 3000 ± 500 Å (large 18_2 vesicles).

Creation of the cationic 18_2 vesicles in the presence of substrate 1 gave vesicles with 1 bound[17] in both endovesicular and exovesicular sites.[8] When desired, exovesicular 1 was removed by gel filtration chromatography of the vesicular solution over a Sephadex G-75 column that had been preequilibrated with empty, substrate-free vesicles. Details of this procedure have been published.[8]

Reactions in CTABr Micelles. The reactions of thiols and 1 were studied in the presence and absence of the surfactant aggregates, with the kinetics followed by monitoring the formation of anion 2 at 412 nm in Tris buffer solution, at 435 nm in CTABr micellar solution, and at 450 nm in 16_2 or 18_2 vesicular solutions. These wavelengths are appropriate to the maxima of 2 when it is free in solution or bound to the several aggregates.[5]

Table I displays pseudo-first-order rate constants for the cleavage of 1 by thiolate ions in Tris buffer and in CTABr micellar solution at pH 8. Generally, thiol/thiolate was present in 2-fold stoichiometric excess over 1. Under these conditions, reaction 1 should be relatively rapid, and the subsequent cleavage of the "mixed" disulfide (EllS-SR) should be rate limiting, so that the observed, well-behaved, pseudo-first-order kinetics of the formation of 2 are best understood as reflecting k_2 in eq 2.[2] Under our conditions,

$$\underset{1}{\text{EllS–SEll}} + \text{RS}^- \xrightarrow{k_1} \text{EllS–SR} + \underset{2}{\text{EllS}^-} \quad (1)$$

$$\text{EllS–SR} + \text{RS}^- \xrightarrow{k_2} \text{RS–SR} + \text{EllS}^- \quad (2)$$

good first-order kinetics were observed *for the appearance of* 2 to >90% of reaction, there was no evidence for kinetic resolution of k_1 and k_2, and the stoichiometry demanded by the *sum* of eq 1 and 2 was manifested.

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Table II. Rate Constants (k_ψ) for the 16_2 Vesicular Cleavage of 1 by Thiols[a]

thiol	concn, M	k_ψ^f, s^{-1} [b]	k_ψ^s, s^{-1} [c]	% fast[d]
7	5×10^{-5}	>500	30.5	70
7	1×10^{-4}	>500	32.3	65
8	5×10^{-5}	>500	180	65
3	5×10^{-5}		0.035	0
9	5×10^{-5}		4.85	<10
10	5×10^{-5}		43.2	<10
TB[e]	5×10^{-5}		5.38	<10
TB	1×10^{-4}		8.8	<10

[a] Vesicles of 16_2 were created at pH 6 in 0.01 M aqueous KCl, in the presence of **1**, by sonication. Reactions were monitored at 25 °C by stopped-flow spectroscopy on a multimix, 3-syringe unit[7] where equal volumes of $1/16_2$, and thiol in pH 8 Tris buffer, were combined in the final mixing step. The final conditions were pH 8, $[16_2] = 5 \times 10^{-4}$ M, $[1] = 2.5 \times 10^{-5}$ M. [b] Rate constant of the fast reaction; >500 s^{-1} indicates that the process exceeds the time resolution of our instrument. [c] Observed rate constant of slow reaction. [d] Percent of fast process; the balance is slow reaction. These values are taken from the measured absorption changes which are in accord with complete reduction of **1** in all cases. Errors are ±5–10%. Entries of <10% indicate little or no fast reaction. [e] TB = thiobutanol.

Owing to the limited solubility of thiocholesterol under our reaction conditions, a 2-fold excess, relative to **1**, was the most that could be achieved. Other thiols were used in the same stoichiometric ratio for purposes of comparison. It is somewhat surprising that these conditions lead to first-order appearance of **2**, but they do. Doubling the ratio of thiol to **1** led to no change in the kinetics or rate constant with thiophenol **7**, whereas with thiobutanol, the kinetics remained first order but k_ψ^s increased from 5.4 to 8.8 s^{-1} (Table II).

The reactive form of RSH in these reactions is the thiolate ion, RS^-,[2] so that the pK_a of RSH is crucial; these data also appear in Table I. Acidity constants for thiophenol (**7**) and thionaphthol (**8**) were spectrophotometrically measured in either 0.01 M Tris/0.01 M KCl, or in 2.5×10^{-3} M micellar CTABr/Tris. Absorbances of **7** or **8** were determined at various pH's ranging from 3 to 10. With 5.5×10^{-5} M **7**, we observed the thiolates at λ 266 nm (in buffer) and 280 nm (in CTABr); the corresponding wavelengths for 5.0×10^{-5} M solutions of **8** were 264 and 272 nm, respectively. These wavelengths represent λ_{max} for the completely dissociated thiols, determined at pH ≥10. pK_a values were then obtained from eq 3, where A

$$pK_a = pH - \log (A - A_{min})/(A_{max} - A) \qquad (3)$$

is the absorbance of RSH/RS^- at any pH, A_{min} is the absorbance of RSH at pH 3, and A_{max} is the absorbance of RS^- at pH 10. Plots of the logarithmic factor vs pH were linear with a slope of unity, and pK_a was read from these plots as numerically equal to the pH at which the logarithmic quantity was zero.

Previous determinations of the pK_a of **7** in CTABr and aqueous solutions, 6.2[20] and 6.8,[20,21] respectively, are in fair agreement with our current values. The apparent pK_a will depend on [surfactant], so that perfect agreement would not be expected. It is clear that both thiophenol and thionaphthol will be completely ionized to their anionic forms in pH 8 cationic micellar or vesicular solutions, i.e., the conditions of our experiments. The lowering of the pK_a's of **7** and **8** by cationic micellar CTABr is well precedented and is principally due to electrostatic stabilization of the anionic thiolate conjugate bases upon binding to the cationic aggregates.[20,21]

The pK_a of TC was determined kinetically from a rate constant–pH profile: rate constants for the cleavage of 2.5×10^{-5} M **1** by 5×10^{-5} M TC in 2.5×10^{-3} M CTABr and 0.01 M Tris/0.01 M KCl were determined as a function of pH over the range 8–11.2. A plot (not shown) of log k_ψ vs pH gave intersecting straight lines with a sharp break point at pH 10.0, that we take as the pK_a of TC when it is solubilized in CTABr micelles. TC will therefore be only ~1% ionized to RS^- under our standard pH 8 reaction conditions.

pK_a values for cysteine and glutathione were taken from the literature;[18,19] see Table I. The latter value was determined kinetically in micellar CTABr, but the pK_a for cysteine was measured in water. We estimate that it would be lower (~8.0) in micellar CTABr or vesicular 16_2 or 18_2. Glutathione will thus be ~6% ionized in pH 8 micellar CTABr, whereas the ionization of cysteine will be ~50%.

A plot of log k_2 vs pK_a for the cleavage of **1** by thiolates **7**–**10** in pH 8 Tris buffer at 25 °C gave a fair Brønsted relation with $\beta = 0.25$. The data were taken from Table I, with k_ψ corrected for [thiolate] as determined from the pK_a values. The Brønsted β value is in reasonable agreement with $\beta = 0.36$ reported by Whitesides et al. for the reactions of mono and dithiols with **1** at 30 °C in 0.05 M, pH 7 phosphate buffer at 30 °C.[2]

The strong rate accelerations anticipated[7,20,22,23] for bimolecular reactions between the anionic reactants, **1** and RS^-, when bound to cationic micellar aggregates are clearly apparent in the last column of Table I; rate constant enhancements of 2–3 orders of magnitude are observed, reaching a maximum of 3810 with glutathione. Much of the apparent enhancement in the observed rate constants (calculated from bulk concentrations) derives from concentration of the reactants into the small reaction volume of the micellar or vesicular polar regions and surfaces.[23b] Both **1**[17] and nucleophiles such as thiophenolate[22] bind very strongly to cationic aggregates. Rate enhancements also accrue from the lowered pK_a of RSH and its augmented ionization to the reactive RS^- when bound to the cationic aggregates. This effect appears to be worth factors of ~2–6. Note that the more hydrophobic nucleophiles, within structurally similar pairs, display larger rate constant enhancements (presumably because of better binding) upon micellization, e.g., **8** > **7** and **10** > **9**.

We lack k_ψ for TC (**3**) in buffer (because of its low solubility) and cannot calculate a rate constant enhancement in CTABr. However, TC is clearly very much less reactive than the other thiols. Some of its low reactivity at pH 8 has to do with its high pK_a, but even if we "adjusted" the pK_a to 9 (a factor of 10 in k_ψ), TC would still be ~4400 times *less* reactive toward **1** than glutathione in pH 8 micellar CTABr. We have no explanation for this effect.

Reactions in 16_2 Vesicles. Table II collects kinetic observations for the reactions of **1** with thiols in sonicated 16_2 vesicles. Here, **1** is located at both exovesicular and endovesicular (or "subvesicular", possibly intercalated[7]) sites. However, the gel to liquid crystalline transition temperature (T_c) is ~25 °C for 16_2 vesicles,[16] so that there is a mobile equilibrium between **1** at either site, with $k_{in} \sim k_{out} \sim 1$–2 s^{-1}.[7]

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Table III. Rate Constants (k_ψ) for the 18_2 Vesicular Cleavage of 1 by Thiols[a]

thiol	concn, M	vesicle type[b]	k_ψ^f, s^{-1} [c]	k_ψ^s, s^{-1} [d]	% fast[e]
7	5×10^{-5}	son	>500	1.22	60
7	5×10^{-5}	son-S	*f*	4.90	*f*
8	5×10^{-5}	son	>500	46.3	60
3	5×10^{-5}	inj	0.12	0.0013	40
3	5×10^{-5}	son	0.18	0.0014	60
3	5×10^{-5}	son-S		0.0009	*f*
3	2.5×10^{-5}	*g*		0.0003	<10
9	5×10^{-5}	inj	14.8	0.001	40
10	5×10^{-5}	inj	74.0	0.004	40
TB	2.5×10^{-4}	son	10.8	0.07	60
TB	2.5×10^{-4}	son-S		0.06	*f*
TB	2.5×10^{-4}	inj	10.3	0.04	40
TO[h]	2.5×10^{-4}	inj	9.3	0.07	40

[a] Vesicles of 18_2 were prepared in the presence of **1**. Concentrations and conditions are analogous to those for 16_2; Table II, note *a*. [b] Son = sonicated, inj = injected; see the Experimental Section for details. S indicates that the 18_2/**1** vesicle preparation was chromatographed over Sephadex G-75 to remove exovesicular **1**.[8] [c] See Table II, note *b*. [d] Cf. Table II, note *c*. [e] Cf. Table II, note *d*. [f] No fast reaction was observed. [g] Inverse addition; **3** was sonicated (N_2 atmosphere) with 18_2, and **1** was added to initiate the reaction. [h] TO = 1-octanethiol.

The results with thiophenol are comparable to our previous findings.[7] Addition of excess **7** (thiophenolate at pH 8) leads to a very rapid reaction ($k_\psi^f > 500$ s^{-1}) in which exovesicularly bound **1** (~65% of total **1**) is reduced. The permeation of **7** is probably not rate limiting,[7] and there is a subsequent, slower ($k_\psi^s \sim 30$ s^{-1}) reaction of the remaining subvesicular **1**. Thionaphthalene (**8**) behaves in a completely analogous manner; the "slow", subvesicular reaction now occurs with $k_\psi^s \sim 180$ s^{-1}. The increase in k_ψ^s, relative to **7**, may reflect the greater hydrophobicity of **8**, and its consequently greater endovesicular/exovesicular distribution that translates into a larger pseudo-first-order rate constant for endovesicular cleavage of **1**.

Different results were obtained with thiols **3**, **9**, **10**, and thiobutanol (TB): little or no fast reaction was observed; instead quantitative reduction occurred with rate constants ranging from 0.03 s^{-1} (**3**) to 43 s^{-1} (**10**). We suggest that the principal reason for the loss of very rapid exovesicular reaction is the low acidity of these nucleophiles relative to **7** and **8**. Thus, at pH 8, **7** and **8** ($pK_a < 6$) are fully ionized, an excess of thiolate floods the vesicular surface, and exovesicularly bound **1** is cleaved with $k > 500$ s^{-1}. With (hydrophilic) **9** ($pK_a \sim 8.5$), and weakly acidic **10** ($pK_a \sim 9$), **3** ($pK_a \sim 10$), or TB ($pK_a \sim 10.7$), much less thiolate will be present at the vesicular surface, and the cleavage of **1** will be slower. Equilibrations of thiol/thiolate and/or substrate between the several vesicular sites will occur rapidly enough to obviate kinetic distinctions between reactions at different loci.

Reactions in 18_2 Vesicles. In Table III, we present kinetic results for the cleavage of **1** in DODAC (18_2) vesicles. The results differ from those obtained in 16_2 vesicles (Table II). Thus, exovesicularly and endovesicularly bound **1**, resulting from the cosonication or coinjection of **1** and 18_2, are *not* in mobile equilibrium (as they are with 16_2 vesicles), and they are cleaved in separate reactions.

We have demonstrated the relative impermeability of 18_2 vesicles to **1** in the ionic dithionite/**1** cleavage reaction.[8] When **1**/18_2 is treated with **7** and **8**, very rapid exovesicular cleavage of **1** occurs, followed by a slower (although still rapid) cleavage of endovesicular **1** (subsequent to the permeation of **7** or **8**, presumably as thiols). If the **1**/18_2 preparation is first chromatographed over Sephadex, thus removing exovesicular **1**, addition of **7** no longer elicits the very fast initial reaction; only the slower, endovesicular reaction of **1** and **7** now remains.[24]

Similar behavior attends the reactions of **1**/18_2 with the other thiolate nucleophiles. The absence of exo/endo equilibration of **1** causes even the weakly acidic thiols to manifest kinetically distinct exovesicular and endovesicular reactions. Here too, the exovesicular reactions can be obviated by Sephadex chromatography (cf. **3** and TB in Table III).

Note that the slow, endovesicular reactions of all of the thiols are very much slower in 18_2 vesicles than are the analogous reactions in the 16_2 vesicles (Table II). This reflects the tighter packing of the alkyl chains in the bilayers of the 18_2 vesicles, which are below T_c, and in their "rigid" gel phase at 25 °C. Consequently, there is much slower permeation of the thiol/thiolate nucleophiles than in the 16_2 vesicles (see also below). However, we note that the slow endovesicular cleavages of **1** associated with (e.g.) **3** and **9** are still 3–4 times faster than the OH^- (buffer) cleavage[6] of **1**/18_2 at pH 8.[8,25] Thus, these thiols can permeate the 18_2 vesicles and react with endovesicular **1**, although the transvesicular reactions appear to be rate limited by permeation.

Note too that although the respective fast and slow rate constants are similar for cleavages by **3** and TB in small (700–800 Å) and large (3000 Å) 18_2 vesicles, there is somewhat more slow reaction in the larger vesicles. This is in keeping with their anticipated greater endovesicular binding capacity.

The thiocholesterol results in Table III show that added TC reacts slowly (0.1–0.2 s^{-1}) with exovesicular **1**, and much more slowly (~0.001 s^{-1}) with endovesicular **1**, after permeation into the 18_2 vesicle. When the TC is first cosonicated with 18_2 *followed* by the addition of **1**, only a single, very slow cleavage reaction is observed; indeed k_ψ^s here is similar to the buffer/18_2 rate constant.[25] Apparently, cosonicated TC becomes an integral part of the 18_2 bilayer and is structurally restricted from rapid reaction with the added **1**, which must remain at exovesicular sites.

These TC/**1**/18_2 studies contrast with experiments in lecithin liposomes[11,12] (see above), where added TC is reported to rapidly permeate to "encapsulated" **1**,[12] and added **1** displays biphasic kinetics with TC/lecithin.[11] The differences might be related to the differing head group structures and anion (i.e., **1**) binding capacities of the 18_2 and lecithin surfactants, as well as to the differing fluidities of their vesicles: at 25 °C, lecithin vesicles are above there T_c and fluid, whereas the 18_2 vesicles are below their T_c and "rigid".[26] It is worthwhile noting that our slow TC reactions cannot solely be functions of TC's low acidity. Thiobutanol, with a similar pK_a reacts 50–60 times more rapidly with either exo- or endovesicular **1**/18_2 (Table III). It is the structure of TC and presumably its particular packing in 18_2 bilayers that determines its unusually low reactivity.

Fluidity of 18_2 Vesicles. The suggestion that the rigidity/fluidity of the 18_2 bilayers controls the permeation, and reactivity of thiols toward endovesicular **1** was examined in more detail with vesicle-incorporated alcohols to modify the fluidity of the 18_2 vesicles. We have demon-

(24) In contrast, Sephadex chromatography does not separate exovesicular and endovesicular **1** in 16_2 vesicle preparations. Vesicles collected in the eluent after the column void volume contain *no* **1**. The mobile exo/endovesicular equilibration of **1** in 16_2 vesicles leads to a complete loss of **1** to the Sephadex column.

(25) This value is given as $k_\psi = 0.00036$ s^{-1} in ref 8.

(26) The T_c's are −5 °C for egg lecithin liposomes[27a] and 36 °C for 18_2 vesicles.[27b] Our present determination of the latter value (see below) is 39 °C.

(27) (a) Huang, C.; Mason, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 308. (b) Fendler, J. H. *Acc. Chem. Res.* **1980**, *13*, 7.

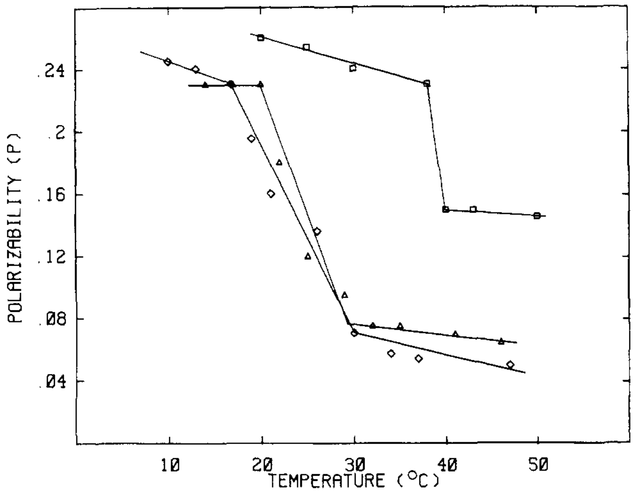

Figure 1. Fluorescence polarization (P) of 1,6-diphenyl-1,3,5-hexatriene in (sonicated) vesicular 18_2 (□), 16_2 (△), and 18_2 + 0.2 wt % 1-hexanol (◇) as a function of temperature. The midpoints of the "transition" regions are taken as T_c.

strated that the addition of 28 wt % ethanol to 18_2 vesicles at 25 °C damages their integrity and strongly enhances otherwise sluggish transvesicular reactions.[8] We now find that longer 1-alkanols are much more effective than ethanol.

Cosonication of 1.0 wt % (135 mM) 1-butanol or 0.2 wt % (20 mM) 1-hexanol with 5×10^{-4} M 18_2 and 2.5×10^{-5} M 1 gave covesicles[28] that were much more reactive toward TC than the vesicles described in Table III. Addition of 5×10^{-5} M TC to these alkanol-doped 18_2 vesicles gave only *single*, quantitative reactions with k_ψ = 0.082 or 0.098 s^{-1} for the butanol or hexanol covesicles, respectively. The distinction between exovesicular and endovesicular 1 was lost in these modified 18_2 vesicles, and the observed rate constants were nearly midway between the exo- and endovesicular rate constants (0.18 and 0.0014 s^{-1}) observed for the TC/1 reactions in native 18_2 vesicles (Table III). Indeed, the reaction in the *modified* 18_2 vesicles resembled that in *native* 16_2 vesicles, where we also observed a quantitative, monoexponential TC/1 reaction, with k_ψ = 0.035 s^{-1}; cf. Table II.

A similar experiment carried out with thiobutanol and 1-hexanol/18_2 afforded a monophasic, quantitative cleavage of 1 with k_ψ = 0.89 s^{-1}. Again, as with TC, kinetic resolution of reactions occurring at distinct vesicular loci was lost, and the observed rate constant was intermediate between those observed for the slow and fast reactions in unmodified 18_2 vesicles.

The hexanol increases the fluidity of the 18_2 bilayers, presumably enhancing the permeation and mutual mobility of both TC and 1. This can be demonstrated by using the fluorescence polarization of 1×10^{-5} M covesicallized 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe of both the gel to liquid crystal phase transition temperature (T_c)[29,30] and the microviscosity of the 18_2 vesicles. Thus, incorporation of 0.2 wt % 1-hexanol lowers T_c from ~39 °C to ~24 °C, almost identical to the T_c of 16_2 vesicles (25 °C). The experimental results are shown in Figure 1, where the fluorescence polarization of the DPH is plotted against temperature.

(28) Dynamic light scattering of the 1-hexanol preparations showed that vesicles (d ~ 1100 Å) were still present, and that the hydrodynamic diameter had not greatly increased from that of the native 18_2 sonicated vesicles (d ~ 700–800 Å).

(29) For details of this method,[30] see ref 15.

(30) Andrich, M. P.; Vanderkooi, J. M. *Biochemistry* **1976**, *15*, 1257.

Table IV. Effects of 1-Hexanol on Vesicles at 25 °C[a]

vesicle	T_c,[b] °C	P[c]	τ,[d] ns	$\bar{\eta}$,[e] cP	$\tau_{1/2}$,[f] s
18_2	39	0.25	5.75	35	19 ± 1
18_2/hexanol[g]	24	0.13	7.34	13	0.8 ± 0.02
16_2	26	0.15	7.51	16	0.2[h]

[a] Vesicles were prepared in 0.01 M aqueous KCl by sonication (see the Experimental Section). [b] Gel to liquid crystal transition temperature from fluorescence polarization studies; see text and Figure 1. [c] Observed fluorescence polarization[30] of 1,6-diphenyl-1,3,5-hexatriene (DPH) at 25 °C. [d] Fluorescence lifetime of DPH.[32] [e] Apparent microviscosity of vesicle bilayers from eq 4. [f] Half-time for permeation of 1,8-anilinonaphthalenesulfonate; see ref 16 for a description of this method. [g] Covesicle with 0.2 wt % 1-hexanol. [h] From ref 16.

Not only does the markedly lower T_c point to an increased fluidity of these 18_2/1-hexanol vesicles at 25 °C, but the apparent microviscosity ($\bar{\eta}$) of the bilayers can be estimated from eq 4.[31] Here, P is the observed fluores-

$$\frac{P_0}{P} = 1 + \frac{kT\tau}{\bar{\eta}v_0} \qquad (4)$$

cence polarization of DPH in 18_2 vesicles at 25 °C, P_0 is the maximum theoretical value of P (P_0 = 0.40 in an infinitely viscous medium), τ is the fluorescence lifetime of DPH in its excited state,[32] v_0 is the effective rotational molar volume of DPH,[33] T is the absolute temperature, and k is the Boltzmann constant. Calculated values of $\bar{\eta}$, and experimental values of P and τ appear in Table IV. Clearly, the apparent microviscosity of the 18_2 vesicles at 25 °C is greatly reduced after covesicallization with 0.2 wt % 1-hexanol. The covesicles have a microviscosity even lower than that of native 16_2 vesicles under similar conditions. It is therefore reasonable that there are marked resemblances between TC/1 reactions in the 18_2/hexanol and 16_2 vesicles.

A similar conclusion follows from stopped-flow experiments in which we measured the half-times for the development of the fluorescence of 1-anilino-8-naphthalenesulfonate (ANS) subsequent to the rapid mixing of aqueous ANS and vesicular solutions. The development of ANS fluorescence can be taken as a kinetic probe of the transport or permeation of ANS across the exovesicular surface and into the bilayer.[16,34] The data in Table IV show that ANS transport is much faster into hexanol-doped 18_2 vesicles than into native 18_2 vesicles; $\tau_{1/2}$ with the doped vesicles approaches that observed with native 16_2 vesicles.

The chemical evidence from the kinetic studies (Table III), and the physical studies (Table IV), together point to the conclusion that the rate and exovesicular/endovesicular resolution of the thiolate/1 reaction depends upon the permeability and fluidity of the vesicular bilayers. Moreover, the covesicallization of small quantities of 1-alkanols with DODAC results in less viscous, more fluid, and more permeable 18_2 vesicles at 25 °C, where the mutual mobilities of (e.g.) TC and **1** are enhanced (relative to native 18_2 vesicles), the rate constants for their reactions are augmented, and the kinetic resolution of distinct, locus-specific vesicular reactions is lost.

Endovesicular to Exovesicular Transport of 1. As shown above, small or large 18_2 vesicles, prepared in the

(31) Jain, M. K.; Wagner, R. C. *Introduction to Biological Membranes*; Wiley: New York, 1980; pp 82–83.

(32) τ was measured with a PRA Laser, Inc., Model 3000 single photon counting fluorescence spectrophotometer.

(33) v_0 is calculated to be 1150 Å^3 from $4/3\pi r^3$, using r = 6.5 Å: Zannoni, C.; Arcioni, A.; Cavatorta, P. *Chem. Phys. Lipids* **1983**, *32*, 179.

(34) Haynes, D. H.; Simkowitz, P. *J. Membr. Biol.* **1977**, *33*, 63.

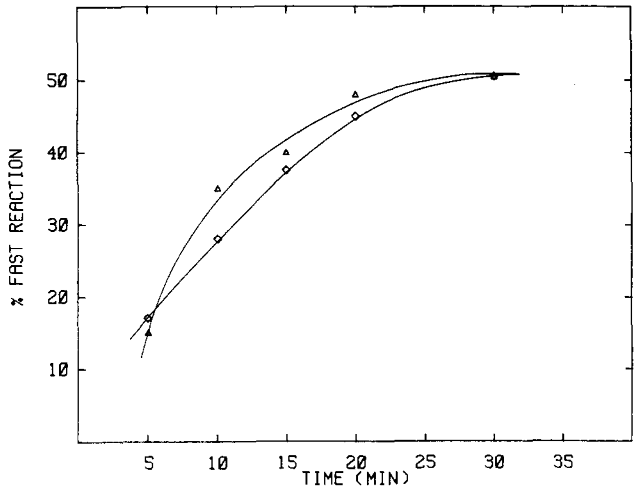

Figure 2. Percent of fast reaction between dithionite ion and 18_2-encapsulated 1 as a function of incubation time at 42 °C. Data is displayed for small, sonicated (◇) and large, injected (△) 18_2 vesicles.

presence of 1, bind this substrate at both exovesicular and endovesicular sites. Gel filtration chromatography removes exovesicular 1, leaving an endovesicular substrate that remains kinetically inaccessible to external reagents such as dithionite ion.[8] Previously, we reported that anion 2 and iodosobenzoate, when separately encapsulated in 18_2 vesicles and therefore unreactive, could be brought into rapid reaction upon warming the vesicle solution above T_c.[8] We have now applied a related regimen to 18_2-encapsulated 1.

Small or large 18_2 vesicles were prepared in the presence of 2.5×10^{-5} M 1 in 0.01 M aqueous KCl at pH 6. Exovesicular 1 was removed chromatographically, and the resulting solution of 18_2-encapsulated 1 was divided into several 2-mL aliquots. Addition of an equal volume of 5×10^{-4} M $Na_2S_2O_4$ in pH 8, 0.01 M Tris/0.01 M KCl to the first aliquot at 25 °C, initiated a very slow reaction with $k_\psi = 3 \times 10^{-4}$ s^{-1}, a reaction that has been attributed[8] to slow permeation of OH^- and subsequent endovesicular hydrolysis[6] of 1. Dithionite ion, which does not readily cross ionic vesicle membranes would react very rapidly with *exovesicular* 1.[7,8]

Next, successive aliquots of 18_2-encapsulated 1 were warmed to 42 °C (above T_c) for varying times, quickly cooled (16–18 °C), warmed to 25 °C, and then reacted with dithionite. In each of these experiments, biphasic reactions occurred. We attribute the initial fast (k_ψ = 37 s^{-1}) reaction to exovesicular 1[7,8] that had been restored to the outer vesicle surface during the heating cycle. The subsequent, slow reaction was the same endovesicular hydrolysis described previously. Importantly, the *distribution* of fast to slow reaction, as measured by the absorbance change corresponding to each kinetic phase, was dependent on the sample's residence time at 42 °C.

Figure 2 displays the percent of fast reaction induced in 18_2-encapsulated vesicular 1 as a function of the warming time at 42 °C. Similar behavior is observed with either small or large 18_2 vesicles. Note that ~15 min of incubation at 42 °C is needed to reach an approximate 50/50 distribution of exo- and endovesicular 1, starting from the all endovesicular state. Least-squares correlations of ln (percent fast reaction) vs time are reasonably linear with $k = 1.7 \times 10^{-3}$ s^{-1} (r = 0.993) and $k = 2.1 \times 10^{-3}$ s^{-1} (r = 0.985) for apparent redistribution rate constants in small and large 18_2 vesicles, respectively. These rate constants describe the approach to exo/endovesicular equilibrium of bound 1 in 18_2 vesicles at 42 °C, starting from endovesicular 1. They can be compared with k_{app} ~3–4 s^{-1} for exovesicular/endovesicular equilibration of 1 in 16_2 vesicles, starting from exovesicular 1 at 25 °C.[7]

The mechanism responsible for the redistribution of 1 in the 18_2 vesicles remains uncertain. Possibly, above the T_c of the vesicles, K^+ 1 ion pairs permeate between the inner and outer surfaces, and k describes this process. Alternatively, in view of the tight binding of 1 to cationic surfactant aggregates,[17] it is possible that 1, electrostatically bound to the head group of an endovesicular 18_2 molecule, is "delivered" to an exovesicular site by "flip-flop" of the surfactant.[35] In liposomes below their T_c, transverse or flip-flop motions of the monomers between inner and outer sites can be very slow,[36] occurring on a time scale of hours to days. This is because flip-flop requires energetically expensive transient interactions of the ionic surfactant head groups with the apolar bilayer interior and of the hydrocarbon chains with water.[35] On the other hand, flip-flop rates are expected to increase at the vesicular T_c, where the highly ordered "gel" phase relaxes to the less ordered, more fluid liquid crystalline phase.[31,35] Presently, we cannot differentiate between the permeation or flip-flop mechanisms for the thermally induced endo to exovesicular redistribution of 1 in 18_2 vesicles. However, analogous experiments with *functional* surfactant vesicles related to 18_2 demonstrate the operation of the flip-flop mechanism.[37]

Conclusions

DODAC (18_2) vesicles can sequester Ellman's reagent (1) in distinct exovesicular and endovesicular sites. Below the T_c of the 18_2 vesicles,[26] these populations of bound 1 can be kinetically differentiated by quantitative cleavages with various thiols to chromophoric anion 2. Exovesicular 1 reacts very rapidly, but endovesicular 1 reacts much more slowly, with (thiol) permeation-limited rates. Differences in thiol acidity and structure influence the rates of permeation and reaction, with thiophenol and thionaphthol the most permeant and reactive reagents, and thiocholesterol (TC) the least permeant and reactive. Small quantities of covesicallized 1-hexanol lower the T_c of the 18_2 vesicles, markedly enhance their fluidity, and so accelerate the reaction of TC and 1. Exovesicular/endovesicular kinetic distinction is thus lost. Finally, incubation of 18_2 vesicles at 42 °C (above T_c) brings about endovesicular to exovesicular redistribution of initially encapsulated 1, either by enhanced permeation of 1 in the more fluid liquid crystalline vesicles, or by enhanced transverse (flip-flop) motion of 18_2–1 surfactant–substrate ion pairs.

Experimental Section

Materials. The surfactants, dioctadecyldimethylammonium chloride,[8] dihexadecyldimethylammonium bromide,[7] and cetyltrimethylammonium bromide[7] were available from previous studies, where their purities were documented. Reagents that were used as received include DL-cysteine hydrochloride monohydrate (Aldrich, 99%), glutathione (Sigma, 98–100%), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, Aldrich, 99%), 8-anilino-1-naphthalene ammonium sulfonate (Aldrich, 97%), 1,6-diphenyl-1,3,5-hexatriene (Aldrich, 98%), 1-butanethiol (Aldrich, 99%), 1-octanethiol (Aldrich, 99%), thiocholesterol

(35) For discussions of dynamic processes in liposomes and vesicles, see: Fendler, J. H. *Membrane Mimetic Chemistry*; Wiley: New York, 1982; Chapter 6, especially p 145*f*. See also ref 31, especially p 110*f*.

(36) Kornberg, R. D.; McConnell, H. M. *Biochemistry* **1971**, *10*, 1111. Litman, B. J. *Biochemistry* **1973**, *12*, 2545. Verkleij, A. J.; Zwaal, R. F. A.; Roelfsen, B.; Comfurius, P.; Kastelijn, B.; van Deenen, L. L. M. *Biochim. Biophys. Acta* **1973**, *323*, 178. Barden, J. A.; Barker, R. W.; Radda, G. K. *Ibid.* **1975**, *375*, 186. Galla, H. J.; Theilen, U.; Hartmann, W. *Chem. Phys. Lipids* **1979**, *23*, 239.

(37) Moss, R. A.; Bhattacharya, S., unpublished work.

(Aldrich),[38] thiophenol (Aldrich, 99%), and 2-thionaphthol (Aldrich, 99%). Buffer and vesicle solutions were prepared from "steam-distilled" water (distilled, U.S.P., Electrified Water Co., Newark, NJ).

Stock solutions (~0.01 M) of thiols and Ellman's reagent were prepared in EtOH (or in THF for thiocholesterol). These solutions were purged and maintained under nitrogen and stored in the dark. Fresh stock solutions were usually prepared on alternate days.

Vesicle Preparation. All vesicle solutions were created in degassed 0.01 M aqueous KCl at pH 6. *Small vesicles* were generated by sonication with the 108 × 19 (diameter) mm probe of Braunsonic Models 1510 or 2000 sonicators, operated at 40 W. Sonication was carried out at 50 °C for 15 min (Model 1510) or for 6 min (Model 2000). Both procedures gave comparable vesicles (dynamic light scattering). The vesicle solutions were allowed to cool slowly to 25 °C and then filtered through 0.8 μM Millipore filters before use. The vesicle size was 700–800 Å by dynamic light scattering.[39]

Large vesicles were generated by slow (1 mL/h) injection using a Sage Instrument Model 341A syringe pump. Typically, 1 mL of 1×10^{-3} M surfactant in $CHCl_3$ was injected into 20 mL of buffer or water at 68–70 °C. Nitrogen was continuously bubbled through the solution during injection to facilitate the removal of the chloroform. After cooling to 25 °C, dynamic light scattering gave the apparent hydrodynamic diameter of these vesicles as 3000 ± 500 Å.

Kinetic Studies. Faster reactions were followed on a Durrum/Dionex Model D-130 stopped-flow spectrophotometer coupled either to a Tektronix Model 5103N storage oscilloscope or, via a custom-built interface, to a Commodore Model 8032 computer. Slower reactions were monitored on a Gilford Model 250 spectrophotometer coupled to a Gilford Model 6051 recorder. Rate constants were obtained from computer-generated correlations of log $(A_\infty - A_t)$ with time. Temperature (±1 °C) was controlled by a circulating-water bath.

Rate constants are tabulated in Tables I–III. All reactions or reaction phases were followed to >90% completion and showed good first-order kinetics ($r > 0.998$). Reproducibilities of the rate constants were better than ±3% in micellar or buffer solutions. Reproducibilities of ±5% were observed in vesicular kinetics runs when the experiments used vesicle solutions derived from the same vesicle preparation. Kinetic reproducibility was poorer (with deviations up to 20% in k_{obsd}) when different vesicle preparations were employed in repetitive runs.

Acknowledgment. We are grateful to the U.S. Army Research Office and to the Charles and Johanna Busch Fund (Rutgers University) for financial support. We thank Professor Larry Romsted for helpful discussions.

(38) Aldrich thiocholesterol (95–100%) had mp 93–94 °C (uncorrected) Aldrich catalogue, 1988–89, gives mp 97–99 °C. TLC on precoated silica gel-polyester gave a single spot, R_f 0.62, when developed with 1:5 $CHCl_3$/MeOH, containing 1% glacial acetic acid.

(39) Experimental details for light scattering appear in ref 8.

Acid-Catalyzed Reactions of Hapalindoles

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Tetracyclic hapalindole isonitriles such as hapalindoles A (**1**), G (**2**), H (**3**), and I (**4**) are converted to the corresponding formamides (**5–8**) with 90% aqueous formic acid at 0 °C or amines (**9–11**) with ethanolic hydrochloric acid at reflux. The tricyclic hapalindole isonitriles C (**12**) and E (**13**), however, give predominantly formamides (**14** and **16**) on formic acid treatment and dihydro-β-carbolines (**15** and **17**), resulting from nucleophilic condensation of the isonitrile carbon at the indole C-2, on strong acid treatment. Treatment of the tricyclic hapalindole C formamide with strong acid leads to a hexahydroindeno[2,1-*b*]indole amine (**21**) as well as hapalindole C amine (**18**), but similar treatment of hapalindole E formamide leads only to hapalindole E amine (**19**). The tetracyclic hapalindole isothiocyanate B (**23**) is recovered unchanged on treatment with ethanolic hydrochloric acid, but hapalindole isothiocyanates D (**24**) and F (**30**) readily cyclize to a mixture of γ-thiolactams (**25–29** and **31–35**) resulting from trans addition of the isothiocyanate and isopropenyl groups to the indole Δ^2 double bond; since the electrophilic addition is initiated by the isopropenyl group, an octahydro-7*H*-benzo[*c*]carbazole is formed.

The hapalindoles are responsible for the antibacterial,[1] antimycotic,[1] and antialgal[2] activities associated with the terrestrial blue-green alga *Hapalosiphon fontinalis* (Ag.) Bornet (Stigonemataceae). All of the hapalindoles that have been isolated and identified to date are isonitriles and isothiocyanates.[1] Formamides and amines, which sometimes accompany isonitriles and isothiocyanates in other isonitrile-producing organisms,[3] have not been found in *H. fontinalis* (strain V-3-1), ATCC 39694). The resemblance of the hapalindoles to the ergot alkaloids, however, prompted us to prepare the corresponding formamides and amines for pharmacological evaluation.[4] During the course of our work, the tricyclic hapalindoles were found to un-

(1) Moore, R. E.; Cheuk, C.; Yang, X. G.; Patterson, G. M. L.; Bonjouklian, R.; Smitka, T. A.; Mynderse, J. S.; Foster, R. S.; Jones, N. D.; Swartzendruber, J. K.; Deeter, J. B. *J. Org. Chem.* **1987**, *52*, 1036.

(2) In a preliminary communication [Moore, R. E.; Cheuk, C.; Patterson, G. M. L. *J. Am. Chem. Soc.* **1984**, *106*, 6456] we reported that our strain of *Hapalosiphon fontinalis* produces an extracellular substance that inhibits the growth of microalgae, including other blue-green algae. The major antialgal substance in this cyanophyte appears to be hapalindole A, which shows antialgal activity in a disk assay (10 μg per 7 mm disk) on an agar plate against *Chlorella vulgaris* and *Aphanocapsa* sp. ATCC 27184.

(3) (a) Sullivan, B. W.; Faulkner, D. J.; Okamoto, K. T.; Chen, M. H. M.; Clardy, J. *J. Org. Chem.* **1986**, *51*, 5134. (b) Gulavita, N. K.; de Silva, E. D.; Hagadone, M. R.; Karuso, P.; Scheuer, P. J.; Van Duyne, G. D.; Clardy, J. *J. Org. Chem.* **1986**, *51*, 5136.

(4) The hapalindole formamides and amines possessed markedly reduced antibacterial and antifungal activity in vitro than do the naturally occurring isonitriles and isothiocyanates. In addition, all compounds displayed a very weak ability to block binding of the appropriate agonists to both the serotonin and dopamine central nervous system receptors in vitro and failed to inhibit prolactin release in vivo when compared to the classic ergot alkaloid-derived drugs. These latter tests were performed under the guidance of J. A. Clemens and N. R. Mason in the CNS Research Division of Lilly Research Laboratories. For a review and leading references on CNS agents, see: *Annual Reports in Medicinal Chemistry*; Bailey, D. M., Ed.; Academic Press: New York, 1985; Vol. 20, Sect. I, Chapters 1–7, pp 1–60.

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