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Insertion of amphiphilic molecules into membranes is catalyzed by a high molecular weight non-ionic surfactant

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We have employed an amphiphilic fluorescent probe to elucidate the mechanism by which a class of oxyethylene-oxypropylene copolymers catalyzes the insertion of hydrophobic or amphiphilic molecules into membranes. The rate of binding can be accelerated by over two orders of magnitude in the presence of the catalyst which does not itself disrupt the lipid bilayer. The rate of probe binding to lipid vesicles does not depend on the lipid concentration in the presence or absence of catalyst but is linearly related to the concentration of the catalyst. Probe binding to the polyol surfactant appears to be a component of the catalytic mechanism and equilibrium binding parameters can be determined; these are used to indirectly establish quantitative binding parameters for the probe to the vesicle membrane. The polyol surfactant is also shown to catalyze insertion of the probe into the outer leaflet of a hemispherical lipid bilayer and the plasma membrane of HeLa cells. The latter were also stained by catalyzed transfer of a fluorescent lipid from lipid vesicles. The permeability of the cell membrane is not significantly altered under any of the catalytic conditions. These data, taken together, suggest that the polyol surfactant extracts a monomeric substrate molecule from its aggregate or microcrystal and passes it to the membrane via a loose and transient contact.

Introduction

Surfactants have become increasingly important in pharmaceutical technology because of their ability to solubilize water-insoluble drugs. Solubilization is, of course, a prerequisite for transport of the drug to the target tissue, but a general danger of the use of surfactants is their tendency to disrupt cell membranes. It is well-known that most surfactants seem to bind to membranes even at low concentrations, and such binding affects the membrane properties in many ways; it can cause alterations in the permeability of the membrane, or at higher surfactant concentrations, more drastic effects such as membrane lysis and fusion [1,2]. The low toxicity of some oxyethylene oxypropylene copolymers toward biological membranes was reported by Nissim [3]. It has been shown that many of the PLURONIC® polyols (F38, F68, F77, F108, F127) do not cause human erythrocyte hemolysis at reasonable concentrations; F68 does not induce lysis even at saturating concentrations [4–6].

The applicability of oxyethylene-oxypropylene copolymers as vehicles for insertion of drugs into

Abbreviations: F127, PLURONIC® F127; MLV, multilamellar lipid vesicles; GUV, giant unilamellar vesicles; DMEM, Dulbecco's modified essential medium; EBSS, Earle's balanced salt solution; FITC-PE, *N*-(fluorescein-5-thiocarbamoyl)dipalmitoyl-L-α-phosphatidylethanolamine; Di-10-ASPPS, 3-[4-(*p*-*N*,*N*-didecylaminostyryl)-1-pyridinium]-propylsulfonate; HB, hemispherical lipid bilayer.

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membranes has been demonstrated with tetrahydrocannabinols, which are not readily soluble in water [7]. Tetrahydrocannabinol was applied to nervous tissue by dissolving it with PLURONIC® F68 polyol in ethanol; the drug binding is accelerated without any apparent surfactant-induced membrane damage. Another oxyethylene oxypropylene detergent, PLURONIC® F127 polyol (F127), was used successfully in studies of axon fluorescence with a relatively water-insoluble merocyanine dye [8]. F127 was added to an ethanol solution of dye, and this mixture was used to stain squid giant axon. Subsequently, these workers used this surfactant routinely in their studies of voltage dependent optical signals from axons stained with a variety of dyes [9–11]; again, no membrane damage was noted.

Detergents can be used to dissolve membrane proteins or lipids which can then be reconstituted in cell free systems upon removal of the detergent by dialysis or dilution to below the critical micelle concentration [12–15]. Recently a detergent was employed at very low concentration to catalyze the insertion of a water soluble channel forming protein into planar lipid bilayers [16]. It occurred to us that it may be possible to devise a reconstitution scheme based on the PLURONIC® polyols, which does not require removal of the surfactant; this goal will first require a thorough characterization of the mechanism by which these polymers promote insertion or removal of molecules from a lipid bilayer. In this work, we employ the potential sensitive membrane probe di-10-ASPPS [17,18] to define the catalytic effect of these surfactants on the insertion of amphiphilic molecules into the lipid bilayer. It was reported by Gibson and Loew [17] that di-10-ASPPS binds very strongly to the membrane but requires long incubation time to achieve a reasonable level of binding (half-life of 10 hours under their conditions). This slow rate, together with the strong fluorescence enhancement of this lipid analogue upon binding, makes it an ideal probe for investigating the mechanism of binding catalysis by F127. In addition, we have explored the ability of this surfactant to promote the benign insertion of lipid molecules into tissue culture cells. The results presented here may provide a basis for new membrane reconstitution schemes.

Experimental procedures

Di-10-ASPPS was synthesized by the aldol condensation procedure as described by Hassner et al. [19]. Oxyethylene oxypropylene polymer F127 was supplied by BASF Wyandotte Chemicals Corp (Wyandotte, MI). Egg phosphatidylcholine (type XI-E), trypan blue, valinomycin and gramicidin were obtained from Sigma Chemical Co. (St. Louis, MO). Rhodamine-6G (laser grade) was obtained from Eastman Organic Chemicals (Rochester, NY); 3,3′-diethylthiodicarbocyanine iodide, di-S-C_3(5), and FITC-PE were obtained from Molecular Probes, Inc. (Junction City, OR). Buffers were prepared from double-glass distilled water. Oxidized cholesterol was prepared according to the procedure described by Tien [20].

Liposomes. Multilamellar liposomes (MLV) were prepared by adding 1 ml of 'K buffer' to 20 mg of egg phosphatidylcholine. 'K buffer' consisted of 50 mM K_2SO_4, 1 mM EDTA and 10 mM Hepes (pH 7.3). The suspension was then vortexed for 10 min and sonicated in a bath-type sonicator (Laboratory Supplies, Hicksville, NY) for 30 min under a nitrogen atmosphere at 0 °C.

Giant liposomes (GUV) with diameters in the 5–50 μm range were prepared by dialyzing the chaotropic ion trichloroacetate from an aqueous lipid suspension [21]. The liposome interior contained 'K′ buffer', which consisted of 100 mM KCl, 100 mM Mops and 0.01 mM EDTA (pH 7.2). 'Na′ buffer' was identical except that NaCl replaced KCl.

Dye binding. Kinetics of di-10-ASPPS binding to MLV were followed by fluorescence using a Perkin-Elmer Model MPF 44B fluorescence spectrometer. Binding of the dye in the presence or absence of F127 can be followed by fluorescence since the three environments (free dye in the aqueous solution (w), dye bound to F127 (p), dye bound to vesicles, (v), impart different fluorescence efficiencies, Q, to the dye total fluorescence, F.

$$F = [\text{dye}]_w Q_w + [\text{dye}]_p Q_p + [\text{dye}]_v Q_v \quad (1)$$

Binding rates of the dye to vesicles were monitored by the large fluorescence enhancement for bound dye (Q_p or Q_v) vs. free dye (Q_w). The

fluorescence was determined at wavelengths corresponding to maximal excitation (467 nm) and emission (595 nm) of the vesicle-bound form. To obtain equilibrium binding parameters, aliquots of F127, from a 50 mg/ml solution in ethanol (the final levels of ethanol did not affect the rate or extent of binding in controls), were used to titrate a 0.5 μM aqueous solution of the dye. To determine vesicle binding constants, aliquots of MLV containing 2 mg/ml or 20 mg/ml lipid were used to titrate 0.5 μM aqueous solution of the dye in the presence of 5 mg/ml F127. These titration data were fit to a one-site saturable binding model, Eqn. 2 [22,23], via nonlinear least-squares analysis.

$$F_c = F_0 + c(F - F_0)/(c + K_d/n) \quad (2)$$

F_c is fluorescence at the titrant concentration c, and K_d/n is the equilibrium constant for dissociation of probe from the binding site per number of binding sites.

Hemispherical lipid bilayers (HB) composed of oxidized cholesterol (30 mg/ml) were used to study the kinetics of binding by monitoring the potentiometric response of di-10-ASPPS. The apparatus and methodology used for measuring the potential-dependent spectroscopic responses of the probe when bound to HB have been described [18,23,24]. HB were formed in a 1 M KCl bathing solution and di-10-ASPPS (5 μM) was introduced with or without F127 on one side of the membrane. Kinetics of dye binding to HB was followed by the development of a transmitted light response, ΔT, to a 100 mV voltage-clamp pulse at 430 nm or 510 nm.

Cell staining. HeLa cells were grown in DMEM (Gibco) on 22 × 30 mm glass cover slips in 60 ml petri dishes. They were gently washed three times with 2 ml EBSS (Gibco) and cells were removed from the bottom side of the cover slip with a Kimwipe. Cells were then bathed in 1.5 ml of EBSS containing either 10 μM di-10-ASPPS, 0.5 mg/ml F127 or 10 μM FITC-PE, 2 mg/ml F127. In the case of di-10-ASPPS, a stock solution of 1 mM dye and 50 mg/ml F127 in ethanol was injected into the EBSS; for FITC-PE, a stock aqueous dispersion was prepared by sonication of 0.5 mM labeled lipid and 100 mg/ml F127 in EBSS. After staining for 10 min, the cover slip was washed gently three times with EBSS and inverted onto a glass slide. A Zeiss universal fluorescence microscope was used to examine the cells and obtain the photomicrographs.

Assays of membrane damage. The procedure of Sims et al. [25] was used to monitor diffusion potentials across the GUV membranes. A 10 μl aliquot of the liposome suspension (15 mg/ml PC) was added to 3 ml of Na$^+$ buffer containing 1 μM of the voltage sensitive dye di-S-C_2(5). The fluorescence response of the dye, first to the formation of a diffusion potential by valinomycin (0.05 nM) across vesicles, and then to the introduction of gramicidin (0.1 μM), was monitored with excitation at 620 nm and emission at 670 nm. The stability of the diffusion potential in the presence of various concentrations of F127 (0.25–2 mg/ml) was used to assess the possibility of detergent-induced permeabilization of the vesicles [26].

The viability of HeLa cells in the presence of F127 was assessed by trypan blue exclusion [27]. Cell suspensions were washed twice with EBSS before staining with 0.4% trypan blue. The relative number of stained cells were counted on a hemocytometer.

Results

Binding kinetics

The kinetics of dye binding to MLV were determined for various lipid : dye molecular ratios (R) at a fixed dye concentration of 0.5 μM. Binding is accompanied by a dramatic enhancement of the fluorescence intensity corresponding to an approx. 1000-fold increase in quantum yield [23]. Kinetic curves are shown in Fig. 1 and suggest that dye binding is independent of lipid concentration. Time-constants for nonlinear least-squares fits of single exponential to the data were evaluated to change in the range 129.6 min for $R = 200$ to 75.5 min for $R = 6400$. These small fluctuations in the rates may be due to the irreproducibility of the MLV preparation with respect to vesicle size distribution and concentration and variability in the degree of dispersion of the dye which is likely to be present in solution as molecular aggegates or microcrystals.

Dye binding to the lipid vesicles can be accel-

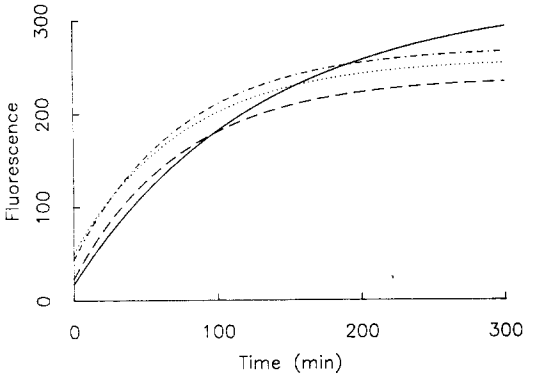

Fig. 1. Kinetics of di-10-ASPPS binding to lipid vesicles at different lipid:dye ratios, R, followed by fluorescence. Lipid vesicles were added at time = 0 to 0.5 μM dye. Time constants for single exponential fits to the data were: 129.6 min for $R = 200$ (———), 76.2 min for $R = 1000$ (– – –), 77.4 min for $R = 3000$ (·····), and 75.5 min for $R = 6400$ (·–·–·). The fluorescence scales in this and all other figures are in arbitrary units and should not be compared to each other.

erated in the presence of F127 (Fig. 2). Time constants for single exponentials fit to the data were evaluated to be 4.2 min for 0.031 mg/ml F127, 2.6 min for 0.062 mg/ml F127 and 1.25 min for 0.125 mg/ml F127 ($R = 200$). Thus, F127 is shown to increase the rate of di-10-ASPPS binding to the membrane by two orders of magnitude and the acceleration is proportional to the polyol concentration. The rate of dye binding to the lipid membrane in the presence of F127 again does not depend on the lipid vesicle concentration; a variation of only a factor of 2 was observed for a 15-fold range of lipid concentration. The kinetics of di-10-ASPPS binding to a hemispherical lipid bilayer is also accelerated by F127. The relative transmittance response for HB stained with di-10-ASPPS indicates a bathochromic shift in the absorption spectrum for a hyperpolarizing voltage pulse with a maximum response at 510 nm [18]; therefore, the transmittance change, ΔT, was recorded at 510 nm in order to follow the kinetics of binding. Kinetic curves were recorded when di-10-ASPPS with or without F127 in ethanol was introduced to the stirred solution (final concentration 5 μM dye, 0.25 mg/ml F127) on the outside of the HB (the final concentration of ethanol never exceeded 1%, and control experiments showed that this level of ethanol did not significantly affect the rate of binding). Again, F127 accelerates dye binding to the hemispherical lipid bilayer (Fig. 3). In addition, this experiment demonstrates that F127 catalyzed binding is confined to the outer lipid leaflet of the bilayer; random insertion into both leaflets would not result in a

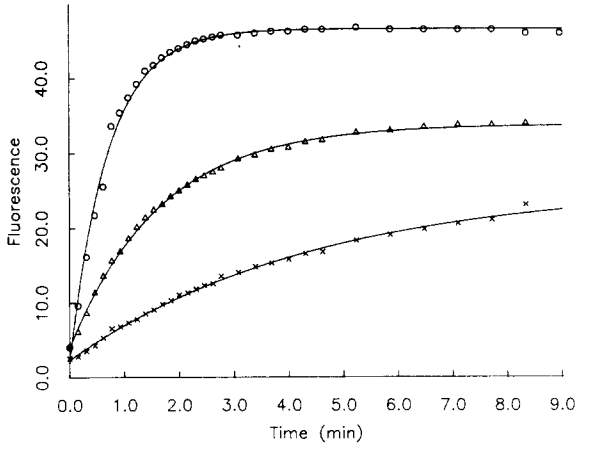

Fig. 2. Kinetics of di-10-ASPPS binding to lipid vesicles in the presence of oxyethylene oxypropylene surfactant F127 ($R = 200$): ×, 0.031 mg/ml; △, 0.062 mg/ml; ○, 0.125 mg/ml F127. Lipid vesicles were added at time = 0 to 0.5 μM dye. Solid curves represent nonlinear least-squares fits of a single exponential to the data. Time constants were calculated to be 4.23 min for 0.031 mg/ml, 2.59 min for 0.062 mg/ml and 1.25 min for 0.125 mg/ml of F127.

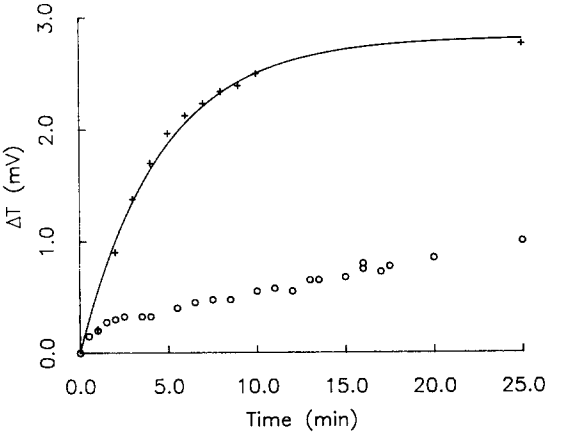

Fig. 3. The development of a spectral response to a voltage-clamp pulse applied across a hemispherical lipid bilayer. The transmitted light response, T, was monitored following the addition of 5 μM di-10-ASPPS to the external medium with (+) or without (○) 0.25 mg/ml F127. The catalyzed rate was fit to a single exponential (solid line) with a time constant of 4.6 min.

spectroscopic response to the applied voltage.

HeLa cells were stained for 10 min with 10 μM di-10-ASPPS in the presence of F127 (0.5 mg/ml) to produce the fluorescence photomicrograph shown in Fig. 4. Notice the 'ring' stain pattern typcial for fluorescent dyes bound only to the plasma membrane. Our attempts to stain HeLa cells with di-10-ASPPS in the absence of F127 for up to 1 hour gave no detectable fluorescence. We have also successfully stained HeLa cells with the

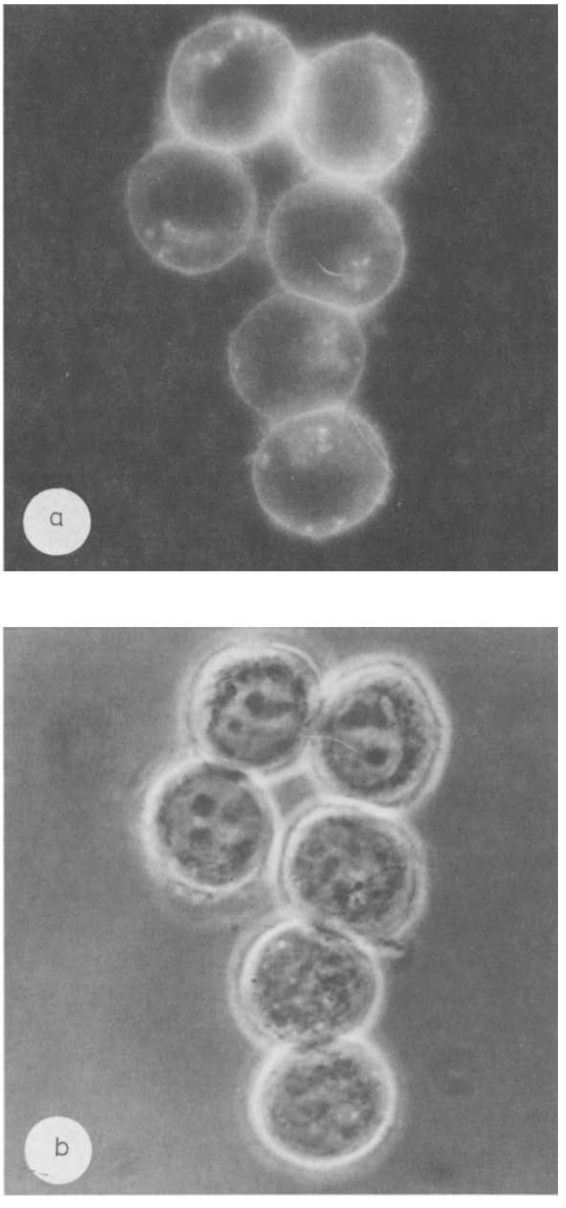

Fig. 4. (a) Fluorescence photomicrographs of HeLa cells stained with 10 μM di-10-ASPPS in the presence of 0.5 mg/ml F127 for 10 min. Attempts to stain the cells in the absence of F127 gave no visible membrane fluorescence. (b) Phase contrast photo of the same field. 1 cm ≡ 6.7 μm.

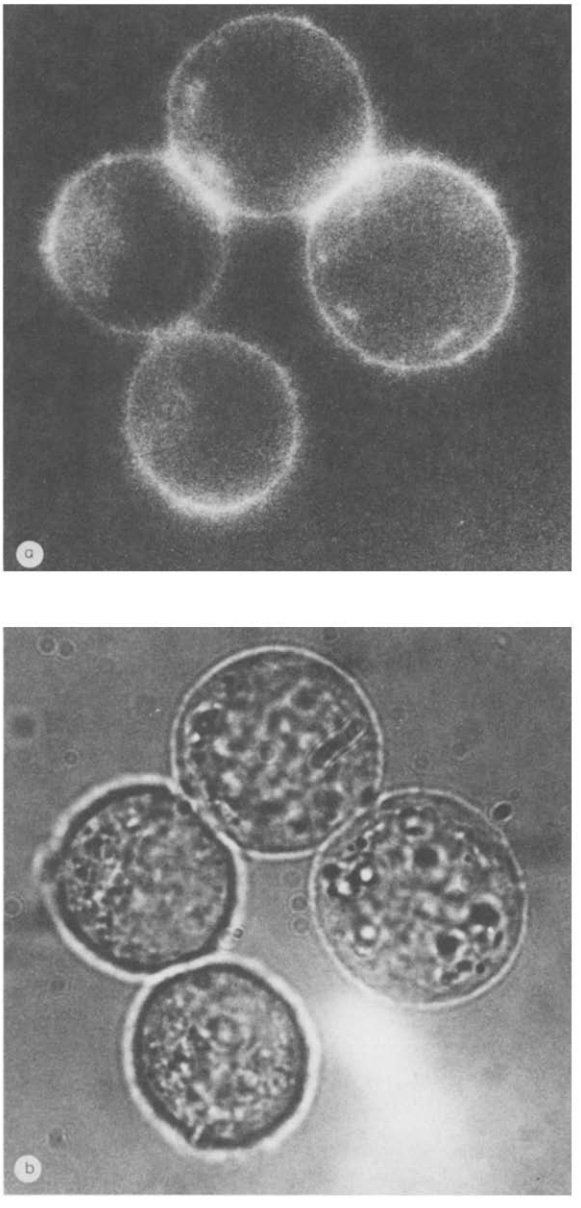

Fig. 5. (a) Fluorescence photomicrographs of HeLa cells stained with 10 μM of *N*-(fluorescein-5-thiocarbamoyl)-dipalmitoyl-L-α-phosphatidylethanolamine (FITC-PE) and 2 mg/ml F127 for 10 min. Staining was only possible in the presence of F127. (b) Phase contrast photo of the same field. 1 cm ≡ 4.4 μm.

fluorescent lipid FITC-PE (Fig. 5). Staining again was possible only in the rpesence of F127. The FITC-PC is dispersed by sonication to form lipid vesicles. The turbid suspension is not clarified by up to 100 mg/ml F127; the surfactant appears, therefore, to catalyze the transfer of the labeled lipid from the vesicle to the cell without solubilizing either membrane. We cannot exclude the possibility that F127 catalyses fusion of the FITC-PE vesicles with the cell membrane instead of the transfer of individual FITC-PE molecules. However, punctate membrane staining, which might be expected from a fusion mechanism, was never observed.

Equilibrium binding

It has been shown [23] that increases in the length of the hydrophobic side chains on the aniline nitrogen of ASP probes (and conjugated homologs) result in tighter binding of the probe to the membrane. Di-10-ASPPS has a rod shaped chromophore with two decyl chains at one end and a fixed negative charge at the other. It is very difficult to titrate di-10-ASPPS with lipid vesicles and to determine the binding parameter K_d/n for the probe directly using Eqn. 2 because of the very slow process of dye binding to the membrane. This parameter provides a quantitative measure of the relative affinities of the probe to the membrane and was determined for other members of the ASP series of probes by Fluhler et al. [23].

This problem can be overcome by taking advantage of the F127 binding catalysis, if one can separate the equilibria which may be involved in the catalytic mechanism. Indeed, by employing high concentrations of F127, binding of the dye to the surfactant and transfer of the dye from the surfactant to MLV can be independently studied. The binding parameter for di-10-ASPPS can then be calculated indirectly on the basis of the cycle:

$$\begin{array}{ccc} [\text{dye}]_v & \overset{K_3}{\rightleftharpoons} & [\text{dye}]_p \\ & {\scriptstyle K_2}\searrow \quad \swarrow{\scriptstyle K_1} & \\ & [\text{dye}]_w & \end{array} \qquad (3)$$

where $[\text{dye}]_w$ = concentration of free dye in aqueous solution, $[\text{dye}]_v$ = concentration of vesicle-bound dye, $[\text{dye}]_p$ = concentration of F127-bound dye.

$$K_2/n_v = (K_3 n_p/n_v)(K_1/n_p) \qquad (4)$$

Di-10-ASPPS titrations with F127 and with lipid vesicles in the presence of F127 (5 mg/ml) are shown in Fig. 6. The results show that $Q_p > Q_v$ (cf. Eqn. 1). Titration data were fit to a one site saturable binding model (Eqn. 2). This model holds for these titrations because the titrants, even

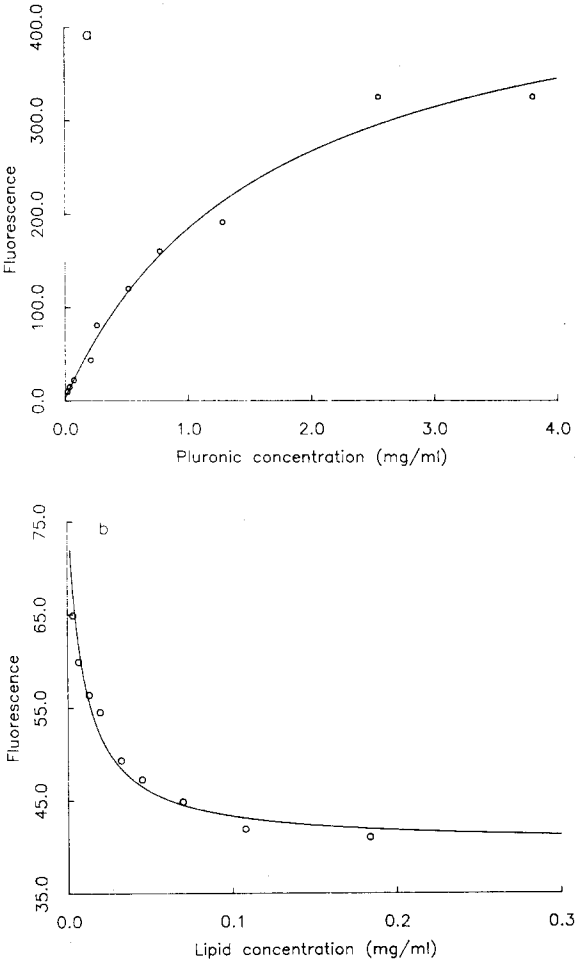

Fig. 6. Di-10-ASPPS titration: (a) with F127; (b) with lipid vesicles in the presence of 5 mg/ml F127. The fluorescence was determined at wavelengths corresponding to maximal excitation (467 nm) and emission (595 nm) of the vesicle-bound form; data were corrected for dilution and fit to a one-site saturable binding model (Eqn. 2) via nonlinear least-squares analysis (solid curves).

at the start of the curves, are at levels higher than 10-times those of the probe; therefore, the binding sites are never filled and essentially do not interact (see Nichols and Pagano [28] for a more thorough treatment of this kind of binding). The binding parameters K_1/n_p and $K_3 n_p/n_v$ were determined to be 1.68 mg/ml and $2.35 \cdot 10^{-3}$, respectively. Thus by Eqn. 4: $K_2/n_v = 3.9 \cdot 10^{-3}$ mg/ml. The values of this binding parameter obtained for di-10-ASPPS is lower than any of the directly determined values obtained for the ASP probes and is consistent with the idea that the binding constant $(1/K_2)$ increases with increasing chain length of the hydrocarbon tails of the aniline nitrogen.

Does F127 perturb the membrane structure? In order to determine if F127 disrupts the lipid bilayer membrane, we have employed the membrane probe di-S-C_2(5) to monitor the stability of a diffusion potential across the membrane of cell-sized unilamellar vesicles (GUV). This is based on the assay for membrane permeabilizing agents developed by Loew et al. [26,29]. F127 concentrations of up to 2 mg/ml have no discernible effect on the ability of the vesicles to support a diffusion potential, although binding of the dye to the surfactant does change baseline fluorescence levels and complicates a quantitative interpretation of the assay. What can be said is that at least 90% of the vesicles maintain their potential for up to 30 minutes in the presence of the highest concentrations of the polyol surfactant.

We are also concerned with the toxicity of the surfactant to cells. A standard assay of cell viability involves the exclusion of trypan blue form healthy cells [27]. The dye was excluded from 71%, 73%, and 76% of HeLa cells in suspension after a 15 min exposure to 5 mg/ml, 2 mg/ml or no F127, respectively. Thus, the polyol surfactant has a minimal effect on cell viability even at concentrations higher than those used to catalyze drug or dye binding. In addition, we found no significant alteration in the membrane potential of HeLa cells in the presence of F127, as determined from the distribution of a permeant cationic dye, rhodamine 6G [30].

Discussion

The PLURONIC® polyols are a series of polyoxypropylene polyoxyethylene condensates with molecular weight range between 1000 to over 15 000. The polyoxyethylene hydrophilic groups on the ends of the molecule can be varied over a wide range to constitute from 10% to 80% of the final molecule. It is possible to obtain members of this class of surfactants to meet various requirements of molecular weight or hydrophilic-lipophilic balance. F127, in particular, contains 70% polyoxyethylene and has a molecular weight of about 11 500. F127 may be represented by the formula:

$$HO(CH_2CH_2O)_{98}(CHCH_3CH_2O)_{67}(CH_2CH_2O)_{98}H$$

We have shown that this nonionic surfactant greatly enhances the rate of amphiphile binding to membranes.

Di-10-ASPPS, having long hydrocarbon chains on the aniline nitrogen, binds tightly to the membrane. As determined by an indirect method in this paper, $K_2/n_v = 3.9 \cdot 10^{-3}$ mg/ml. This may be compared to the weaker binding of other members of the ASPPS series determined by Fluhler et al. [23] (e.g. $K_2/n_v = 1.5 \cdot 10^{-2}$ mg/ml for di-6-ASPPS). On the other hand, the rate of binding is very slow for di-10-ASPPS compared to its shorter chain analogues. We have noted this pattern of behavior for dialkyl cyanine dyes, which are available with a wide variety of chain lengths, as well. Two experimental observations provide evidence for a suggestion which can reconcile the apparently inconsistent trends in binding rate and binding strength. The time constants for binding of 0.5 μM probe to lipid vesicles were in the range 130 min to 76 min – less than a factor of 2 variation in rate despite a 30-fold change in vesicle concentration. Secondly, the rates actually decrease at higher dye concentrations; Gibson and Loew [17] reported a 10 hour time constant for binding when the probe concentration was 5 μM. These results suggest that di-10-ASPPS exists as a stable aggregate or micelle in aqueous media and that the kinetics of binding to membranes is limited by the dissociation of monomers.

From Figs. 1–3, it can be seen the dye binding to the membrane can be accelerated by a factor of 100 in the presence of 0.125 mg/ml of F127. The rate of dye binding, again, does not depend on the lipid vesicle concentration, but it does depend on

the F127 concentration (Fig. 2). Fig. 6 demonstrates that at high concentrations the surfactant can form stable associations with an amphiphile. At all the concentrations employed in this work, F127 is likely to exist as micelles in solution since the critical micelle concentration of lower molecular weight polyols is reported to be as low as 3 μM [31]. On the basis of our results, the following conclusions can be drawn: the rate limiting step for catalysis is the formation of the F127-probe complex; at very high concentrations of F127, the probe transfer from F127 to lipid vesicles can become rate limiting. These ideas together with those derived from the uncatalyzed kinetics are summarized in Fig. 7. It is likely that more than one molecule of probe can be associated with a F127 molecule or micelle and that conceivably the entire probe aggregate may be complexed. Also, the data do not provide any insights on the precise mode of transfer of the probe from the F127 to the membrane; it may be that the probe dissociates as a monomer from the F127 and then associates with the bilayer, or it is possible that F127 is directly involved as a shuttle vehicle for the probe.

In addition,experiments in which HeLa cells were stained with di-10-ASPPS as well as with the fluorescent lipid FITC-PE (Figs. 4 and 5, respectively), indicate that F127 accelerates binding of lipids as well as lipid-like dye molecules to the plasma membrane of tissue culture cells. It is important to emphasize the observation that FITC-PE is in the form of a vesicle suspension which is not solubilized by the polyol surfactant; thus, F127 is capable of catalyzing lipid transfer from one membrane to another without a gross disruption of the bilayer. We have performed experiments employing giant unilamellar vesicles and HeLa cells to check for a more subtle influence of F127 on membrane structure. Both of the experiments indicate that F127 (up to 2 mg/ml) does not alter the membrane permeability. The extraction from or delivery of molecules to the target membrane appears, therefore, to involve a weak and shallow association of the surfactant with the bilayer surface. It is compelling to speculate on

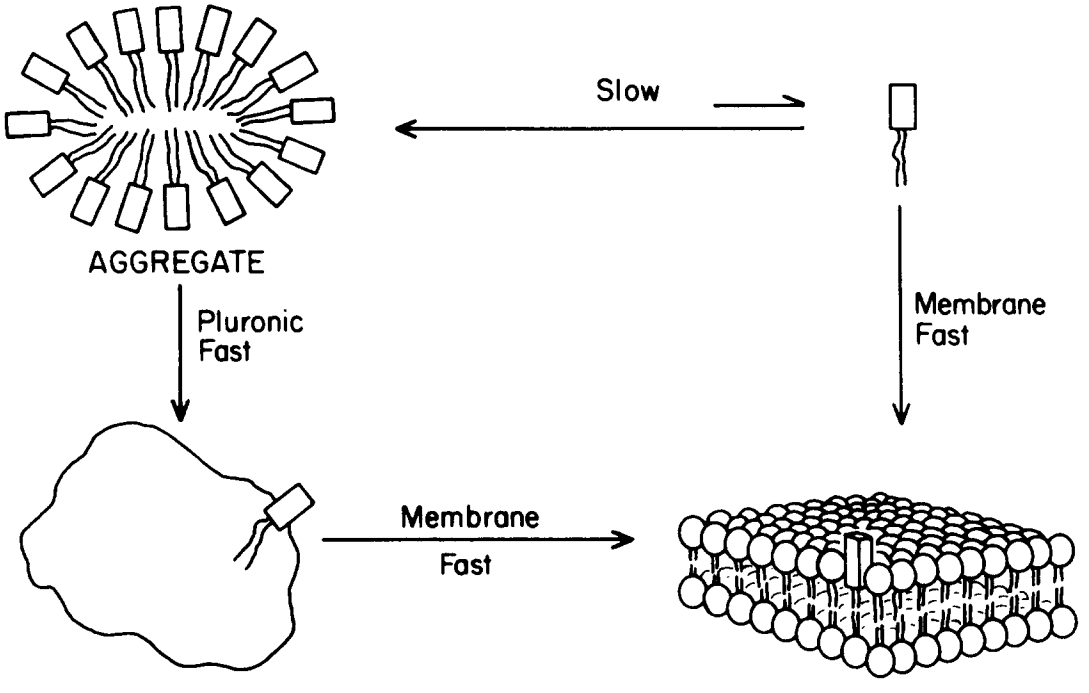

Fig. 7. Proposed mechanism for catalysis of probe binding to a lipid bilayer. Mechanism of PLURONIC catalysis.

possible applications to membrane reconstitution studies where one may hope, for example, to extract a loosely bound integral protein from a cell membrane (at very high polyol concentration) and achieve its incorporation into a model membrane in a single step.

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