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Lipid exchange between mixed micelles of phospholipid and triton X-100

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Abstract

If phospholipase catalyzed hydrolysis of phospholipid dissolved in a detergent mixed micelle is limited to the phospholipid carried by a single micelle, then hydrolysis ceases upon exhaustion of that pool. However, if the rate of phospholipid exchange between micelles exceeds the catalytic rate then all of the phospholipid is available for hydrolysis. To determine phospholipid availability we studied the exchange of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine between mixed micelles of phospholipid and non-ionic Triton detergents by both stopped-flow fluorescence-recovery and nuclear magnetic resonance-relaxation techniques. Stopped-flow analysis was performed by combining mixed micelles of Triton and phospholipid with mixed micelles that contained the fluorescent phospholipid 1-palmitoyl-2-(12-[{7-nitro-2-1,3-benzoxadiazo-4-yl}amino]dodecanoyl)-*sn*-glycero-3-phosphocholine (P-2-NBD-PC). The concentration dependence of fluorescence recovery suggested a second-order exchange mechanism that was saturable. The true second-order rate constant depends on the specific mechanism for exchange, which was not determined in this study, but the rate constant will be on the order of 10^6 to 10^7 $M^{-1}\,s^{-1}$. Incorporation of 1-palmitoyl-2-(16-doxylstearoyl)phosphatidylcholine into micelles increased the rate of proton relaxation and gave a limiting relaxation time of 1.3 ms. The results demonstrate that phospholipid exchange was rapid and that the phospholipid content of a single micelle did not limit the rate of phospholipid hydrolysis by phospholipases.

Keywords: Lipid exchange; Mixed micelle; Phospholipase; Phospholipid; Triton

1. Introduction

Phospholipases are a class of enzymes that hydrolyze phospholipids at one or more of the ester bonds [1–3]. These enzymes are found widely in nature and are diverse in their molecular architecture, site of attack, and mechanism of action. One feature they hold in common, though, is the water insoluble char-

Abbreviations: A, acceptor micelle; D, donor micelle; DM-NBD-PE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazole-4-yl); δ_{mic}, the limiting proton chemical shift in micelles; δ_{mono}, the proton chemical shift of the monomer in dilute solution; δ_{obs}, measured chemical shift; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; M_r, micelle molecular weight; O-2-NBD-PC, 1-oleoyl-2-(12-[{7-nitro-2-1,3-benzoxadiazole-4-yl}amino]dodecanoyl)-*sn*-glycero-3-phosphocholine; OPE-9, 1-(1,1,3,3-tetramethylbutyl)-phenoxy-4-nonaoxyethylene; P-2-NBD-PC, 1-palmitoyl-2-(12-[{7-nitro-2-1,3-benzoxadiazo-4-yl}amino]dodecanoyl)-*sn*-glycero-3-phosphocholine; PDPC, 1-palmitoyl-2-(16-doxylstearoyl)phosphatidylcholine; PX, pre-associated complex; X, phospholipid

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acter of their substrate, the phospholipid. All phospholipases therefore share the property of an interfacial interaction with phospholipid. There are, however, polymorphic states of phospholipid that will influence the enzyme's interaction with the substrate interface. This problem, coupled with the fact that classic enzyme kinetics is based on the use of soluble and not particulate substrates, has complicated the study of phospholipases.

Investigators who study phospholipases have used various approaches to overcome the problems of substrate heterogeneity [4,5]. The goal of these approaches have been to have a uniform interface that allows the development of detail kinetic analysis of phospholipid hydrolysis. Three systems have been used primarily, each having certain advantages yet presenting limitations.

First, single shelled vesicles that are of a defined diameter provide such an interfacial system. The surface concentration of substrate can be estimated for a single vesicle as well as for the entire suspension of vesicles. Jain and coworkers [6] have developed a burst kinetic model for this system that they termed the 'scooting mode'. It is proposed in this model that an enzyme molecule binds to the surface of a vesicle and hydrolyzes the outer monolayer of substrate. The rate is measured as a burst of hydrolysis relative to the secondary rate, 'hopping', in which the enzyme dissociates from the hydrolyzed vesicle, the rate determining step of the process, followed by association with a new vesicle. To accurately measure phospholipid hydrolysis in vesicles it is important that the 'hopping' of the enzyme molecule between vesicles is slow relative to the rate of hydrolysis. Practical considerations for measuring the rate of hydrolysis using vesicles requires setting the ratio of enzyme molecules to the number of vesicles at 1:5 or less, but with sufficient enzyme to convert an easily measurable amount of substrate in a relatively short period of time. A large amount of often precious enzyme is required to achieve these large enzyme to vesicle ratios. This system also requires that the vesicles remain intact and vesicle fusion or other physical changes do not occur.

A second assay system with a well defined interface employs monomolecular films of phospholipid [7,8]. This system allows careful regulation of substrate packing in the film and of the nature of the interface. Lipid mixtures can be used that permit the study of lipid–lipid, and lipid–protein, and protein–protein interactions as they effect interfacial binding and catalysis. Also, by using the appropriate trough, the reaction displays zero-order kinetics, an advantage lacking in other approaches. While this is a very powerful technique, it requires special instrumentation and does have the limitation that relatively little of the enzyme binds to the film.

A third commonly used approach involves the dispersion of phospholipid substrate in detergent to form mixed micelles. A useful model for kinetic analyses has been developed by Dennis and coworkers [9,10] that permits the calculation of interfacial as well as the active-site binding by the enzyme. The advantages of the mixed micellar system are that the amount of enzyme required is less than with pure phospholipid vesicles and that there is greater flexibility in the assay conditions. Drawbacks of the mixed micellar system are complications that arise from the enzyme's affinity for the detergent used and the possibility that substrate and/or enzyme exchange between micelles could be rate limiting. The first problem can be solved by the measurement of enzyme–detergent binding while the second problem remains controversial, but has been measured by detergent–enzyme studies [11]. Studies by our group [12] and by that of Soltys and Roberts [13] argue that the exchange of phospholipid molecules between Triton X-100 micelles is as fast as or faster than the rate of catalysis by the phospholipase under investigation. These estimates of phospholipid exchange rate range from the hundreds of microseconds to a few milliseconds. The group of Jain, Gelb and Berg, on the other hand, have argued that the exchange rate occurs in seconds, which would mean that the rate limiting step in the observed hydrolysis would be substrate exchange, not catalysis. The points raised by this group are based on the studies of Nichols [14] who used bile salt mixed micelles. These arguments are summarized in the 1995 Annual Review of Biochemistry [15].

The purpose of the study presented here is to resolve this controversy over phospholipid exchange rates in mixed micelles. We have studied the exchange rate of phospholipid between Triton X-100 (or OPE-9) micelles using stopped-flow fluorescence

recovery and nuclear magnetic resonance (NMR) techniques. We confirm and extend the previous findings by our group that the rate of phospholipid exchange was much faster than the reported catalytic rates of known phospholipases. The kinetics of fluorescence recovery was second order, suggesting that exchange took place by collisions between micelles, a mechanism for transfer that does not involve dissolution of a naked phospholipid molecule from the micelle into the aqueous medium.

2. Materials and methods

1-(1,1,3,3-Tetramethylbutyl)phenoxy-4-nonaoxyethylene (OPE-9) was prepared by the method of Robson and Dennis [16]. The identity and purity of the product was checked by thin-layer chromatography, gas chromatography–mass spectrometry, and elemental analysis (Atlantic Microlab, Norcross, GA). All reagents were of the highest grade commercially available.

The critical micelle concentration (cmc) of OPE-9 was determined at 25°C in distilled water, and in 50 mM and 200 mM Tris–HCl buffer, using the UV absorption method of Gratzer and Beaven [17]. Absorption data were obtained from either a Hewlett Packard 8452A diode array or a Milton Roy Spectronic 1201 spectrometer.

Stopped-flow fluorescence studies were carried out on a Bio DX-17MV Sequential Stopped-Flow ASVD spectrofluorometer from Applied Photophysics. Samples were irradiated at 460 nm with a 2-mm slit and fluorescence measured at >530 nm through a sharp cut-off filter. The fluorescent reporters, 1-palmitoyl-2-(12-[{7-nitro-2-1,3-benzoxadiazo-4-yl}amino]dodecanoyl)-*sn*-glycero-3-phosphocholine (P-2-NBD-PC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazole-4-yl) (DM-NBD-PE), 1-oleoyl-2-(12-[{7-nitro-2-1,3-benzoxadiazole-4-yl}amino]dodecanoyl)-*sn*-glycero-3-phosphocholine (O-2-NBD-PC), were from Avanti. Studies were carried out at room temperature (24°C) at several different micelle concentrations. The concentration of the fluorescent reporters in mixed micelles of 1,2-dioleyl-*sn*-glycerophosphocholine (DOPC) (Avanti) and Triton X-100 (Fisher Scientific) or OPE-9 was varied to optimize instrument response. Static fluorescence measurements were carried out on an SLM Model 8000 spectrofluorometer.

Proton NMR analyses were recorded on a Bruker AM-300 FT NMR spectrometer at 24°C. Chemical shifts are reported in D_2O (Aldrich) with respect to 0.0038% sodium 3-(trimethylsilyl)propionic acid (Aldrich). Routine NMR spectra, 32 scans of 16-K blocks, were acquired using a $\pi/2$ pulse with a 0.5-s delay. Measurements of T_1 were obtained using a standard inversion–recovery sequence. T_2 measurements were estimated from changes in the peak width at half-height. The spin-label exchange analyses were conducted in 40 mM OPE-9 or Triton X-100 solutions containing 50 mM Tris–HCl, pH 7.5. The concentration of DOPC was 5 mM and the concentration of 1-palmitoyl-2-(16-doxylstearoyl)phosphatidylcholine (PDPC) (Avanti) was varied but never exceeded 70 μM.

The weight average molecular mass, M_r, and Stokes radius, R_s, were obtained by static and dynamic light scattering analyses, respectively, using a Brookhaven Instruments BI-200SM light scattering goniometer equipped with a photon counting detector and a Spectra Physics 127 He–Ne laser as described [18]. Sample scattering intensities were expressed relative to a benzene standard. Sample temperature was maintained at 25 ± 0.1°C with a recirculating bath. Refractive index was measured with a Price–Phoenix differential refractometer. Sucrose solutions were used for reference calibration ($\Delta n/\Delta d = K$).

3. Results and discussion

3.1. cmc

The commercial detergent Triton X-100 is a mixture with an average length of approximately 9.5 oxyethylene units per detergent molecule. To more precisely define the detergent micelles a detergent having nine oxyethylene units per molecule, OPE-9, was synthesized in good yield by the method of Robson and Dennis [16]. The general structure of Triton is shown in Fig. 1 for OPE-9, $n = 8$, along with the NMR spectrum of OPE-9. The cmc was determined from NMR spectra of OPE-9 at several concentrations in 50 mM Tris pH 7.5 (D_2O). Plots of the

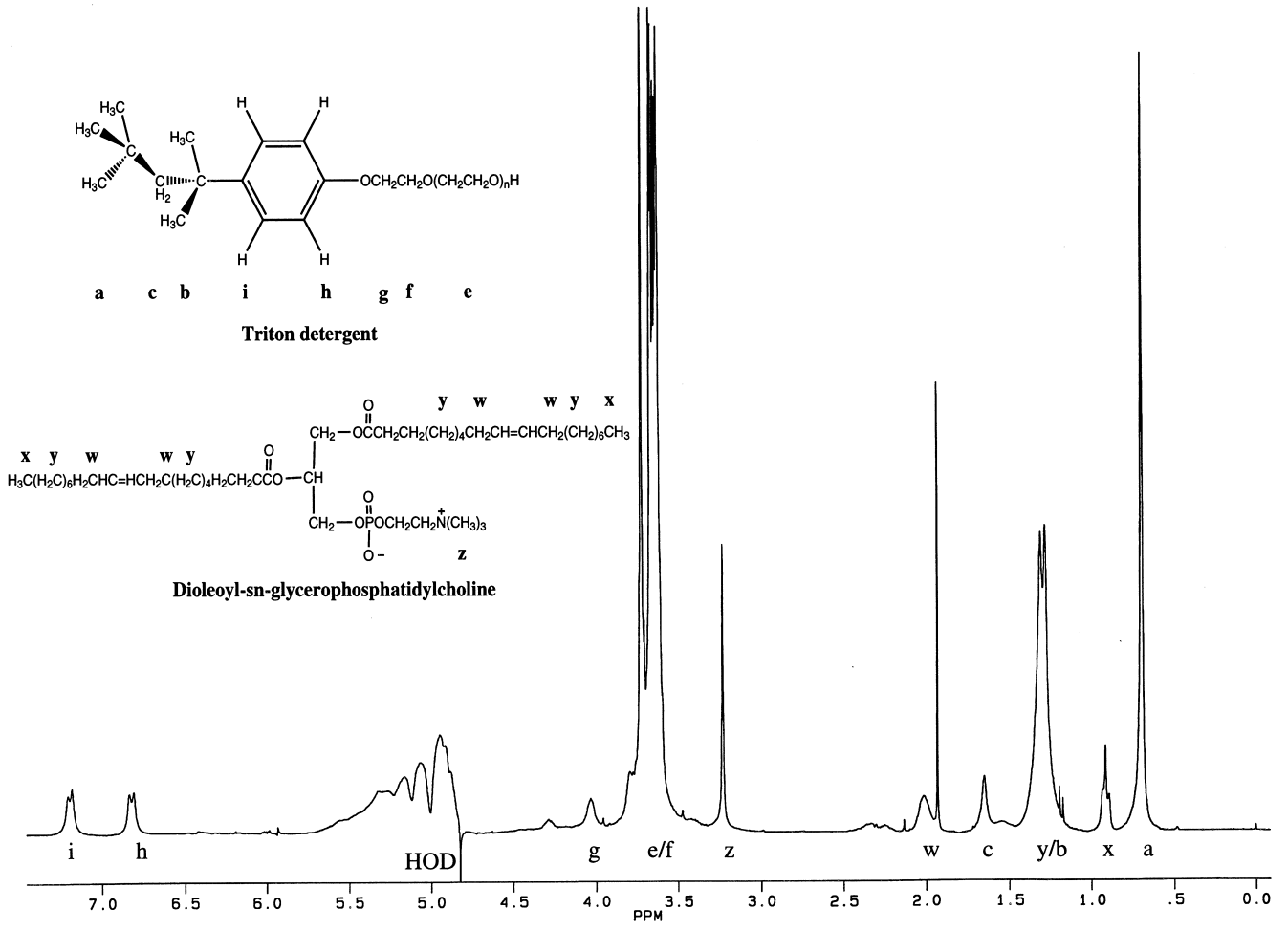

Fig. 1. 300 MHz proton NMR spectrum of 1-(1,1,3,3-tetramethylbutyl)phenoxy-4-nonaoxyethylene (OPE-9) and 1,2-dioleyl-*sn*-glycerophosphocholine (DOPC) mixed-micelle preparation (40 mM and 5 mM, respectively) in D_2O. The structures of OPE-9 ($n=8$) and DOPC are inset in the figure. NMR signals and structural motifs are correlated with letters.

chemical shift, δ_{obs}, for protons a to c versus 1/(OPE-9 concentration), shown in Fig. 2A,B, gave an average cmc of 0.31 ± 0.02 mM. The cmc was taken to be the intersection of the extrapolated lines. Similar plots were made for $1/T_1$ versus 1/(OPE-9 concentration), data not shown, gave a cmc of 0.33 ± 0.01 mM. The cmc of OPE-9 was confirmed by optical studies in three different solvents, distilled water, 50 mM Tris (pH 8.5), or 200 mM Tris (pH 7.4), by the spectrophotometric method of Gratzer and Beaven [17]. Plots of absorption at 276 or 286 nm versus the OPE-9 concentration showed a break point at 0.31 ± 0.04 mM (data not shown) for all of the solvents. The mean value for the cmc determined by the several different methods used in this study, 0.32 ± 0.04 mM, is in excellent agreement with that reported by Robson and Dennis [16], cmc = 0.30 ± 0.02 mM.

3.2. Micelle aggregation number and size

3.2.1. Light scattering: static and dynamic measurements

The molecular mass, M_r, of Triton X-100 micelles was determined in the absence or presence of 50 mM Tris (pH 7.5), using static light scattering techniques. The mean anhydrous M_r of Triton X-100 was 73 ± 4 kg/mol or approximately 120 molecules per micelle over the concentration range of 1.7 mM to 50 mM Triton X-100. Micelles formed by 23.7 mM OPE-9 were somewhat smaller, 63 kg/mol, about 104 molecules per micelle. Kushner and Hubbard [19], using

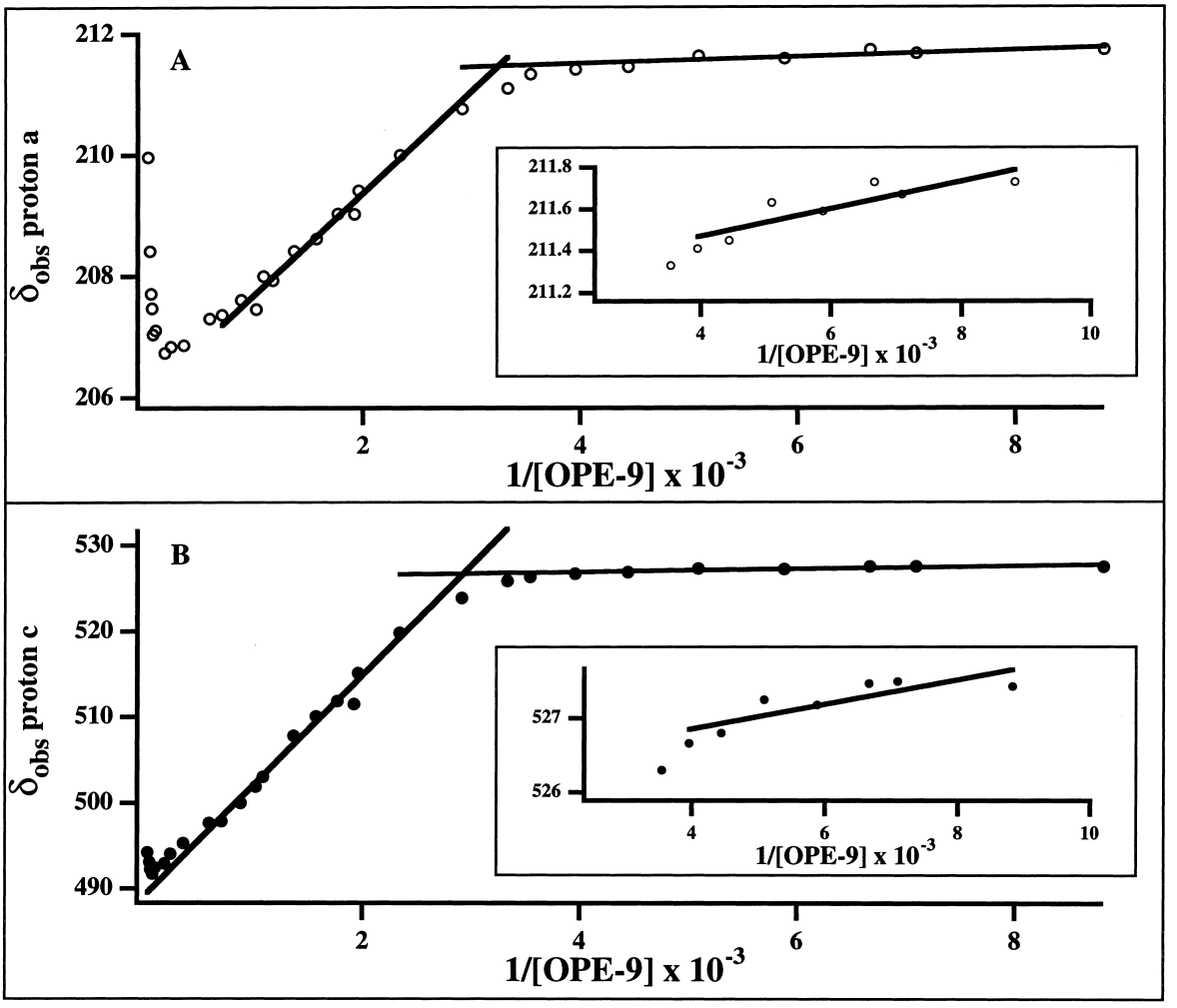

Fig. 2. (A,B) Plots of the changes in chemical shift, δ_{obs}, for protons a and c versus 1/(OPE-9 concentration). The cmc was estimated from the intersection of the extrapolated lines and gave a mean value for the cmc of 0.31 ± 0.02 mM. Values for δ_{mono} and δ_{mic} were obtained from these plots: for proton a δ_{mono} = 211.73 Hz, δ_{mic} = 211.28 Hz for the low concentration region and 206.37 Hz for the higher concentration region; for proton c δ_{mono} = 527.47 Hz, δ_{mic} = 526.26 Hz for the low concentration region and 490.52 Hz for the higher concentration region. Two relationships were obtained from the slopes of the two linear portions of the curves below 4 mM. The first showed a cmc of 0.33 mM while the second indicated a pre-association state at 0.13 mM.

viscometry and turbidometry, reported that the average micelle M_r was approximately 90 kg/mol (140 molecules) at 25°C over a concentration range of 3 mM to 20 mM Triton X-100. Using static light scattering techniques Corti et al. [20] found similar M_rs at 9 mM and 70 mM Triton X-100: 102 kg/mol (158 molecules) and 98 kg/mol (152 molecules), respectively. Ultracentrifugation gave M_rs for Triton X-100 that were in good agreement with the light scattering measurements (summarized by Robson and Dennis [21]). In this study dynamic light scattering measurements gave a mean radius for hydrated Triton X-100 micelles of 59 ± 4 Å. This experimental radius was larger than the average radius, 48 Å, reported by others using the same technique [22,23]. A much smaller radius, 32 Å, was found using gel filtration analysis of OPE-9 micelles [16]. For a spherical particle the Stokes radius, r, and M_r are related by the equation, $4\pi r^3/3 = M_r v/N_o$, where v is the partial reduced volume of OPE-9, 0.91 ml g^{-1} [16], and N_o is Avogadro's number. Triton micelles contain approximately 1.2 g of water per g of detergent [19,22]. Assuming a mean hydrated M_r of 16.1 kg/mol for Triton-X100 micelles, the particles would

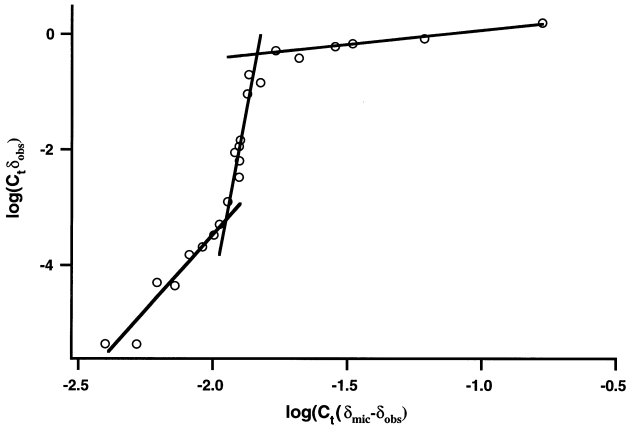

Fig. 3. Plots of $\log(C_t\delta_{obs})$ versus $\log(C_t(\delta_{mic}-\delta_{obs}))$ for the a protons of OPE-9. Below the cmc the plot gave a slope of approximately 5. Above the cmc the slope was approximately 25.

have a Stokes radius of 38.7 Å. The large difference between the experimental, 58.7 Å, and calculated Stokes radius implies that the hydrated micelles were highly asymmetric, in keeping with their characterization as ellipsoidal by Robson and Dennis [16].

3.2.2. Nuclear magnetic resonance

Proton NMR analysis of OPE-9 dissolved in D_2O showed that the chemical shift, δ_{obs}, of several detergent protons was a function of the OPE-9 concentration. The chemical shift of the alkyl protons a to c decreased from the lowest concentration studied, 0.108 mM (9259 M^{-1}), to approximately 3 to 4 mM (250 M^{-1}) OPE-9 with most of the change occurring between 0.33 mM (3030 M^{-1}) and 4 mM. However, above 4 mM the chemical shift began to increase. Fig. 2A,B show these changes in δ_{obs} for protons a and c. The increase of δ_{obs} demonstrates that above 4 mM detergent δ_{obs} was the average of two different micellar environments, i.e., that the ordering of components in the micelle was changing. Using the pseudophase model the following equation relates the observed chemical shifts to the cmc below 4 mM [24–26], where C_t is the total OPE-9 concentration, δ_{mic} the limiting proton

$$\delta_{obs} = \delta_{mic} - \{cmc/C_t\}(\delta_{mic} - \delta_{mono})$$

chemical shift in micelles, and δ_{mono} the proton chemical shift of the monomer in dilute solution. Plots of δ_{obs} versus $1/C_t$ for protons a or c, Fig. 2A and B, gave two different slopes (= $cmc(\delta_{mic}-\delta_{mono})$): 1.54×10^{-4} from 0.108 mM to 0.25 mM OPE-9 and 1.21×10^{-2} from 0.43 mM to 4.24 mM, respectively. Substituting the chemical shift values gave apparent cmcs of 0.13 mM and 0.33 mM, respectively.

Using the equation shown below for the single-step equilibrium model, where K is the equilibrium constant, it is possible to calculate the number of OPE-9 monomers, N,

$$\log(C_t\delta_{obs}) = N\log[C_t(\delta_{mic}-\delta_{obs})] + \log K + \log N - (N-1)\log\delta_{mic}$$

incorporated into the micelle [27]. Plots of $\log(C_t\delta_{obs})$ versus $\log[C_t\ (\delta_{mic}-\delta_{obs})]$ for protons a and c gave a slope, N, of approximately 4 to 5 below the cmc, Fig. 3. Above the cmc to approximately 4 mM OPE-9, the aggregation number showed considerable experimental variation with N ranging from 25 to 50 detergent molecules/micelle. This composition is noticeably smaller than the 104 detergent molecules/micelle measured by static light scattering at 23.7 mM OPE-9.

These results suggest that OPE-9 begins to form small aggregates around 0.13 mM OPE-9 and micelles above the cmc. However, at detergent concentrations greater than 4 mM the environment of

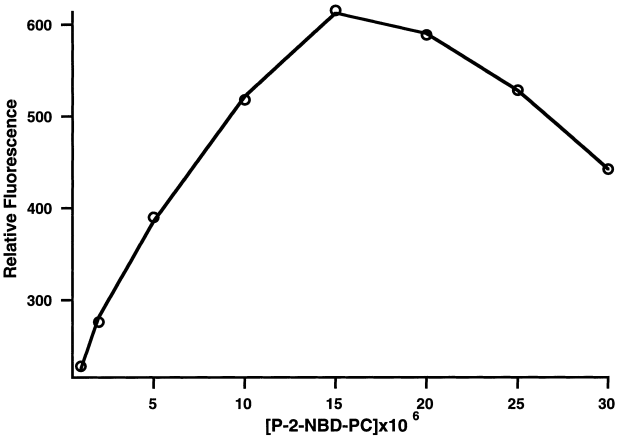

Fig. 4. The fluorescence intensity of 1-palmitoyl-2-(12-[{7-nitro-2-1,3-benzoxadiazo-4-yl}amino]dodecanoyl)-*sn*-glycero-3-phosphocholine, P-2-NBD-PC, in a solution of 4 mM dioleoylphosphatidylcholine/40 mM Triton plotted against the total solution concentration of P-2-NBD-PC. The fluorescence does not increase above 15 μM P-2-NBD-PC.

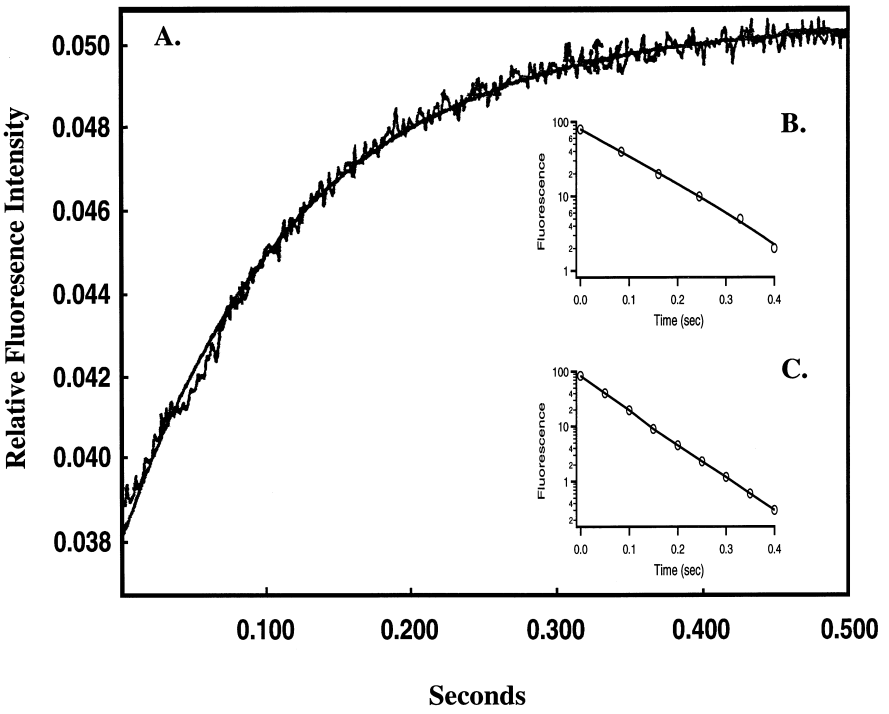

Fig. 5. (A) Stopped-flow fluorescence trace showing increase in fluorescence when donor micelles loaded with 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazole-4-yl) (DM-NBD-PE) were rapidly mixed with acceptor micelles that do not contain DM-NBD-PE. The concentrations of the acceptor and donor solutions before mixing were 0.94 mM Triton+0.12 mM DOPC and 0.5 mM Triton+0.05 mM DOPC+0.012 mM DM-NBD-PE, respectively, in 200 mM Tris (pH 7.4). The mixing ratio was 1:10 donor to acceptor. (B) A log plot of ln((maximum fluorescence signal−fluorescence signal at time *t*) versus time for the trace shown in a at an acceptor micelle to donor micelle ratio of 33.6 after mixing. The concentrations of the acceptor and donor solutions before mixing were 0.94 mM Triton+0.12 mM DOPC and 0.5 mM Triton+0.05 mM DOPC+0.01 mM DM-NBD-PE, respectively, in 200 mM Tris (pH 7.4). The mixing ratio was 1:10 donor to acceptor. (C) A log plot of ln((maximum fluorescence signal−fluorescence signal at time *t*) versus time for DM-NBD-PE at an acceptor micelle to donor micelle ratio of 1.7 after mixing. The concentrations of the acceptor and donor solutions before mixing were 0.94 mM Triton+0.12 mM DOPC and 4.0 mM Triton+0.4 mM DOPC+0.1 mM DM-NBD-PE, respectively, in 200 mM Tris (pH 7.4). The mixing ratio was 1:10 donor to acceptor.

protons a and c was altered and became more like the monomer as the OPE-9 concentration increased. Nonionic micelles are reported to increase in size above the cmc [18,28,29]. Small increases in micelle volume with OPE-9 concentration that were not detected by us using static light scattering may explain the change in the proton environment. We surmise that above 4 mM the more hydrophobic residues of OPE-9 are forced out of the hydrophobic core of the micelle into a region that contained a large fraction of the more 'hydrophilic' oxyethylene chains. Because diffusion between these regions would be fast, the chemical shift was averaged and the observed chemical shift increased as more OPE-9 molecules were forced into the hydrated region.

3.3. *Phospholipid exchange between mixed micelles using fluorescent phospholipids*

1-Palmitoyl-2-(12-[{7-nitro-2-(1,3-benzoxadiazo-4-yl}amino]dodecanoyl)-*sn*-glycero-3-phosphocholine, P-2-NBD-PC, is a hydrophobic, fluorescent phospholipid with a fluorescence lifetime of about 6 to 9 ns [30], that undergoes self-quenching at high concentration. The fluorescence of solutions containing 4.0 mM DOPC/40 mM Triton X-100 (or OPE-9), Fig. 4, maximized at 15 μM P-2-NBD-PC, a concentration that gives approximately 1 molecule of P-2-NBD-PC per 22 micelles. Above 15 μM P-2-NBD-PC self-quenching substantially reduces luminescence suggesting that P-2-NBD-PC was rapidly moving among micelles.

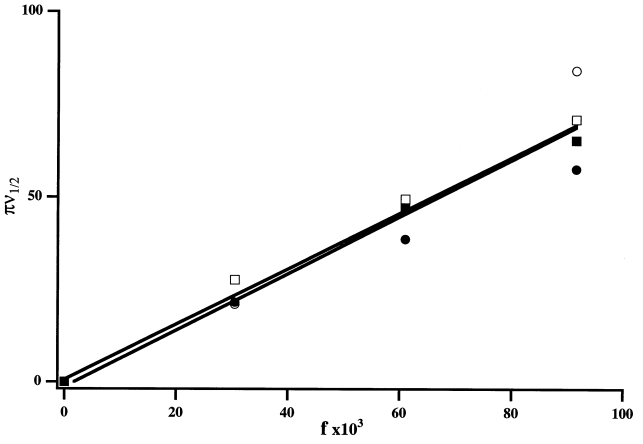

Fig. 6. A plot of $\pi\upsilon_{1/2}$ (π times (the peak width at half-height with spin label−the peak with at half-height without spin label) versus the mole fraction, f (moles PDPC/moles micelles), for several concentrations of spin-label 1-palmitoyl-2-(16-doxylstearoyl)phosphatidylcholine (PDPC) in 40 mM Triton/5 mM DOPC. The filled symbols are for OPE-9 and open symbols for Triton-100 mixed micelles. The circles are for proton a of the detergent molecule. The squares are for the choline methyl signal of DOPC.

The rate of exchange was measured using the fluorescence-dequenching technique of Nichols [14]. This technique employs two samples of micelles one of which contains a small amount of a fluorescent phospholipid at a concentration where fluorescence is self-quenched. After rapidly mixing the two samples, the fluorescent phospholipid diffuses from the loaded micelles to acceptor micelles that do not contain fluorescent phospholipid. Because self-quenching is either reduced or eliminated the sample fluorescence increases in proportion to the amount of fluorescent phospholipid that has exchanged into the acceptor micelles. For these studies, two preparations of micelles prepared from DOPC and detergent were rapidly mixed in a stopped-flow spectrofluorometer having a mixing dead time of 1.34 ms.

Solutions containing the fluorescent reporters O-2-NBD-PC or 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-(1,3-benzoxadiazole-4-yl), DM-NBD-PE, and acceptor were mixed with an acceptor to donor ratio of 10 to 1. The ratio of Triton X-100 (or OPE-9)/DOPC/O-2-NBD-PC was 8:0.8:0.2 and 8:1:0 in the donor and acceptor, respectively, with approximately 15 molecules of DOPC per micelle. From the mole ratio of detergent to DOPC and average micelle size we estimate that there were on average three fluorescent phospholipids per micelle distributed such that 80% of the donor micelles contain two or more fluorescent molecules. A typical trace for O-2-NBD-PE fluorescence obtained by rapidly mixing 0.5 mM Triton/DOPC/O-2-NBD-PE micelles with 0.94 mM Triton X-100/DOPC at a 8:1 ratio is shown Fig. 5A. The fluorescence increased exponentially and a plot of ln(maximum fluorescence signal−fluorescence signal at time t) versus time was linear for the first five half-lives (Fig. 5B). The experimental first-order rate constants, k_{obs}, are given in Table 1. When the acceptor micelle concentration was kept constant and the donor micelle concentration varied, k_{obs} was independent of the donor concentration for both DM-NBD-PE (Table 1) and O-2-NBD-PC (data not shown). The mean values of k_{obs} for different acceptor concentrations are shown in Table 2.

Table 1
Typical set of observed rate constants (k_{obs}) for several donor/acceptor micelle concentrations

Starting donor Triton conc.		4 mM	2 mM	1 mM	0.5 mM
Final donor micelle conc.		2.8 μM	1.3 μM	0.52 μM	0.14 μM
Starting acceptor Triton-100 conc.	Final acceptor micelle conc.	k_{obs} (s^{-1})			
40 mM	300 μM	219.0 ± 6.1	213.6 ± 1.4	226.2 ± 11.6	235.8 ± 15.8
10 mM	73 μM	93.1 ± 19.4	85.8 ± 0.5	84.4 ± 3.0	83.1
0.94 mM	4.7 μM	15.4 ± 0.9	13.3 ± 1.9	9.6 ± 0.8	8.1 ± 0.1

Unlabeled, acceptor micelles containing Triton and DOPC in 200 mM Tris (pH 7.4) and DM-NBD-PE-labeled, donor micelles containing Triton, DOPC, and DM-NBD-PE in 200 mM Tris (pH 7.4) were mixed in a ratio of 10:1. The mole ratio of Triton to DOPC was 8:1 while the mole ratio of DOPC to DM-NBD-PE was 4:1. The starting Triton concentrations and final micelle concentrations are listed.

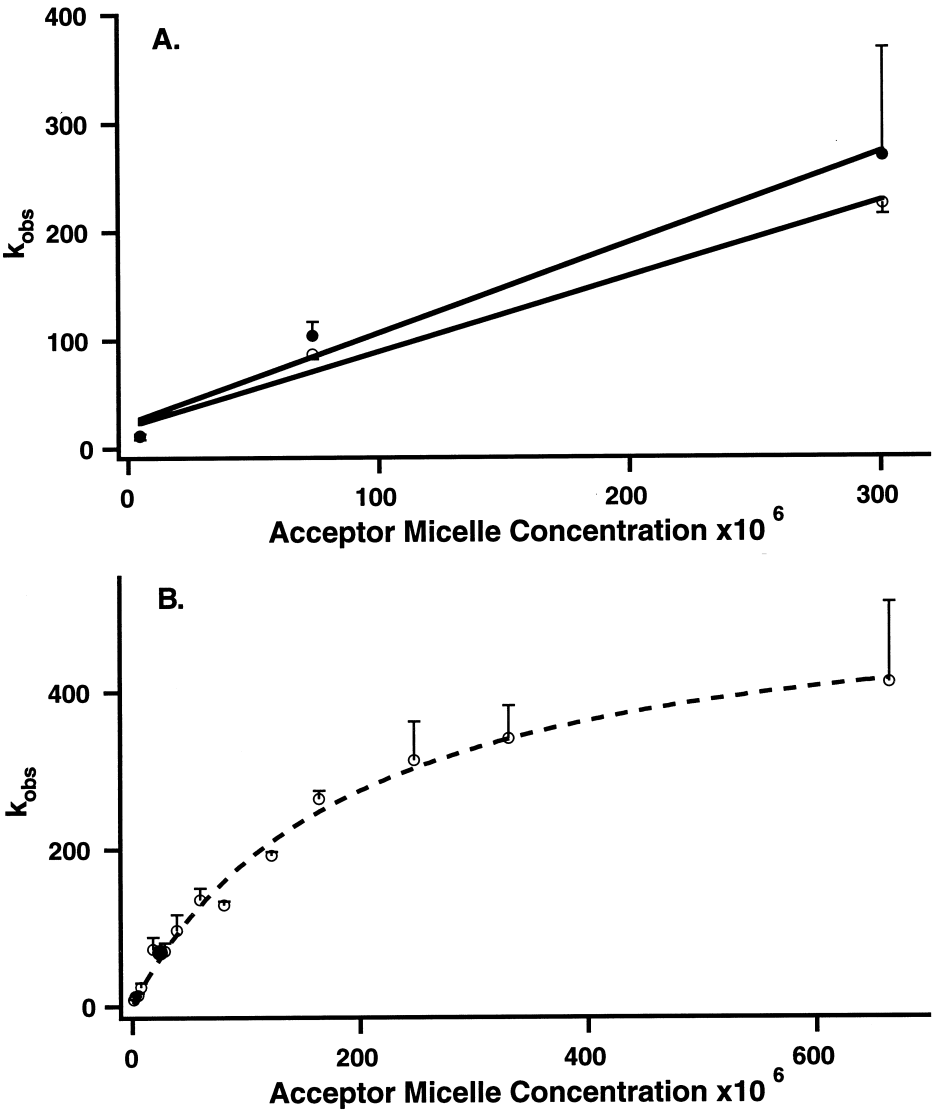

Fig. 7. (A) A plot of the mean k_{obs} (±S.D.) versus the acceptor detergent concentration for donor micelles loaded with either 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazole-4-yl) (DM-NBD-PE; open circles) or 1-oleoyl-2-(12-[{7-nitro-2-1,3-benzoxadiazole-4-yl}amino]dodecanoyl)-*sn*-glycero-3-phosphocholine (O-2-NBD-PC; filled circles). The fitted line assumes a linear relationship between k_{obs} and acceptor concentration. (B) A plot of the mean k_{obs} (±S.D.) versus acceptor micelle concentration for DM-NBD-PE-loaded micelles. The fitted line assumes a hyperbolic relationship between k_{obs} and acceptor concentration. The filled circle is a data point taken from a similar study by Soltys and Roberts [13].

3.4. Phospholipid exchange between mixed micelles using spin-labeled phospholipids

We employed steady-state NMR methods to evaluate the exchange rate. 1-Palmitoyl-2-(16-doxylstearoyl)phosphatidylcholine, PDPC, was included in the phospholipid to rapidly relax the protons of DOPC and OPE-9. In these experiments 1 out of 32 micelles to 1 out of 10 micelles contained a molecule of PDPC. However, in all cases a single resonance was observed for the protons at position a of Triton X-100, positions a and c of OPE-9, and positions d, x, and z of DOPC, see Fig. 1 for proton positions. If the exchange of phospholipid between micelles had been slow, the observed proton signals from DOPC protons would have had contributions from both slowly relaxing and rapidly relaxing nuclei, e.g., the NMR signal would consist of a sharp signal (no

Table 2
Average observed rate constants (k_{obs}, s^{-1}) for exchange for the different acceptor concentrations listed in Table 1

Fluorescent lipid	k_{obs} at acceptor micelle conc. of:		
	300 μM	73 μM	4.7 μM
O-2-NBD-PC	268.1 ± 98.3	104.2 ± 12.7	11.9 ± 1.9
DM-NBD-PE	223.7 ± 9.6	86.6 ± 4.5	11.6 ± 3.3

The donor micelle concentrations ranged from 2.8 to 0.14 μM.

paramagnetic relaxation) superimposed on a smaller, broad signal caused by paramagnetic relaxation. Because only peak broadening was observed the results were analyzed using the equation for fast exchange, $1/T_{2P} = f/(T_{2M}+\tau)$, where T_{2P} is the measured relaxation time, T_{2M} is the paramagnetic relaxation time of protons near PDPC, and $1/\tau$ is the rate of exchange between micelles [31]. The variable f was the fraction of micelles containing a single PDPC. Fig. 6 shows a plot of $1/T_{2P}$ versus f (= moles PDPC/moles micelles) that gave ($T_{2M}+\tau$) equal to 1.3 ms. The slopes for both the a and z protons were similar in both detergents and the mean of two slopes for each proton in the different detergents were equal within experimental error. Because T_M should vary among different chemical shifts the similarity of the slopes for different protons suggests, but does not prove, that τ was larger than T_M.

3.5. Mechanism

A rapid rate of phospholipid exchange was suggested from the measurements of fluorescence self-quenching and NMR line broadening. Most of the stopped-flow experiments were performed under pseudo-first-order conditions, i.e., the concentration of the acceptor, A, was at least 20-fold greater than the concentration of the donor, D. The A to D ratio varied from 107 to 1.7 in the first column of Table 1 to 2143 to 33.6 for the last column. Fluorescence recovery for all of the stopped-flow experiments was first order at both high and low A to D ratios, e.g., the analysis with A/D = 1.7, ([A] = 4.7 μM and [D] = 2.8 μM) was linear for at least 7-half lives, inset C of Fig. 5. Table 1 shows that the rate of exchange did not depend on the concentration of D at starting concentrations of 10 mM or 40 mM A. The rate of exchange increased as A increased, but there was not a one-to-one correlation between the increase in [A] and the increase in rate.

The results were analyzed in terms of the approach to equilibrium after a small perturbation using equations developed by King [32]. This method of analysis does not assume a mechanism for the exchange process, but the form of the equations may suggest a mechanism. Eq. 1 shows the equilibrium condition that is being measured by the stopped-flow experiments where the micelle, M, containing DOPC, X, equilibrates with a donor micelle containing the fluorescent phospholipid, X*, and X. Because studies of ionic micelles suggested a pre-associated complex (PX) the term Q was used in equations to represent either D or a pre-associated state complex of Triton and phospholipid, Eq. 2.

$$MX + MX* \rightleftharpoons MX* + MX \quad (1)$$

$$AX + QX* \rightleftharpoons AX* + QX \quad (2)$$

For small changes in concentration the relaxation time, τ ($= 1/k_{obs}$), obtained from a plot of ln(fluorescence) versus time, will be equal to $k_f\{1/[AX]_e + 1/[QX^*]_e\} + k_r\{1/[AX^*]_e + 1/[QX]_e\}$ [32], where the subscript e means at equilibrium, k_f is the rate constant for the forward reaction giving AX*, while k_r is the rate constant for the reverse reaction giving QX*. Plots of k_{obs} from the last column of Table 1 versus ($[AX]_e$) or ($[AX]_e + [DX^*]_e$) gave slopes of $k_f = 9.6 \times 10^5$ $M^{-1}\,s^{-1}$ for DM-NBD-PE ($r = 0.83$). The results for ($[AX]_e$) are shown in Fig. 7A. Because there is a historical precedent for the participation of a pre-micellar state ($[AX]_e + [PX^*]_e$) was plotted versus k_{obs} and gave a line similar to the others (data not shown). The concentration of PX* was estimated by assuming that at equilibrium X* was uniformly distributed among AX and QX. All of the plots show that k_{obs} does not increase linearly with the concen-

tration of acceptor micelle and appears to be approaching a maximum.

Because plots of k_{obs} versus ($[AX]_e$) or ($[AX]_e+[DX^*]_e$) showed a slight downward curvature, a series of experiments were conducted in which the acceptor concentration was varied at constant donor concentration of 4 mM DM-NBD-PE. The k_{obs} from these experiments were fitted to a simple equation for a hyperbola $k_{obs} = V[AX]/(K+[AX])$. The results are shown in Fig. 7B. The good fit suggests that exchange approaches saturation at concentrations of Triton normally used for the study of phospholipase activity. The fit of the points gave $V = 535$ and $K = 1.9 \times 10^{-4}$. The results suggest an exchange mechanism like that depicted by Eq. 3.

$$AX + QX^* \rightleftharpoons M \rightleftharpoons AX^* + QX \quad (3)$$

The kinetic analysis did not identify a unique mechanism, but showed that the number measured by stopped-flow or NMR analysis was the product of a rate constant and a concentration. The rate of exchange was independent of [D] suggesting either a pseudo-first-order process or the presence of a constant concentration of a pre-associated complex. Assuming that a pre-associated complex composed of five detergent molecules and phospholipid was present at 0.13 mM suggests a rate constant for exchange, k_e, of approximately 2.1×10^7 $M^{-1}\,s^{-1}$ if $V = k_e[A]$, approximately 20-fold larger than the calculated second-order rate constant. Because in our NMR experiments we did not prove that τ dominated T_{2M}, the estimated rate, $1/(T_{2M}+\tau) = 769$ s^{-1}, is only a minimum rate for phospholipid exchange. Irrespective of the mechanism of exchange this work shows that exchange among micelles was extremely fast.

In summary, these studies demonstrate that the phospholipids in Triton-phospholipid mixed micelles rapidly exchange among micelles as previously reported by Kucera et al. [12]. Because the rate depended on the concentration of A, k_{obs} is probably the product of a rate constant and concentration. The rate constants calculated using the concentrations employed in this study were on the order of 10^6 to 10^7 $M^{-1}\,s^{-1}$. In a study similar to that reported here, Soltys and Roberts [13] reported a first-order rate constant of 69 s^{-1} for the exchange of fluorescent phospholipid, 1-hexadecanoyl-2-(1-pyrenedecanoyl)phosphatidylcholine with pure Triton micelles. The final concentration of Triton X-100 was assumed to be the average of 1.45 mM Triton-X 100 mixed micelles and 5 mM Triton-X 100 micelles. When this point was placed on Fig. 7B it fell on the fitted line.

The mechanism of exchange may involve rapid collisional transfer of material between micelles and pre-micellar aggregates. This mechanism is similar to one proposed for phospholipid exchange between mixed micelles composed of phospholipid and ionic detergents such as deoxycholate [14]. In these systems it was suggested that the phospholipid undergoes either a 'naked' transfer between micelles or an assisted transfer by 'submicellar aggregates' formed from lysophospholipid and detergent salts [33–35].

The impetus for these studies was our continued interest in measuring phospholipase activity with a reproducible assay system. Several phospholipases have been studied in a mixed-micelle system of Triton X-100-phospholipid under conditions where the number of micelles exceeded the number of enzyme molecules by 10^4 and substrate availability might have limited hydrolysis. If the enzyme was limited to the phospholipid carried by a single micelle, hydrolysis would cease when that pool was exhausted. However, if the phospholipid exchange between micelles exceeds the catalytic rate, all of the phospholipid in solution is available for hydrolysis. For example, a phospholipase A_1 with a turnover of 90 mol phospholipid s^{-1} (mol enzyme)$^{-1}$ hydrolyzes 0.73 nmol phospholipid s^{-1} [12]. Using the slowest rate constant for exchange, the concentration of the pre-micellar species, and the concentration of enzyme used in our experiments, the calculated rate of exchange was 260 nmol phospholipid s^{-1}. Therefore, the rate of exchange was more that 100 times faster than the rate of hydrolysis. This conclusion differs, for example, from that of Jain et al. [36]. They state that the intrinsic catalytic turnover by phospholipases A_2 is on the order of 300 s^{-1}, a rate much slower than we report here for exchange. From this they calculated that '20% of the substrate molecules in a micelle would be hydrolyzed in 30 ms'. Their calculations assumed less than 50 phospholipid molecules per micelle that is in reasonable agreement with our findings. However, our results conclusively demonstrate that the exchange rate was more rapid

than hydrolysis catalyzed by most, if not all, phospholipases and that phospholipid availability was not rate limiting in the Triton X-100 mixed-micelle assay system.

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