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Magnetic Nonequivalence within the Fatty Acyl Chains of Phospholipids in Membrane Models: ^{1}H Nuclear Magnetic Resonance Studies of the α-Methylene Groups†

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ABSTRACT: The existence of a large chemical shift difference between the α-methylene groups of the two fatty acyl chains of phospholipids in Triton X-100/phospholipid mixed micelles has been demonstrated using ^{1}H NMR [Roberts, M. F., & Dennis, E. A. (1977) *J. Am. Chem. Soc. 99,* 6142]. This difference between the two α-methylene groups in the Triton mixed micelle system (0.09 ppm) is now compared with that observed for synthetic short-chain phospholipids which exist as monomers (0.03 ppm for dihexanoylphosphatidylcholine) and those which form micelles (0.09 ppm for dioctanoylphosphatidylcholine). Ionic and zwitterionic detergents, such as cetyltrimethylammonium bromide, sodium dodecyl sulfate, sodium deoxycholate, and 3-(dimethyltetradecylammonio)propane-1-sulfonate, which form mixed micelles with dipalmitoylphosphatidylcholine, also were found to promote the large chemical shift difference of the two phospholipid α-methylene groups. Phospholipid line widths are much narrower in these latter mixed micelles, and the magnetic nonequivalence of the two protons on the *sn*-2 α-methylene carbon is observable. The spectra of the α-methylene protons are analyzed by decoupling the β-methylene protons and treating the resultant *sn*-2 multiplet as an AB system. The absolute magnitude of J_{AB} is always about 16 Hz, while the degree of the magnetic nonequivalence of the two protons depends on the detergent. In micelles with Triton X-100, only phospholipids containing short-chain fatty acids display line widths narrow enough to show the AB pattern. These results suggest that phospholipid molecules adopt a unique conformation in all micellar environments, be they pure phospholipid micelles or mixed micelles with nonionic or ionic detergents. In this conformation, the *sn*-1 α-methylene protons have indistinguishable chemical shifts and are in a more hydrophobic (shielded) environment than the strongly differentiated protons of the *sn*-2 α-methylene group. This pronounced difference in the two chains, which is not observed for monomeric phospholipid, is discussed in terms of phospholipid conformation and susceptibility to phospholipase A_2, an enzyme which catalyzes the hydrolysis of phospholipids specifically at the *sn*-2 carbonyl.

Proton NMR spectroscopy has been used extensively to examine the structure and packing of phospholipids in model membrane systems such as multibilayers and sonicated vesicles (Finer et al., 1972; Lee et al., 1972; Feigenson & Chan, 1974; Michaelson et al., 1974). We have used this technique to study phosphatidylcholine in mixed micelles with the nonionic detergent Triton X-100, a polydisperse preparation of *p-tert*-octylphenylpolyoxyethylene ethers (Ribeiro & Dennis, 1975). In these mixed micellar structures, subtle differences in the environment or conformation of the α-methylene groups of the two fatty acyl chains of various phospholipids are detected as chemical shift differences in the ^{1}H NMR spectra (Roberts & Dennis, 1977). This finding is of particular interest because mixed micelles serve as ideal substrates for phospholipase A_2 (Deems et al., 1975). This enzyme acts specifically to catalyze the hydrolysis of phospholipid by reaction at the carbonyl

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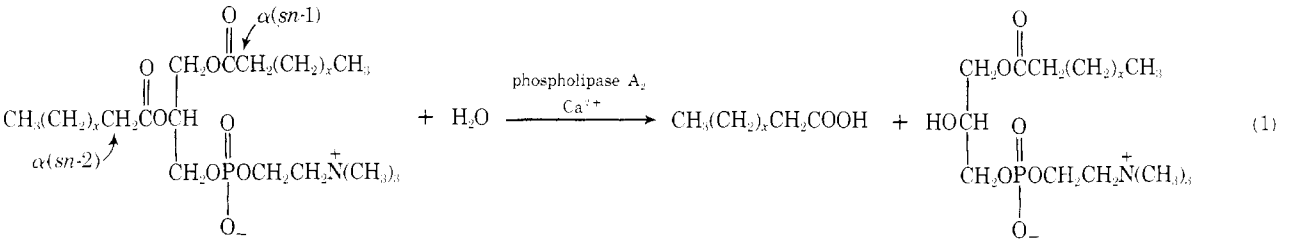

(1)

carbon adjacent to the α-methylene group of the *sn*-2 fatty acyl chain (Deems & Dennis, 1975) as shown in eq 1.

It is important to determine whether the magnitude of the observed chemical shift difference between the two α-methylene groups is unique to long-chain phospholipids in the Triton X-100 mixed mixed micelle system, or is a property of any packed phospholipid structure. ^{1}H NMR studies of phospholipid in vesicles, multibilayers, or liposomes have not revealed differences in the α-methylene groups, but chemical shift differences of a similar magnitude would be obscured by the large line widths of the phospholipid in these structures. X-ray studies of crystalline dilaurylphosphatidylethanolamine have shown that the α-methylene groups of the two fatty acyl chains occupy distinctly different environments (Hitchcock et al., 1974). Similarly, deuterium quadrupole coupling magnetic resonance studies of specifically deuterated dipalmitoylphosphatidylcholine suggest that a difference in chain environment also occurs for phospholipid in multibilayer preparations (Seelig & Seelig, 1974).

We have now examined the ^{1}H NMR spectra of several synthetic short-chain phospholipids above and below their critical micelle concentrations, both in the presence and absence of the detergent Triton X-100. We have also examined mixed micellar systems formed by dipalmitoylphosphatidylcholine and other detergents, and find the difference in the two fatty acyl chains always present. Only monomeric phospholipid has nearly identically shifted α-methylene group signals. Furthermore, in ionic and zwitterionic detergent systems, narrower phospholipid line widths allow us to investigate a further shift difference of the two protons on the *sn*-2 α-carbon. From the activities of phospholipase A_2 toward phospholipid in these different mixed micellar systems, we have confirmed that the environmental or conformational difference in the *sn*-1 and *sn*-2 chains is necessary but not sufficient for efficient catalysis.

Experimental Procedure

Materials. Dipalmitoylphosphatidylcholine[1] and bovine brain sphingomyelin were obtained from Calbiochem. β-Dipalmitoylphosphatidylcholine was the gift of Dr. Sunney Chan, California Institute of Technology. Dihexanoylphosphatidylcholine was provided by Dr. Michael Wells, University of Arizona. Dioctanoylphosphatidylcholine and 1-palmitoyl, 2-[2′-$^2H_2$]palmitoylphosphatidylcholine were synthesized by Dr. Thomas G. Warner, University of California, San Diego, by procedures summarized elsewhere (Roberts & Dennis, 1977). Nonionic detergents employed in the formation of mixed micelles included polydisperse Triton X-100 (Triton) (Rohm and Haas) and two homogeneous compounds: *p*-(1,1,3,3-tetramethylbutyl)phenoxynonaoxyethylene glycol (OPE-9) synthesized in our laboratory (Robson & Dennis, 1978), and *n*-dodecyloctaethylene ether ($C_{12}EO_8$) (Nikol Chemical). Other detergents used include cetyltrimethylammonium bromide (CTAB) (Sigma), sodium dodecyl sulfate ($NaDodSO_4$) (Eastman), sodium deoxycholate (DOC) (Sigma), and 3-(dimethyltetradecylammonio)propane-1-sulfonate (DTAPS), provided by Dr. Amnon Gonenne, University of California, San Diego.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were obtained at 220 MHz and 40 °C with a Varian HR-220/Nicolet TT-100 pulse Fourier transform system. Mixed micelles were prepared by adding solutions of detergent in D_2O to dry phospholipid; mixing was achieved by a few strokes with a Potter-Elvejhem homogenizer. Samples were not degassed. Short-chain phospholipids were dissolved in D_2O and examined as pure phospholipid monomers or micelles or as mixed micelles with Triton X-100 added. Phospholipid concentrations were 6 mM and detergents were 24 mM, except where indicated. This gives mixed micelles with a detergent/phospholipid molar ratio of 4:1. Line widths were measured as the full-width at half-height maximum intensity; field inhomogeneity was taken to be the line width of the HOD peak and this was subtracted from the reported values. Chemical shifts are reported relative to TSP included in some samples. Average errors in the reported chemical shift differences between peaks were ±5 to 10%.

Results

Shift Difference of the α-Methylene Groups in Mixed Micelles with Polydisperse and Homogeneous Nonionic Surfactants. Previous results suggested that the chemical shift difference is not due to slow exchange between two micellar environments (Roberts & Dennis, 1977). However, Triton X-100 is a polydisperse material and forms a bimodal distribution of micelle sizes when the ratio of Triton/phospholipid is low (Dennis, 1974). Because it was still possible that this is the cause for the two observed signals in the α-methylene region, we employed two homogeneous surfactants, *n*-dodecyl octaethylene ether ($C_{12}EO_8$), and *p*-1,1,3,3-(tetramethylbutyl)phenoxynonaoxyethylene glycol (OPE-9), to solubilize dipalmitoylphosphatidylcholine. The latter detergent has been shown to form a single well-defined mixed micelle species at all Triton/phospholipid ratios investigated (Robson & Dennis, 1978). In both of these detergents, the α-methylene pattern of the phospholipid was found to be identical with that for Triton X-100, as illustrated in Figure 1. The assignment (Roberts & Dennis, 1977) of the narrower peak at 2.35 ppm to the *sn*-2 α-methylene group, and the broader peak at 2.26 ppm to the *sn*-1 α-methylene group was reconfirmed by observing the spectrum of the phospholipid in which the *sn*-2 α-methylene group was substituted with deuterium in mixed micelles with $C_{12}EO_8$ (Figure 1D).

Dihexanoylphosphatidylcholine: Comparison of the α-Methylene Shift Difference in Monomers and Mixed Micelles with Triton X-100. The short-chain phospholipid, dihexanoyl

[1] Abbreviations used: diacyl phosphatidylcholine, 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine; β-dipalmitoylphosphatidylcholine, 1,3-dipalmitoyl-*sn*-glycerol-2-phosphorylcholine; Triton, Triton X-100; OPE-9, *p*-(1,1,3,3-tetramethylbutyl)phenoxynonaoxyethylene glycol; $C_{12}EO_8$, *n*-dodecyloctaethylene ether; CTAB, cetyltrimethylammonium bromide; $NaDodSO_4$, sodium dodecyl sulfate; DOC, sodium deoxycholate; DTAPS, 3-(dimethyltetradecylammonio)propane-1-sulfonate; TSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$; cmc, critical micelle concentration.

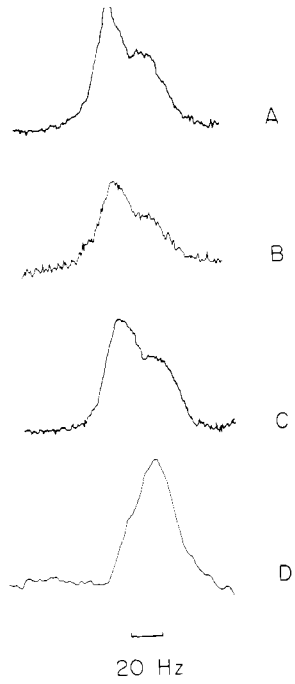

FIGURE 1: ^1H NMR spectra of the α-methylene region of dipalmitoylphosphatidylcholine in mixed micelles with (A) Triton X-100, (B) OPE-9, and (C) $C_{12}EO_8$. In D, the spectrum of the α-methylene region of 1-palmitoyl, 2-[2′-$^2H_2$]palmitoylphosphatidylcholine in mixed micelles with $C_{12}EO_8$ is shown.

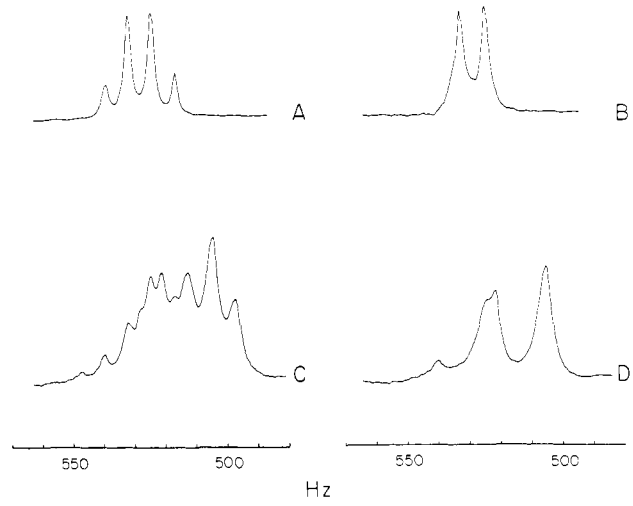

FIGURE 2: ^1H NMR spectra of the α-methylene region of dihexanoylphosphatidylcholine as monomers (A) without and (B) with the β-methylene groups decoupled and as mixed micelles at a molar ratio of Triton/phospholipid of 4:1 (C) without and (D) with the β-methylene groups decoupled.

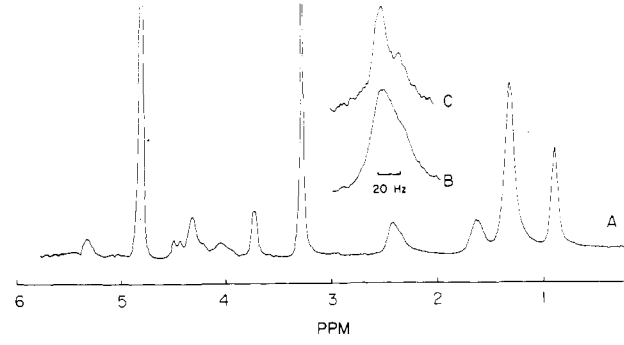

FIGURE 3: ^1H NMR spectrum of dioctanoylphosphatidylcholine micelles in 0.2 M LiI (A). The α-methylene region is shown with an expanded scale in B and the same region with decoupling of the β-methylene protons is shown in C.

phosphatidylcholine, has a high cmc (12–14 mM) and should exist as a monomer when dissolved in water below that concentration (Tausk et al., 1974a,c). The α-methylene region has been described previously (Hershberg et al., 1976) and is composed of a doublet of triplets separated by 7–8 Hz (0.03 ppm) as shown in Figure 2A. Irradiation of the β-methylene protons collapses this pattern to two singlets (Figure 2B). This small chemical shift difference reflects the different environments of the two α-methylene groups in the monomer phospholipid conformation. We find a similar small chemical shift difference for dipalmitoylphosphatidylcholine dissolved in organic solvents such as $CDCl_3$ or CD_3OD (3.5–7 Hz, 0.02–0.03 ppm) as has been observed previously (Birdsall et al., 1972). When sufficient Triton X-100 is added to a 6 mM dihexanoylphosphatidylcholine solution (detergent/phospholipid 4:1), the cmc of the zwitterionic short-chain compound is apparently lowered, so that almost all of the phospholipid is in the Triton micelle (Figure 2C). Such a decrease in the phospholipid cmc is likely, because when the cmc of an ionic surfactant is higher than that of a nonionic one, the cmc of the mixture decreases rapidly as the content of the nonionic detergent increases (Moroi et al., 1975).

The complicated splitting pattern of the α-methylene region can be simplified by irradiation of the β-methylene peaks (Figure 2D). When the phospholipid is micellized, the two α-methylene group signals are separated by 17–18 Hz. The peak centered at 2.38 ppm appears to show residual structure, i.e., a partial quartet in which the three low-field peaks are visible, and the remaining peak is obscured by the *sn*-1 singlet. This could represent slow exchange between two environments for that particular α-methylene group, or it could represent magnetic nonequivalence of the two protons on the *sn*-2 α-methylene carbon.

Thus, when phospholipid goes from a monomer in aqueous solution to mixed micelles with Triton, the chemical shift difference of the α-methylene groups is enhanced from 7 to 8 Hz to an average of 17–18 Hz. One can also compare the chemical shifts of the two α-methylene groups upon forming mixed micelles: the *sn*-2 peak shifts upfield an average of 9–11 Hz, while the *sn*-1 peak shifts upfield twice that amount, about 20 Hz.

Dioctanoylphosphatidylcholine: Comparison of the α-Methylene Groups in Phospholipid Micelles and Mixed Micelles with Triton X-100. Unlike the longer chain phosphatidylcholines investigated, the synthetic short-chain compound, dioctanoylphosphatidylcholine, forms micelles when dispersed in water without surfactants (Tausk et al., 1974a). Phospholipase A_2 will readily hydrolyze these pure phospholipid micelles (Roberts et al., 1978). The apparent molecular weight of dioctanoylphosphatidylcholine above the cmc (0.3 mM) has been determined by light scattering and is very large and concentration dependent, spanning the range 5×10^6 to 20×10^6 (Tausk et al., 1974b). This phospholipid tends to form cloudy two-phase solutions in water, but this phase separation can be suppressed by the addition of 0.2 M LiI (Tausk et al., 1974b). The full spectrum of dioctanoylphosphatidylcholine in D_2O is shown in Figure 3 and the chemical shifts for the protons in these micelles are given in Table I based on previous assignments for phosphatidylcholine in organic solvents (Hague et al., 1972; Birdsall et al., 1972). The inserts in Figure 3 show an expansion of the α-methylene region and the effect

TABLE I: ^{1}H NMR Chemical Shifts for Dioctanoylphosphatidylcholine (10 mM) in 0.2 M LiI.

Resonance	δ (ppm)
ω-CH_3	0.89
$(CH_2)_n$	1.31
β-CH_2	1.62
α-CH_2	2.33, 2.43
$N^+(CH_3)_3$	3.28
$CH_2N^+(CH_3)_3$	3.74
CH_2O	
CH	
CH_2OP	4.06
$CH_2CH_2N^+(CH_3)_3$	4.32
CH_2O	4.43, 4.49
CH	
CH_2OP	
CH_2	
CH	5.33
CH_2	

TABLE II: Chemical Shift Differences between the Two α-Methylene Groups of Phosphatidylcholine in Monomers, Micelles, and Mixed Micelles with Triton.[a]

Phospholipid	Monomer (Hz)	Micelle (Hz)	Mixed micelle (Hz)
Dihexanoyl-PC	7-8		18[b]
Dioctanoyl-PC		18-20	20[b]
Dipalmitoyl-PC	3.5-7[c]		20

[a] Phosphatidylcholine is abbreviated PC. [b] The average value for the chemical shift of the *sn*-2 peak is taken as the midpoint of the residual pattern observed upon decoupling the β-methylene protons. [c] Dipalmitoylphosphatidylcholine dissolved in CD_3OD or $CDCl_3$. The phospholipid in these solvents may actually be in small aggregates or inverse micelles.

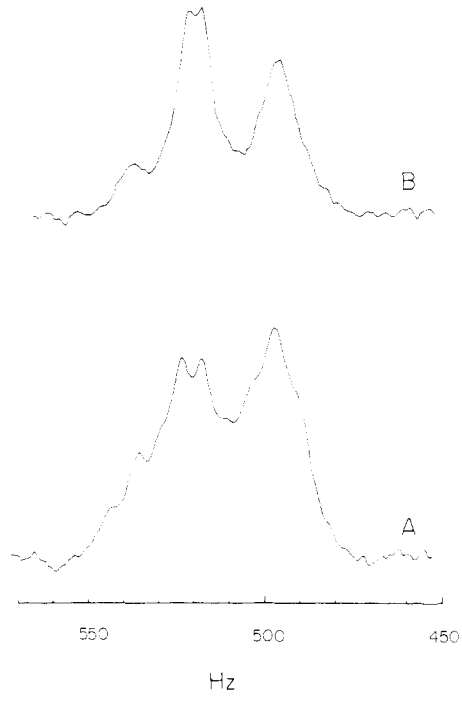

FIGURE 4: ^{1}H NMR spectra of the α-methylene region of dioctanoylphosphatidylcholine in mixed micelles with Triton X-100 at a molar ratio of 4:1 Triton/phospholipid (A) without and (B) with β decoupling.

of irradiation of the β-methylene protons on the line widths of the α-methylene peaks.

The line widths are larger than for the corresponding resonances of Triton/phospholipid mixed micelles. Yet, the α-methylene region presents the same, albeit much broadened, pattern seen with dipalmitoylphosphatidylcholine/Triton mixed micelles. The larger line widths make it difficult to resolve accurately the two separate peaks even with β decoupling, although the separation appears to be 18-20 Hz.

When dioctanoylphosphatidylcholine is inserted into Triton micelles, the line widths of observable resonances narrow to values consistent with other phosphatidylcholines in Triton. Most notable is the improvement in resolution in the α-methylene region; there are clearly two distinct resonance regions with nearly the same chemical shifts as for dipalmitoylphosphatidylcholine (Figure 4A). Multiplet patterns are observed for what correspond to *sn*-2 and *sn*-1 α-methylene protons. The β-methylene decoupled spectrum of this region is shown in Figure 4B. Extra splitting of the *sn*-2 α-methylene group is evident as was observed for dihexanoylphosphatidylcholine in Triton micelles. In these mixed micelles, the average chemical shift difference between the α-methylene groups is 18-20 Hz. A comparison of the difference of the two fatty acyl chains for phospholipid monomers, pure phospholipid micelles, and Triton mixed micelles is shown in Table II.

Other Choline-Containing Phospholipids in Triton X-100 Mixed Micelles. As was shown earlier (Roberts & Dennis, 1977), both a saturated and a natural phosphatidylcholine containing unsaturated fatty acid exhibit the same chemical shift pattern for the α-methylene groups of the two fatty acyl chains when solubilized in Triton X-100. The present results for short-chain phospholipids suggest that this chemical shift difference is considerably enhanced over that observed for monomer phospholipid, and that in micellar structures it is independent of fatty acid chain length. The α-methylene group of the *sn*-1 chain is in a more hydrophobic environment than that of the *sn*-2 chain; the latter is near the site of phospholipase A_2 hydrolysis. Two other choline containing phospholipids were examined for a correlation of α-methylene chemical shifts and susceptibility to enzymatic hydrolysis. The symmetric phospholipid, β-dipalmitoylphosphatidylcholine, is a substrate for phospholipase A_2 (M. F. Roberts & E. A. Dennis, unpublished results). When inserted into the Triton micelle it shows a single peak in the α-methylene region with chemical shift (2.32 ppm) and line width (17-18 Hz) analogous to the *sn*-2 α-methylene group of phosphatidylcholine in Triton. Sphingomyelin, with only one fatty acid linked to the backbone by an amide rather than an ester bond at the 2 position, is not a substrate for phospholipase A_2 (Roberts et al., 1978). The chemical shift of the α-methylene group in $CDCl_3$ is 2.13 ppm from tetramethylsilane (a value considerably upfield of the ester-linked group of phosphatidylcholine). Solubilization by Triton X-100 results in a chemical shift for the α-methylene group nearly identical with that of the *sn*-1 chain in phosphatidylcholine mixed micelles (2.26 ppm). Although the chemical shift may be indicative of the amide, it may also be that the α-methylene group in sphingomyelin is in a more hydrophobic environment compared with the *sn*-2 α-methylene group of phosphatidylcholine. Although the lack of phospholipase A_2 activity toward sphingomyelin is probably due to

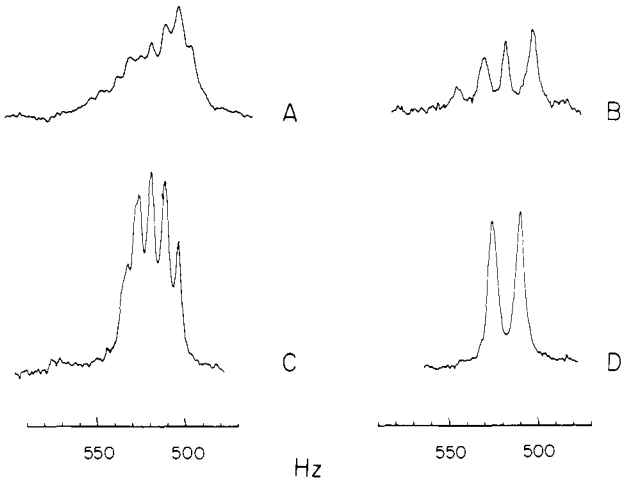

FIGURE 5: ^{1}H NMR spectra of the α-methylene region of dipalmitoylphosphatidylcholine in mixed micelles with cetyltrimethylammonium bromide (CTAB) at a molar ratio of 8:1 CTAB/phospholipid (A) without and (B) with the β-methylene groups decoupled and with sodium dodecyl sulfate ($NaDodSO_4$) at a molar ratio of 4:1 $NaDodSO_4$/phospholipid (C) without and (D) with the β-methylene groups decoupled.

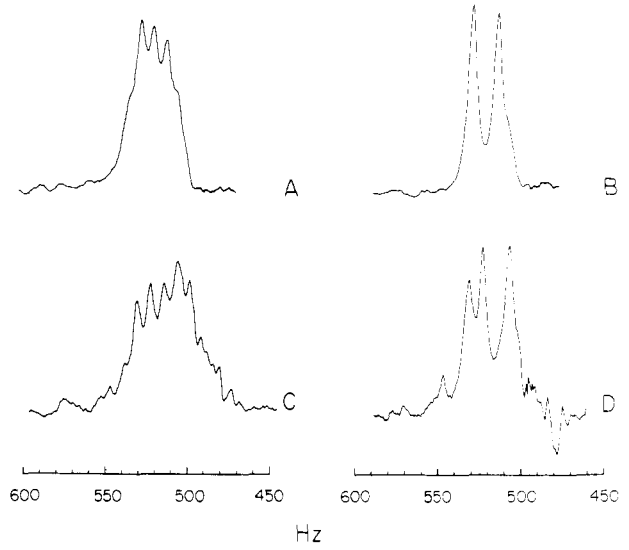

FIGURE 6: ^{1}H NMR difference spectra computed for the α-methylene region of dipalmitoylphosphatidylcholine in micellar systems where detergent resonances partially overlap phospholipid resonances: mixed micelles with sodium deoxycholate (DOC) at a molar ratio of 4:1 DOC/phospholipid (A) without and (B) with the β-methylene groups decoupled, and with 3-(dimethyltetradecylammonio)propane-1-sulfonate (DTAPS) at a molar ratio of 4:1 DTAPS/phospholipid (C) without and (D) with decoupling of the β-methylene groups.

enzymatic specificity for esters rather than amides, the possibility that it is due to the inaccessibility of the amide must now also be considered.

Dipalmitoylphosphatidylcholine in Ionic and Zwitterionic Detergent Systems. The nearly universal adoption of a phosphatidylcholine conformation in which the α-methylene groups of the two fatty acyl chains are in distinct environments in Triton mixed micelles prompted us to examine the α-methylene protons of dipalmitoylphosphatidylcholine in other micelle systems formed with cationic, anionic, and zwitterionic detergents. In all these charged micelle systems, phosphatidylcholine α-methylene line widths are considerably narrower than in the nonionic oxyethylene detergents.

CTAB was used to form the cationic system. This detergent bears some resemblance to the choline moiety and also has a long alkyl chain. Coupled and β-methylene decoupled spectra of the α-methylene region in 8:1 CTAB/phospholipid micelles are shown in Figures 5A,B. When mixed micelles were prepared with the *sn*-2 deuterated analogue of dipalmitoylphosphatidylcholine, only the decoupled peak at 2.28 ppm was observed, assigning it to the *sn*-1 α-methylene group (the same ordering as for the nonionic detergents). A partial quartet is observed for the decoupled *sn*-2 α-methylene group of dipalmitoylphosphatidylcholine.

Both $NaDodSO_4$ and DOC were used to form an anionic micelle matrix. The α-methylene regions of the phospholipid in these detergents are shown in Figures 5C,D and 6A,B. Because DOC possesses resonances that partially overlap the phosphatidylcholine α-methylene groups, difference spectra where the pure detergent spectrum was subtracted from the mixed micellar one were computed. The extra splitting of the *sn*-2 peak observed in both Triton and CTAB systems appears to be present in the $NaDodSO_4$ mixed micelles, but could not be quantitated and was not apparent in the computer subtracted DOC mixed micelle spectrum. Irradiation of the β-methylene protons in DOC mixed micelles produces an apparent singlet for the *sn*-2 α-methylene protons (Figure 6B). The separation of the α-methylene groups of the two fatty acyl chains is about 16 Hz (0.07 ppm).

DTAPS is a zwitterionic detergent that readily solubilizes dipalmitoylphosphatidylcholine. The ^{1}H NMR spectrum of the α-methylene phospholipid groups is also complicated by overlapping detergent resonances. Difference spectra were computed to obtain spectral parameters of the phospholipid α-methylene groups without and with irradiation of the β-methylene protons (Figure 6C,D). Again, what appears to be part of a quartet was observed for the *sn*-2 α-methylene group.

The residual structure of the phospholipid *sn*-2 α-methylene group signals observed with β-CH_2 decoupling in CTAB and DTAPS mixed micelles can be analyzed in terms of a simple AB system where the two protons on the *sn*-2 carbon are magnetically nonequivalent. The parameters derived from this treatment are shown in Table III. $|J_{AB}|$ is constant, 15–16 Hz, while the chemical shift difference between the two protons depends on the specific detergent.

The synthetic short-chain phospholipids, dihexanoylphosphatidylcholine and dioctanoylphosphatidylcholine, when inserted into Triton micelles also show residual coupling of the *sn*-2 α-methylene protons. This can also be interpreted as an AB splitting pattern; resulting parameters are summarized in Table III. Longer chain phospholipids micellarized with Triton show much broader resonances, and the residual structure in the *sn*-2 group cannot be resolved.

Other Choline-Containing Phospholipids in CTAB Mixed Micelles. Because CTAB promoted the largest chemical shift difference in the *sn*-2 α-methylene protons of phosphatidylcholine, we chose this detergent to solubilize β-dipalmitoylphosphatidylcholine and sphingomyelin. These two phospholipids appeared to show a correlation of susceptibility to phospholipase A_2 with the environment of the fatty acid chains (as determined by the chemical shift of the α-methylene group). β-Dipalmitoylphosphatidylcholine shows a simple triplet structure for its α-methylene group; irradiation of the β-methylene protons collapses this to a singlet 2.35 ppm from TSP (Figure 7A). The two α-methylene protons on both fatty acid chains are not detectably chemically shifted. The observation of a single decoupled peak further strengthens the AB splitting argument for the nonsymmetrical dipalmitoylphosphatidylcholine, as opposed to an explanation involving different micelle environments in slow exchange.

TABLE III: Shift Differences between α-Methylene Groups of Phosphatidylcholine in Different Mixed Micellar Systems.[a]

Detergent/phospholipid	$\delta_{sn\text{-}2}$ α-CH_2 (ppm) H_A	H_B	$\delta_{sn\text{-}1}$ α-CH_2 (ppm)	$\lvert J_{AB}\rvert$ (Hz)	$\Delta_{A\rightarrow B}$ (Hz)	$\Delta_{sn\text{-}2\rightarrow sn\text{-}1}$ (Hz) H_A	H_B
Triton/dipalmitoyl-PC	2.35[b]		2.26			20[b]	
Triton/dioctanoyl-PC	2.39	2.34	2.26	16	12	28	16
Triton/dihexanoyl-PC	2.40	2.36	2.30	15.8	10	23	13
DTAPS/dipalmitoyl-PC	2.43	2.35	2.30	15.7	18	29	11
CTAB/dipalmitoyl-PC	2.43	2.33	2.28	15.6	23	33	10
$NaDodSO_4$/dipalmitoyl-PC	2.40		2.32			17	
DOC/dipalmitoyl-PC	2.39		2.32			15	

[a] Phosphatidylcholine is abbreviated PC. [b] Only one broad *sn*-2 α-CH_2 peak is observed for dipalmitoyl-PC in Triton mixed micelles.

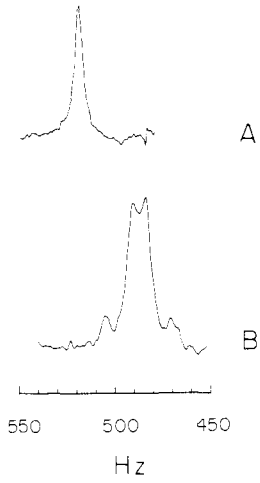

FIGURE 7: ^1H NMR spectra of the phospholipid α-methylene region (with decoupling of the β-methylene protons) in CTAB mixed micelles containing (A) β-dipalmitoylphosphatidylcholine at a molar ratio of 6:1 CTAB/phospholipid and (B) sphingomyelin at a molar ratio of 8:1 CTAB/phospholipid.

The α-methylene group of sphingomyelin, on the other hand, shows a complicated splitting pattern in CTAB. This collapses to an AB quartet upon irradiation of the β-methylene protons (Figure 7B). An analysis of the AB quartet gives $|J_{AB}| = 15$ Hz, while the chemical shifts of the two nonequivalent protons are 2.26 and 2.19 ppm. One should keep in mind that the α-methylene group of this phospholipid is in an analogous position to the *sn*-2 α-methylene group of phosphatidylcholine. Thus the packing of this phospholipid in mixed micelles induces an observable magnetic nonequivalence of the two α-methylene protons in the β-irradiated molecule.

Discussion

Shift Difference of α-Methylene Groups in Phospholipids. The two α-methylene groups in phosphatidylcholine are environmentally distinguishable in micellar structures. From the ^1H NMR analysis of these phospholipid groups solubilized in nonionic, ionic, zwitterionic, or pure phospholipid micelles, several generalizations can be made: (i) The two α-methylene groups in phosphatidylcholine are differently shielded in all micelles. Although monomer phospholipid shows a slight shift between these groups, the difference is magnified when phospholipid is packed in micelles. (ii) The two *sn*-2 α-methylene protons have observably different shieldings in most mixed micelle systems; this difference in shieldings is not observed for monomer phospholipid. The magnitude of the shift difference is detergent dependent. (iii) The *sn*-1 α-methylene protons maintain their observably equivalent shieldings in micelles. They occupy a relatively more hydrophobic environment than the *sn*-2 α-methylene protons.

An interesting difference exists for long-chain phospholipids micellarized in nonionic oxyethylene detergents compared with ionic or zwitterionic detergents. Dipalmitoylphosphatidylcholine in Triton X-100 displays large line widths for the α-methylene groups, with a characteristic pattern of the *sn*-1 group appearing broader than the *sn*-2 group (15–17 Hz vs. 8–9 Hz for β-methylene decoupled spectra). In other detergents, the phospholipid α-methylene resonances are quite narrow (5–6 Hz), even when the cmc is low and the micelle size is relatively large like Triton as is the case for DTAPS (Herrmann, 1966; Ernst & Miller, 1978). A classical structure for Triton mixed micelles would place the phospholipid α-methylene groups down in the oxyethylene cascade region perhaps near the aromatic ring (Robson & Dennis, 1977). The oxyethylene chains may effectively restrict phospholipid chain movement. Furthermore, the *sn*-1 α-methylene protons may be subject to more relaxation by protons in the hydrocarbon-like environment near the start of this fatty acyl chain compared with the less hydrophobic *sn*-2 α-methylene protons. In other charged detergent systems, the hydrophilic/hydrophobic interface is well defined, and the α-methylene groups would be at or near the micelle surface. Repulsive interactions between the charged detergent molecules might allow for looser packing of phospholipids, hence, greater phospholipid mobility and narrower line widths. For a shorter chain phospholipid such as dihexanoylphosphatidylcholine in Triton X-100, the line widths of the α-methylene groups are as narrow as in the charged detergent systems.

The nonequivalence of the *sn*-2 α-methylene protons must be explained in terms of phosphatidylcholine conformation. The chemical shift differences between these two α-methylene protons are bigger or smaller depending on the nature of the detergent: anionic detergents ($NaDodSO_4$ and DOC) produce little or no shift, although the *sn*-2 α-methylene protons are at least somewhat shifted in the undecoupled spectrum of $NaDodSO_4$. CTAB, with its choline head group, induces the largest shift; the nonionic and zwitterionic detergents are intermediate. These differences could be caused by micelle surface charge which could affect the chemical shift of the protons through direct charge interactions and might cause differential shielding of the two protons. The same phospholipid structure would be adopted in each micelle system, but the electronic microenvironment, and hence NMR parameters, would vary because of the detergent. Differences in solvent exposure would also be important in this case. Alternatively, the gross phospholipid conformation could be similar but not identical in all micelles. The phospholipid would adopt a conformation where the beginnings of the *sn*-1 and *sn*-2 chains are physically distinct and the *sn*-1 chain always more hy-

drophobic, but the details of the conformation vary. Phospholipid would have a preferred conformation in each detergent.

Structure of Phosphatidylcholine in Mixed Micelles and Correlation with Phospholipase A_2 Activity. X-ray diffraction studies of dilaurylphosphatidylethanolamine (Hitchcock et al., 1974) and ^2H NMR studies (Seelig & Seelig, 1974, 1975) indicate that in the bilayer crystal the beginning of the *sn*-2 chain is oriented parallel to the bilayer surface, while the *sn*-1 chain is perpendicular to the surface. A model based on the x-ray data is shown in Figure 8. The orientations of the two α-methylene groups are quite different, and if a similar structure occurs in mixed micelles, it would not be surprising for them to give rise to such dramatically different chemical shifts. The accessibility of the *sn*-2 carbonyl group to the surface is particularly notable.

^2H NMR studies of dipalmitoylphosphatidylcholine specifically deuterated at the *sn*-2 α carbon showed two quadrupole splittings for that group (Seelig & Seelig, 1975). This was interpreted as the result of two long-lived and almost energetically equivalent conformations of the lipid with two different orientations of the *sn*-2 chain, although the lack of "motional equivalence" of the two deuterons was an alternate explanation. In a previous ^1H NMR study of dihexanoylphosphatidylcholine above the cmc, the α-methylene region consisted of a complicated splitting pattern (similar to that which we observe for dihexanoylphosphatidylcholine in Triton X-100) which was attributed to slow exchange between two micellar environments (Hershberg et al., 1976). In light of the different shielding and magnetic nonequivalence of the two *sn*-2 α-methylene protons observed here for phospholipid in mixed micelles, it is quite likely that this same differential shielding was responsible for the above cited observations in bilayers and in pure phospholipid micelles, rather than the presence of two conformations or more than one micellar environment. It is easy to see in Figure 8 that a specific conformation of the choline head group, specific solvent interactions, etc. could easily affect the two protons on the *sn*-2 α carbon in a differential fashion. The magnitude of the geminal coupling constant between the α protons of the *sn*-2 chain should be a function of the rotational angle about the CH_2CO bond (Pople & Bothner-By, 1965). From representative values of J observed in open chain and cyclic esters, one may deduce an approximate relation $J \simeq -12 - 6 \sin^2 \theta$, where θ is the angle between the interproton axis and the nodal plane of the π bond of the carbonyl group. For $|J| = 15.5$–16, this yields $\theta = 50$–$55°$ which is not significantly different from the 43° deducible from the x-ray structural parameters given for dilaurylphosphatidylethanolamine (Hitchcock et al., 1974; Elder et al., 1977).

It is worthwhile to consider whether these conformational features of the packed phospholipid molecule have an effect on phospholipase A_2 activity. Phospholipase A_2 specifically hydrolyzes the ester at the *sn*-2 position. Part of the reason for this specificity may be the accessibility of the carbonyl (and neighboring α-methylene group) at the interface. In going from monomer phospholipid to a packed structure, this area of the molecule may become exposed for optimal binding of the enzyme. Cobra venom phospholipase hydrolyzes Triton/dihexanoylphosphatidylcholine (4:1) micelles 17 times faster than monomer dihexanoylphosphatidylcholine (Roberts et al., 1978). The monomer to micelle transformation accentuates the difference in the two fatty acyl chains, placing the *sn*-1 chain in a more hydrophobic environment. Yet the presence of this chain difference does not guarantee that phosphatidylcholine in a mixed micelle will be hydrolyzed by phospholipase. Preliminary results indicate that dipalmitoylphosphatidylcholine in the nonionic Triton, zwitterionic DTAPS, and anionic DOC systems serves as a suitable substrate for cobra venom phospholipase A_2 (M. F. Roberts & E. A. Dennis, unpublished results). In cationic CTAB and anionic $NaDodSO_4$, phospholipase activity is not observed. Thus, other factors such as micelle surface charge must also play a part in phospholipase action, even though the phospholipid may be packed in an analogous fashion with regard to the two fatty acyl chains. It is interesting to compare the susceptibility of a mixed micelle to phospholipase with the extent of observed shift of the phospholipid *sn*-2 α-methylene protons. DOC does not give rise to an observable AB splitting pattern, and yet forms mixed micelles with phospholipid which are susceptible to phospholipase hydrolysis. CTAB, which induces the largest changes, does not form hydrolyzable phospholipid micelles (although because CTAB has a choline head group, it might preferentially bind as an inhibitor to the enzyme). β-Dipalmitoylphosphatidylcholine in Triton is a substrate; yet the α-methylene protons are equally shielded in all detergents. Sphingomyelin, on the other hand, is not a substrate for phospholipase, yet one sees shifts between the α-methylene protons in CTAB and other detergents. Thus differential shielding of the two protons on the *sn*-2 carbon is not a prerequisite for enzymatic hydrolysis by phospholipase A_2.

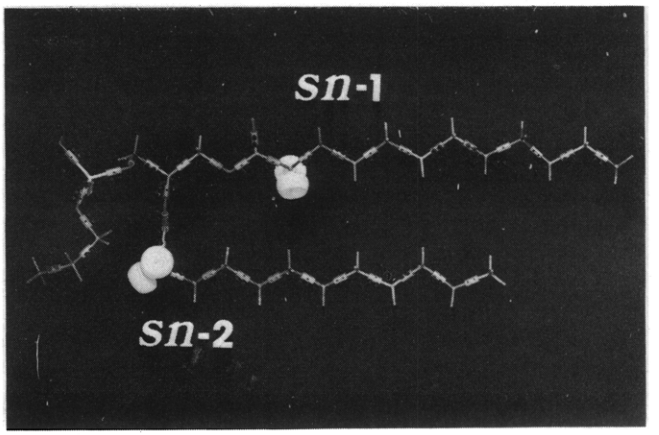

FIGURE 8: Model of dilaurylphosphatidylethanolamine based on the reported torsional angles in the x-ray crystal structure (Hitchcock et al., 1974; Elder et al., 1977). The *sn*-1 and *sn*-2 α-methylene protons are indicated by white balls. The conformational difference of the α-methylene groups of the two fatty acyl chains is quite pronounced.

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Kinetic Analysis of the Acid–Alkaline Conversion of Horseradish Peroxidases†

Tsunehisa Araiso* and Isao Yamazaki

ABSTRACT: The nature of the acid–alkaline conversion of horseradish peroxidases was studied by measuring four rate constants in reactions, $E + H^+$ (k_1) $\rightleftharpoons$ (k_2) EH^+ and $E + H_2O$ (k_3) $\rightleftharpoons$ (k_4) $EH^+ + OH^-$, where EH^+ and E denote the acid and alkaline forms of the enzymes. The values of k_1, ($k_2 + k_3$), and k_4 were obtained by measuring the relaxation rates of the acid → alkaline and alkaline → acid conversions by means of the pH jump method with a stopped-flow apparatus. The value of k_3 could also be obtained by measuring the rate of reactions between hydrogen peroxide and peroxidases at alkaline pH. The measurements were conducted with four peroxidases having different pK_a values: peroxidase A (pK_a = 9.3), peroxidase C (pK_a = 11.1), diacetyldeuteroperoxidase A (pK_a = 7.7), and diacetyldeuteroperoxidase C (pK_a = 9.1). The value of k_1 was about 10^{10} M^{-1} s^{-1} in the reaction of the four enzymes while k_4 was quite different between the enzymes. The pK_a was determined by k_3 and k_4 for the natural peroxidases and by k_1 and k_2 for the diacetyldeuteroperoxidases. The mechanism of the acid–alkaline conversion was discussed in comparison with that of metmyoglobin.

High-spin hemoproteins, such as methemoglobin, metmyoglobin, and plant peroxidases, change their electronic and magnetic properties at alkaline pH (Theorell, 1947; Keilin & Hartree, 1951). The change has been attributed to the proton dissociation of an iron-bound water molecule. This idea seems to conform to the fact that the rates of the acid–alkaline conversion are very fast in methemoglobin and metmyoglobin (Ilgenfritz & Schuster, 1971; Iizuka et al., 1976). On the other hand, the slow rate of the conversion in the case of horseradish peroxidase C has recently been interpreted in terms of the involvement of a conformation change of the protein (Epstein & Schejter, 1972; Iizuka et al., 1976). Evidently, this mechanism contradicts the classical one. The evidence that supports the new mechanism, however, seems insufficient.

This paper reports a detailed investigation of the kinetics of the acid–alkaline conversion of horseradish peroxidases A and C containing a proto- or diacetyldeuterohemin; the pK_a values are reported to be much different between these peroxidases (Makino & Yamazaki, 1972). The present paper also deals with a general rule in the relation between the pK_a and four rate constants involved in the acid–alkaline conversion.

Materials and Methods

Horseradish peroxidases were prepared from wild horseradish root by the method of Shannon et al. (1966) with slight modification. Peroxidase isoenzymes used in this experiment were peroxidase A and peroxidase C, according to nomenclature by Paul (1958) and Shannon et al. (1966). Peroxidase A was a mixture of A_1 and A_2; the difference of catalytic and chemical properties between A_1 and A_2 has not been determined, although they possess different charge properties (Kay et al., 1967; Shin et al., 1971). Artificial peroxidases, containing 2,4-diacetyldeuterohemin in the place of protohemin IX of natural peroxidases, were prepared by the method of Makino & Yamazaki (1972, 1973).

All measurements were performed at 20 ± 0.2 °C and the

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