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Characterization of Short-Chain Alkyl Ether Lecithin Analogues: ^{13}C NMR and Phospholipase Studies†

R. A. Burns, Jr.,‡ J. M. Friedman, and M. F. Roberts*

ABSTRACT: Several short-chain ether-linked lecithin analogues (*rac*-1,2-dihexyl-, *rac*-1,2-diheptyl-, and *rac*-1,2-dioctyl-*sn*-glycero-3-phosphocholine and L-1,2-diheptyl-*sn*-glycero-3-phosphocholine) have been synthesized and characterized. When dispersed in aqueous solution, these synthetic phospholipids form micelles (not bilayers) and can be used to investigate phospholipase action. Critical micellar concentrations are 1.5- to 2-fold lower than those of the comparable chain length diacyllecithins. This critical micelle concentration difference corresponds to the methylene ether being approximately 200 cal/mol more "hydrophobic" than an ester moiety. This value is compatible with the solvent free energy transfer potentials for ester/ether substitution in model compounds. ^{13}C NMR has been used to characterize the conformation and mobility of short-chain lecithins as monomers and micelles [Burns, R. A., Jr., & Roberts, M. F. (1980) *Biochemistry 19*, 3100]. ^{13}C T_1 relaxation times, chemical shift differences generated in the monomer/micelle transition, and interchain magnetic shift nonequivalence generated by micellization are similar at corresponding carbon positions in ester and ether lecithins. However, T_1 relaxation times do indicate greater fluidity near the terminal methyl end of ether lecithin chains. These data suggest that the carbonyl groups make little contribution to overall lipid conformation and mobility. Ester and ether lecithins form a useful assay system for the phospholipases because substrates and inhibitors can be comixed with minimal change in the characteristics of the interface. Racemic and chiral diheptylphosphatidylcholines are equivalent inhibitors of phospholipase A_2 from *Naja naja naja*. If Michaelis–Menten kinetics are assumed, $K_I \sim 0.2K_m$ of the corresponding diester lecithin. The *sn*-2 ester carbonyl, although the site of hydrolysis, does not dominate the enzyme–lecithin binding interaction. Racemic and chiral diheptyllecithins are extremely poor substrates and/or inhibitors of phospholipase C from *Bacillus cereus*. Enzymatic activity against the ether lecithins could not be detected in the assay system used, nor do the ether lecithins affect the rate of hydrolysis of diheptanoylphosphatidylcholine in a mixed micellar system. This kinetic result suggests a requirement of *B. cereus* phospholipase C for fatty acyl carbonyl groups for efficient substrate binding.

Lecithins are the major lipids in a variety of membranes. Many physical studies have elucidated the conformation, structure, and packing of 1,2-diacyl-*sn*-glycero-3-phosphocholines and -ethanolamines (Seelig & Niederberger, 1974; Yeagle, 1978; Seelig & Browning, 1978; Buldt et al., 1978; Elder et al., 1977; Burns & Roberts, 1980). Yet, the extent to which phospholipid structural features affect chain conformation or aggregation is not well established. Modified lecithins occur in several membranes and may have distinct roles in altering membrane structure. In particular, phospholipids where one or both acyl groups are replaced by alkyl ether moieties are found in a variety of systems (Mangold, 1979). In diacyllecithins the two fatty acyl chains are conformationally nonequivalent: the *sn*-2 carbonyl and first few carbons of the chain are oriented parallel to the interface, while the *sn*-1 chain is perpendicular to the surface. This packing

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could in part be due to the polarity of the carbonyl group or to the rigidity of the ester linkage. If the carbonyl does contribute to lecithin conformation, then it may affect the activity and specificity of several interfacial enzymes, such as the phospholipases.

Recently, we found that short-chain lecithins yield very informative high-resolution ^{13}C NMR spectra (Burns & Roberts, 1980). Detailed spectral analyses of monomer and micelle are possible because all carbons are resolved. For the diacyllecithins, differences in the chemical shifts of comparable *sn*-1 and *sn*-2 carbons depend on the lecithin aggregation state: micellization enhances acyl chain magnetic nonequivalence. Comparison of ^{13}C relaxation behavior of micelles and ^{2}H relaxation in bilayers suggests that motions in the acyl chains are the same (Burns & Roberts, 1980; Brown et al., 1979). Therefore, these micellar systems offer a rapid way to screen chemically modified lipids and compare them to diacyllecithin aggregate structures.

The importance of the lecithin carbonyls has been examined by characterizing dialkyl ether short-chain lecithins. The ^{13}C magnetic nonequivalence of alkyl chains and comparable relaxation behavior of these molecules compared to ester-linked lipids suggest that the carbonyl groups are not required for chain packing and orientation of lipid aggregates. Because the two lecithins are so similar, they should form well-behaved mixed micelles for kinetic studies with phospholipases. In this way we can assess the contribution of the lipid carbonyl to enzyme binding and the existence of surface dilution kinetics (Dennis, 1973).

Experimental Procedures

Materials. Diheptanoylphosphatidylcholine (diheptanoyl-PC)[1] was synthesized by the fatty acid imidazolide method and purified by silicic acid chromatography (Burns & Roberts, 1980). Phospholipid purity was monitored by thin-layer chromatography in $CHCl_3/CH_3OH/H_2O$ (65:24:4) or in $CHCl_3/CH_3OH$/10.5 M NH_4OH (60:35:8) and ^{13}C NMR spectroscopy.

Dihexanoylglyceride was synthesized as follows: 7.7 ng of phospholipase C was added to 140 μmol of dihexanoyl-PC in 0.1 M Tris-HCl, pH 7.9, and allowed to react for 3 days under a N_2 atmosphere. The reaction mixture was extracted 3 times with $CHCl_3/CH_3OH$ (3:1). The pooled organic phase was then washed three times with distilled water. Thin-layer chromatography on silica plates in $CHCl_3/CH_3OH$/10.5 M NH_4OH (60:35:8) showed a single spot, R_f ~0.96), indicating the absence of dihexanoyl-PC (R_f ~0.40) or Tris buffer (R_f ~0.25). The sample showed two spots in petroleum ether/diethyl ether/glacial acetic acid (70:30:1) of R_f ~0.2 and ~0.25, possibly the 1,2- and 1,3-diglyceride isomers. The dihexanoylglyceride was dried under a stream of N_2 and evacuated at low pressure for 24 h.

Hexanoic acid (Aldrich, 99.5%) and hexanol (Aldrich, 98%) were used without further purification.

The general scheme followed for synthesis of diether lecithins is discussed below.

*3-Benzyl-1,2-isopropylidene-*L*-glycerol and 3-Benzyl-1,2-isopropylidene-rac-glycerol.* Solketal (1,2-isopropylidene-*rac*-glycerol), obtained from Aldrich Chemical Co., and 1,2-isopropylidene-L-glycerol (IPG), prepared by the method of LeCocq & Ballou (1964) from 1,2,5,6-diisopropylidene-D-mannitol (Baer, 1952), were benzylated by using a phase-transfer method comparable to that of DeCesarre & Grosse (1976) for glycoside diols. IPG (0.16 mol) was dissolved in 650 mL (4 L/mol) of benzene containing a 1.5-fold excess (0.25 mol) of benzyl chloride and 0.016 mol of tetrabutylammonium bromide. Subsequently, 650 mL of NaOH (50% w/w) was added with stirring to the benzene solution. The reaction was complete in 6–8 h. The benzene phase was separated, washed 3 times with Millipore-filtered distilled water, and dried over Na_2SO_4. Benzene was removed under reduced pressure. Vacuum distillation of the resulting syrup over zinc dust yielded 35.11 g of 3-benzyl-1,2-isopropylideneglycerol (98% yield): bp 92–95 °C (0.05 mm); $[\alpha]^{25}_D$ +14.7° for the chiral compound.

Deacetonation and Alkylation. Deacetonation to 3-benzylglycerol was performed by the method of Howe & Malkin (1951) with vigorous stirring and refluxing. The reaction took 40–60 min for completion. Distillation over zinc dust yielded 96% product.

The success of the phase-transfer technique for benzylation suggested a modified method for formation of the diether glycerol using the alkyl bromide as the alkylating agent and the basic phase-transfer methodology above. The reaction was monitored by thin-layer chromatography of the organic phase in ethyl ether/petroleum ether (1:1). For six- to eight-carbon alkyl chains, the reaction appeared to proceed with an intermediate buildup of the monoalkylglycerol (R_f 0.3–0.5) followed by formation of the dialkylated product (R_f 0.8–0.9).

Hydrogenation and Head-Group Coupling. Hydrogenation by the method of Kates et al. (1963) with the Pd/C catalyst of Hessel et al. (1954) produced the dialkyl-*O*-glycerols. The coupling of these compounds to phosphocholine followed the Brockerhoff & Ayengar (1979) procedure. Low yields of the final products (5–15% overall) were due to (i) the increased solubility of the final short-chain lecithin analogue in water and (ii) the propensity of toluenesulfonate to associate with the lecithin in inverse micelles. Purification usually required several silicic acid columns and, in the case of the dihexyl compound, preparative TLC. Products were characterized by multinuclear NMR; ^{1}H NMR spectral features for diheptyl-PC in $CDCl_3$ are as follows (peaks ppm downfield from internal Me_4Si): 0.84 (t, 6 H, terminal CH_3), 1.23 (s, 16 H, alkyl chain C-3,4,5,6), 1.50 (m, 4 H, alkyl chain C-2), 3.30 (s, 9 H, $N(CH_3)_3$), 3.31 (t, 2 H, *sn*-1 alkyl C-1), 3.46 (m, 2 H, *sn*-2 alkyl C-1), 3.35, 3.48, 3.79 (m, 2 H each, belonging to CH_2O and CH_2OP groups), 3.74 (broad s, 2 H, CH_2N), 4.26 (m, 1 H, CHO).

Determination of Critical Micelle Concentrations. The critical micelle concentrations (cmc's) of *rac*-diheptyl-, *rac*-dioctyl-, L-diheptyl-, L-diheptanoyl-, and L-dioctanoyl-3-glycerylphosphocholine were determined by using the fluorescent dye 8-anilinonaphthalene-1-sulfonate (Horowitz, 1977). The fluorimeter used was a Perkin-Elmer MPF-4 fluorescence spectrometer.

The cmc of *rac*-dihexyl-PC was determined from the concentration dependence of ^{13}C chemical shifts in 10 mM potassium phosphate–1 mM EDTA, pD 7.4, with internal [2-^{13}C]acetate. Phosphate analyses were done for each sample to ensure an accurate micellization curve based on lecithin concentration.

Enzymatic Assays. Phospholipase A_2 (*Naja naja naja*) and phospholipase C (*Bacillus cereus*) were purified as described elsewhere (Roberts et al., 1977; Little et al., 1975). Enzymatic hydrolysis of lecithin micelles was measured by pH-stat (pH 8 end point) (Dennis, 1973) at 25 °C. Assay mixtures contained 5–10 mM ester lecithin, 0–10 mM ether lecithin, and

[1] Abbreviations used: diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; cmc, critical micelle concentration; T_1, spin–lattice relaxation time; IPG, 1,2-isopropylidene-L-glycerol.

Table I: Critical Micelle Concentrations for Short-Chain Lecithins

phospholipid	cmc (mM)
rac-dihexyl-PC	8
L-dihexanoyl-PC	14[a,b]
rac-diheptyl-PC	1.1
L-diheptyl-PC	0.85
L-diheptanoyl-PC	1.6
rac-dioctyl-PC	0.10
L-dioctanoyl-PC	0.25

[a] Data from Tausk et al. (1974a,b). [b] R. A. Burns, Jr., M. F. Roberts, R. Dluhy, and R. S. Mendelsohn, unpublished results.

5 mM $CaCl_2$ for phospholipase A_2. Lipid samples were cosolubilized directly in the assay vessels.

NMR Spectroscopy. ^{13}C NMR spectra were obtained at 67.9 MHz with a Bruker 270 spectrometer equipped with a Nicolet 1080 data system. Spin–lattice relaxation times were measured as described previously (Burns & Roberts, 1980). Aqueous samples contained 7–80 mM lecithin, 50 mM potassium phosphate, and 1 mM EDTA in D_2O, pD 7.4, 303 K.

Results

Critical Micelle Concentrations and Physical Properties. Substitution of ether linkages for esters in the diglyceride portion of the lecithin yields lipids with consistently 1.5-2.0-fold lower cmc's (Table I). *rac*-Diheptyl- and L-diheptyl-PC have experimentally identical cmc's. Both dihexyl-PC and diheptyl-PC form optically clear solutions at high lecithin concentrations. In contrast, the eight-carbon ether-linked lecithin, dioctyl-PC, tends to separate into phases at higher concentrations. Similar behavior is seen with dioctanoyl-PC (Tausk et al., 1974b), but its phase separation can be suppressed by the addition of 0.2 M KSCN. Addition of KSCN to "salt-in" dioctyl-PC produces optically clear two-phase solutions. Analysis (by aqueous/organic extraction and phosphate assay (Eaton & Dennis, 1976)) of upper and lower phases of dioctyl-PC samples prepared in the NMR buffer shows that both phases contain similar concentration of phosphate buffer, but the upper phase contains greater than 95% of the dioctyl-PC.

^{13}C NMR Studies. Figure 1 shows the spectra of diheptyl-PC in D_2O and CD_3OD. Identification of carbon resonances (Table II) was made by comparison with diester lecithins and Lindeman–Adams calculations for alkyl chain carbons (Klein & Kemp, 1977). Spin–lattice relaxation times were used to differentiate glyceryl backbone and alkyl chain CH_2O resonances; the backbone CH_2O is expected to have the shorter T_1. All backbone and head-group carbon atoms are resolved in the ^{13}C spectra.

For monomers in CD_3OD or dihexyl-PC in D_2O, only the *sn*-1 and *sn*-2 C-1 and C-2 are nonequivalent (for example, see Figure 1). Upon aggregation of the lipid, other alkyl carbons become detectably nonequivalent. Similar *sn*-1/*sn*-2 magnetic chain nonequivalence is seen for diheptanoyl-PC as monomer and micelle (Table II). Chemical shift changes generated by the monomer to micelle transition for head-group and alkyl chain carbons of dihexyl-PC are similar to those for dihexanoyl-PC (Table III). These similarities in ^{13}C parameters suggest that chain packing and conformation are similar in the ester and ether lecithin micelles.

Table IV shows ^{13}C NMR T_1 relaxation times for monomeric and micellar diheptyl-PC. Comparison of the values for micellar diheptyl-PC with those for micellar diheptanoyl-PC (Burns & Roberts, 1980) shows that nearly all corresponding carbon atoms in the ester and ether lecithins have similar T_1 values (excluding the carbonyl vs. methylene difference at chain position 1). However, as shown in Figure 2, T_1s for the ether lecithins are significantly longer than those in the ester lecithin at alkyl chain carbons five and six.

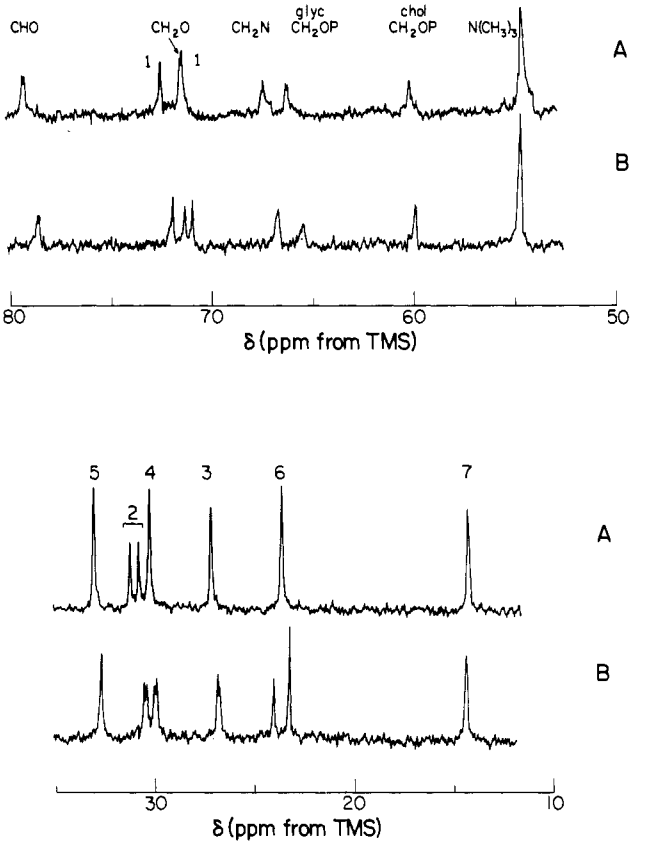

FIGURE 1: ^{13}C NMR spectra (67.9 MHz) of 80 mM diheptyl-PC in (A) CD_3OD and (B) D_2O. The resonance at 24.1 ppm in (B) is internal [2-^{13}C]acetate.

Table II: ^{13}C NMR Chemical Shifts of Monomeric and Micellar L-Diheptyl-PC (ppm from Me_4Si) and Comparison of *sn*-1/*sn*-2 Carbon Nonequivalence with Diheptanoyl-PC

carbon atom	monomer δ (CD_3OD)	Δ *sn*-1/*sn*-2[a]	micelle δ_c (D_2O)	Δ *sn*-1/*sn*-2[a] ether	ester[b]
head group					
$N(CH_3)_3$	54.70		54.85		
CH_2N	67.44		66.88		
CH_2OP	60.41		60.16		
backbone					
CH_2OP	66.19		65.66		
CHO	79.33		78.84		
CH_2O	71.64		71.56		
chains					
1	72.41	0.87	72.19	0.95	
	71.54		71.24		
2	31.23	0.38	30.55	0.12	0.11
	30.85		30.43		
3	27.26	0.0	26.89	0.11	0.15
			26.78		
4	30.35	0.0	30.06	0.12	0.14
			29.94		
5	33.09	0.0	32.72	0.0	0.06
6	23.73	0.0	23.36	0.0	0.0
7	14.48	0.0	14.57	0.06	0.04
			14.51		

[a] The absolute chemical shift difference (ppm) of *sn*-1 and *sn*-2 carbon atoms. [b] Data from Burns & Roberts (1980).

Phospholipase Activity toward Dialkyllecithins. Phospholipase A_2 catalyzes the hydrolysis of phospholipid *sn*-2 ester bonds. Substitution of an ether linkage at this site should

Table III: ^{13}C NMR Chemical Shifts (ppm from Internal [2-^{13}C]Acetate) of Aqueous *rac*-Dihexyl-PC and Changes upon Micellization

carbon atom	δ_C (monomer)	Δ(micelle - monomer) *rac*-dihexyl-PC	Δ(micelle - monomer) L-dihexanoyl-PC[a]
head group			
$N(CH_3)_3$	30.72	0.02	0.00
CH_2N	42.75	−0.02	0.00
CH_2OP	36.10	−0.06	−0.08
backbone			
CH_2OP	41.55	−0.08	−0.23
CHO	54.03	0.65	−0.21
CH_2O	47.20	−0.02	0.33
chains			
1	48.31[b]	−0.19	−3.89[c]
	46.28	1.02	−3.81[d]
2	5.71[b]	0.56	0.06[c]
	5.30	0.83	−0.04[d]
3	1.67	0.68	0.24[c]
		0.59	0.35[d]
4	7.59	0.62	0.61[d]
			0.47[c]
5	−1.30	0.50	0.45
6	−9.96	0.42	0.29

[a] R. A. Burns, Jr., and M. F. Roberts, unpublished results. [b] The first peak in each pair is the most downfield peak. [c] *sn*-2 carbon atom, identified previously [Burns & Roberts (1980)]. [d] *sn*-1 carbon atom, identified previously [Burns & Roberts (1980)].

Table IV: ^{13}C NMR T_1 Relaxation Times (s) for Monomeric (CD_3OD) and Micellar *rac*-Diheptyl-PC and L-Diheptyl-PC[a]

carbon atom	monomer *rac*-diheptyl-PC (CD_3OD)	micelle *rac*-diheptyl PC (D_2O)	micelle L-diheptyl-PC (D_2O)
head group			
$N(CH_3)_3$	0.95 (0.04)	0.77 (0.06)	0.83 (0.01)
CH_2N	0.75 (0.07)	0.47 (0.02)	0.53 (0.08)
CH_2OP	0.65 (0.05)	0.51 (0.02)	0.58 (0.07)
backbone			
CH_2OP	0.50 (0.03)	0.21 (0.03)	0.27 (0.04)
CHO	0.81 (0.04)	0.28 (0.02)	0.29 (0.04)
CH_2O	0.58 (0.02)	0.18(0.01)	0.19 (0.03)
alkyl chains[b]			
1	1.29 (0.08)	0.46 (0.03)	0.38 (0.07)
	1.02 (0.19)	0.32 (0.04)	0.42 (0.04)
2	1.35 (0.08)	0.41 (0.05)	0.48 (0.01)
	1.40 (0.03)	0.52 (0.02)	0.56 (0.02)
3	1.91 (0.06)	0.61 (0.02)	0.79 (0.10)
		0.54 (0.04)	0.63 (0.02)
4	2.37 (0.10)	0.51 (0.06)	0.70 (0.01)
		0.60 (0.04)	0.78 (0.01)
5	3.68 (0.13)	1.07 (0.06)	1.02 (0.07)
6	4.50 (0.15)	1.69 (0.08)	1.65 (0.12)
7	4.72 (0.45)	2.41 (0.15)	2.69 (0.35)
		2.53 (0.05)	2.81 (0.20)

[a] Numbers in parentheses represent standard deviations in reported T_1 values. [b] The first value in each pair is for the most downfield peak.

generate a competitive inhibitor. As seen in Table V, the diether lecithin, either racemic or chiral, is an effective inhibitor of ester hydrolysis. In a simple Michaelis–Menten model, the phospholipase A_2 kinetics are consistent with K_I (ether) $\sim 0.2K_M$ usually found for these short-chain lecithins (Bonsen et al., 1972).

Phospholipase C catalyzes the hydrolysis of the diglyceride phosphate ester bond of phospholipids. Substitution of alkyl

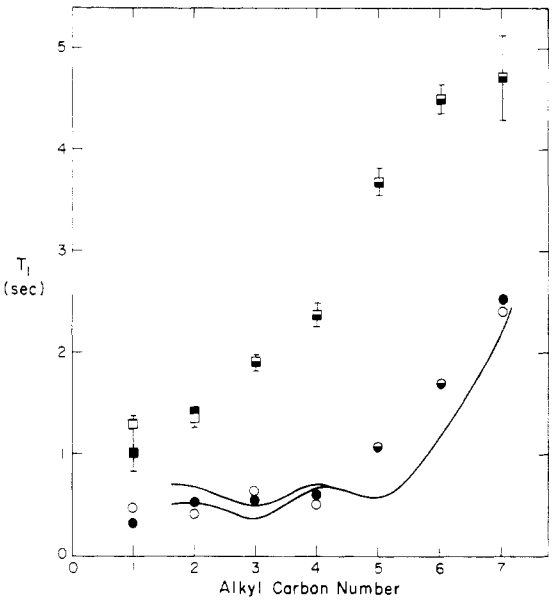

FIGURE 2: ^{13}C–T_1 profile for the alkyl chain carbons of diheptyl-PC as micelles (circles) and monomers (squares) in CD_3OD. The solid line shows the T_1 profile for diheptanoyl-PC micelles. Filled symbols represent the most downfield peak for an *sn*-2/*sn*-1 differentiated carbon; open symbols are for the upfield carbon. Where the two chains are equivalent, a half-filled symbol is used.

Table V: Phospholipase Activity toward 5 mM Diheptanoyl-PC and Mixed Micelles with Diheptyl-PC[a]

micelle system	phospholipase A_2 (μmol min^{-1} mg^{-1})	phospholipase C (μmol min^{-1} mg^{-1})
5 mM diheptanoyl-PC	213 (3)	1650 (90)
+2 mM *rac*-diheptyl-PC	60 (12)	1650 (45)
+5 mM *rac*-diheptyl-PC	40 (1)	
+2 mM L-diheptyl-PC	81 (2)	2000 (240)
+5 mM L-diheptyl-PC	46 (3)	1560 (10)
+5 mM diglyceride[b]		1250 (10)
+2 mM hexanoate		1390 (160)
+2 mM hexanol		1230 (10)
5 mM *rac*-diheptyl-PC	0	<4
10 mM *rac*-diheptyl-PC		<1
5 mM L-diheptyl-PC	0	<3

[a] Samples contained 5 mM $CaCl_2$ and were assayed at pH 8.0; numbers in parentheses are standard deviations. [b] 1,2-dihexanoylglycerol, prepared as described under Experimental Procedures.

ether for acyl moieties is a structural alteration three and four chemical bonds from the site of hydrolysis. Diheptyl-PC is not a good substrate of phospholipase C from *B. cereus* (Table V); activity could not be detected in the pH-stat assay used. In addition, the presence of racemic or L-diheptyl-PC does not markedly affect the observed specific activity against a fixed concentration of diheptanoyl-PC. Thus, diheptyl-PC appears to be neither a substrate nor an inhibitor. Lipophilic molecules such as 1,2-dihexanoylglycerol and hexanoic acid detectably inhibit phospholipase C (25% inhibition by 5 mM diglyceride, 16% inhibition by 2 mM hexanoic acid). These kinetics suggest that diheptyl-PC is not hydrolyzed because it does not bind to the active site of phospholipase C. Thus, the ester carbonyls must make a significant contribution to phospholipid binding to the enzyme.

Discussion

Alterations in the structure of a surface-active substrate can affect interfacial enzyme activity in two ways: (i) through normal enzyme–substrate binding and catalysis or (ii) by

altering the characteristics of the interface. Variation in the interface can change available surface area, affect protein conformation, alter water and ion composition near the interface, or change substrate mobility in the surface. A reasonable approach to maintaining fixed interfacial characteristics and yet retain the useful characteristics of a multicomponent kinetic system is to use molecules with similar structures. The short-chain ether and ester lecithins form a system where substrate and/or inhibitor concentrations can be varied without drastically changing interfacial characteristics.

Replacement of ester bonds with ether linkages does not appear to affect micellar lecithin behavior drastically, as judged by physical studies. Differences in cmc between analogous ether/ester lecithins are small. With the nomenclature of Tanford (1973), if monomer lipid is in equilibrium with micelles of the same mean aggregation number (m), then the average cmc difference between ester and ether lecithins yields a $\Delta\Delta\mu^\circ$ [$\Delta\mu^\circ$(ether) − $\Delta\mu^\circ$(ester)] equal to −380 (± 120) cal/mol. Micelles are favored over monomer by an average of −200 cal/mol for each methylene ether substituted for an ester in the lecithin molecule. Estimates based on solvent transfer data (Tanford, 1973; Davis et al., 1974) of model compounds are compatible with these values; i.e., the cmc difference is that expected for substitution of two methylene ethers for ester groups. If ether lecithins are approximately 400 cal/mol more "hydrophobic" than the corresponding ester lecithins, then a slightly higher gel to liquid crystalline phase transition temperature might be expected for long-chain ether lecithins. Both slightly higher and slightly lower phase transition temperatures have been reported for equivalent synthetic lecithins and synthetic ether lecithins, although all studies agree that phase transition temperature differences for corresponding ester/ether lecithins are small, on the order of a few degrees (Lee & Fitzgerald, 1980; Sunder & Bernstein, 1978; Paltauf et al., 1971).

Both ^{13}C NMR chemical shift and T_1 relaxation studies of the monomer/micelle transition are consistent with similar conformations and mobilities for ester- and ether-linked lecithins. Monomer/micelle chemical shift differences for corresponding carbon atoms in dihexyl-PC and dihexanoyl-PC chains and head groups are similar (Table III), although they are considerably larger for the initial part of the ether alkyl chain. For the ester lecithin, micellization shifts arise from solvent transfer to a less hydrogen bond forming environment (head group and backbone), a slight increase in the probability of trans conformers for micellized acyl chains, and a possible conformational change in the glycerol backbone (R. A. Burns, Jr., M. F. Roberts, R. Dluhy, and R. S. Mendelsohn, unpublished results). A notable exception to the similarity between ether and ester micellization shifts is the ether backbone region, in particular the CHO which shifts in the opposite direction to the corresponding ester lecithin carbon. However, these carbons are close to the ester/ether substitution site. In dihexanoyl-PC the presence of the carbonyl may generate an electric field effect which dominates the observed shift of the CHO. Alternatively, solvent transfer alone can model the ether lecithin CHO data, indicating little, if any, conformational change in the glyceryl backbone upon micellization. An analysis of ^1H coupling constants for backbone protons will aid in comparing ether and ester lecithin conformation.

The absolute value of the interchain magnetic nonequivalence generated by micellization is quite similar for diheptyl-PC and diheptanoyl-PC; both the magnitude of the shift differences (Table II) and the T_1 values for most comparable carbon atoms in diheptyl-PC and diheptanoyl-PC are within experimental uncertainty (Burns & Roberts, 1980). However, ether lecithin T_1s at chain positions five and six are considerably longer than T_1s for the corresponding ester lecithin chain positions. Comparison of T_1 values for dioctanoyl-PC (Burns & Roberts, 1980) with those of diheptyl-PC (both chains having the same number of methylene units) shows that ether lecithin T_1s are still 1.5 times the values for the corresponding ester positions. Calculations available elsewhere (Wennerstrom et al., 1979) suggest that this arises from differences in fast motional contributions to T_1. Further studies of the cause for this difference in T_1 behavior will center on determination of the micelle sizes for the corresponding ester and ether lecithins. The differences in T_1s could represent packing constraints in the center of the micelle caused by the structure of the micelle itself.

Because all of these physical parameters suggest that the motion and conformation of short-chain ether and ester lecithins in micelles are quite similar, we can interpret kinetics of phospholipase hydrolysis of these mixed micelles in terms of substrate binding requirements. The kinetics of diheptanoyl-PC hydrolysis in the presence or absence of *rac*- or L-diheptyl-PC show that ether lecithins are effective inhibitors of cobra venom phospholipase A_2. Pure competitive inhibition or Michaelis–Menten kinetics have not been unambiguously established for this system, but the differences in K_I and K_M are small enough to represent simple differences in binding energy. The small difference in K_M and K_I suggests that the ester carbonyls are not dominant binding determinants for the phospholipase A_2–lecithin interaction.

rac- and L-diheptyl-PC are neither effective substrates nor inhibitors of phospholipase C from *B. cereus*. This phenomenon must arise from poor binding of diheptyl-PC to the enzyme active site, since the presence of the ether lecithin does not affect observed specific activities toward diheptanoyl-PC (Table V). Thus, in contrast to phospholipase A_2, the ester carbonyls are an important binding determinant for the phospholipid–phospholipase C (*B. cereus*) interaction. Such behavior may be specific to the phospholipase C from *B. cereus*. Rosenthal & Pousada (1966a,b) showed that a nonhydrolyzable isosteric ether lecithin analogue with a C–P bond was an extremely potent inhibitor of the enzyme isolated from *C. perfringens*. Since the ester carbonyls make a significant contribution to the enzyme–lecithin binding interaction of the *B. cereus* enzyme, product inhibition should be much more effective by diglycerides than by phosphorylated base head groups. The failure of such compounds as glycerylphosphorylcholine, choline phosphate, etc. (Litvinko et al., 1977), to inhibit phospholipase C from a variety of sources is known. Product inhibition by diglyceride is observed in our assay system (Table V) and has also been observed for phospholipase C from *Cl. perfringens*. Bangham & Dawson (1962) noted that phospholipase C activity ceases completely when 60–70% of the substrate has been hydrolyzed. While they attributed this effect to changes in aggregate net charge, enzyme inhibition by diglyceride could also explain the data. It should be noted that hexanoic acid and hexanol also inhibit phospholipase C to comparable extents.

The lack of effect of diheptyl-PC on diheptanoyl-PC hydrolysis by phospholipase C suggests that this mixed micelle system is ideal to investigate "surface dilution" kinetics for that enzyme. Diheptyl-PC is, in effect, the ideal noninteracting surface diluter. While preliminary results tend to indicate that a rate-limiting interfacial binding step distinct from the catalytic step does not occur (i.e., the rate for diheptanoyl-PC diluted with 1:1 with diheptyl-PC is 0.94 the hydrolysis rate

of pure ester lecithin), a detailed kinetic investigation should be definitive.

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On the Ratio of the Proton and Photochemical Cycles in Bacteriorhodopsin†

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ABSTRACT: The ratio of protons released per M-412 intermediate formed in illuminated purple membrane sheets of *Halobacterium halobium* varies from 0.3 to 3.0 as a function of the ionic strength and light intensity. The ratio increases with decreasing light intensity. At high ionic strength the illumination time affects the monophasic rate of proton uptake and biphasic rates of the M-412 decay. The influence of the ionic strength on the ratio is discussed on the basis of Gouy–Chapman theory as a consequence of pK_a shifts of light-activated proton binding groups.

Under conditions of low oxygen tension in the presence of light, *Halobacterium halobium* develops a membrane system, the purple membrane (PM),[1] which acts as a light-driven proton pump converting light energy into electrochemical potential energy ($\Delta\mu_{H^+}$) acrosss the bacterial membrane [for summary, see Stoeckenius et al. (1979)]. $\Delta\mu_{H^+}$ can drive ATP synthesis (Oesterhelt, 1974; Danon & Stoeckenius, 1974) and other energy-linked cellular functions (Lanyi, 1978). The energy transduction is due to the function of a single chromoprotein, bacteriorhodopsin, which in a complex of about 10 lipid molecules/protein associates to form the purple membrane in the bacterial plasma membrane (Stoeckenius et al., 1979).

Functional analysis of the isolated purple membrane revealed the occurrence of a photochemical cycle coupled to a release of protons (Oesterhelt & Hess, 1973). This phenomenon also could be observed in the intact bacterial cell (Oesterhelt & Stoeckenius, 1973; Oesterhelt, 1974). It was shown that upon illumination protons are liberated directly from the purple membrane in a time course which correlates with or lags behind the photochemical formation of a stable intermediate absorbing at 412 nm (M-412) (Oesterhelt & Hess, 1973; Lozier et al., 1975; Chance et al., 1975; Lozier

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[1] Abbreviations used: PM, purple membrane; M-412, intermediate absorbing at 412 nm.

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