Methyl Branching in Short-Chain Lecithins: Are Both Chains Important for Effective Phospholipase A_2 Activity?†

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ABSTRACT: Several seven-carbon fatty acyl lecithins with varied acyl chain branching have been synthesized and characterized as potential phospholipase A_2 substrates. Micellar bis(4,4-dimethylpentanoyl)phosphatidylcholine, bis(5-methylhexanoyl)phosphatidylcholine, bis(3-methylhexanoyl)phosphatidylcholine, and bis(2-methylhexanoyl)phosphatidylcholine are poor substrates for phospholipase A_2 (*Naja naja naja*). These branched lecithins also inhibit the hydrolysis of diheptanoylphosphatidylcholine by the enzyme with K_i values comparable to or smaller than the apparent K_m of the linear compound. The terminally branched lecithins are excellent substrates for another surface-active hydrolytic enzyme, phospholipase C from *Bacillus cereus*. When only one acyl chain bears a methyl group, the hybrid lecithins 1-heptanoyl-2-(2-methylhexanoyl)-phosphatidylcholine and 1-(3-methylhexanoyl)-2-heptanoylphosphatidylcholine are substrates comparable to diheptanoylphosphatidylcholine. Analysis of micellar structure and dynamics by ^1H and ^{13}C NMR spectroscopy, quasi-elastic light scattering, and comparison of critical micellar concentrations indicates little significant difference in the conformation and dynamics of these seven-carbon fatty acyl lecithin micelles, even when the methyl groups are adjacent to the carbonyls. Phospholipase A_2 UV difference spectra induced by phospholipid binding imply different enzyme conformations or aggregation states caused by linear-chain and asymmetric-chain lipids compared to bis(methylhexanoyl)phosphatidylcholines. The differences in hydrolytic activity of phospholipase A_2 against the branched-chain micellar lecithins can then be attributed to an enzyme–lipid interaction at the active site. The species with both fatty acyl chains branched bind to phospholipase A_2 but are not turned over rapidly. Since poor enzymatic activity only occurs for lecithins with both chains methylated, the interaction of both chains with the enzyme must be important for catalytic efficiency.

Short-chain lecithin micelles have been used extensively to characterize the activity of water-soluble phospholipases (Verger & DeHaas, 1976; Wells, 1974; Little, 1977). A common feature of these esterases is their dramatically enhanced hydrolysis rates toward micellar or aggregated phospholipid compared to the monomeric substrate. They often show sensitivity to lecithin structural modifications remote from the site of catalysis (Burns et al., 1981). For enzymes with soluble, monomeric substrates, chemical modification of the substrate molecule can often be correlated with enzyme binding requirements. For surface-active enzymes, such as phospholipases, the added complication of altered aggregate structure must also be considered.

In an attempt to understand the kinetics of phospholipases, we have synthesized and characterized a series of lecithins with seven-carbon fatty acyl chains: diheptanoyl-PC,[1] bis(2-methylhexanoyl)-PC, bis(3-methylhexanoyl)-PC, bis(5-methylhexanoyl)-PC, bis(4,4-dimethylpentanoyl)-PC, 1-heptanoyl-2-(2-methylhexanoyl)-PC, and 1-(3-methylhexanoyl)-2-heptanoyl-PC. Branching of lecithin fatty acyl chains could perturb chain orientation and therefore packing and micelle characteristics (as well as monomer conformation). Alterations in phospholipase activity toward these systems might be caused by altered micelle surface properties or altered phospholipid structures and not reflect steric constraints at the enzyme active site.

Examination of critical micellar concentrations, ^1H NMR glycerol backbone coupling constants, ^{13}C NMR relaxation times, and hydrodynamic radii (by quasi-elastic light scattering spectroscopy) of these lipid micelles suggests that micellar structure and dynamics are not significantly different when the acyl chains have branching methyl groups. Yet compared to diheptanoyl-PC, the lecithins with both chains branched are poor substrates and good inhibitors of phospholipase A_2 and nearly equivalent substrates for phospholipase C. When only one acyl chain bears a methyl group, the hybrid lecithin is easily hydrolyzed by phospholipase A_2. This occurs even when the added methyl group is adjacent to the lipid ester bond that is to be cleaved. Because micellar structure and behavior and lipid conformation are not radically different in these systems, we postulate that the inhibition of phospholipase A_2 by branched acyl chain lecithins is due to a steric interaction of both bulky fatty acyl chains with the enzyme that prevents a catalytically efficient change in the protein aggregation state or conformation. This is supported by results of UV absorption difference spectra. The importance of both fatty acyl chains and steric constraints at the enzyme active site are discussed in terms of the crystal structure of *Crotalus atrox* phospho-

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[1] Abbreviations: diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; QLS, quasi-elastic light scattering; cmc, critical micelle concentration; T_1, spin–lattice relaxation time; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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lipase A_2 (Keith et al., 1981).

Experimental Procedures

Materials. Diheptanoyl-PC was obtained from Avanti Biochemicals. Symmetric branched seven-carbon chain lecithins were synthesized by the fatty acyl imidazolide method using 2-methylhexanoic acid (Saber Laboratories), 3-methylhexanoic acid (Saber Laboratories), 5-methylhexanoic acid (Pfaltz and Bauer), and 4,4-dimethylpentanoic acid (Saber Laboratories) (Burns, 1982; Burns et al., 1983; Boss et al., 1975). Lipid purity was monitored by thin-layer chromatography as described previously (Burns & Roberts, 1980). ^{1}H NMR spectral features for bis(5-methylhexanoyl)-PC in CD_3OD are as follows (peaks ppm downfield from internal Me_4Si): 0.90 (d, 6 H, terminal CH_3), 1.21 (m, 4 H, 4-CH_2), 1.56 (m, 2 H, 5-CH), 1.62 (m, 4 H, 3-CH_2), 2.30 and 2.32 (2 t, 2 H each, *sn*-1 and *sn*-2 2-CH_2), 3.22 [s, 9 H, $N(CH_3)_3$], 3.64 (br t, 2 H, CH_2N), 3.99 (m, 2 H, glycerol CH_2OP), 4.27 (m, 2 H, choline CH_2OP), 4.16 and 4.42 (m, 2 H, CH_2O), and 5.22 (m, 1 H, CHO). ^{1}H NMR spectral features for acyl chain groups of bis(4,4-dimethylpentanoyl)-PC in CD_3OD are as follows: 0.90 and 0.91 (2 s, 9 H each, $C(CH_3)_3$], 1.52 and 1.54 (2 t, 4 H, 3-CH_2), and 2.30 and 2.32 (2 t, 2 H each, 2-CH_2). Backbone and headgroup resonances have chemical shifts identical with those of bis(5-methylhexanoyl)-PC. Because the 2-methylhexanoate and 3-methylhexanoate used in lipid synthesis are racemic mixtures, the ^{1}H NMR spectra showed complications indicative of the different isomers. A full characterization of the ^{1}H NMR features of these compounds will be given elsewhere.

Synthesis of asymmetric seven-carbon lecithins was achieved by imidazole-activated acylation of the appropriate lysolecithin obtained by incubation of the corresponding diacyl symmetric lecithin with immobilized phospholipase A_2 (Burns & Roberts, 1980). The reaction of the enzyme with diheptanoyl-PC goes to completion, and the heptanoyl lysolecithin was separated from free fatty acid by precipitation of the lyso compound from cold ethyl ether (4 °C). Acylation with 2-methylhexanoic acid yielded 1-heptanoyl-2-(2-methylhexanoyl)-PC. The phospholipase A_2 hydrolysis of bis(3-methylhexanoyl)-PC under comparable conditions (100 mM Tris-HCl, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.5, 50 °C) did not produce more than 40–50% lyso-PC. To separate the branched fatty acyl lyso compound from unreacted lecithin, phospholipase C (*Bacillus cereus*) was added to convert lecithin to diglyceride and phosphocholine (Little, 1977). When lecithin was no longer detected by thin-layer chromatography, the reaction mixture was adjusted to pH 3, lyophilized, then resuspended in chloroform. Incubation with 10-fold excess of heptanoic acid reacted with 1,1-carbonyldiimidazole yielded 1-(3-methylhexanoyl)-2-heptanoyl-PC. Both of these asymmetric lecithins were checked for purity by ^{13}C NMR spectroscopy. The ^{13}C chemical shifts of the acyl chain carbons are diagnostic for *sn*-1/*sn*-2 position (Burns & Roberts, 1980) and identity of the fatty acid. For 1-(3-methylhexanoyl)-2-heptanoyl-PC the acyl chain carbon chemical shifts in CD_3OD are as follows (ppm from Me_4Si): for the *sn*-1 chain, 41.6 [C(2)], 30.4 [C(3)], 29.3 [C(4)], 20.1 [C(5)], 13.5 [C(6)], 19.1 [C(3′)]; for the *sn*-2 chain, 34.3 [C(2)], 25.2 [C(3)], 29.0 [C(4)], 31.7 [C(5)], 22.8 [C(6)], and 13.6 [C(7)]. For 1-heptanoyl-2-(2-methylhexanoyl)-PC, the acyl chain shifts in CD_3OD are as follows (ppm from Me_4Si): for the *sn*-1 chain, 34.1 [C(2)], 25.0 [C(3)], 28.9 [C(4)], 31.7 [C(5)], 22.6 [C(6)], and 13.9 [C(7)]; for the *sn*-2 chain 40.3 [C(2)], 32.3 [C(3)], 29.5 [C(4)], 21.9 [C(5)], 16.8 [C(6)], and 17.2 [C(2′)]. Confirmation of these structures was obtained by complete phospholipase A_2 hydrolysis of each asymmetric lecithin, purification of fatty acid from lysolecithin via silicic acid chromatography, and subsequent analysis by ^{13}C NMR spectroscopy. Each enzymatically liberated fatty acid [heptanoic acid from 1-(3-methylhexanoyl)-2-heptanoyl-PC; 2-methylhexanoic acid from 1-heptanoyl-2-(2-methylhexanoyl)-PC] was judged at least 95% pure.

Sample preparation for NMR and quasi-elastic light-scattering experiments has been described previously (Burns & Roberts, 1980; Burns et al., 1983). The activities of phospholipase A_2 purified from cobra venom (Roberts et al., 1978) and phospholipase C purified from *B. cereus* (Little et al., 1975) toward short-chain lecithins were measured by the pH-stat technique (Dennis, 1973) with a pH 8 end point.

NMR Spectroscopy. ^{13}C NMR spectra were obtained at 67.9 MHz with a Bruker 270 spectrometer. Samples contained 80 ± 10 mM lecithin in 50 mM potassium phosphate and 1 mM EDTA in D_2O, pD 7.4, 30 °C. Spin–lattice relaxation times were measured as described previously (Burns & Roberts, 1980). ^{1}H NMR spectra at 270 MHz (Bruker 270) were used to measure critical micelle concentrations of lecithins. Analysis of glycerol backbone coupling and fatty acyl chain nonequivalence was done at 500 MHz on a home-built spectrometer at the Francis Bitter National Magnet Laboratory. For several of the branched-chain lecithins, a two-dimensional *J*-resolved experiment (Aue et al., 1976; Bax, 1982) was used. The pulse sequence for the latter is T–$(\pi/2)_x$–$t_{1/2}$–$(\pi)_y$–$t_{1/2}$–ACQ. Data reduction of the two-dimensional data set used a Gaussian window function to enhance resolution. Error in measuring coupling constants in these micellar systems with this technique is 0.3 Hz.

Quasi-Elastic Light Scattering. The use of QLS to study micellar systems has been described in detail (Missel, 1981; Missel et al., 1980; Mazer et al., 1976). This technique measures time-dependent fluctuations in the scattered light to form an autocorrelation function from which is extracted a diffusion coefficient. The mean hydrodynamic radius, $\bar{R}_h$, is then calculated from the mean diffusion coefficient with the Stokes–Einstein relation. For a polydisperse system, the width of the distribution is characterized by the variance, the standard deviation of the mean decay constant. Several linear short-chain lecithins have already been examined by QLS (Burns et al., 1983) and provide a basis for comparing the effects of methyl branching on micelle size.

UV Difference Spectroscopy. Difference spectra were obtained with a Perkin-Elmer Lambda 3 spectrophotometer. Both sample and reference cuvettes contained 0.5 mg mL^{-1} phospholipase A_2 in 100 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA, pH 7.5. Buffered lipid solution was pipetted into the sample cuvette while an equal volume of buffer was added to the reference cuvette. The effect of Ba^{2+} on these spectra was assessed by adding 5 mM metal ion to both sample and reference cuvettes. Scans were taken from 340 to 250 nm.

Results

Critical Micelle Concentrations of Short-Chain Lecithin Micelles. Table I shows critical micellar concentrations (cmc's) determined by ^{1}H NMR spectroscopy for the short-chain lecithins in D_2O. cmc values increase as acyl chain branching increases in the series. The value for diheptanoyl-PC is in good agreement with the cmc determined by Tausk et al. (1974a). Tanford (1973) has shown the free energy of transfer from hydrocarbon to water, $\mu^\circ_{HC} - \mu^\circ_W$, is a linear function of the number of methylenes and methyl groups in the chain for linear hydrocarbons only. Branched-chain hydrocarbons do not follow such a simple rule. Using the

Table I: Critical Micellar Concentrations and Differences Calculated from Hydrocarbon Solubility Data

lecithin	cmc (mM)[a]	cmc/cmc$_0$[b] calcd[c]	cmc/cmc$_0$[b] obsd
diheptanoyl-PC	1.6 (0.1)		1.0
bis(5-methylhexanoyl)-PC	3.4 (1.1)	2.1	2.1
bis(3-methylhexanoyl)-PC	2.9 (1.0)	1.8	1.8
bis(2-methylhexanoyl)-PC	3.3 (1.3)	2.1	2.1
bis(4,4-dimethylpentanoyl)-PC	4.3 (1.3)	3.8	2.7
1-heptanoyl-2-(2-methylhexanoyl)-PC	1.4 (0.3)	1.4	0.9
1-(3-methylhexanoyl)-2-heptanoyl-PC	3.8 (0.5)	1.5	2.4

[a] Values in parentheses represent standard deviations in the cmc measurements. [b] The ratio of the indicated lecithin cmc to that of linear diheptanoyl-PC (cmc$_0$). [c] Calculations of the cmc derived from hydrocarbon solubility data are described in the text.

aqueous hydrocarbon solubility data of McAuliffe (1966), we can find hydrocarbons that differ in the same fashion as the acyl chains of the three lecithins. Differences in aqueous solubility of hexane (mole fraction solubility in water $X_w = 2.0 \times 10^{-6}$), 2-methylpentane ($X_w = 2.9 \times 10^{-6}$), 3-methylpentane ($X_w = 2.7 \times 10^{-6}$), and 2,2-dimethylbutane ($X_w = 3.9 \times 10^{-6}$) suggest that the differences in cmc's for the lecithins are due to differences in aqueous solubility of acyl chains. Table I shows the relative cmc values expected if $\mu^\circ_{HC} - \mu^\circ_W$ is altered by an amount equal to $2(\mu^\circ_{HC} - \mu^\circ_W)$ for the corresponding two hydrocarbons. This model assumes equilibrium between monomers and micelles of the same size. A "cratic" (entropy of dilution) correction was not applied to the hydrocarbon solubility data. This correction is small (~0.3% of total) and similar for all the hydrocarbons. All symmetric lecithins have experimental cmc's in good agreement with predicted values. On the other hand, the two asymmetric lecithins deviate from prediction in an interesting fashion. When only the *sn*-2 chain has a methyl group adjacent to the carbonyl, there is little difference in the cmc of that lipid from diheptanoyl-PC. In contrast, when only the *sn*-1 chain is branched, the perturbation on the observed cmc is much larger than one predicts. The difference in these two asymmetric lecithins implies that the two chains are not identical in their aqueous exposure in going from monomer to micelle. This is consistent with the long-chain lecithin structure generated by X-ray (Pearson & Pascher, 1979) and neutron (Buldt et al., 1978) diffraction work and other NMR studies (Roberts et al., 1978; Seelig & Browning, 1978; Burns & Roberts, 1980).

QLS of Branched-Chain Lecithins. Figure 1 shows the dependence of the hydrodynamic radius, $\bar{R}_h$, on lecithin concentration for linear diheptanoylphosphatidylcholine and the symmetric branched-chain lecithins. At concentrations approaching the cmc, $\bar{R}_h$ of all these seven-carbon lecithin micelles approaches approximately 20 Å. Above the cmc, $\bar{R}_h$ increases, more rapidly for diheptanoyl-PC and bis(5-methylhexanoyl)-PC and less rapidly the closer the branch methyl is to the carbonyl group. For any given concentration, micellar size is decreased by branching and placing the methyl group close to the interface. For comparison, the dihexanoyl-PC micelle ($\bar{R}_h \sim 20$ Å) does not increase in size with increasing lipid concentration (P. J. Missel and G. B. Benedek, unpublished results; Tausk et al., 1974b). Therefore, all the symmetric seven-carbon branched-chain lecithins form micelles intermediate in size between diheptanoyl-PC (60 Å at 50 mM) and dihexanoyl-PC (20 Å).

^{1}H NMR and ^{13}C NMR Analysis of Lecithin Conformation. The geminal and vicinal coupling constants of the lecithin glycerol backbone yield information about the conformation of the molecule (Hauser et al., 1980). In CD_3OD, dihexanoyl-PC, diheptanoyl-PC, bis(5-methylhexanoyl)-PC, bis-

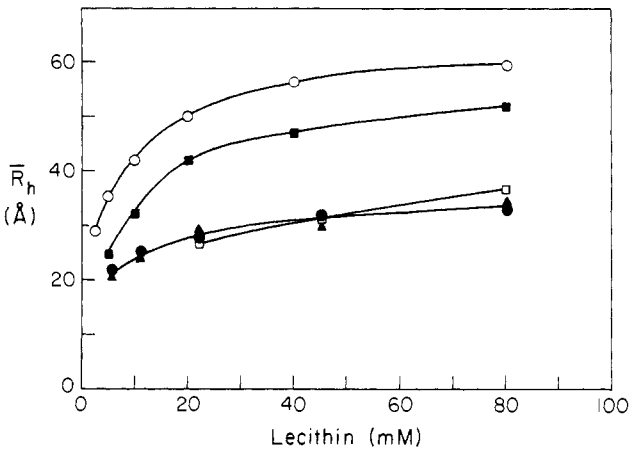

FIGURE 1: Mean hydrodynamic radius, $\bar{R}_h$, of seven-carbon lecithin micelles as a function of lecithin concentration: (○) diheptanoyl-PC; (■) bis(5-methylhexanoyl)-PC; (●) bis(3-methylhexanoyl)-PC; (▲) bis(2-methylhexanoyl)-PC; (□) bis(4,4-dimethylpentanoyl)-PC.

Table II: ^{1}H NMR (500 MHz) Magnetic Nonequivalence of Fatty Acyl Methylene and Methyl Protons of Seven-Carbon Short-Chain PC's

lecithin	$\Delta_{sn\text{-}1/sn\text{-}2}$ (Hz) CD_3OD (monomer)	$\Delta_{sn\text{-}1/sn\text{-}2}$ (Hz) D_2O (micelle)
diheptanoyl-PC		
2-CH_2	12	35
3-CH_2	0	*a*
7-CH_3	0	4
bis(5-methylhexanoyl)-PC		
2-CH_2	11.5	35.2
3-CH_2	<1	8.8
4-CH_2	0	2.5
5-CH	<1	6.6
6-CH_3	0	4.4
bis(4,4-dimethylpentanoyl)-PC		
2-CH_2	12	34.4
3-CH_2	7.8	13.2
5-CH_3	3.0	8.8

[a] The nonequivalence is difficult to measure by selective decoupling since this group is also coupled to 4-CH_2 and is partially overlapping with the bulky methylene peak.

(4,4-dimethylhexanoyl)-PC, and 1-heptanoyl-2-(2-methylhexanoyl)-PC show nearly equivalent glycerol coupling constants as measured from 1-D spectra ($^2J_{AB} = 12.1 \pm 0.2$, $^3J_{AC} = 6.8 \pm 0.1$, $^3J_{BC} = 3.2 \pm 0.1$). Upon micellization in D_2O, changes in ^3J values occur that are consistent with fatty acyl chains on average kept more eclipsed ($^3J_{AC} = 8.3 \pm 0.3$, $^3J_{BC} = 2.9 \pm 0.3$). Because racemic fatty acids were used to synthesize the 2-methyl and 3-methyl lecithins, the ^{1}H NMR spectra are extremely complex, representing compounds with both L or D and mixed L,D pairs. Slight chemical shift differences among these species make a simple one-dimensional analysis of backbone coupling constants very difficult. To measure these coupling constants, two-dimensional *J*-resolved spectra were acquired. A comparison of micellar coupling constants for these compounds with those for diheptanoyl-PC determined by a similar *J*-resolved experiment ($^2J_{AB} = 12.8$ Hz, $^3J_{AC} = 8.1$ Hz, and $^3J_{BC} = 3.4$ Hz) shows very similar values (±0.1 Hz) for all lecithins. The overall conclusion from examining backbone coupling constants is that adding bulky groups to chains does not dramatically affect lecithin glycerol conformation.

Another way of comparing chain orientation/conformation in the terminally branched lecithins is to examine the chain nonequivalence of *sn*-1 and *sn*-2 methylene and methyl protons (Table II) (Roberts et al., 1978). For monomeric dihepta-

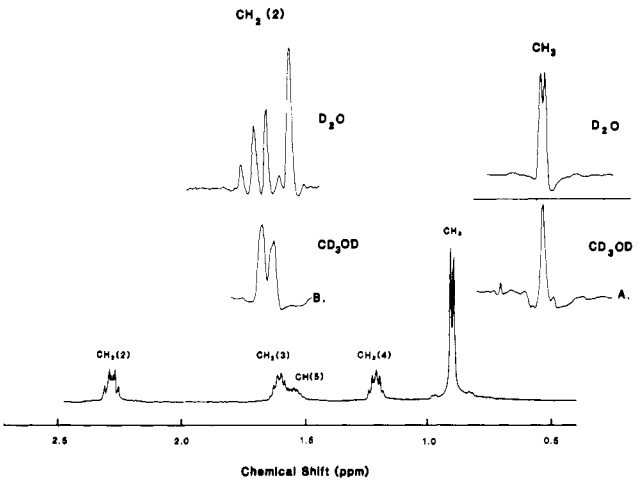

FIGURE 2: The 500-MHz ^1H NMR spectrum of the acyl chain region of bis(5-methylhexanoyl)-PC in CD_3OD; the identity of resonances are indicated above. Inset A shows the CH-decoupled terminal CH_3 in CD_3OD (a singlet) and in D_2O (*sn*-1/*sn*-2 nonequivalent peaks), and inset B shows the 3-CH_2-decoupled nonequivalent 2-CH_2 in CD_3OD (two singlets) and in D_2O as micelles (AB quartet for *sn*-2 group, singlet for *sn*-1).

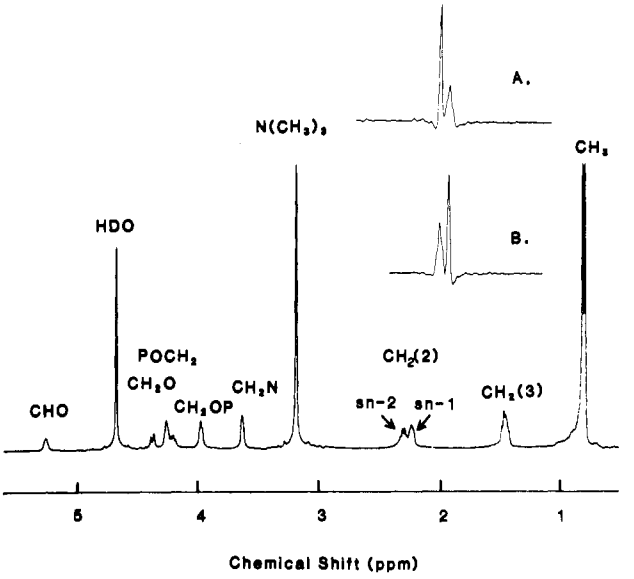

FIGURE 3: The 500-MHz ^1H NMR spectrum of bis(4,4-dimethylpentanoyl)-PC (80 mM) in D_2O. The inset shows the 3-CH_2 region when the *sn*-2 2-CH_2 AB pattern is irradiated (A) and when the *sn*-1 2-CH_2 group is irradiated (B).

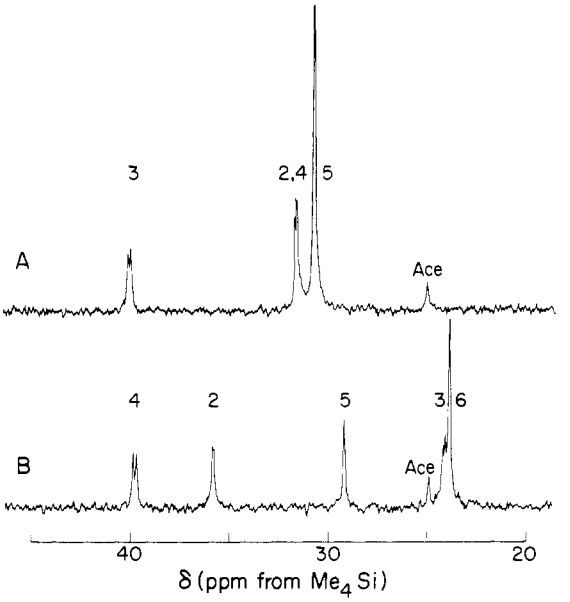

FIGURE 4: ^{13}C NMR spectra of the alkyl chain region of aqueous (A) bis(4,4-dimethylpentanoyl)-PC (80 mM) and (B) bis(5-methylhexanoyl)-PC (80 mM). Carbon resonances are identified by number from the acyl chain carbonyl.

noyl-PC, only the methylene protons adjacent to the carbonyl are observed to be nonequivalent. For micelles, this nonequivalence is propagated down the chain; two overlapping triplets that can be collapsed to two singlets by irradiation of the bulk CH_2 indicate the terminal methyl groups of the chains are magnetically distinct in micelles. For bis(5-methylhexanoyl)-PC (spectrum of all chain protons in CD_3OD shown in Figure 2), the C(2) methylene is nonequivalent in CD_3OD (Figure 2B); in D_2O, this group is observed as an *sn*-2 AB quartet and an *sn*-1 singlet when the adjacent methylene is irradiated. The micellar chain nonequivalence is also seen at the terminal methyl group (Figure 2). In contrast to the linear and singly branched compounds, bis(4,4-dimethylpentanoyl)-PC shows *sn*-1/*sn*-2 nonequivalence of all chain protons in monomer as well as micelle state (Table II), this magnetic nonequivalence increases further in the micellar solution. Two terminal methyl resonances are observed indicating *sn*-1/*sn*-2 nonequivalence in CD_3OD and D_2O. When the 2-CH_2 is irradiated selectively, the 3-CH_2 region appears as two resonances; by irradiating the 2-CH_2 *sn*-2 downfield peak, the downfield peak of 3-CH_2 increases in intensity (Figure 3A); irradiation of the *sn*-1 peak correlates with the upfield 3-CH_2 peak (Figure 3B). If the observed nonequivalence reflects a slight change in chain orientation [perhaps increased chain order or a conformational change involving the glycerol CHO as suggested by ^{13}C chemical shift studies of the dihexanoyl-PC monomer-to-micelle transition (Burns et al., 1982b)], then branching the acyl chains at the terminus must encourage similar packing even in monomeric solutions.

An analysis of the ^{13}C chemical shifts of alkyl carbons shows that for monomers of diheptanoyl-PC, bis(5-methylhexanoyl)-PC, bis(3-methylhexanoyl)-PC, and bis(4,4-dimethylpentanoyl)-PC, only the *sn*-1 and *sn*-2 C(2) methylenes are magnetically nonequivalent (0.18 ppm). As can be seen in the ^{13}C NMR spectra in Figure 4, this nonequivalence is propagated upon micellization down the chain with *sn*-1/*sn*-2 chemical shift differences comparable to the linear lecithin: for bis(5-methylpentanoyl)-PC, 0.06 ppm [C(2)], 0.12 ppm [C(3)], and 0.17 ppm [C(4)]; for bis(4,4-dimethylpentanoyl)-PC, ≤0.06 ppm [C(2)], 0.14 ppm [C(3)], and 0.08 ppm [C(4)]. When the branch methyl occurs near the interface, the magnetic nonequivalence of the two chains is more pronounced in monomer and micelle states (Figure 5). In bis(2-methylhexanoyl)-PC, the *sn*-1/*sn*-2 shift difference observed for the micellar alkyl carbon signals is larger than that observed for the other branched compounds: 0.62 ppm [C(2)], 0.26 ppm [C(3)], and 0.18 ppm [C(2′)]. For bis(3-methylhexanoyl)-PC, comparable chain differences are 0.14 ppm [C(2)], ≤0.08 ppm [C(3)], and 0.28 ppm [C(4)]. In monomer spectra, shift differences are observable at the C(2) position (0.18 ppm) for bis(3-methylhexanoyl)-PC and at C(2) and the 2-methyl group (0.18 and 0.18 ppm) for bis(2-methylhexanoyl)-PC. Part of the monomeric (and to some extent the micellar) shift differences observed in the bis(2-methylhexanoyl)-PC and bis(3-methylhexanoyl)-PC spectra reflect the racemic nature of the phospholipids. Because they have been synthesized from racemic fatty acids, there will be a mixture of isomers. A D or L configuration can occur on each acyl chain; hence, there are D,D, L,L, D,L, and L,D possible *sn*-1, *sn*-2 combinations. This isomer heterogeneity might help to

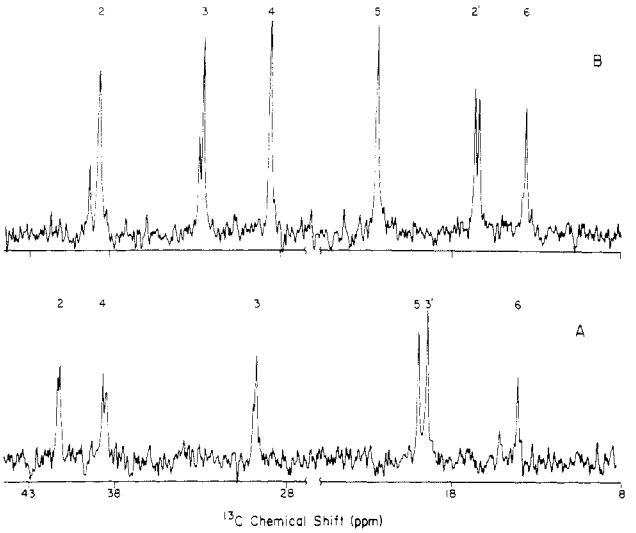

FIGURE 5: ^{13}C NMR spectra of the alkyl chain regions of aqueous (A) bis(3-methylhexanoyl)-PC (90 mM) and (B) bis(2-methylhexanoyl)-PC (90 mM). Carbons are identified with C(2) being the α-carbon and numbers increasing toward the methyl end. The terminal methyl carbons (but not other carbons) are partially saturated in these spectra.

account for the intensity differences for *sn*-1, *sn*-2 split peaks. For example, in bis(2-methylhexanoyl)-PC (Figure 5B), if one or more of the isomers has the 2'-methyl group shielding the *sn*-2 2-CH and 3-CH_2, the resulting upfield shift could superimpose it on the normal *sn*-1 position. Thus, the splitting usually associated with *sn*-1, *sn*-2 chain differences may be altered because of differential methyl shielding among the possible isomers. It should be noted that this is not as pronounced for bis(3-methylhexanoyl)-PC.

^{13}C Relaxation Times. Backbone, head-group, and carbonyl T_1 values of branched-chain lecithins are nearly identical with those of micellized diheptanoyl-PC (Burns & Roberts, 1980). This is not surprising since all particles are similar in size and the lecithin conformation is unaltered. Carbon atoms undergoing similar motions are expected to have different T_1 values related by NT_1 if N is the number of directly bonded protons. The NT_1 profile should indicate any unique motions in the micelle caused by acyl chain branching and packing. Acyl-chain NT_1 values are shown in Figure 6 along with T_1 values for diheptanoyl-PC and dihexanoyl-PC (Burns & Roberts, 1980). Micellar dihexanoyl-PC was studied at a concentration that was only 4–5 times the cmc. Therefore, monomeric dihexanoyl-PC contributes to the micellar T_1 values, leading to values that are somewhat higher than those for the branched-chain lecithins. However, all lecithins show quite similar characteristics: a plateau region near the ester linkage and then a dramatic increase in T_1 for the last two carbons. A similar "profile" is provided by looking at branched methyl T_1's in different lecithins (Figure 6B). For simple ^{13}C dipolar relaxation, T_1 is inversely proportional to solution viscosity (Levy et al., 1974). A given position along the acyl chain experiences the same relative viscosity and environment, since methyl groups and linear-chain carbons behave similarly.

Activities of Phospholipases against Branched Chain Lecithins. Table III shows hydrolytic activities of phospholipase A_2 (*N. naja naja*) and phospholipase C (*B. cereus*) against seven-carbon lecithin micelles. Phospholipase C shows reasonably similar activities against linear and methyl short-chain lecithin micelles. In contrast, acyl chain branching at any position on the short-chain lecithins causes these symmetric lecithins to become significantly poorer substrates for phospholipase A_2. These large differences in observed specific

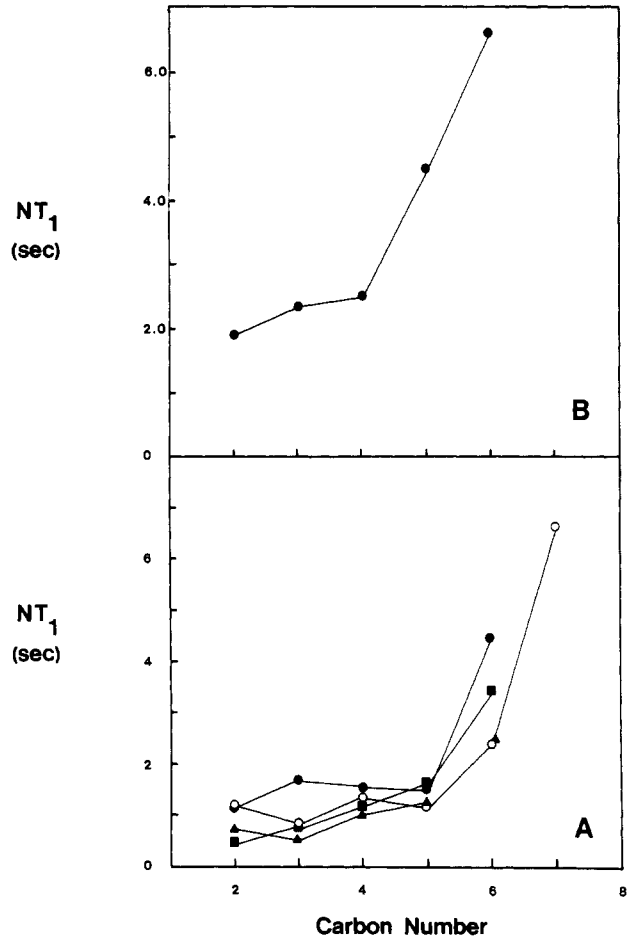

FIGURE 6: ^{13}C NMR spin–lattice relaxation times multiplied by the number of directly bonded protons for alkyl chain carbons of micellar short-chain lecithins. (A) NT_1 values for all but branching methyls are plotted: diheptanoyl-PC (○); bis(5-methylhexanoyl)-PC (■); bis(3-methylhexanoyl)-PC (●); bis(2-methylhexanoyl)-PC (▲). (B) NT_1 is plotted for branch methyl groups at each position in the different compounds.

Table III: Activity of Phospholipase A_2 (*N. naja naja*) and Phospholipase C (*B. cereus*) toward Linear and Branched Short-Chain Lecithins

lecithin[a]	act. (μmol min^{-1} mg^{-1})[b] phospholipase A_2	phospholipase C[c]
diheptanoyl-PC	700 (200)	1900 (300)
bis(5-methylhexanoyl)-PC	70 (60)	2300 (100)
bis(3-methylhexanoyl)-PC	45 (40)	
bis(2-methylhexanoyl)-PC	10 (10)	
bis(4,4-dimethylpentanoyl)-PC	0.9 (0.3)	2000 (300)
1-heptanoyl-2-(2-methylhexanoyl)-PC	600 (14)	
1-(3-methylhexanoyl)-2-heptanoyl-PC	600 (20)	

[a] Lecithin concentration in assays is 10 mM. [b] Values in parentheses represent standard deviations from assays done in triplicate. [c] Phospholipase C appears to need the carbonyl group for efficient binding to the enzyme; hence, we are only comparing its activity for terminally branched lecithins to the linear compound. Lecithins with branching at position 2 or 3 could be inhibitory because of the enzyme's steric requirements and slight changes in lecithin conformation or packing.

activities are not simply due to differences in the phospholipase A_2–substrate binding interaction. Figure 7 shows that the terminally branched chain lecithins influence phospholipase A_2 activity against mixed lecithin micelles in a similar fashion. The observed specific activity is reduced by approximately 50% when the mixed micelle is 50% branched chain lecithin. For comparison, an equimolar mixture of branched-chain fatty acid

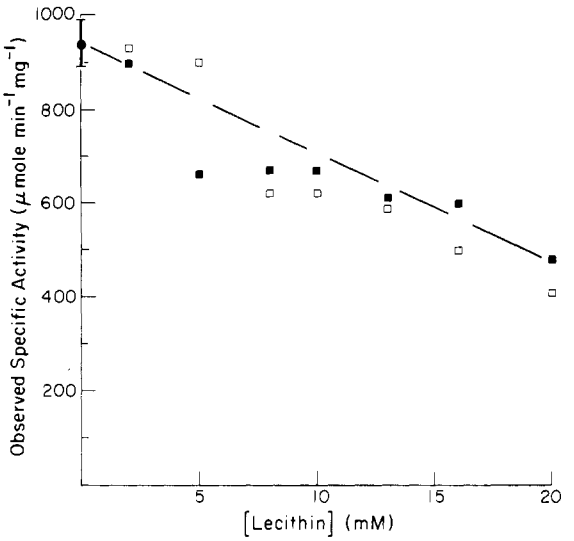

FIGURE 7: Surface dilution experiments for diheptanoyl-PC/branched-chain PC mixed micelles. Plot of observed phospholipase A_2 specific activity for 20 mM diheptanoyl-PC with varying concentrations of bis(5-methylhexanoyl)-PC (■) or bis(4,4-dimethylpentanoyl)-PC (□).

and diheptanoyl-PC shows no inhibition of the lecithin hydrolysis. Since the phospholipase A_2 activity against pure branched-chain micelles is so low, the observed activity in mixed systems is apparently a concentration-weighted average of the pure micelle activities.

When the methyl group is near the carbonyl group of the fatty acyl chain, the lecithin is a more potent inhibitor of diheptanoyl-PC hydrolysis. For example, with 9 mM diheptanoyl-PC and 2 mM methylated lecithin, bis(2-methylhexanoyl)-PC decreases the enzyme specific activity to 0.29 of the diheptanoyl-PC value; bis(3-methylhexanoyl)-PC reduces it further to 0.15 of the diheptanoyl-PC value. Bis(5-methylhexanoyl)-PC mixed in the same proportion with diheptanoyl-PC would decreases enzyme activity to 0.80 of the single-species value.

To address the question of whether both fatty acyl chains must be methylated for branched-chain lecithins to be poorly turned over by phospholipase A_2, we examined two asymmetric hybrid lecithins. When the lecithin contains only one methylated fatty acid, the hybrid lipid becomes an excellent substrate for phospholipase A_2. Furthermore, there appears to be no enzymatic difference for the branched chain occurring at either the *sn*-2 [1-heptanoyl-2-(2-methylhexanoyl)-PC] or *sn*-1 [1-(3-methylhexanoyl)-2-heptanoyl-PC] position. These kinetic results suggest that when both fatty acyl chains contain branch methyl groups, the lecithin must bind differently to the enzyme than linear- or mixed-chain species.

This large difference in phospholipase A_2 activity toward bis(methylhexanoyl)-PC and linear species is lost in longer chain lecithins with methyl groups at C(12). Bis(12-methylstearoyl)-PC, 1-palmitoyl-2-(12-methylstearoyl)-PC, and egg yolk lecithin solubilized in Triton X-100 mixed micelles are all comparable substrates (C. D. DeBose and M. F. Roberts, unpublished results).

It has been suggested that lecithins can activate phospholipase A_2 toward other phospholipids (such as phosphatidylethanolamine) by binding to a site on the enzyme distinct from the catalytic site (Roberts et al., 1979; Adamich et al., 1979). Given the poor substrate but good inhibitor properties of the branched-chain lecithins, we decided to examine their ability to activate phospholipase A_2 toward egg phosphatidylethanolamine in Triton X-100 mixed micelles (Table IV). The

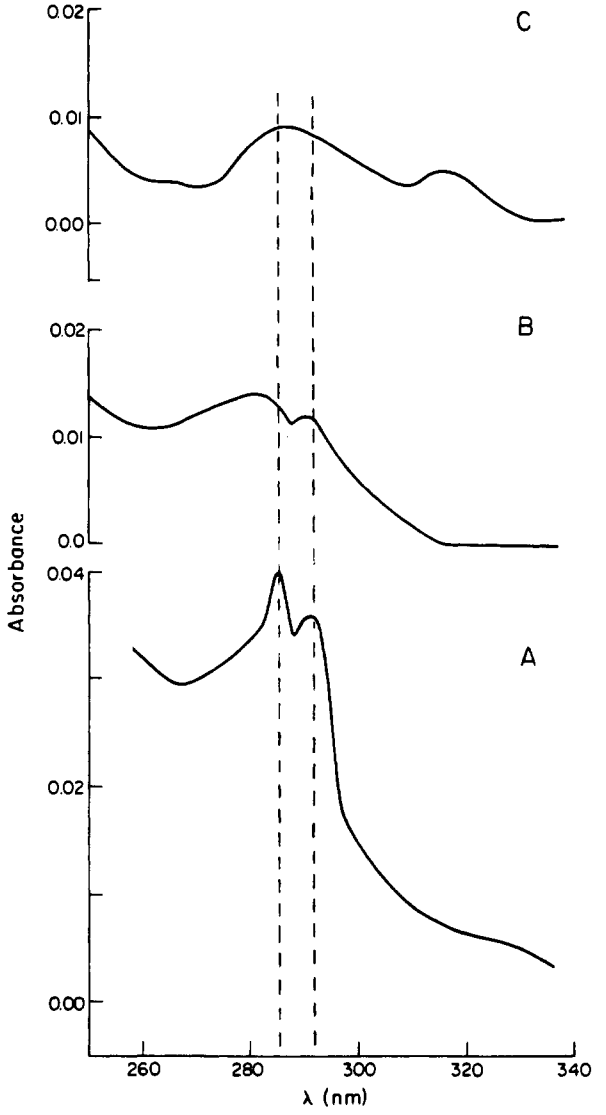

FIGURE 8: UV difference spectra of 0.5 mg/mL phospholipase A_2 in the presence of short-chain lecithins: (A) 1.0 mM diheptyl-PC; (B) 1.6 mM 1-(3-methylhexanoyl)-2-heptanoyl-PC; (C) 1.6 mM bis(3-methylhexanoyl)-PC.

branched-chain phosphatidylcholines are as effective as the linear compound in increasing the rate of phosphatidylethanolamine hydrolysis by the enzyme.

Binding of Branched-Chain Lecithins to Phospholipase A_2. While Ca^{2+} is needed for phospholipase A_2 activity, it is not a prerequisite for lipid binding to the enzyme (DeBose & Roberts, 1983). Therefore, in solutions with EDTA, we can examine the strength of lecithin binding to phospholipase A_2 and any conformational changes caused by this binding. When apophospholipase A_2 binds monomeric lecithin analogues, a characteristic UV difference spectrum is produced. With the ether-linked substrate analogue diheptyl-PC (Burns et al., 1981; DeBose & Roberts, 1983), two distinct maxima occur at 285 and 293 nm (Figure 8A); K_D is estimated to be 0.2 mM. A similar but weaker UV difference spectrum (with maxima at 282 and 292 nm) is induced when the hybrid lecithin 1-(3-methylhexanoyl)-2-heptanoyl-PC binds to phospholipase A_2 (Figure 8B); a K_D of 0.2 mM is estimated. The addition of Ca^{2+} to the ether PC solution (DeBose & Roberts, 1983) and Ba^{2+} to the hybrid lecithin solutions shifts these maxima slightly. In contrast to these spectra, bis(3-methylhexanoyl)-PC produces a very weak altered pattern with poorly characterized peaks (Figure 8C, a broad maxima centered at 287 nm.) Bis(5-methylhexanoyl)-PC yields the same UV

Table IV: Effect of Branched-Chain Lecithins on Hydrolysis of Egg Phosphatidylethanolamine (PE) in Triton X-100 Mixed Micelles[a]

added lecithin	PE hydrolysis (μmol min^{-1} mg^{-1}) −PC	+PC	lecithin (mM)	normalized activity[b]
diheptanoyl-PC	20	58	0.023	1.00
bis(2-methylhexanoyl)-PC	15	44	0.017	1.03
bis(3-methylhexanoyl)-PC	20	38	0.013	1.16
bis(5-methylhexanoyl)-PC	18	61	0.024	1.01

[a] Assay conditions are 5 mM egg phosphatidylethanolamine, 20 mM Triton X-100, and 5 mM Ca^{2+}, pH 8.0. [b] Added amounts of lecithin varied slightly; to take this into account we have taken the observed activated activity, normalized it to a concentration of lecithin equivalent to the linear species [for instance, bis(2-methylhexanoyl)-PC's adjusted activity would be 59], and compared that value with 58 μmol min^{-1} mg^{-1} observed for 0.023 mM diheptanoyl-PC.

difference spectrum. Addition of Ba^{2+} does not affect the difference spectrum caused by these symmetric methylated lipids.

Discussion

Enzyme kinetics against aggregated substrates can be divided conceptually into two parts: (i) binding of the enzyme to the aggregated substrate (interfacial phenomena) and (ii) kinetic processing of the substrate. In mixed micellar systems, changes in the composition or components can affect either (or both) part of interfacial enzyme kinetics. The substitution of a cationic for an anionic detergent in a mixed micellar system will obviously affect the interfacial characteristics of the micelle, but it may also influence the enzyme kinetics by differential competitive inhibition, etc. As proposed in an earlier study (Burns et al., 1981), a rational approach to analyzing the kinetics of surface active enzymes is to (i) use components that can be used in any proportions without drastically altering the characteristics of the interface, (ii) study the micelles carefully for any altered physical properties, and (iii) analyze the enzyme kinetics in light of the observed micelle physical chemistry.

The linear and branched short-chain lecithins are useful substrates for studying the kinetics of phospholipases at surfaces. While surface area per molecule and charge densities in the pure and mixed micelles should be very similar, the methyl groups and in particular the bulky *tert*-butyl groups at the end of the acyl chains could dramatically alter chain packing and conformation. Differences in the cmc values of the symmetric short-chain lecithins are understandable in terms of the differential solubilities of the hydrophobic portion of the molecules. These differences allow fairly accurate prediction of the cmc for all the symmetric branched-chain compounds presented here. This implies that the observed cmc's in the branched compounds are related to the cmc of the linear compound simply by $\Delta G_{transfer}$ (free energy of transferring a group from aqueous to hydrocarbon phase). The difference expected appears due to substitution of a methyl and methine group for a methylene. In the case of the singly branched PC [1-heptanoyl 2-(2-methylhexanoyl)-PC], the deviation in cmc from the value predicted by simple solubility arguments may reflect the fact that the C(2) position of the *sn*-2 chain in micellar PC resides at the interface (Roberts et al., 1978). The $\Delta G_{transfer}$ contributed by a methyl group at the C(2) *sn*-2 position then will not be the full value contributed by a methyl group positioned lower on the acyl chains where it is in a hydrocarbon environment. Under the assumption that the C(2) *sn*-2 methyl does not contribute to the $\Delta G_{transfer}$, the compound would be expected to have a cmc much like the unbranched compound, which is what is observed. The cmc for the singly branched PC is 1.4 mM as compared to 1.6 mM for the unbranched compound. The observed cmc (3.8 mM) for 1-(3-methylhexanoyl)-2-heptanoyl-PC is, by contrast, closer to what is predicted (2.9 mM) assuming that $\Delta G_{transfer}$ reflects the presence of the methyl group in a hydrocarbon environment. This further suggests that branching at C(2) or C(3) on the *sn*-1 chain contributes more effectively to $\Delta G_{transfer}$ than does similar branching on the *sn*-2 chain.

^1H coupling constant analysis of glycerol backbone protons suggests the average conformation of the backbone, and hence acyl chain orientation, is basically the same for the linear and branched phospholipids. Quasi-elastic light scattering studies show these branched seven-carbon lecithins form micelles intermediate in size between seven- and six-carbon straight-chain lecithins. The position of chain branching is an important factor in the aggregate physical behavior of these lecithins. When the methyl group is near the chain terminus, where chains are less ordered, there is little difference from the straight-chain lecithin. However, when the methyl group disrupts the ordered chains near the acyl group, there is a much larger effect on micellar size. The bis(5-methylhexanoyl)-PC is closer to diheptanoyl-PC in aggregate physical characteristics, while bis(2-methylhexanoyl)-PC with methyl branching close to the acyl linkage and presumably the micelle–water interface is similar to dihexanoyl-PC. The two linear species (dihexanoyl-PC and diheptanoyl-PC) are excellent substrates for phospholipase A_2 while all the symmetric branched-chain species are poor substrates. Therefore, there is no correlation between micelle size and susceptibility to phospholipase A_2 hydrolysis.

Given the similarity in lecithin conformation of diheptanoyl-PC and methylated species, we can explain the low activity of phospholipase A_2 toward branched-chain lecithins in terms of enzyme–phospholipid processing. The terminally branched chain lecithins bind to the enzyme with approximately the same affinity as diheptanoyl-PC because inhibition is proportional to the mole fraction of the branched-chain lecithin. These branched-chain species bind but are not turned over rapidly by phospholipase A_2. There are several possible explanations for slow turnover by the enzyme: unproductive substrate binding, product inhibition, slow release of either or both products of hydrolysis, or incorrect enzyme conformation or aggregation state. Moderate amounts (up to 25 mol %) of branched fatty acid do not inhibit enzyme activity toward substrate and therefore cannot be the cause of low phospholipase A_2 activity. Branched-chain lysolecithin may also be ruled out as a potent rate-determining product inhibitor because 1-(3-methylhexanoyl)-2-heptanoyl-PC is a good substrate and cleavage results in branched 1-(3-methylhexanoyl)-PC. Product inhibition may be a small factor in the difference in inhibition of diheptanoyl-PC hydrolysis seen with bis(2-methylhexanoyl)-PC, bis(3-methylhexanoyl)-PC, and bis(5-methylhexanoyl)-PC. When the methyl group is near the carboxyl group, the lecithin is a more potent inhibitor of diheptanoyl-PC hydrolysis. This does not reflect a binding affinity difference to phospholipase A_2. In fact, the bis(methylhexanoyl)-PC species bind slightly weaker to the enzyme than the linear species (Table VI). This suggests that however the symmetric methylated lecithins are bound to the enzyme; upon hydrolysis, the lyso with the methyl group near the glycerol backbone is "stickier" than other lyso species.

One of the remaining explanations for the low phospholipase A_2 activity toward methylated lecithins is that the enzyme cannot undergo a conformational change (enzyme aggregation

or an alteration of peptide chain) necessary for efficient catalysis. Binding of the lecithins with both chains branched either prevents or does not facilitate the same change in the enzyme that good substrates cause. Thus, poor substrate lecithins cannot easily induce the appropriate enzyme orientation for rapid hydrolysis. A comparison of UV absorption difference spectral data shows that the binding of linear diheptanoyl-PC induces an optical change in the enzyme that is quite different from that induced by lecithins with both chains methylated. The asymmetric lecithin 1-(3-methylhexanoyl)-2-heptanoyl-PC induces a change in the enzyme similar to that of the linear lipid but of a smaller magnitude. Both linear and asymmetric lipids are good substrates. Their interaction with the enzyme clearly differs from that of lecithins with both chains branched. The enzyme changes implied by the spectra are clearly the results of two distinctly different enzyme–lipid interactions, one associated with good substrate binding and the other with poor substrate binding. Because both chains must be branched for a lecithin to be a poor substrate, a steric interaction misaligning the lecithin may be suggested, although another explanation could be that the symmetric branched-chain lecithins may not induce aggregation of the enzyme. Cobra venom phospholipase A_2 at milligram per milliliter concentrations forms aggregates up to tetramer (Lewis et al., 1977). At low concentrations similar to those used in kinetic assays, this enzyme is a monomer, although substrates or analogues can induce enzyme aggregation (Roberts et al., 1977). The UV difference spectra generated in this work used phospholipase A_2 at concentrations where it is already aggregated. Hence, the difference in UV spectra seen with linear and asymmetric vs. symmetric branched-chain lecithins more likely reflects an enzyme conformational change rather than an altered aggregation state of the enzyme.

Keith et al. (1981) noted that (barring some kind of conformational change) the catalytic site seems inaccessible as presented in the crystal structure of another snake venom enzyme, *Crotalus atrox* phospholipase A_2. The catalytic center is enclosed in a central cavity in the dimer structure. They speculate that binding Ca^{2+} and possibly substrate to one promoter is structurally linked to functional access of substrate to the active center of the other promoter. If a similar structure is taken by the cobra venom enzyme, we would suggest that the linear and asymmetric short-chain lecithins bind and facilitate the needed conformational change effectively but the dibranched-chain lecithins do not induce this change in the enzyme. The increased bulkiness of the chains could hinder the conformational rearrangement of the enzyme by preventing the lipid molecule(s) from gaining entry into the active center or by sterically filling up the active site and blocking the necessary conformational change. If, in fact, two molecules of substrate are required to bind for catalysis, as has been suggested for the cobra venom enzyme (Roberts et al., 1977, 1978), and both bind in the same vicinity, the active center may be too crowded to accommodate a second branched lecithin molecule after one branched-chain lecithin is bound.

Branched short-chain lecithins activate phospholipase A_2 toward egg phosphatidylethanolamine as effectively as linear short-chain lecithins. This is in keeping with the idea that effective binding to this functionally distinct (but not necessarily spatially distinct) site is dominated by the phosphocholine moiety (Adamich et al., 1979). Occupation of the activator site must affect interactions at the catalytic site. If our UV difference results represent branched-chain lecithins binding to the activator site, yet the same methylated species are good inhibitors of the enzyme, it suggests that binding to the catalytic site occurs as well. An explanation consistent with this is that the catalytic and activator sites are not spatially separate but form a combined site for two phospholipid molecules.

These results strongly suggest that both fatty acyl chains are important in determining the rate of phospholipase A_2 hydrolysis of a lipid. The bis(methylhexanoyl)-PC molecules are poor substrates because of altered enzyme conformation or aggregation state, not because of altered phospholipid conformation.

Registry No. Bis(5-methylhexanoyl)-PC, 94944-00-0; bis(3-methylhexanoyl)-PC, 94944-01-1; bis(2-methylhexanoyl)-PC, 94944-02-2; bis(4,4-dimethylpentanoyl)-PC, 94944-03-3; 1-heptanoyl-2-(2-methylhexanoyl)-PC, 94944-04-4; 1-(3-methylhexanoyl)-2-heptanoyl-PC, 94944-05-5; diheptanoyl-PC, 35387-75-8; phospholipase A_2, 9001-84-7; phospholipase C, 9001-86-9.

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Hypoxanthine–Guanine Exchange by Intact Human Erythrocytes

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ABSTRACT: The uptake and release of [^{14}C]hypoxanthine by human erythrocytes, suspended in a tris(hydroxymethyl)aminomethane (Tris)–glucose–NaCl isotonic medium (pH 7.4), have been studied at 37 °C. The uptake of hypoxanthine, mediated by its incorporation into inosine 5′-monophosphate (IMP), was markedly stimulated by preincubating the cells in phosphate-buffered saline. After a lag time, [^{14}C]-IMP-enriched erythrocytes released [^{14}C]hypoxanthine in the medium. Formycin B, at concentrations known to inhibit purine nucleoside phosphorylase in intact erythrocytes, affected hypoxanthine uptake and release and led to an increase in the intracellular concentration of inosine, suggesting that the main catabolic path of IMP is the sequential degradation of the nucleotide to inosine and hypoxanthine. The addition of guanine to a suspension of [^{14}C]IMP-enriched erythrocytes led to an increase in the rate of [^{14}C]hypoxanthine release, which was unaffected by the presence of formycin B. During the guanine-induced hypoxanthine release, guanine was taken up by the cells as GMP. These results suggest that the presence of guanine in the incubation medium activates a catabolic path in human erythrocytes leading to IMP degradation without formation of inosine.

Human erythrocytes appear to be important in carrying purines from organs with a purine surplus to organs with a purine requirement (Lowy & Lerner, 1974). De novo purine synthesis does not occur in mature red cells (Lowy et al., 1961); thus, the turnover must result from continual entry and release of purines into the plasma.

Human erythrocytes take up adenine, guanine, hypoxanthine, and xanthine and convert them into nucleotides, but hypoxanthine and xanthine are the only purines released in vitro (Mager et al., 1966). The release of purines by human red cells is mediated by prior conversion of the various purine nucleotides to inosine 5′-monophosphate (IMP), which can be either sequentially degraded to inosine and hypoxanthine or converted to xanthosine 5′-monophosphate (XMP) (Mager et al., 1966). Xanthine formation from the latter mononucleotide does not appear to occur significantly in vivo owing to the conversion of XMP to guanosine 5′-monophosphate (GMP) in the presence of the relatively high glutamine levels prevailing in the blood (Hershko et al., 1967). Therefore, purine catabolism of human erythrocytes in vivo appears to be mainly directed via IMP and inosine by hypoxanthine, which is the chief base released (Murray, 1971). In this paper, hypoxanthine release from mature human red cells has been studied.

Materials and Methods

[8-^{14}C]Hypoxanthine (59 Ci/mol) and [8-^{14}C]guanine (55 Ci/mol) were obtained from Radiochemical Centre, Amersham. All other reagents were high-purity commercial samples from Merck or Sigma.

Human erythrocytes were prepared from blood freshly drawn in heparin and washed twice with ice-cold 0.9% NaCl with removal of white cells by aspiration. The packed erythrocytes were suspended in an equal volume of phosphate-buffered saline containing 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.4), 5 mM glucose, 0–12 mM sodium phosphate, and the appropriate amount of NaCl to give an isotonic solution and incubated for 15–60 min at 37 °C. At the end of the incubation period, the cells were washed twice in buffered saline not containing phosphate (50 mM Tris-HCl, 5 mM glucose, and 12.8 mM NaCl, pH 7.4). The packed cells were transferred to an equal volume of the last medium containing cold or ^{14}C-labeled hypoxanthine (0–12 μM), cold or ^{14}C-labeled guanine (0–70 μM), and formycin B (0–8 mM). At regular intervals, samples of the incubation mixture were removed. The medium, separated from the cells by centrifugation, was employed for liquid scintillation counting and paper chromatography (Gerlach et al., 1965). The cell count in the incubation media (either containing or not containing phosphate) ranged from 4.2 to 4.7 million/mm^3.

The radioactive compounds present within the erythrocytes were identified by paper chromatography as previously described (Giacomello & Salerno, 1979). Intracellular concentration of radioactive compounds was determined from the relative amplitude of radioactive peaks obtained by paper chromatography. Intracellular 5-phosphoribosyl 1-pyrophosphate concentration was determined according to published procedures (Hershko et al., 1967).

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