proton relaxation studies suggested either that all of the metal coordination sites were occupied by protein ligands or that any first coordination sphere H_2O ligands were hindered from fast proton exchange with the solvent.[2] A high-resolution three-dimensional X-ray structure of any α-lactalbumin species has yet to be published, but it can be predicted that the calcium site will contain all oxygen ligands. This study and several complementary solution conformation techniques can describe protein conformation in much structural detail. The long-awaited X-ray results would also be valuable in comparing the precise three-dimensional structure of the α-lactalbumin site with the quite well understood EF hand domains of the several calcium binding proteins reported to date (Kretsinger, 1980).

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[2] This was also suggested from ^1H water proton relaxation studies at 60 MHz with Gd(III)–α-lactalbumin where enhancements slightly less than 1.0 were measured (L. J. Berliner and H. Gilboa, unpublished results).

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Articles

Photolysis of Cholesteryl Diazoacetate in Small Unilamellar Vesicles†

Sue A. Keilbaugh and Edward R. Thornton*

ABSTRACT: Cholesteryl diazoacetate (**1**), a potential membrane photolabeling reagent, has been incorporated into dipalmitoylphosphatidylcholine (DPPC) small unilamellar vesicles (SUV). Immobilization of the photolabel within the bilayer matrix was demonstrated by ^{13}C and ^1H NMR and was found to be closely analogous to that of cholesterol. SUV composition was verified by integration of proton NMR resonances from $CDCl_3$ solutions of chloroform extracts of vesicle preparations. Photolysis of the label in DPPC SUV resulted in C–H insertion into the choline head group of DPPC, O–H insertion into water, and also production of cholesterol. These intermolecular C–H and O–H insertions indicate that the photogenerated carbene from **1** is situated at the aqueous interface of the membrane, analogously to the known orientation of the OH group of cholesterol. Therefore, by these criteria, **1** appears to behave as a cholesterol analogue in DPPC bilayers and may be a useful membrane photolabeling reagent.

Cholesterol, the major sterol of mammalian cells, regulates membrane fluidity (Schreier-Muccillo et al., 1973; Demel & de Kruijff, 1976; Lindblom et al., 1981), influences enzymatic activity (Klappauf & Schubert, 1977; Klein et al., 1978; Madden et al., 1979), passive transport (Schreier-Muccillo et al., 1973; Benz & Cros, 1978), immune response (Inbar & Shinitzky, 1974; Shinitzky et al., 1979), and phospholipid fatty acyl chain composition (Dahl et al., 1980), complexes with polyene antibiotics (Bittman et al., 1981), cytochrome $P\text{-}450_{scc}$ (Lambeth et al., 1980), and lysophosphatidylcholine (Rammy & Brockerhoff, 1982), and mediates bilayer structure (de Kruijff et al., 1979). Many of these effects seem to be the result of the modulation of phospholipid behavior by cholesterol. According to differential scanning calorimetry data, the preference of cholesterol for different phospholipids is sphingomyelin ≫ phosphatidylserine, phosphatidylglycerol > phosphatidylcholine ≫ phosphatidylethanolamine, except in mixtures of PS–PE,[1] PS–PC, and PC–PG where the lower

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[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; SM, sphingomyelin; PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; EtOAc, ethyl acetate; CI, chemical ionization; *hν*, light; IR, infrared spectoscopy; TLC, thin-layer chromatography; Me_4Si, tetramethylsilane; MeOH, methanol.

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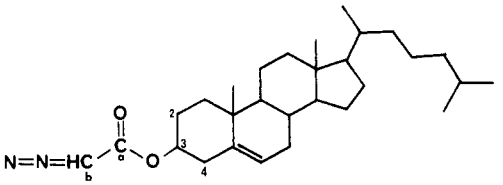
melting lipid is preferred (van Dijck, 1979; Estep et al., 1979). However, two recent reports have indicated no difference in cholesterol affinity for SM and PC (Calhoun & Shipley, 1979; Lange et al., 1979).

The interaction of cholesterol with phospholipid appears to be primarily hydrophobic. Attempts to prove any direct interaction between the two components have been unsuccessful. Proposed model systems rely heavily on van der Waals interactions between the steroid nucleus and the phospholipid chains (de Kruijff, 1978; Presti et al., 1982). Photoaffinity labeling, which has become quite useful in the elucidation of biological interactions (Chowdhry & Westheimer, 1979; Khorana, 1980), would be capable of probing sterol–lipid associations. Cholesteryl diazoacetate (**1**) was developed for



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this purpose. The synthesis and photoreactivity of **1** in model systems were reported previously (Keilbaugh & Thornton, 1983). This particular photolabel was designed to cause little or no perturbation of the membrane from the glycerol backbone through the hydrocarbon region of the bilayer. Our next objective was to compare the membrane properties of **1** with those of cholesterol. The present paper demonstrates that **1** incorporates into DPPC SUV analogously to cholesterol. Carbon and proton NMR spectra of the SUV indicate that the immobilization of cholesteryl diazoacetate is similar to that of cholesterol. Photolysis of **1** in DPPC SUV was also carried out, resulting in C–H insertion into the choline head group, O–H insertion into water, and the production of cholesterol. These insertions indicate that the photogenerated carbene from **1** is situated at the aqueous interface of the membrane, as is the OH group of cholesterol (de Kruijff, 1978; Presti et al., 1982).

Materials and Methods

Materials. All reagents used were ACS grade or better and were used as purchased unless noted below. For flash chromatography columns Merck silica gel 60 (40–63 μm particle size) was used. Packing for gel permeation chromatography columns was Sephadex LH-20-100 (25–100 μm particle size). The gel filtration column was 2.5 cm in diameter with 115 cm of bed height. Cholesteryl diazoacetate was synthesized as reported earlier (Keilbaugh & Thornton, 1983). Dipalmitoylphosphatidylcholine (DPPC) was prepared according to procedures developed by Gupta et al. (1977) up to the final purification step. The original silica gel column purification was changed to a reusable flash column (packing 2.5 cm diameter × 13.75–15 cm height) (Still et al., 1978). The column was loaded with 1.5–2.0 mmol of crude DPPC in $CHCl_3$, followed by 300 mL of $CHCl_3$. The eluant was changed to $CHCl_3$/MeOH azeotrope to elute DPPC. Once the DPPC was removed, the column was stripped with 50/50 (v/v) MeOH, 300 mL, and then reequilibrated with $CHCl_3$ before it was reused. It should be noted that first-time use of this column may require several liters of azeotrope before elution of DPPC; alternatively, the column may be partially deactivated by elution of 150 mL of MeOH followed by reequilibration with $CHCl_3$ before sample application.

Physical Methods. Infrared spectra were obtained on a Perkin-Elmer 735 spectrophotometer. Proton and carbon NMR spectra (structure determination) were obtained on a Bruker WM 250 spectrometer (250 and 62.9 MHz, respectively). All NMR chemical shifts are reported in parts per million (ppm) downfield from internal Me_4Si unless otherwise noted. ^1H NMR spectra used for integration were run at a 30° pulse width, 8 total scans, and a 5-s repetition time. Although full data are given in the supplementary material (see paragraph at end of paper regarding supplementary material), agreement of corresponding NMR absorptions for the steroid nucleus and the fatty acid chain was very close for the compounds studied, except where noted below. The statement that the absorptions for a particular compound conformed to the steroid nucleus or the fatty acid chain means agreement generally within ±0.02 ppm for ^1H and 0.5 ppm (±1.5 ppm for olefin peaks only) for ^{13}C NMR spectra. TLC plates (Merck silica gel 60) were visualized by using phosphomolybdic acid solution, 35 g in 1 pt of ethanol (Kritchevsky & Kirk, 1952). Elemental analyses on samples dried under vacuum for 12 h were performed by Galbraith Analytical Laboratories, Knoxville, TN.

Vesicle Preparation. Vesicles were prepared by dispersing 10, 20, or 30 mol % of **1** (or cholesterol) in DPPC with $CHCl_3$ and then reducing the solution to dryness by rotary evaporation. Final traces of solvent were removed under vacuum overnight. The residue was suspended in buffer (0.1 M KNO_3 and 0.002 M K_2HPO_4) at a ratio of 75 mg:1 mL. Sonication of the suspension was carried out at ca. 72 W for 1 h on pulse (0.5 s on, 0.5 s off) while immersed in an ice bath. Then continuous sonication was maintained for 2 min with the ice bath plus a further 10 min without the bath. The temperature of the preparation was not permitted to exceed 65 ± 2 °C during sonication. After sonication, the mixture was centrifuged at 17 000 rpm for 15 min and immediately filtered through a 0.45-μm filter into a heated (52 ± 1 °C) reaction vessel for photolysis. From the point of continuous sonication without the ice bath through the filtration, the vesicle mixture was maintained at a temperature above the phase transition of DPPC.

Multilayer Preparation. Multilayer preparation was identical with that used for vesicle preparation up to the point of sonication. Instead of sonication, the mixture was stirred overnight at 52 ± 1 °C in the dark. The suspension was then transferred to a heated reaction vessel for photolysis.

Photolysis Reaction. Prior to photolysis all reaction mixtures were warmed to 52 ± 1 °C and purged of oxygen by bubbling oxygen-free N_2 (Fieser & Fieser, 1967) through the solution for a minimum of 5 min. The reaction vessel was a 40 cm long quartz tube (2.5 cm i.d.), the contents of which were agitated by magnetic stirrer. Temperature was maintained during the reaction by a flow of forced air at 52 ± 1 °C. Photolysis took place under N_2 for 60 min (vesicles) in a Rayonet RPR-100 photoreactor using 16 254-nm lamps (Rayonet RPR-2537 A).

Photolysis of multilayer preparations was carried out in the same reaction vessel with the addition of a glass rod (1-cm diameter) in the center. Stirring was achieved by rotation of the glass rod. Photolysis was continued until it could be shown by TLC that **1** had completely reacted (ca. 16 h).

Control samples, not photolyzed, showed negligible decomposition of **1** and DPPC in each case. Exposure to laboratory light can cause nonnegligible decomposition of **1** within 6 h.

Products. After photolysis, the reaction mixture was freeze-dried, and the residue was extracted with $CHCl_3$ to

remove the buffer salts. The $CHCl_3$ extract was then applied to a flash column packed in $CHCl_3$. Then the column was eluted with 500 mL of $CHCl_3$, 1000 mL EtOAc/$CHCl_3$, 50/50 (v/v), and 100 mL of $CHCl_3$/MeOH azeotrope. The $CHCl_3$ fraction contained minor amounts of nonpolar products plus any residual **1**. Cholesteryl hydroxyacetate (**2**) and cholesterol (**3**) were in the EtOAc/$CHCl_3$ (50/50) fraction. A DPPC–**1** coupled compound mixture (**4**) was isolated from the $CHCl_3$/MeOH azeotrope fraction.

Separation of **2** and **3** was achieved by flash chromatography with EtOAc/hexane, 1/4 (v/v). Final purification of **4** was achieved by gel permeation chromatography with $CHCl_3$/MeOH, 50/50 (v/v), as the mobile phase.

Cholesteryl Hydroxyacetate (**2**). Spectra conformed to the steroid nucleus with the following additions: yield 20% of **1** photolyzed; mp 148–151 °C; IR (KBr) 3440 (s), 1730 (s), 1250 (s), 1110 (m) cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.74 (m, 1, H_3), 4.13 (s, 2, H_b), and 2.49 (br s, 1, H_c); ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 75.79 (C_3), 172.91 (C_a), and 61.03 (C_b); CI mass spectrum (NH_4^+), *m/e* (relative intensity) 462 ($[M + 18]^+$, 6), 386 (4), 370 (28), 369 (100), 368 (48), 353 (5), 255 (4), 247 (5), 147 (11). Anal. Calcd for $C_{29}H_{48}O_3$: C, 78.32; H, 10.88. Found: C, 78.36; H, 10.78.

Cholesterol (**3**). Identification was achieved by the identity, within experimental error, of ^1H NMR and IR spectra. Additionally, TLC R_f's when run on the same plate for **3** and authentic cholesterol were equivalent.

DPPC–1 Adduct Mixture (**4**). Spectral data conformed to the steroid nucleus plus typical DPPC resonances. ^{13}C NMR spectral data conform to those of cholesterol and DPPC except for resonance shifts in the 50–70 ppm range. Further exceptions are the following: ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 76.46, 77.35 (**4a** and **4b** or **4c**, C_3), 178.24 (C_a), 37.12 (**4a**, C_c), and ca. 38.3 (**4b** or **4c**, C_c).

Vesicle NMR. Vesicles were prepared as described and filtered into heated NMR tubes. ^1H NMR samples were run at 52 ± 1 °C on a Bruker WM 250 spectrometer with a 0.3-s relaxation delay, a 30° pulse width, and 400 total scans. ^{13}C NMR samples were run at 52 ± 1 °C on a JEOL PFT-100 (25 MHz) spectrometer by using a 1-s relaxation delay, a 45° pulse width, 8K memory, and 24-h accumulation.

Results

Vesicle Characterization. Incorporation of **1** into DPPC SUV was achieved by sonication (Huang, 1969; Sheetz & Chan, 1972). Centrifugation and filtration of the vesicle suspension removed unincorporated DPPC and **1** as well as multilamellar vesicles (MLV) from the extravesicular solution. The ^{13}C and ^1H NMR spectra of the sonicated preparation demonstrated that SUV were prepared (Yeagle et al., 1977; Chatterjie & Brockerhoff, 1978). Spectra were obtained of SUV at 10, 20, and 30 mol % **1** in DPPC and compared to SUV with 10, 20, and 30 mol % cholesterol in DPPC (actual ^{13}C NMR spectra in supplementary material). Cholesterol causes the phospholipid chain resonances to broaden due to an increase in chain order. The choline methyl proton resonance is unaffected by cholesterol or **1**; however, the main methylene peak decreases in height and broadens considerably with increasing concentrations of steroid. Cholesterol also broadens the ^{13}C NMR phospholipid resonances, yet does not add any isolated resonances from its own structure to the spectrum. As yet cholesteryl diazoacetate and 4,4-dimethylcholesterol are the only other sterols known to reproduce this effect at 25 MHz (Yeagle et al., 1977). It should be noted, however, that with the increased sensitivity and resolution afforded by a 63-MHz field actual resonances attributable to cholesterol can be observed (C_6, C_5, C_{18}, C_{19}, and C_{21}) (Brainard & Cordes, 1981). The spectra generated do indicate that **1** is immobilized in vesicles in a manner similar to that of cholesterol. Additionally, if **1** were immobilized in the bilayer in a manner very different from cholesterol such as epicholesterol or lanosterol, some resonances from the steroid structure should be observable (Yeagle et al., 1977). It should be emphasized that it is not just the similarity of these NMR properties of **1** to those of cholesterol but also the fact that *cholesterol has special NMR properties relative to almost all other sterols studied* (Yeagle et al., 1977), which strongly indicates that **1** is immobilized in the bilayer very similarly to cholesterol.

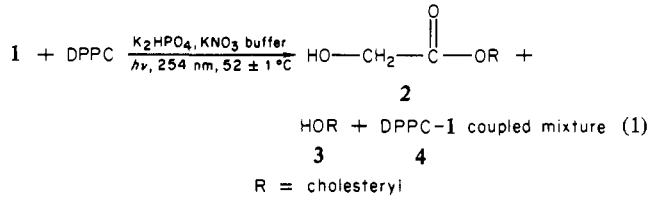
Table I: Vesicle Composition: ^1H NMR Integration

composition	experimental choline/olefin	theoretical choline/olefin
mol % cholesterol in DPPC		
10	80.7	81
20	33.3	36
30	20.3	21
mol % 1 in DPPC		
10	76.6	81
20	38.7	36
30	20.8	21

The composition of the SUV was confirmed by integration of ^1H NMR spectra. After filtration the vesicle preparation was freeze-dried, extracted with $CHCl_3$, and reduced to dryness, and then the proton NMR was taken in $CDCl_3$. Since the choline methyl resonance of DPPC and the olefin resonance of the steroid ring are isolated, they can be integrated. Table I presents the results of this procedure.

Photolysis in SUV. Photolysis of **1** was previously shown to proceed by first-order kinetics in both hydrocarbon and alcoholic solvents (Keilbaugh & Thornton, 1983). Furthermore, it was demonstrated that **1** readily inserted into C–H and O–H bonds, regenerated cholesterol, and produced little Wolff rearrangement. Insertion into O–H bonds was favored over C–H insertion by 16:1.

The photolabel, **1**, was incorporated into DPPC vesicles at 30 mol %. Photolysis of the resulting SUV was performed to determine the ability of the photolabel to insert into phospholipid. Reaction of **1** produced three noteworthy products (eq 1): cholesteryl hydroxyacetate (**2**), cholesterol (**3**), and,



most important, C–H insertion into the choline head group of DPPC (**4**). A negligible amount (ca. 4%) of the Wolff rearrangement product, HO_2CCH_2OR, was noted by TLC.

The predominant product was **2**, which is the result of carbene insertion into an O–H bond of water. Spectral data were uniquely consistent with the proposed structure. Its yield was 20% of the diazo compound actually photolyzed.

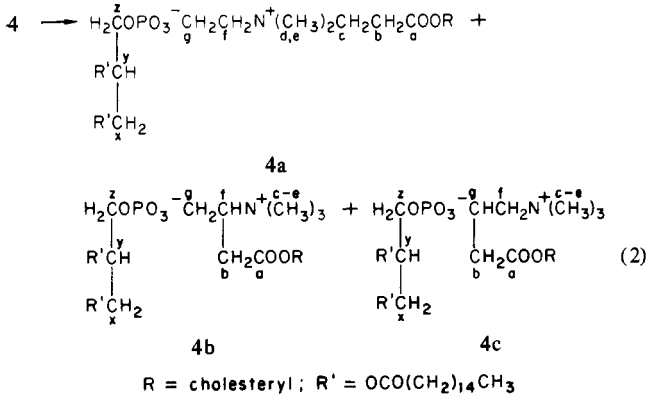
Identification of **3** was made by comparison of IR and ^1H NMR spectra with those of cholesterol plus comparison of R_f values for **3**, cholesterol, and a mixture of **3** plus cholesterol. The yield, approximated by TLC, was found to be 5% of **1** photolyzed. This is reasonable considering the yield of cho-

lesterol found when **1** was photolyzed in cyclohexane or methanol (Keilbaugh & Thornton, 1983).

The yield of couple mixture, **4**, was found to be 5% of **1** photolyzed. The total yield of currently isolated products is 35% of **1** photolyzed. Under the assumption that 20% of the products are lost during isolation because of peak shaving, transfer losses, etc., then ca. 55% of the photolabel has been recovered. TLC indicates there are at least 10 other products generated, but each is present in less than 5% yield. According to gel filtration of a mixture of all the reaction products, *none of the remaining products has a molecular weight greater than that of DPPC.*

Characterization of **4** began with gel filtration of the product through Sephadex LH-20. DPPC, PEG 1500 [poly(ethylene glycol) M_r 1500], and cholesterol were used as standards. **4** was found to have a molecular weight considerably higher than that of DPPC and equal to or less than that of PEG 1500. Integration of the proton NMR of **4** provides further support that it is an adduct consisting of one molecule of steroid plus one molecule of DPPC. Proton and carbon NMR spectra exhibit resonances that are attributable to both the steroid ring system and DPPC. *Thus, **4** is a DPPC–**1** coupled compound.*

The isolate **4** is believed to be a mixture of two or possibly three compounds (eq 2). All three adducts are the result of



(2)

insertion into the choline head group of DPPC. One is the result of carbene insertion into one of the choline methyl groups (**4a**) while the others are the products of insertion into either of the two methylene groups of the head group (**4b**, **4c**). Proton and carbon NMR have been used to partially elucidate the components of the mixture.

Proton NMR spectra of various preparations of **4** demonstrate readily that it is a steroid–DPPC adduct and that a mixture of compounds exists. Figure 1 is a typical example of such a spectrum where the major steroid and phospholipid peaks have been noted. Integration of the spectrum indicated a total of 126 H's, with the well-resolved olefin proton of the steroid ring system in a 1:1 ratio with the well-resolved C_y proton of the glycerol backbone. Thus, the adduct is a molecule consisting of one steroid and one phospholipid molecule.

Supporting evidence for the presence of a mixture arises from the chemical shift of the choline methyl peak and the integration of two sections of the proton spectra. The chemical shift for the choline methyl peak, generated by several different preparations, was found to cover a range of 3.282–3.517 ppm. Other sharp peaks in the spectrum (from steroid and DPPC) vary less than 0.02 ppm from preparation to preparation. This chemical shift variance is attributed to a change in the concentration of the components of the mixture. Detailed efforts have been made to resolve the choline peak resonance of each component; however, with our current level of resolution only one peak was observed.

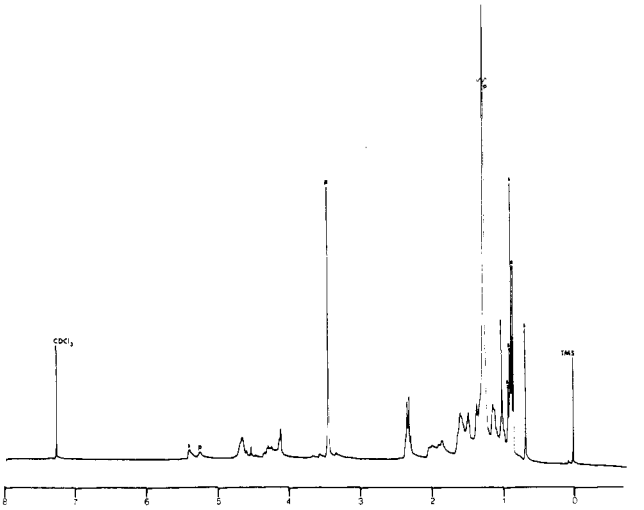


FIGURE 1: ^{1}H NMR spectrum of **4** (250 MHz, 8 scans, 5-s relaxation delay, and 35 mg in 0.4 mL of $CDCl_3$) where s denotes peaks from steroid ring system and p denotes peaks of phospholipid.

Integration of the choline methyl peak further supports the presence of a mixture. The range of values is from 7.19 H's to 8.62 H's (average 7.85 H's), relative to the well-resolved cholesteryl alkene H_6 as 1.0 H. If **4a** were the sole product, the integration of the choline methyl peak should be equivalent to 6 H's, while if insertion occurred at any other point in DPPC, the choline methyl resonance should integrate to 9 H's (or more if insertion results in a fortuitous chemical shift). The intermediate value found demonstrates partial insertion into one of the choline methyl groups, **4a**. It should be emphasized that these integrations are highly reproducible for spectra run on the same sample, because they involve ratios of areas of well-resolved peaks. Therefore, the differences observed from one run to another are real. We believe they result from differences in chromatographic peak shaving, not from the formation of different product ratios in different runs.

The only other portion of the ^{1}H NMR that does not integrate to the expected number of hydrogens is the very broad multiplet from 4 to 5 ppm. It covers a range of 8.81–10.99 H's (average 10.11 H's). Protons attached to C_x, C_z, C_g, C_f, C_c, and C_3 absorb in this region and should total 11 H's if **4a** is the sole product. If insertion has occurred at any other position in the head group (other than the choline methyl positions) or glycerol C_x or C_z, the multiplet should integrate to 8 H's. [The integration should equal 9 H's if insertion has occurred in the acyl chains or at glycerol C_y, but these possibilities are ruled out by the ^{13}C NMR (see below) and the observation of a clearly resolved C_y proton resonance, respectively.]

Further information can be derived from the carbon NMR of **4**. Resonances for the fatty acyl chains in the adduct correspond to those of DPPC. The steroid resonances correspond to cholesterol with the exception of that for C_3 which is shifted to 76.46 ppm in **4a** (72.00 ppm for cholesterol). A second resonance attributable to C_3 in **4b** and/or **4c** is at 77.35 ppm. Resonances for the glycerol backbone (C_x, 63.10; C_y, 70.66; C_z, 63.42 in DPPC) have been assigned to the following: C_x, 61.85; C_y, 69.73; C_z, 62.41 in the adduct (Figure 2). This is an approximate upfield shift of 1 ppm for each resonance. The intensity of these peaks and the lack of any new resonances downfield[2] that are not readily accounted for by other carbons

[2] Attachment of the carbene to a saturated carbon by C–H insertion would give a significant chemical shift change, on the order of 8 ppm downfield (Wehrli & Wirthlin, 1976).

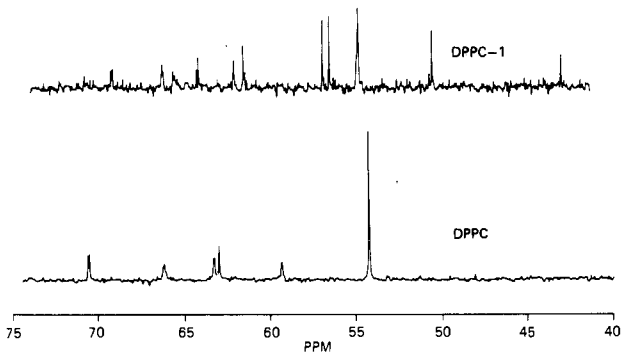


FIGURE 2: ^{13}C NMR, 50–70 ppm range, of **4** (DPPC–**1** adduct) and DPPC. Solvent was $CDCl_3$. DPPC–**1** spectrum: 44 mg in 0.5 mL, 3-s relaxation delay, and 12169 scans at 62.9 MHz. DPPC spectrum: 100 mg in 0.5 mL, 2.5-s relaxation delay, and 3505 scans at 62.9 MHz. Note ^{31}P splittings of the 70.66 and 63.42 ppm resonances of DPPC and the 69.73 ppm resonance of DPPC–**1** (Murari et al., 1982).

of the head group show that the glycerol carbons are not the subject of C–H insertion.

The remaining peaks in the 50–70 ppm range could be assigned by analogy to DPPC (Birdsall et al., 1972; Murari et al., 1982) in the following manner: 54.91, $C_{d,e}$ of **4a** plus C_{c-e} of **4b** and/or **4c**; 64.60, C_c of **4a**; 66.06, C_f of **4a**; 66.67, C_f of **4c** or C_g of **4b**; 61.73, C_g of **4a**; a small broad peak at ca. 63, C_f of **4b** or C_g of **4c**. A peak attributable to C_b of **4a** is observable at 37.12 ppm while C_b for **4b** and/or **4c** is at 38.20 ppm. This particular rationale for the peak assignments would allow **4** to be a mixture of either **4a** plus **4b**, **4a** plus **4c**, or **4a** plus **4b** plus **4c** since the resonances for the choline head group of **4b** and **4c** could be essentially the same. Due to the low intensity of some of these peaks and their broadness further specification of the components of the mixture is not possible at this time.

Photolysis in MLV. Incorporation of **1** into DPPC multilayers at 30 mol % has also been achieved. Photolysis of the resulting MLV requires considerably longer reaction times due to the turbidity of the reaction mixture, 16 h vs. 1 h. The product mixture contains, according to TLC, the same components as those derived from photolysis of DPPC plus **1** SUV. Preliminary NMR spectra indicate that the DPPC–**1** adduct mixture from photolysis of MLV is also some mixture of **4a**, **4b**, and **4c**.

Conclusion

Cholesteryl diazoacetate readily incorporates into DPPC vesicles. Its effect on the ^1H NMR spectra of vesicles closely resembles that of cholesterol.

Photolysis of **1** in DPPC SUV produces a steroid–phospholipid (1:1 molecular ratio) adduct mixture, cholesterol, and cholesterol hydroxyacetate, the result of carbene insertion into an O–H bond of water. The major products from photolysis result from insertion into water and choline. Therefore, the photogenerated carbene is most probably situated in the aqueous interface region, i.e., at the surface of the membrane, as is the OH group of cholesterol (de Kruijff, 1978; Presti et al., 1982).

The NMR and photochemical results, taken together, provide evidence that the nonpolar portion of cholesteryl diazoacetate affects the interior of the membrane similarly to cholesterol and that the polar portion (the photogenerated carbene) is at the membrane surface. While absolute criteria of exact membrane orientations of lipids are not available, we feel that the present evidence very strongly supports the contention that cholesteryl diazoacetate is both oriented and immobilized in the membrane analogously to cholesterol.

Since **1** has been shown to incorporate into model membranes and to label phospholipids in the head-group region, it appears that cholesteryl diazoacetate could be a valuable probe of lipid–lipid and lipid–protein interaction in bilayers. Its utility could probably extend to a variety of model systems, to natural membranes, and also to high-density and low-density lipoprotein complexes. The observed competition between insertion into choline and water would be expected to be altered with significant organizational changes at the membrane surface, and thus the ratio of these products could provide a measure of perturbations which alter the accessibility of the carbene to choline vs. water. Competition between choline groups, as in membranes containing both phosphatidylcholine and sphingomyelin, would also be expected to provide information about preferential association of one or the other with cholesteryl diazoacetate.

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Supplementary Material Available

Tables II–IV of ^1H and ^{13}C NMR chemical shifts of cholesteryl diazoacetate and its photolysis products and Figure 3 of ^{13}C NMR of 10, 20, and 30 mol % DPPC plus **1** and DPPC plus cholesterol SUV (4 pages). Ordering information is given on any current masthead page.

Registry No. **1**, 85135-66-6; **2**, 86863-09-4; **3**, 57-88-5; DPPC, 2644-64-6.

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Kinetic Studies of the Micellar to Lamellar Phase Transition of 1-Stearoyllysophosphatidylcholine Dispersions†

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ABSTRACT: Steady-state 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence depolarization anisotropy, ^{31}P nuclear magnetic resonance (^{31}P NMR), and high-sensitivity differential isothermal calorimetry were performed to investigate the kinetics of the micellar to lamellar phase transition of 1-stearoyllysophosphatidylcholine dispersions at temperatures below the lamellar → micellar transition temperature (T_m). Around the supercooling temperature of 25 °C below T_m, the kinetic process of this phase transition was found to consist of an initial lag time followed by a cooperative step of lamellar formation. The cooperativity of this lamellar formation was strongly negatively temperature dependent. Within experimental error, no positive concentration dependence was found for the micellar → lamellar transition with lysophospholipid concentrations in the 0.01-100 mM range. Immediately following the completion of lamellar formation, the lipid aggregates were shown, by ^{31}P NMR, to be large extended lamellar structures. The chaotropic salt NaSCN was shown to slow down the rate of formation of the large extended lamellar structures. However, the lamellar formation detected by either calorimetry or DPH anisotropy was not affected by chaotropic salts. The mechanism of the transition of the lysophospholipid micelles to lamellae at a constant supercooling temperature is discussed in terms of two-dimensional nucleation and growth processes. The heat of formation for the lamellar phase was found to be much lower than that expected from consideration of the melting enthalpy of the lamellar to micellar transition. We suggest that the packing of the lysophospholipid acyl chain in micelles at the supercooling temperature is probably tighter than that of normal micelles. This different micellar structure in conjunction with the driving force associated with supercooling is believed to play a major role in the enhancement of the rate of lamellar formation.

In a recent paper (Wu et al., 1982), it was shown by quasi-elastic light scattering, Raman spectroscopy, and high-sensitivity differential scanning calorimetry that 1-stearoyllysophosphatidylcholine in excess water can undergo a sharp, cooperative lamellar → micellar transition. This transition has a phase transition temperature, T_m, centered around 26.2 °C and a transition enthalpy, ΔH, of 7 kcal/mol. It was further observed that on immediate cooling of the micellar solution to a temperature slightly below T_m the lysophospholipids did not return to the lamellar structure. This indicated that the lamellar → micellar transition is irreversible at temperatures near the T_m. However, the lamellar structure of 1-stearoyllysophosphatidylcholine could be formed by prolonged annealing of the lysophospholipid micellar solution at 0 °C. This phenomenon raises an interesting question as to how lysophospholipid micelles are converted into lamellae at the supercooling temperature, a temperature which is far below T_m. The lamellar structure of 1-stearoyllysophosphatidyl-

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