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Sites of intermolecular crosslinking of fatty acyl chains in phospholipids carrying a photoactivable carbene precursor

(membranes/lipid–lipid interactions/synthetic phospholipids/photolysis/electron impact and field desorption mass spectrometry)

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ABSTRACT **Sonicated vesicles of 1-fatty acyl-2-ω-(2-diazo-3,3,3-trifluoropropionoxy) fatty acyl *sn*-glycero-3-phosphorylcholines were shown recently to form intermolecular crosslinks by insertion of the photogenerated carbene into a C—H bond of a neighboring hydrocarbon chain. We now report that photolysis of multilamellar dispersions gives a second series of products in which carbene insertion is accompanied by elimination of a molecule of hydrogen fluoride. The sites of crosslinking in the latter compounds have been studied by mass spectrometry using phospholipids with varying chain lengths of the fatty acyl groups carrying the carbene precursor. The patterns observed show that the point of maximum crosslinking is consistent with the recent conclusion that in phospholipids the *sn*-2 fatty acyl chain trails the *sn*-1 chain by 2–4 atoms.**

In a chemical approach to the study of phospholipid–phospholipid and phospholipid–protein interactions in biological membranes, the synthesis of phospholipids containing photoactivable carbene precursors as parts of their fatty acyl groups has recently been described (1, 2). Photolysis of unilamellar vesicles prepared from such phospholipids was demonstrated to give intermolecularly crosslinked products, as expected from carbene insertion into C—H bonds (Fig. 1, series A) (2). We now find that photolysis of the phospholipids as multilamellar dispersions gives products in which intermolecular carbene insertion is accompanied by elimination of one hydrogen fluoride molecule (Fig. 1, series B). The presence of the double bond allylic to the point of crosslink in the latter products makes it possible to analyze the sites of crosslinking by mass spectrometry. A study has therefore been carried out by using phospholipids with varying chain lengths of the fatty acyl groups carrying the carbene precursor. The results obtained on the distribution of crosslinks show a correlation between the sites of crosslinking and the length of the hydrocarbon chain carrying the carbene precursor, the point of maximum crosslinking being consistent with the recent conclusion that the *sn*-2 fatty acyl chain trails the *sn*-1 chain in phospholipids by 2–4 carbon atoms (3, 4).

MATERIALS AND METHODS

Materials. 12-Hydroxylauric acid, 16-hydroxyhexadecanoic acid, *tert*-butylchlorodiphenylsilane, 4-dimethylaminopyridine, and *N,N*-diisopropylethylamine were bought from Aldrich. 12-Hydroxystearic acid was obtained from Eastman Kodak. C^2H_3OH was purchased from Merck, Sharp & Dohme (Montreal, Quebec, Canada).

General Methods. Methods for separation of phospholipids and their characterization and for Sephadex LH-20 chromatography were described (1, 2).

Preparation of Phospholipids for Photolysis. The method for preparation of unilamellar vesicles has been described (1) except that, after evaporation of chloroform under N_2, any residual solvent was removed by continued suction *in vacuo* for 3–4 hr. After addition of the buffer (1), the suspension was vortexed for 5–7 min and, when required, sonicated for varying lengths of time under protection from light. The sonication temperature was 35–45°C. Photolysis was performed as described (2) by using RPR 3500-Å lamps. An aqueous 2% potassium hydrogen phthalate solution was used as the filter. The extraction of the photolyzed mixtures and their separation and subsequent transesterification were as described (2).

^{19}F NMR Spectrometry. The spectra were measured on a Bruker 270 MHz instrument operated at 254.01 MHz using trifluoroacetic acid as external reference. Chemical shifts are reported in ppm downfield from trifluoroacetic acid.

Gas Chromatography–Mass Spectrometry. These analyses were carried out using a 6 foot × 2 mm (inside diameter) glass column packed with 3% OV-1 on 100/120 mesh Gas Chrom Q, temperature programmed from 170–270°C at 12°C/min. The Perkin–Elmer 990 gas chromatograph was interfaced via a glass frit to a Hitachi RMU-6L low resolution mass spectrometer. An IBM 1800 computer was used for data acquisition and processing. Electron energy was 70 eV, accelerating voltage was 3050 V, and ion source temperature was 200°C.

High-resolution electron impact (EI) mass spectra were recorded on Ionomet photoplates by using a CEC 21-110B instrument (Du Pont Instruments, Monrovia, CA). Electron energy was 70 eV, accelerating voltage was 8 kV, and ion source temperature was 200°C. The plates were read on a comparator (David W. Mann Co., Burlington, MA) interfaced to the IBM 1800 computer.

Field desorption (FD) mass spectra were obtained by using a Varian MAT 731 double-focusing instrument with a combined FD/FI/EI source (FI = field ionization). The field anode was held at +8 kV and the counter electrode was at −3 kV, spaced at a distance of 1.5 mm. Ion source temperature was 90°C. Desorption of the diesters was optimal at 18–20 mA and of the phospholipids, at 28–30 mA. Spectra were recorded by scanning at a resolution of 1000. The emitters were 10-μm tungsten wires activated as described (5). Samples were transferred to the emitter by dipping the latter into a chloroform solution of the diester and a chloroform/methanol (2:1) solution of the phospholipid. The FD spectra of the diesters are described below (see *Discussion*). The FD spectra of the phospholipids were consistent with those reported by Wood *et al.* (6), having diagnostic fragment ions, protonated molecular ions, and cluster ions. The accurate peak intensity data on which Fig. 6 and Table 1 are based were obtained from low-resolution EI mass spectra of the photolysis products recorded by using slow

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Abbreviations: NMR peaks shown as s = singlet, d = doublet, t = triplet, and m = multiplet; IR, infrared; FD, field desorption; EI, electron impact.

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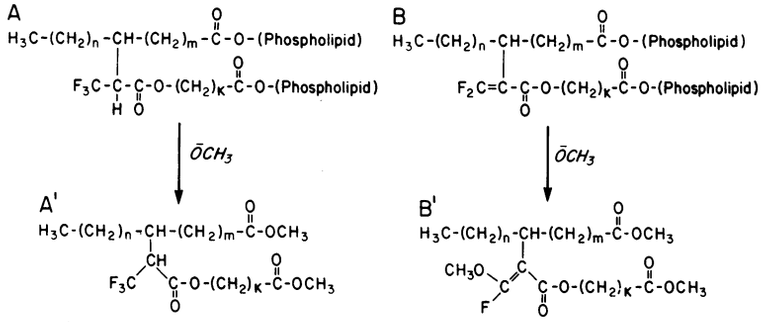

FIG. 1. General structures of the two series of crosslinked products obtained on photolysis of phospholipids containing ω-(2)diazo-3,3,3-trifluoropropionyloxy) groups in the *sn*-2 fatty acyl chain, series A and B, and the products obtained after transesterification, series A′ and B′.

scans (2048 sec/decade) on this instrument. The S/N ratio for the peaks corresponding to Cleavage A of Fig. 6, whose intensities were used to determine the insertion point, was at least 70:1.

Chemical Syntheses. 11-Hydroxyundecanoic acid was prepared by treatment of methyl 11-bromoundecanoate with sodium benzoate in dimethylformamide followed by alkaline hydrolysis. Methyl 12-ketostearate was prepared by oxidation of methyl 12-hydroxystearate with pyridinium chlorochromate (7), and methyl 7-ketopalmitate was synthesized as described (8). 7,7-Dideuteropalmitic acid and 12,12-dideuterostearic acid were made following the standard transformations of keto groups to dideuteromethylenes (9, 10). The extent of incorporation of deuterium into the fatty acid esters as determined by mass spectrometry was more than 93% in the $^2H_2$ form.

Two methods were used for the synthesis of phospholipids (Fig. 2) containing ω-trifluorodiazopropionyl group in *sn*-2 position. One method (1) involved the preparation of ω-trifluorodiazopropionoxy fatty acids and acylation of 1-acyl lysolecithins. A second method, now developed, involves the preparation of 1-acyl-2-ω-hydroxyl fatty acyl lecithins and their acylation with trifluorodiazopropionyl chloride. The method is exemplified by the following synthesis:

(*i*) *12-tert-Butyldiphenylsilyloxy lauric acid.* Methyl 12-hydroxyllaurate (2 mmol) was treated with *tert*-butyldiphenylchlorosilane (2.2 mmol) and imidazole (2.1 mmol) in anhydrous dimethylformamide (2.0 ml) at 25°C for 2 hr. The reaction mixture was poured onto ice water and the product was extracted three times with ether. The ether extracts were washed with water and dried over $MgSO_4$. The solvent was removed and the residue was dried under reduced pressure.

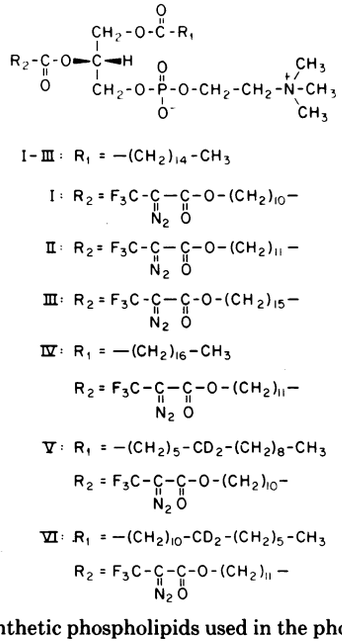

FIG. 2. Synthetic phospholipids used in the photolysis studies.

The crude ester was treated with 1.0% aqueous sodium hydroxide solution (vol/vol) in 30% aqueous methanol (15 ml) at room temperature for 20 hr. The solvents were removed and the residue was dissolved in water and acidified to pH 2 with 1 M HCl. After extraction (three times) with ether, the combined extracts were dried over anhydrous $MgSO_4$. Removal of the solvent gave a syrup, which was purified by silica gel column chromatography (ether/hexane as eluant). The yield of the lauric acid was 80%. Infrared $(IR)_{max}$ cm^{-1} 1710 (C=O); NMR (C^2HCl_3)δ: 10.26 (broad hump, 1H, exchangeable with ^2H_2O), 7.13–7.83 (m, 10H), 3.66 (t, J = 7 Hz, 2H), 2.30 (t, J = 7 Hz, 2 H), 1.16–2.0 (m, 18H), 1.05 (s, 9H).

(*ii*) *1-Stearoyl-2-(12-tert-butyldiphenylsilyloxy)lauroyl-sn-glycero-3-phosphorylcholine.* The above acid was converted to the corresponding anhydride and the latter was used to acylate 1-stearoyl-*sn*-glycero-3-phosphorylcholine as described (1). The product was purifed by preparative thin-layer chromatography, the yield being 76%. NMR (C^2HCl_3)δ: 7.1–7.83 (m, 10H), 5.2 (m, 1H), 3.77–4.66 (m, 8H), 3.66 (t, J = 7 Hz, 2H), 3.43 (δ, 9H), 2.33 (m, 4H).

(*iii*) *1-Stearoyl-2-(12-hydroxylauroyl)-sn-glycero-3-phosphorylcholine.* The preceding compound (0.1 mmol) was treated with 0.25 M tetra-*n*-butylammonium fluoride/0.25 M pyridinium fluoride in pyridine (1.2 ml) at room temperature for about 6 hr (11). The solvent was removed and the residue was dissolved in a 5:4:1 mixture (10 ml) of methanol/chloroform/water and treated with prewashed Rexyn I-300 resin (5.0 ml). After removal of the resin by filtration and of the solvent under reduced pressure, the product was purified by preparative thin-layer chromatography (yield, 70%). IR (Nujol) ν_{max} cm^{-1}: 1740 (C=O); NMR (C^2HCl_3)δ: 5.23 (m, 1H), 3.46–4.73 (m, 10H), 3.40 (m, 4H). FD spectra: MH^+, *m/e* 750, calculated for $C_{40}H_{80}NPO$: *m/e* 749.

(*iv*) *1-Stearoyl-2-(12-trifluorodiazopropionyl)lauroyl-sn-glycero-3-phosphorylcholine.* To an ice-cold solution of the above phospholipid (0.033 mmol) in anhydrous methylene chloride (1.0 ml) were added 3,3,3-trifluoro-2-diazopropionylchloride (0.1 mmol) and anhydrous pyridine (0.12 mmol). The reaction mixture was gradually allowed to warm to room temperature and then kept at this temperature for 22 hr. After removal of the solvent, the product was purified by preparative thin-layer chromatography followed by chromatography on a Sephadex LH-20 column. The yield was in the range of 25–40%. IR (Nujol) ν_{max} cm^{-1}: 2140 (N_2) and 1740 (C=O); NMR (C^2HCl_3)δ: 5.2 (m, 1H), 3.4 (s, 9H).

RESULTS

Isolation and Characterization of Crosslinked Fatty Acid Esters. Sonicated vesicles or aqueous dispersions of phospholipids (Fig. 2) were photolyzed. After separation of the photolysis products on a Sephadex LH-20 column (1), the crosslinked phospholipids (series A or B in Fig. 1) were transesterified (1). Products of general structures A′ and B′ (Fig. 1) were thus obtained. Assignment of the elemental composition was based on mass spectrometric measurements as described below.

Photolysis of multilamellar phospholipid dispersions above the transition temperature (30–45°C) consistently gave products of structure B, the latter being converted to B′ under transesterification conditions. In contrast, photolysis of unilamellar vesicles prepared by extensive sonication (about 1 hr at above transition temperature) uniformly gave insertion products of structure A. Prolonged sonication followed by photolysis at low temperature (below transition) also gave products of the B series. As expected, intermediate conditions led to mixtures of both types of compounds.

Mass Spectrometry of Crosslinked Fatty Acid Esters. FD mass spectra of both Series A′ and B′ (Fig. 1) contained singly and doubly charged molecular species and no fragment ions. In series A′, the MH^+ ion was dominant and the M^+ was not observed (Fig. 3*A*). For series B′, the M^+ was the most abundant ion, but both $(M-H)^+$ and MH^+ were also present (Fig. 3 *B* and *C*). More opportunity for charge delocalization probably exists in the B′ series, allowing the formation of several stable species. Because the FD spectra were much simpler than the EI spectra, it was convenient to examine the product mixtures first by FD, in order to determine the relative amounts of the A′ and B′ products and to ensure that further studies were carried out on samples containing only one type of product. The molecular weights and molecular formulae of the diesters were determined by high-resolution mass spectrometry with an average error of 2 ppm in the exact mass measurement. The mass spectra of compounds of series A′ had ions of relatively high intensity corresponding to loss of CH_3O and $CH_2CO_2CH_3$ from M^+. The other prominent ions observed in the spectra were those derived from McLafferty rearrangements. However, ions characteristic for the site of crosslinking were not observed in the spectra.

Compounds of series B′ consistently were eight mass units smaller than the corresponding compounds of series A′. Low-resolution EI spectra of two of these are shown in Fig. 4. Series of ions whose relative abundance could be related to the distribution of crosslinking points were observed (Table 1) and permitted an assessment of the pattern (see *Discussion*).

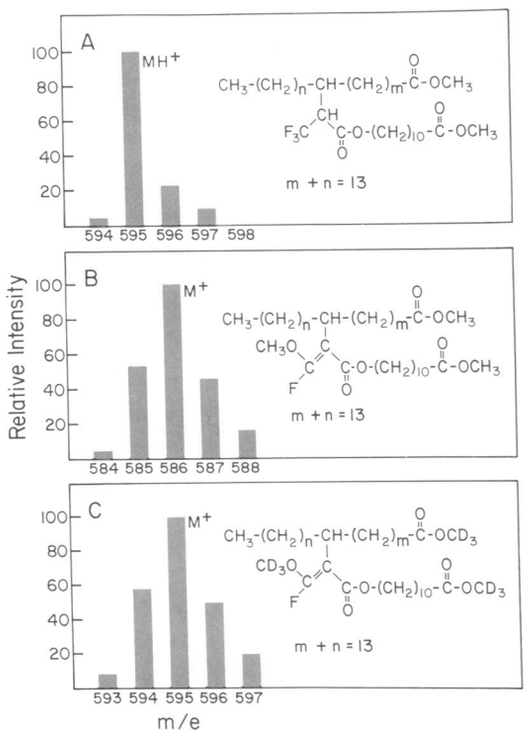

FIG. 3. FD mass spectra of the crosslinked diester A′ obtained from phospholipid I (M_r 594) (*A*), the crosslinked diester B′ from phospholipid I (M_r 586) (*B*), and the crosslinked diester B′ from phospholipid I after transesterification in C^2H_3OH (M_r 595) (*C*).

DISCUSSION

Photocatalyzed crosslinking of phospholipids carrying a photosensitive carbene precursor was shown to yield two series of products, depending on whether unilamellar vesicles or multilamellar phospholipid dispersions were used. Structural work was performed after methoxide-catalyzed transesterification which yielded products of the A′ and B′ series (Fig. 1), respectively. Assignment of the structures, especially to the latter compounds, was made mainly on the basis of mass spectrometric and ^{19}F-NMR measurements.

The nominal mass of the molecular ion of the B′ series varied with an alteration in the chain length of either *sn*-1 or *sn*-2 fatty acyl chain. This general result showed that both acyl chains of the photolyzed phospholipids were present in the above products. For the product obtained from phospholipid IV (Fig. 2), measurement of the exact mass (m/e 628.4749) of the molecular ion suggested the elemental composition $C_{36}H_{65}O_7F$ (calculated m/e 628.4714) and homologous compositions for the other members of the series. The difference between the molecular species in series A′ and B′, therefore, would be consistent with the loss of 2 mol of HF and the addition of 1 mol of methanol. The major features of the mass spectra could be rationalized by the fragmentation pattern shown in Fig. 5 for the above product. (Analogous ions were present in the high-resolution mass spectrum of each compound in the B′ series.) However, the exact mass observed for the molecular ions of the series can accommodate a second elemental composition. Thus, for example, the product B′ obtained from phospholipid I will have the composition $C_{33}H_{59}O_7F$ (calculated m/e 586.4245), but $C_{34}H_{57}O_4F_3$ (calculated m/e 586.4236) is also within experimental limits of the observed value (m/e 586.4240). Only the former structure should involve the incorporation of a molecule of methanol in addition to the two in ester groups. Therefore, transesterification was carried out with $NaOC^2H_3/C^2H_3OH$. Use of trideutero methanol should increase the molecular weight by six mass units if two methoxy groups were taken up during transesterification, or by nine mass units if 3 mol of methanol were utilized. The mass spectrum of the crosslinked transesterified product (m + n = 13; k = 10) from phospholipid I (Fig. 2) indeed showed an increase of nine, thus eliminating the alternative composition ($C_{34}H_{57}O_4F_3$).

Further support for the proposed structure was provided by the ^{19}F-NMR data. The two peaks observed (in a 1:2 ratio) had chemical shifts in agreement with *cis* and *trans* isomers of structure B′ (12). Only one fluorine atom was present, neither ^{19}F-^1H nor ^{19}F-^{19}F coupling being detected.

Formation of products (B′) from B involved the incorporation of a molecule of methanol in addition to the two required for transesterification. This showed the presence of a functional group sensitive to the methoxide ion. This is the case for the structure assigned to B (Fig. 1). A Michael-type addition of a methoxide ion in methanol followed by abstraction of a proton from the enol hydroxyl group and loss of a fluoride ion would lead to B′. Similar additions of alkoxide anions across the olefinic bonds followed by loss of hydrogen fluoride are known (13).

The formation of products A or B depended on the state of the phospholipids. Lecithins in multilamellar systems have been shown to be highly ordered (4, 14). In contrast, small vesicles formed on sonication of lecithins have less order (14–16). In the present phospholipids, the ordered structure of acyl chains in multilamellar dispersions may be enhanced by the localization of the polar photoreactive groups in the middle of the bilayer. The highly ordered structure may then enable the interaction of two carbene intermediates with one methylene acceptor

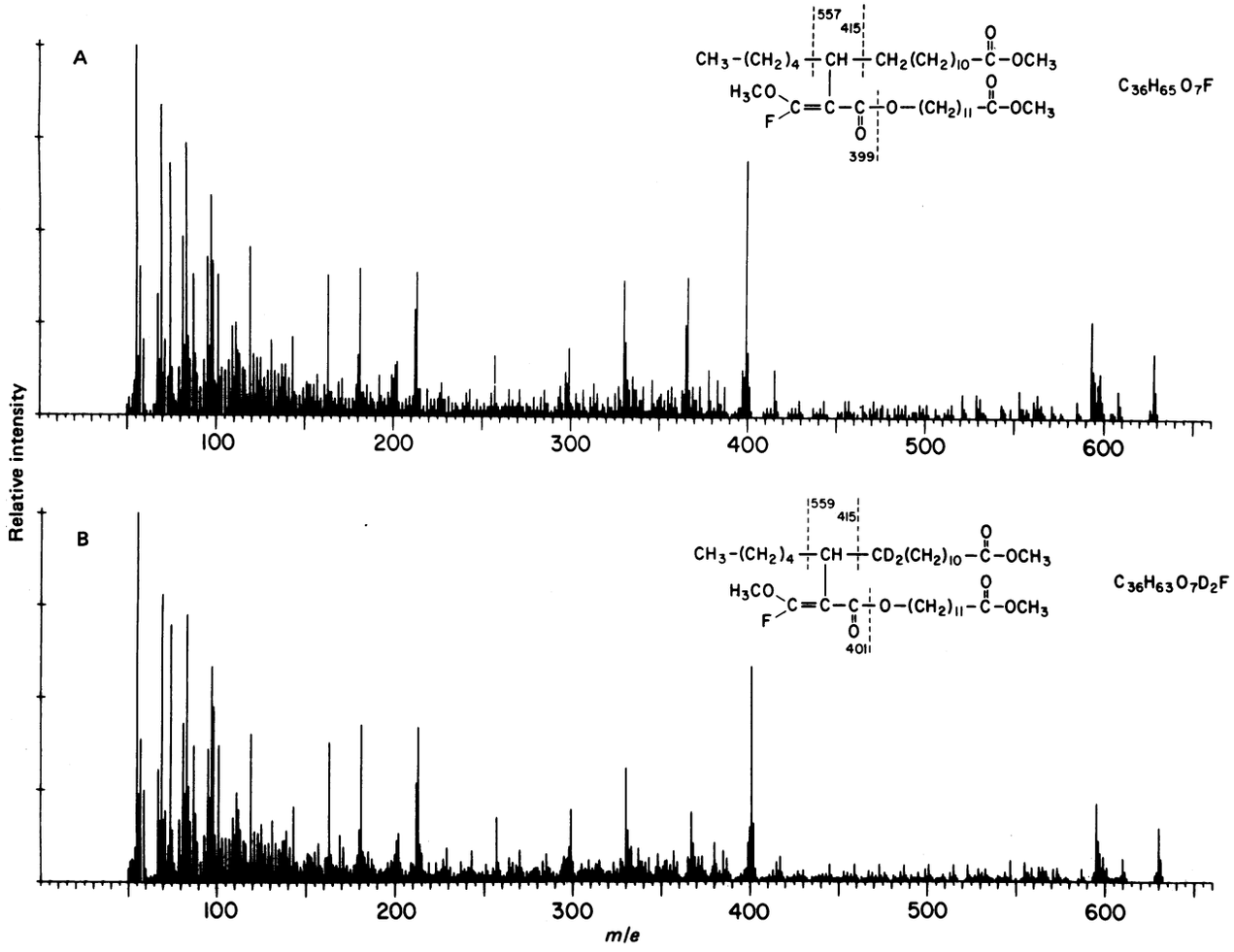

FIG. 4. EI mass spectra of the isomeric diesters derived from phospholipids IV and VI, M_r 628 (*A*) and M_r 630 (*B*), respectively.

group, perhaps forming a six-membered transition state. Consequently, the crosslinking of the hydrocarbon chain carrying the carbene intermediate with the acceptor chain, the accompanying abstraction of a proton from the acceptor carbon, and the elimination of a fluoride ion may occur in a concerted fashion. In single-walled vesicles, the normal insertion reaction is favored because the carbene intermediates are sufficiently separated from each other so as to preclude the cooperative effect observed in the multilamellar structures.

Formation of the B′ products, which also arose via an insertion mechanism, made it reasonable then to look for a distribution pattern among the intensities of fragments that reflected the linkage site. It had not been possible to observe such a pattern in the spectra of the A′ products. However, a pattern was found for the B′ products, as described below. That the carbene insertion should occur at a unique site is unlikely. Rather, a mixture of isomers varying in the points of crosslinks would be expected. To determine the distribution of the insertion points in each product mixture, ions in the mass spectra characteristic of crosslinking points had to be selected for comparison.

As can be seen in Fig. 5, in addition to the molecular ion, the ions $(M - OCH_3)^+$, $(M - HF)^+$, and $(M - [HF + CH_3])^+$ will be shifted upon changes in both acyl chains but will be independent of the position of insertion. The *m/e* of the ions derived from cleavage C will shift with changes in the *sn*-1 chain but will be independent of the insertion point. Cleavage A leads to ions that vary with the *sn*-2 chain, but their *m/e* is affected by the site of the crosslink. Although similar ions can arise by losses of $(CH_2)_xCO_2CH_3$ along the *sn*-2 chain, part of the series can be distinguished in the spectra of the deuterium-labeled crosslinked products from phospholipids V and VI. Cleavage B yields a series of ions that shift with both acyl chains and also with the position of insertion. Although losses of the fragment $(CH_2)_xCO_2CH_3$ are possible by cleavage of any $—CH_2—CH_2—$ bond along the ester chains, it seems reasonable that cleavage A would be favored because it involves an allylic bond. Ions resulting from this cleavage in compounds of series B′ are, therefore, sufficiently abundant to give a ratio indicative of the relative proportion of the isomers differing in crosslinking sites.

Fig. 6 has been constructed on the basis of intensity mea-

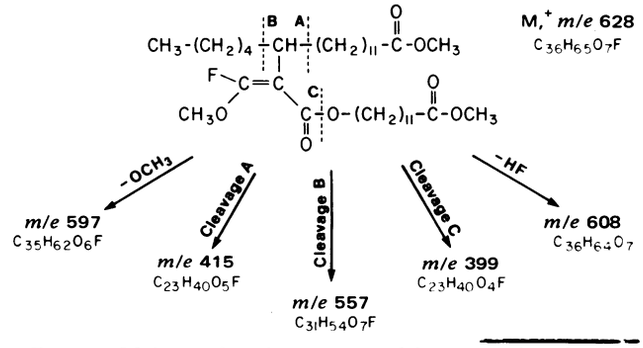

FIG. 5. Major modes of mass spectral fragmentation observed for compounds of the B′ series.

Table 1. Relative intensities of the ions resulting from cleavage A in the mass spectra (Fig. 4 *A* and *B*) of the products formed from IV and its deuterated analog VI, respectively.

Linkage site	$[M - (CH_2)_nCOOCH_3]^+$ *m/e*	Relative intensity Fig. 4*A*	Fig. 4*B*
C-6	513	25	
	515		18
C-7	499	30	
	501		19
C-8	485	43	
	487		21
C-9	471	40	
	473		22
C-10	457	46	
	459		21
C-11	443	50	
	445		21
C-12	429	58	
	430/431		15/07
C-13	415	100	100
C-14	401	72	75
C-15	387	75	43
C-16	373	66	44
C-17	359	46	38
C-18	345	19	13

Although the relative intensities listed in the last two columns must represent the sum of the abundances of ions formed by cleavage A (Fig. 5) and of the ions due to cleavage of the same C—C bonds in all the other isomers, one can assume for reasons outlined in the text that that isomer for which this fragment represents cleavage A will be by far the major contributor. Under any circumstances, the contributions of the other isomers will be relatively the same for each C—C bond and thus cancel out. If these contributions would be taken into account (which would only be possible if the spectrum of a pure specific isomer were available), the distributions shown in Fig. 6 and this table would be even more pronounced, rather than less. It should be noted also that for the labeled compound (Fig. 4*B*) the ion resulting from this fragmentation would contain only one deuterium atom and appear at *m/e* 430 when the linkage site is C-12.

surements on the low-resolution mass spectrum for the fragments in each series. Relative intensities of the appropriate ions in the spectra of B′ (m + n = 15; k = 11) and B′ (m + n = 15, d_2 at C_{12}; k = 11) are given in Table 1 and their spectra are given in Fig. 4 *A* and *B*. In the few cases where the nominal mass of interest was not a single elemental composition, the intensity used here was obtained by assigning the contribution of each isobar on the basis of its relative abundance in the high-resolution spectrum. A second series of homologous ions corresponding to cleavage B is less intense but follows the same distribution. From the total results with the B′ series, it is apparent that there is a distribution of crosslinking sites, with the maximum being observed at C-12 when the *sn*-2 acyl chain of the phospholipid is undecanoyl (Fig. 6*A*) and C-13 when the *sn*-2 acyl chain is lauroyl (Fig. 6 *B* and *C*). Extension of the *sn*-2 acyl chain beyond the *sn*-1 acyl chain does not give the same type of pattern (Fig. 6 *D*).

The main significance of the present findings derives from the fact that the points of maximum crosslinking for different phospholipids are consistent with the recent conclusions regarding the molecular structure of the phospholipids. Thus, both the x-ray diffraction work (3) and the neutron diffraction studies (4) indicate that the molecular conformation of the phospholipids in the gel crystalline state is such that the *sn*-2 acyl chain is shorter than the *sn*-1 acyl chain by 2–4 carbon atoms. Hopefully, the correlation discovered will prove useful in studies of the topography of membrane-embedded proteins using phospholipids that carry carbene precursors at varying distances from the polar head group.

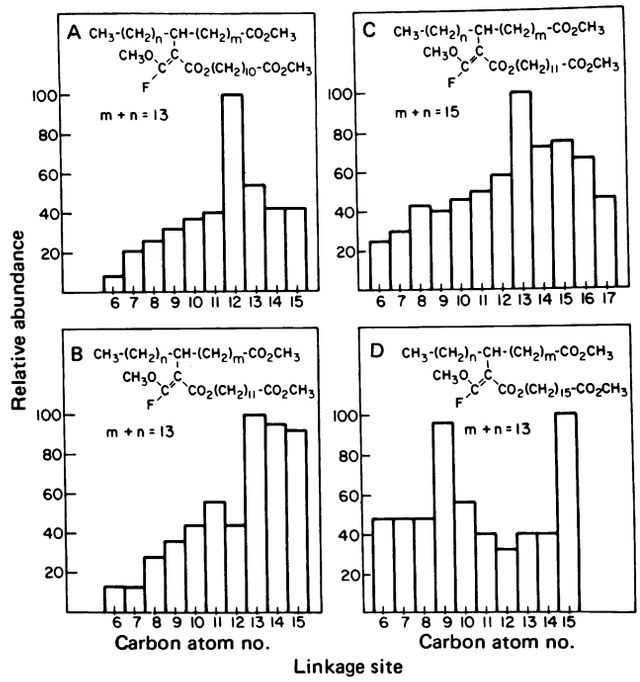

FIG. 6. Distribution of linkage sites for crosslinking in the B′ diesters, calculated on the abundance of the fragment ions arising via cleavage A (Fig. 5).

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