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A New Approach to the Study of Phospholipid–Protein Interactions in Biological Membranes. Synthesis of Fatty Acids and Phospholipids Containing Photosensitive Groups†

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ABSTRACT: In a general approach to the study, in vivo and in vitro, of the interactions between phospholipids and proteins in biological membranes, a variety of fatty acids containing photosensitive groups in different positions in the alkyl chains has been synthesized. The fatty acids synthesized include: 16-azidopalmitelaidic acid, 12-azidooleic acid, 6-, 9-, 11-, and 12-azidostearic acid, 12-*O*-(ethyl-2-diazomalonyl)stearic and -oleic acids, 12-*O*-(4-azido-2-nitrophenyl)stearic and -oleic acids, and 12-oxo-10-octadecenoic acid. Some of the above synthetic fatty acids were also prepared in the radioactively labeled form. For in vitro studies, many of the above fatty acids were used to acylate the 2 position in the preparation of a number of mixed acylphosphatidylcholines and mixed acylphosphatidylethanolamines. On sonication, the synthetic phospholipids formed sealed vesicles. Intermolecular cross-linking of the fatty acyl chains in phospholipids was demonstrated on photolysis of the vesicles.

A central problem in membrane biochemistry is the understanding of the specific interactions between phospholipids and proteins possessing different biological functions. A variety of approaches, physicochemical, biochemical, and chemical, has been used with varying degrees of scope and success. Thus, physicochemical techniques, such as electron spin resonance, infrared, circular dichroism, nuclear magnetic resonance (NMR), and fluorescence spectroscopy (see, e.g., Chapman and Dodd, 1971; Jost et al., 1971; Wallach and Winzler, 1974; Fleischer and Packer, 1974), have given valuable information on general properties of biological membranes as well as in studies of specialized membrane functions. Biochemical approaches have aimed at the isolation and study of defined proteins possessing specific membrane functions [for selected reviews see Fleischer and Packer (1974); Coleman (1973); Rothfield and Romeo (1971); and Racker et al. (1975)]. Studies of reconstituted systems have frequently given significant information on the specificity of phospholipid requirements. Further, the use of bacterial mutants with a requirement for exogenously supplied fatty acids has allowed the manipulation of the phospholipid composition in the membranes and thus to study its effect on the function of different membrane enzymes and of certain transport systems. More recently, a number of mono- and bifunctional agents has been increasingly used in studies of the protein–protein or protein–lipid interactions. The reagents may or may not penetrate the phospholipid bilayer and information may therefore be gained regarding the surface or the interior of the membrane bilayers. While most of the reagents used aim at standard types of chemical reactions with functional groups in proteins, noteworthy reagents recently used are those which utilize photocatalyzed formation of nitrene intermediates (Staros and Richards, 1974; Staros et al., 1974; Klip and Gitler, 1974).

Development of new organochemical approaches to the study of phospholipid–protein interactions in biological membranes would be highly desirable and the work described in the present paper represents a start in this general area. Some of the considerations taken into account in formulating the present approach are as follows. Firstly, the most prominent and unique feature of integral membrane proteins is their hydrophobic character and, presumably, their hydrophobic surface, which allows strong and specific interaction with the phospholipids. Therefore, the principal aim of new approaches should be the study of hydrophobic

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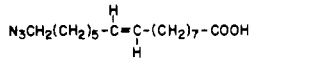
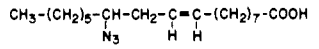
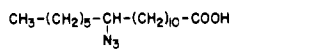
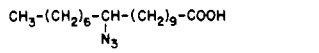
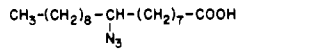
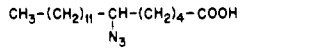
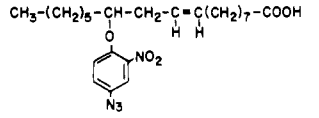
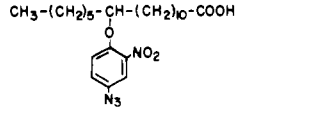
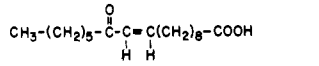
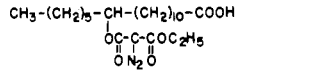
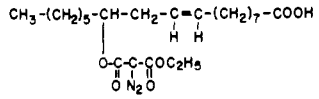
Table I:

Compound No.	Name	Structure
I	16-Azidopalmitelaidic acid	
II	12-Azidooleic acid	
III	12-Azidostearic acid	
IV	11-Azidostearic acid	
V	9-Azidostearic acid	
VI	6-Azidostearic acid	
VII	12-O-(4-Azido-2-nitrophenyl)-oleic acid	
VIII	12-O-(4-Azido-2-nitrophenyl)-stearic acid	
IX	12-Oxo-10-octadecenoic acid	
X	12-O-(Ethyl-2-diazomalonyl)-stearic acid	
XI	12-O-(Ethyl-2-diazomalonyl)-oleic acid	

interactions between proteins and phospholipids. Secondly, as described above, the currently available approaches involve adding to the membranes reagents which may serve as noncovalently linked probes or as covalent cross-linking agents. Since these reagents added from outside may unduly perturb the normal hydrophobic contacts, it would be clearly preferable to have "built-in" activable groups in fatty acids themselves at the outset. This approach would have a broad scope for systematic exploration of the hydrophobic interactions, since by synthetic design, the "reactive" groups could be introduced one by one at every one of the carbon atoms in the fatty acid chains and, furthermore, such fatty acids could be incorporated into positions 1 or 2, or both, of the glycerol moiety in phospholipids. Thirdly, since the aim is to explore the interaction or points of contact between the chemically inert fatty acid chains and, presumably, the exposed hydrophobic amino acid side chains in proteins, clearly the large body of chemistry which utilizes the customary functional groups is excluded. Instead, the types of groups which would be promising would be the photoactivable groups, groups which can generate the highly reactive nitrenes or carbene intermediates (Knowles, 1972). Further, the groups to be used should be as small as possible so as to cause minimal perturbation of the normal functions of the membranes in vivo or of isolated proteins.

In the present paper, a number of saturated and monounsaturated fatty acids has been synthesized with photosensitive groups in them. The fatty acids synthesized are shown in Table I. The synthetic fatty acids can be used in two general types of experiments. Firstly, in in vivo experiments, it may be hoped that they may support the growth of bacterial fatty acid auxotrophs. Should this prove to be the case, then a powerful approach of wide scope could become available for the intended studies. Separately, it is being reported that a substantial number of the synthetic fatty acids listed in Table I does in fact support the growth of an *Escherichia coli* auxotroph (Greenberg et al., 1975). Similarly, encouraging results have been obtained for an unsaturated fatty acid auxotroph of a *pseudomonad* in collaboration with Drs. N. Tsukagoshi and R. M. Franklin of Basel University. The organism serves as the host for the virus PM2 which contains a phospholipid bilayer (Franklin, 1974).

For in vitro studies of reconstitution of defined membrane functions, the synthetic fatty acids could be incorporated synthetically into phospholipids of completely defined structure. The latter could then be tested for their efficiency in various reconstitution systems as well as in the reactivation of delipidated membrane enzymes which require phospholipids for their normal functions. A large number of defined systems for such studies in vitro has recently become available through the efforts of many laboratories (Racker et al., 1975; Rothfield and Romeo, 1971; Coleman, 1973; Fleischer and Packer, 1974).

With these studies in view, syntheses have been carried out of a number of phospholipids containing photosensitive groups. The synthetic phospholipids fall into three groups. Two classes belong to the phosphatidylcholine–phosphatidylethanolamine series in which the fatty acids present in the 2 position carry different photosensitive groups. In the third group, the photosensitive groups are present on the amino groups of dipalmitoylphosphatidylethanolamine. This last type of synthesis was performed in order to extend the interesting results obtained previously with *N*-acetylphosphatidylethanolamine (Knowles et al., 1975).

While detailed studies with the synthetic phospholipids will be reported later, two basic experiments are now described which are encouraging for further work. Firstly, in standard sonication experiments performed in the presence of [^{14}C]glucose, the synthetic phospholipids were demonstrated to form sealed vesicles. Secondly, photolysis of the vesicles resulted in the formation of higher molecular weight products in which intermolecular cross-linking of fatty acid chains was demonstrated.

A preliminary report of some of the present results has previously been made (Chakrabarti et al., 1974).

Materials and Methods

The following fatty acids were purchased from NuChek Preparations, Inc., Minnesota: 6-, 9-, 11- and 12-hydroxystearic acids and 12-hydroxyoleic acid (ricinoleic acid). β-Hydroxymyristic acid was purchased from Analab and 16-bromopalmitelaidic acid from Aldrich Chemicals. L-α-Dipalmitoylphosphatidylcholine and L-α-dipalmitoylphosphatidylethanolamine were purchased from Calbiochem Corporation. L-α-Dipalmitoylphosphatidylcholine-*1*-^{14}C was obtained from Applied Science Laboratories. Crude snake venom (*Crotalus adamanteus*) from Ross Allen's reptile farm was used as the source of phospholipase A_2. *sn*-Glycero-3-phosphorylcholine was prepared from the purified soya lecithin essentially by the procedure of Chada (1970).

Table II: Thin-Layer Chromatography of Phospholipids, Fatty Acids, and Their Methyl Esters with Photoactivable Groups.

Compound	Solvent A (R_f Rel to Palmitic Acid)	Solvent B (R_f Rel to Palmitic Acid)	Solvent C (R_f Rel to Methyl Palmitate)	Solvent D (R_f Rel to Methyl Palmitate)	Solvent E (R_f Rel to XV and XX)
Palmitic acid	0.61	0.53			
I–VI	0.66	0.46			
VII–VIII	0.35				
IX	0.6	0.4			
Methyl palmitate			0.77	0.78	
Methyl ester of the acids (I–VI)	0.85		0.77	0.68	
Methyl Ester of IX	0.65		0.73	0.023	
XV–XIX					0.51
Lysophosphatidylcholine					0.24
XX–XXIII					0.75
Lysophosphatidylethanolamine					0.49
N-t-Boc-PE[a]					0.92
Lyso-*N-t*-Boc-PE					0.86

[a] *t*-Boc, *tert*-butyloxycarbonyl; PE, phosphatidylethanolamine.

Thin-layer chromatography (TLC) of fatty acids, their methyl esters, and various phospholipids was performed on silica gel plates or strips using the following solvent systems: solvent A, benzene–ligroin–ethyl acetate–acetic acid (100:10:4:1, v/v); solvent B, petroleum ether (bp 60–80°)–diethyl ether–acetic acid (8:2:0.1, v/v); solvent C, petroleum ether (bp 60–80°)–diethyl ether (9:1, v/v); solvent D, petroleum ether–acetone (50:0.7, v/v); and solvent E, chloroform–methanol–water (70:25:4, v/v). Solvents A and B were used for fatty acids, solvents C and D for fatty acid methyl esters, and solvent E for phospholipids. Visualization of the various compounds on TLC was carried out by using the following reagents: (1) iodine vapor for unsaturated compounds; (2) 2′,7′-dichlorofluorescein (0.2% in ethanol) for fatty acids and esters; (3) Molybdenum Blue for phospholipids; (4) ninhydrin spray for compounds containing free amino groups; and (5) Draggindorff reagent for choline containing compounds. R_f values are given in Table II. Uv, ir, and NMR spectra were recorded in Zeiss uv spectrophotometer (Model PM4-Q111), Perkin-Elmer spectrophotometer (Model 567), and Varian (T-60) spectrometer, respectively.

General Methods for the Synthesis of Azido-Substituted Fatty Acids. In one method (Figure 1), methyl ester of the hydroxy fatty acid (2 mmol) was treated with methanesulfonyl (mesyl) chloride (2.1 mmol) in pyridine (10 ml) at room temperature. After evaporation of the solvent, the residue was extracted with ether, the ether extract was washed with water and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the mesyl ester thus obtained was treated with sodium azide (10 mmol) in dimethylformamide–water (40 ml:3 ml) for 40–50 hr at room temperature. The conversion to the azido derivative was usually complete and the product was purified by passage through a column of silicic acid using chloroform as the solvent.

During the preparation of the aromatic azide derivatives, all operations were carried out under dim daylight or under a red safelight.

For preparation of the aromatic azides (Figure 1), the hydroxy fatty acid (1 mmol) was treated with potassium *tert*-butoxide (2.5 mmol) in dry ether and then with 4-fluoro-3-nitrophenyl azide (1.2 mmol) for 36–40 hr at room temperature. The azido-substituted fatty acid was purified by preparative thin-layer chromatography (E. Merck, silica gel F-254, 0.5-mm thickness plates) using the solvent benzene–ligroin–ethyl acetate–acetic acid, 100:10:14:1 (v/v).

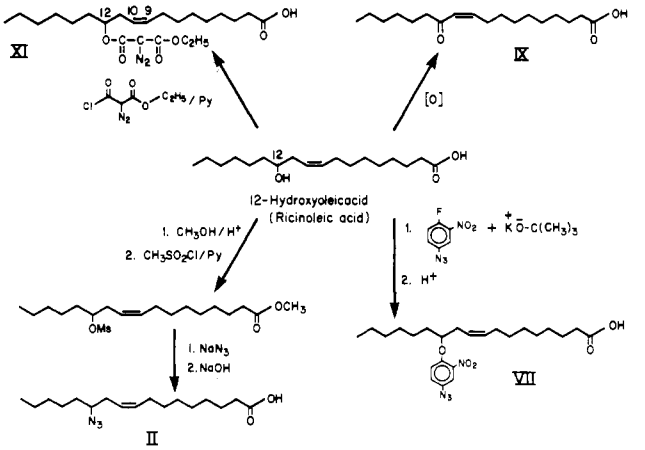


FIGURE 1: The preparation of different photosensitive derivatives of 12-hydroxyoleic acid (ricinoleic acid): (a) preparation of II, the 12-azido derivative; via the mesyl ester; (b) preparation of the aromatic azido derivative (VII), by reaction with 14-fluoro-3-nitrophenyl azide; (c) preparation of the diazomalonyl derivative (XI); and (d) the α,β-unsaturated ketone (IX) by oxidation.

Synthesis of Diazomalonyl-Substituted Fatty Acids. Acylation (Figure 1) of the hydroxy fatty acids (1 mmol) was performed with ethyl diazomalonyl chloride (5 mmol) in dry pyridine (3 ml) at 0° for 0.5 hr followed by 5 hr at room temperature. Ethyl diazomalonyl chloride was prepared according to the procedure of Vaughan and Westheimer (1969). The diazomalonyl-substituted fatty acids were also purified by preparative TLC. The procedure was the same as described above for the separation of aromatic azido-substituted fatty acids. In these syntheses also all operations were carried out under dim laboratory light.

Synthesis of ^{14}C-Labeled Fatty Acids. For the identification of the membrane components after cross-linking, the use of radioactively labeled fatty acid would be helpful. A general approach was developed for the introduction of ^{14}C-label in the C_1 position of the fatty acids. This is illustrated in Figure 2 in the synthesis of [1-^{14}C]-12-hydroxyoleic acid.

General Methods for the Synthesis of Mixed Acyl-3-

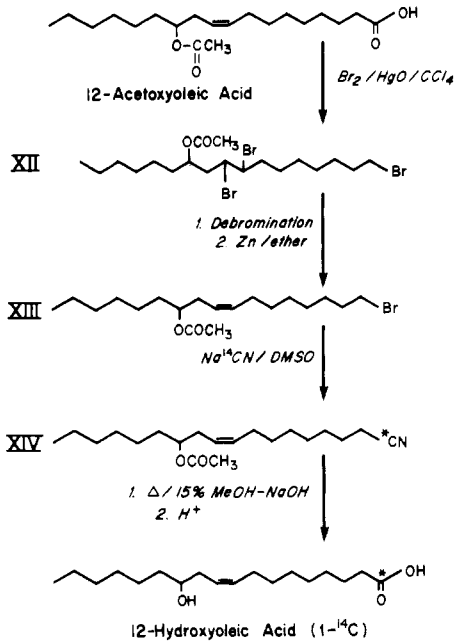


FIGURE 2: Steps in the synthesis of the ^{14}C-labeled (at C_1) 12-hydroxyoleic acid.

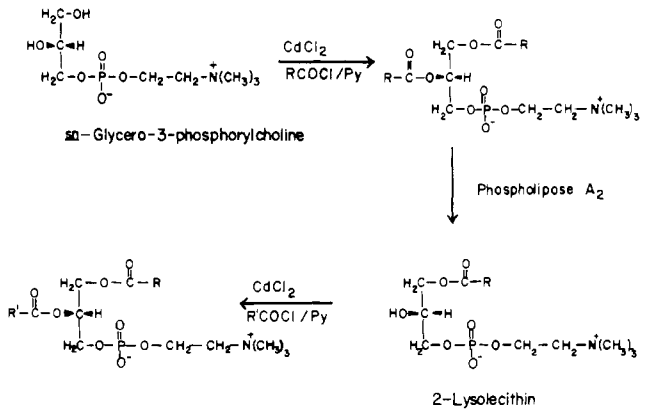


FIGURE 3: Steps in the synthesis of mixed acylphosphatidylcholines.

sn-phosphatidylcholines. The steps used are shown in Figure 3 and the general route is based on the methods of Baer and Buchnea (1959) for the synthesis of symmetrical diacyl-3-*sn*-phosphatidylcholines and on the procedure of de-Hass and van Deenen (1960) for the preparation of mixed acylphosphatidylcholines. Thus, after acylation of the *sn*-glycero-3-phosphorylcholine to the corresponding 1,2,-diacyl derivative, the 2 acyl group was selectively removed by the use of phospholipase A_2 present in the crude snake venom. The product as its $CdCl_2$ (1 mmol) complex was treated with the appropriate fatty acid chloride (12 mmol) in dry chloroform (7 ml, ethanol-free) and pyridine (1 ml) and the required mixed acyllecithin (Figure 3) was purified either by column chromatography over silicic acid using a gradient of chloroform–methanol to methanol or by preparative thin-layer chromatography using the solvent chloroform–methanol–H_2O, 65:35:5 (v/v).

General Methods for the Synthesis of the Mixed Acyl-3-sn-phosphatidylethanolamines. The steps used are shown in Figure 4. Thus, the starting material was the commercially available dipalmitoylphosphatidylethanolamine. The amino group was first protected by *tert*-butyloxycarbonyl

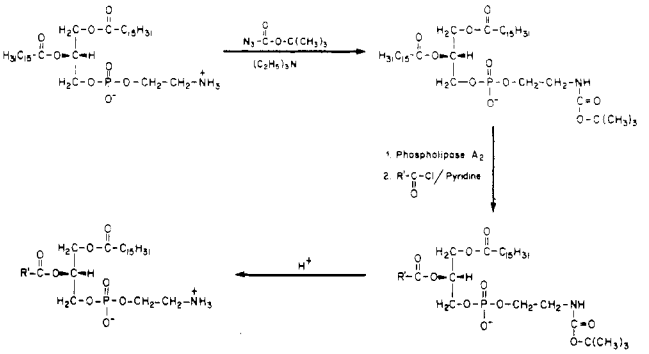


FIGURE 4: Steps in the synthesis of mixed acylphosphatidylethanolamines.

group. The subsequent steps for the preparation of the mixed acyl phospholipid were as described for the lecithin derivatives above. The *tert*-butyloxycarbonyl group was removed by a mild acidic treatment.

Characterization of Mixed Acyl Phosphatidylcholines and Phosphatidylethanolamines. Mixed acylphosphatidylcholines and phosphatidylethanolamines synthesized with photoactivable groups were characterized by (1) cochromatography with standard dipalmitoyl-3-*sn*-phosphatidylcholine (R_f in solvent E, 0.51) or dipalmitoyl-3-*sn*-phosphatidylethanolamine (R_f in solvent E, 0.75), (2) spectrophotometric measurement (uv, ir, NMR), (3) successive treatment with phospholipase A_2 yielding the corresponding 2-lysophospholipid and the fatty acid with the photoactivable group, and (4) treatment with tetramethylammonium hydroxide in methanol to give *sn*-glycero-3-phosphorylcholine and *sn*-glycero-3-phosphorylethanolamine and the methyl esters of the fatty acids.

General Procedure for the Reaction of Phospholipase A_2 with Phosphatidylcholines and Phosphatidylethanolamines. Phosphatidylcholine (1.2 mmol) was dissolved in a mixture of ether (196 ml) and methanol (12 ml) and the solution was stirred vigorously in the presence of borate buffer (49 ml of 0.1 *M*, pH 7.4) containing $CaCl_2$ (0.72 m*M*) and 5 mg of the crude rattle snake venom (*Crotalus adamanteus*) at 37° for 3 hr. The reaction was monitored by TLC (solvent A). After completion of the reaction, the organic layer, which contained the fatty acid, and the aqueous layer, which contained lysophosphatidylcholine and other water-soluble materials, were separated. The aqueous layer was washed with ether to remove any fatty acid and then lyophilized. The residue was extracted with chloroform–methanol (2:1, v/v) and centrifuged. On evaporation of the clear supernatant, lysophosphatidylcholine was obtained in about 90% yield. Thin-layer chromatography of lysophosphatidylcholine in solvents A and C showed that it was free from diacylphosphatidylcholine and fatty acid. If fatty acid was present, this was removed by crystallization of the lyso compound from ethanol–ether.

Conditions for phospholipase A_2 reaction with phosphatidylethanolamine analogs were as follows: diacylphosphatidylethanolamine (72 μmol) in 5 ml of Tris buffer (pH 9, 0.017 *M*) containing $CaCl_2$ (83 μ*M*) and 0.1 mg of crude venom in 10 μl of 0.02 *M* borate buffer was sonicated for 2–3 min. After addition of ether (20 ml), the mixture was vigorously shaken at 37° for 3 hr. Similar procedure as described in the case of phosphatidylcholines was followed afterwards. R_f values of lysophosphatidylethanolamines in solvent A are given in Table II.

Methanolysis of 2-Lysophosphatidylcholines and 2-Lysophosphatidylethanolamines. Treatment (2.5 μmol) of 2-lysophosphatidylcholines or 2-lysophosphatidylethanolamines in ether (400 μl) with tetramethylammonium hydroxide in methanol (25%, 40 μl) for 2 hr at room temperature yielded methyl palmitate (TLC, R_f in Table II and GLC) and *sn*-glycero-3-phosphorylcholine and *sn*-glycero-3-phosphorylethanolamine, as shown by comparison with authentic samples (paper chromatography, ascending technique). The solvent system used was methanol–88% formic acid–water (80:15:5, v/v). Treatment with phospholipase A_2 and tetramethylammonium hydroxide established the positional purity of the acyl substituents in the phospholipids with photoactivable fatty acids.

Similar procedure was used for methanolysis of diacylphosphatidylcholines and diacylphosphatidylethanolamines.

Experimental Procedures and Results

Syntheses of Modified Fatty Acids

12-Azidooleic Acid (II). Ricinoleic acid (12-hydroxyoleic acid) (566.6 mg, 1.9 mmol) was treated with 3% methanolic hydrogen chloride (30 ml) at room temperature for 3 hr. After evaporation of the solvent in vacuo, the residue was extracted with ether; the extract was washed with aqueous sodium bicarbonate (5%) and saturated sodium chloride, and was then dried. The yield of the dry residue was 560 mg (98%). TLC (solvent A) showed complete conversion of ricinoleic acid to the methyl ester. [NMR (CCl_4) δ 3.6 (s, 3 H, $COOCH_3$), 5.4 (m, 2 H, olefinic protons), 3.5 (broad signal overlapping with ester signal, 1 H, *H*-C-OH), and there was no signal due to carboxyl proton.]

A dry pyridine (10 ml) solution of the above methyl ester (624 mg, 2 mmol) was treated with mesyl chloride (244.0 mg, 2.1 mmol) for 3 hr at room temperature. After evaporation of the solvent, the residue was extracted with ether and the ether extract washed with water and then dried over anhydrous Na_2SO_4. The ir spectrum (CCl_4) showed strong bands at 1740 ($COOCH_3$) and at 1340 and 1175 cm^{-1} ($-SO_2O-$). [NMR (CCl_4) δ 2.91 (s, 3 H, $COSO_2CH_3$), 4.6 (broad multiplet, 1 H, $HCOSO_2CH_3$), 3.6 (s, 3 H, $COOCH_3$), and 5.43 (broad, 2 H, olefinic protons).]

The above mesyl ester (390 mg, 1 mmol) in a mixture of dimethylformamide (18 ml) and water (1.5 ml) was stirred with sodium azide (325 mg, 5 mmol) for 50 hr at room temperature. The reaction mixture was evaporated to dryness, diluted with water, and extracted with ether. The ether extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The yield was 303 mg (90%). Thin-layer chromatography (solvents C and D) showed the complete conversion of the mesyl ester to the azido ester. The ir and NMR data were characteristic for the azido derivative and the absence of the mesyl group signals showed the completion of reaction. The ir (CCl_4) spectrum showed a sharp band at 2100 cm^{-1} due to the azide group (N_3) and at 1735 cm^{-1} for the ester group ($COOCH_3$). The NMR (CCl_4) spectrum showed a broad signal at δ 3.28 (1 H, HCN_3) and a singlet at 3.6 (3 H, $COOCH_3$).

The above azido ester (337 mg, 1 mmol) was hydrolyzed with 1 *N* methanolic sodium hydroxide (5 ml) for 24 hr at room temperature. The reaction mixture was diluted with water, acidified with 1 *N* HCl, and extracted with ether. The ether extract was washed with water, dried over anhydrous Na_2SO_4, and then evaporated in vacuo. Thin-layer chromatography (solvents A and B) and ir and NMR data showed complete deesterification. Yield of the gummy residue was 305 mg (95%). The ir spectrum showed sharp bands at 2100 (N_3) and 1710 cm^{-1} (COOH). The NMR spectrum showed a broad signal at δ 10.2 for one carboxyl proton (COOH) and no signal for methyl ester ($COOCH_3$). The uv spectrum showed an absorption maximum at 285 nm (ε 220).

Other Azido Fatty Acids. The following azido fatty acids (Table I) were prepared by using the procedure described above for 12-azidooleic acid; 12-azidostearic acid (III) from 12-hydroxystearic acid; 11-azidostearic acid (IV) from 11-hydroxystearic acid; 9-azidostearic acid (V) from 9-hydroxystearic acid; 6-azidostearic acid (VI) from the corresponding hydroxy acid. All the products were characterized by NMR and ir spectra.

16-Azidopalmitelaidic Acid (I). The commerically available 16-bromopalmitelaidic acid was converted to the methyl ester by treatment with 3% methanolic hydrogen chloride. The methyl ester (460 mg) was purified by column chromatography on a silica gel column (14 g). Elution with benzene gave a single compound (TLC) corresponding to the methyl ester. The methyl ester (426 mg, 1.22 mmol) in methyl ethyl ketone (25 ml) was heated under reflux with potassium iodide (306 mg, 1.84 mmol) for 26 hr. The reaction mixture was evaporated to dryness and diluted with ether and the ether extract was washed with water and dried over anhydrous Na_2SO_4. On evaporation in vacuo, a gum (454 mg; 94% of theoretical) was obtained. The R_f values of both the starting bromo compound and the product, iodo ester, were the same. However, the NMR spectrum (CCl_4) of the iodo ester was characteristic and showed complete conversion of the bromo ester to the iodo ester. The spectrum (CCl_4) showed a singlet at δ 3.63 for the ester group ($COOCH_3$) and a triplet at δ 3.08 (2 H, J = 7.5 Hz) for the two methylene protons attached to the iodo group (ICH_2CH_2). The upfield shift of these methylene protons by 15 Hz compared to the bromo ester showed the formation of the corresponding iodo ester.

The above iodo ester (376.2 mg, 0.95 mmol) in a mixture of dimethylformamide (18 ml) and water (1.5 ml) was treated with sodium azide (92.3 mg, 1.42 mmol) for 30 hr at room temperature. The desired azido ester was obtained in 90% yield. The ir spectrum (CCl_4) showed strong band at 2100 cm^{-1} (N_3); the NMR spectrum showed δ 3.21 (t, 2 H, $N_3CH_2CH_2$) and downfield shift of these methylene protons by 5 Hz compared to the iodo ester.

The above azido ester was hydrolyzed to 16-azidopalmitelaidic acid (Table I) as described above. The ir spectrum in CCl_4 showed a sharp band at 2100 cm^{-1} (N_3), and broad absorption around 3500–2500 and 1710 cm^{-1} (COOH). The NMR spectrum (CCl_4) showed δ 3.21 (t, 2 H, $N_3CH_2CH_2$) and broad signal at 11.92 (1 H, COOH).

12-(4-Azido-2-nitrophenoxy)oleic Acid (VII). 12-Hydroxyoleic acid (298 mg, 1 mmol) in dry ether (3 ml) was stirred with potassium *tert*-butoxide (280 mg, 2.5 mmol) for 4 hr at room temperature. Stirring was continued for 36–40 hr at room temperature after addition of a dry (Na_2SO_4) ether solution (1 ml) of 4-fluoro-3-nitrophenyl azide (218.4 mg, 1.2 mmol). The mixture was acidified with ice-cold 1 *N* HCl and the product was extracted with ether. The ether extract was washed with water and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was dissolved in chloroform (1 ml) and the product sep-

arated by preparative TLC (E. Merck, silica gel F-254, 0.5-mm thickness plates) using the solvent system: benzene–ligroin–ethyl acetate–acetic acid (100:10:14:1, v/v). The first band from the origin corresponded to the unreacted starting material. The second main band (uv absorbing) represented the product. An orange-yellow liquid was obtained in 40% yield after elution of this band with chloroform. The ir spectrum (CCl_4) of the compound showed two strong bands at 2100 (N_3) and 1710 (COOH) cm^{-1}. The NMR spectrum (CCl_4) showed δ 4.28 (broad multiplet, 1 H, *H*COAr), 5.4 (broad multiplet, two olefinic protons), 7–7.4 (three aromatic protons), and 11.2 (broad signal, 1 H, COOH). The signals due to the methyl and methylene protons of the fatty acyl chain were observed in the region δ 0.9–2.3. The compound showed an absorption maximum at 460 nm (ϵ 5000).

12-(4-Azido-2-nitrophenoxy)stearic Acid (*VIII*). This was prepared from 12-hydroxystearic acid following the above procedure.

Synthesis of 12-Oxo-10-octadecenoic Acid (*IX*) (*Figure 1*). Chromic oxide reagent (Jones' reagent) was prepared as follows: CrO_3 (267 mg) was dissolved in 0.23 ml of concentrated H_2SO_4 and 0.77 ml of H_2O. Methyl ricinolate (312.3 mg; 1.1 mmol) was dissolved in 15 ml of acetone, and to the stirred solution was added 0.09 ml of the above chromic oxide solution at 5–10°. The addition was completed in 1 min and the stirring was continued for another 5 min. Nitrogen was bubbled through all the solvents, reagents, and solutions before and during the oxidation reaction in order to prevent hydroperoxide formation. The mixture was diluted with ether; the ethereal solution was washed with aqueous 5% $NaHCO_3$ and water and was dried over anhydrous Na_2SO_4. The solvent was evaporated in vacuo, the yield of the dry residue thus obtained being 290 mg (92%). Thin-layer chromatography of the product in solvents A and B showed a single spot with R_f different from that of 12-hydroxyoleic acid. The ir spectrum (CCl_4) showed bands at 1740 (ester carbonyl) and 1720 (keto carbonyl) cm^{-1} and no band for free hydroxyl group, thus showing the complete conversion of the hydroxy compound to the corresponding keto compound. The NMR spectrum (CCl_4) showed δ 5.46 (m, 2 H, *H*C=C*H*), 3.6 (singlet, 3 H, $COOCH_3$), and 3.06 and 2.97 [2 H, methylene protons α to both double bond and keto group (-C(O)C*H*$_2$CH=CH)].

The above unsaturated compound was isomerized to the conjugated form either by acidic or basic catalysis. (a) A methanolic solution (3 ml) of the above keto ester (50 mg) was heated on a steambath for 5 min with a catalytic amount (50 μl) of 10% methanolic potassium hydroxide in an atmosphere of nitrogen. The reaction mixture was neutralized with dilute acetic acid, evaporated to dryness, and then extracted with ether. The ether extract was washed with water, dried, and evaporated. (b) The keto ester (50 mg) in benzene solution (5 ml) was heated with *p*-toluenesulfonic acid (1 mg) at 80° for 0.5 hr and then washed with sodium bicarbonate (5%) and water. After drying over anhydrous Na_2SO_4, the solvent was removed under reduced pressure. This operation was also carried out in an atmosphere of nitrogen.

Methyl 12-oxo-10-octadecenoate obtained by (a) or (b) above gave the following spectra. The uv spectrum (MeOH) showed an absorption maximum at 218 nm (ϵ 4450) and the ir spectrum (CCl_4) showed a broad band in the region 1735–1695 cm^{-1} due to the overlapping of the bands due to ester and α,β-unsaturated keto groups. The NMR (CCl_4) spectrum showed δ 3.6 (s, 3 H, $COOCH_3$) and 3.22 (s, two olefinic protons; presumably due to the resonance of the conjugated system, the two protons were equivalent and equally shielded). The signal for the olefinic protons is at higher field than is usually the case. Taylor and Fuller (1969) also obtained identical NMR data for this compound. Further, from the characteristics of the ir spectrum, the cis configuration for the α,β-unsaturated ketone is deduced (Table I). This conclusion is consistent with the extensive observations and conclusions of Noack and Jones (1961), but is at variance with the conclusion of Taylor and Fuller (1969).

To reconfirm the structure of the above α,β-unsaturated ketone, the compound was converted to the corresponding dibromo derivative using a procedure selective for the bromination of the double bond only, namely, treatment with 1 equiv of 2% solution of bromine in CCl_4 at 0–5°. The NMR (CCl_4) spectrum of the product after evaporation of the solvent showed no signal at δ 3.22 (due to olefinic protons) but showed two overlapping multiplets in the region δ 4.2–5.0 due to two protons attached to carbon-bearing bromine atoms (Br-CH-CH-Br).

Hydrolysis of the above methyl ester (310 mg, 1 mmol) with methanolic sodium hydroxide (5 ml, 0.1 *N*) overnight under N_2 gave the free 12-oxo-10-octadecenoic acid (IX).

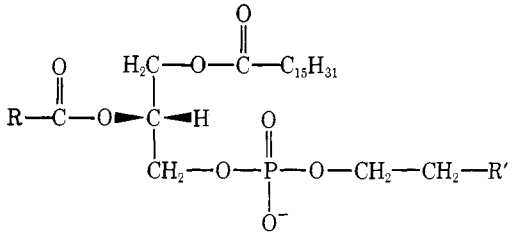
Direct oxidation of ricinoleic acid with Jones' reagent as described above followed by acid- or base-catalyzed isomerization also afforded the α,β-unsaturated keto fatty acid (IX).

12-O-(Ethyl-2-diazomalonyl)stearic Acid (*X*). Ethyl 2-diazomalonyl chloride was prepared from ethyl diazoacetate as follows: A solution of phosgene in Dry Ice–cold benzene (5 ml) was prepared by condensing it through a cold finger (Dry Ice and acetone) and ethyl diazoacetate (570 mg, 5 mmol) was added to this ice-cold solution with stirring. The mixture was allowed to warm up to room temperature and stirring was continued for 2–4 hr until nitrogen evolution ceased. Then, the solvent was distilled off under reduced pressure and the residue in dry benzene (1 ml) was added to an ice-cold solution of 12-hydroxystearic acid (300 mg, 1 mmol) in dry pyridine (3 ml) and the mixture was stirred at 0° for 0.5 hr and then at room temperature for 5 hr. The reaction was terminated by the addition of some ice, and the solution was evaporated to dryness. The residue was extracted with ether; the extract was washed with water and dried over anhydrous Na_2SO_4. After evaporation of the ether solution in vacuo, the product was isolated and purified by preparative TLC using silica gel plates (0.5 mm thick) with the solvent system benzene–ligroin–ethyl acetate–acetic acid (100:10:6:1, v/v). The compound (uv absorbing and the second band from the origin) was eluted with chloroform and evaporated. The yield of the pure product was 30%. The compound showed an absorption maximum at 254 nm (ϵ 5000) in the uv spectrum and at 2125 (-C=N$^+$=N$^-$) and 1710 (COOH) cm^{-1} in the ir spectrum (CCl_4). The NMR spectrum of the compound showed a broad multiplet at δ 4.8 due to a proton attached to carbon bearing diazomalonyl group (*H*-C-O-C(O)-C(N_2)C(O)-OC_2H_5) and a broad signal at δ 11.8 for one proton of the carboxyl group.

12-O-(Ethyl-2-diazomalonyl)oleic Acid (*XI*). This was prepared from 12-hydroxyoleic acid following the procedure described above for the stearic acid analog.

Preparation of 1,8,9-Tribromo-11-acetoxyheptadecane (*XII*) (*Figure 2*). A solution of bromine (640 mg, 4 mmol)

Table III: Synthetic Phosphatidylcholines and Phosphatidylethanolamines.



Phosphatidylcholines	Phosphatidylethanolamines
XV–XIX; R′ = $-\overset{+}{N}(CH_3)_3$	XX–XXIII; R′ = $-\overset{+}{N}H_3$
XV; R = $C_{15}H_{31}$(palmitoyl)	XX; R = $C_{15}H_{31}$ (palmitoyl)
XVI; R = $CH_3(CH_2)_5\overset{O}{\overset{\|}{C}}\overset{H}{\underset{H}{C}}=\overset{H}{\underset{H}{C}}(CH_2)_8-$	XXI; R = $CH_3(CH_2)_5C(N_3)HCH_2\overset{H}{\underset{H}{C}}=\overset{H}{\underset{H}{C}}(CH_2)_7-$
XVII; R = $CH_3(CH_2)_6\underset{N_3}{CH}(CH_2)_9-$	XXII; R = $N_3CH_2(CH_2)_5\overset{H}{\underset{H}{C}}=\overset{H}{\underset{H}{C}}(CH_2)_7-$
XVIII; R = $CH_3(CH_2)_5\underset{N_3}{CH}HCH_2\overset{H}{\underset{H}{C}}=\overset{H}{\underset{H}{C}}(CH_2)_7-$	XXIII; R = $CH_3(CH_2)_5\overset{O}{\overset{\|}{C}}-\overset{H}{\underset{H}{C}}=\overset{H}{\underset{H}{C}}(CH_2)_8-$
XIX; R = $N_3CH_2(CH_2)_5\overset{H}{\underset{H}{C}}=\overset{H}{\underset{H}{C}}(CH_2)_7-$	XXIV; R = $C_{15}H_{31}$; R′ = $-NH-\overset{O}{\overset{\|}{C}}-\underset{N_2}{\overset{O}{\overset{\|}{C}}}-\overset{O}{\overset{\|}{C}}-OC_2H_5$
	XXV; R = $C_{15}H_{31}$; R′ = NH–(p-C_6H_4)–N_3, NO_2

in 1 ml of dry CCl_4 was added slowly to a warm stirred mixture of acetylricinoleic acid (680 mg, 2 mmol) and red mercuric oxide (1.076 mg) in 3 ml of dry CCl_4 and the mixture was heated under reflux for 1 hr (Davis et al., 1965). The mixture was filtered, the residue was washed with 5 ml of CCl_4, and the combined filtrate was washed with 5% NaOH solution followed by water and then dried over anhydrous Na_2SO_4. The product was purified by column chromatography over silicic acid (30 g). Elution with petroleum ether–benzene (1:1) gave the product in 91% yield. The ir spectrum (CCl_4) showed absorption at 1740 cm^{-1} corresponding to an acetoxy group and no free carboxyl group. The NMR spectrum (CCl_4) showed no signals for olefinic proton, δ 4.8 (1 H, br multiplet, *H*-C-OAc), 4.2 (2 H, br multiplet, $-C(Br)_2-CH_2-$), 3.33 (2 H, triplet, $-CH_2$-Br), and 2 (3 H, s, $COOCH_3$).

Preparation of 1-Bromo-11-acetoxy-8-heptadecene (XIII) (Figure 2). A solution of the above tribromide (XII) (500 mg, 0.91 mmol) in dry ether (10 ml) was refluxed with freshly activated Zn powder (400 mg, 6.1 mmol) and glacial acetic acid (0.25 ml) for 10 hr, the reaction mixture was filtered, and the filtrate was washed with sodium bicarbonate (5%) and water and dried over Na_2SO_4. The yield was 300 mg (80%). The NMR (CCl_4) spectrum showed δ 5.33 (2 H, multiplet, $-CH=CH-$) and 3.33 (t, 2 H, $-CH_2Br$), and the signal due to $BrCH$-CHBr had disappeared. Similar result was obtained when HBr (48%, 100 μl) was used in place of acetic acid in the above zinc treatment.

Preparation of the [1-^{14}C]Nitrilo-11-acetoxy-8-heptadecene (XIV) (Figure 2). A solution of the monobromide (0.5 mmol) in dry dimethyl sulfoxide (0.5 ml) was stirred with $Na^{14}CN$ (15 mg, 0.3 mmol, specific activity 53.5 Ci/mol) keeping the mixture in an oil bath at 90°. The reaction was monitored by TLC and was complete in 3 hr. The reaction mixture was then diluted with $CHCl_3$; the solution was washed with saturated NaCl solution and dried over anhydrous Na_2SO_4. The product was purified by preparative TLC using benzene as the solvent. In the NMR spectrum (CCl_4), triplet at δ 3.3 due to $-CH_2Br$ disappeared and a triplet at δ 2.3 (2 H) due to $-CH_2CN$ appeared. Specific activity was found to be 45 Ci/mol.

Preparation of [1-^{14}C]-12-Hydroxyoleic Acid (Ricinoleic Acid) (Figure 2). The above nitrile (XIV) on refluxing with 15% methanolic KOH for 10 hr and subsequent acidification and extraction with ether gave ricinoleic acid identified by TLC and ir with an authentic sample. Radioactively labeled compounds II and IX (Table I) were synthesized from [1-^{14}C]-12-hydroxyoleic acid following the procedure described before. Phosphatidylcholine (XVI) (Table III) was synthesized using the fatty acid (IX) (Table I) following the methods described above.

12-Methoxyoleic Acid. 12-Hydroxyoleic acid (298 mg, 1 mmol) in dry ether (4 ml) was stirred with potassium *tert*-butoxide (246 mg, 2.2 mmol) for 4 hr at room temperature. Stirring was continued for 70 hr at room temperature after addition of methyl iodide (4.56 g, 32.1 mmol). The reaction mixture was acidified with ice-cold hydrochloric acid (0.05 *N*) and the product was extracted with ether. The ethereal extract was washed with water and dried over anhydrous Na_2SO_4. After evaporation of the solvent, a gummy residue was obtained in 93% yield (290 mg). The product, 12-*O*-methyloleic acid, was found to be pure by thin-layer chromatography (R_f in solvent A, 0.48). The ir spectrum (CCl_4) showed absorption at 1710 cm^{-1} (COOH) and no absorption for the free hydroxyl group. The NMR spectrum ($CDCl_3$) showed signals at δ 3.37 (s, 3 H, H-C-OCH_3) and 10.63 (broad, 1 H, COOH).

Synthesis of Modified Phospholipids

Preparation of sn-Glycero-3-phosphorylcholine. Commercially available soya lecithin was subjected to the fol-

lowing purification steps: (1) washing with acetone for removal of accompanying triglycerides, (2) acetylation with acetic anhydride and triethyl- or trimethylamine at pH 9.5 at room temperature for 15 min and extraction of *N*-acetylphosphatidylethanolamine with acetone, (3) deacylation of the acetone-insoluble residue in anhydrous ether and tetramethylammonium hydroxide in methanol (25%) for 2 hr at room temperature, (4) formation of the cadmium chloride complex of the residual glycerophosphorylcholine and several crystallizations of the resulting complex from water–ethanol (1:4, v/v) at 0°, and (5) the removal of cadmium chloride by passing the aqueous solution through a mixed ion exchange resin (Rexyn 1-300, H-OH). The aqueous solution was lyophilized and the residue was dissolved in absolute ethanol and crystallized after addition of ether to the ethanolic solution.

1,2-Dipalmitoyl-sn-glycero-3-phosphorylcholine (XV) (Figure 3). A solution of palmitoyl chloride (4.5 g, 20 mmol) in anhydrous ethanol-free chloroform (10 ml; freshly distilled over P_2O_5) was slowly added to a dry powder of the $CdCl_2$ complex of *sn*-glycero-3-phosphorylcholine (1 mmol), which was kept in ice bath, under dry nitrogen atmosphere. This was followed by the addition of a solution of anhydrous pyridine (2.5 g, 22 mmol) in anhydrous chloroform (6 ml). The mixture was stirred at 0° for 1 hr and then at room temperature for 3 hr. After evaporation of the solvent in vacuo, the residue was dissolved in 20 ml of chloroform–methanol–water (5:4:1, v/v) and passed through a mixed ion exchange resin (Rexyn 1-300, H-OH) to remove cadmium chloride. After evaporation of the solvent, the residue was dissolved in chloroform (20 ml) and passed through a column (1.8 cm dia. × 20 cm) of silicic acid (Bio-Sil-HA). The column was thoroughly washed with chloroform until free from the fatty acid (TLC) and then eluted with a gradient of chloroform–methanol (1:1) to pure methanol. The elution was monitored by TLC. The eluted fractions showing the mobility corresponding to dipalmitoylphosphatidylcholine were combined and evaporated to dryness. The product was crystallized from chloroform–petroleum–ether and was identified as dipalmitoylphosphatidylcholine (XVI) by comparison with an authentic sample (ir and NMR). The overall yield starting with *sn*-glycero-3-phosphorylcholine was 50–60%.

1-Palmitoyl-sn-glycero-3-phosphorylcholine (Figure 3). 1,2-Dipalmitoylphosphatidylcholine (900 mg, 1.2 mmol) was subjected to crude venom treatment as mentioned in the general methods, and 1-palmitoyl-*sn*-glycero-3-phosphorylcholine was obtained in 89% yield (518 mg).

Cadmium Chloride Adduct of 1-Palmitoyl-sn-glycero-3-phosphorylcholine (Figure 3). A solution of $CdCl_2 \cdot 2.5\ H_2O$ (700 mg) in water (0.5 ml) and absolute ethanol (18 ml) was added gradually with stirring to a solution of the above 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (600 mg) in absolute ethanol (50 ml). The mixture was kept at 0° for 30 min, and the precipitate formed was collected by centrifugation, washed twice with ether, and dried in vacuo over P_2O_5 at 40–45°. The weight of the residue corresponded to a 90% yield.

Preparation of Acid Chlorides. (a) The different azido-substituted fatty acids were treated with an excess (about threefold) of freshly distilled thionyl chloride at room temperature under nitrogen. Subsequent removal of thionyl chloride in vacuo gave the acid chlorides which were characterized by the sharp bands in the ir spectrum (CCl_4) at 1800 (COCl) and 2100 cm^{-1} (N_3). The NMR spectra showed the disappearance of the signal due to the carboxyl proton.

(b) 12-Oxo-10-octadecenoyl Chloride. The acid chloride from 12-oxo-10-octadecenoic acid (IX) was prepared by treating with freshly distilled thionyl chloride (two–threefold excess) at 0° for 2.5–3 hr in a nitrogen atmosphere. Removal of the excess of thionyl chloride under reduced pressure at 5° furnished the corresponding acid chloride which was characterized by ir [1800 cm^{-1} (COCl) and 1710 cm^{-1} (C=O)], NMR (no carboxylic proton), and by preparation of methyl ester (NMR, δ 3.68, 3 H, s, $COOCH_3$) on treatment with dry methanol at 0°.

1-Palmitoyl-2-(12-oxo-10-octadecenoyl)-sn-glycero-3-phosphorylcholine (XVI) (Table III). A solution of freshly prepared 12-oxo-10-octadecenoyl chloride (12 mmol) in freshly distilled anhydrous chloroform (7 ml) was slowly added to the chilled powdered $CdCl_2$ adduct of 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (1 mmol) with stirring under nitrogen atmosphere. This was followed by the addition of a solution of dry pyridine (1 ml, about 10 mmol) in dry chloroform. The reaction mixture was stirred at 0° for 1 hr and at room temperature for 3 hr. Isolation and purification of the product were carried out as in the case of the symmetrical 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (XV). The yield was 50%. The ir ($CHCl_3$) spectrum showed a broad band in the region 1735–1700 cm^{-1} (ester and keto groups). R_f value of XVII is given in Table II (solvent E).

1-Palmitoyl-2-(11-azidostearoyl)-sn-glycero-3-phosphorylcholine (XVII) (Table III). Acylation of 1-palmitoyl-*sn*-glycero-3-phosphorylcholine (2-lysolecithin) with 11-azidostearoyl chloride was performed as described above, and it furnished the mixed diacyl product (XVII). The yield was 50%. The ir spectrum ($CHCl_3$) showed a sharp band at 2095 cm^{-1} (N_3) and a broad band at 1740 cm^{-1} (ester groups).

1-Palmitoyl-2-(12-azidooleoyl)-sn-glycero-3-phosphorylcholine (XVIII) (Table III). Acylation of 1-palmitoyl-*sn*-glycero-3-phosphorylcholine with 12-azidooleoyl chloride was performed and the product was purified as described above for XV. The yield was 60%. The R_f value in solvent E is given in Table II. The ir spectrum ($CHCl_3$) showed bands at 2090 (N_3) and 1740 cm^{-1} and the NMR spectrum ($CDCl_3$) showed signals at δ 0.95–1.3 (terminal methyl and methylene groups of fatty acyl chains), 2.3 ($-CH_2CH{=}CHCH_2-$ and CH_2COOR), 5.5 (broad m, $-CH{=}CH-$), 4.0 (broad, methylene group of glycerol backbone and choline moiety), and 3.3 (broad s, $N(CH_3)_3$).

1-Palmitoyl-2-(16-azidopalmitelaidyl)-sn-glycero-3-phosphorylcholine (XIX) (Table III). Acylation of 1-palmitoyl-*sn*-glycero-3-phosphorylcholine with 16-azidopalmitelaidyl chloride was performed and the product was purified as described above. The yield was 50%. The ir spectrum ($CHCl_3$) showed a sharp band at 2100 cm^{-1} (N_3) and a broad band at 1740 cm^{-1} (ester groups). The R_f value is given in Table II (solvent E).

1,2-Dipalmitoyl-sn-glycero-3-(N-tert-butyloxycarbonylaminoethyl) phosphate (Figure 4). A solution of 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine (XX) (Table III) (872.5 mg, 1.26 mmol) in dry chloroform (25 ml) was prepared by heating under reflux and then cooling to room temperature. This was stirred with *tert*-butyloxycarbonyl azide (1.046 g, 7.25 mmol) in the presence of triethylamine (1.27 g, 12.6 mmol) for 96–100 hr at room temperature. After removal of the solvent, the residue was again dis-

solved in chloroform and the solution thoroughly washed with water, the layers being separated by centrifugation. The organic layer was dried and the residue was crystallized from cold methanol. The product showed a single spot in TLC (Table II, solvent E) and gave a negative ninhydrin test. The ir spectrum ($CHCl_3$) showed bands at 1745 cm^{-1} (ester carbonyl) and 1690 cm^{-1} (amide). The NMR spectrum ($CDCl_3$) showed a singlet at 1.48 for the *tert*-butyl group [$C(CH_3)_3$].

1-Palmitoyl-sn-glycero-3-(N-tert-butyloxycarbonylaminoethyl) Phosphate (Figure 4). The N-protected 1,2-dipalmitoylphosphatidylethanolamine (Figure 4) (550 mg, 0.69 mmol) was dissolved in an ether (110 ml) and methanol (0.6 ml) mixture. This was stirred in Tris buffer (pH 9.0) (60 ml of 0.02 *M*), containing calcium chloride (2.4 m*M*) with the crude rattle snake venom (700 μl of a 10-mg/ml solution in 0.2 *M* borate buffer) for 5 hr at 37°. The ether layer was separated from the aqueous layer by centrifugation. A precipitate which formed at the interface due to the calcium salt of palmitic acid was removed by filtration. The aqueous layer, after lyophilization, was thoroughly extracted with chloroform, and the chloroform extract was combined with the residue obtained from evaporation of the ether layer. The chloroform solution was passed through a short column of silicic acid (Bio-Gel-HA) to remove a small amount of calcium salt of palmitic acid still present in the solution. The yield of 1-palmitoyl-*sn*-glycero-3-(*N-tert*-butyloxycarbonylaminoethyl) phosphate was 90%. The R_f value is given in Table II.

1-Palmitoyl-2-(12-azidooleoyl)-sn-glycero-3-(N-tert-butyloxycarbonylaminoethyl) Phosphate (Figure 4). A solution of freshly prepared 12-azidooleoyl chloride (4.9 mmol) in dry freshly distilled chloroform (5 ml) was added to a chilled solution of 271 mg, 0.49 mmol, under nitrogen atmosphere. The mixture was stirred for 1 hr in ice bath, for 6 hr at room temperature under nitrogen. After evaporation of the solvent in vacuo, the residue was taken up in ether; the solution was thoroughly washed with water, dried, and evaporated. The residue was dissolved in benzene and adsorbed on a silicic acid column (1.8 cm dia. × 10 cm). The column was first thoroughly washed with benzene–chloroform (3:1) to remove free fatty acid. Elution of the column with chloroform–methanol (3:1, v/v) gave after evaporation the desired product as a gummy residue (435 mg, yield 80%). This showed only one spot in TLC in solvent E (Table II), and the product was ninhydrin-negative but gave a positive test with phosphate reagent (Molybdenum Blue). The ir spectrum ($CHCl_3$) showed a broad absorption at 3500–3400 (hydroxyl and phosphate), 2090 (N_3) 1740 (ester carbonyl), and 1690 cm^{-1} (amide). The NMR spectrum ($CDCl_3$) showed the following signals: δ 0.9–1.26 (terminal methyl and methylene groups of fatty acyl chain), 5.2 (broad, CHOCO), 3.91–4.4 (broad, CH_2OCO and CH_2OPO), 3.2 (broad, CH_2NHCO), and 1.48 [s, 9 H, $C(CH_3)_3$]. The latter signals were thus due to the glycerol backbone and polar head group.

1-Palmitoyl-2-(12-azidooleoyl)-sn-glycero-3-phosphorylethanolamine (XXI) (Figure 4). The removal of the N-protecting group, *N-tert*-butyloxycarbonyl, from the N-protected phosphatidylethanolamine (0.3 mmol) was accomplished by treatment with anhydrous trifluoroacetic acid (200 μl) as the solvent at 0° for 0.5 hr. The solution was evaporated in vacuo at 0° and the residue was dissolved in chloroform–methanol (2:1 v/v) and passed through a short column of Amberlite IR 45 (OH^-). After evaporation of the solvent, the residue was found to be homogeneous in TLC (Table II, R_f value similar to that of XX). The yield was 80%. The ir spectrum showed bands at 2090 (N_3), 1740 (ester), and 1650 cm^{-1} (CNH_2).

1-Palmitoyl-2-(16-azidopalmitelaidyl-sn-glycero-3-phosphorylethanolamine (XXII) (Table III). Acylation of 1-palmitoyl-*sn*-glycero-3-(*tert*-butyloxycarbonylaminoethyl) phosphate with 16-azidopalmitelaidyl chloride was performed as described above. Subsequent removal of the N-protecting group with CF_3COOH yielded XXI in 85% yield. The ir spectrum showed bands at 2095 (N_3), 1740 (ester), and 1650 cm^{-1} (CNH_2).

1-Palmitoyl-2-(12-oxo-10-octadecenoyl)-sn-glycero-3-phosphorylethanolamine (XXIII) (Table III). Acylation of 1-palmitoyl-*sn*-glycero-3-(*N-tert*-butyloxycarbonylaminoethyl) phosphate with 12-oxo-10-octadecenoyl chloride was performed as described above. Purification of the product by chromatography on silicic acid and subsequent removal of the N-protecting group with CF_3COOH furnished XXIII in 80% yield. The ir spectrum ($CHCl_3$) showed broad bands in the region 1735–1700 cm^{-1} (ester and keto group). The R_f value is given in Table II (solvent E).

1,2-Dipalmitoyl-sn-glycero-3-(N-diazomalonylaminoethyl) Phosphate (XXIV) (Table III). Diazomalonyl chloride was prepared by treating ethyl diazoacetate (171 mg, 1.5 mmol) in dry benzene (0.5 ml) with phosgene (1.26 mmol, 1 ml of 12.5% phosgene in benzene). The reaction mixture was evaporated to dryness and the residue was dissolved in dry chloroform (0.5 ml) and added to a solution of dipalmitoylphosphatidylethanolamine (XX, 300 mg, 0.43 mmol) in dry chloroform (15 ml) containing triethylamine (1.28 mmol). The mixture was stirred at room temperature for 12 hr. Thin-layer chromatography (solvent E) showed complete conversion of XX to a compound with mobility similar to that of 1,2-dipalmitoyl-*sn*-glycero-3-(*N-tert*-butyloxycarbonylaminoethyl) phosphate. The compound gave a negative ninhydrin test, but gave a positive test with Molybdenum Blue. The reaction was terminated by adding some ice. The solvent was evaporated to dryness and residue was dissolved in chloroform; the solution was washed with water and dried. The residue was thoroughly washed with petroleum ether to remove unreacted ethyl diazoacetate and diazomalonic acid. The solid residue contained a single compound by TLC. The ir spectrum ($CHCl_3$) showed bands at 2095 ($C{=}N^+{=}N^-$), 1754 (ester), and 1690 cm^{-1} (amide). The uv spectrum (EtOH) showed absorption maximum at 254 nm (ϵ 4090).

^{14}C-Labeled diazomalonyl derivative (XXIV) of dipalmitoylphosphatidylethanolamine (XX) was synthesized using radioactive phosgene ($[^{14}C]COCl_2$, specific activity 10 Ci/mol) in the following proportions: phosgene (0.1 mmol in 0.1 ml of benzene), ethyl diazoacetate (0.15 mmol in 0.5 ml of benzene), dipalmitoylphosphatidylethanolamine (0.15 mmol in 5 ml of dry chloroform), and triethylamine (50 μl). The product was worked up as described before and purified by preparative TLC (E. Merck, silica gel F-254 0.5 mm thickness) using the solvent $CHCl_3$–MeOH–H_2O (65:35:5, v/v). The area containing the radioactive band was scraped and eluted with chloroform and the solution concentrated. Specific activity was found to be 8.5 Ci/mol.

1,2-Dipalmitoyl-sn-glycero-3-(N-4-azido-3-nitrophenylaminoethyl) Phosphate (XXV) (Table III). 1,2-Dipalmitoylphosphatidylethanolamine (XX) (692 mg, 1 mmol) was dissolved in dry chloroform (25 ml) by warming. The solution was brought to room temperature. After adding trieth-

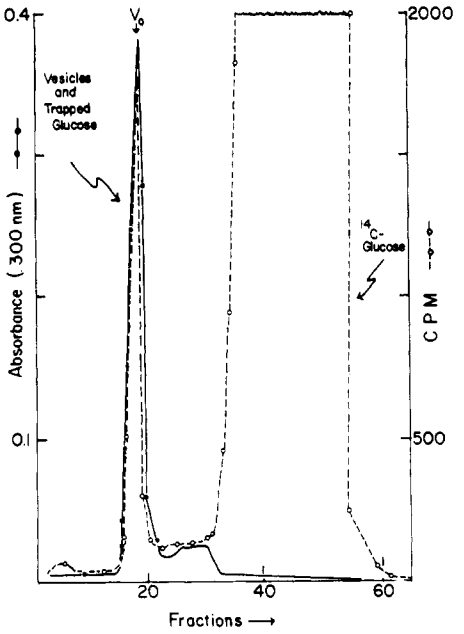


FIGURE 5: Flow-through separation of the sonication product of 1-palmitoyl-2-(12-oxo-10-octadecenoyl)phosphatidylcholine. [^{14}C]Glucose was added prior to sonication. Separation was on a Sephadex G-50 column (29 cm × 1 cm) at a flow rate of 0.23 ml/min. Elution was with 0.15 *M* KCl, fractions being collected every min.

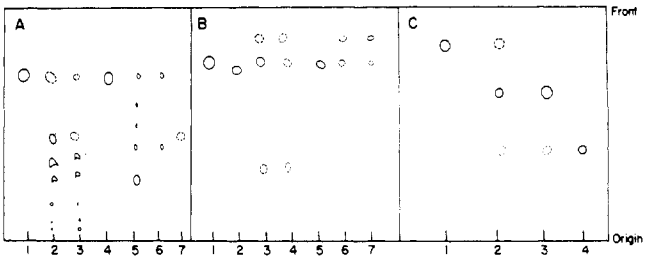


FIGURE 6: Photolysis of mixed acylphosphatidylcholines and phosphatidylethanolamine. (A) Spot 1, marker of 1-palmitoyl-2-(12-oxo-10-octadecenoyl)phosphatidylcholine (XVI); spot 2, the same phospholipid (XVI) after 6 hr of irradiation as described in text; spot 3, after 24 hr; spot 4, marker of 1-palmitoyl-2-(11-azidostearoyl)phosphatidylcholine (XVII); spot 5, the same phospholipid (XVII) after 6 hr of photolysis; spot 6, the same after 24 hr of irradiation; spot 7, 1-palmitoyllysophosphatidylcholine. The solvent for TLC was solvent E. (B) The irradiation products were subjected to methanolysis and the methyl esters of the cross-linked fatty acids were analyzed by TLC using solvent: hexane–diethyl ether–acetic acid (165:30:1, v/v). Spot 1, methyl palmitate as marker; spot 2, methyl 12-oxo-10-octadecenoate (methyl ester of IX); spot 3, methyl esters of cross-linked fatty acids after 6-hr irradiation of XVI; spot 4, same as spot 3, but after 24-hr irradiation; spot 5, methyl azidostearate as marker; spot 6, methyl esters of cross-linked fatty acids after 6-hr irradiation; and spot 7, after 24-hr irradiation of XVII. (C) Photolysis of 1-palmitoyl-2-(12-azidooleoyl)-*sn*-glycero-3-phosphorylethanolamine (XXI). Conditions for photolysis and for TLC of the phospholipid products were as in (A) above. Spot 1, marker of XXI; spot 2, after 30 min of irradiation; spot 3, after 1 hr of irradiation; spot 4, lysophosphatidylethanolamine as marker.

ylamine (202 mg, 2 mmol) and 4-fluoro-3-nitrophenyl azide (182 mg, 1 mmol), the mixture was stirred at 40° for 4 days. The solution was filtered from a small amount of white solid (1,2-dipalmitoylphosphatidylethanolamine). The orange filtrate was evaporated to dryness in vacuo at room temperature. The residue was dissolved in chloroform (5 ml) (20 cm × 1.8 cm) over silicic acid (Bio-Sil-HA). Elution with chloroform yielded 4-fluoro-3-nitrophenyl azide and elution with chloroform–methanol (3:1, v/v) yielded a deep orange product. The product showed a single spot in TLC (Table II, solvent E, R_f 0.9) with Molybdenum Blue reagent and gave a negative ninhydrin test. The ir ($CHCl_3$) spectrum showed a strong absorption at 2120 cm^{-1} (N_3) and a broad absorption at 1730 cm^{-1} (ester).

Vesicles from Synthetic Phosphatidylcholines. A chloroform–methanol (2:1, v/v) solution of the synthetic phosphatidylcholines (30 μmol) was evaporated to dryness under a stream of nitrogen. To the residue was added 0.400 ml of 0.15 *M* KCl, and the mixture, after being flushed with N_2, was sonicated at 35° using an ultrasonic bath type sonicator (80 W, 80 kHz at 3.2 A). After 10 min, when an opalescent to optically clear solution was obtained, the contents was applied to a Sephadex G-50 column (29 cm × 1 cm dia.) and elution was performed with 0.15 *M* KCl, 0.25-ml fractions being collected every minute. Absorbance at 300 nm was recorded as well as phosphorus content and radioactivity when [^{14}C]glucose was also included before sonication. Typical pattern obtained is shown in Figure 5.

An alternative assay of liposome formation was used and this involved retention of the liposomes on Millipore filters (HA 0.45 μ*M*). For this purpose, an aliquot of the sonicated solution (10 μl) to which [^{14}C]glucose had been added prior to sonication was diluted with 2.5 ml of 0.15 *M* KCl and then passed through the filter in a total time of 30 sec–1 min followed by two washes with 5 ml each of the KCl solution. The radioactivity retained on the filter paper was measured. Under the conditions used above for liposome formation, 1.1% of the total radioactivity appeared with the liposomes. Thus, in a typical experiment, of a total of 1,108,700 cpm applied, 12,350 cpm were on the filter paper and 1,093,580 cpm were in the filtrate. In control experiments in which the synthetic lecithins were incubated with [^{14}C]glucose at 35 min without sonication, only 640 cpm (0.056%) were on the filter paper.

Vesicle Preparation from Synthetic Phosphatidylethanolamines. The procedure was similar to that described above, except that Tris-HCl (pH 9) (100 μl of 0.017 *M*) replaced the KCl solution. Sonication was at 30–35° for 5–10 min.

Millipore filter paper assay as in the case of phosphatidylcholine vesicles showed that 1.2% of the total radioactivity was retained on the filter paper. Thus, in a typical experiment, of the 4,160,750 cpm applied, 53,780 cpm were on the filter paper and 4,114,380 cpm were in the filtrate.

Photolysis Experiments. Photoactivation of the vesicles prepared from the synthetic phosphatidylcholines in 0.15 *M* KCl (100 μl) by sonication was carried out in quartz NMR tubes (4 mm internal dia.) placed in a Rayonet photochemical reactor (Model #RPR-100) equipped with 16 symmetrically placed RRR-2530 Å lamps (each 75 W). Photolysis was done in a nitrogen atmosphere for different lengths of time at 35–40°. For analysis, (1) the products were extracted with chloroform–methanol (2:1) and examined by direct TLC, and (2) the products were subjected to methanolysis (tetramethylammonium hydroxide in methanol, 25%) and the resulting methyl esters were extracted in ether and were examined by TLC.

Photolysis of 1-Palmitoyl-2-(12-oxo-10-octadecenoyl)-sn-glycero-3-phosphorylcholine (XVI). The conditions and results are shown in Figure 6A and B. With increasing time, gradual disappearance of the starting phospholipid (Figure 6A) was observed and a number of slower moving products (presumably oligomers) were observed.

After methanolysis (Figure 6B) of the photolysis products, none of the original photosensitive 12-oxo-10-octadecenoate was present, and four new fatty acid ester spots were observed. One spot corresponded to methyl palmitate, and three new products were present.

Characterization of the Photolysis Products. The products obtained in the preceding experiment were characterized by mass spectrometry after separation of the methyl esters by TLC (see Discussion).

Photolysis of 1-Palmitoyl-2-(12-oxo-10-octadecenoyl)-sn-glycero-3-phosphorylcholine in the presence of [^{14}C]dipalmitoyl-sn-glycero-3-phosphorylcholine. Photolysis of vesicles prepared from the modified phosphatidylcholine (XVI) in the presence of increasing amount of [^{14}C]dipalmitoyllecithin ([^{14}C]pamitic acid) was carried out as in the previous experiment. The lipid mixture was subjected to thin-layer chromatography (solvent E) and the thin-layer chromatogram was then scanned for radioactivity (Figure 7). Increasing amount of radioactivity was found to be associated with more polar compounds which were originated due to photolysis of the phospholipid (XVI).

The photolysis product was subjected to methanolysis and then analyzed by TLC as described in Figure 6B. Two radioactive spots were identified, one of them corresponding to methyl palmitate and another one with R_f value 0.25. High-resolution mass spectral analysis of the different components after separation by preparative TLC were carried out.

Photolysis of Liposomes Prepared from 1-Palmitoyl-2-(11-azidooctadecenoyl)-sn-glycero-3-phosphorylcholine (XVII). This was performed exactly as described above using the 253.7-nm lamps. The photolysis products obtained are shown in Figure 6A and B. Ir spectrum of the fatty acid methyl esters obtained showed the total disappearance of the azide band at 2100 cm^{-1}.

Photolysis of 1-Palmitoyl-2-(12-azidooleoyl)-sn-glycero-3-phosphorylethanolamine (XXI). Liposome prepared from the mixed-acid phosphatidylethanolamine (XVIII) in tricine buffer (10 m*M*, 100 μl) by sonication was subjected to photoinactivation (photolysis apparatus, mini-reactor, Model RMR-400, with one centrally placed RPR-2530-Å lamp) for different lengths of time, as mentioned in the legend to Figure 6. The result was analyzed by TLC (Figure 6C). Disappearance of a number of slower moving bands was observed as in the case of phosphatidylcholine analogs.

Discussion

This paper has reported on an approach which, it is hoped, will prove to be of general use for the study of hydrophobic interactions between proteins and phospholipids in biological membranes. The organic groups which provide "preactivation" for cross-linking are built into fatty acids. For in vivo studies, the latter may directly be used for uptake by growing cells, whereas for in vitro studies of specific membrane functions and their reconstitution, the fatty acids are first incorporated into phospholipids.

Some questions of general interest, to which unequivocal answers are as yet not available, are the following. While lateral mobility is an understandably important property of both phospholipids and proteins in membranes, are there, nevertheless, specific associations between membrane proteins and phospholipids which are not disturbed by the fluidity or lateral motion in membranes? Are there functional units in membranes which possess organization at a supramolecular level? Thus, it is logical to consider that in functions such as the phosphotransferase system studied by Kundig and Roseman (1971), energy-coupled transport of amino acids studied extensively by Kaback and coworkers (1974) or the machinery responsible for the chemotactic response in *Escherichia coli* (Larsen et al., 1974; Berg, 1975), the coordinated structures containing phospholipids and multiple proteins are never disturbed during perturbations in the membranes. Indeed, one important aim of the present studies is to be able to develop "nearest neighbor analysis" in membrane components.

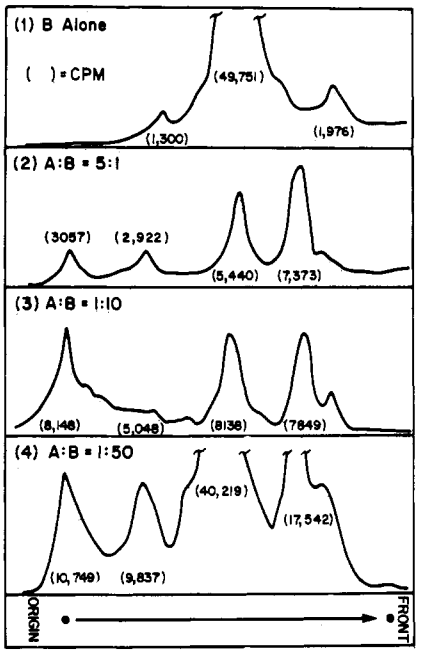


FIGURE 7: Radioactivity scans of the products obtained on photolysis of mixtures in different proportions (see insets) of the mixed acylphosphatidylcholine (XVI) and [^{14}C]dipalmitoylphosphatidylcholine (XV). Details are in text. The ratios of the radioactive and nonradioactive phospholipids are shown in the inset to the different scans.

If specific associations do, in fact, exist between proteins and phospholipids, then is the specificity directed toward the polar head group or to the fatty acid side chains or sometimes to both? In in vitro studies, evidence for polar head group specificity has frequently turned up (Coleman, 1973; Fleischer and Packer, 1974; Kundig and Roseman, 1971), and there are examples of specificity for fatty acid side chains as well (Rothfield and Romeo, 1971; Gazzotti et al., 1974; Grover et al., 1975). Further, the concept of nonexchangeable boundary lipid has also been advanced. Studies of this important question at in vivo level are very desirable. If specificity for polar head groups can be demonstrated, then the question arises: does this specific interaction with the polar head group occur only in areas exterior to the hydrophobic bilayer?

Another major problem which awaits an experimental attack is the manner of folding of, especially, the hydrophobic parts of the membrane proteins and the nature of the specific interactions with fatty acid chains. A part or all of a membrane protein may be designed to "live" in the hydrophobic mileau and clearly the stabilization by interaction with the lipid bilayer is extremely important. The folding and the consequent tertiary structure must obviously be responsible for the interactions since the available information on the amino acid composition of the membrane proteins does not reveal any striking overall abundance of hy-

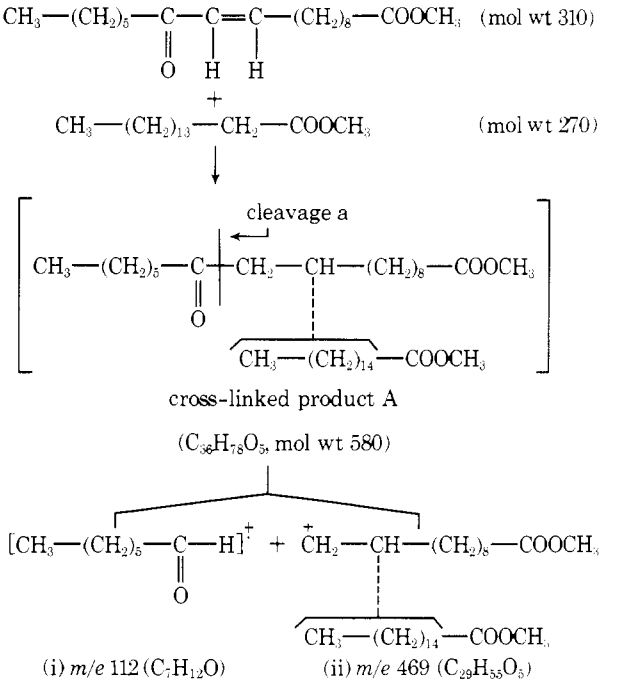
drophobic amino acids. The strong interactions between the integral membrane proteins (Singer, 1971) and the phospholipids is evidenced by the tenacious association of a relatively large amount of phospholipid with the proteins during purification of the membrane proteins and the frequently rapid aggregation or inactivation when attempts are made to remove the phospholipid. The present approach would aim at studies of specific membrane functions by reconstitution from completely phospholipid-free homogeneous membrane proteins and completely defined synthetic phospholipids. Following successful reconstitution, attempts would be made to bring about cross-linking using a variety of phospholipids. Analysis of the sites on the polypeptide backbone where cross-linking occurred should provide a start on understanding the nature of the hydrophobic regions.

In principle, the scope of the present approach is very large. Thus, a variety of activable groups may be used. Secondly, each one of these groups may be placed, one at a time, at every one of the carbon atoms in a fatty acid chain. Thirdly, fatty acids containing such groups may be used in position 1 and/or 2 of the glycerol moiety in the synthetic phospholipids. Thus, the synthetic possibilities for systematic investigation of the hydrophobic contacts between phospholipids and proteins are enormous.

Another promising aspect of the present approach is indicated by the syntheses of the two N-substituted (with photosensitive groups) phosphatidylethanolamines. The use of these may be of interest in exploring the nature of the contacts on the surface of the vesicles in reconstitution systems. It may be recalled that the use of *N*-acetylphosphatidylethanolamine in studies of the reconstitution of ionic pumps gave highly interesting findings (Knowles et al., 1975).

The results of the cross-linking experiments on the phospholipid vesicles are encouraging in that they show that the two fatty acyl chains do in fact approach each other so that cross-linking can occur. The high resolution mass spectral analysis of one (R_f 0.87, Figure 6B) of the methanolysis products of irradiated phosphatidylcholine (XVII) showed the presence of the following ions: (i) *m/e* 112 ($C_7H_{12}O$), (ii) 467 ($C_{29}H_{55}O_4$), (iii) *m/e* 439 ($C_{27}H_{51}O_4$), (iv) *m/e* 495 ($C_{31}H_{59}O_4$) and (v) *m/e* 523 ($C_{33}H_{63}O_4$). The ions (i) and (ii) could be derived by fragmentation from a cross-linked product of higher mass (A) obtained by coupling between α,β-unsaturated keto acid (X) and palmitic acid as shown below. It is well known that in case of fatty acids with branching or substitution like keto, hydroxyl groups, etc., in the chain, molecular ions are not observed in their mass spectra. This was also observed in the present case. The two ion fragments (i) and (ii) were obtained by cleavage of the cross-linked product A next to the keto group (cleavage a). The ion at *m/e* 112 ($C_7H_{12}O$) is the keto group containing fragment with loss of one hydrogen atom and the ion at *m/e* 467 ($C_{29}H_{55}O_4$) is the diester containing fragment.

Perhaps, the most encouraging evidence for the usefulness of the present approach comes from the two lines of in vivo investigation which are to be reported separately. Using an unsaturated fatty acid auxotroph of *E. coli*, it has been demonstrated (Greenberg et al., 1975) that the majority of the azido and α,β-unsaturated keto fatty acids prepared support very well the growth of the auxotroph. The fatty acids used have been shown to be incorporated in the expected 2 position of the glycerol moiety in phospholipids.



Similarly, an unsaturated fatty acid auxotroph of a marine organisim, *pseudomonus BAL 31*, which is the host for an interesting phospholipid bilayer containing DNA virus (PM2) (Tsukagoshi et al., 1975) has been shown to grow on the azido fatty acids. In turn, the virus multiplies well on the auxotroph thus grown. The azido fatty acids have been shown to be incorporated in the 2 position of the phospholipids. A new approach thus appears to be available for structural studies of the viral components.

The in vivo work reported above can be extended to other systems such as yeast, neurospora (Keith et al., 1974), and mammalian cells (Horwitz et al., 1974; Williams et al., 1974; Ferguson et al., 1975). In all of these systems, techniques have recently been developed for the uptake of exogenously supplied fatty acids and for the study of RNA tumor viruses which often contain membranes.

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Poly(8-aminoguanylic acid): Formation of Ordered Self-Structures and Interaction with Poly(cytidylic acid)†

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ABSTRACT: Poly(8-aminoguanylic acid) has in neutral solution a novel ordered structure of high stability. The 8-amino group permits formation of three hydrogen bonds between two residues along the "top", or long axis, of the purines. The usual hydrogen bonding protons and Watson–Crick pairing sites are not involved in the association. The bonding scheme has a twofold rotation axis and is hemiprotonated at N(7). Poly($8NH_2G$) is converted by alkaline titration (pK = 9.7) to a quite different ordered structure, which is the favored form over the range ~pH 10–11. The bonding scheme appears to be composed of a planar, tetrameric array of guanine residues, in which the 8-amino group does not participate in interbase hydrogen bonding. Poly($8NH_2G$) does not interact with poly(C) in neutral solution because of the high stability of the hemiprotonated G·G self-structure. Titration to the alkaline plateau, however, permits ready formation of a two-stranded Watson–Crick helix. In contrast to the monomer $8NH_2GMP$, poly($8NH_2G$) does not form a triple helix with poly(C) under any conditions. The properties of the ordered structures are interpreted in terms of a strong tendency of the 8-amino group to form a third interbase hydrogen bond, when this possibility is not prevented by high pH.

In ordered structures of nucleic acids and polynucleotides the amino groups perform a vital function in forming the hydrogen bonds upon which specificity of pairing depends. We have investigated the role of amino groups in determining the geometry and stability of base-pairing interactions by introducing additional amino groups into the purine rings of both monomers and polynucleotides (Howard et al., 1966a,b; Ikeda et al., 1970; Hattori et al., 1975a). These studies have shown that when a new hydrogen bond can be formed by the introduced amino group there is a marked elevation of the transition temperature of the complex formed by the modified purine (Howard et al., 1966a,b; Ikeda et al., 1970; Hattori et al., 1975a). Conversely, when a new hydrogen bond can be formed in only one of several possible bonding schemes, formation of such a bond can be used to elucidate the geometry of pairing (Ikeda et al., 1970; Hattori et al., 1975a).

We have introduced an 8-amino group into poly(G) and report here the synthesis of the polymer and the effect of this chemical perturbation on the ordered structures which

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