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Chemical Studies of Biological Membranes[1]

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INTRODUCTION

George Kenner and I first met in Vladimir Prelog's laboratory at the Eidgenoessische Technische Hochschule in Zurich in August 1948 where we happened to arrive almost simultaneously from England. George arrived from Cambridge and I arrived after finishing my Ph.D. work in Alexander Robertson's department at Liverpool.[2] We were assigned adjoining benches and we both began to investigate the structure of erythrina alkaloids, work on which had been started there 2 years earlier with unusual vigor by Karl Wiesner. George and I soon became close friends, and I saw a lot of George during that year. I learned an enormous amount from him about practically everything, in particular, about chemistry and even about British chemists. With his innate gifts, his excellent schooling (Manchester Grammar School and The University of Manchester with its excellent School of Chemistry), and the influence of his father (an eminent chemist) and of Professor (later, Lord) Todd, George had had an exceptionally good start. I considered myself exceedingly fortunate to be able to spend a great deal of time with him, both in and out of the laboratory. Prelog, in his typically perceptive manner, immediately recognized and appreciated George's intellectual strengths, and I vividly remember that he would spend unusually long periods of time at George's bench to discuss his own ideas and research, to seek out George's criticisms and advice, and even to exchange views on different scientific personalities of the time.

My own association with George, in addition to meaning so much to me, was soon to assist in bringing about a turning point in my life. On my return to India after the year with Prelog, I was unable to find any employment there and it was George who came to my rescue. He was able to persuade (Lord) Todd to offer me a fellowship out of the Nuffield Grant which had just been awarded to the department for work on ACTH and on the chemistry of peptides. Thus it was that

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[2] George Kenner was later (1957) to succeed Robertson in the same chair.

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I again joined George—this time to help him start peptide research, which was to become his major life-long commitment. George, I am sure, would have approved the material in this article. After a span of about 25 years[3] (*1*) I have again become deeply interested in proteins—this time membrane proteins, in particular. During his last 10 years of research, George's strategy for the total synthesis of lysozyme had involved him in step-by-step construction of protected fragments of increasing chain length representing different portions of the enzyme. These studies had necessitated clean separation of synthetic polypeptide mixtures bearing very hydrophobic protecting groups. New and powerful methods of high resolution were being constantly developed, methods which would resolve the different species in completely deaggregated forms. At Christmastime in 1977, only 6 months before his tragic death, I visited him in order to receive advice on problems of handling hydrophobic proteins. It was stimulating to see George's laboratory at the forefront of methodology in the field—the use of new chaotropic organic solvents, such as trifluoroethanol, and of new column materials, such as hydroxypropylsephacryl for use in gel permeation methods for separation of hydrophobic polypeptides. The efforts described below on membrane proteins and their hydrophobic fragments routinely use the principles of separation that George's laboratory was using.

Biological membranes and cell surfaces form my current research interests. Although in the past the importance of biological membranes has often been grossly underestimated, it has become clear in recent years that membranes hold a central position in a multitude of biological phenomena of great importance, including neural function, cell and nuclear division, biological transport, energy metabolism, and macromolecular synthesis. Indeed, the existence of the plasma membrane and other organelle membranes is a basic foundation of modern cellular biology. Similarly, the surface of a cell is one of its most important components. Through the surface, the cell communicates with the outside environment and other cells. The study of the cell surface plays a major role in essentially all aspects of cell biology, including growth control, hormonal activity, immunology, development, differentiation, and many other processes. For example, in the transformation of the normal cell to a malignant state, critical changes and alterations in cell surface morphology and properties have been demonstrated to occur.

New organobiochemical approaches to the study of biological membranes would clearly be desirable. They would be complementary to those that are available at this time and would, in fact, be necessary in order to obtain deeper insights into membrane structure and function. As a start, attention was, and is, being focused on two different types of problems. In the first, the aim is the study of biologically important cell surface components. In the present work, the lipopolysaccharide of gram-negative bacteria, having a variety of important biological functions, has been investigated. The second area of study aims at developing new approaches to the study of interactions between phospholipids

[3] Except for the interlude with work on the genetic code, which involved studies on protein biosynthesis and on polypeptides formed by synthetic messengers.

and between phospholipids and membrane proteins. The latter, as will be evident below, vary widely in their structures and functions and in the nature of their association with the membrane phospholipid bilayer. While the work on the study of such interactions is only at initial stages, a review of the membrane proteins selected and the status of their chemistry and biochemistry should be of interest.

LIPOPOLYSACCHARIDE, A MAJOR SURFACE COMPONENT OF THE GRAM-NEGATIVE BACTERIA

The unicellular organisms, *Escherichia coli, Salmonella,* and related enteric bacteria, are bounded by a complex "envelope" which consists of three distinct layers with very different chemical structures and functions. The innermost layer enclosing the cytoplasm contains the cytoplasmic membrane. This is responsible for active transport and other energy-dependent processes and contains the bulk of "membrane-bound" biosynthetic enzymes. The next outer layer is that of the rigid cell wall containing the peptidoglycan polymer. The outermost layer is that of the outer membrane. This membrane contains phospholipid, some major proteins, and a large number of minor proteins, which serve as receptor proteins. A prominent component of this membrane is lipopolysaccharide (LPS) which possesses a very large number of diverse biological functions. Structural investigation, especially of LPS from *Salmonella, Escherichia coli,* and related bacteria, has shown them to consist of three distinct regions (*2, 3*) (Fig. 1).[4] The outermost region consists of a polysaccharide chain with repeating oligosaccharide units of variable length and constitutes the O-antigen region. The latter is connected to the outer core polysaccharide chain, which is relatively invariant within certain classes of bacteria. The outer core polysaccharide is, in turn, linked to the "backbone" polysaccharide containing a unique eight-carbon sugar, 3-deoxy-D-mannooctulosonate (KDO). The latter is connected to a complex hydrophobic component, called lipid A (Fig. 1). Anchored in the outer membrane, lipid A is a derivative of D-glucosamine disaccharide and thus differs greatly in structure from the ubiquitous glycerophospholipids. Genetic blocks at different stages in the stepwise biosynthesis of core lipopolysaccharide can result in the conversion of the wild-type (smooth) bacterial strains to rough mutants which lack the O-antigen and a part or all of the outer core or to deep rough mutants which lack, in addition, a part of the backbone.

Each of the three regions in LPS is endowed with multiple but specific biological functions (*3, 4*). The O-antigen, the determinant of the antigenic specificity of the organism, also functions as a receptor for bacteriophages. The core region, which can also function as a bacteriophage receptor, plays a role in maintaining the integrity of the outer membrane. Thus, in deep rough mutants

[4] Compounds or structures in the figures are numbered independently of other figures. Thus, references to, or discussions of, the structures in different figures are in the context of the specific figures.

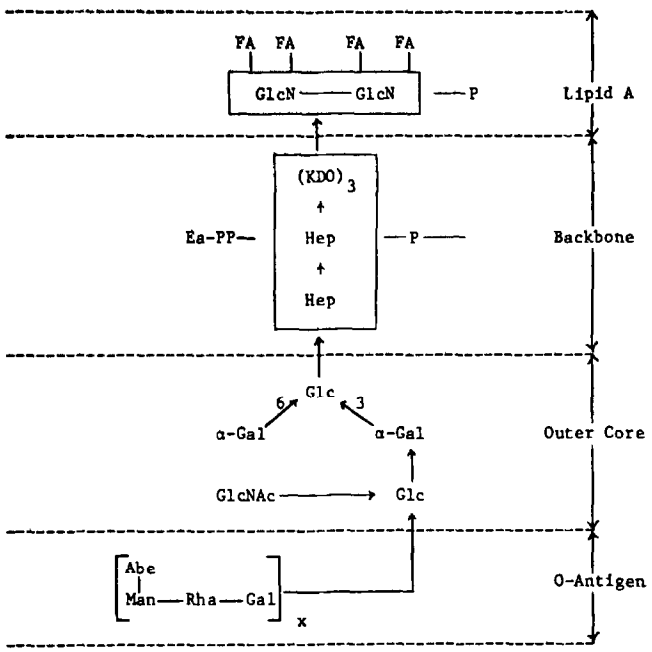


FIG. 1. General structure of the lipopolysaccharide of *Salmonella typhimurium*. Abbreviations: FA, fatty acid; GlcN, glucosaminyl; (KDO), 2-keto-3-deoxyoctonyl-(3-deoxy-D-mannooctulosonyl); Hep, L-glycero-D-mannoheptosyl; EaPP, pyrophosphorylethanolamine; Glc, glucosyl; Gal, galactosyl; GlcNAc, *N*-acetylglucosaminyl; Rha, L-rhamnosyl; Man, mannosyl; Abe, abequosyl(3,6-dideoxy-D-galactose). All sugars of the the D-configuration. As shown, there are four distinct parts of the highly complex structure: *Lipid A*, which is shown very schematically and is mainly discussed in this article, is linked to the *backbone* polysaccharide. The latter is linked to the *outer core* polysaccharide which is linked, in turn, to the *O-antigen* which may consist of 15 or more repeats of a tri- or tetrasaccharide structure.

which lack most of the core oligosaccharides, there is observed an increase in permeability to hydrophobic compounds concomitant with a decrease in protein content of the outer membrane (*5–7*). A similar effect is observed following the partial release of LPS from the membrane on treatment with the chelating agent, EDTA (*8*). Lipid A is the primary agent responsible for the endotoxicity of gram-negative bacteria and displays mitogenic stimulation, complement activation, and pyrogenic induction, in addition to a number of other biological functions (*3*). Lipid A as well as KDO appears to be indispensable to the outer membrane of gram-negative bacteria. Thus, although mutants lacking the O-antigen and core region can be readily isolated, no bacterial mutant lacking lipid A has yet been identified. The only mutants defective in the synthesis of KDO that have been observed are of the temperature-sensitive type (*9–11*).

Elucidation of the Structure of Lipid A in a Heptose-less Mutant of E. coli K-12

While the structures of the core and O-antigen polysaccharide regions have been elucidated in a number of bacterial strains, including *Salmonella* and *E. coli*

(*3*), detailed structures of the lipid A component have until recently remained unclear. Mainly through the efforts of Westphal, Lüderitz, and their colleagues, lipid A of *Salmonella typhimurium* is known to be composed of a D-glucosamine disaccharide backbone which carries up to six long-chain fatty acyl residues, two phosphate residues, and, occasionally, 4-aminoarabinose and phosphorylethanolamine (*12*). Until recently, LPS was believed to be an oligomer consisting of D-glucosamine disaccharide subunits interlinked by means of phosphate groups. Both pyrophosphate (*12*) and phosphodiester bonds (*13*) have been postulated at one time or another to be present in *Salmonella* LPS. Very recently, from a ^{31}P nmr study (*14*), *Salmonella* lipid A was concluded to be only a monomer of glucosamine disaccharide containing monoesterified phosphate groups; in some strains the latter groups were found to be derivatized to form phosphodiester and/or pyrophosphate linkages but not resulting in oligomers of the glucosamine disaccharide.

Because of the above conflicting results on the structure of lipid A, its many important biological functions, and its interactions with cell surfaces, the principal aim of our work has been to clarify the structure of lipid A. An *E. coli* K-12 deep rough mutant, isolated and studied by Boman and co-workers (*15*), was chosen as the source of lipopolysaccharide. This mutant is unable to add any of the sugar residues beyond the first unit, an exotic sugar, KDO, to the lipid A portion. Consequently, the molecule is largely hydrophobic, and it was considered that this feature would facilitate structural studies on lipid A. Three distinct types of studies were pursued. The first approach, investigated by Drs. Marsha Rosner, Israel Barsilay, and Jiunn-Yann Tang, involved stepwise chemical degradation and characterization of the products (*16*). In the second approach, the chemical nature of the phosphate groups in LPS and its degradation products was studied by Drs. Marsha Rosner and Arnold Satterthwait using ^{31}P nmr spectroscopy (*17*). Finally, the use of enzymes for specific cleavages of the molecule was investigated. This approach has played an important role in the past in the structural elucidation of complex molecules of biological interest. In the present work, carried out by Dr. Rosner and Mr. Reynold Verret, use of the slime mold, *Dictyostelium discoideum*, which is a natural scavenger of *E. coli*, resulted in the discoveries of two highly interesting long-chain fatty acyl amidases (*18*).

Stating the structural conclusion of the present studies at the outset, the *E. coli* K-12 mutant contains two species of LPS designated [LPS]-I and [LPS]-II (Fig. 2). Both are derivatives of a single glucosamine disaccharide unit but differ, in the main, in the nature of the phosphate groups present at the glycosyl positions. Thus, [LPS]-I contains two phosphomonoester groups, one at the 4′-hydroxyl group and one at the glycosyl position. [LPS]-II also contains a phosphomonoester group at the 4′-hydroxyl group, but carries a pyrophosphate group at the glycosyl position. The glucosamine amino groups in both LPS species are acylated uniquely by β-hydroxymyristoyl groups, while the hydroxyl groups were esterified by lauryl, myristoyl, and β-hydroxymyristoyl groups. It has also been determined that the extent of acylation of the different hydroxyl groups in lipid A can vary (*16*). The exact assignment of different fatty acyl groups to specific hydroxyl groups in the glucosamine rings has not been carried out. Both types of

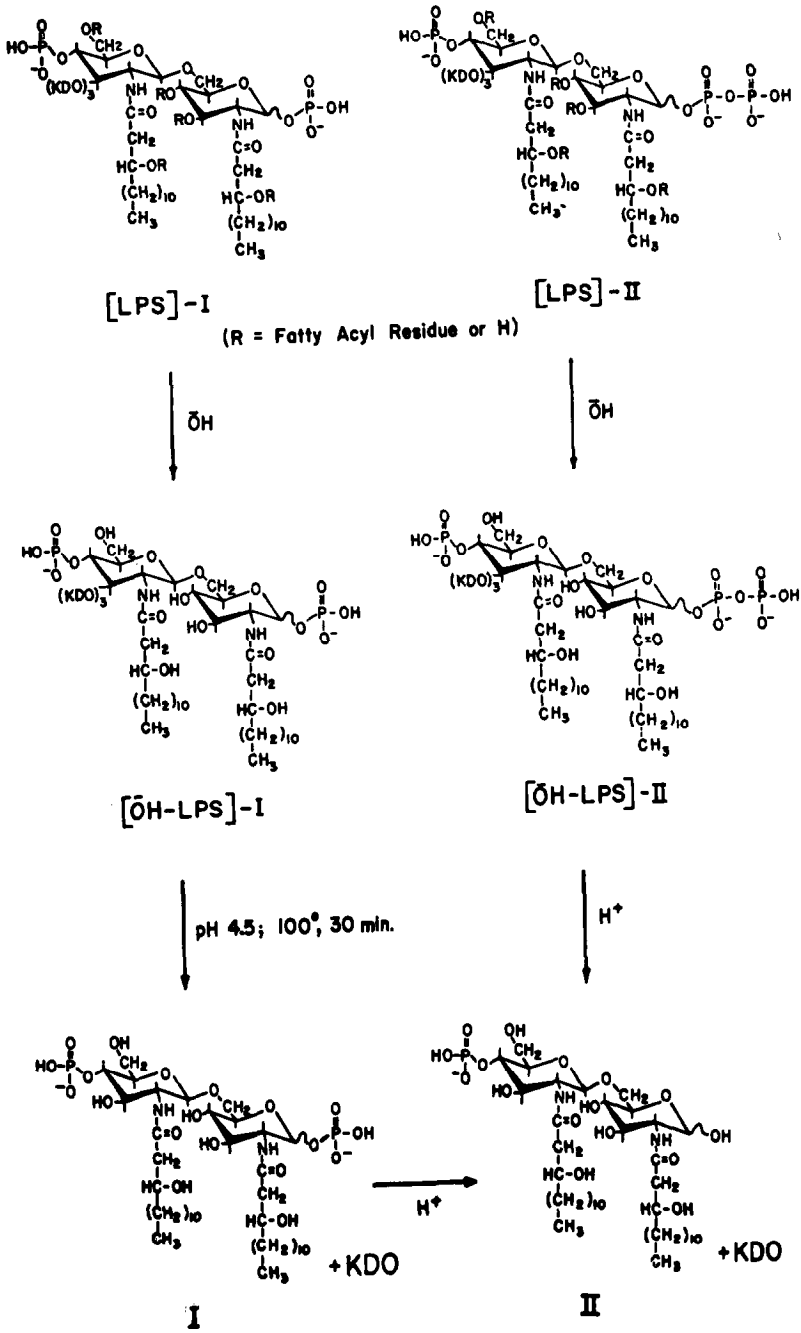


FIG. 2. Structure of the two species of lipopolysaccharide [LPS]-I and [LPS]-II identified in *E. coli* D31m4 and the products obtained from them on successive treatments with alkali and acid. R stands for a long-chain fatty acyl residue or simply H. The acyl groups are myristic acid, lauric acid, and β-hydroxymyristic acid. On alkaline treatment, [LPS]-I gives [ŌH-LPS]-I, while [LPS]-II gives [ŌH-LPS]-II. Treatment of [ŌH-LPS]-I at pH 4.5 and 100°C yields Compound I, which can be converted to II on 0.1 *N* HCl treatment. Compound II is also obtained from [ŌH-LPS]-II on 0.1 *N* HCl treatment.

LPS carry the KDO groups. However, the most important conclusion is that lipid A, in the mutant used in the present work, contains monosubstituted monophosphate and pyrophosphate groups only and there is no evidence for the presence of any oligomeric or polymeric structures containing glucosamine disacchride units linked covalently to one another.

Chemical Studies

Treatment of the total LPS with alkali at room temperature gave products which were concluded to be [ŌH-LPS]-I and [ŌH-LPS]-II as shown in Fig. 2. Thus, in the conversion of [LPS]-I to [ŌH-LPS]-I and of [LPS]-II to [ŌH-LPS]-II, only base-catalyzed removal of the fatty acyl groups was concluded to occur. If, as was previously believed, pyrophosphate or phosphate diester bonds were to be present in LPS, then there would have been the possibility of their cleavage by neighboring hydroxyl group participation. The consequence would be the formation of phosphomonoester groups accompanied by phosphoryl group migration. Pyrophosphate and phosphate diester bond hydrolyses by neighboring hydroxyl group participations in naturally occurring phosphate esters have been well established for some time (*19*). Indeed, this possibility was examined in great detail. In one experiment, [ŌH-LPS] was given a mild acidic treatment to free the 3′-hydroxyl group for possible neighboring group participation and was again treated with alkali under various conditions. An increase in the phosphomonoester groups should thus have resulted and fragmentation of the molecule would have been observed. None of this was actually observed. It should also be noted that during the alkaline treatment of LPS, no significant release of inorganic phosphate was observed, indicating that [ŌH-LPS]-I was not derived from [ŌH-LPS]-II by cleavage of the pyrophosphate group. The result is also consistent with the absence of a free neighboring group at the 2 position adjacent to the glycosyl pyrophosphate bond.

Following alkaline treatment of LPS, a very mild acidic (pH 4.5) treatment was given which removed, essentially selectively, the KDO groups (Fig. 2). From the existing knowledge of the hydrolytic behavior of different types of phosphate esters, neither pyrophosphate nor phosphomonoester groups would be expected to migrate or hydrolyze under the conditions used in this step (*19*). Phosphate groups located at glycosyl positions could undergo some hydrolysis and this was observed. The main product thus obtained from [ŌH-LPS]-I was Compound I (Fig. 2).

Controlled acidic treatment can also be used to selectively hydrolyze the acid-labile phosphate groups located at glycosyl bonds without affecting those present at any of the other hydroxyl groups in the hexopyranose rings. In this way, Compound II (Fig. 2) was produced as the sole product from both [ŌH-LPS]-I and [ŌH-LPS]-II, the KDO groups being removed simultaneously (see also below). Further, cleavage of the Cl phosphate in Compound I occurred concomitantly with the generation of a free reducing group. Treatment of [ŌH-LPS]-II at pH 4.5 also partially generated inorganic pyrophosphate and Compound II. The result of the hydrolytic experiment showed that two-thirds of the total phosphate in [ŌH-LPS]-

II was at the glycosidic position. The presence of the glycosyl pyrophosphate in [ŌH-LPS]-II was also shown by nmr studies reviewed below (*14*).

Several further lines of evidence indicated that LPS contains only a single disaccharide unit. The ratio of phosphate to glucosamine in Compound I was 1 : 1. Since both phosphate groups were monoesters and the ratio of reducible to nonreducible glucosamine residues was also 1 : 1, a disaccharide structure is compatible with the data (see also below).

Of the several alternative modes of degradation and characterization used in the identification of Compound I, those illustrated in Figs. 3 and 4 are reviewed here. The intermediates and the accompanying products shown in Fig. 3 were completely characterized. Thus, treatment with the bacterial alkaline phosphatase hydrolyzed 50% of the total phosphorus and gave Compound III. The latter on mild acidic treatment released all of the remaining phosphorus (from the rate of release, this must be present as glycosyl phosphate) and the organic product formed was Compound IV. Alternatively, the sequence of degradation of Compound I was reversed and Compound IV was obtained via Compound II. (As described below, enzymatic degradation made it possible to complete mild and selective degradation down to completely unacylated glucosamine disaccharide.)

The degradation described in Fig. 4 used Moffatt oxidation of Compound I to produce, along with the keto groups, the aldehyde in β-position (6′) to the 4′-phosphate group. Subsequent base treatment brought about β-elimination of the phosphate groups and more than 50% of the phosphorus was released as inorganic phosphate.

The Use of ^{31}P nmr Spectroscopy

^{31}P nmr spectroscopy, a physical method which has been used with great advantage in the study of variously substituted organic phosphate esters, was applied in the present investigation of the LPS structure. The spectra and a detailed account have been given elsewhere; here, it is sufficient to state the main conclusions.

LPS. The spectrum of the mixture of the two LPS species ([LPS]-I, [LPS]-II, Fig. 2) showed both phosphomonoester and monoesterified pyrophosphate residues. However, no signals corresponding to phosphodiester or P^1, P^2-diesterified pyrophosphate groups were present, thus ruling out the possibility of oligomer formation between disaccharide units through phosphate or pyrophosphate bridges.

[ŌH-LPS]-I. [ŌH-LPS]-I showed at pH 8.7 two singlets of equal intensity that resembled the spectrum of Compound I, a derivative of [ŌH-LPS]-I (see below). The spectrometric titration curves for the singlets at -3.93 and -2.48 ppm (relative to 85% phosphoric acid) yielded pK_a values of 6.76 and 7.38, respectively. These parameters correspond to the pK_a values obtained for Compound I. This result again demonstrates that both signals arise from phosphomonoester residues in [ŌH-LPS]-I. The singlet at -2.48 ppm was identified as the glycosidic phosphate resonance by analogy to the corresponding signal in the spectrum of

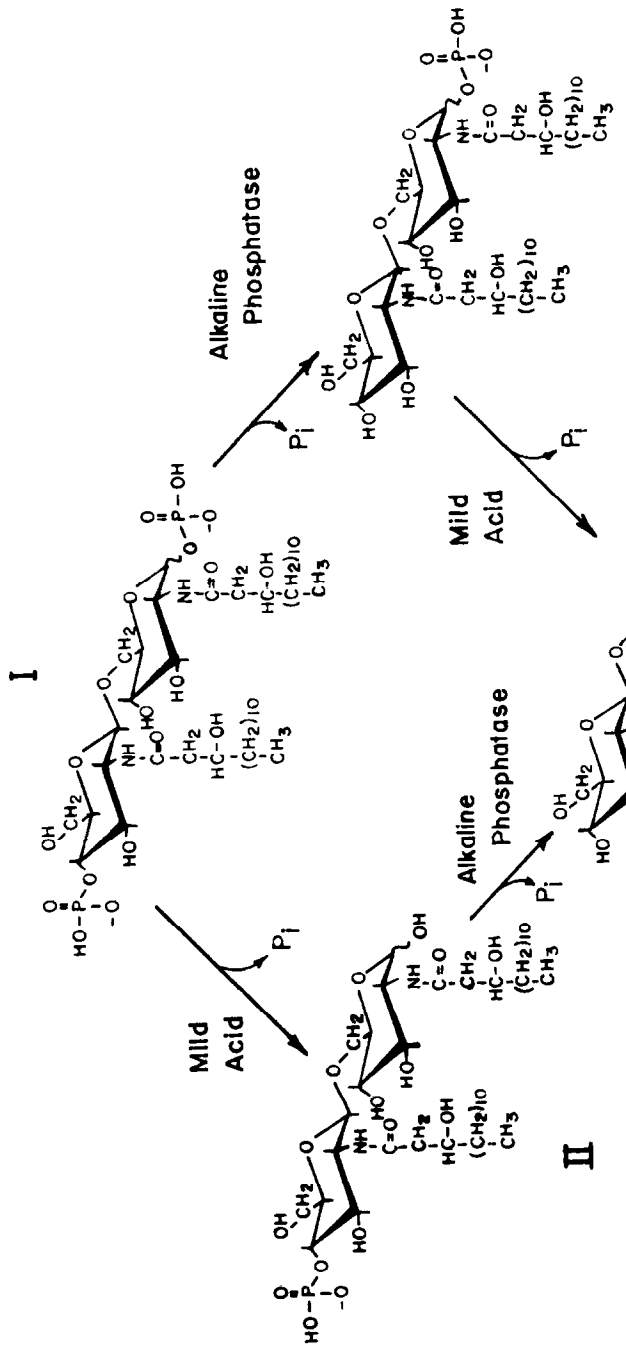


FIG. 3. Summary of chemical and enzymatic degradations of Compound I.

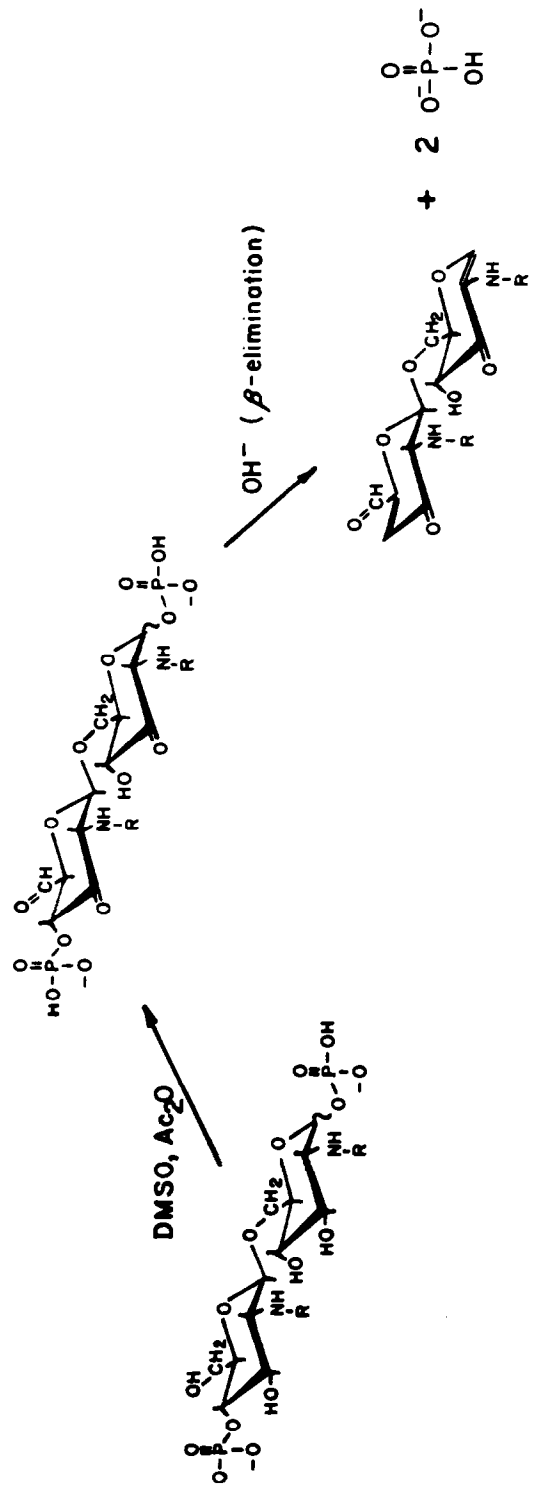


FIG. 4. Scheme depicting oxidation of the appropriate hydroxyl groups and base-catalyzed elimination of the phosphate groups in Compound I.

Compound I. The singlet at −3.93 ppm, however, was shifted 0.20 ppm upfield from the nonglycosidic phosphate resonance in Compound I. In addition, the proton–phosphorus coupling constant ($J_{H-P} \leq 3$ Hz) was lower than that of the nonglycosidic phosphate residue in Compound I (see below). The difference must be attributed to the fact that [ŌH-LPS]-I contains three KDO residues next to 4′-phosphate while Compound I does not.

[ŌH-LPS]-II. The ^{31}P nmr spectrum of [ŌH-LPS]-II at pH 8.7 showed a singlet at −3.93 ppm and two doublets centered at 5.03 and 11.74 ppm ($J_{P-P} = 2$ Hz). Integration of the spectrum showed that the phosphate groups giving rise to the singlet and each of the doublets were present in an equimolar ratio (*17*). The signal at −3.93 ppm appeared to be in a position identical to that for the nonglycosidic phosphomonoester group in [ŌH-LPS]-I. This was confirmed directly when the ^{31}Pnmr spectrum of a mixture of [ŌH-LPS]-I and [ŌH-LPS]-II was examined. Further, the proton–phosphorus coupling constant ($J_{H-P} \leq 3$ Hz) and pK_a of 6.76 obtained for the phosphomonoester resonance in [ŌH-LPS]-II were identical to those of the nonglycosidic phosphate group in [ŌH-LPS]-I. The doublets at 5.03 and 11.34 ppm were identified unambiguously as the resonances of a pyrophosphate monoester group on the basis of chemical shifts, pK_a, and coupling constants. A comparison of chemical shifts and phosphorus–phosphorus coupling constants for ADP, UDP-glucose, and [ŌH-LPS]-II (*17*) strongly indicated that [ŌH-LPS]-II contained a monoesterified pyrophosphate residue. The pK_a of the group was 7.3, indicating a monoester linkage. Further, the changes in chemical shift of the doublets upon titration mirrored those observed for a model pyrophosphate monoester, ADP. Thus, the lower-field doublet (5.0 ppm at pH 9.2) moved 5.1 ppm upfield with decreasing pH as expected for conversion to a monoanion, identifying it as the resonance of the β-phosphate. In contrast, the α-phosphate doublet (11.7 ppm at pH 9.2) showed only a small change in chemical shift reflecting protonation of the terminal phosphate dianion. These assignments were confirmed by proton–phosphorus coupling data. In the proton-coupled ^{31}P nmr spectrum of [ŌH-LPS]-II, the doublet at +11.34 ppm (α-phosphate) was split into a doublet of a doublet, indicating coupling to a single proton with $J_{H-P} = 8$ Hz; no proton coupling to the β-phosphate was observed.

Since two-thirds of the total phosphate groups in [ŌH-LPS]-II were shown to be acid labile (see above), the pyrophosphate monoester residue must be in a glycosidic linkage. The coupling data presented above are consistent with the conclusion from the hydrolytic studies. The nmr data further support the conclusion that [ŌH-LPS]-I and [ŌH-LPS]-II differ only in having a phosphoryl or pyrophosphoryl group at the glycosidic position.

Compounds I and II. The ^{31}P nmr spectrum of Compound I, taken at pH 8.7, showed two singlets with chemical shifts of −4.48 and −2.34 ppm. Integration of the resonances indicated that the two types of phosphate groups are present in a 1 : 1 molar ratio.

The two singlets were identified as resonances from phosphomonoester groups. Thus, spectrometric titration curves for these phosphoryl groups yielded pK_a values of 6.80 and 7.24, respectively. The observed change in chemical shift of approximately 4 ppm upon change in ionization is characteristic of phosphate

monoester groups. Diesters of phosphoric acid or P^1,P^2-disubstituted pyrophosphates would not ionize in this pH range (*20*).

The ^{31}P nmr spectrum of Compound II, the product of further acidic treatment of Compound I, showed a single resonance with a chemical shift of −4.48 ppm at pH 8.7. Since the glycosidic phosphate group in Compound I is lost during the formation of Compound II (Fig. 2), this signal must arise from the nonglycosidic phosphomonoester. Therefore, the singlet at higher field (−2.34 ppm) must be the signal from the glycosidic phosphate group. A comparison of the above chemical shifts with those for model compounds supports the assignments. In particular, the chemical shift for a nonglycosidic phosphate, e.g., −4.50 ppm in D-glucose 6-phosphate, and that for a glycosyl phosphate, e.g., −2.36 ppm for α-D-glucose 1-phosphate, were identical with the corresponding shifts observed for Compound I.

The position of the nonglycosidic phosphate residue was concluded to be C4 using both chemical and proton–phosphorus coupling data. In the proton-coupled ^{31}P nmr spectrum of Compound I, both phosphorus signals were split into doublets with a coupling constant J_{H-P} of 7 Hz, indicating that each phosphorus nucleus is coupled to a single proton. This result is consistent with one phosphomonoester group being located on the glycosyl position (C1) and the second phosphate being limited to positions C2, C3, or C4. The chemical studies reviewed above showed that the nonglycosidic phosphate could only be at positions C4 or C6. Since a phosphorus at C6 would be coupled to two protons, giving a triplet, the nonglycosidic phosphate residue must be attached to C4.

Two Long-Chain Fatty Acyl Amidases and Specific Cleavages of β-Hydroxymyristoyl Residues in LPS

At the outset of this investigation, it seemed worthwhile to make a search for enzymes that might lead to specific and structurally useful cleavages of the LPS molecule. The source selected was the slime mold, *Dictyostelium discoideum*, which is a natural scavenger of *Escherichia coli*. Although reports on the presence of fatty acid esterase and amidase activities in this mold had previously appeared (*21–23*), significant amidase activity had not been demonstrated nor was anything known on the mode of action of any of the enzymes detected. In our own experiments, the presence of esterases, amidases, and phosphatases in extracts of *D. discoideum* was readily demonstrated. However, attention was focused on the isolation and characterization of the amidases, the use of which clearly was complementary to the studies described above and which, therefore, would be useful in completing the structural work.

Briefly, two amidases have now been purified from the slime mold, and their action on LPS derivatives, especially Compound I, has been studied. Amidase I acts on Compound I (Fig. 5) so as to cleave the β-hydroxymyristoyl group at the glycosyl phosphate end of the molecule. The product thus formed, Compound V, then serves as a substrate for Amidase II, which removes the β-hydroxymyristoyl group at the distal end of the molecule yielding Compound VI (Fig. 5). The two resulting compounds, containing one or both free amino groups, apart from their

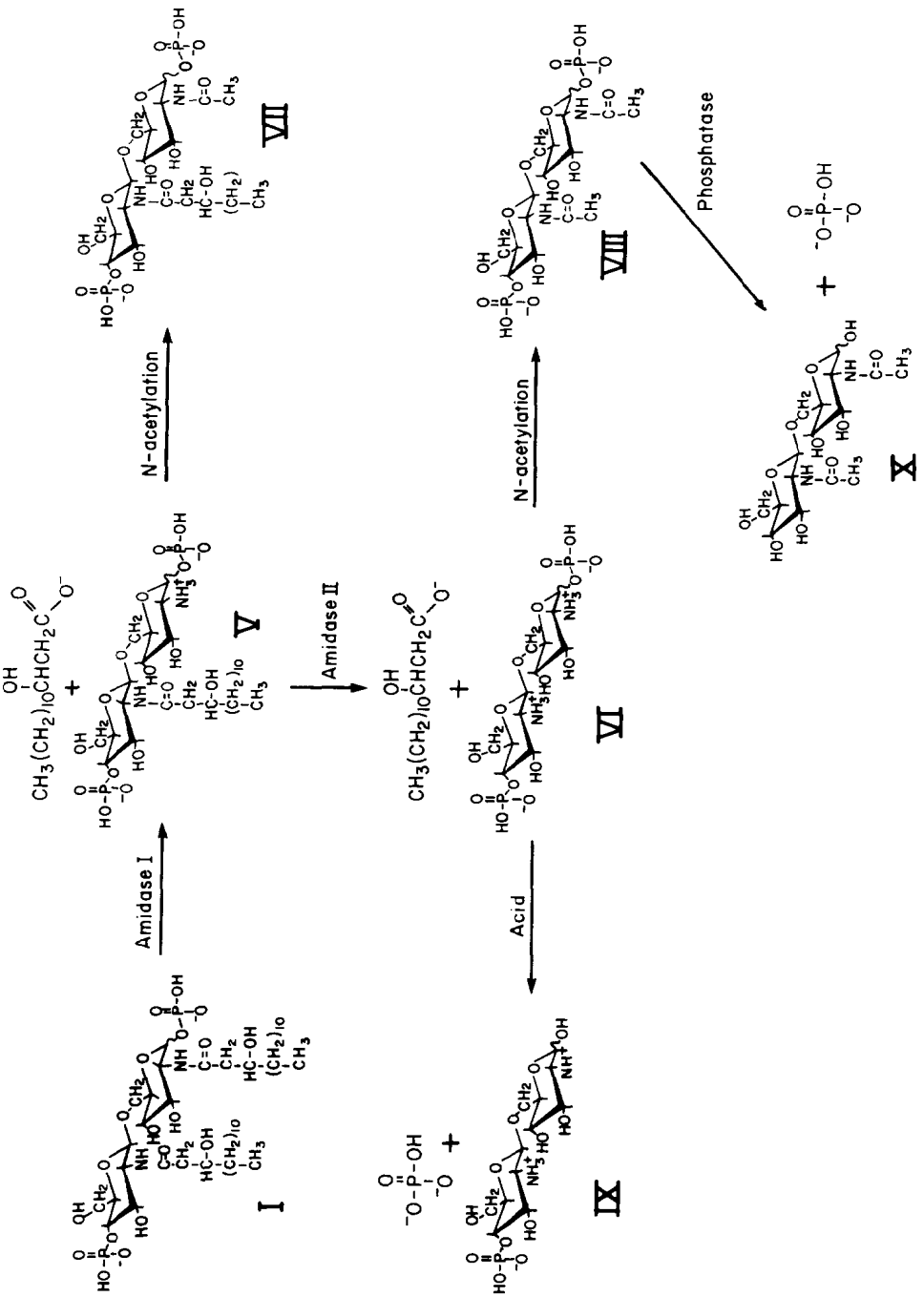


FIG. 5. Successive degradation of Compound I by Amidase I and Amidase II. Characterization of products, V and VI, by N-acetylation and acidic and phosphatase treatment.

value in concluding the structural work on the LPS, promise to be very useful for further work in studies of structure–function relationships. The role of the fatty acids in the various biological activities of lipid A and LPS has remained unclear. The reactions now described provide opportunities for the specific alteration of the ester or the amide groups for biological studies.

Stepwise Action of the Amidases and Conclusion of the Structural Problem

Amidase I. Treatment of ^{32}P-labeled Compound I with Amidase I gives quantitatively a single new ^{32}P-labeled product (V). None of the phosphate groups are lost. Further, the use of Compound I labeled in the β-hydroxymyristoyl groups with ^{14}C (*18*) in this experiment showed that about 50% of the ^{14}C radioactivity was released as β-hydroxymyristic acid. To show that the free amino group produced was adjacent to the glycosyl phosphate group (V), deamination with nitrous acid was performed. Inorganic [^{32}P]phosphate was indeed produced as a new product. The release of inorganic phosphate must be as a result of the transformation of the hexose ring to the furanose ring following breakdown of the diazonium salt (*18*). Further support for the location of the free amino group at C2 adjacent to the glycosyl phosphate in Compound V was forthcoming from the rate of acidic hydrolysis of [^{32}P]Compound V. The presence of the free amino group at C2 is known to stabilize dramatically the glycosyl phosphate linkage to acidic hydrolysis (*24*). This was indeed found to be the case. Thus, under the conditions necessary for the complete hydrolysis of Compound I, less than 15% inorganic phosphate was released from Compound V. However, after *N*-acetylation of [^{32}P]-Compound V, the kinetics of hydrolysis were typical for a glycosyl phosphate bond.

Action of Amidase II on Compound V. No degradation was observed when Compound I was incubated with Amidase II. On the other hand, treatment of Compound V with this enzyme resulted in degradation. Again, a single ^{32}P-labeled product (Compound VI, Fig. 5) was produced from [^{32}P]Compound V. Treatment of [^{14}C]Compound V, labeled with ^{14}C in the fatty acids, with Amidase II released most of the radioactivity as β-hydroxymyristic acid, no radioactivity being found in Compound VI, the second product formed.

Evidence that Compound VI contains two amino groups was shown in a variety of ways. These included mobility on electrophoresis at selected pH values, both before and after acidic hydrolysis. Further characterization in regard to the nature of the glycosidic linkage was carried out by the sequence of the reactions shown in Fig. 5 (**VI** → **VIII** → **X**). The behavior of the resulting di-*N*-acetyl disaccharide was compared with authentic samples of β-(1 → 4) and β-(1 → 6)-di-*N*-acetyl-D-glucosamine disaccharides by tlc on silica gel. In the three solvents tried, the ^{14}C-labeled product comigrated with the marker β-(1 → 6) disaccharide and was different from the sample of the (1 → 4) compound.

The anomeric configuration of the glycosyl linkage in the glucosamine disaccharide. The anomeric configuration was determined by the use of β-*N*-acetylglucosaminidase. Conditions were determined first for the complete cleavage of an authentic sample of β-(1 → 6)-di-*N*-acetyl-D-glucosamine disaccharide by the

enzyme. Under these conditions, the ^{14}C-labeled glucosamine disaccharide isolated as described above was cleaved completely to *N*-acetylglucosamine. The result indicates that the disaccharide in the *E. coli* lipid A is a β-(1 $\rightarrow$ 6)-linked D-glucosamine disaccharide. Thus, the use of the amidases followed by well-established reactions enabled the completion of the structural work. The structures of [LPS]-I and [LPS]-II are as shown in Fig. 2. The only unknown feature of the two structures is the configuration of the glycosyl phosphate or pyrophosphate group in these molecules. Indeed, this aspect should be of considerable interest from the standpoint of biosynthesis.

THE STUDY OF PROTEIN–PHOSPHOLIPID INTERACTIONS IN BIOLOGICAL MEMBRANES: A GENERAL ORGANOCHEMICAL APPROACH

Biological membranes perform an amazing variety of functions. The functions may be broad or very specialized. In *Escherichia coli,* a unicellular organism, functions, such as phospholipid synthesis, DNA synthesis, cell division, active transport, and energy metabolism, are all performed by one membrane. In the extremely halophilic bacteria, e.g., *Halobacterium halobium,* while the membrane carries out a large number of functions, a specialized function, light-dependent proton pumping, is performed by a single protein. Sarcoplasmic reticulum is an example of a mammalian membrane which performs a single function, namely, regulation of calcium concentration in the muscle fibers. What is of utmost fundamental importance is to understand the structure and dynamics of the proteins that carry out these functions within the membranes. Broadly, the problem is one of protein–protein and protein–phospholipid interactions.

If the membrane proteins could be crystallized in the presence of phospholipid molecules and studied by X-ray diffraction, as has been done in the case of *wet* crystals of water-soluble proteins, a great deal would clearly be learned about the functional form of membrane proteins. This, however, has not proved possible so far, although there would appear to be, theoretically, no insurmountable difficulty. Physical methods have been used extensively in membrane studies but, on the whole, the information obtained so far has been, and is likely to remain, rather limited. A third general possibility would be the development of organochemical approaches which would aim at bringing about specific covalent crosslinks between the phospholipid and protein molecules. Although, as mentioned below, a number of reagents have been proposed for the purpose [see reviews by Bayley and Knowles (*25*), Peters and Richards (*26*), and Gitler and Klip (*27*)] it is necessary to state clearly what the requirements are for this type of approach.

First, 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 interactions between proteins and phospholipids. Second, often in the approaches proposed crosslinking reagents are added from outside and these partition between the

interior of the bilayer and the aqueous phase. The reagent can freely diffuse into the membrane and it can react quite unspecifically with groups on the surface of the proteins or even fatty acyl chains, depending upon its reactivity. There is, therefore, complete lack of specificity in regard to the sites of crosslinking; further, reagents added from outside are likely to cause rather large perturbation. In the approaches explored in this laboratory, the aim has been to use "built-in" activable groups in fatty acyl chains at the outset (*28*). The location of the activable groups could be varied along the hydrocarbon chains (at *sn*-1 or *sn*-2) of the phospholipid, and it would be hoped that site-specific crosslinks would thus take place and the study of the crosslinked proteins would reveal meaningful information concerning the topology of the hydrophobic regions. Finally, 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 (*29*). 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.

The Synthesis of Mixed Acyl Glycerophospholipids

In the present studies, mild and efficient methods for the chemical synthesis of 1,2-diacyl and mixed diacyl glycerophospholipids are essential because the synthetic photoreactive fatty acids would not be easily accessible and, in addition, may be sensitive to harsh conditions. A mild and highly effective method for the catalysis of the acylation reactions has been developed in the present work by Gupta *et al.* (*30*), and it is illustrated by the three general reactions in Fig. 6. Of the different approaches to the synthesis of glycerophospholipids (*31–35*), the one involving acylation(s) of a preformed phospholipid backbone, such as *sn*-glycero-3-phosphorylcholine(I) (GPC) is particularly attractive. The diacyl product may, when necessary, be converted to the 2-lyso-phospholipid by treatment with phospholipase A_2, and subsequent acylation of the 2-OH group (Fig. 6) then yields the mixed diacyl phospholipid. Commonly used reagents for acylation of GPC and of lysophospholipids (III) are fatty acyl chlorides (*36*), anhydrides (*37, 38*), or mixed anhydrides (*34, 39*). The reagents are usually used in large excess (3- to 10-fold), especially because the acylation of the 2-OH group is particularly sluggish. Further, the use of fatty acyl chlorides may be accompanied by the formation of significant amounts of several side products (*40*). Acylation with anhydrides requires relatively vigorous conditions, e.g., 48 hr at 80°C (*37, 38*). Finally, the yields have been most often unsatisfactory.

In the present work, it has been found that *N,N*-dimethyl-4-aminopyridine, which has been shown to be a powerful catalyst in many acylation reactions in organic synthesis (*41, 42*), is also very efficient in phospholipid synthesis. Acylations of GPC, lysolecithins, N-protected lysophosphatidylethanolamines,

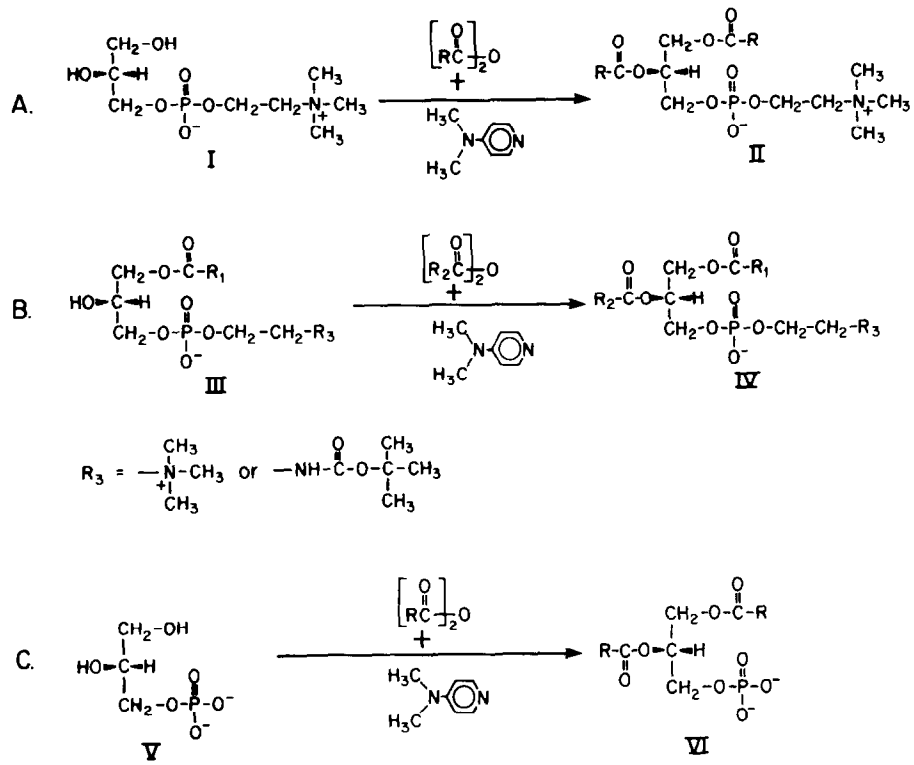


FIG. 6. *N,N*-Dimethyl-4-aminopyridine-catalyzed acylation reactions in the synthesis of phospholipids. (A) Acylations of *sn*-glycero-3-phosphorylcholine (GPC). (B) Acylation of 1-fatty acyl-*sn*-glycero-3-phosphorylcholines and N-protected ethanolamines. (C) Synthesis of phosphatidic acids by acylation of *sn*-glycero-3-phosphate.

and *sn*-glycero-3-phosphate (Fig. 6) all could be accomplished in 75–90% yields at room temperature with moderate amounts (1.2–1.5 mol eq per OH group) of fatty acyl anhydrides.

Of the many syntheses carried out using this method, representative examples, which are of interest in the study of crosslinking reactions, are shown in Fig. 7.

Crosslinking Studies with Photoactivable Groups Generating Nitrenes or Carbenes

A number of laboratories have studied the use of photosensitive reagents which generate nitrenes. Two principles have been used. In one, the nitrene precursor is added to a model membrane or a membrane from outside. Two reagents investigated in this way are azidobenzene (*43*) and iodonaphthyl azide (*44*). In our work, a variety of phospholipids were synthesized by Drs. Chakrabarti (*28*) and Radhakrishnan (unpublished results; (*45*)) with azido groups present in saturated or unsaturated fatty acyl groups located at *sn*-2 of the phospholipids (for example, VIII and IX of Fig. 7). Extensive photolytic studies with residues

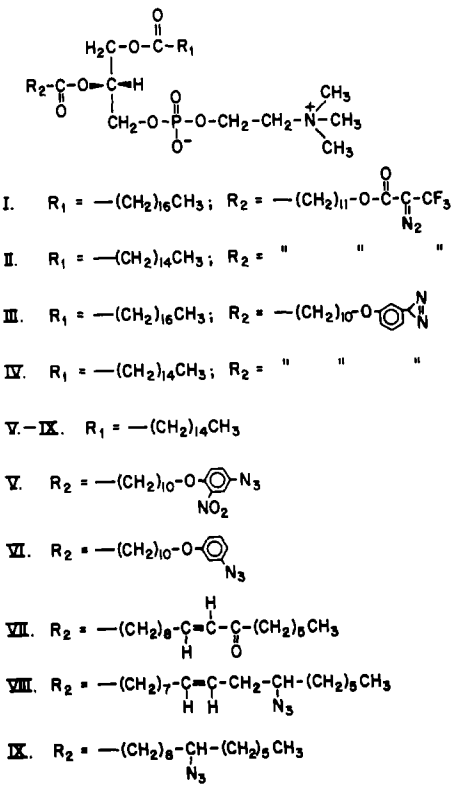


FIG. 7. Synthetic mixed 1,2-diacylphosphatidylcholines carrying different photoactivable groups in the acyl substituents on the C2-hydroxyl group of the glycerol backbone. The phospholipids were prepared from 1-acyl-*sn*-glycero-3-phosphorylcholines by acylation with fatty acyl anhydrides containing photoactivable groups in the hydrocarbon chains.

prepared from these phospholipids showed that the nitrene intermediate displayed very little, if any, intermolecular insertion reactions into saturated carbon chains. With iodonaphthalene azide, extensive utilization of the reagent in covalent reactions has been reported, due undoubtedly to a long half-life of the nitrene intermediate, but no experimental evidence for the occurrence of crosslinking with saturated alkyl chains has been provided. It is likely that the nitrene reacts with double bonds in unsaturated fatty acids and with sulfhydryl amino and other functional groups present in the proteins. Similarly, the crosslinking reactions reported by Stoffel and co-workers (*46*) with azidooleic acid are probably due to free radical mechanism rather than insertion by the nitrene group.

Intermolecular Crosslinking Reactions in Phospholipids Using Carbene Precursors

Two types of photoactivable groups which generate carbenes have proved to be the most useful so far. One group, investigated by Dr. Gupta, contains the α-diazo-β-trifluoropropionyl group, as exemplified by phospholipids I and II in Fig.

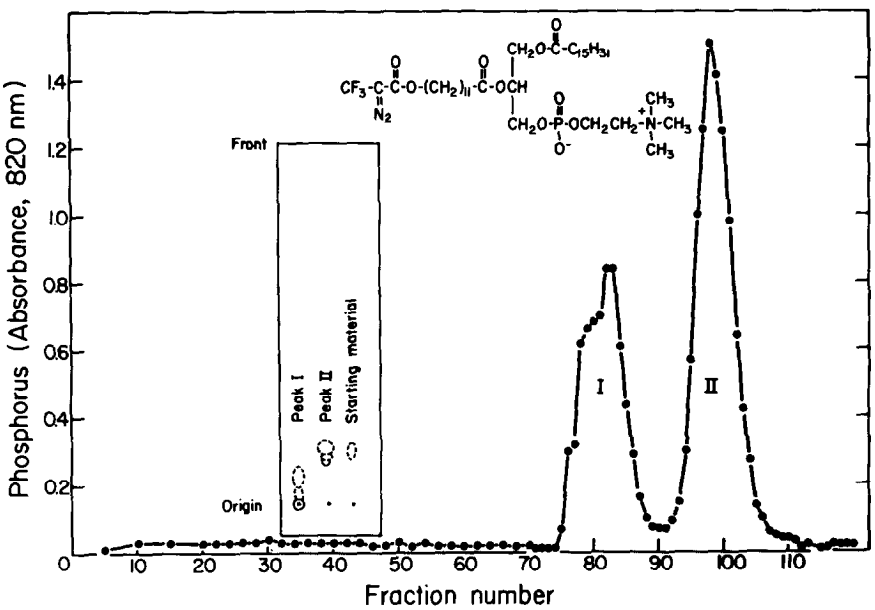


FIG. 8. Flow-through pattern from a Sephadex LH-20 column (2.5 × 100 cm) of the products obtained from the photolysis of 1-palmitoyl-2ω-(trifluorodiazopropionyl)lauroyl-phosphatidylcholine. Peak I accounted for 38% of the total phosphate-containing material.

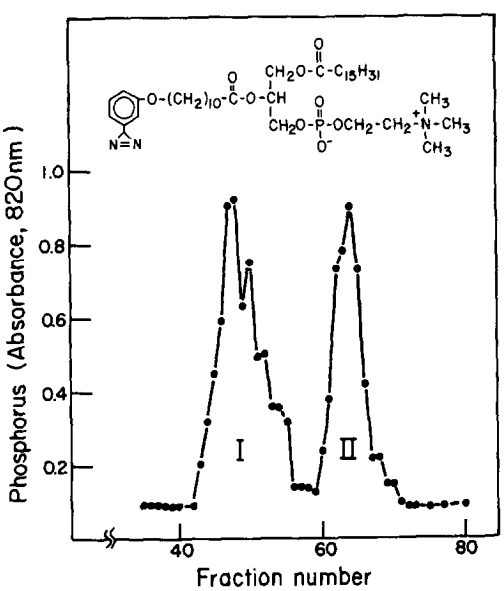


FIG. 9. Flow-through pattern from a Sephadex LH-20 column (2.5 × 100 cm) of the products obtained from the photolysis of 1-palmitoyl-2ω-(diazirinophenoxyl)-undecanoyl-phosphatidylcholine. Peak I accounted for 55% of the total phosphate-containing material.

7. The second group, investigated by Dr. Radhakrishnan, is the diazirinophenoxy group as in Structures III and IV (Fig. 7). The two groups are different in regard to their chemical character and may, in fact, prove to be complementary in many ways. The diazirine group has one important advantage, namely, that of higher extinction coefficient and, especially in the case of the phenyl derivatives (III and IV, Fig. 7), the absorption is shifted markedly in the desired (longer wavelength) direction.

With both sets of the above phospholipids, sealed vesicles can be prepared by sonication and these appear to consist of regular phospholipid bilayer structures. Photolysis at 315 nm and above gives rise to crosslinked products which may be characterized after separation on gel permeation columns. Two representative separations are shown in Figs. 8 and 9 (*45*). The positions of the first main peaks show these peaks of molecular weights higher than those of the starting materials. The average yield of crosslinked products in the case of the trifluorodiazophospholipids is 40–45%, while that in the case of diazirinophenoxy phospholipids is 50–60%. Several lines of evidence establish that crosslinking occurs mainly between two phospholipid molecules and that it involves insertion of the photogenerated carbene into the *sn*-1 fatty acyl chain of the second molecule.

Evidence for Intermolecular Crosslinking Reactions using Mixtures of Phospholipids

Further experiments carried out by Drs. Radhakrishnan and Gupta (*45*) used mixtures of the photoactivable phospholipids with radioactively labeled phospholipids such as [^{14}C]dipalmitoylphosphatidylcholine (DPPC), which can serve only as the "acceptor" of the generated carbene. Crosslinking to the radioactive phospholipid should give a radioactively labeled dimer which can be characterized. Results of a typical photolysis experiment are shown in Fig. 10. (Conditions for the maximal formation of the radioactive dimer by adjusting the ratio of the two phospholipids have not been worked out.) Peak I contains the dimer which has been studied by a number of degradative methods as shown in Fig. 11. The ratios of distribution of the radioactivity in the multiple products formed are as would be expected. In particular, attention should be drawn to the products formed on methoxide-catalyzed transesterification of the crosslinked products. As expected, [^{14}C]methylpalmitate and the ^{14}C-labeled dimeric fatty acid ester were produced and their ratio, as judged by radioactivity, was 1 : 1 (Fig. 12).

The results of corresponding experiments with the diazirinophenoxy phospholipids were identical.

Further experiments on demonstration of intermolecular crosslinking were carried out by including [^{14}C]cholesterol in phospholipid vesicles. The results are described below.

From these studies and those reported below, the general structures of the crosslinked products, after transesterification to remove the phospholipid resi-

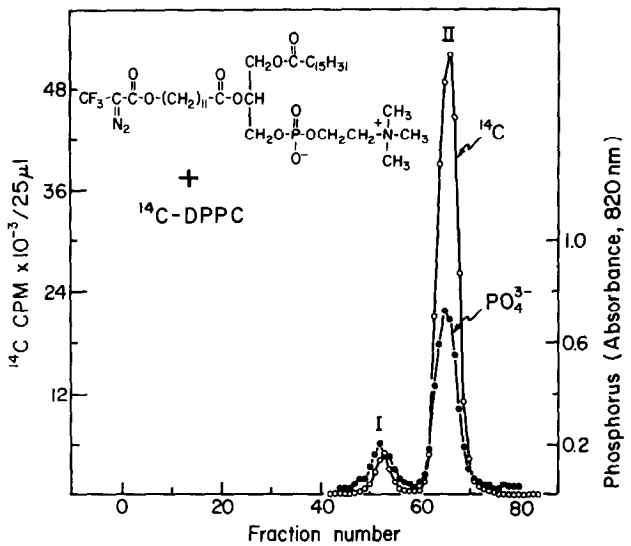


FIG. 10. Sephadex LH-20 column elution pattern of the products obtained from the photolysis of vesicles prepared by using an equimolar mixture of 1-stearoyl-2ω-trifluorodiazopropionyl-phosphatidylcholine and [^{14}C]dipalmitoylphosphatidylcholine (DPPC). Peak I accounted for 7.5–8% of the total ^{14}C.

dues, have been concluded to be as shown in Figs. 13A and B, for the diazo and diazirino derivatives, respectively.

Determination of the Sites of Crosslinks in Intermolecular Reactions

Having demonstrated the occurrence of intermolecular insertion reactions, it is important to ask if the points of crosslinking are related to the lengths of the two

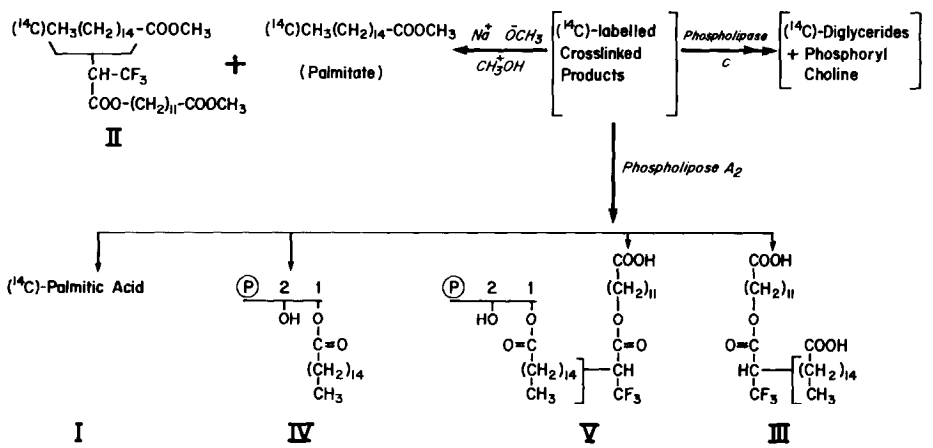


FIG. 11. Methods of degradation used for characterization of crosslinked phospholipids (peak I of Fig. 10). Only the [^{14}C]palmitic acid and products containing these radioactive residues are shown. Ⓟ stands for phosphorylcholine.

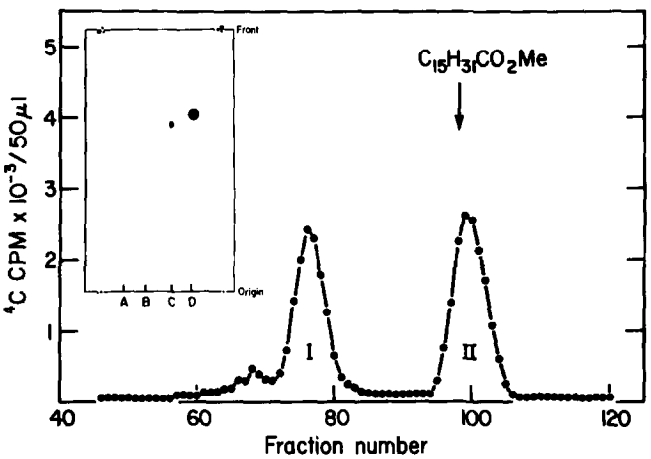


FIG. 12. Sephadex LH-20 chromatogram of the products obtained after transesterification of peak I (Fig. 10). Fractions 72–81 (peak I) and 95–105 (peak II) were pooled. The inset is the autoradiogram of silica gel tlc (solvent B) of peaks I and II. (A) Mixture as present in peaks I and II, (B) peak II, (C) peak I, (D) authentic methylpalmitate.

hydrocarbon chains involved. Because of the cooperativity in hydrophobic interactions between the fatty acyl chains, a correlation may indeed hold because of the ordered packing. If a relationship could be seen between the sites of insertion reactions in the acceptor and the carbene precursor chains, then, in principle, a potentially useful approach to the study of topography of interactions between membrane components would become available. As described below, a correlation has indeed been demonstrated with respect to the sites of crosslinks for both the diazo- and diazirinophospholipids and, further, the results are consistent with the recent conclusion that the *sn*-1 and *sn*-2 fatty acyl chains are out of step by two to four carbon atoms (*47*, *48*).

Experimental work on this study required the synthesis of a variety of

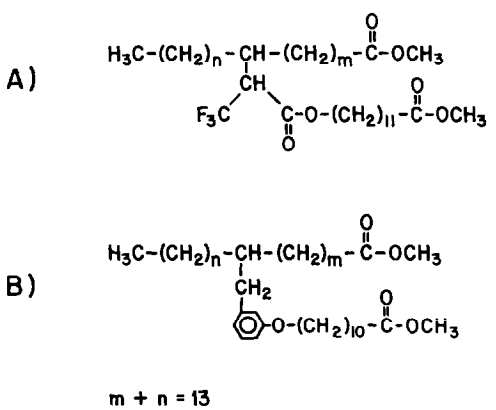


FIG. 13. General structures of the crosslinked fatty acid esters obtained using (A) the diazo and (B) phenyldiazirino systems.

phospholipids belonging to each series in which the lengths of the chains (at *sn*-2) carrying the photoactivable group and those of the acceptor chain (at *sn*-1) are systematically varied. Then there is required a satisfactory method for the analysis of the crosslinked products. For this formidable task mass spectrometry has provided the method of choice. We are deeply grateful to Professor K. Biemann and Dr. C. Costello for assistance and collaboration in this work. The fragmentation patterns of the general crosslinked structures shown in Fig. 13 would be difficult to interpret or would be indecisive (for Series B). In work with the trifluorodiazophospholipids, the following observation enabled the use of mass spectrometry reliably. Thus, it was found (*49*) that when photolyses are performed using the highly ordered multilamellar structures, the primary products formed are exclusively those shown in Series B (Fig. 14), while photolysis of unilamellar vesicles prepared by sonication gives the adducts shown in Series A (Fig. 14), also shown in Fig. 13. The products shown in Fig. 13 would arise from phospholipids of Series A (Fig. 14) by transesterification. It is obvious that the presence of the double bond in Series B would determine the fragmentation pattern and the spectra would be more readily interpretable.

In the case of the crosslinked products obtained from the diazirinophenoxy group, the general protocol used was to prepare the "acceptor" fatty acyl chains (at *sn*-1) such that in the expected region of crosslinking one carbon at a time carried a $-CD_2-$group. In fact, CD_2 groups were introduced, one at a time, at C_7—C_{12} in stearic acid which was then used to acylate the C1 position. Following photolysis, depending upon the sites of crosslinking, more or less deuterium would be found in the benzyl group belonging to the *sn*-2 fragment.

Using these methods, quite extensive analyses have been made which allow the conclusion stated above. It is important to add that, as expected, crosslinking does not occur to a unique site—rather, there is a distribution with the maximum being at the position theoretically expected from the molecular structures (*47, 48*) of phospholipids in bilayer. These studies need to be extended further but the results offer promise for crosslinking reactions at approximately predetermined distances from the exterior of the bilayer.

Crosslinking of Phospholipids Carrying Photoactivable Groups with Cholesterol in Vesicles

Initial experiments by Drs. Gerber, Radhakrishnan, and Gupta show that when [^{14}C]cholesterol is included in vesicles prepared from the two types of phospholipids by sonication, and photolysis is performed by the standard procedure, crosslinked products, as shown in Fig. 15, are indeed obtained. Thus, phospholipid I, an example of the diazophospholipid, gives a product of the structure III. Similarly, diazirinophenoxyphospholipid (phospholipid II) gives the crosslinked product, phospholipid IV. Although the exact nature of the site(s) of crosslinking in these reactions has not been determined, the method offers a great deal of potential for further studies of the structure of cholesterol in biomembranes.

Typical experimental results are shown in Figs. 16 and 17. Figure 16 shows the elution pattern from a Sephadex LH-20 column using chloroform–methanol as the

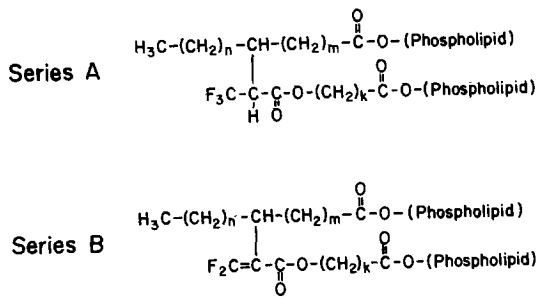


FIG. 14. General structures of the two series of crosslinked products obtained on photolysis of phospholipids containing the ω-(2-diazo-3,3,3-trifluoropropionyloxy) group in the *sn*-2 fatty acyl chain.

eluting agent. At least two ^{14}C-containing products are formed. Both of these products move in the area of phospholipid monomer and dimer. A further study of the main crosslinked product is shown in Fig. 17. The product (designated cholesterol-PC) is eluted as shown when simply rerun on this column. The positions of the phospholipid dimer (PC dimer), the monomer (PC), and the lysophosphatidylcholine (lyso PC) are shown. After phospholipase A_2 treatment, the released product (I, Fig. 18) now appears as shown. The results with the diazirinophenoxyphospholipids and [^{14}C]cholesterol are identical. It should also be noted that the results further confirm the occurrence of intermolecular crosslinks in the present photolytic reactions.

Phospholipids Containing Carbene Precursors in the Study of Membrane Properties (50)

Crosslinking in Mixed Phospholipid Systems

An important consideration in the development of these photochemical reagents as probes of membrane structure is whether functional groups such as double bonds can "scavenge" carbenes in the bilayer and thus prevent random insertion into membrane components. A series of competition experiments was

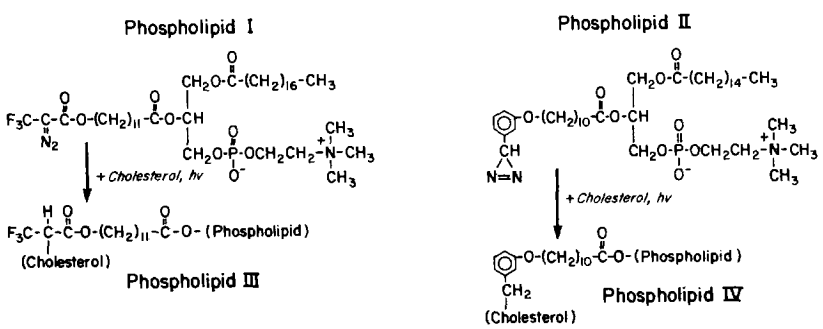


FIG. 15. Structures of crosslinked products formed from phospholipids containing diazo (I) and diazirino (II), groups.

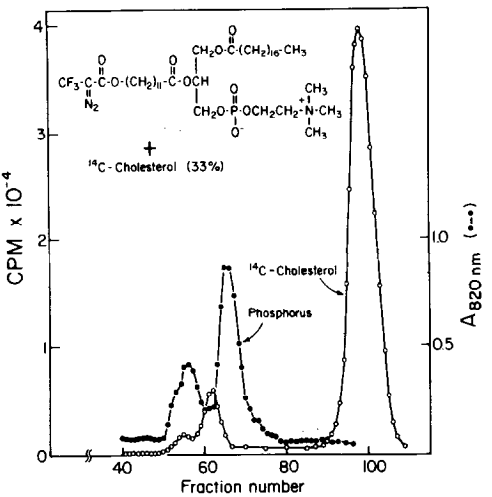


FIG. 16. Sephadex LH-20 column elution pattern of the products obtained from the photolysis of vesicles prepared by using an equimolar mixture of 1-stearoyl-2ω-trifluorodiazopropionyl-phosphatidylcholine and [^{14}C]cholesterol.

therefore performed to determine the extent of such effects. A typical experiment is shown in Fig. 19. Competition between dipalmitoyl lecithin (DPL) and dioleyl lecithin (DOL) for crosslinking to the diazirine phospholipid (IV of Fig. 7) was studied by crosslinking various mixtures of the diazirine phospholipid/[^{14}C]DPL/DOL and diazirine phospholipid/DPL/[^{14}C]DOL. The results show that there is some preference for crosslinking to DOL, presumably due to some specificity of the carbene for the double bond in the 9–10 position of the acyl chain in DOL. However, significant crosslinking to DPL is also observed

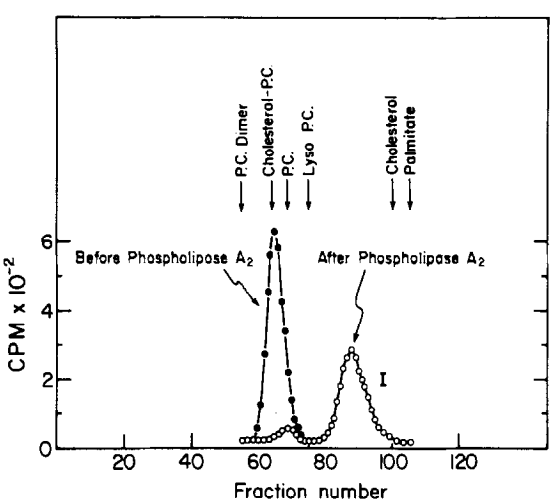


FIG. 17. Flow-through patterns of the crosslinked products (Fig. 15) after treatment with phospholipase A_2. P.C. stands for phosphatidylcholine.

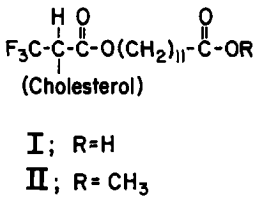


FIG. 18. Structure (I) of the product (last peak of Fig. 17) after phospholipase A_2 treatment of the crosslinked phospholipid in Fig. 15.

in the presence of DOL. The results of similar experiments with the trifluorodiazophospholipid (I of Fig. 7) were almost identical. Phospholipids containing linolenoyl acyl chains (double bonds in 9–10, 12–13, and 15–16 positions) or acyl chains with ω-phenyl groups have also been shown not to act as carbene scavengers. Thus, the crosslinking phospholipid probes now described appear to be promising for random insertion into membrane components.

Crosslinking in Phase Separation Studies

Synthetic diacyl phospholipids are known to undergo a phase transition at a characteristic temperature commonly known as the order–disorder transition. This transition consists of the cooperative melting of the bilayer acyl chains from a relatively ordered to a more disordered state. Differential scanning calorimetry (DSC) recordings are presented in Fig. 20 which illustrates the order–disorder transitions of the diazophospholipid (I of Fig. 7), DPL, and a 1 : 1 (mole/mole) mixture of the diazophospholipid and DPL. The 1 : 1 diazophospholipid : DPL

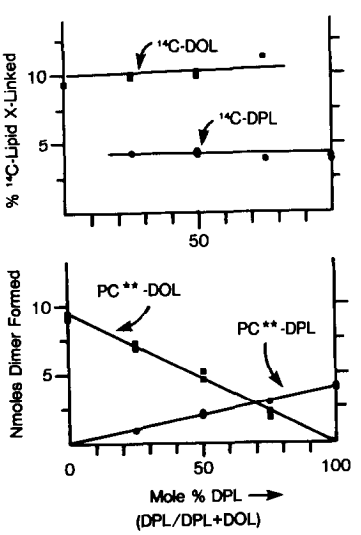


FIG. 19. Competition between DPL and DOL for crosslinking to the diazirine phospholipid. The mole ratio of diazirine phospholipid to (DOL + DPL) was 1 : 1 in all cases. *Top panel:* percentage of DPL or DOL crosslinked as a function of (DPL : DPL + DOL). *Bottom panel:* nanomoles of dimeric products produced.

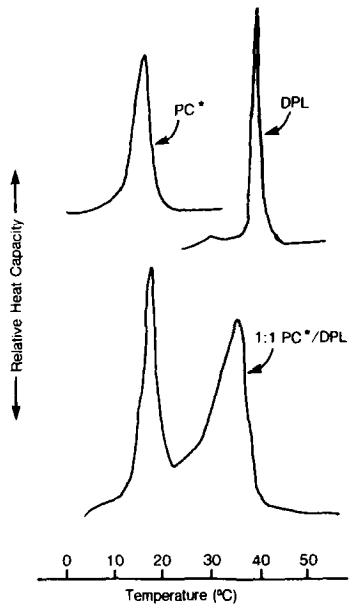


FIG. 20. Differential scanning calorimetry (DSC) recordings of diazophospholipid (PC*), DPL, and a 1 : 1 mixture of diazophospholipid and DPL. The heating rate was 5°C/min.

system exhibits calorimetric behavior which indicates that lateral phase separation (a two-phase zone) occurs at certain temperatures. Thus, as the 1 : 1 mixture is cooled from 50°C, a broad transition occurs over the range 28–38°C which consists of acyl chain solidification in a DPL-rich phase, accompanied by lateral separation of a fluid diazophospholipid-rich phase. As the temperature is decreased further, the acyl chains in the diazophospholipid-rich phase solidify at ~18°C. In order to determine whether the distribution of carbene insertion products is sensitive to the physical states of the phospholipids, the temperature dependence of crosslinking was studied for a 1 : 1 mixture of diazophospholipid and DPL. The results are shown in Fig. 21. Above ~35°C, the distribution of

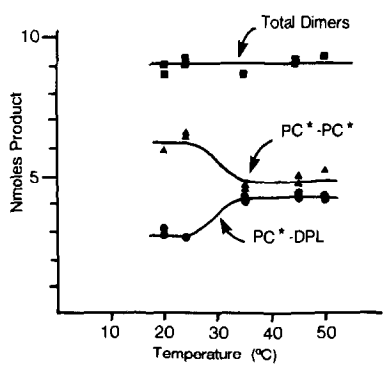


FIG. 21. Distribution of crosslinked dimeric products generated by photolysis of a 1 : 1 mixture of diazophospholipid (PC*) and DPL, as a function of temperature.

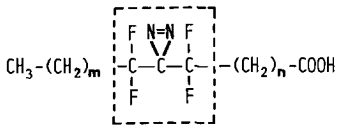


FIG. 22. A fatty acid containing a diazirine group at a carbon atom in the chain, flanked by two fluorine atoms on each of the adjoining carbon atoms. The total units shown in the dashed box could be moved up and down the fatty acyl chain to desired locations.

dimeric products remains invariant with temperature. However, at temperatures below ~35°C, the production of diazophospholipid–DPL dimer decreases and diazophospholipid–diazophospholipid dimer increases. This is consistent with the calorimetric results described above and indicates that the crosslinking method is capable of detecting phase separations previously observed only by physical methods. In more complicated mixtures of phospholipids, the crosslinking method has also been used as a probe to detect lateral phase separation and to indicate which components are separating. These encouraging experiments indicate the possible utility of the photolabeled phospholipids as probes for the lateral organization of biological membranes.

Attemps at Design of More Versatile Carbene-Generating Groups

While the encouragement derived from the results described above using the β-trifluoro-α-diazopropionyl group were encouraging, attempts were made to design new groups which, in addition to having the properties of maximal insertion into C–H bonds and minimal intramolecular rearrangements, would offer the flexibility of being accommodated anywhere along the fatty acyl chains. In the studies reported above with the phospholipids containing the diazo or diazirino groups, the latter groups are at the ω positions of the fatty acyl chains and their positions

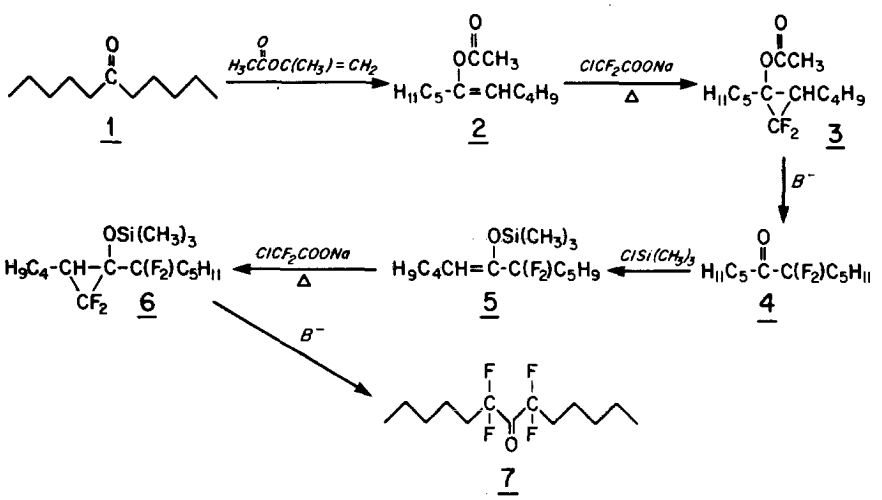


FIG. 23. Steps in the synthesis of the tetrafluoroketone, 7, from di-*n*-pentylketone, 1.

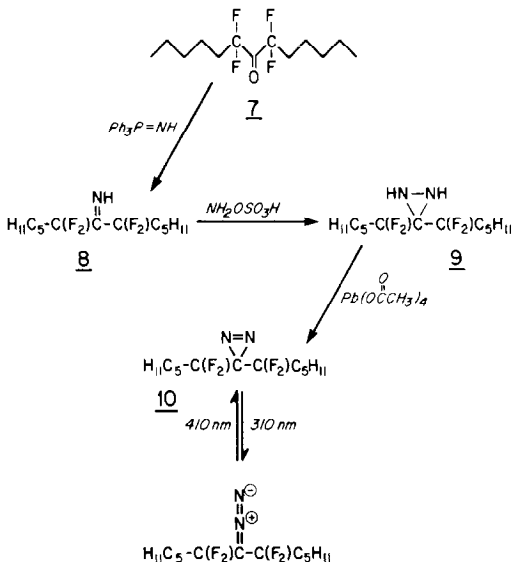
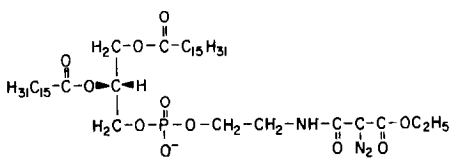


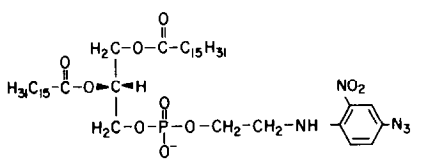
FIG. 24. Preparation of the tetrafluorodiazirine, **10**, from the tetrafluoroketone, **7**. Irradiation of **10** at 310 nm undergoes isomerization to the diazo compound shown. Irradiation of the latter at 410 nm reforms the diazirine. In both photolyses, side products are formed as a result of the rearrangements of the presumed intermediate carbene.

can be varied only by altering the length of the fatty acyl chain which carries them. Ideally, it should be possible to retain the desired length in the fatty acyl chain but move the photoactivable group up and down the chain as required.

Other important considerations are those of perturbation in the packing of hydrocarbon chain and of polar character such as that in the ester group; both of these should be kept to a minimum. A basic structure chosen as a goal for synthesis (unpublished work of Dr. B. Erni) is shown in Fig. 19. As supporting



I



II

FIG. 25. Structure of phosphatidylethanolamines carrying photoreactive polar head groups.

evidence for the choice of this basic structure, it should be added that the studies on the trifluorodiazopropionyl group by Chowdhry *et al.* (*51*), as well as those on perfluorodiazo compounds by Krespan and Middleton (*52*), all demonstrated that the photogenerated carbenes from these systems insert into C–H bonds of the solvent without intramolecular rearrangement involving fluorine migration.

As an initial goal, synthesis of a short hydrocarbon chain containing the structure shown in Fig. 22 (dashed box) was undertaken. Of the various routes investigated, the one which proved successful is illustrated in Figs. 23 and 24. Di-*n*-pentyl ketone (**1**) was converted to the corresponding enol acetate **2**. The latter was reacted with difluorocarbene to form the difluorocyclopropyl compound **3** by the method of Crabbé (*53*). Base-catalyzed hydrolysis then gave the α-difluoroketone (**4**) which was converted to the trimethylsilyl enol ether **5**. Reaction with difluorocarbene as described above for **3** gave the cyclopropyl compound (**6**). The latter, under basic catalysis, afforded the tetrafluoroketone (**7**), a key intermediate in the present synthesis. The next step, i.e., conversion to the imine (**8**), was carried out by a Wittig-type reaction using triphenylphosphineimine (Fig. 24). The imine was converted to the diaziridine (**10**) by reaction with hydroxylamine-*O*-sulfonic acid. Oxidation with lead tetraacetate afforded the required diazirine (**10**).

Surprisingly, no intermolecular insertion into C–H bonds of cyclohexane or O–H bonds of methanol could be demonstrated. Irradiation at 310 nm brought about isomerization of the diazirine to the diazo compound (Fig. 24) to an extent of more than 50%. Intramolecular reactions, i.e., alkyl migration and cyclopropane formation, accounted for the remainder of the products. Further, the interesting observation was made that upon irradiation at 410 nm, where there is a weak absorption band, the diazo compound reverted to the diazirine (**10**). This process is also accompanied by partial intramolecular rearrangements.

Carbene and Nitrene Precursors as Probes for Interactions between Polar Head Groups and Nonembedded Portions of Membrane Proteins

Phospholipids in membranes contain a variety of polar head groups. Certain polar head groups are characteristic of certain specific membranes (e.g., cardiolipin in *E. coli* membrane and in mitochondrial membrane) but there is great heterogeneity in the nature of head groups in most membranes. The meaning(s) of this diversity is far from clear. However, it is also known that some membrane enzymes show absolute or varying degrees of specificity with respect to polar head groups (*54*). Thus, the inner mitochondrial enzyme, β-hydroxybutyrate dehydrogenase shows absolute specificity for the choline head group (*55, 56*).

It would be very desirable to have available a set of derivatives of phospholipids which contain carbene or nitrene precursors as parts of their polar head groups. A list of derivatives that can be envisaged would be extensive but two which were prepared sometime ago by Dr. Chakrabarti (*28*) are shown in Fig. 25. One of these derivatives has already proved useful in the study of phospholipase A_2 from the snake venom (*57*).

Studies of Interactions between Membrane Proteins and Phospholipids

In the era of biochemical development, when the preoccupation was with the elucidation of metabolic pathways and characterization of the enzymes involved in the enormous number of different reactions, enzymes that could not be solubilized were simply designated particulate enzymes. The latter are now usually known to be associated with membranes. It has also become clear that the latter enzymes vary in the strength of their association with the membrane. Thus, peripheral proteins (*58*) have a relatively weak association and can be separated from membranes relatively easily by salt, urea, or related treatments. On the other hand, there are proteins designated by Singer (*58*) as the integral proteins, which have strong hydrophobic association with membranes and are usually very difficult to isolate. For solubilization without inactivation, they require the use of carefully chosen detergents and they bind a substantial amount of phospholipid which is very difficult to remove completely. Without the phospholipid (sometimes detergents may substitute for the phospholipid), membrane enzymes often lose their activity. Adding back phospholipids under appropriate conditions often restores the activity. Dramatic conformational changes have been reported during the reconstitution of the activity upon addition of phospholipids. It is clear from the above that membrane proteins display strong interactions with membrane phospholipids. Since these proteins are capable of lateral motion and they may, and often do, interact with other proteins in the membranes (e.g., the activation of adenyl cyclase after the formation of the hormone–receptor complex and the

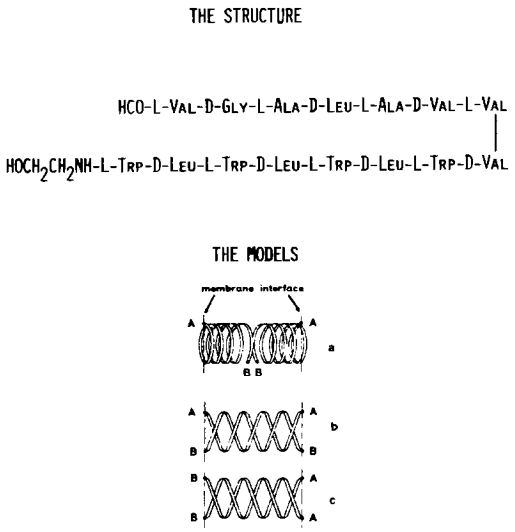


FIG. 26. The primary structure of gramicidin A and the proposed models for its action as ionophore. As seen in the sequence, the N-terminal valine carries a formyl group while at the C-terminus, L-tryptophan is linked to β-aminoethanol. In the model proposed by Urry and co-workers (*62*), two molecules of gramicidin align as shown to form the channel. The N-formyl terminus is B, while the C-terminal ethanol is A. In the second model proposed by Veatch, Blout, and co-workers (*63*), parallel or antiparallel helixes formed from two molecules span the membrane to form the channel.

transduction of signals from the cell surface to the inside of the cell involving the cytoskeleton), it is clear that the study of interactions between phospholipids and proteins and of the dynamics of membrane proteins is of paramount importance in the understanding of membrane functions. The main hope of the initial organochemical approaches would be to obtain some insights regarding the topology of the hydrophobic parts of the membrane-embedded proteins, the nature of the surface contacts between the phospholipids and the proteins and, finally, the nature of interactions between proteins in the membranes.

This section describes the different membrane protein systems which form the topics of our current studies, studies which are for the most part at initial stages. A number of considerations influenced the choice of each one of the systems. First, a general and overriding consideration in all of the systems chosen has been the availability of proteins in a homogeneous state so that structural studies and the reconstitution of its function by the use of defined phospholipids can be performed. Second, as will be evident, the proteins chosen are such that essentially all the different types of membrane-associated proteins are represented. Third, the study of each of the chosen proteins will hopefully yield important insights into membrane function. Finally, an important requirement in the studies being initiated here is the primary amino acid sequence of the proteins concerned. Including the primary structure work reported below, this requirement is met for all the polypeptides and proteins chosen for study.

Gramicidin A

Ionophores, which bring about permeability changes in membranes for different ions may be divided into channel formers and carriers (*59–62*). While both classes of ionophores are extremely interesting and pertinent to the study of membranes, initially we have chosen Gramicidin A, the hydrophobic polypeptide, shown in Fig. 26. Gramicidin A forms a channel which increases permeability to monovalent cations such as Na^+, K^+, and Rb^+. Evidently two molecules of the polypeptide participate in the formation of one channel. Our choice of this system for study is timely because a number of defined chemical experiments with modified Gramicidin A, as well as a number of physicochemical experiments, have already been performed with this system, and two clear-cut alternative models of its action have been proposed (*63, 64*). These models are shown in Fig. 26 (see legend for details). By reconstituting ionophore function in phospholipids containing the above-described photoactivable groups, it is hoped to obtain a set of data on the sites of crosslinking in the polypeptide chain and this, in turn, should be consistent with one or the other model. In experiments to date, crosslinking of the synthetic phospholipids to Gramicidin A has been demonstrated by Dr. Majumdar. The sites of crosslinks are under investigation.

Bacteriophage M-13 Coat Protein: Its Structure in the Cytoplasmic Membrane of E. coli and Its Association With the Newly Synthesized DNA

Upon infection of *E. coli* by the filamentous virus M-13, the single-stranded DNA enters the cell but the coat protein remains in the cytoplasmic membrane

(*65, 66*). As infection continues, more and more of the coat protein is synthesized within the cell and it also enters the cytoplasmic membrane. The coat protein represents up to about 25% of the total protein of the membrane (*67*). In both types of events, i.e., entry of the coat protein into the membrane from outside or from inside, the protein maintains the same asymmetric orientation. Thus, the amino terminus is always on the exterior of the cytoplasmic membrane while the carboxyl end is on the cytoplasmic side. The primary structure of the coat protein as determined by Braunitzer and Konigsberg and their co-workers (*68, 69*) is shown in Fig. 27. In further elegant studies by Wickner (*70, 71*), conditions for reconstitution of the protein into defined phospholipids with *natural* orientation have been defined. Temperatures close to the transition temperature of the phospholipid are required for correct orientation. At temperatures considerably below the transition temperature, both termini of the protein project, at least partly, outside of the vesicles. At temperatures above the transition of the phospholipid, no incorporation of the protein into the phospholipid vesicles occurs. The method used for these reconstitution studies was the cholate dilution procedure devised by Racker *et al.* (*72*).

In work carried out in collaboration with Professor G. Robert Greenberg of the University of Michigan, Ann Arbor, reconstitution following Wickner's methods has been performed using radioactive phospholipids containing a carbene-generating precursor. Further, upon photolysis of the reconstituted vesicles, Dr. Greenberg has shown the formation of protein–phospholipid crosslinked product(s). The next stage is the determination of the sites of crosslinks.

Cytochrome b_5, Cytochrome b_5 Reductase, and Stearyl-Coenzyme A Desaturase

In higher organisms, saturated fatty acids are the precursors of unsaturated fatty acids. A pathway for this conversion has been characterized in liver microsomes. It involves an electron transport sequence of cytochrome b_5 reductase to cytochrome b_5 to the terminal cyanide-sensitive oxidase, the desaturase, which brings about the conversion of stearyl-coenzyme A to oleyl-coenzyme A (Fig. 28). Mainly through the efforts of Strittmatter and co-workers (*73–76*), a great deal of progress in biochemical characterization of this system has been made. Some of the features are as follows: (1) both cytochrome b_5 reductase and cytochrome b_5 are proteins which interact on the exterior surface of the

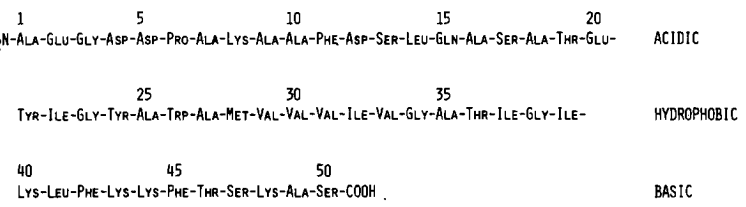


FIG. 27. The primary structure of the major coat protein of the filamentous DNA virus M-13. The hydrophobic region is shown. When in the *E. coli* membrane, the α-amino terminus protrudes to the exterior of the membrane while the carboxyl terminus is on the cytoplasmic side.

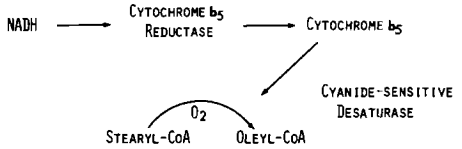


FIG. 28. The microsomal electron transport pathway for unsaturated fatty acid synthesis in higher organisms.

microsomes but they are anchored into the microsomes by virtue of hydrophobic polypeptide fragments (tails); (2) the desaturase is an integral enzyme, well embedded in the membrane; (3) it has been possible to reconstitute systems containing cytochrome b_5 and cytochrome b_5 reductase into liposomes, and an important result is that the two proteins do interact and that fluidity of the liposomes is necessary for the lateral motion and consequent interaction of the two proteins; (4) similarly, all three proteins have been recombined to bring about O_2 and NADH-dependent desaturation of stearyl-coenzyme A to oleyl-coenzyme A; (5) the amino acid sequence of the hydrophobic segment of cytochrome b_5 is known for the protein from three different sources (*77–79*) and preliminary models of the secondary structures of these segments within the membranes have been proposed (*77–80*).

Because of the above advances in the biochemistry of the above system and the ready availability of the proteins, the system is highly attractive for further studies of the interactions between phospholipids and membrane-embedded segments. Cytochrome b_5 from rabbit liver has been purified in this laboratory, and because of distinct advantages which this offers for our crosslinking studies, the amino acid sequence of its hydrophobic segment has been determined (unpublished work

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          91                  96                  101                 106                 110
PORCINE   ILE ALA LYS PRO SER GLU THR LEU ILE THR THR VAL GLU SER ASN SER SER TRP TRP THR...

HORSE     ILE ALA LYS PRO VAL GLU THR LEU ILE THR THR VAL ASP SER ASN SER SER TRP TRP THR...

BOVINE    ILE THR LYS PRO SER GLU SER ILE ILE THR THR ILE ASP SER ASN PRO SER TRP TRP THR...

RABBIT    LEU SER LYS PRO MET GLU THR LEU ILE THR THR VAL ASP SER ASN SER SER TRP TRP THR...

          111                 116                 121                 126                 131
PORCINE   ..ASN TRP VAL ILE PRO ALA ILE SER ALA LEU VAL VAL SER LEU MET TYR HIS PHE TYR THR SER GLU ASN

HORSE     ..ASN TRP VAL ILE PRO ALA ILE SER ALA VAL VAL VAL ALA LEU MET TYR ARG ILE TYR THR ALA GLU ASP

BOVINE    ..ASN TRP LEU ILE PRO ALA ILE SER ALA LEU PHE VAL ALA LEU MET TYR HIS LEU TYR THR SER GLU ASP

RABBIT    .. ASN TRP VAL ILE PRO ALA ILE SER ALA LEU ILE VAL ALA LEU MET TYR ARG LEU TYR MET ALA ASP ASP
```

FIG. 29. Comparison of the primary sequences of the hydrophobic termini of the cytochrome b_5 from pig, horse, beef, and rabbit livers. The sequences from the first three sources were elucidated by Ozols, Strittmatter, and their colleagues. The sequence of the cytochrome b_5 from rabbit liver was performed by Takagaki *et al., J. Biol. Chem.* **255**, 1536–1541 (1980). The sequences are aligned to show homologics. The amino acids underlined are those where variation is found in one or more of the four species.

of Dr. Takagaki). This sequence, as well as the ones determined by Ozols and colleagues, is shown in Fig. 29.

In work directed toward exploring the interaction between phospholipids and cytochrome b_5, it was found by Dr. Takagaki that the latter can be inserted into liposomes consisting of phospholipid I (Fig. 7) and furthermore, on photolysis, significant covalent crosslinking of the phospholipid to the hydrophobic segment of the protein takes place. Determination of the site(s) of crosslinking is in progress.

Glycophorins

The red cell membrane and its ghost have been studied extensively, especially with regard to proteins and phospholipids. Primary structures of several proteins and their orientations, as well as interactions between different proteins, have been worked out or are being actively investigated (*81, 82*). Thus, asymmetry in proteins and phospholipids was first demonstrated for this membrane (*83, 84*). Here, attention is focused on glycophorin A which is the major sialoglycoprotein of the red cell and is a blood group determinant. Through the efforts of Marchesi, Furthmayr, and their colleagues (*81, 85–87*) the total primary sequence of glycophorin is known (Fig. 30). The glycoprotein traverses the membrane. It has a substantial polypeptide part (at the carboxyl terminus) projecting on the cytoplasmic side and a large portion starting from the amino terminus and carrying the carbohydrate chains (60% of the total mass of glycophorin A) at the external side of the membrane. Finally, there is a hydrophobic region of about 20 amino acids (roughly, amino acids 73–95), which is believed to be embedded in the membrane.

The above properties and the chemical progress being made make glycophorin highly attractive for further chemical studies.

Only the initial progress in our experiments on bringing about crosslinks between the phospholipids carrying the photoactivable diazirinophenoxy group and the reconstituted glycophorin may be mentioned. Dr. Alonzo Ross has successfully carried out the reconstitution of glycophorin A with synthetic phospholipids and, following photolysis, has demonstrated the formation of covalent linkage(s) between a radioactively labeled phospholipid and glycophorin. Further, it has been found that more than 90–95% of the crosslinking occurs with the hydrophobic tryptic peptide T_6 (residues 62–96). Sequence work on determining the site(s) of crosslinking is in progress.

Phospholipid exchange proteins. In the past 10 years, a number of proteins which bring about specific or nonspecific exchange of phospholipids between phospholipid vesicles and/or membranes have been discovered (*88–91*). Purification has been reported of some of the proteins. The purified proteins seem to bind one mole of the phospholipid substrate. Aside from their biological function, the exchange proteins provide very valuable tools for a number of studies, e.g., for the specific modulation of the phospholipid composition of a single leaflet and for the determination of flip–flop or the exchange of phospholipid from one leaflet to the other (*92, 93*).

Clearly, these proteins offer promise for insertion of phospholipids containing

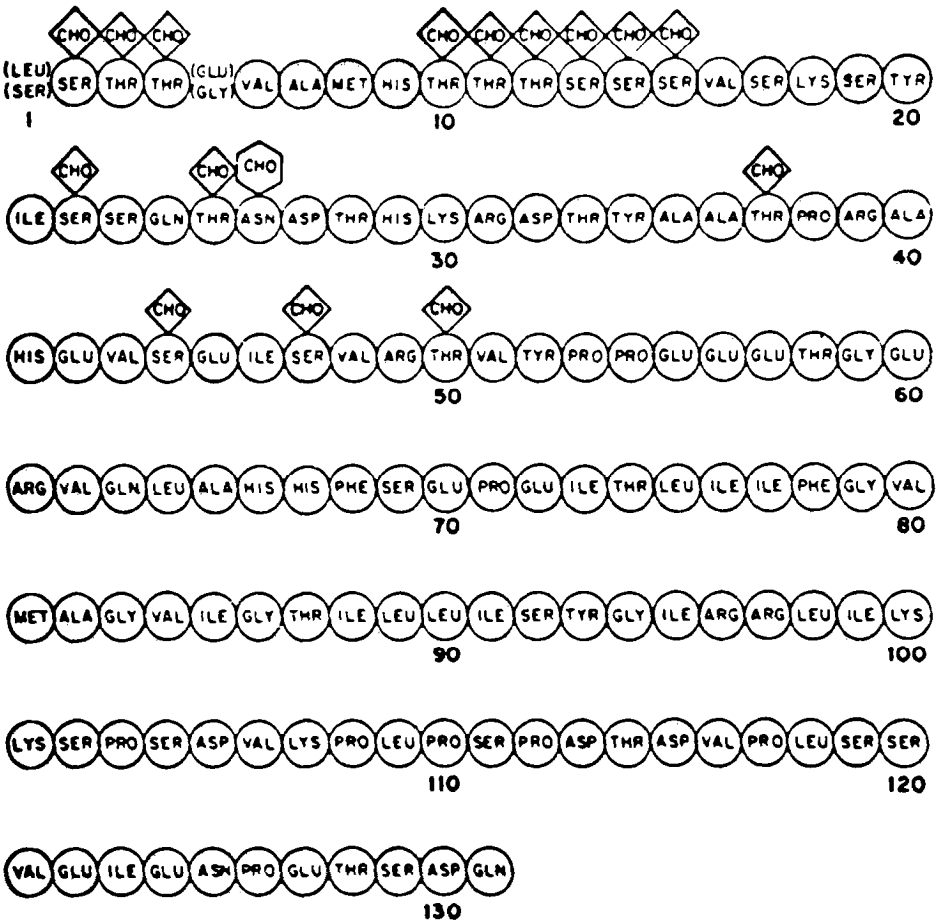

FIG. 30. Amino acid sequence of human erythrocyte glycophorin A. Boxes above certain residues indicate attachment of oligosaccharides at these sites (*86*).

photoactivable groups specifically into one of the leaflets. With a protein or proteins inserted into the bilayer, crosslinking experiments should yield valuable information regarding the extent of penetration of the membrane proteins into the bilayer.

In a second study being performed jointly with Dr. K. Wirtz of Utrecht University, Holland, an original discoverer of the exchange protein, the phospholipid binding site on the protein molecule is being explored by photolysis of the protein–phospholipid complex formed by using radioactively labeled photoactivable phospholipids.

β-Hydroxybutyrate Dehydrogenase and Its Specific Activation by Choline-Phospholipids

This enzyme was the first one to be identified by David Green and co-workers in the early sixties as a phospholipid-requiring mitochondrial enzyme. The specific

requirement for phospholipids containing choline head groups was established (*94*). The independent studies of Hammes (*55*) and Fleischer (*56*) and their respective colleagues have confirmed this specificity. Furthermore, elegant kinetic studies of the phospholipid–protein interactions have been carried out using delipidated enzyme. Furthermore, both groups have reported on methods for purification of the enzyme to virtual homogeneity. Because of biochemical characterization, physicochemical studies, and the important feature of polar head group specificity in addition to the requirement of the fatty acyl chains (*54, 55, 94*), this enzyme has been chosen for studies by crosslinking methods. In addition to incorporating photoactivable groups in fatty acyl chains, analogs of the choline head group containing photoactivable carbene precursors should be of interest for the study of the active site of the enzyme.

Ca^{2+}/Mg^{2+} ATPase of Sarcoplasmic Reticulum

Sarcoplasmic reticulum which regulates the concentration of Ca^{2+} ions in muscle fibrils, thereby enabling the filaments to contract or to relax, has only about seven proteins and a proteolipid in its membrane. While all the proteins have been characterized, the major component, the Ca^{2+}/M^{2+} ATPase, has received the most attention. Methods for its purification have been described by a number of workers (e.g., (*96–99*)). The protein is of about 100,000 MW. Approximately one-half of the total protein may be embedded in the membrane. Rapid progress in elucidation of the primary sequence is currently being made, especially by Allen and co-workers (*100*). There is believed to be an ionophore in association with the ATPase. The possibility that a polypeptide fragment of the ATPase may function as the ionophore has been proposed by Shamoo, MacLennan, and co-workers (*101*), while Racker and co-workers ascribe to the proteolipid the function of the ionophore (*102*). However, a recent report from the latter group ascribes the Ca^{2+} binding property to a hydrophobic fragment of the ATPase (*103*).

On purification, the protein retains a large amount (about 50–60 mol/mol of protein) of the phospholipid. Methods for delipidation have been proposed in connection with attempts at reconstitution of the ATPase activity as well as of the Ca^{2+} pumping activity. Reconstitution using purified ATPase has been studied by a number of groups, especially by Racker and co-workers (*104, 105*) and Metcalfe and his colleagues (*98*), and a detailed mechanism for Ca^{2+} transport has been proposed (*106*).

The vectorial transport of a divalent ion using a homogeneous protein and synthetic phospholipids provides a nicely defined system. Indeed, using purified and completely delipidated enzymes, full restoration of the ATPase activity by addition of synthetic phospholipids carrying photoactivable groups has already been demonstrated by Dr. Takagaki in this laboratory. Studies on the reconstitution of the Ca^{2+} pump and on the structural work by the crosslinking approach should prove to be highly interesting.

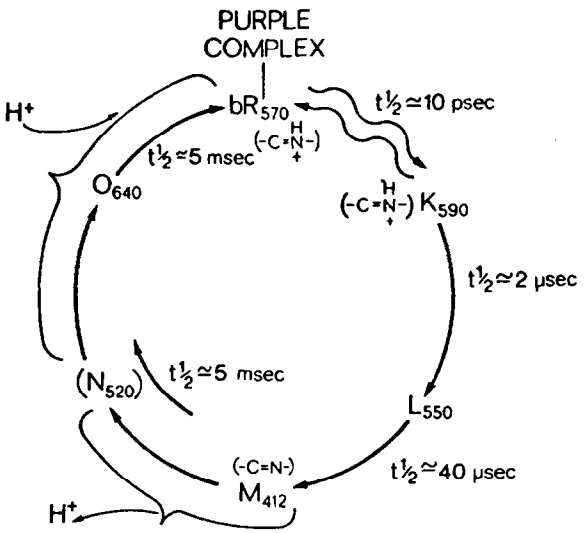

FIG. 31. A current model showing the intermediates detected in the photochemical cycle of the purple membrane by low-temperature and flash spectroscopy (*109*). One proton is released and taken up again, near the positions shown. The protonation states of the chromophore are also shown. They are derived by resonance Raman spectroscopy. A similar series of intermediates is well known in the visual pigment, rhodopsin, which also contains the chromophore retinal, in a 1 : 1 complex with the protein. Reproduced, with permission, from R. Henderson, *Annu. Rev. Biophys. Bioeng.* **6**, 87–109 (1977), Annual Reviews Inc., Palo Alto, Calif.

Bacteriorhodopsin: Purple membrane, the biological proton pump

Some extremely halophilic bacteria, such as *Halobacterium halobium*, can use normal processes such as respiration and glycolysis for energy production. However, when grown in light and under low oxygen tension they can produce and insert into their regular membrane patches of a purple membrane (*107, 108*) which uses light energy for the synthesis of ATP. This is accomplished by the agency of a retinylidene complex of a protein named bacteriorhodopsin. The latter is a protein of ~26,000 MW and is largely (~80%) embedded in the purple membrane. When exposed to light, a cyclic process resulting in the pumping of protons from the interior to the exterior of the cell is set in motion and the resulting gradient is utilized in the synthesis of ATP by the cell (*109*).

Of the total information available, only the following aspects may be mentioned. (1) In the light-catalyzed cycle leading to the ejection of a proton from inside to outside, the intermediates shown in Fig. 31 have been discerned spectroscopically (*110*). A model for the mechanism of the proton pump which is consistent with these observations has been proposed (*111*); (2) electron and X-ray diffraction studies of the highly organized protein in the purple membrane by Henderson and co-workers show the protein to contain seven rods, presumably α-helixes, each of which spans the membrane and is largely embedded in it (*112, 113*); (3) some information has been forthcoming on the characteristics of the two faces of the purple membrane (*114, 115*); (4) the orientation of the protein

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                5                  10                  15                  20                  25
<GLU ALA GLN ILE THR GLY ARG PRO GLU TRP ILE TRP LEU ALA LEU GLY THR ALA LEU MET GLY LEU GLY THR LEU
               30                  35                  40                  45                  50
TYR PHE LEU VAL LYS GLY MET GLY VAL SER ASP PRO ASP ALA LYS LYS PHE TYR ALA ILE THR THR LEU VAL PRO
               55                  60                  65                  70                  75
ALA ILE ALA PHE THR MET TYR LEU SER MET LEU LEU GLY TYR GLY LEU THR MET VAL PRO PHE GLY GLY GLU GLN
               80                  85                  90                  95                 100
ASN PRO ILE TYR TRP ALA ARG TYR ALA ASP TRP LEU PHE THR THR PRO LEU LEU LEU LEU ASP LEU ALA LEU LEU
              105                 110                 115                 120                 125
VAL ASP ALA ASP GLU GLY THR ILE LEU ALA ILE VAL GLY ALA ASP GLY LEU MET ILE GLY THR GLY LEU VAL GLY
              130                 135                 140                 145                 150
ALA LEU THR LYS VAL TYR SER TYR ARG PHE VAL TRP TRP ALA ILE SER THR ALA ALA MET LEU TYR ILE LEU TYR
              155                 160                 165                 170                 175
VAL LEU PHE PHE GLY PHE THR SER LYS ALA GLU SER MET ARG PRO GLU VAL ALA SER THR PHE LYS VAL LEU ARG
              180                 185                 190                 195                 200
ASN VAL THR VAL VAL LEU TRP SER ALA TYR PRO VAL VAL TRP LEU ILE GLY SER GLU GLY ALA GLY ILE VAL PRO
              205                 210                 215                 220                 225
LEU ASN ILE GLU THR LEU LEU PHE MET VAL LEU ASP VAL SER ALA LYS VAL GLY PHE GLY LEU ILE LEU LEU ARG
              230                 235                 240                 245
SER ARG ALA ILE PHE GLY GLU ALA GLU ALA PRO GLU PRO SER ALA GLY ASP GLY ALA ALA ALA THR SER
```

FIG. 32. Primary structure of bacteriorhodopson: ⟨Glu (pyroglutamic) is at the amino terminus.

in the purple membrane as shown by Gerber and colleagues in this laboratory (*114*) is such that the carboxyl end is on the cytoplasmic side of the membrane with a run of at least 20 amino acids exposed into the cytoplasm (*114*); (5) more recently, the total amino acid sequence of the protein was determined at MIT (*116, 117*) and in Ovchinnikov's laboratory (*118*). This sequence is shown in Fig. 32. Sequence determination in our hands (no details of any experimental work or the methods employed by the Russian workers have appeared) necessitated the development of new methodology for handling the hydrophobic protein and its fragments. Noteworthy features are the use of gel permeation with organic solvents and of reverse-phase high-pressure liquid chromatography for separation of the fragments and of a well-balanced combination of gas chromatograph–mass spectrometry and automated Edman degradation for sequencing as well as sequencing of hydrophobic fragments after attachment to a functionalized porous glass.

The immediate tasks are to determine the sites and orientation of the turns in helixes which protrude out of the membrane.

In attempts at reconstitution of the purple membrane with synthetic phospholipids, the purple membrane has been delipidated to the extent of 90–95% in this laboratory (*119*). Partial delipidation has also been described by Overath's laboratory (*120*).

With the elucidation of the primary amino acid sequence, the problems coming into sharp focus are the nature of the turns in the polypeptide chains, the three-dimensional structure of the protein in the membrane, and the chemistry of the cycle of changes it undergoes in the total process of transporting a proton from the inside to the outside medium.

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