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Studies on the Biosynthesis and Functions of Lipids in Bacterial Membranes

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The atmosphere pervading the current scene in membrane research is, in many ways, reminiscent of that existing a decade ago when much of the excitement in the biological sciences was focused on molucular biology, and the mechanisms of transcription and translation of the genetic code. Then—as now—workers with training in diverse areas of the biological sciences reoriented their research efforts to meet a new challenge.

Why is there all the current excitement and research activity dealing with membranes? Why are so many biochemists, biophysicists, and, ironically, molecular biologists interested in membranes? I think that the answer to this question lies in the fact that all life is typified by the presence of membranes and yet the elucidation of their structure and functions remains as one of the major challenges in the field of biochemistry. All cells have a cytoplasmic membrane that serves to isolate them from the "outside world." Moreover, within the cell, especially the more highly evolved nucleated eukaryotic cells, membranes serve to separate and compartmentalize the multitude of processes that together are essential for the maintenance of the life of the cell.

We understand most of the metabolic processes operating within the cell, and the techniques of electron microscopy and X-ray crystallography have afforded a rather clear picture of the molecular structure of many of the macromolecules found in cells. By comparison, our understanding of the structure and function of membranes, be they plasma, nuclear, mitochondrial, or other cellular membranes, is primitive.

Why is it that so little is known about such a vital component of all cells? The answer is directly related to the complex chemical composition of the membrane. This complexity is not apparent when one observes that the overall composition of membranes, on a weight basis, is approximately 50% lipid and 50% protein. Some idea of the degree of complexity becomes apparent, however, when one refines this statement and realizes that the lipid component is composed of at least a half-dozen different classes of lipids, and that within each class there are at least a half-dozen individual molecular species resulting from structural variations in the fatty acid chains.

Considering the protein component the numerology is equally, if not more, staggering. No one knows how many different proteins are present in a membrane, but it is clear from analysis of membrane proteins by techniques that dissociate them into polypeptide chains that most membranes contain at least 50 to 100 different polypeptide chains.

Finally, the full complexity of membranes is revealed when one realizes that all of these components interact, presumably in some orderly fashion, to produce a supramolecular structure that not only serves as a *selective* permeability barrier, but contains a host of enzymes that are involved in both anabolic and catabolic processes. Moreover, at least in the bacteria, it is likely that the cytoplasmic membrane is responsible for synthesis of at least part of itself, since the enzymes of phospholipid biosynthesis are an integral part of it.

The study of such a complex system offers challenges that biochemists and biophysicists have been unable to meet with techniques that were developed for analysis of cellular components. The biochemists' forte has been purification. Techniques to disrupt cells and isolate and characterize a specific macromolecule in its native form are well developed and highly successful. Biochemists now routinely deal with multimolecular complexes of enzymes and with more complex supramolecular structures such as ribosomes and bacteriophages. These components exist in an environment that is, for the most part, aqueous and the techniques to isolate and study them involve the use of aqueous media. But the membrane and therefore the molecules that comprise it exist in a nonaqueous, hydrophobic environment. As a consequence, many of the biochemical techniques devised during the past several decades simply cannot be applied to the analysis of membrane structure.

Extensive use has been made of biophysical techniques in studying the structure of membranes. Ana-

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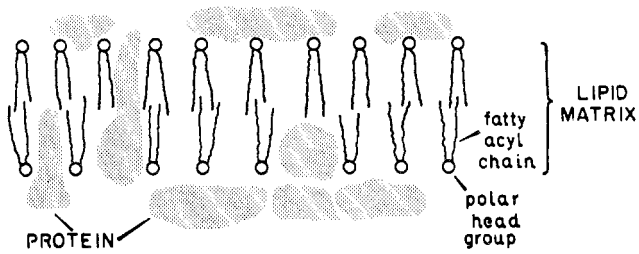

Figure 1. Bilayer model for membrane structure.

lytical tools such as nmr, ORD, CD, esr, and calorimetry, which have provided useful information on the structure of pure proteins or lipids in an aqueous environment, have afforded some information on the structural organization of these components in membranes, but the complexity of the system under study severely limits interpretation of the results of such studies. These techniques have been applied with more success in simplified model membrane systems lacking the protein components. In an aqueous environment lipids, especially phospholipids, spontaneously form bilayers. Physical studies on such bilayer systems have been informative and many parallels with natural membranes have been observed, but the fact remains that these are model membranes, not natural ones.

Electron microscopy and, more recently, X-ray diffraction techniques have been applied to membrane structures. Taking into account the results of these studies, as well as all the other chemical and physical chemical studies, it appears clear that the basic structure of the membrane approximates the working model of Danielli and Davson[1] proposed over 30 years ago, that is, a matrix consisting of a bilayer of lipid molecules, the polar ends of which are at the periphery of the bilayer. Until recently it was thought that the proteins were all found on the exterior of this lipid bilayer, but more recent evidence, summarized by Singer,[2] suggests that some proteins may be partly or completely bound within the hydrophobic interior of the bilayer (Figure 1).

Approaches to the Study of Bacterial Membrane Structure and Function

I turn now to a consideration of some aspects of the current state of knowledge of membrane structure, strictly limiting the discussion to bacterial membranes, since they have been the main focus of our research. As will be evident, the primary emphasis will be on the lipids and lipid-synthesizing enzymes of the membranes. The major questions that are of interest to us are: (1) what is the structure of the membrane, and how is it synthesized? (2) What is the role of the lipids in the structure, synthesis, and functions of the membrane? These are multifaceted questions for which there are, as of yet, no simple answers. By way of introduction

(1) J. F. Danielli and H. Davson, *J. Cell Comp. Physiol.*, **5**, 495 (1934–1935).
(2) S. J. Singer and G. L. Nicholson, *Science*, **175**, 720 (1972).

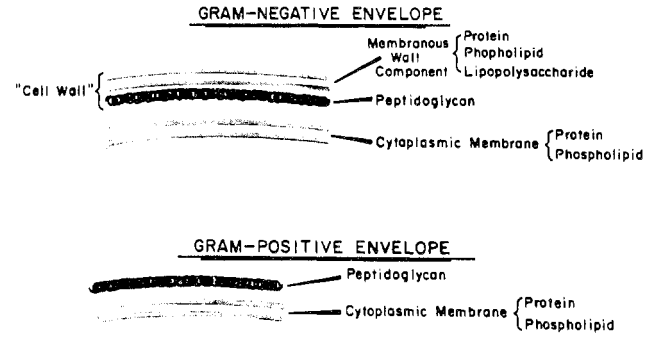

Figure 2. Structure of the cell envelope of gram-negative and gram-positive bacteria.

it is worthwhile to try to categorize the different approaches taken by various workers in order to obtain answers to them.

One approach is strictly analytical and involves isolation and characterization of the components of the membrane. This is not only important *per se*, but essential if one wishes to study the question of membrane synthesis and the possibility that the membrane is formed by a self-assembly process. Therefore it is important not only to characterize the components of the membrane, but also to devise means to isolate them in the native form, or in a form that does not preclude reversion to the native state.

A second approach also requires a knowledge of the constituents of the membrane but relies primarily on studies of the effect of various reagents or probes on the structure and function of the intact membrane. Many probes have been used, ranging from treatment with proteolytic enzymes to introduction of spin-labeled fatty acyl chains into phospholipids.

A third approach is to effect controlled change in a single molecular component of the membrane by genetic means. Techniques for variation in the components of the membrane have, until recently, been limited to environmental alterations resulting from changes in growth media, oxygen supply, temperature, etc. However it is clear that a much more powerful tool for *selective* alteration in membrane composition is genetics. Gradually, a number of techniques for the isolation of membrane mutants are being developed, and the potential of this apprach, as witnessed by its utility throughout the history of modern biochemistry, is obvious.

Enzymes of Phospholipid Synthesis in the Envelope of *Escherichia coli*. Because so many aspects of the metabolic and genetic activities of *E. coli* have been studied, it is not surprising that a great deal of the work on bacterial membranes has centered on this organism. In fact, however, gram-negative bacteria, including *E. coli*, have a much more complex cell envelope than the gram-positive bacteria. Only recently has a coherent picture of its structure emerged. As shown in Figure 2, it is believed that the cell envelope of *E. coli* is composed of two membranous components: the outer wall, which contains protein, phospholipid, and lipopolysaccharide, and is associated with the rigid

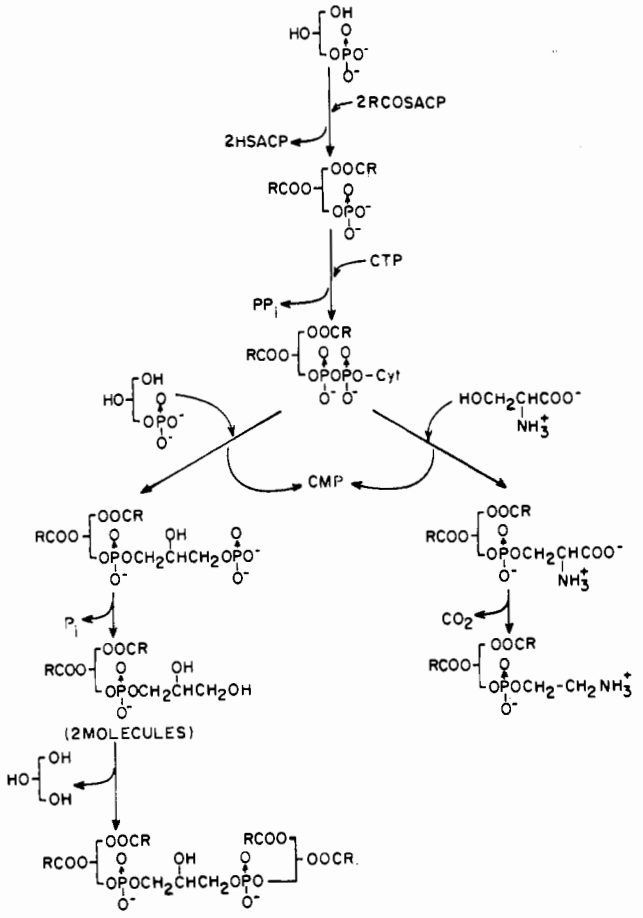

Figure 3. Pathways of biosynthesis of the common bacterial lipids.

peptidoglycan layer which gives the cell its structural stability; and the inner, cytoplasmic membrane. Methods for separating and isolating the membranous wall from the cytoplasmic membrane have been devised,[3,4] and each has been examined by electron microscopy.[3] Both components show the trilaminar structure which is typical of most membranes and which is usually interpreted in terms of the classical bilayer model for membrane structure.

The protein compositions of the two membranous fractions differ considerably. The membranous wall appears to contain fewer proteins than the membrane, and one polypeptide (or one class of polypeptides of identical molecular weight) comprises over 50% of the protein of the wall.[3] In contrast, in the cytoplasmic membrane, no one protein is predominant and a wide range of the polypeptide chains of differing molecular weights are observed.

The phospholipids, which comprise the major fraction of the total lipids of the unfractionated envelope, consist primarily of phosphatidylethanolamine (*ca.* 75%), with lesser amounts of phosphatidylglycerol and diphosphatidylglycerol. With the availability of isolated wall and cytoplasmic membrane it has been possible to perform comparative analyses of these sub-

(3) C. A. Schnaitman, *J. Bacteriol.*, **104**, 890 (1970).
(4) D. A. White, W. J. Lennarz, and C. A. Schnaitman, *ibid.*, **109**, 686 (1972).

Table I
Distribution of Enzymatic Activities for Phospholipid Synthesis in Envelope Fractions[a]

	Envelope	Wall	Membrane	Membrane/Wall
Glycerol phosphate acylation	11.1	1.5	64.0	42.5
CDP-diglyceride synthesis	13.5	2.2	53.0	24.0
Phosphatidylglycerol synthesis	98.8	8.4	293.1	34.7
Phosphatidylserine decarboxylation	17.1	6.7	19.4	13.5
Phosphatidylserine synthesis	10.8	1.9	2.3	1.2

[a] Values expressed as specific activity in nmoles of product/mg of protein per 20 min.

fractions of the envelope.[4] Both contain phospholipid, although the proportion of total phospholipid to protein is greater in the membrane than in the wall. The wall contains lipopolysaccharide, which is absent from the cytoplasmic membrane. Conversely, coenzyme Q, and succinic dehydrogenase, both components of the respiratory chain, are almost exclusively localized in the cytoplasmic membrane. With regard to the phospholipid composition of each membranous component, the differences are quantitative rather than qualitative[4] (see below).

Earlier studies from a number of laboratories defined the pathway of biosynthesis of the three major lipids of *E. coli* and showed that the enzymes involved in these reactions were particulate, and therefore probably part of the cell envelope.[5] As shown in Figure 3 the basic pathway is *via* acylation of glycerol 3-phosphate to phosphatidic acid which is converted to cytidine diphosphate (CDP) diglyceride. This serves as a common activated precursor of phosphatidylethanolamine and phosphatidylglycerol. Diphosphatidylglycerol is formed by a novel transphosphatidylation reaction between two molecules of phosphatidylglycerol.[6]

Because both membranous wall and cytoplasmic membrane contain the same phospholipids it was of interest to determine the site of synthesis of these lipids. As shown in Table I, with one exception, all the biosynthetic enzymes studied are localized in the inner, cytoplasmic membrane.[7] These findings have been corroborated by Bell, *et al.*,[8] who have also shown that diphosphatidylglycerol synthetase is localized in the inner membrane. The one exception among these anabolic enzymes is phosphatidylserine synthetase, which apparently is associated with the ribosomes.[9] It is possible, of course, that *in vivo* the ribosomes themselves are associated with the cytoplasmic membrane, but in any event it is clear that this enzyme is atypical with regard to its mode of interaction with the membrane.

(5) Studies cited by W. J. Lennarz in "Metabolism and Function of Lipids," S. Wakil, Ed., Academic Press, New York, N. Y., 1970, p 155.
(6) C. B. Herschberg and E. P. Kennedy, *Proc. Nat. Acad. Sci., U. S.*, **69**, 648 (1972).
(7) D. A. White, F. R. Albright, W. J. Lennarz, and C. A. Schnaitman, *Biochim. Biophys. Acta*, **249**, 636 (1971).
(8) R. M. Bell, R. D. Mavis, M. J. Osborn, and P. R. Vagelos, *ibid.*, **249**, 628 (1972).
(9) C. Raetz and E. P. Kennedy, *J. Biol. Chem.*, **247**, 2008 (1972).

These findings on the asymmetric distribution of the enzymes of phospholipid synthesis evoke a consideration of the origin of the phospholipids of the outer membranous wall. Is there a specific transport mechanism to translocate phospholipids synthesized in the inner membrane to the outer membrane? Or are there regions in the envelope where these two membranes are contiguous, and thus allow flow of the newly formed lipids to the outer membrane? There is some evidence for regions of contiguity[10] and for mobility of lipids in membranes (see below), so perhaps the latter hypothesis is more likely to be correct.

Mutants of *E. coli* as Tools in Studying Membrane Structure. As noted above one approach to understanding membrane structure is the study of the effect of mutational changes on the properties of the membrane or its component enzymes. So far, the most useful mutants are those first isolated by Vagelos and coworkers[11] and found to be defective in synthesis of unsaturated fatty acids. Since unsaturated fatty acids added to growth medium are almost exclusively incorporated into phospholipids, with such auxotrophs it has been possible to study a number of questions related to the role of phospholipids in various membrane functions. Although it is beyond the scope of this review to discuss these studies in detail, a few salient findings can be mentioned. It is clear that, when various membrane-specific processes, *e.g.*, respiration or transport of glycosides, are studied as a function of temperature, biphasic Arrhenius plots are observed.[12] Moreover, the transition temperature of these biphasic curves varies as a function of the exogenous fatty acid supplied, being lowest for cis polyunsaturated, intermediate for cis monounsaturated, and highest for trans unsaturated fatty acids. Thus, these membrane functions are correlated with the degree of chain–chain interaction of the acyl chains in the phospholipids. Wilson and Fox[12] have taken advantage of this phenomenon in studying the question of whether or not synthesis and introduction of an inducible transport protein into a membrane require synthesis of new lipid for functional expression of the transport activity. Their conclusion, based on shifts from one type of fatty acid to another, and the effect of this on the temperature characteristic of the transport process, were that there was a requirement for new lipid synthesis. However, studies by Overath, *et al.*,[13] do not support this contention and, moreover, indicate rapid randomization of the lipid phase of the membrane. These findings, of course, do not necessarily mean that certain proteins in the membrane do not have an affinity for a specific phospholipid class or a given molecular species of phospholipid within that class. In fact, studies by Esfahani, *et al.*,[14] and by Mavis and Vagelos[15] on the effect of different fatty acids on the temperature dependence of the activity of various membrane bound enzymes are consistent with this possibility.

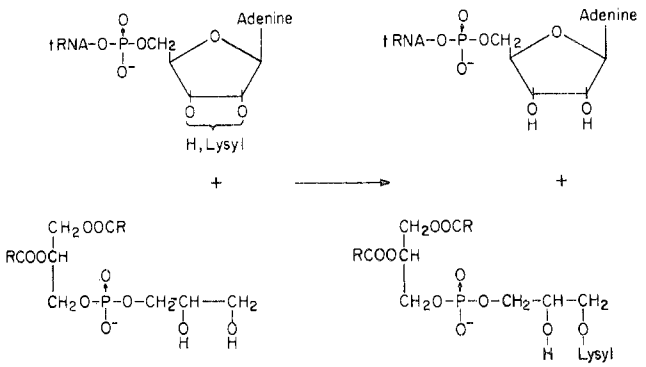

Figure 4. Transaminoacylation reaction involved in biosynthesis of lysylphosphatidylglycerol from lysyl-tRNA.

Studies on the Function of Lipids in Membranes of Gram-Positive Bacteria

As noted above, in general the gram-positive bacteria have a simple cell envelope consisting of a single, inner cytoplasmic membrane bounded by the rigid peptidoglycan layer (Figure 2). Because isolation of the cytoplasmic membrane ghosts can be readily accomplished by enzymatic removal of the peptidoglycan, much of our research effort has been devoted to a study of the metabolism and function of the lipids and lipid-synthesizing enzymes in these organisms. Two classes of lipids that, shortly after their discovery, attracted our interest with regard to a possible function in cell membranes were the aminoacyl derivatives of phosphatidylglycerol and the glycosyl diglycerides.

Aminoacyl Derivatives of Phosphatidylglycerol. Our initial efforts were concerned with the mechanism of biosynthesis of lysylphosphatidylglycerol and the other commonly occurring aminoacyl derivative, alanylphosphatidylglycerol. Not unexpectedly, the formation of these compounds proceeded by aminoacylation of phosphatidylglycerol, but it was a surprise to find that the aminoacyl donor was aminoacyl-tRNA.[16] Prior to this work the only known instance of participation of aminoacyl-tRNA in processes other than protein synthesis was in the formation of the polypeptide cross-links in the cell wall peptidoglycan.[17] However, in the case of aminoacylphosphatidylglycerol the reaction is clearly a transesterification, involving transfer of the aminoacyl group from the diol system of the terminal ribosyl moiety of tRNA to the diol system of the glyceryl moiety of phosphatidylglycerol (Figure 4). The transferases that catalyze this reaction are associated with the cytoplasmic membrane, whereas the synthetases involved in formation of the various aminoacyl-tRNAs are found in the cytoplasm. Specificity studies

(10) C. A. Schnaitman, *J. Bacteriol.*, **108**, 545 (1971).
(11) D. F. Silbert and R. R. Vagelos, *Proc. Nat. Acad. Sci. U. S.*, **58**, 1579 (1967).
(12) G. Wilson and C. F. Fox, *J. Mol. Biol.*, **55**, 49 (1971).
(13) P. Overath, H. U. Schairer, and W. Stoffel, *Proc. Nat. Acad. Sci. U. S.*, **67**, 606 (1970).
(14) M. Esfahani, A. R. Lumbrick, S. Knutton, T. Oka, and S. J. Wakil, *ibid.*, **68**, 3180 (1971).
(15) R. D. Mavis and P. R. Vagelos, *J. Biol. Chem.*, **247**, 652 (1972).
(16) W. J. Lennarz, J. A. Nesbitt, and J. Reiss, *Proc. Nat. Acad. Sci., U. S.*, **55**, 934 (1966).
(17) M. Matsuhasi, C. P. Dietrich, and J. L. Strominger, *ibid.*, **54**, 587 (1965).

have revealed that the membrane-associated transferases responsible for synthesis of lysylphosphatidylglycerol and alanylphosphatidylglycerol recognize both the aminoacyl and the polyribonucleotide moieties of the aminoacyl-tRNA.[18,19]

Since the discovery of aminoacyl derivatives of phosphatidylglycerol there has been much speculation on their function, primarily because the linkage in aminoacylphosphatidylglycerol, like that in aminoacyl-tRNA, is a "high-energy" bond. In turning our efforts to the question of function we showed that the lysyl group of lysylphosphatidylglycerol did undergo turnover in growing cells.[20] However, a comparison of the rate of turnover with the rate of lysine uptake by cells indicates that the turnover process must not serve as a mechanism for lysine transport, since its rate is much lower than the transport rate. Moreover, chase experiments revealed that the lysine released from lysylphosphatidylglycerol enters the free lysine pool, thus making it unlikely that the lysylphosphatidylglycerol serves as a direct lysyl group donor in any biosynthetic process. This possibility was also specifically excluded in the case of incorporation of the lysyl group found in the pentapeptide side chain of the cell wall peptidoglycan; *in vitro* studies showed that lysylphosphatidylglycerol does not replace lysine and ATP, the previously reported substrates for this reaction.[21]

As earlier reported[22] and confirmed in our studies,[20] the proportion of the two major lipids found in *S. aureus*, phosphatidylglycerol and lysylphosphatidylglycerol, is dependent on the pH of the growth medium. As compared to neutral pH, the level of negatively charged phosphatidylglycerol decreases at low pH, whereas that of the positively charged lysylphosphatidylglycerol remains almost constant.[20] The net effect is that the effective charge of the membrane lipids becomes more positive at low pH. It had earlier been suggested that this charge change would serve to permit growth at low pH (high external proton concentration) because proton entry through the membrane would be minimized.[22] Two different approaches were used to test this idea. First, we turned to model membrane bilayers, prepared from positively charged lysylphosphatidylglycerol, neutral glycolipids, and negatively charged phosphatidylglycerol, and studied the permeability of these to protons, as well as to other positively charged cations.[23] These studies with a model system clearly showed that bilayers with a net positive surface charge were, in fact, less permeable to protons. A second approach was to search for mutants of *S. aureus* that were unable to grow at low pH, in order to see if such mutants lacked the ability to synthesize lysylphosphatidylglycerol.[24] Although a number of mutants unable to grow at low pH (pH 5.2) were obtained, the correlation between pH sensitivity and lysylphosphatidylglycerol content was tenuous. Moreover, this study revealed that the alteration in the lysylphosphatidylglycerol to phosphatidylglycerol ratio was rather complex and not simply a function of the pH of the growth medium. Although subsequent studies by others[25] are consistent with the original hypothesis on control of membrane permeability to protons, it remains unproven.

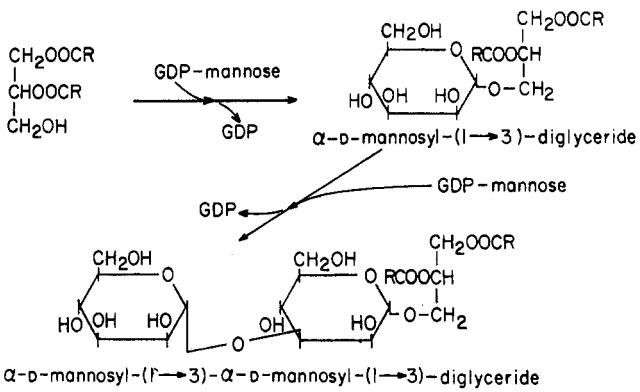

Figure 5. Biosynthesis of mannosylmannosyl diglyceride.

Glycolipids. As noted above, the other class of lipids found in gram-positive bacteria that intrigued us with regard to function were the glycosyl diglycerides. Following the initial preliminary characterization of mannosyldiglyceride in *M. lysodeikticus* by MacFarlane,[26] we investigated the structure and biosynthesis of this compound.[27] As shown in Figure 5, the glycolipid actually is mannosylmannosyl diglyceride, and it is formed in a two-step process involving glycosylation of diglyceride to form mannosyl diglyceride, which is converted to mannosylmannosyl diglyceride. The biosynthetic origin of the diglyceride substrate is uncertain, but recent studies indicate that it is formed by action of a phosphatase on phosphatidic acid.[28] Thus it appears that in gram-positive bacteria phosphatidic acid serves as a branch point, either retaining its phosphate group upon conversion to CDP diglyceride (which serves as precursor of both phosphatidylglycerol and phosphatidylethanolamine; *cf.* Figure 3), or being dephosphorylated and subsequently glycosylated.

The function of the glycosyl diglyceride remains obscure. Attempts to demonstrate that mannosylmannosyl diglyceride serves as a mannosyl donor in some membrane-associated biosynthetic process were fruitless, the rate of turnover of the mannosyl moieties being extremely slow compared to the growth rate.[29]

(18) J. A. Nesbitt III and W. J. Lennarz, *J. Biol. Chem.*, **243**, 3088 (1968),
(19) R. M. Gould, M. P. Thornton, V. Liepkalns, and W. J. Lennarz, *ibid.*, **243**, 3096 (1968).
(20) R. M. Gould and W. J. Lennarz, *J. Bacteriol.*, **104**, 1135 (1970).
(21) J. A. Nesbitt III and W. J. Lennarz, cited in ref 19.
(22) U. M. T. Houtsmuller and L. L. M. Van Deenen, *Biochim. Biophys. Acta*, **84**, 96 (1964).
(23) U. Hopfer, A. L. Lehninger, and W. J. Lennarz, *J. Membrane Biol.*, **2**, 41 (1970).
(24) C. M. Kent, S. Krag, and W. J. Lennarz, manuscript in preparation.
(25) C. W. M. Haest, J. De Gier, J. A. F. Op Den Kamp, P. Bartels, and L. L. M. Van Deenen, *Biochim. Biophys. Acta*, **255**, 720 (1972).
(26) M. G. MacFarlane, *Biochem. J.*, **80**, 45P (1961).
(27) W. J. Lennarz and B. Talamo, *J. Biol. Chem.*, **241**, 2707 (1965).
(28) S. Krag and W. J. Lennarz, unpublished studies.
(29) W. J. Lennarz, unpublished studies.

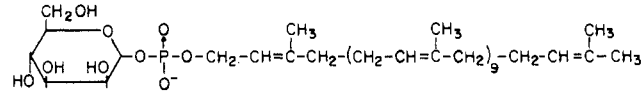

Figure 6. Structure of mannosyl-1-phosphorylundecaprenol.

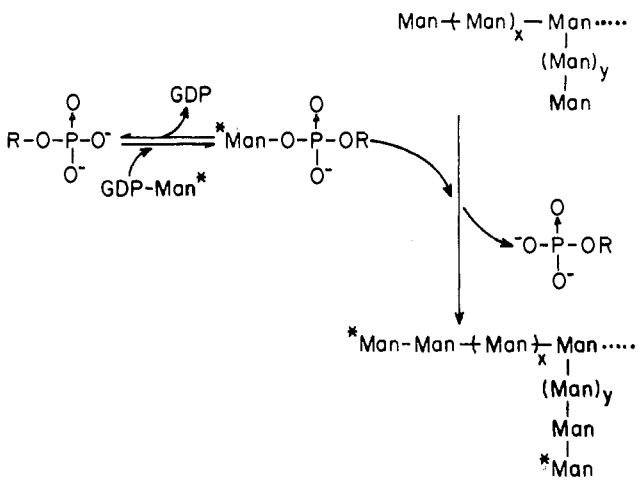

Figure 7. Role of mannosyl-1-phosphorylundecaprenol in mannan synthesis. R represents the undecaprenyl residues.

However, during the process of studying the *in vitro* biosynthesis of the mannosyl diglycerides, the formation of a third, mannose-containing lipid with very different chemical properties was detected.[27] Chemical analysis, along with mass spectrographic and nmr studies, proved that it was mannosylphosphorylundecaprenol, the structure of which is shown in Figure 6.[30]

Since the lipophilic moiety as well as the general structure of mannosylphosphorylundecaprenol was found to be similar to that reported to be involved in peptidoglycan[31] and O-antigen biosynthesis,[32] it seemed reasonable that it served as a mannosyl group carrier. Earlier studies by MacFarlane[33] had shown that the cytoplasmic membrane of *Micrococcus lysodeikticus* contains a mannan, and it was possible to demonstrate the *in vitro* formation of mannan-^{14}C in the presence of a membrane preparation and guanosine diphosphate mannose-^{14}C.[34] More detailed studies revealed that this reaction proceeds within the membrane in two steps.[35,36] In the first step the mannosyl group of guanosine diphosphate mannose is transferred to undecaprenyl phosphate in a freely reversible reaction. This lipophilic activated derivative of mannose then acts as a mannosyl donor, transferring the hexose to terminal nonreducing ends of molecules of mannan

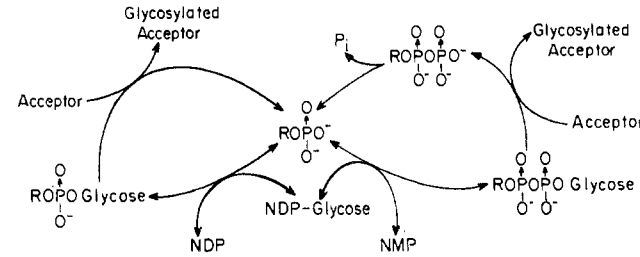

Figure 8. Role of undecaprenyl phosphate as a carrier in glycan synthesis. R represents the undecaprenyl residue and N represents nucleoside.

associated with the membrane. The overall process is outlined in Figure 7. Whether or not the *de novo* synthesis of mannan also involves mannosylphosphorylundecaprenol is not yet known.

It is now known that a variety of processes involving biosynthesis of glycans that exist within or beyond the hydrophobic permeability barrier are mediated by lipid intermediates.[37] Of course, in each case different sugars are involved and in some instances the hexose 1-phosphate, rather than the hexose, is transferred to undecaprenyl phosphate, with production of hexose pyrophosphorylundecaprenol. However, in each instance the glycosyl lipid intermediate ultimately serves as a glycosyl donor in the synthesis of the glycan, and the undecaprenyl phosphate released or regenerated upon hydrolysis of undecaprenyl pyrophosphate is reutilized as carrier in another cycle of the biosynthetic process (Figure 8). Thus despite many speculations over the years on roles for various lipids as "carriers" in the membrane, undecaprenyl phosphate constitutes the only definitive example of such a role. Clearly it serves to convert sugars activated in the form of hydrophilic sugar nucleotides to hydrophobic, but still activated, forms that can permeate and function within the cell membrane.

Studies on the Membranes of Bacilli

Recently we have undertaken a detailed study of the membranes of *Bacilli*. Two different approaches have been taken, but the ultimate aim of both of them is to investigate the reassembly of membrane components *in vitro*.

Dissociation and Reaggregation of Ghosts. Purified ghosts of *Bacillus megaterium* have been isolated and shown to contain all the enzymes necessary for synthesis of the two major phospholipids of this organism, phosphatidylethanolamine and phosphatidylglycerol, from the common precursor, phosphatidic acid (*cf.* Figure 3).[38] In searching for procedures that can be used to solubilize membranes under conditions whereby the dissociated components can be reaggregated, we have found that treatment with sodium dodecyl sulfate (SDS) in the presence of 7.5 *M* urea shows the most

(30) M. Scher, W. J. Lennarz, and C. C. Sweeley, *Proc. Nat. Acad. Sci. U. S.*, **59**, 1313 (1968).
(31) Y. Higashi, J. L. Strominger, and C. C. Sweeley, *ibid.*, **57**, 1878 (1967).
(32) A. Wright, M. Dankert, P. Fennessey, and P. W. Robbins, *ibid.*, **57**, 1798 (1967).
(33) M. G. MacFarlane in "Metabolism and Physiological Significance of Lipids," R. M. C. Davson and D. N. Rhodes, Ed., John Wiley, New York, N. Y., 1964, p 399.
(34) M. Scher, K. Kramer, and W. J. Lennarz, 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1967, Abstract No. D 43.
(35) M. Scher and W. J. Lennarz, *J. Biol. Chem.*, **244**, 2777 (1969).
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(37) Studies cited in W. J. Lennarz and M. Scher, *Biochim. Biophys. Acta*, **265**, 417 (1972).
(38) P. Patterson and W. J. Lennarz, *J. Biol. Chem.*, **246**, 1062 (1970).

promise.[39] The rationale of this procedure, using a combination of two protein denaturants, is to avoid, or at least minimize, irreversible denaturation by the stepwise removal of the denaturants. The SDS, which frequently irreversibly denaturates proteins, is removed first by means of ion exchange resin, followed by removal of urea by dialysis. Under these conditions over 85% of the proteins and 95% of the phospholipids originally present in the membrane reaggregate in the presence of Mg^{2+}. Subsequent to the initiation of our studies, Weber and Kuter[40] reported that several soluble, purified enzymes that normally are irreversibly inactivated by SDS can be reactivated by this two-step procedure.

Because the enzymes of phospholipid synthesis are responsible for synthesis of over one-third of the weight of the membrane, it seems likely that they play a key role in the structural organization of it. Consequently we have focused our efforts on a study of the recovery of activity of these enzymes after dissociation–reaggregation. So far, the results of this study are very encouraging, and it has been possible to recover activity in the enzymes responsible for synthesis of CDP-diglyceride, phosphatidylserine, and phosphatidylglycerol.[41] In the presence of added phospholipids, which perhaps serve as a core or matrix for reaggregation of the enzymes into an active form, over 90% of the original activity of these enzymes can be recovered in the reaggregate. Currently we are trying to develop procedures for the fractionation and isolation of the solubilized form of these enzymes as well as the other proteins of the membrane. Concurrently, we are studying the reaggregation process by electron microscopy in order to determine if the reaggregated material, in the absence or presence of exogenous phospholipid, shows the trilaminar morphology characteristic of the original ghosts.

Mutants with Osmotically Fragile Protoplast Membranes. Another approach, involving the use of mutants, is being used to study the structural organization of the membrane of the *Bacillus subtilis*.[42] However, in contrast to previous genetic approaches in which the selection of mutants was based on screening for a defect in a specific enzyme required for synthesis of a membrane component (*e.g.*, unsaturated fatty acids), we undertook to screen for structurally defective membranes. The criterion arbitrarily chosen for selection was osmotic fragility. That is, we searched for mutants the protoplasts of which were osmotically unstable under conditions where protoplasts of the wild type were stable. The membranes of these mutants were examined by disc gel electrophoresis for alterations in protein composition by use of a double isotope technique after admixture with wild-type membranes. Several mutants were found to contain alterations in the content of one or more proteins. However, the relationship of these alterations to the osmotic fragility of the protoplasts of these organisms remains to be established.

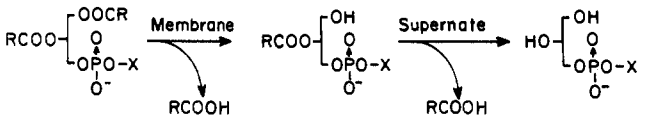

Figure 9. Proposed catabolism of phospholipids in *Bacillus subtilis* CMK 33.

In addition to proteins, the membranes of the mutants having osmotically fragile protoplasts have been compared with the wild type in terms of phospholipid and fatty acid composition. With one exception, the phospholipids, as well as their component fatty acids, are qualitatively unchanged in all mutants studied. This one exception, however, may prove to be most interesting in regard to control of phospholipid catabolism. Intact cells of mutant 33 appear to have a normal phospholipid composition. However, upon conversion to protoplasts there is a rapid breakdown of phospholipid, with concomitant lysis. This breakdown has been shown to result from the combined action of a phospholipase A_1 in the membrane and a lysophospholipase in the cytoplasm (Figure 9). Protoplasts or isolated membranes of the wild type do not manifest such catabolic activity. Most interesting, however, is the fact that the wild type contains an inhibitor of the phospholipase activity of the mutant. Thus, our working hypothesis is that mutant 33 is defective in synthesis of an inhibitory protein whose function is to regulate the activity of the phospholipase. A study of this inhibitor should be most enlightening, since relatively little is known about the control of catabolism of phospholipids in bacteria.

Concluding Comments

Our knowledge of the detailed structural organization of the membrane is still very limited. As is often the case in research, progress has not been limited by lack of interest, but rather by lack of appropriate experimental methods. Only when general biochemical techniques to deal with membrane proteins are developed, and when physical chemical methods such as electron microscopy and X-ray analysis are further refined, will a clear picture of the structure of biological membranes emerge.

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