CHEMISTRY OF PHOSPHOLIPIDS IN RELATION TO BIOLOGICAL MEMBRANES

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ABSTRACT

The quantitative determination of molecular species of natural phospholipids gave new information about the pairing of the fatty acid chains in a given lipid class. Cells appear to be equipped with enzymes which control the composition and pairing of hydrocarbon chains of phospholipids and display regulatory mechanism(s) which allow for adaptation of physical properties of membrane lipids to alteration in environmental conditions. Phospholipids containing various types of fatty acid combinations encountered in membranes have been prepared by chemical synthesis. Examination of these compounds in artificial membrane systems demonstrated that chain-length, degree of unsaturation, and the type of pairing of hydrocarbon chains determine the rate of diffusion of non-electrolytes and efficiency of carrier mediated transport across the hydrocarbon barrier. Comparison with natural membranes of different lipid composition revealed a close similarity with the model systems. This endorses the conclusion that the detailed chemical make-up of the lipid dictates the permeability behaviour of the region of the biological interface.

The diversity of polar headgroups of phospholipids is demonstrated by the polyglycerol phospholipids of bacterial membranes. Detailed information about the structure of amino acyl and glucosamine derivatives of phosphatidyl glycerol has been obtained by combination of chemical synthesis and enzymatic methods.

I. INTRODUCTION

Phospholipids are essential constituents of all living cells. By a combination of lipophilic and hydrophilic groups within one molecule, physical properties are attained which make them particularly suitable compounds to serve as major constituents for biological interfaces. Although the basic chemical structure of a phospholipid is a relatively simple one, the variations encountered in the chemical make-up of phospholipids in biological membranes offer a vast field for investigation not only to chemists of natural products but to biochemists and molecular biologists as well. Confining this discussion to one class of phospholipids (*Figure 1*), the so-called phosphoglycerides, it can be stated that nature produces phospholipids with one to at least four hydrocarbon chains, but that the compounds with two apolar tails dominate. Lipid–lipid association involving London–van der Waals interactions

between the apolar residues of the phospholipids contributes to the arrangement of a palisade alignment so as to give lipid barriers which separate aqueous compartments. On the other hand, it has been suggested during recent years that hydrophobic interactions between the hydrocarbon chains of lipids and apolar regions of the proteins are of paramount importance

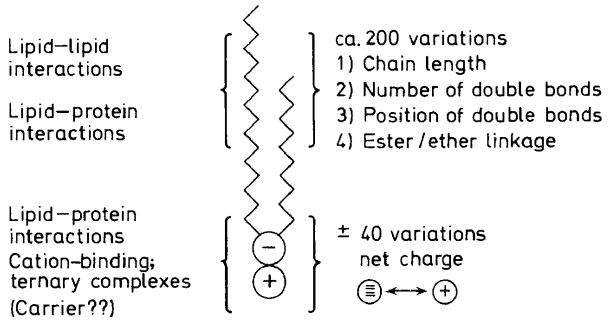


Figure 1. Phospholipid skeleton in relation to its functions

for the integrity of the lipoproteins in biological membranes. A bewildering variation in chemical nature of the hydrocarbon chains provokes many questions with respect to a possible rationale between structure and function of this part of the phospholipid molecule. Similarly, most conspicuous chemical differences have been found in the hydrophilic moiety of phospholipids. Polar headgroups containing e.g. amino alcohols, cyclitols, aminoacids, hexoses, hexosamines and a varying number of phosphate residues have been detected. As a result a great variation in net charge of phospholipid molecules can be encountered in many membranes. Electrostatic forces may bind polar groups of lipids to oppositely charged groups of proteins and perhaps of other lipid species, while in addition, cations may be involved in linking negatively charged groups of different molecules so as to form ternary complexes. Different views have been expressed about the importance of the electrostatic interactions between lipids and proteins and as a result highly conflicting models for biological membranes have been proposed. However, a certain degree of non-uniformity in membrane structure is likely in view of the multiple functions of biological interfaces. The chemical heterogeneity of both lipid and protein constituents also favours the opinion that within the framework of one interface different molecular arrangements involving various types of lipid–lipid and lipid–protein may contribute to the correct functioning of the various regions of the membrane concerned.

The chemistry of phospholipids in combination with enzymology and well-defined artificial model systems can contribute towards solving some of the compelling problems around the molecular architecture and dynamic behaviour of biological interfaces.

II. MOLECULAR SPECIES OF PHOSPHOGLYCERIDES

The analytical data on the fatty acid composition of phospholipids which have been accumulated during the past decade could easily fill a sizeable volume of an encyclopedia. Some two hundred different fatty acid and aldehyde constituents have been recognized and many facts have been discovered about their occurrence in a great variety of cells[1]. The composition of the apolar moiety of phospholipids present in a given membrane often is a reflection of the capacities for fatty acid biosynthesis of the organism concerned. In addition it has been clearly demonstrated that environmental factors can influence to a significant extent—but not randomly—the make-up of the apolar part of phospholipids. A considerable amount of work was invested to separate the various classes of phospholipids containing different polar headgroups, and to determine their fatty acid composition. In this way, many preferential combinations between particular headgroups and hydrocarbon chains were detected, which raised many questions not only with respect to function, but also concerning the biosynthetic machinery responsible for attaining these associations. The next logical step was the quantitative determination of the pairing of fatty acid constituents within one phospholipid class.

1. Analysis of molecular species

A phospholipid such as lecithin (phosphatidylcholine) present in a given membrane cannot be considered as one chemical entity but rather must be thought of as a family of related species which have in common the polar headgroup but vary with respect to the nature of the apolar tails. The lecithin preparation isolated from one membrane may contain some ten different fatty acid constituents and these can be combined theoretically in a great number of pairs. Phospholipase A_2 hydrolysis will furnish information about the location of the constituents at the C_1 and C_2 fatty acid ester position, but usually the information obtained does not warrant a complete molecular description of the phospholipid (*Figure 2*). It is possible to unravel a phospholipid family by using chromatography on silicic acid impregnated with silver nitrate[2]. Although a certain degree of subfractionation can be achieved by subjecting the phospholipids directly to the chromatographic procedure a higher degree of resolution can be obtained by abolishing the polar character of the phospholipid[3]. For many purposes, this can be easily done by hydrolyses with phospholipase C and fractionating the diglycerides produced in this manner (*Figure 2*). The separation of the species appears to depend on the degree of unsaturation of the fatty acid constituents, the position of the double bonds and the distribution of the various acyl constituents among both fatty acid ester positions. Information about the localization of the different fatty acid constituents can be obtained by hydrolysing the separated diglyceride fractions with pancreatic lipase, which enzyme exhibits a specific action on the C_1 fatty acid ester position. As an example, some earlier results are presented on lecithin from human erythrocyte membrane, which could be analysed in terms of at least twenty

major molecular species[4] (*Figure 3*). By further refinements of the analyses many minor species were identified[5].

The lecithin species can be subdivided into four classes, which will be discussed briefly.

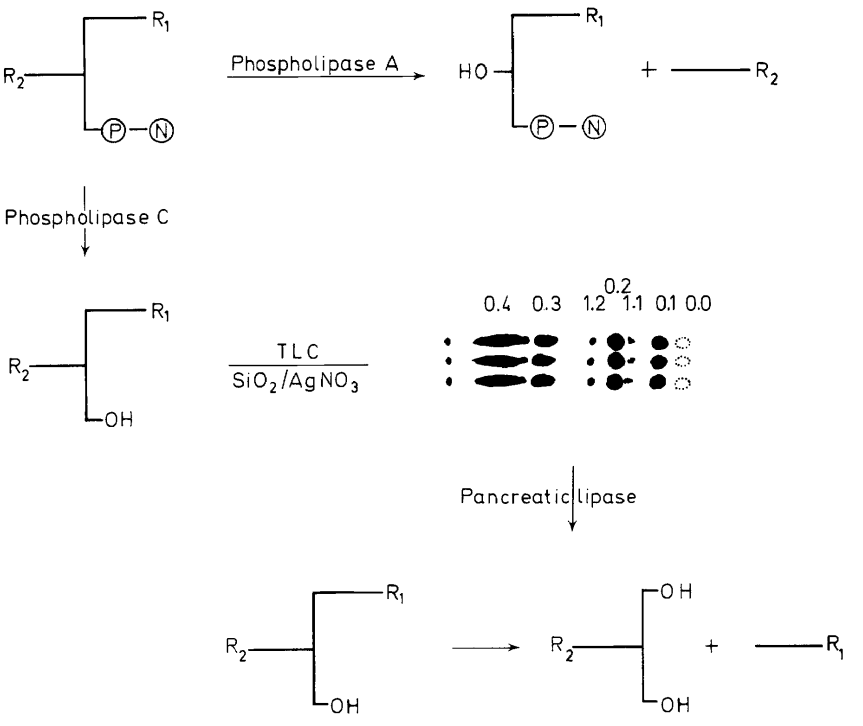


Figure 2. Principles for the analysis of molecular species of phosphoglycerides

A. Di-saturated species

Analysing the data from *Figure 3*, it can be said that a fully saturated species abundant in the lecithin of the human erythrocyte is 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (dipalmitoyl-GPC). Remarkably, the closely related species such as distearoyl or dimyristoyl-GPC are hardly more than minor species if present at all. A mixed-acid species viz. 1-stearoyl-2-palmitoyl-*sn*-glycero-3-phosphorylcholine appears to be present.

B. Di-unsaturated species

The total amount of species containing either two identical or two different unsaturated fatty acids cannot be ignored. Most conspicuous in this group are the species containing at least one mono-unsaturated chain, e.g. 1-oleoyl-2-linoleoyl-GPC and dioleoyl-GPC. However, species containing two poly-unsaturated fatty acids appear to be extremely rare.

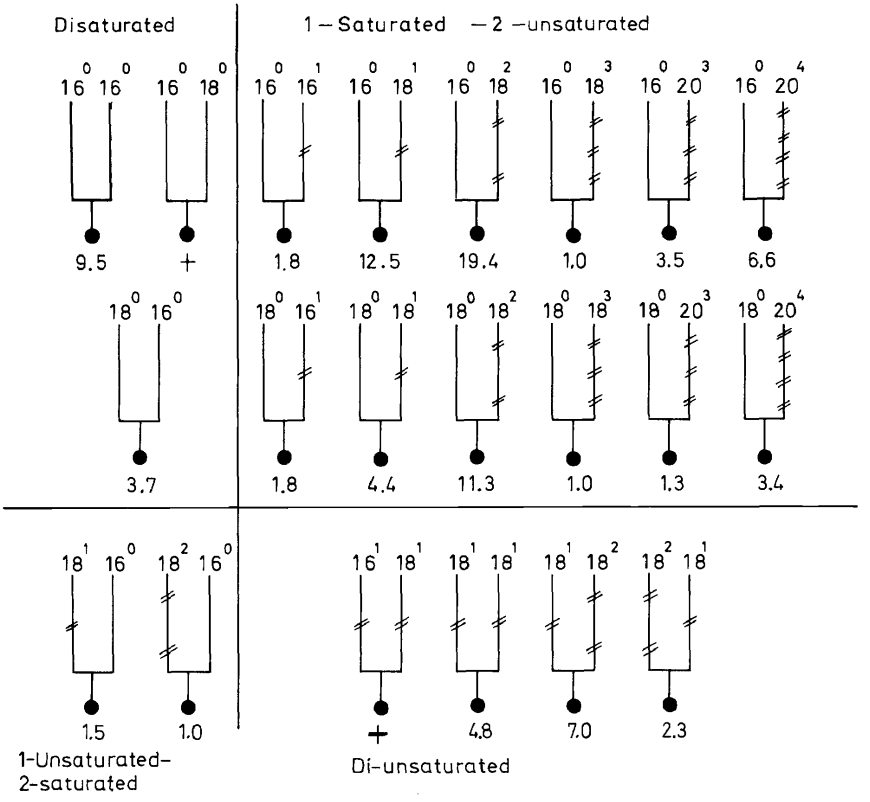


Figure 3. Major molecular species of lecithin from human erythrocyte membrane[4]

C. Unsaturated–saturated species

As could be expected on the basis of previous studies on the fatty acid distribution with the aid of phospholipase A_2 the category of species having an unsaturated fatty acid constituent at the 1-position and a saturated at the 2-position (e.g. 1-oleoyl-2-palmitoyl-GPC) are in the minority.

D. Saturated–unsaturated species

The mixed-acid lecithins containing a saturated and an unsaturated chain at the C_1 and the C_2 positions respectively appear to be predominant in this membrane. The saturated fatty acid constituents are palmitate and stearate mainly, while the unsaturated ones include oleate, linoleate and arachidonate.

Analyses performed on other phospholipid classes found in the erythrocyte membrane (phosphatidyl ethanolamine, phosphatidyl serine, inositol phospholipids, sphingomyelin etc.) reveal a similarly complex pattern. Taking into account the occurrence of saturated and unsaturated ether linkages in phospholipids, one arrives at an estimate of several hundreds of chemically different lipid molecules in the red cell membrane. The phospholipids of certain microorganisms appear to be somewhat more simple in terms of

species composition[6]. Although more work has to be done in this area, the comparative analyses carried out to date appear to support the conclusion that with evolution the molecular composition of phospholipids has become more complex. As will be discussed later in this paper chemically different phospholipid molecules may be rather similar with respect to their physical properties and may be able to a given extent to fulfil a similar function in a biological membrane.

Limiting this discussion to lecithin from mammalian origin the question can be raised whether the data presented on the molecular species of lecithin from human erythrocyte membrane are a safe guide line for further exploration of the possible connections between structure and functions. Species analysis on some thirty lecithin preparations[7] from different tissues of several mammals enable us to give a positive answer to this question, although some further considerations are necessary. It is relevant to note that in the lecithin families significant quantitative differences exist in the ratio of different lecithin molecules from various organs of one animal species, while in addition also qualitative variations have been found to occur. As an example, some species analyses of lecithin from a number of organs from pig (*Figure 4*) are presented. Concerning the disaturated species it can be stated again that

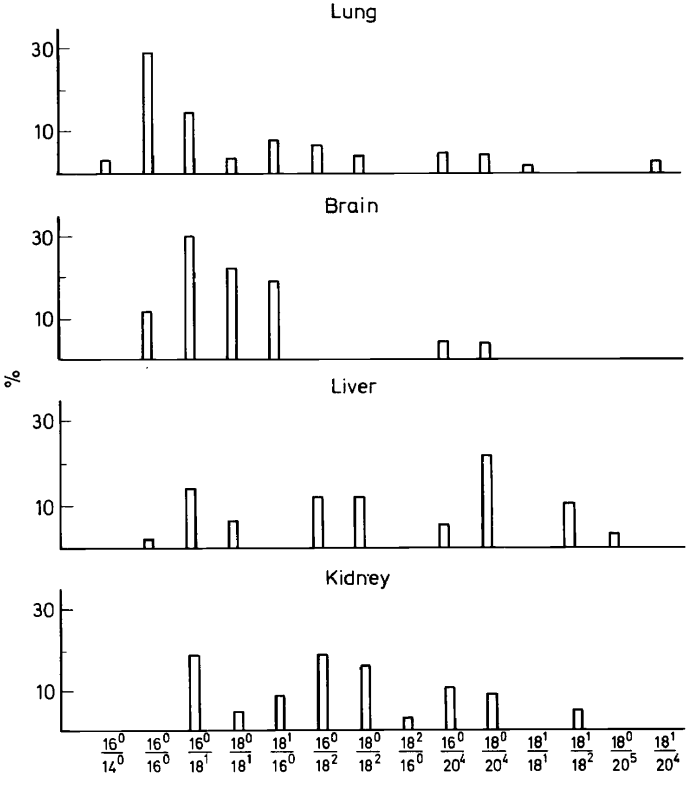


Figure 4. Variations in molecular species composition of lecithin of several organs from pig[7]

the most prominent member in this subclass is the dipalmitoyl-GPC. In all samples tested so far distearoyl-GPC was barely present. This may have significant meaning with respect to the properties desired by the membrane of its phospholipid constituents. The absence of lecithin species such as dilauroyl- and dimyristoyl-GPC is interesting in view of the finding that phospholipids such as the former (and even more didecanoyl-GPC) cause a rapid lysis of erythrocytes[8,9]. The significant quantity of dipalmitoyl-GPC found in lung tissue is not unique for pig. This species is also present in a high quantity in lung tissue from other mammals (*Figure 5*) and this phenomenon may be

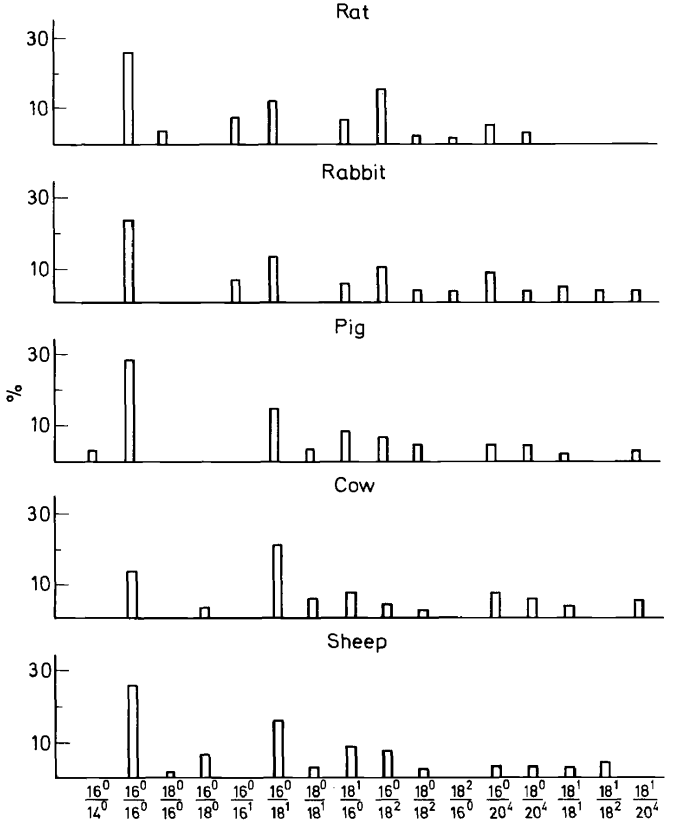


Figure 5. Major molecular species of lecithin from lung tissue from different mammals[4,1]

related to the presence of this saturated lecithin in the alveolar lining of the lung. Although notable quantities of species with two identical or two different unsaturated acids were found, the species containing two poly-unsaturated fatty acid constituents seem to be rather rare in phospholipids in mammalian tissues. While in general the make-up of the species found in the lecithins of various organs follows the trends deduced from the analysis of

lecithin from red cell membrane, some exception has to be made with respect to the kidney. In this organ notable quantities of the unsaturated-saturated subclass (e.g. 1-linoleoyl-palmitoyl-GPC) can be found[7, 10, 11]. However, in all lecithins studied the species having saturated fatty acid at the C_1 position and poly-unsaturated fatty acids at the C_2 position prevail.

It must be emphasized that the molecular species composition of lecithins of mammalian organs, particularly of liver, is greatly dependent on environmental circumstances. Reference can be made to studies with diets devoid of essential fatty acids[11, 12]. Under these conditions the fully saturated lecithin species increase to only a slight extent but species with linoleate and arachidonate are replaced by species containing eicosatrienoate at the C_2 position. Furthermore, the absence of unsaturated fat in the diet leads to an

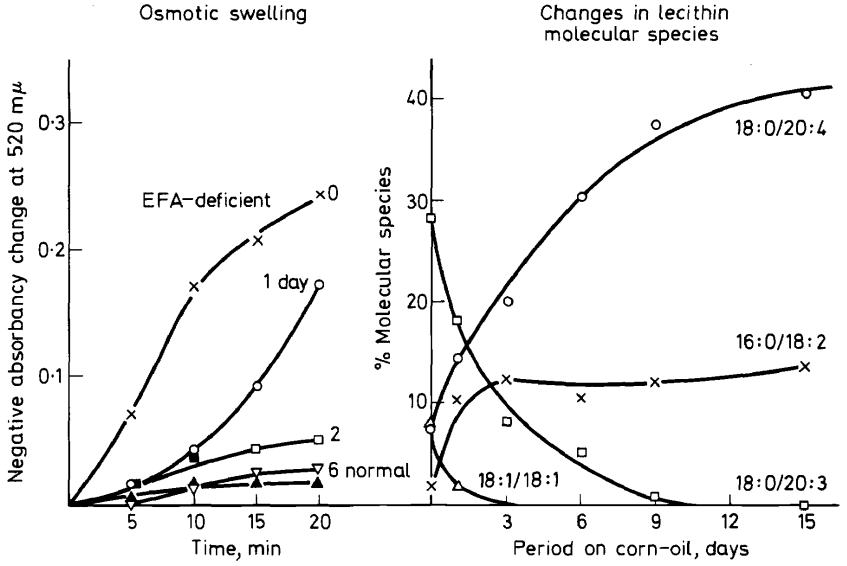


Figure 6. Changes induced in swelling of rat liver mitochondria (left) and in species composition of lecithin (right) by feeding of corn oil to EFA-deficient rats as a function of time[12, 14]

augmentation of those species containing mono unsaturated fatty acid constituents. It appears that the shifts in molecular composition are not random and that the organism makes an attempt to maintain as much as possible certain physical characteristics of the phospholipids. In the case of EFA deficiency this reaction apparently is not fully adequate. This is demonstrated also on the level of membrane properties in as much as mitochondria isolated from the liver of EFA-deficient rats exhibited a high tendency to swell[13, 14]. Feeding of a linoleic acid containing diet brings about a rapid replacement of the 'abnormal species' by those containing linoleate and arachidonate as well as a normalization of the quantities of various other species (*Figure 6*). The feeding of corn oil for 48 h to the EFA-deficient rats

reduced the high rate of swelling of mitochondria nearly to the normal level. Studies of this type indicate the importance of the molecular composition of phospholipids for membrane properties (see also section II, 4) and indicate the existence of a metabolic machinery which includes regulatory mechanisms so as to provide the membrane with suitable phospholipids.

2. Metabolic pathways of molecular species

The analysis of molecular species of phospholipids and the increasing notion that there exists an intimate relation with membrane function challenges many investigators concerned with lipid metabolism. The ultimate make-up of the apolar moiety of phospholipids appears to be controlled at the level of both fatty acid biosynthesis and of that of the enzymes catalysing phospholipid biosynthesis, which have to select from the fatty acyl-CoA pool chains of different apolarity in order to attain particular combinations.

The preferential distribution of saturated and poly-unsaturated fatty acid constituents among the C_1- and C_2- positions of e.g. rat liver lecithins has been the subject of much investigation. Several groups tackled the question at which stage of phospholipid metabolism this particular distribution of fatty acids was introduced. Various possibilities can be envisaged (*Figure 7*). The *de novo* synthesis of lecithins proceeds via acylation of glycerophosphate so as to give phosphatidic acid[15]. After dephosphorylation

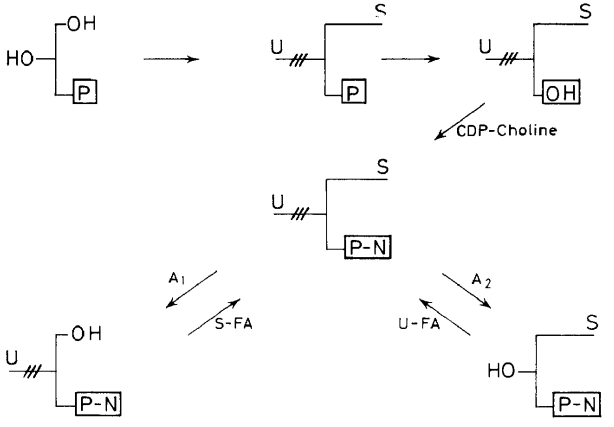


Figure 7. Pathway of phosphoglyceride biosynthesis and renewal of fatty acid constituents. Control of positional distribution of saturated (S) and unsaturated (U) fatty acid constituents

of this key-intermediate by phosphatidic acid phosphatase, the diglycerides accept phosphorylcholine from cytidine diphosphorylcholine with the participation of a choline phosphotransferase[16,17]. The lecithins produced by this pathway may be subject to enzymatic hydrolysis by phospholipase A, which cleaves one fatty acid ester linkage so as to give monoacyl-glycerophosphorylcholine (lysolecithin). It was found that in mammalian tissues such as rat liver two lipolytic activities are present denoted as phospholipases A_1 and A_2 which are specific in hydrolysing the fatty acid ester linkages at

C_1 and C_2 so as to form a 2-acyl-*sn*-glycero-3-phosphorylcholine and 1-acyl-*sn*-glycero-3-phosphorylcholine respectively[18,19] (*Figure 7*). It had been previously established that rat liver was capable of reacylating both monoacyl phosphoglycerides and that these conversions displayed a specificity compatible with the non-random fatty acid distribution found in lecithin[20,21] (and phosphatidyl ethanolamine). Earlier *in vitro* experiments indicated that the acylation of glycerophosphate may proceed in a random manner[22]. On the basis of these observations it was argued that phosphoglycerides such as lecithin produced by the *de novo* pathway at first would consist of a mixture of species not having the characteristic fatty acid distribution, and that the deacylation–reacylation cycle having the isomeric lysolecithins as intermediates was responsible for introducing the preferential localization of saturated and unsaturated fatty acid constituents. This, however, appears to be a rather uneconomic sequence of events[23].

Recent studies have demonstrated that the origin of the non-random distribution of fatty acids is achieved to a great extent during the first step of the *de novo* synthesis of phosphoglycerides. One research group was able to demonstrate that in microsomal preparations from rat liver a high degree of selectivity of incorporation of saturated and unsaturated fatty acids into the C_1 and C_2 positions of phosphatidic acid occurs[23,24,25] (*Table 1*). Another

Table 1. Positional distribution of radioactive fatty acids incorporated into phosphoglycerides of rat liver microsomes

Fatty acid	*Phosphatidylcholine*		*Phosphatidylethanolamine*		*Phosphatidic acid*	
	1-pos.	*2-pos.*	*1-pos.*	*2-pos.*	*1-pos.*	*2-pos.*
16:0	83	17	87	13	72	28
18:0	90	10	86	14	86	14
18:2	6	94	22	78	16	84
18:3	12	88	32	68	20	80

group did not arrive at this conclusion with respect to the microsomal system, but demonstrated that in liver slices the incorporation of fatty acids into phosphatidic acid occurs in a non-random manner[26]. Two other groups established that in diglyceride biosynthesis the preferential distribution of saturated and unsaturated fatty acids is attained as well[27,28].

The view that unsaturated fatty acids are preferentially incorporated into the C_2 position and saturated fatty acids predominantly at the C_1 position in the first step of phosphoglyceride synthesis was supported by analysis of the trace amounts of phosphatidic acid found in rat liver, which revealed a positional distribution similar to that of the end products[25] (*Table 2*). However, the overall fatty acid composition of the precursor appears to be quantitatively rather different from that of the end products. Most striking is the fact that phosphatidic acid has less than half of the amount of arachidonate when compared with lecithin. This difference could be brought about in theory by a specificity of choline phosphotransferase (assuming that phosphatidic acid phosphatase is non-specific). A preferential use of those diglycerides containing arachidonic acid, however, has not been established.

By contrast, it was found that the synthesis of lecithin by rat liver microsomes in the presence of [^{14}C]-CDP choline was stimulated to the same extent by diglycerides containing one, two and four unsaturated double bonds[29]. When differently labeled diglycerides of varying degrees of unsaturation were incubated in different ratios with unlabeled CDP-choline the lecithins

Table 2. Composition and fatty acid distribution in phosphatidic acid and lecithin isolated from rat liver

	Phosphatidic acid		*Lecithin*	
16:0	27		26	
18:0	23		25	
18:1	19		8	
18:2	22		17	
20:4	9		24	
	1-pos.	*2-pos.*	*1-pos.*	*2-pos.*
16:0	46	8	48	2
18:0	38	2	47	1
18:1	11	30	5	12
18:2	5	35	1	35
20:4	—	25	—	47

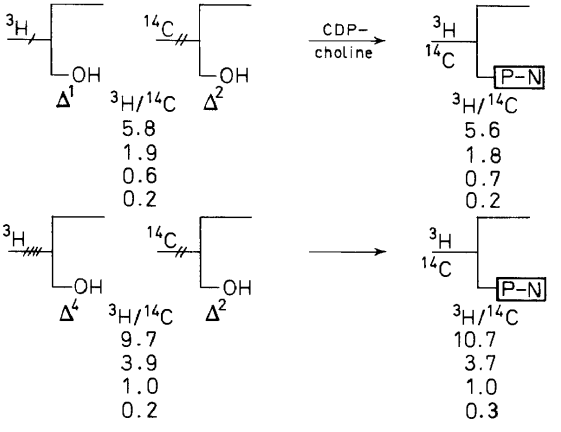


Figure 8. Utilization of diglyceride species in lecithin biosynthesis by rat liver microsomes[29]

produced reflected an identical alteration in isotopic ratio[29] (*Figure 8*). This lack in specificity of choline phosphotransferase from rat liver with respect to various molecular species of diglycerides *in-vitro* makes it unlikely that this enzyme is responsible for the introduction of arachidonic acid during *de novo* synthesis sufficient to account for the amount actually found in lecithin (and phosphatidyl ethanolamine). Another candidate for this function could be the deacylation–acylation cycle (*Figure 7*).

As mentioned before, it was demonstrated that 1-acyl-glycero-3-phosphoryl-choline preferentially reacts with an unsaturated fatty acyl group[20]. The acyl residue in the 1-acyl-GPC appeared to have less influence on the rate of acyl transfer than the nature of the acyl group on the CoA ester[30,31]. On the other hand, 2-acyl-GPC preferentially stimulates the uptake of saturated acyl chains. Experiments with synthetic[32,33,34] 2-acyl-GPC and 1-acyl-GPC having differently labeled acyl chains enabled one to follow their conversion into molecular species of lecithin[35,36]. The results were in accordance with the view that 1-acyl-GPC is preferentially acylated with unsaturated fatty acids, whereas 2-acyl-GPC is better acylated with saturated fatty acids (*Figure 9*). The labeled acyl constituents were recovered

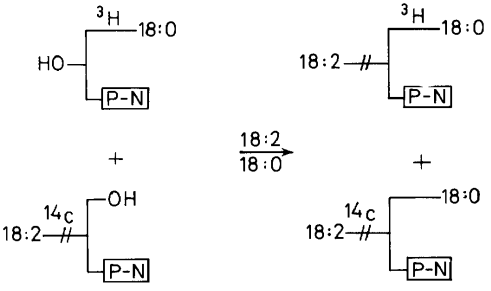


Figure 9. Selective acylation of isomeric lysolecithins by rat liver microsomes[36]

in newly formed lecithins at essentially the same positions as those at which they were originally located in the lysolecithins[35,36]. The action of phospholipase A_2 and A_1 in liver will give rise to the formation of endogenous 1-acyl-GPC containing predominantly saturated acids and of 2-acyl-GPC with unsaturated acids mainly[9]. The selective acylation of these endogenous compounds will yield lecithin species with a fatty acid distribution comparable to that occurring in the natural lecithins. Various observations appear to support the view that the deacylation–reacylation cycle not only maintains the positional fatty acid distribution but that this pathway may make final adjustments in the molecular species composition. That arachidonate is more readily introduced into lecithin than into phosphatidic acid species is in agreement with the observation that poly-unsaturated-CoA esters are better incorporated into 1-acyl-GPC than into 1-acyl-glycerophosphate[37]. As regards the acylation of 1-acyl-GPC a most significant conversion into tetraenoic species was observed[38]. The dietary experiments dealt with in the previous section suggested that different molecular species participate to a different extent in the metabolic conversions under discussion[12,39]. It was argued that replacement of eicosatrienoic species by those containing arachidonate may be accomplished to a great extent by a deacylation–reacylation cycle, while production of linoleate containing species was more dependent on the *de novo* synthesis. *In vitro* experiments with several labeled precursors appear to support this conclusion[40].

The analytical data raise many questions about the contributions of several pathways to the metabolism of individual species of phosphoglycerides in many tissues. For instance, the high content of dipalmitoyl-lecithin encountered in lung tissue poses an interesting problem. Current studies indicate that during *de novo* synthesis an appreciable quantity of this lecithin species may be formed. However, it was most striking that rat lung microsomes in contrast to liver microsomes in the presence of 1-acyl-GPC incorporated palmitate to at least the same extent as linoleate[41]. Thus the deacylation–reacylation cycle may be potentially capable of contributing to the 1,2-dipalmitoyl-GPC formation in lung as well.

The study of the enzymatic processes of phospholipid species is not only important with respect to questions of the design of tailormade membrane constituents but also intimately related to problems of biogenesis of membranes and the ending of their sometimes short life-span. This area of research involves also the transport of lipids from one membrane to another, as well as the translocation of lipids within one single membrane. Furthermore, lipids firmly bound at a given membrane may be subject to intermediary conversions concerning either their polar headgroups or hydrocarbon chains. Such conversions affecting charge distributions and hydrophobic associations can induce motion at the membrane site concerned and may contribute to the dynamic behaviour of the biological interface. These reactions may be important to the theme of rapid transformations between different lipid–protein arrangements perhaps overlapping several conflicting proposals, which incorrectly consider the membrane as a static structure.

3. Chemical synthesis of phosphoglyceride species

The analytical and biochemical investigations demonstrate that cells are equipped with enzymes which are responsible for attaining a particular molecular make-up of membrane phospholipids. The analytical data on phospholipids still have to be interpreted in terms of precise functions of these molecules in biological interfaces. If one makes a comparison with the area of nucleic acids and protein synthesis it seems that we have reached in phospholipid chemistry a stage comparable to that achieved in the field of nucleic acids around 1950.

Well-defined phospholipids and other complex lipids have to be subjected to physical examination using a great variety of techniques. Although it is possible now to analyse all naturally occurring phospholipids in terms of molecular species it is not possible to isolate all the individual members from a given phospholipid class in a pure form. This, however, can be achieved by chemical synthesis. This area of research has attracted during several decades only relatively few research groups. Various contributions were made to the preparation of phospholipids with different polar headgroups but often the synthesis was limited to fully saturated compounds, which differ from natural phospholipids and are often less suitable for physical experiments. Later, several research groups concentrated on the chemical syntheses of unsaturated phosphoglycerides. During the past decade the so-called mixed-acid phosphoglycerides having combinations of two different fatty acid constituents as found in natural phosphoglycerides became available by chemical synthesis. As an example, the pathways of the first reliable syntheses

of lecithin and phosphatidyl ethanolamine having a saturated fatty acid at C_1 and an unsaturated fatty acid constituent at C_2 are given[42,43,44] (*Figure 10*). Not only lecithins with many combinations of fatty acid constituents including poly-unsaturated ones have been prepared, but also phosphatidyl ethanolamines, phosphatidic acids and

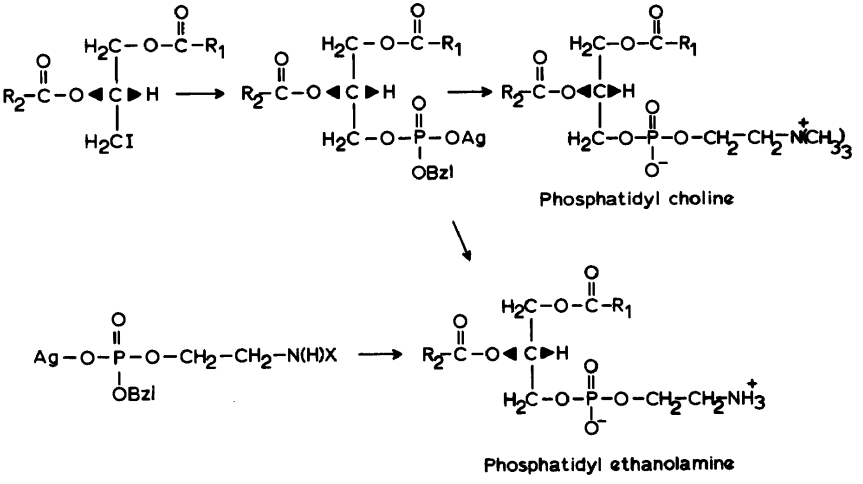


Figure 10. Principles for the chemical synthesis of mixed-acid phosphoglycerides[45,46]

phosphatidyl glycerols and various related phospholipids (see also section III) were synthesized in the mixed-acid form. For a detailed discussion of the merits of different methods of synthesis, the use of new protecting groups and various modifications, reference has to be made to two reviews which are complimentary in time[45,46]. The synthetic phosphoglycerides with defined localization of two different fatty acid constituents were of great value in the determination of the mode of action of phospholipase A. In the period 1954 to 1963 quite different opinions were expressed with respect to the question about the site of attack of this enzyme. A distinction between the various possibilities, namely *a.* a positional unspecific hydrolysis which depends on the nature of fatty acid constituents *b.* a specific action on either the C_1 or C_2 fatty acid ester linkages could be made on the basis of several isomeric pairs of synthetic mixed-acid phosphoglycerides[47–51]. It was found that phospholipase A from snake venom and pancreas hydrolyses exclusively the fatty acid ester linkage at C_2 of *sn*-3-phosphoglycerides irrespective of the nature of the fatty acid constituents (*Figure 11*). The mode of action of this enzyme—now denoted as phospholipase A_2—offered another attractive route[52,53] towards the synthesis of mixed-acid lecithin which is frequently employed. A synthesis of lecithins containing two identical fatty acid constituents can be achieved readily by acylation of *sn*-glycero-3-phosphorylcholine (GPC prepared by deacylation of natural lecithin). Degradation with phospholipase A_2 produces 1-acyl-GPC, which then can

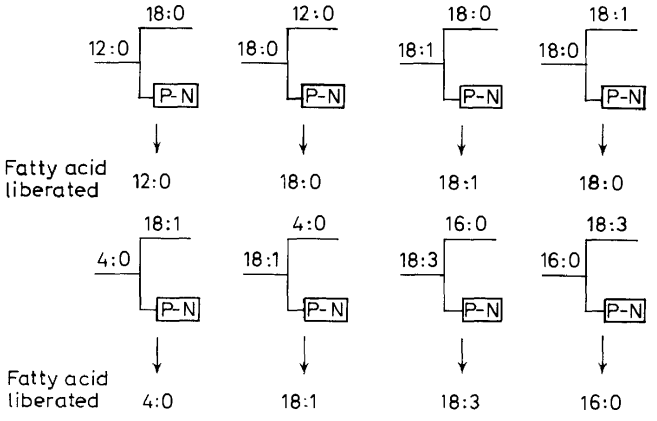


Figure 11. Specificity of action of phospholipase A_2 on synthetic phosphoglyceride species containing two different fatty acid constituents

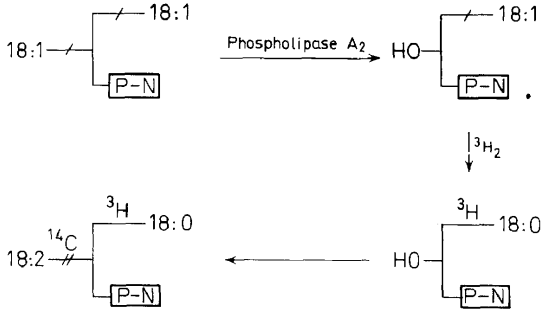


Figure 12. A partial synthesis of a mixed-acid lecithin, containing two differently labeled fatty acid constituents

be reacylated chemically with a different fatty acid constituent. The principle of this synthesis is illustrated with the preparation of a doubly-labeled lecithin (*Figure 12*). Compounds of this category have been important in establishing the occurrence of phospholipases acting on either the C_1 or C_2 position (see section II, 2) and in characterizing phospholipases localized in subcellular structures. With such substrates it has been found that also pure lipase preparations could act at the C_1 position of phosphoglycerides[54]. This finding offered a possibility for the preparation of unsaturated 2-acyl-glycero-phosphorylcholine[34] (*Figure 13*) an isomer which had escaped the chemical synthesis of the full range of lysolecithins. Because of its wide applications the substrate specificity of phospholipase A_2 has been verified with a great number of synthetic compounds[51]. In studies on intact membranes, use can now be made of pure preparations of phospholipase A isolated from snake venom or pancreatic tissue. The latter phospholipase A_2 was found to occur in the form of an inactive precursor[55] and its amino acid sequence was recently elucidated[56].

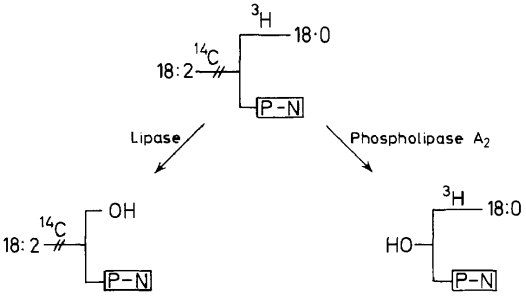


Figure 13. Preparation of isomeric lysolecithins by enzymatic hydrolysis of a synthetic lecithin

These examples are quoted to demonstrate briefly that combinations of methods of organic chemistry and enzymology are useful also in the field of phospholipids and allow the preparation of molecular species of precisely the same structure as natural phospholipid families.

4. Properties of phospholipids in model systems

In order to evaluate the properties of distinct phospholipid species in relation to their function in biological membranes use can be made of methods of surface chemistry, e.g. monomolecular layers and bimolecular lipid membranes which may serve as restricted but in some respects as valuable models for natural membranes. In this manner, important data can be provided to the molecular architect attempting to depict functional models of lipid and protein associations for the complex biological interfaces. In this respect it may be useful to include not only the synthetic phospholipids, tailormade according to the pattern provided by nature, but to study related structures not found in nature and to assess why their biosynthesis and incorporation into membranes is avoided by the cell. Conspicuous differences are often found in the chemical make-up of phospholipids in one membrane under different conditions as well as between different membranes. Whereas in many cases such structural variations may be reflected by different properties of these membrane constituents it is very possible that chemically dissimilar lipids are rather similar with respect to their physical properties.

The simple system of monomolecular layers of phospholipids which had a great impact on concepts of membrane structure for more than forty years showed considerable variations in the molecular orientation of different molecular species of phospholipids such as lecithin and phosphatidyl ethanolamine[57,58].

The mean molecular area occupied by a lecithin molecule at the air/water interface appears to increase when the saturated hydrocarbon tails become shorter (*Figure 14*). This shift from a condensed to a more expanded type of film can be explained by assuming that a decrease in London–van der Waals interaction allows a greater mobility of the chains. This is in contrast to the behaviour of different lysolecithins which revealed little difference in surface area, below the collapse pressure[59]. Introduction of an unsaturated fatty acid constituent into the lecithin also leads to considerable expansion of the film, and the space occupied by the phospholipid molecule appears to increase

with increasing unsaturation (*Figure 14*). In particular, the introduction of the first unsaturated fatty acid constituent (or a shorter saturated one) giving an 'asymmetric' molecule appears to have a significant effect on the molecular interactions. In this system there is a fair amount of similarity between phosphoglyceride species having at C_1 a saturated long-chain fatty acid and at C_2 either a mono-unsaturated, a cyclopropane or a saturated fatty acid constituent of medium chain-length[1] (C_{10}, C_{12}, C_{14}). It is intriguing

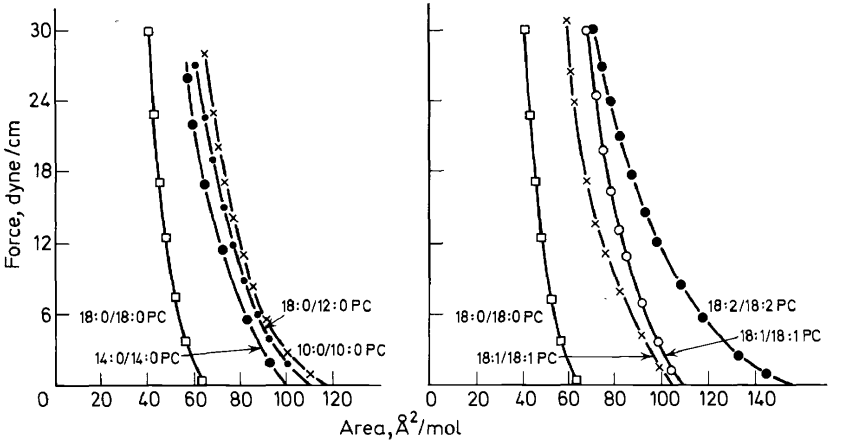


Figure 14. Force/area characteristics of monomolecular films of synthetic lecithins with different fatty acid constituents[58]. (18 :0/18 :1 PC stands for 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoryl-choline)

to note that unicellular organisms have an ability to interchange such fatty acid constituents in their phospholipids provoking the suggestion that under different conditions the physical properties of the membrane phospholipids can be maintained. Cells appear to avoid the biosynthesis of phospholipids containing two saturated fatty acids of medium chain length, e. g. didecanoyl or dilauroyl lecithin. It is of interest to note that synthetic lecithins of this type were found to be highly lytic[8] and are apparently not suitable membrane constituents.

Although some relationships between the behaviour of phospholipids in monolayer and biological phenomena can be seen, a more relevant approach may be a study of the permeability properties of bilayers of phospholipid species. For this purpose, excellent possibilities are given by liposomes, which consist of a composite array of multiple. concentric closed bimolecular membranes[60]. The liposomes behave as osmometers[61] allowing one to obtain information on the permeability of non-electrolytes with the same methods as previously applied to e.g. erythrocytes and mitochondria (*Figure 15*). As for erythrocyte, the penetration rate into the liposomes is highly dependent on temperature and on the molecular dimensions of the solute (e.g. glycol > glycerol > erythritol). Using liposomes made of various synthetic lecithin species it could be demonstrated that increase in chain

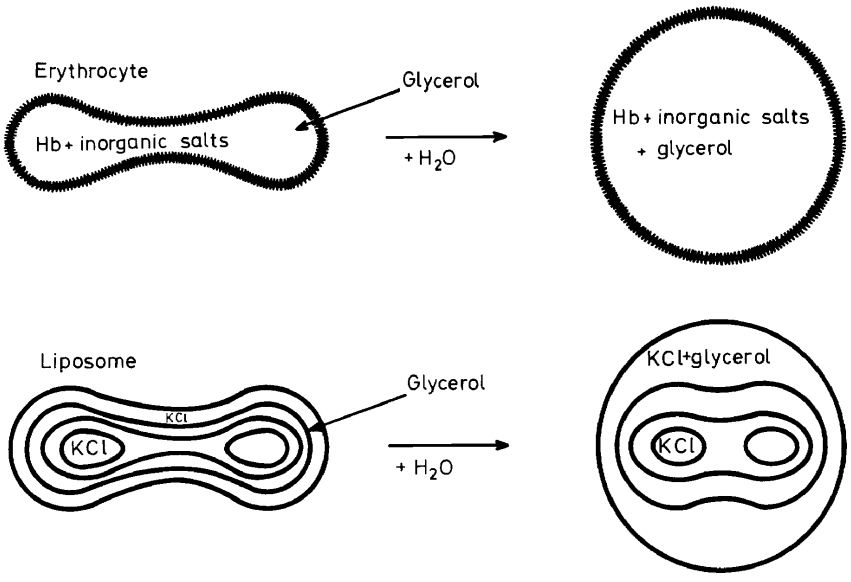


Figure 15. Osmotic behaviour of erythrocytes and liposomes

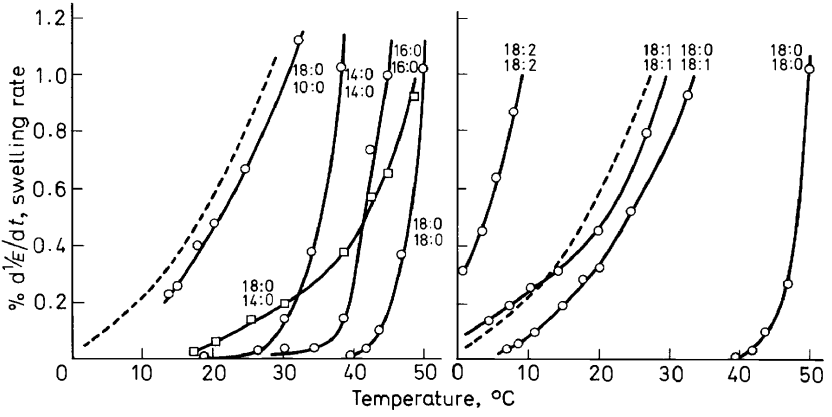


Figure 16. Effects of chain length and unsaturation on glycerol permeability of liposomes of synthetic lecithins. Initial swelling rate in isotonic glycerol as a function of temperature. The broken line represents the swelling of egg lecithin[62]

length considerably reduces the penetration rate by glycerol molecules[62] (*Figure 16*). It is of interest to note that at physiological temperature a conspicuous difference exists between dipalmitoyl-GPC and distearoyl-GPC, the former is known to be abundant in membranes, whereas the quantity of distearoyl-GPC is very low (section II, 1). One is inclined to conclude that a tight alignment formed by distearoyl-GPC is not appropriate

for normal membrane function. In addition, a comparison of the permeability of the liposomes of 1-stearoyl-2-decanoyl-GPC and 1-stearoyl-2-myristoyl-GPC with those obtained from 1,2-dimyristoyl-GPC and 1,2 dipalmitoyl-GPC respectively, suggests that the model structures of phospholipids with two different fatty acid constituents are much more permeable than those of lecithins with two chains of equal length and the same number of paraffin carbon atoms. In accordance with the expectations from the monolayer studies, introduction of double bonds in the hydrocarbon chains causes an increase of permeability (*Figure 16*). It appears that the permeability of the liposomes from lecithins with one saturated and one mono-unsaturated chain is very close to those of lecithins with a saturated long-chain and a saturated medium-length chain. Liposomes of lecithin species containing two polyunsaturated fatty acids, which species are very rare in mammalian cells (section II, 1) appear to be very leaky. The observations on the influence of the fatty acid constituents of the phospholipids on glycerol and erythritol permeability are in agreement with data on the glucose leak from liposomes[63].

These model experiments on systems composed of phospholipids only, suggest that 'simple diffusion' through a lipid barrier may be highly dependent on the degree of packing and thermal motion of the apolar chains. Although in biological membranes the situation is more complex, the various observations suggest that in these interfaces a selection of the make-up of the lipid species may contribute so as to regulate the properties of a membrane. For example, the observation made with model systems, that an increase in the number of unsaturated lipid species enhances permeability, is of interest in relation to the adaptation of organisms towards lower environmental temperatures. For *E. coli* it has been demonstrated that when the temperature of growth decreases there is a marked decrease in saturated and an increase in unsaturated and cyclopropane fatty acid constituents[64,65]. The permeability of liposomes of the bacterial phospholipids revealed differences to be expected from the work with synthetic phospholipids suggesting that the bacteria attempt to counteract a decrease in permeability at lower temperature by increasing the degree of unsaturation of membrane lipids[65] (*Figure 17*).

Interesting possibilities are offered by the isolation of mutants of *E. coli* which require an unsaturated fatty acid for growth[66]. Fatty acids of quite different structure can be incorporated into the phospholipids indicating that the membrane is rather tolerant in this respect. However, it was recently demonstrated that these mutants also control the fatty acid composition of their lipids to a notable extent[67]. When, for instance, the degree of unsaturation of the supplemented *cis*-fatty acid was increased, relatively more saturated hydrocarbon chains and fewer unsaturated chains were found to be incorporated into the phospholipids. With a series of *cis*-mono-unsaturated fatty acids of different chain length it was found that the percentage of unsaturated fatty acid constituents of the phospholipids increases with increasing chain length. The conclusion that there exists a regulatory mechanism which has to maintain the physical properties of the membrane lipids within certain limits appears to be supported by current studies on monolayers and bilayers of the phospholipids from these mutants[68].

The impact of the nature of phospholipid species for the physical state and properties of the natural membrane—as suggested by model studies—in

principle should be demonstrated also by direct comparisons between cells of different lipid composition and the behaviour of liposomes. Good possibilities are offered by *Mycoplasma laidlawii* which has relatively simple species composition of the membrane lipids which can be altered to a given extent by supplementing different fatty acids to the medium[69]. Measurement

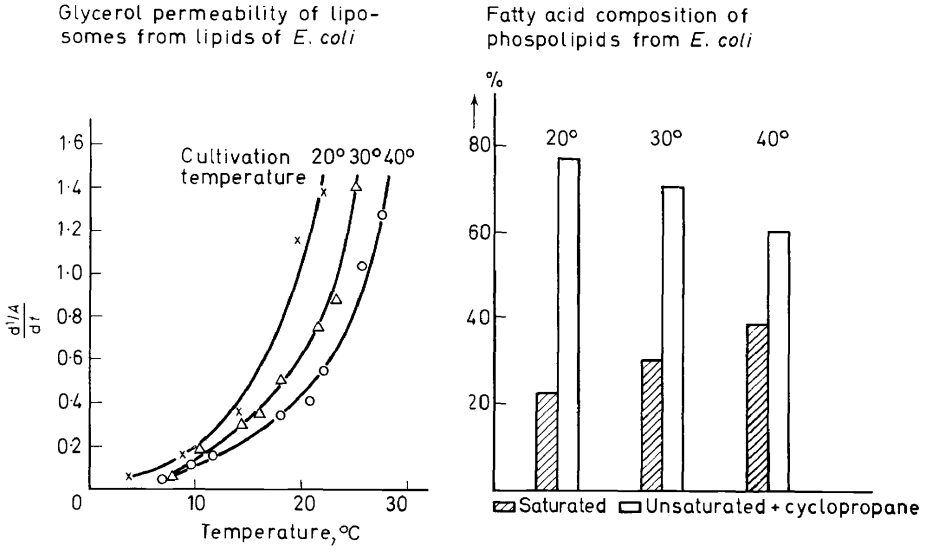


Figure 17. Effect of growth temperature of *E. coli* on the fatty acid composition of the phospholipids (right) and the permeability behaviour of liposomes[65] (left)

of glycerol diffusion into intact cells and liposomes from the *Mycoplasma* lipids showed that in both the natural and artificial membranes permeability for glycerol depends on the fatty acid composition of membrane lipids, and that the shifts in permeability observed in *Mycoplasma* cells and liposomes are in good agreement[70] (*Figure 18*). These studies confirm that there is a certain degree of tolerance in fatty acid make-up of membrane lipids but on the other hand, the composition can vary only between given limits without effecting the viability of the cells. There appear to be restrictions with respect to the degree of both unsaturation and saturation of the phospholipid species.

The barrier properties of phospholipids as dictated by the chemical nature of the apolar chains may not only contribute to the control of permeation of non-electrolytes but perhaps also affect the rate of other transport processes. This suggestion is made on the basis of observations on the valinomycin induced Rb^+ leak from liposomes[71]. It was found that the promoting effect of valinomycin is determined by the degree of unsaturation of the phospholipid species present in the bilayers (*Figure 19*). The concept that within the cell, carrier mediated transport may be dependent on the nature of the lipid constituents is being tested with biological membranes of different lipid composition.

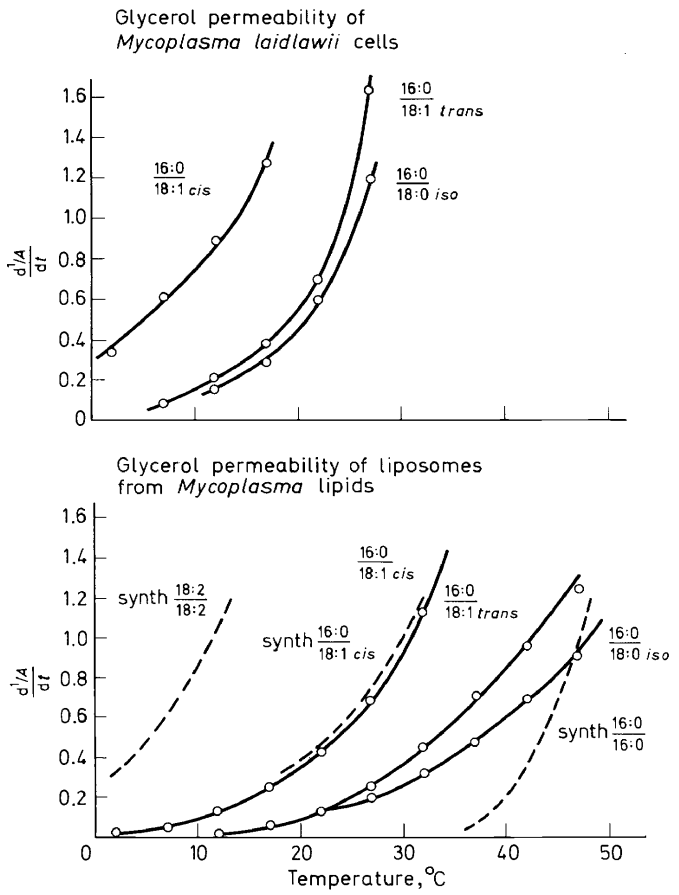


Figure 18. Effect of different fatty acid composition on the glycerol permeability behaviour of intact cells of *Mycoplasma laidlawii* (top) and liposomes of membrane lipids[70] (bottom)

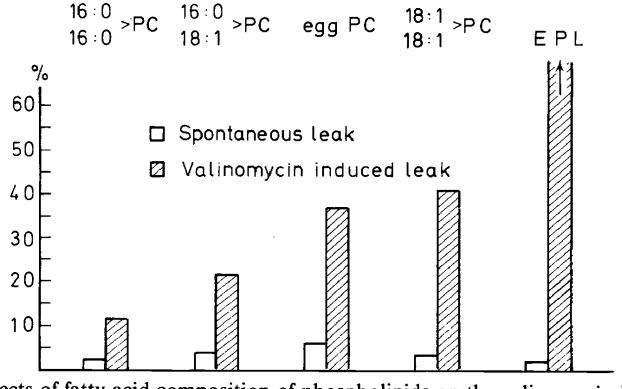


Figure 19. Effects of fatty acid composition of phospholipids on the valinomycin induced leak of $^{86}Rb^{+}$ from liposomes[71]

Apart from phospholipids, other membrane constituents can control the diffusion across biological interfaces. Experiments on monomolecular layers[57, 58] and bilayers[62, 63, 72] of well-defined phospholipid species demonstrated an effect of cholesterol. Liposomes of mixtures of phospholipid and cholesterol normally demonstrate a decrease in permeability which is proportional to the concentration of cholesterol. The presence of cholesterol appears to limit the penetration of glycerol and erythritol, the diffusion of glucose through the bilayers, but also to reduce valinomycin induced permeability of Rb^+ [71]. Extrapolation of this finding to natural membranes suggesting that the prevailing effect will be again a restriction of permeation in cholesterol rich regions of the membrane is supported by experiments with erythrocytes and *Mycoplasma.* The erythrocyte which has a molar ratio of phospholipid/cholesterol close to one can be depleted by part of its sterol. After removal of part of the cholesterol the erythrocytes exhibited a considerable increase in osmotic fragility and glycerol permeability[73]. Conversely, cells of *Mycoplasma* which contained cholesterol in the membrane revealed a decrease in glycerol permeability when compared with cells devoid of cholesterol[70].

Although many correlations can be made between model systems and natural membranes such observations do not lead of course to the conclusion that the membranes can be considered as continuous lipid-bilayers coated with protein. While some regions of a membrane may have such structures it can also be envisaged that a considerable degree of interpenetration of the lipid core by protein exists. The temperature dependence of the glycerol permeation into liposomes of total lipid (phospholipid plus cholesterol) from erythrocytes and the behaviour of the intact erythrocytes show some differences[74] indicating that the proteins also control the degree of mobility of the structural elements in the natural hydrophobic barrier. Recombination experiments with the complex mixture of proteins solubilized from erythrocyte membranes indicated that their association with lipids involves for initial association polar interactions followed by the formation of apolar bonds[75, 76]. These experiments endorse the view that a heterogeneity exists in the binding of lipids to proteins in the erythrocyte membranes. Attempts to reconstitute *in vitro* membrane structures containing lipids and proteins from erythrocytes which have the same permeability characteristics as intact erythrocytes so far were not successful. Experiments with single protein components isolated from the membrane may be more rewarding.

III. POLYGLYCEROLPHOSPHOLIPIDS

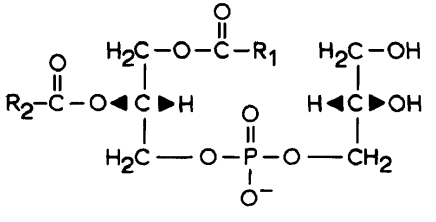
To date, the polar headgroups of phospholipids have been thoroughly investigated in a great number of biological membranes. Considerable variations in phospholipid composition exist, not only between biologically distinct membranes but sometimes also between functionally identical membranes[77]. Most membranes contain a number of phospholipids which differ with respect to the nature of differently charged polar headgroups. This heterogeneity in hydrophilic groups has given support to the idea that the polar headgroups of phospholipids may be involved in a number of functions,

viz. interaction with charged sidegroups of proteins, binding of cations and merely speculatively, as carriers in transport phenomena.

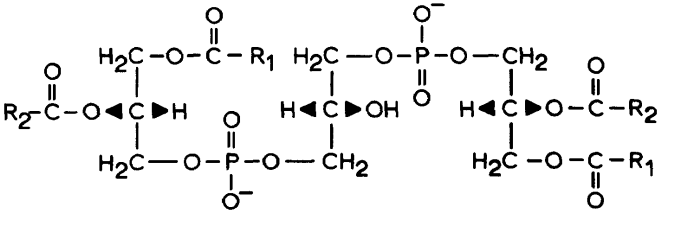
In many mammalian membranes the proportions of phospholipids with different headgroups are relatively constant for a given membrane and appear to be genetically determined. More flexibility is often revealed by bacterial membranes. In recent years, several new phospholipids have been detected in bacteria and some examples of this area of natural product chemistry will be discussed briefly.

Phosphatidylglycerol

This phospholipid which was first detected in algae is an important constituent of chloroplasts[78]. This compound is present in minor amounts in mammalian tissues but is abundant in many bacteria. The stereochemical configuration (*Figure 20*) was established to be 3-*sn*-phosphatidyl-1′-*sn*-glycerol[3], this being in agreement with the biosynthetic pathway[79] involving



Phosphatidyl glycerol



Di-phosphatidyl glycerol
(Cardiolipin)

Figure 20. Two major polyglycerol phospholipids

a reaction with CDP-diglyceride and glycerol-3-phosphate producing as an intermediate 3-*sn*-phosphatidyl-1′-glycerol-3′-phosphate. The stereochemical configuration was confirmed by chemical synthesis[80].

Diphosphatidylglycerol

This phospholipid usually denoted as cardiolipin is by no means unique for bacteria. It is well established that it is a quantitatively important constituent of the inner membrane of mitochondria, but its precise function

is unknown. Cardiolipin has been the subject of many investigations and several structures have been proposed. A chemical synthesis of diphosphatidylglycerol and several related compounds confirmed that beef heart cardiolipin is identical to diphosphatidylglycerol[81] (*Figure 20*). A conclusive proof was obtained by breakdown of synthetic and natural phospholipid with phospholipase C, which gave in both cases, 1,2-diacylglycerol and glycerol-1,3-diphosphoric acid. It is not to rule out, however, the occurrence in nature of other polyglycerol phospholipids closely related to diphosphatidylglycerol. Some indications were obtained that in a bacterium an acyl derivative of diphosphatidylglycerol may occur[82,83].

Amino acid esters of phosphatidylglycerol

A class of phospholipids which seems so far to be unique for bacterial membranes is represented by *O*-aminoacyl derivatives of phosphatidylglycerol[84,85]. In gram-positive bacteria, L-lysine and L-alanine appear to be the predominant amino acids, but in *Mycoplasma* both D- and L-alanine were reported to be linked to phosphatidylglycerol[86]. The lysine ester of phosphatidylglycerol was isolated in a pure form and by chemical and enzymatic hydrolysis, the stereochemical configuration was established (*Figure 21*). It could be concluded that the compound was 3-*sn*-phosphatidyl-1′-(2′ or 3′-*O*-L-lysyl)-*sn*-glycerol[87], but the position of alkaline labile linkage

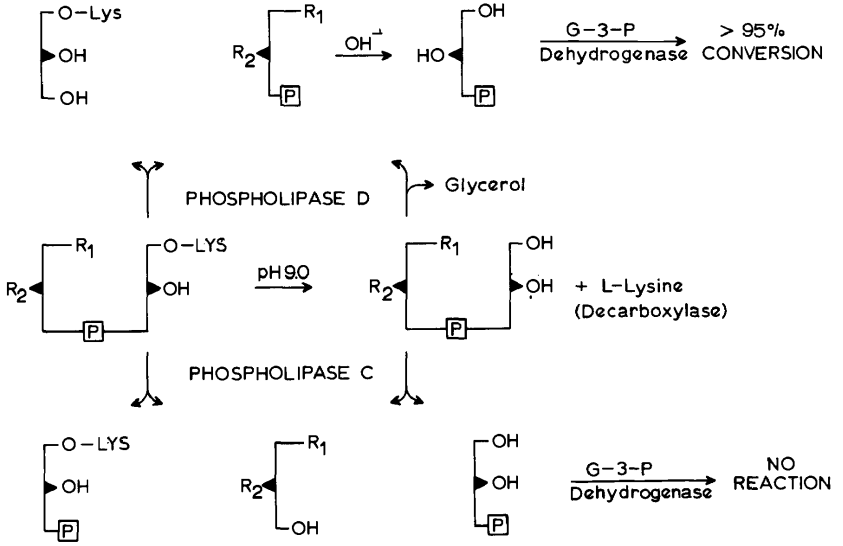


Figure 21. Enzymatic degradations utilized for the structural comparison of natural and synthetic *O*-lysyl-phosphatidylglycerol[87,91]

of the lysyl moiety remained uncertain (*Figure 22*). This question was tackled by various approaches. Several groups undertook the chemical synthesis of this phospholipid class[88,89,90]. As an example, the synthesis of 3-*sn*-phosphatidyl-1′-(3′-*O*-L-lysyl)-*sn*-glycerol is given (*Figure 23*)[91]. The synthetic

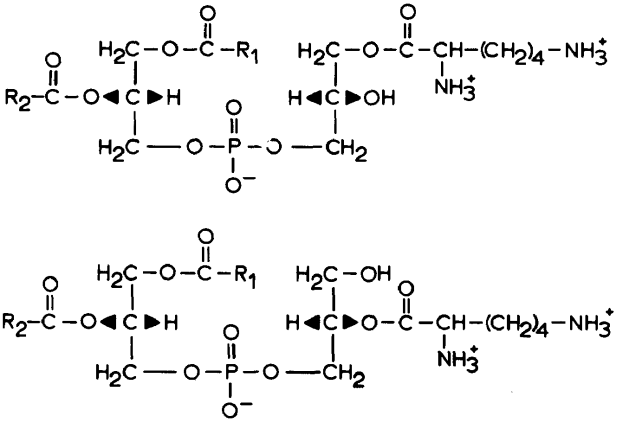


Figure 22. Isomers of *O*-lysyl-phosphatidylglycerol

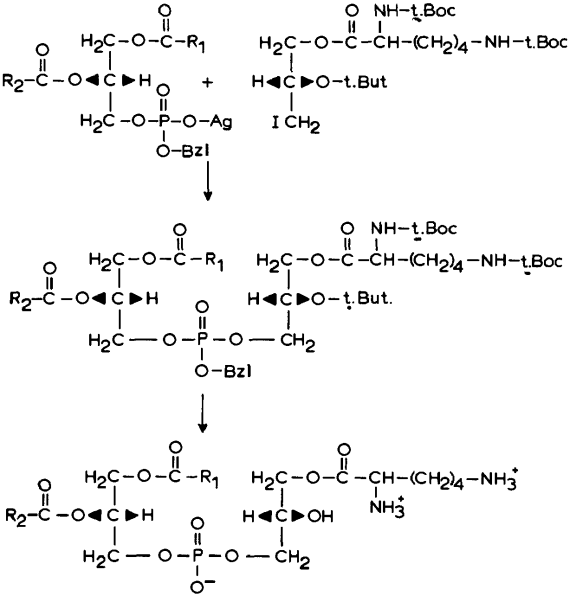


Figure 23. A chemical synthesis of 3-*sn*-phosphatidyl-1′-(3′-*O*-L-lysyl)-glycerol[91]

compound was compared with the natural product and it was found that both substances were completely identical. Of particular interest is the similar enzymatic hydrolysis of both compounds by phospholipases C and D (*Figure 22*). The first enzyme gave a complete hydrolysis into a 1,2-diglyceride and a water-soluble product identical to synthetic *O*-L-lysyl-glycerol-phosphate. Phospholipase D action produced phosphatidic acid and one water-soluble compound identical to synthetic 1-*O*-lysyl-glycerol[91]. The

latter observation indicates that in the natural product the lysine was esterified to the primary hydroxyl function just as in the synthetic phospholipid. The possibility of a migration of the lysyl residue from the 2′ to the 3′ position during the isolation of the phospholipid or its enzymatic hydrolysis was not ruled out. However, one research group active in this field synthesized isomers having the lysyl moiety linked to either the primary or secondary hydroxyl function (*Figure 22*) and found that migration from the 2′ to the 3′ position does not occur during chromatography on silicic acid in acidic and neutral systems[92]. Furthermore they found that both isomers displayed a different chromatographic behaviour. These joint observations endorsed the view that the phospholipid isolated from *St. aureus* occurs in the bacteria as 3-*sn*-phosphatidyl-1′-(3′-*O*-L-lysyl)-*sn*-glycerol.

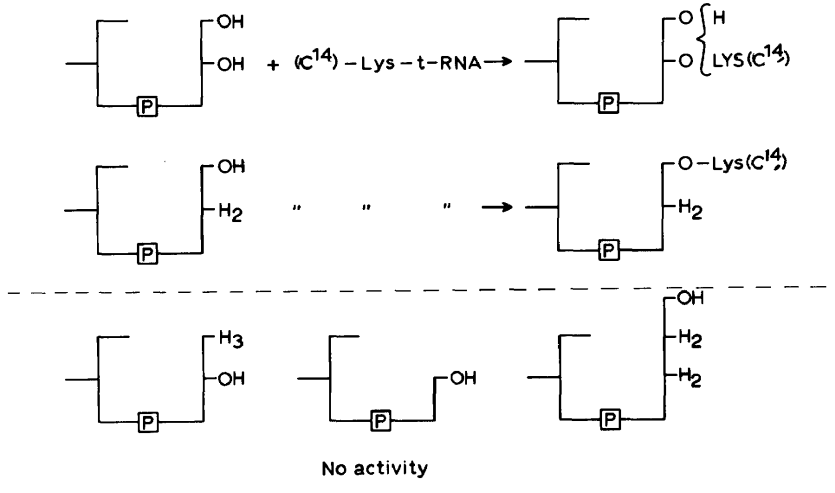


Figure 24. Lipid specificity in the transfer of lysine from [^{14}C]lysyl-*t*-RNA to phospholipid[94]

A biosynthetic pathway was found to involve a transfer of the lysyl moiety from lysyl-t-RNA to phosphatidylglycerol[93]. A number of potential acceptors for the lysyl group were tested (*Figure 24*) and only 3-*sn*-phosphatidyl-1′-(2-deoxy)-glycerol appears to be active[94]. The isomeric 3-*sn*-phosphatidyl-1′-(3′-deoxy)-glycerol was inactive endorsing the view that the position of enzymatic esterification of phosphatidylglycerol is the 3′- rather than the 2′-hydroxyl group. When the conclusion is made that this biosynthetic pathway initially leads to 3-*sn*-phosphatidyl-1′-(3-*O*-L-lysyl)-*sn*-glycerol it remains possible that subsequently a migration occurs so as to give a mixture of 2′ and 3′ amino acyl esters of phosphatidylglycerol. In this respect it is of interest to note that recently, in *Streptococcus faecalis*, in addition to 3-*sn*-phosphatidyl-1-(3′-*O*-L-lysyl)-*sn*-glycerol, a lysyl containing phospholipid was detected[83] which revealed chromatographic properties similar to those of synthetic 2-*O*-lysyl ester of phosphatidylglycerol[92]. Furthermore, it was suggested that a 2′,3′-dilysyl derivative of phosphatidylglycerol may occur in this bacterium[83] which raises many interesting questions about the metabolic relations among these amino acid containing phospholipids.

Glucosamine derivatives of phosphatidylglycerol

Recent reports on the occurrence of glucosaminyl-phosphatidylglycerol in *Bacillus megaterium*[95,96] and *Pseudomonas ovalis*[97] enlarged the series of phospholipids derived from phosphatidylglycerol. A compound isolated

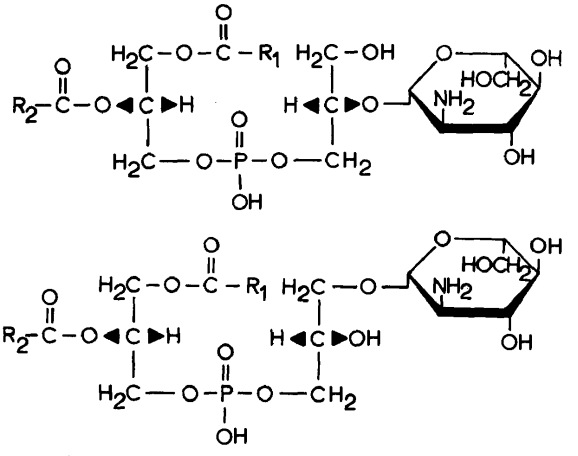


Figure 25. Isomers of glucosaminyl-phosphatidylglycerol

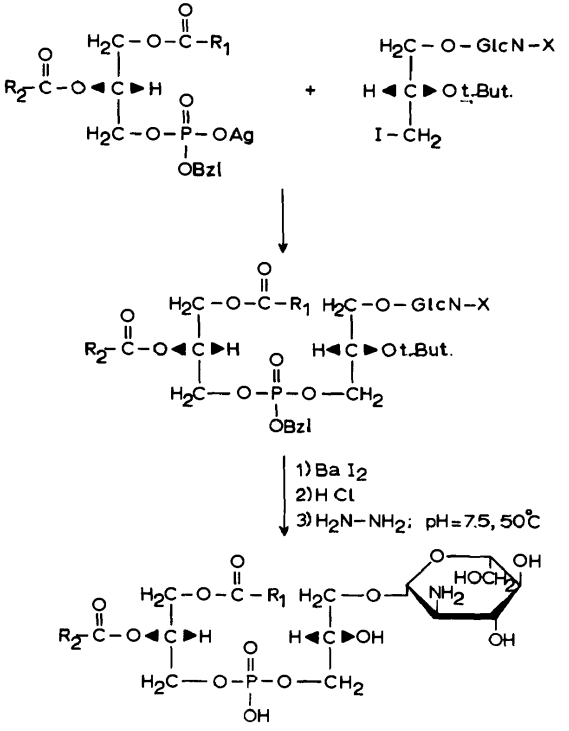


Figure 26. Chemical synthesis of a glucosaminyl-phosphatidylglycerol[102]

from a *B. megaterium* (MK 10D) was subjected to a variety of chemical and enzymatic hydrolysis procedures and was proven to be identical to 3-*sn*-phosphatidyl-1′-[2-(2-amino-2-deoxy-β-D-glucopyranosyl)]-*sn*-glycerol[98]. This structure was confirmed by chemical synthesis[99]. However, evidence was presented that apart from the 2′-isomer also the 3′-glucosaminyl derivative of phosphatidylglycerol occurs in this bacterium[100,101] (*Figure 25*). Both compounds were subsequently synthesized[102] (compare *Figure 26*) and a comparison with the pure isolated compounds by chemical and enzymatic procedures demonstrated their identity[103]. The ratio of the 2′ and 3′-glucosamine derivative of phosphatidylglycerol appeared to be dependent on the growth conditions. The biosynthesis of these compounds is not elucidated but it can be suggested that a reaction between UDP-glucosamine and phosphatidylglycerol may be involved.

Functional aspects

As regards the functions of the amino acid and glucosamine containing phospholipids one could speculate about a function in transport or a role as donors in the biosynthesis of macromolecules. However, no conclusive evidence has come forward to prove that these functions can be attributed to these phospholipid classes. Recent results even appear to argue against such functions[104]. Alternatively, the possibility exists that these phospholipids are mainly structural components essential for particular molecular arrangements within the plasma membrane or intracellular membranes of bacteria. Particularly the lysine ester(s) of phosphatidylglycerol are conspicuous because these phospholipids are unique in having at normal cell pH a net positive charge. It may be that the positively charged endgroup is of paramount importance for interaction with protein constituents or for donating a given surface charge to the membrane. Both functions are not mutually exclusive. A possible structural function of these lipids finds some support in observations that the ratio of amino acyl phosphatidylglycerol to the negatively charged phosphatidylglycerol can increase under certain environmental conditions such as an acidic pH[86,87,104]. It can be speculated that this is a physiological reaction to counteract the increased proton concentration at the cell surface so as to preserve the integrity of the membrane. Actually it has been known for several years that in membrane model systems such as liposomes the presence of positively charged surfactants causes a further restriction of the diffusion of cations through the lipid barrier[60]. Recently, it was reported that bilayer membranes made of lysyl phosphatidylglycerol were more permeable to chloride ions than to protons, whereas, the reverse was true for membranes composed of phosphatidylglycerol[105]. This selectivity of thin lipid barriers composed of phospholipid with different polar headgroups is in agreement with observations made on liposomes composed of various bacterial phospholipids. In addition it was found that the valinomycin mediated exchange of cations is strongly reduced in liposomes containing lysyl phosphatidylglycerol[106]. The suggestion that the chemical make-up of the phospholipid headgroup controls not only the simple diffusion, but also the rate of carrier-mediated transport of ions needs to be verified on natural membranes.

CONCLUDING COMMENTS

Progress has been made with respect to the precise chemical characterization of membrane lipids. Details have been elucidated about the lipid composition of a great variety of biological membranes. Molecular species of various phospholipid classes have been chemically synthesized. Studies on monolayers and bilayers continue to contribute to the evaluation of barrier properties relevant to their function in biological membranes. This approach may be particularly useful when combined with the induction of chemical variation in the lipid components of membranes of cells and measurements of changes in properties. The chemical nature of the hydrocarbon tails of phospholipids appears to determine to a significant extent the diffusion processes but likely also, the rate of carrier-mediated transport across cell membranes. Membranes appear to tolerate a certain degree of flexibility in the chemical nature of the apolar moieties but depending on the cell type this variation is limited. Enzyme systems involved in the biosynthesis of individual molecular species of phospholipids display specificities which control the physical properties of phospholipids within certain limits. A function of the polar headgroups of phospholipids in regulating surface properties and associations with protein constituents is likely but this area of research needs further exploration, also with respect to the understanding of catalytically active proteins which depend on the presence of particular lipid constituents for their activity. The advances made in lipid chemistry and membrane model systems can be expected to promote in the near future a better understanding of the electrostatic and hydrophobic associations between lipids and proteins in biological interfaces.

ACKNOWLEDGEMENTS

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