PHOSPHOLIPIDS AND BIOMEMBRANES

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I. INTRODUCTION

THOUGH a sorption theory has been maintained by Troshin[477] to explain the differences in concentration of ions and other substances outside and in the cell, most investigators favour the existence of a limiting membrane, which preserves the integrity of the cells as a whole and regulates the selective transport processes. The presence of lipids in the surface membranes of living cells was already suggested at the end of the nineteenth century by Overton[380] in order to account for the observed relations between lipid solubility of substances and the velocity of penetration into the cell. This concept was severely attacked;[475] Ruhland[418] arrived at the conclusion that differences in the molecular size brought about the observed distinction in penetration velocity, thereby favouring the idea that a membrane acts as a molecular sieve. An attempt to abolish several difficulties inherent to both theories was made by Collander[84] who combined several of the principles of both controversial views into one model. Various approaches made it clear that a simple lipid membrane could not account for certain of the properties of cell surfaces. While Gorter and Grendel[180, 181] pointed out that the amount of lipid present in the red cell was just sufficient to provide a bimolecular lipid leaflet concerning this cell surface, Danielli and Harvey[99] concluded that proteins participate in forming together with lipids the cell boundary. The various observations and hypotheses led to the formulation[98, 104] of a "paucimolecular theory", which survived till the present, though other molecular arrangements are subject of discussion as well (Section III).

In all current models on membranes, lipids form an integral part of a protein–lipid network, while among the lipids concerned a fundamental role is attributed to the class of phospholipids. The ubiquitous occurrence of phospholipids as indispensable components of all living organism led early to surmise that these substances must play a vital role in living cells. Mayer and Schaeffer[346] already observed that the lipid–phosphorus content of most organs did not change markedly under a diversity of conditions such as overfeeding or inanition. The observation that these compounds are abundant in materials identical or related to cell membranes and the recognition of the peculiar physical properties combined within the phospholipid molecule greatly influenced the developments in this difficult field. Based on colloid-chemical studies on coacervates Bungenberg

de Jong[49, 66, 67] and his school pictured models of the cell membrane, involving a tricomplex system between phospholipids, proteins and cations. Many other approaches further endorsed the importance of phospholipids for attaining the physical and chemical arrangements required to give the bio-interfaces their remarkable properties. Sub-cellular components, consisting of membranes which are believed not to be fundamentally different in several respects from the cytoplasmic membrane, have been demonstrated to be extraordinarily rich in phospholipids. The mitochondrial phospholipids are now known to play an indispensable role in the energy transducing functions of these cell particles.[185]

The problem of the structure and function of membranes challenges investigators from many disciplines, who are forced mostly to take into consideration the phospholipids with their remarkable interfacial properties. Recent progress in the chemistry of phospholipids clearly demonstrated the complexity in the chemical composition and the numerous structural variations of this class of lipids, thus unmasking the over-simplification to consider the membrane phospholipids merely as just a lipid type consisting of apolar side-chains and a polar headgroup. While the exact molecular structure of membranes as derived from electron microscopy and X-ray analysis still is subject to question, it is recognized that membrane lipids exhibit a multiplicity of chemical variations which may influence significantly the organization and properties of the protein–lipid network. Although phospholipids undoubtedly are physico-chemically involved in the maintenance of interfaces they must not be considered, however, to act exclusively as static building-stones. The high degree of organization of surfaces is maintained through a continuous expenditure of energy by the cell, supplied by metabolic reactions proceeding at the interface. Apart from energy-consuming reactions, e.g. the active transport, the metabolic activity of the membrane may be directed towards a continuous renewal of certain of its structural components. Thus phospholipids can be regarded as dynamic components of membranes which maintain a fairly constant pattern through delicately balanced cycles of anabolic and catabolic reactions. Theoretically, this pattern nevertheless may alter when the functional aspects of the membrane involved are forced to change. Considering phospholipids as dynamic membrane constituents the question arises whether these compounds are intimately involved in functional processes of the membrane such as active transport.

It is the purpose of the present contribution to evaluate some facts about the chemistry and metabolism of phospholipids relevant to biomembranes. Such an approach is too limited for dealing adequately with a complex problem like that of biomembranes, which requires the efforts of many disciplines. On the other hand the recent explosion of literature on phospholipids in relation to membranes even forced limitation of this review to such topics currently of interest in the author's laboratory.*

* The survey of the data pertaining to this review roughly covers the literature available to December 1963.

II. Lipid Composition of Membranes

A detailed knowledge of the chemical composition of the membranous building-stones forms a prerequisite for a fair understanding of the molecular architecture of the membrane. As regards the cytoplasmic membrane, progress still is limited because of the difficulties involved in obtaining uncontaminated membrane material in sufficient quantity to allow accurate analyses. Membranes of cells lacking certain intracellular structure are believed to escape most of these difficulties and with the advancement in analytical methods the knowledge about the lipid composition of the membrane from erythrocytes and bacterial protoplast is gaining rapidly, although the insights are still rather poor about protein–lipid interaction in these membranes. The progress in cytology provided by the combined efforts of electron microscopy and the isolation of subcellular components by differential centrifugation enhanced the studies on subcellular interfaces. These membranous structures from inside the cell are readily accessible now for analytical purposes and the data accumulating on membranes from nuclei, mitochondria and the endoplasmic reticulum supply valuable information about relations between structure and function of lipids.

A. Total lipid and phospholipid content

1. Cell membranes

(*a*) *Erythrocytes.* The non-nucleated erythrocytes offer the possibility of obtaining a post-haemolytic residue or a "ghost" which is generally believed to resemble the original protoplasmic membrane very closely. Although the mature mammalian red cell permits determination of the quantitative distribution of lipids in its cellular boundary, this approach is not without difficulties. According to Parpart,[385] the ghost of erythrocytes cannot be separated from the surrounding fluid by centrifugal force alone; methods using a precipitation between pH 4·5 to 5·5 have been employed frequently. Using a medium saturated with carbon dioxide, ghosts were obtained which contained no more than 0·01 per cent of haemoglobin and retained practically all of the red cell lipids.[120, 385] On the other hand, lipid loss during the preparation of red cell ghost by other methods has been reported as well.[120] Dawson *et al.*[113] using haemolysis in water reported that about 40 per cent of the lipid phosphorus was not to be recovered in the sediment even after high speed centrifugation. Investigating the effects of pH and ionic strength of the haemolysing solution, Dodge *et al.*[132] introduced a method for the preparation of haemoglobin-free ghost by haemolysis in 20 milliosmolar phosphate buffer at pH 7·4. This brilliant white ghost material, according to experiences in the laboratory of Dr. Elbers reveals upon electron microscopy intact membrane structures (Fig. 1), and contains essentially all of the red cell lipids. Apparently, the methods used in the ghost preparation can influence the recovery of the lipids in this material to a significant extent, while on the

other hand the various methods may furnish materials different in protein composition. For this reason the amount of lipids can vary from 30 to 50 per cent on basis of dry ghost weight depending on the method utilized. Phospholipids represent about 55–65 per cent of the total lipids from the red cell surface.[120] Some differences have been demonstrated in the lipid content between various species, but the accuracy of the methods applied do not allow any final decisions. As discussed recently in a comprehensive survey,[120] significant variations still

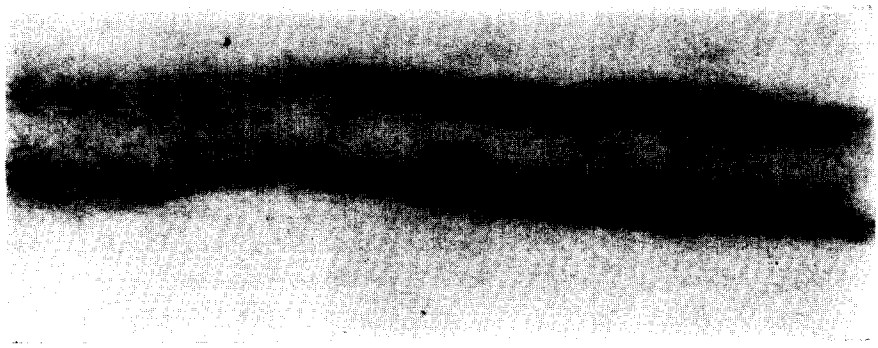


FIG. 1. Electron micrograph of rabbit erythrocyte ghosts[132] fixed by calcium permanganate (× 320,000). [By courtesy of Dr P. Elbers and Mr A. Montfoort.]

are to be noted between the data reported by several research groups on the total lipid and phospholipid content of the human erythrocyte.

(*b*) *Liver cell membrane.* The difficulties involved in the isolation of the cell surface from mammalian tissues recently have been discussed by Weiss,[502] who concluded that pure specimens of cell membranes have not yet been prepared. Neville[370] described a method for the isolation of a cell-membrane fraction from rat liver, using centrifugation and flotation techniques. This method has been applied for studies on the role of phospholipids in transport processes.[476] Electron microscopy showed that such preparations consisted essentially of cell membranes, but also a few dense granules were observed to be present. A lipid content of about 40 per cent of dry weight (22 per cent of acetone and ethanol-diethylether soluble lipids + 18 per cent of a so-termed phosphatido-peptide fraction) was reported by Tria and Barnabei,[476] who obtained 10–16 mg dry weight of cell membranes from 16–24 g of rat liver. The possible contamination with microsomes[502] rich in phospholipid puts some limitations on these data.

(*c*) *Skeletal muscle cell membrane.* A membrane structure from the cells of rat skeletal muscle containing a collagen-like protein was found to contain 15 per cent lipid on a dry weight basis.[301] Most of the lipid was demonstrated to belong to the class of phospholipids and was extracted by ether only on acidification.

(*d*) *Myelin.* The nerve myelin sheath, frequent object of electron microscopic[148] and X-ray diffraction studies,[152] is known to be rich in lipids. For a detailed review of lipids of the nervous system reference can be made to the recent monograph of Ansell and Hawthorne.[8] Applying ultracentrifugation in sucrose solutions Nussbaum *et al.*[376] were able to obtain fractions of myelin sheath from rat brain. Comparison with subcellular particles demonstrated that the greater amount of phospholipids is to be found in the myelin sheaths (50 per cent). Quite recently Autilio *et al.*[17] obtained a myelin preparation of high purity (Fig. 2) and ascertained a lipid content of about 75 per cent. Phospholipids represented over 40 per cent of the total lipids of the purified myelin.

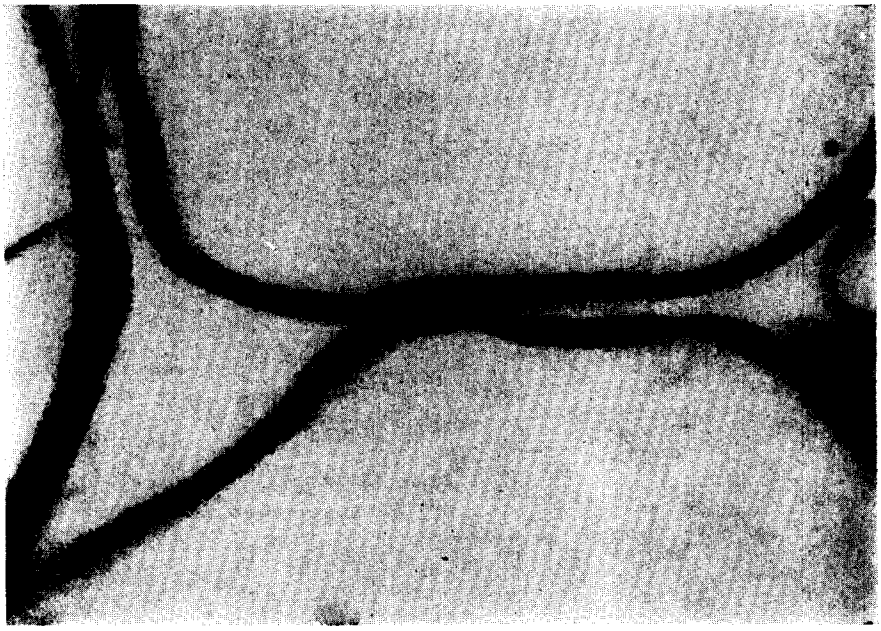


FIG. 2. Electron micrograph of purified myelin membranes from beef brain[17] (× 193,000). [Reproduced with the kind permission of Drs L. A. Autilio, W. T. Norton and R. D. Terry.]

(*e*) *Bacterial protoplast membrane.* Under the rigid outer coat of bacteria, the cell wall, lies a distinct membrane referred to as the cytoplasmic or plasma membrane (Fig. 3). Bacterial protoplasts of *Bacillus megaterium* were first prepared by Weibull[498] and Tomcsik[473] through the action of lysozyme. Bacterial protoplasts act as osmometers[499] and may be lysed mechanically or by other means to produce a ghost which may be identified with the protoplast membrane. The delicate protoplasmic membrane which acts as an osmotic barrier appears to retain, however, also many enzymic functions of the cell.[353]

In the ghost of *B. megaterium* Weibull and Bergström[501] found that 12 per

cent of the dry weight was lipid; 55–75 per cent of the lipid of *B. megaterium* can be accounted for as membrane lipid.[500] According to Yudkin[524, 525] the protoplast membrane of *B. megaterium* represents 7·5 per cent by weight of the cell. After removal of poly-β-hydroxy butyrate which sedimented with the membrane, this investigator found that extracted neutral lipid and phospholipid account for 12·9 per cent and 10·0 per cent respectively of the dry weight of the membrane from *B. megaterium KM*. The particulate fraction of protoplasts from *Sarcina lutea*

FIG. 3. Electron micrograph demonstrating the fine structure of a cell of *Clostridium welchii* (× 100,000). W, cell wall; pm, plasma membrane; n, nuclear material. [By courtesy of Dr. A. M. Glauert; by permission of the *British Medical Bulletin*.]

was reported to contain about 29 per cent of lipids.[65] The membrane of *Micrococcus lysodeikticus* isolated by Gilby *et al.*,[175] representing 8·6 per cent of the bacterial dry weight, appeared to contain about 28 per cent of lipid of which 80 per cent was a polyglycerol phospholipid. Further studies of Macfarlane[331] showed that the lipid from whole cells of *M. lysodeikticus* was only little more than that of the protoplast membranes alone. Identifying a small particle fraction of disrupted cells with the protoplast membrane, Mitchell and Moyle[354] reported this membrane of *Staphylococcus aureus* to contain about 40 per cent protein and 20 per cent lipid. Investigating the lipid components of *Staphylococcus aureus* and *Salmonella typhimurium*, Macfarlane[332] established a preponderance of phospholipids in the lipid extracts of these organisms.

Confirming the observations that the membrane accounts for most of the phospholipid content of whole cells Kolb *et al.*[299] considered the possibility that the membrane may be the sole seat of bacterial phospholipid. Experiments

on *Streptococcus faecalis* even led these investigators to propose that lipid phosphorus may serve as a fair index for membrane substance.

2. Subcellular particles

(*a*) *Nuclei.* A recent comparative account[411] of the methods applied for the isolation of nuclei outlines that many preparations are grossly contaminated with other cytoplasmic material, the degree of purity of the nuclei isolated being highly dependent on the techniques utilized. Hence, the lipid content of nuclei of rat liver as reported by several investigators reveals most striking differences (compare the recent reviews by Roodyn[411] and by Ansell and Hawthorne[8]). There is little doubt, however, that the cell nucleus contains only a very minor part of the cellular lipids. According to Ansell and Hawthorne[8] only about 1 per cent of the lipid phosphorus in the liver is present in the nuclei and the amounts of the other types of lipids are very small as well. Contamination of the separated nuclei with other cell components rich in lipids can be eliminated by the use of media containing citric acid or sucrose–citric acid or 2·2 M sucrose with certain additions. Highly interesting observations were made by Gurr *et al.*,[188] who found that liver nuclei isolated in 2·2 M sucrose revealed a ratio between lipid-P and DNA-P of 0·13 whereas this value was decreased to 0·049 when the nuclei were isolated in a citric acid medium. These differences which agreed fairly well with isolated observations of other investigators using different procedures could be explained only by a loss of lipid material from the surface of the nuclei during their isolation in the citric acid medium. Actually, examination by electron microscopy showed that in this medium the outer of the membranes was lost whilst in the nuclei obtained in the sucrose medium a double membrane was observed.[188]

(*b*) *Mitochondria.* Numerous investigations dealt with the lipids of the mitochondrial fraction, particularly from (rat) liver cells. The highly organized framework of these cell organelles is known to consist of a substantial amount of lipids. Analysis on isolated whole mitochondria indicate between 20 per cent and 30 per cent of lipids (dry weight) to be present, the predominant part being formed by phospholipids.[2, 79, 304, 336, 433, 450, 464] Whereas the values reported on the part occupied by phospholipid in liver mitochondria range between 50–90 per cent of total lipid, investigations on heart mitochondria indicated that in excess of 90 per cent of the total lipid is phospholipid.[185, 343] During the last few years investigators pursued subfractions of these cell particles in order to obtain mitochondrial membranes and other sub-units involved in the energy transducing functions of these cell particles. Siekevitz and Watson[439] disrupted isolated mitochondria from liver by treatment with deoxycholate. Fractions separated by centrifugation revealed under the electron microscope partially disrupted mitochondria and membrane elements, both showing succinate oxidase and cytochrome oxidase activity. The phospholipid content turned out to be about 38 per cent of the protein and lipid total weight, this pointing to a

localization of the electron transducing system along with phospholipids. A different approach applied earlier by Ball and Cooper[23] involved removal of all water-soluble components from minced tissue with rupture of the mitochondria and then separation of the particulate matter containing the cytochromes from the remainder of the insoluble material. Using a precipitation with ammonium sulphate these investigators obtained a preparation which upon electron microscopy showed a homogeneous distribution of thin-walled vesicles with membranous structures. The mitochondrial membrane preparation from beef heart muscle, being enzymically active, contained over 40 per cent of lipids which consisted of 91 per cent phospholipids.[24] The intimate association of phospholipids with the energy converting and releasing machinery of the living cell has been endorsed by numerous investigations during the past few years, particularly by D. E. Green and co-workers.[157, 185] The mitochondrion has been fragmented into elementary particles,[44] being the seat of enzymes from the electron-transfer chain and structural protein,[185] accounting for some 60 per cent of the total protein. The mitochondria is pictured by Green as a structural protein–phospholipid matrix to which are affixed many thousands of elementary particles. The lipid was demonstrated to be associated largely both with the elementary particles and the structural protein sandwich layer thus containing together all the lipid of the mitochondrion. The essentiality of phospholipids for the electron transfer has been demonstrated for three segments of the electron-transfer chain. (Compare Section III.)

(*c*) *Microsomal fraction.* The endoplasmic reticulum presenting an accumulation of membranous material as revealed by electron microscopy is believed to contain the greater part of cellular phospholipids. For instance, the microsomal fraction obtained from liver high speed centrifugation has been demonstrated by Getz *et al.*[168, 170] to contain 44 per cent of the phospholipids of the cell, while 9 per cent was recovered in the mitochondrial fraction. On a weight basis 55 per cent of rat liver microsomes was found to be lipid. From the cytoplasmic extract of calf thymus a fraction has been obtained which contained 1–4 per cent RNA and 35 per cent of lipids.[216] The heterogeneity of the microsomal fraction[416] still makes it difficult to evaluate precisely the lipid content of the cytoplasmic membranes concerned.

(*d*) *Chloroplasts.* Although the present contribution emphasizes mainly the part played by phospholipids in membranes of mammalian tissues, there are good reasons to include some pertinent facts about the chemical composition of lipids from chloroplasts which show, when viewed in thin section by electron microscopy, defined lamellar structures embedded in a matrix. The separated lamellae contain the chlorophylls and perform the light reactions and electron transfer reactions of photosynthesis. Progress made about the structural organization of plastids by electron microscopy and X-ray analysis[305] was accompanied by an increased knowledge about the chemical composition of the structural proteins[348, 494] and the lipids of the subunits of the chloroplast. Confining our discussion to some recent reports, reference can be made to the work of Park and

Pon,[384] who obtained highly purified chloroplast lamellae from spinach and ascertained a lipid-to-protein weight ratio of about 1. As stated by Lichenthaler and Park[323] an increasing lipid-to-protein ratio can be noticed in the analysis of the photosynthetic apparatus during the past 20 years. As regards the chemical composition of the true lipids, particularly the work of Benson[31, 32, 33, 36] and co-workers, led to the recognition that phospholipids and glycolipids are important constituents of the photosynthetic apparatus. A further evaluation of the lipid data available on the representative distribution of substances in chloroplast lamellae was recently made by Lichenthaler and Park.[323] Apart from chlorophylls, carotenoids and quinones, the glycolipids and to a lesser extent the phospholipids fairly contribute to these membrane structure of the photosynthetic unit (see above). Significant differences are known to exist in the lipid composition of chloroplasts from different origins. Wintermans[512] established a molar ratio of chlorophyll:phospholipid of 2·0 for spinach chloroplasts, of 1·05 for beet chloroplast and calculated from literature data values of 5·0 and 2·2 for rye and lettuce respectively. Apart from the lamellae, also spheroidal bodies with varying diameters have been observed in many types of plastids. The lipids of these globules, containing only a trace of chlorophyll and devoid of β-carotene, recently have been characterized by Greenwood *et al.*[187] and Bailey and Whyborn.[22]

3. Implications of the phospholipid content

Despite the many efforts, part of which have been summarized above, much still remains to be done before an absolutely certain answer can be given to the simple question: How much lipid is present in a given membrane? Even in the case of the mature mammalian erythrocyte which lost practically all subcellular structures and readily renders its envelope for analytical purposes, one can determine a different lipid content per dry weight ghost without knowing which one of the preparative methods used did yield a material resembling most closely the native membrane. It may create much argument to pose that the preparation containing the highest (phospho)lipid content represents the most reliable material, but in various articles, sometimes hidden between the lines, one can find support for such a view. The uncertainties about the purity in terms of native membrane structures make it difficult to evaluate the possible meaning of differences in lipid content varying at present greatly between membranes from different origin. In any case all biological membranes investigated contain a most significant amount of lipids, particularly of those types which carry next to the bulky paraffinic chains a hydrophylic group rendering these molecules pronouncedly surface-active.

In this context attention may be paid to the work of Gorter and Grendel[180, 181] which deeply influenced the concepts on membrane structure for the past 40 years. The first sentence of their paper[180] reads: "We propose to demonstrate in this paper that the chromocytes of different animals are covered by a layer of lipoids just two molecules thick." This unique approach involved the extraction

by large quantities of acetone of the lipids from erythrocytes, which were then spread from a solution of benzene onto a Langmuir–Adam trough. Measuring the surface area occupied by the monomolecular layer of the red cell lipids at a pressure of 3 dynes per cm, the results were found: "to fit in well with the supposition that the chromocytes are covered by a layer of fatty substances that is two molecules thick" (Fig. 4). Although this original estimate can be

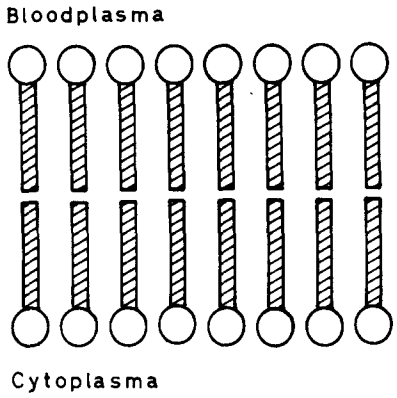


FIG. 4. Diagrammatic illustrations of the concept of Gorter and Grendel describing the limiting membrane of the erythrocyte as a bimolecular lipid leaflet.

criticized, the idea of a bimolecular lipid leaflet is maintained until the present in the unit-membrane theory. (Compare Section III.) The calculation of cell surface area by Gorter and Grendel, as well as their preparation of the lipid extracts and the film experiments, may be subject of criticism. However, as suggested by a current study in the authors' laboratory, some of the errors involved may counterbalance each other. On the other hand, the observation that approximately the right amount of lipid is afforded by the membrane of course does not necessarily mean that the lipids are organized in a bimolecular layer. Nevertheless, this approach, when carefully handled and combined with the results of other measurements, can supply useful information. This is demonstrated by a recent study of Gurr *et al.*[188] on the membrane of the liver cell nucleus. As quoted already above, the lipid content (mainly phospholipid and cholesterol) of the nuclei depends on the isolation procedure, being 2·65 times greater for nuclei isolated in sucrose than for nuclei obtained in a citric acid medium. Deducing the amount of lipid per nucleus and assuming that the lipid components of the nuclear membrane form layers similar to those obtained by a close-packed film on a Langmuir trough, it was found when the data were related to the surface area of the nucleus that in the citric acid nuclei sufficient lipid was present to cover the whole surface of the nucleus with 2·1 monolayers. This value, suggesting again a bimolecular lipid layer, becomes for the sucrose isolated nuclei 5·6. Assuming that parts of the endoplasmic reticulum are still

attached to nuclei isolated in sucrose, the concept of a double membrane involving two bimolecular lipid layers still is tenable. Actually, the electron micrographs showed a double layer which is often interpreted as two unit membranes, whereas the nuclei isolated in citric acid though having a boundary present did not reveal such a double layer. Though not conclusive in the sense of the molecular arrangement of the lipids, this approach indicated it to be likely that the phospholipids of the nucleus are concentrated in the membrane.

B. Cholesterol–phospholipid ratio

1. The distribution of cholesterol in membranes

Apart from the associations between phospholipids and proteins the various molecular species of lipids present in the lipid core of the membrane will interact with each other. Lipid–lipid interactions have frequently been demonstrated to play an important part in the interaction between lipids and proteins during different physiological processes. (See the review by Rapport.[392]) Also in biological membranes the interaction between lipids may contribute in several ways to the properties of these interfaces and will improve the stability of the molecular arrangements. In this latter respect cholesterol and other sterols may fulfil special functions. In animal cells, certain protozoa, algae and fungi considerable quantities of sterols are found. Only the eubacteria examined so far appear to be devoid of sterols.[14] However, when comparing the sterol content of membraneous structures from the animal cell, significant quantitative difference are to be noted, thus raising questions about the physiological significance of the cholesterol content. Red cell membranes and the nervous tissue contain a high level of cholesterol, whereas mitochondria from liver and heart contain only small quantities.

Winkler and Bungenberg de Jong[511] already visualized cholesterol as stabilizing arrays of the phospholipid molecules in the red cell membrane. The red cell has been extensively examined for its cholesterol content.[120] In the human erythrocyte unesterified cholesterol has been found to be present in an amount nearly equal on a molar basis to that of total phospholipids. Also the red cells of other mammalian species are known to exhibit a ratio of cholesterol to phospholipids rather closely to unity.[120] However, fowl red cells showed a higher proportion of phospholipids, which was suggested by Kates and James[266] to be attributable to an additional contribution of phospholipids from the nucleus. It is noteworthy that in mammalian erythrocytes the phospholipid–cholesterol ratio is fairly constant in spite of the differences in the composition of the phospholipid classes between these animal species (Fig. 5). By contrast to the concentrations of these lipids in the serum, showing a high degree of variability dependent, e.g. on nutritional factors, the cholesterol and phospholipid content of red cells appear to be rather constant. As reviewed recently,[120] numerous reports indicate that an elevated level of serum cholesterol in man and animal is not reflected by

a corresponding augmentation in the red cell. However, guinea-pig erythrocytes were found by Ostwald and Shannon[379a] to respond considerably to feeding with cholesterol in their cholesterol and phospholipid contents, this being accompanied by anaemia. Since cholesterol is known to interchange rapidly between the red cell and the serum,[192,327] its relative constancy in concentration in normal erythrocytes points to a rather fixed position in the cell membrane. The high content of cholesterol in nervous tissue is well established. (See the data compiled by Ansell and Hawthorne.[8]) As in the red cell membrane, in white matter

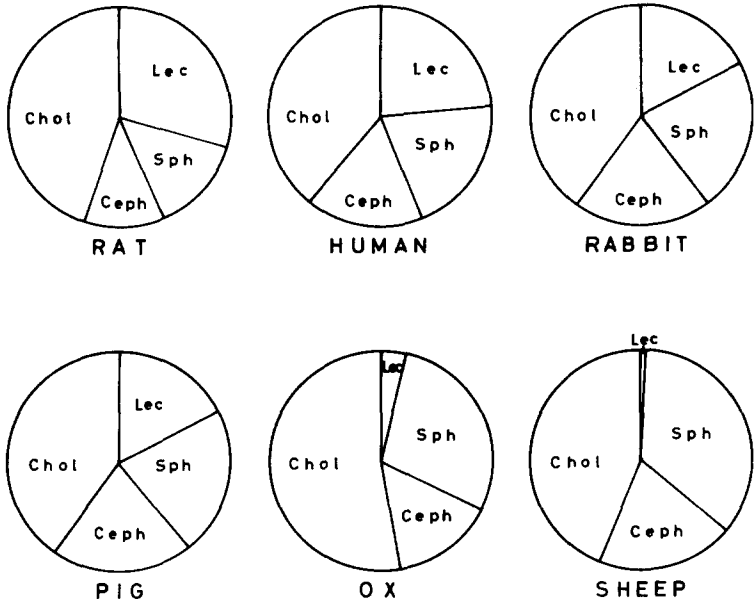


FIG. 5. Molar ratios of cholesterol and major phospholipid classes in erythrocyte ghosts from several animal species.[172] Chol, cholesterol; lec, lecithin; sph, sphingomyelin; ceph, cephalin.

of brain tissue of adult man the phsopholipid–cholesterol ratio is rather close to one, although a greater amount of other lipid species is present than in the red cell. In purified myelin preparations Autilio *et al.*[17] found molar ratios very close to 4:2:3 for cholesterol:galactolipids:phospholipids.

2. Cholesterol–phospholipid interaction

It is well known that the physical properties of monomolecular layers formed by mixed lipids may be quite different from those of the films of the single lipid components. In this way the interaction between several lipid classes, e.g. fatty acids and triglycerides, cholesterol and fatty acids, glycerides and phospholipids, has been established. (Compare the survey by Dervichian.[126]) As regards the interaction between cholesterol on phospholipids Leathes[313] has already shown

that the presence of cholesterol, which is assumed to have a practically invariable molecular cross-section, causes a decrease of the area occupied by the lecithin molecules. This condensing effect of cholesterol has been investigated most thoroughly in the laboratory of Dervichian. Measurements were made by de Bernard[37] on mixtures of 38 different proportions ranging from 100 per cent of cholesterol to 100 per cent of egg lecithin, The molecular area occupied by a lecithin molecule in the film was found to be reduced by the addition of cholesterol at all ratios of both partners (Fig. 6). The curve describing the mean molecular

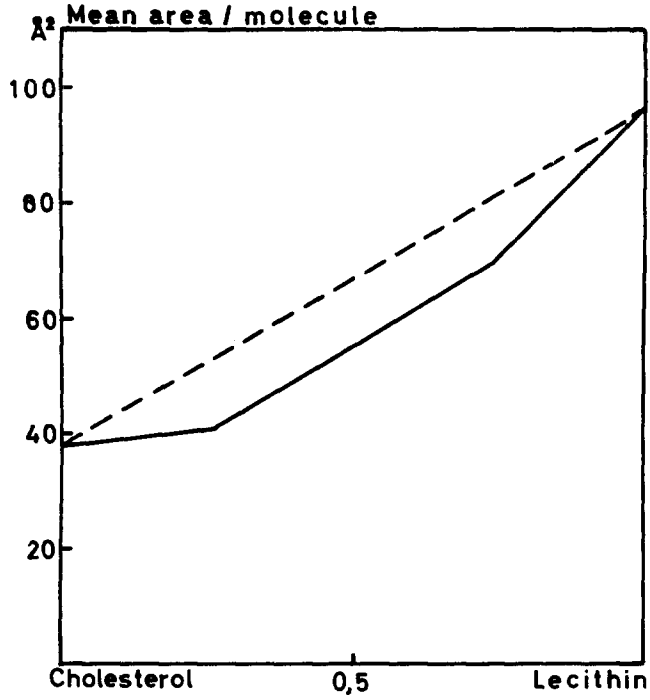


Fig. 6. Mean area per molecule in mixed films of cholesterol and egg lecithin according to de Bernard and Dervichian.[126] The experimental values are shown by the solid line; the dotted line indicates values from composition without interaction. [Reproduced with the kind permission of Prof. Dervichian.]

area as a function of the composition showed two breaks at mixtures corresponding to cholesterol–lecithin ratioso f 3:1 and 1:3 respectively. Making the legitimate assumption that the area of cholesterol undergoes little variation, by extrapolation it was derived that in mixtures containing less than 25 per cent of lecithin, the phospholipid occupies an area of 50 Å^2 only. The cross-section of egg lecithin in a single film at the pressure concerned was 96 Å^2. This significant diminution of the lecithin area was less significant for mixtures containing over 75 per cent of lecithin. It has been outlined that molecular complexes may

be formed at the given stoicheometric proportions.[37, 126] Similar studies carried out recently by Demel in this laboratory with the aid of defined synthetic phospholipids confirmed the condensing effect of cholesterol. As was expected the magnitude of the interaction appeared to depend largely on the nature of the fatty acid constituent of the phospholipid (Fig. 7). A number of synthetic lecithins when mixed with cholesterol at various proportions did not reveal any

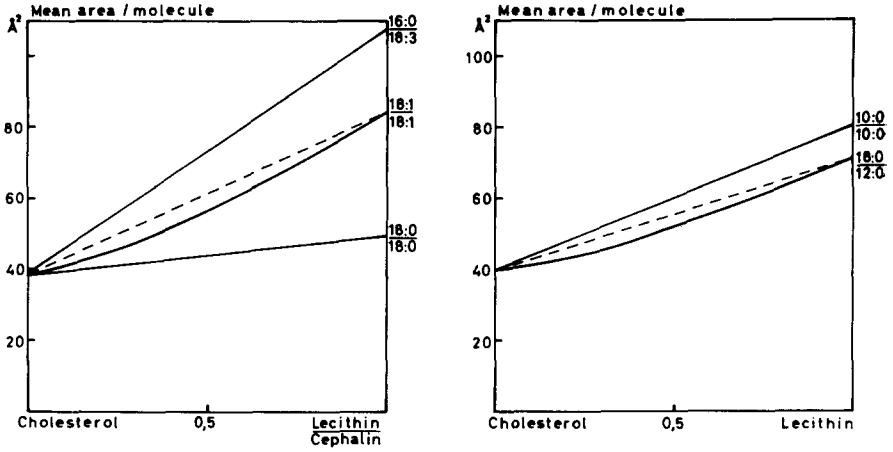


FIG. 7. Interaction of synthetic phosphoglycerides and cholesterol in monomolecular films. The experimental values are indicated by the solid line; the dotted line indicates values from composition without interaction. The phosphoglycerides are indicated by means of their fatty acids, e.g. 18 : 0/12 : 0 stands for (stearoyl-lauroyl)-L-α-lecithin; one cephalin is included, *viz.* (palmitoyl-linolenoyl)-L-α-phosphatidyl ethanolamine.

measurable reduction of these cross-sectional areas. The mean area per molecule in mixed films of cholesterol with, for example, (distearoyl)-L-α-lecithin did practically follow the simple additivity rule, as indicated by the straight line obtained. This result is not surprising in as much as the given lecithin forms already a condensed film itself. However, a saturated lecithin with fatty acids of intermediate chain length, *viz.* (didecanoyl)-L-α-lecithin, which is known to give an expanded film,[125] also refused to evoke this effect, though the lecithin was able to solubilize cholesterol in an aqueous medium. It can be argued that the number of CH_2 groups provided by this lecithin at the air–water interface is insufficient to give appropriate London–Van der Waals' attraction necessary to attain a fair cohesion of both molecular species. A definite reduction of the cross-sectional area occupied by phospholipids in the monolayers was demonstrated for a number of phospholipids giving themselves a rather expanded film[125] but having at least one long-chain fatty acid constituent. The condensing effect of cholesterol was evident with films of (γ-stearoyl-β-myristoyl)-L-α-lecithin, (dimyristoyl)—DL-α-lecithin, (γ-stearoyl-β-oleoyl)-L-α-lecithin (Fig. 7)

and the corresponding ethanolamine analog, as well as with isolated pure sphingomyelin. However, in the expanded films of synthetic lecithins and phosphatidyl ethanolamine containing linoleic or linolenic acid as fatty acid constituent no appreciable reduction of the phospholipid cross-section was induced by cholesterol. At present it cannot yet be precluded that oxidation of these fatty acid constituents was involved. On the other hand it can be envisaged, however, that also in this case the London–van der Waals' dispersion energies in the monolayer films, due to the peculiar structures of these acyl chains, are of a magnitude too low to warrant interaction between cholesterol and the paraffin chains. It has to be emphasized, however, that this result obtained at the air–water interface is not to be extrapolated to the cell boundary. The poly-unsaturated phospholipids concerned were demonstrated to be highly suitable to interact in micellar form with cholesterol. Saunders *et al.*[423] found that synthetic (dilinoleoyl)-L-α-lecithin giving clear and irreversible dispersions in water was capable of solubilizing cholesterol. By contrast to the results obtained by de Bernard[37] on a phospholipid of molecularly heterogeneous composition, so far no clear indications for the formation of cholesterol–phospholipid complexes at proportions 1:3 or 3:1 were found with the synthetic substances, this difference being under investigation now. The behaviour of the various defined phospholipid in mixed films certainly endorses the view that the interaction between phospholipids and cholesterol is governed by London–van der Waals' forces, thus being dependent on the nature of the paraffinic side-chain of the phospholipid.

3. Cholesterol–phospholipid associations in the membrane

Considerations of chemical characteristics of cell membranes in relation to known physico-chemical properties are of great value to develop insights on the molecular organization of the biological membranes. The importance of cholesterol–phospholipid interaction recognized by earlier investigators has been visualized by Finean,[150] who proposed a very attractive model of this lipid–lipid complex (Fig. 8). It accounts for the presence of large quantities of cholesterol in highly organized structures like the myelin layer and depicts the attainance of an orderly and compact arrangement of the phospholipids facilitated by the rigid structure of the cholesterol molecule. As a particular feature of this model the curling of the ionic end-group of the phospholipid may be noted. This bend of the phospholipid headgroup involves a hydrogen bond between the N-terminal group of the phospholipid and the hydroxyl group of cholesterol, thereby preventing contact between cholesterol and the outer layer of the membrane and exposing the phosphate group ready for interaction with non-lipid components. The arguments for this "walking stick" configuration were in part suggested by X-ray observations on myelin sheath and the model introduced explained the observed thickness of the lipid layer.[152]

Whereas no doubt has been expressed with respect to the interaction of the

paraffinic chains with the cholesterol molecule, some arguments have been raised against the bending of the polar headgroup to the phospholipid.[508] Quite recently Vandenheuvel[482] proposed a somewhat modified model based on considerations with stereomodel projections.[481] Reproduced in a simplified form (Fig. 8), it may be seen that the bending of the polar headgroup of the phospholipid is less pronounced in this model. For complete structural details

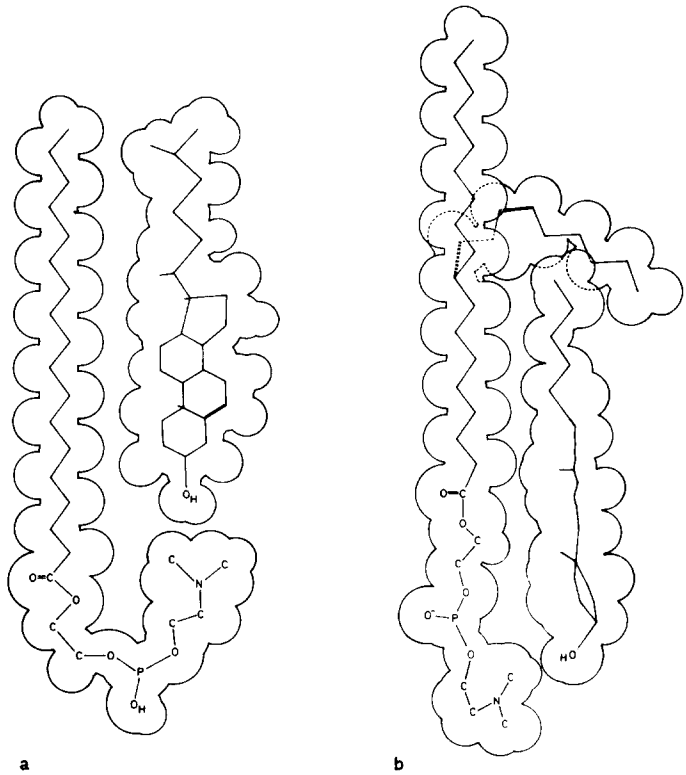


FIG. 8. Schematic illustration of possible molecular interactions between cholesterol and phospholipids. (a) See Finean.[150] (b) See Vandenheuvel[482]

the reader has to be referred to the original papers. The principle applied by Vandenheuvel involves that of maximal London–van der Waals' attraction forces. Both with lecithins containing an unsaturated fatty acid as well as with sphingomyelin an arrangement in a bimolecular lipid leaflet was proposed that did meet the distances ascertained in human myelin by Finean.[152] Although monolayer studies may be helpful for judging between both models varying in an important detail, other approaches certainly will be necessary to warrant an ascertainment of the exact alignment of cholesterol and phospholipid in biomembranes. In any case all evidence available at present points to an important

role of cholesterol in tightening the molecular packing of the lipid core of these interfaces by maintaining the apolar side-chains together by means of London–van der Waals' dispersion forces. It is attractive to speculate that the relatively high rigidity of the membrane from circulating red cells, having to withstand many turbulences and shears, is due at least in part to the high and constant cholesterol content, providing for each pair of flexible paraffinic chains nearly one rigid sterol skeleton. Although reaching the stage of oversimplified speculation one is tempted to ask whether the fragility of the bacterial protoplasma membrane, demonstrated after removal of the protective cell wall, is attributable to the lack of sterols.

Finally some attention may be paid to the derivatives of cholesterol. Whereas in the serum lipoproteins cholesterol esters are prominent partners to phospholipids and even dominate unesterified cholesterol, the red cell membrane is practically devoid of esterified cholesterol.[120] Also white and grey matter of normal adult brain tissue lack cholesterol esters, and it is important to note that in a number of demyelinating diseases cholesterol esters occur in considerable quantities in the pathological membranes. A reasonable explanation for an apparent difference in suitability between cholesterol and cholesterol esters for the membrane structure perhaps can be derived from model studies.

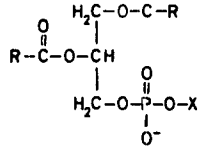
C. Composition of phospholipids

1. Variability in chemical structure

The phosphorus-containing lipids or phospholipids are derivatives of either glycerol or sphingosine. The former components unambiguously can be denoted as phosphoglycerides, while there is a strong tendency to reserve the term phosphatides for this phospholipid type as well. Natural phosphoglycerides have been demonstrated to be derivatives of L-α-glycerophosphate (according to Baers' terminology[19]) or D-1-phosphoryl glycerol (Benson and Maruo[35] and Brown *et al.*[63]) or glycerol-3-phosphate (according to the nomenclature suggested by Hirschmann,[217] which avoids the confusing D,L-terminology altogether). So far no evidence has been obtained for the occurrence in natural materials of the enantiomeric compounds neither for those derivatives having the phosphoryl-containing group attached to the β- or 2-position of glycerol.

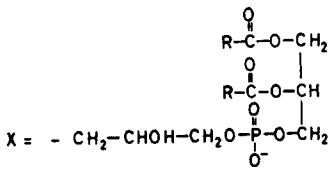
The simplest member of the phosphoglyceride class is phosphatidic acid (I; Fig. 9), which has been detected in small concentrations only, but is known to be a highly dynamic key-intermediate in the biosynthesis of other phosphoglycerides and glycerides (Section IV). The term phosphatidic acid (diacylglycerol phosphate) serves as a useful and rational basis for naming phosphoglycerides. Thus the most prominent phospholipid of nearly all mammalian membranes, lecithin, is denoted as phosphatidyl choline (II). Apart from this widely distributed choline-containing phosphoglyceride, a β-methyl choline analog was reported to be produced under certain conditions by the black

blowfly.[39] During recent years evidence accumulated on the existence of N-mono- and dimethyl-ethanolamine-containing phosphoglycerides (III and IV) which were demonstrated to act as intermediates in the conversion of phosphatidyl ethanolamine (V) into phosphatidyl choline (II).[13, 54, 55, 171, 178, 193] Phosphatidyl serine (VI), isolated by Folch[158, 159] from ox brain, was the first amino acid-containing phosphoglyceride to be recognized. More recently threonine has been identified as a constituent of phosphoglycerides, resulting in the isolation of phosphatidyl threonine (VII) from tunny muscle.[246] Pursuing the idea that other hydroxy amino acids may serve as constituents of phosphoglycerides, phosphatidyl hydroxyproline[21] and the 2-amino-2-methylpropanol analog[20] have been synthesized in anticipation of their isolation from natural sources. On the other hand, various hydroxyamino acids have been detected in so-termed lipo-peptides, which have been repeatedly reported to occur in nature.[163, 177, 214, 215, 243, 431, 520] In most cases, however, it is not clear yet whether such compounds have the phosphatidyl moiety linked through covalent

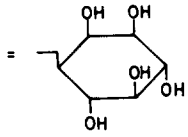


X = - H	phosphatidic acid	(I)
= $-CH_2-CH_2-\overset{+}{N}(CH_3)_3$	phosphatidyl choline or lecithin	(II)
= $-CH_2-CH_2-N(CH_3)_2$	phosphatidyl (N-dimethyl)-ethanolamine	(III)
= $-CH_2-CH_2-NH(CH_3)$	phosphatidyl(N-methyl)-ethanolamine	(IV)
= $-CH_2-CH_2-NH_2$	phosphatidyl ethanolamine	(V)
= $-CH_2-CH(NH_2)-COOH$	phosphatidyl serine	(VI)
= $-CH(CH_3)-CH(NH_2)-COOH$	phosphatidyl threonine	(VII)
= $-CH_2-CH(OH)-CH_2OH$	phosphatidyl glycerol	(VIII)
= $-CH_2-CH(OH)-CH_2-O-C(=O)-CH(NH_2)-R$	O-amino acid ester of phosphatidyl glycerol	(IX)
= $-CH_2-CH(OH)-CH_2O-PO_3H_2$	phosphatidyl glycerophosphate	(X)

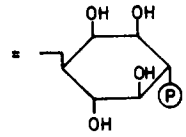
FIG. 9. Structures of natural phosphoglycerides.



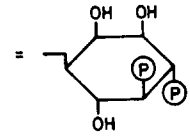
diphosphatidyl glycerol (XI)



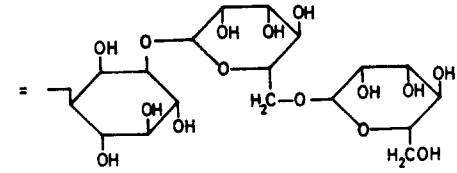
phosphatidyl (myo) inositol (XII)
or monophosphoinositide



phosphatidyl (myo) inositol-4phosphate (XIII)
or diphosphoinositide



phosphatidyl(myo) inositol-4,5 diphosphate (XIV)
or triphosphoinositide



phosphatidyl (myo) inositol-dimannoside (XV)

FIG. 9. (*cont.*) Structures of natural phosphoglycerides.

bonds to a peptide chain or whether complexes are involved between recognized phospholipids and peptides, which are held together by ionic and hydrophobic attraction forces.

Fresh progress was made in this difficult field when a new class of amino acid carrying phosphoglycerides from bacteria was characterized as O-amino acid esters of phosphatidyl glycerol.[236, 333] The parent molecule phosphatidyl glycerol (VIII) was not known until 1957–1958 when Benson[35] and co-workers detected this phospholipid in chloroplasts. The compound, which was shown to contain one unesterified glycerol unit, is known to be present in relatively low amounts in animal tissues, but also contributes considerably to the phospholipid fraction of many bacteria. The suggestion that the two glycerol units have an opposite stereochemical configuration recently was conclusively supported by biosynthetic studies of Kennedy *et al.*,[287] as well as by the results obtained in this laboratory on the enzymic hydrolysis of phosphatidyl glycerol.[205, 206] Macfarlane[333] was the first to report on the amino acid derivatives of this polyglycerol phospholipid (IX), which were first obtained from *Cl. welchii* and other bacteria as fractions containing a heterogeneity of amino acids. Some single

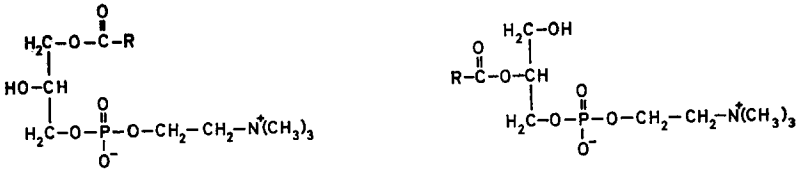
specimens containing the amino acids ornithine and lysine meanwhile have been isolated by Macfarlane[334] and in this laboratory.[236, 237, 238] The results of both independent studies strongly support the structure IX, but further work is necessary to give confirmatory evidence and to determine the exact position of the amino acids. Recent experiences in this laboratory make it highly probable that in addition to this class of compounds other amino acid and peptide-containing phospholipids occur in bacteria, which substances also may have a covalent linkage but differ in chemical structure from IX. Phosphatidyl glycerophosphate (X) was recently detected by Kennedy *et al.*[287] in liver to act as an intermediate in the biosynthesis of phosphatidyl glycerol and an accumulation of this phosphorylated precursor was obtained with sulphydryl poisons. According to Kennedy[278] phosphatidyl glycerol is likely to be an intermediate in the biosynthesis of cardiolipin. The latter polyglycerol phospholipid, first isolated and characterized by Pangborn,[382, 383] was demonstrated by Macfarlane and co-workers[330, 335, 338] and Faure and Coulon-Morelec[90, 91, 145] to be identical to diphosphatidyl glycerol (XI). Recently, however, Rose[412a] reopened the question about the chemical structure of cardiolipin.* Marinetti *et al.*[342] have recognized the presence of this lipid in the mitochondria of heart muscle and from other sources.

Challenging many investigators, the complex class of inositol-containing phosphoglycerides during the past few years revealed their chemical structures. Apart from comparative investigations on mono-phospho-inositides, demonstrated to be identical to 1-phosphatidyl L-myo-inositol (XII), the polyphosphate inositides from brain tissue were firmly characterized. The "diphospho-inositol" fraction of Folch was found to be heterogeneous and to contain a triphospho-inositide as was shown by independent work of Brockerhoff and Ballou[60, 61, 474] and Dittmer and Dawson.[112, 130] On the assumption that only the glycerol portion is acylated with fatty acid residue Brockerhoff and Ballou[60] could identify the substances as 1-phosphatidyl-L-myo-inositol-4-phosphate (XIII) and 1-phosphatidyl-L-myo-inositol-4,-5-diphosphate (XIV). The work of Dittmer and Dawson[112, 130] as well as studies of Ellis and Hawthorne[137] and Wagner *et al.*[489, 490] also led to the conclusion that both polyphosphate inositides are present in brain. Santiago *et al.*[421] reported about a phospho-inositide containing 4 phosphates per inositol† which was different in properties from an inositol phosphoglyceride complex containing an oligosaccharide isolated by Klenk and Hendricks,[292] also having a mole ratio of inositol to phosphate of 1:4. Mycobacteria have been demonstrated to contain phosphatidyl-inositol-oligo-glycosides, e.g. phosphatidyl-myo-inositol-dimannoside (XV) and analogs which structures were established recently.[25]

* A structural comparison of ox-heart cardiolipin and synthetic diphosphatidyl glycerol, however, showed the identity of both compounds (G. H. de Haas and L. L. M. van Deenen, in the press 1965).

† Further studies, however, failed to confirm the occurrence of this phospho-inositide (Dr. Hokin, personal communication).

Although the enlisted structures of phosphoglycerides are probably far from complete, one may be struck already by the enormous variations in the nature of the polar headgroup. The possibilities for variations in phospholipid structure can be multiplied by a considerable factor when taking into account the variability of the apolar side-chains. Dealing later on with the chemical structure of the fatty acids, the phosphoglycerides containing only one acyl residue, the so-termed lyso-phosphoglycerides, have to come into consideration. Recent studies affirmed that the lyso-derivatives encountered usually in small amounts in many lipid extracts have not to be considered as artifacts.[371, 496] As will be discussed in Section IV, these compounds are now believed to act as key intermediate in certain metabolic events of the concerning diacyl analogs. As regards their chemical structure, two isomers differing in the position of the fatty acid ester linkage have to be envisaged (Fig. 10). The isomer containing

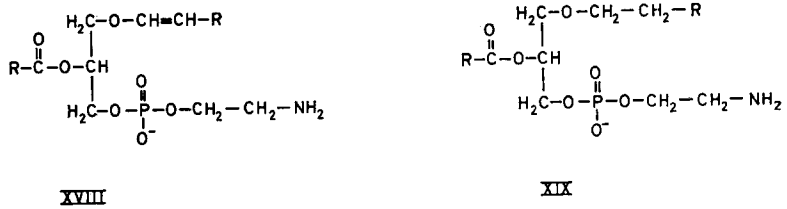


XVI XVII

FIG. 10. Isomeric lysolecithins of the L-α series.

the fatty acid in 1 (or γ)-position (XVI) may arise by the action of phospholipase A (EC 3.1.1.4), an enzyme which catalyses specifically the hydrolysis of the fatty acid ester bond at the 2-position of the phosphoglyceride molecule.[190, 198, 465] Direct investigations on the structure of naturally occurring lysophosphoglycerides hardly have been made; recently it was reported[321] that lysolecithin isolated from yeast was identical to 1-acyl-glyceryl-3-phosphoryl-choline (XVI). Recent studies favour the view that also lyso-derivatives identical to structure XVII occur in living cells (Section IV).

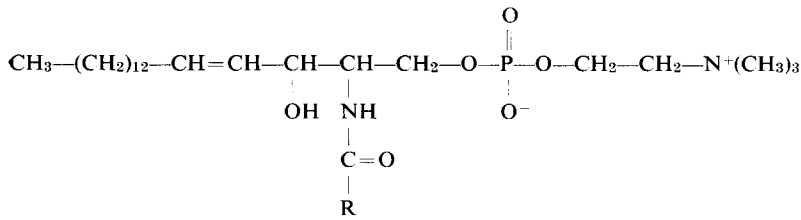
An important class of phosphoglycerides, particularly of the animal kingdom, are the plasmalogens which contain one aldehydogenic chain linked to glycerol as an α,β-unsaturated ether (Fig. 11). The correct structure (XVIII) has been



XVIII XIX

FIG. 11. Phosphoglycerides containing ether linkages.

established in recent years and it appears to be generally accepted that the fatty acid ester bond is present at position 2. The plasmalogens are known to carry different polar end-groups and for more details on their distribution and properties reference can be made to recent surveys.[291, 399] In the past few years a further break-through has occurred in the knowledge of the phosphoglycerides containing a saturated ether linkage (XIX). Glyceryl ether-containing phospholipids with ethanolamine as nitrogeneous base have been detected in several sources; e.g. egg yolk, brain tissue, ox heart, bovine erythrocytes, bovine red marrow (compare the review by Hanahan and Thompson[202]). The quantitative importance of this type of phospholipid structure is clearly demonstrated by studies of Hanahan and coworkers[200, 201] who observed that the ethanolamine lipids which make up 30 per cent of the total phospholipids from bovine erythrocytes are for the greater part present as glyceryl ether derivatives. Choline-containing[392, 401] lipids and polyglycerol phospholipids[268] have been reported to contain saturated glyceryl ether linkages as well.[341] Furthermore phosphoglycerides containing two ether linkages also have been encountered.[268, 341]



XX

Sphingomyelin (XX) is the only fully characterized phospholipid from the class of sphingolipids. It may be noted, however, that also dihydrosphingosine has been detected in preparations of animal phospholipids[70] and evidence has been claimed that this compound may be part of the sphingophospholipid from horse spinal cord.[162] Several studies suggested that a compound analogous to sphingomyelin but with the choline substituted by ethanolamine may occur. The misleading results which can be obtained because of the interference of the very stable glyceryl-ether phospholipids are recognized now.[9] Nevertheless, it has been maintained that in the housefly a sphingomyelin type compound occurs in which ethanolamine replaces choline.[92, 93] Further indications of the possible complexity of the sphingophospholipid class are given by reports on sphingosine phosphate fatty acid esters[306] and sphingoplasmalogens.[295]

It needs no argument that an exact determination of the composition of a natural phospholipid mixture is no sinecure. Progress in this difficult field was possible by the development of chromatographic techniques including columns with silica or aluminium oxide,[196] paper impregnated with silica or otherwise pretreated,[341] thin-layer chromatography[417] and application of various hydrolysis techniques combined with chromatographic resolution of the split products,[109]

as well as determinations of the polar end-groups. Depending on the composition of a given mixture of phospholipids, combinations and modifications of methods are often necessary to gain a fair impression of the phospholipid pattern. Extensive data concerning the occurrence of phospholipids have been compiled in several reviews quoted above as well as by Dittmer.[129] The following pages will deal with some examples only, believed to be sufficient to show the differences which exist in the phospholipid distribution between several membranes, and to demonstrate the gaps in our understanding of the significance of a given phospholipid pattern for the membrane.

2. Phospholipid distribution

(*a*) *Erythrocyte membrane.* The mature mammalian erythrocyte attracted many investigators since the lipids from this cell are believed to come from one uniform membrane. According to the experiences of about ten research groups during the past few years, the phospholipid fraction of human erythrocytes consists of lecithin (about 30–40 per cent), phosphatidyl ethanolamine, including the plasmalogen type (23–28 per cent), phosphatidyl serine (2–15 per cent), sphingomyelin (22–30 per cent) and smaller amounts of phosphatidic acid, inositol-phosphoglycerides, lyso-derivatives and unidentified compounds.[120] Several discrepancies are to be noted between the results of various studies, even between subsequent reports from single laboratories. These differences appear to depend to some extent on the different analytical methods utilized, each of which has its own imperfections. In addition variations in the recovery of extracted lipids as well as degradation of certain compounds are likely to be involved. A significant part of the discrepancies existing is to be attributed to variations in the amount of phosphatidyl serine recorded in the various reports. Recent results of several laboratories pursuing this work confirm that a substantial amount of this phosphoglyceride is present indeed, and a reliable knowledge about the phospholipid composition of this membrane is within reach now.[493]

Interesting results emerged from comparative studies on the phospholipids from erythrocytes of various mammalian species. Before 1940 it was already known that erythrocytes from different animals exhibit great differences in their permeability behaviour for certain lipid-soluble substances. At that time Parpart and Dziemian[387] already attempted to correlate such differences with a distinction in the lipid composition of the concerning membranes. However, the methods available for the analysis of phospholipids did not enable one to establish clear-cut differences. In 1957–1958 Turner *et al.*[478] examined the phospholipids extracted from erythrocytes of different species and observed at the chromatograms developed on silica impregnated paper that lecithin was either absent or present in low concentration only in the red cell of certain ruminants. Trying to relate the susceptibility of red cells towards snake venom with the phospholipid pattern of the membrane, it was found, however, that not all ruminants revealed this peculiar feature, e.g. camel erythrocytes[479] turned out to be fairly rich in

lecithin. A quantitative comparison of the phospholipid composition of mammalian erythrocytes carried out in several laboratories with the use of different methods confirmed that most significant variations exist.[113, 172, 202] Results obtained by de Gier and van Deenen[172] are illustrated in Fig. 5 and indicate that for the series rat, rabbit, human, pig, ox and sheep the lecithin content of the erythrocyte membrane decreased progressively from 56 per cent in the rat to 1 per cent in the sheep, while there was a concurrent increase in sphingomyelin content from about 25 per cent to 63 per cent. This counterbalancing effect between lecithin and sphingomyelin was also demonstrated by Dawson *et al.*,[113] who found that ruminant red cell membranes contain less than 10 per cent lecithin as compared with about 30 per cent in the non-ruminants studied. Further studies in this laboratory showed that the lecithin content in sheep erythrocytes can vary between 1 and 12 per cent of the total phospholipids.[174] The investigations carried out so far allow the conclusion that the sum of choline-containing phospholipids is relatively constant for the mammalian erythrocyte. Concerning the cause of these striking differences in the proportion of lecithin and sphingomyelin between the various erythrocytes. A satisfactory explanation cannot yet be afforded. Studies on the phospholipid composition of hematopoietic bone-marrow carried out by Thompson and Hanahan[469] and by Mulder *et al.*[365] showed in good agreement that lecithin was present as a major phospholipid in the marrow of both ruminants and non-ruminants. Furthermore, our dietary studies involving a by-pass of the action of the rumen in sheep demonstrated that alterations occur in the fatty acid pattern of the erythrocytes without effecting, however, a significant alteration in the phospholipid composition.[174]

The question can be raised whether such differences in the phospholipid patterns between the erythrocytes are related to differences in other properties of the concerned membranes. When making a comparison between the lecithin content and the data of Jacobs *et al.*[249] on the permeability behaviour of the red cells for compounds like glycerol we were struck to find a high degree of parallelism between both characteristics. (See Section III.) However, the coincidences noted certainly do not permit any conclusion about a direct relationship between phospholipid composition and the permeability properties. As will be discussed later in this paper, significant differences also exist in the fatty acid constituents of the phospholipids from different mammalian species. Further efforts of lipid biochemists made it clear that fundamental structural differences occur between the ethanolamine-containing phosphoglycerides of erthrocytes. As demonstrated by Farquhar,[142] 60 to 70 per cent of the ethanolamine phosphoglycerides of the human erythrocytes belong to the class of the plasmalogens (Fig. 11; XVIII). Bovine erythrocytes were found by Hanahan and co-workers[200, 201] to contain the ethanolamine lipids predominantly in the form of saturated glyceryl ethers (XIX). The biosynthetic as well as the functional aspects of both structurally related phospholipids are not yet elucidated.[201]

Erythrocytes of different mammalian species are known to differ greatly with respect to their content of several cations. Red blood corpuscles of, for

example, man, rat and rabbit have a low sodium content when compared with the plasma concentration, whereas in the cow and sheep the reverse is true. Therefore, it is tempting to investigate whether such differences are related to the apparent distinction in the phospholipid composition of the membrane. Some preliminary investigations were made in collaboration with Dr M. Vaughan and Dr J. Hoffmann (N.I.H. Bethesda) on sheep erythrocytes differing in potassium-sodium ratio. No differences were found in the lecithin content of both types of erythrocytes, but it will be of interest to examine more thoroughly the various acidic types of phosphoglycerides.

(*b*) *Mammalian organs: Liver.* As in most mammalian tissues lecithin is the predominant phospholipid in liver. The phospholipid fraction of rat liver[169, 199, 342] consists in the order of decreasing concentration of: lecithin, phosphatidyl ethanolamine, sphingomyelin, polyglycerol phospholipids, phosphatidylserine and inositol phospholipids. Lysolecithin has been found to be present as well. Although the comparative studies on the distribution of liver phospholipids are confined to few species, it can be recorded that liver of ox,[199] sheep[109] and mouse[369] revealed about the same pattern, all having over 50 per cent of phosphatidyl choline. This is consistent with the view that little differences exist in the nature and composition of phospholipids from the same kind of tissue, with some striking exceptions like the erythrocytes.

Stimulated by the desire to obtain knowledge about the particular functions of phospholipd classes, descriptive studies on the phospholipid distribution within the micro-cosmos of the cell have been extensively undertaken. The nuclei, which contain only a very small part of the cellular phospholipids, were found by Gurr *et al.*[188] to have a phospholipid pattern very similar to that of the cell as a whole. However, nuclei appear to be devoid of cardiolipin. On the other hand, Biezensky *et al.*[40] reported that nuclei are characterized by a higher lipid serine than ethanolamine content, while also large amounts of unidentified phospholipids were deduced to be present.

Since the phospholipids of the animal cells so far investigated are largely concentrated in the mitochondria and endoplasmic reticulum or microsomes, it is not surprising that the distribution of phospholipids in these membranous structures reflects that of whole tissues. The mitochondria of rat liver contain a high amount of lecithin, accounting for approximately half of the lipd phosphorus present. As indicated by a compilation of Getz *et al.*[170] some pertinent differences in the relative proportions of lecithin and cephalin from rat-liver mitochondria are to be noted in the literature. According to Getz *et al.*[170] the ratio lecithin: cephalin is always higher in the isolated microsomal fraction than in the isolated mitochondria. As calculated by these investigators the microsomes contain about 60 per cent of the liver lecithin. Apart from the distinction in lecithin–cephalin ratio between the mitochondrial and microsomal fraction, the most unique difference in intracellular distribution is exhibited by cardiolipin. As supported by many studies mitochondria contain most, if not all, of the cell cardiolipin. Plasmalogens could not be detected in rat liver

mitochondria or microsomes, but were found to be present in whole ox liver.[182]

Heart. In as much as mitochondria isolated from heart are frequently used now to assess the importance of phospholipids in the functional processes of their membranes some recent analytical data will be included.

A mitochondrial membrane fraction of heart muscle was found to be rich in phospholipids and the lipids in this enzyme preparation turned out to be concentrated about threefold over their amount in fresh beef heart.[24] Since on the order of 65 per cent of the total lipids of heart muscle appear to be localized in the mitochondrial membrane fraction, it is not surprising that the major phospholipids turned out to be identical with phosphatidyl choline and phosphatidyl ethanolamine.[343] A characterization of the phospholipids from pig heart mitochondria and cytochrome oxidase by Marinetti *et al.*[343] showed little difference between both phospholipid patterns. Fleischer *et al.*[157] have fragmented beef heart mitochondria and determined the phospholipid distribution in the purified enzyme-containing fragments. Some conspicious quantitative differences, e.g. in the content of cardiolipin, are to be noted.

The mitochondria of heart, when compared with liver and brain, are unique because of their high plasmalogen (choline) content. According to Green and Fleischer[185] no distinct functional significance is apparent, since plasmalogen-free phospholipid could substitute beef heart mitochondrial phospholipids in restoring the enzymic activity of lipid-free mitochondria.

Nervous tissue. The diversity of lipids in brain and other nervous tissues, comprising complex lipids like sulphatides, gangliosides and other glycolipids, is probably greater than in any other animal tissue This holds true also for the class of phospholipds which is more abundant in brain than in other tissues, thus supporting the view that the major function of these compounds lies in their participation in membranous structures. Numerous studies on the comparative aspects of phospholipids in brain and other nervous tissues have been carried out and have shown that the cephalin fraction dominates the lecithin fraction. However, the former fraction contains five or six different components. Based on recent analysis, making possible a better distinction of the different types of phospholipids, it becomes clear that phosphatidyl choline is quantitatively the most prominent individual phospholipid also in brain. Because of the relatively high concentration of phosphatidyl serine, the amount of this phospholipid together with phosphatidyl ethanolamine is equal to or even dominates that of lecithin, this depending on the particular material investigated. A certain preference of brain tissue for negatively charged phospholipids is also demonstrated by the occurrence of several members of inositol-phosphoglycerides. The di- and tri-phospho inositides recently have been demonstrated in liver and other tissue.[282] Sphingomyelin concentration has been found to differ in the brain of several animal species. It is interesting to note that during the myelinization process at an early age of the brain, the concentration of phospholipids increases and this effect is most conspicuous for the sphingophospholipid.[252]

Since it is not possible to survey in detail the increasing knowledge about the anatomical (phospho)lipid distribution in nervous tissue, only some appealing facts about their partition in subcellular fractions will be considered. In the phospholipid fraction of cell nuclei from human brain cortex, the proportions of lecithin, sphingomyelin and cephalins were found to be similar to whole tissue, only the content of cerebrosides appeared to be higher.[480] The nuclei of brain cortex and cerebellum have been reported to exhibit a large degree of similarity in phospholipid composition.[116] The composition of mitochondrial phospholipids has been found to be rather similar to that of the microsomal fraction and the total homogenate of rat brain.[41] The cephalin fraction, which by contrast to the brain lecithin, consists of an appreciable amount of plasmalogens was found to be rather uniform for both cellular fractions as well.

Interesting observations have been made on the distribution of phospholipids in the myelin sheath fraction which contains a higher amount of phospholipids than mitochondria, microsomes, nuclei and cytoplasmic liquid respectively. The myelin sheath was found by Nussbaum *et al.*[376] to have an amount of cephalins approximately twice as high as that of lecithin. Furthermore, the myelin sheath turned out to hold a substantial amount of sphingomyelin but not all of it. Considerable quantities of this phospholipid were recovered in the fractions of mitochondria, microsomes and nuclei as well. Thus the investigators quite correctly concluded that its abundance in intracellular membranes makes necessary at least partial revision of the view that the increase of sphingomyelin during growth related primarily to myelination.

(*c*) *Chloroplasts.* The lipid fraction of photosynthetic tissue is extremely complex and difficult to separate into the component classes, but recently marked progress has been made by the application of paper and thin-layer chromatography. Apart from the pigments, the lipids from chloroplast appear to differ from other plant sources in having a high proportion of glycolipids and certain phospholipids and a low triglyceride content (Table 1). The glycolipids, e.g. mono- and digalactosyl diglycerides (XXI and XXII), which represent a considerable amount of the lipid fraction of the green plant appear to be intimately related with the photosynthetic unit. According to Benson[32] these molecules may be oriented in the lamellar membranes together with phospholipids their galactose moieties facing the enzymes of carbohydrates synthesis. Another polar lipid, *viz.* an anionic sulpholipid[34] structurally related to the galactosyl diglycerides has been characterized by Benson and co-workers as a derivative of 6-sulpho-D-quinovose[96, 521] (XXIII; Fig. 12).

The phospholipids of chloroplasts from higher plants and algae now have been extensively studied with various chromatographic techniques. Fractionation on columns appeared not to give perfect resolution of the phospholipids and galactolipids,[528] although recently this technique enabled the isolation of mono- and digalactosyl diglycerides, lecithin[422] and phosphatidyl glycerol[204] from leaves.

A break-through in the characterization of the phospholipids from chloroplasts became possible by their separation on silica impregnated paper and

chromatography of the water-soluble phosphodiesters obtained by deacylation. It was by the latter technique that Benson and co-workers[35] discovered phosphatidyl glycerol and found it to be specifically linked with the photosynthetic unit. Phosphatidyl glycerol has been recently isolated in a pure state from spinach leaves in this laboratory and the structure and configuration as ascertained by Benson were completely confirmed.[205, 206] The phospholipid fraction from spinach leaves[512] and runner beans[264] have been demonstrated to contain (in the order of decreasing concentration) lecithin, phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidyl inositol. Beet leaves[512] were found to have a

Table 1. Representative distribution of lipids in spinach chloroplast lamellae on basis of minimum molecular weight of 960,000 per mole of manganese (after Lichenthaler and Park[323])

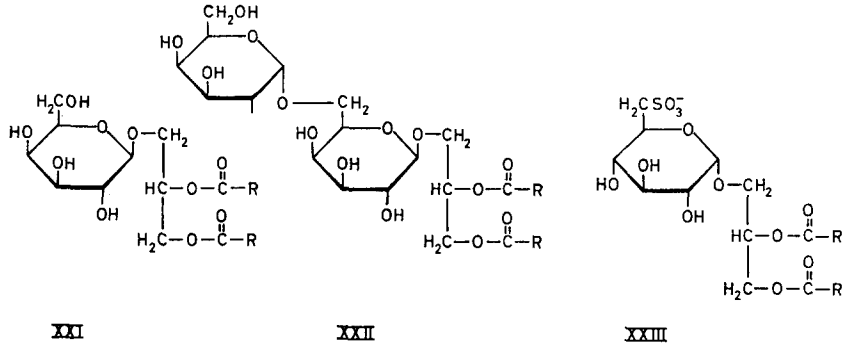
Lipid (composition moles/mole Mn)		
115 chlorophylls	103,200	
24 carotenoids	13,700	
23 quinone compounds	15,900	
58 phospholipids	45,400	
72 digalactosyldiglycerides	67,000	
173 monogalactosyldiglycerides	134,000	
24 sulpholipid	20,500	
? sterols	7500	
unidentified lipids	87,800	
		495,000
Protein		465,000
Lipid + protein		960,000

higher content of phosphatidyl ethanolamine, while in cabbage leaves this compound was found to be the most dominant phospholipid and turned out to be accompanied by the serine analog.[505] Algae lipids have been demonstrated to contain also the conventional types of phospholipids including phosphatidyl glycerol, though Benson[36] found evidence that the composition is rather different for individual species of *Rhodophyta* and *Chlorophyta*. After deacylation the derivative of cardiolipin was detected in some algae species. Plasmalogens have not been found in algae or plants and it is interesting to note that by contrast animal flagellates and amoeba appear to produce these substances.[191]

Confirming the importance of phosphatidyl glycerol for the photosynthetic apparatus, Wintermans[512] found this phospholipid to be concentrated together with galactosyl lipids in the chloroplast, whereas lecithin, phosphatidyl inositol, phosphatidyl ethanolamine and the sulpholipid were not specifically present in the photosynthetic unit. Quite recently, Nichols,[373] who examined the lipids from total leaves and chloroplast qualitatively by thin-layer chromatography, observed that only a small proportion of the leaf phospholipids was present in the

chloroplast. Phosphatidyl glycerol together with a dominating amount of mono- and digalactolipids and a novel lipid were recovered in the plastids, and this investigator suggested that lecithin and phosphatidyl ethanolamine may be concentrated in the mitochondria or cell nuclei.

The rapid labeling of phosphatidyl glycerol and the galactolipids during photosynthesis[149] led Benson to the conclusion that these compounds are dynamically involved in this process. It may be noted that a high turnover of this phospholipid has recently been observed in bacteria as well.[237, 261] A comparison of lipid patterns in photosynthesizing and non-photosynthesizing cells of *Euglena gracilis* indicated that in addition to significant alterations in sulpho- and galactolipids, also the phospholipid concentration and composition



XXI XXII XXIII

FIG. 12. Glycolipids of chloroplast. Monogalactosyl diglycerides (XXI), digalactosyl diglyceride (XXII) and the plant sulpholipid, sulphoquinovosyl diglyceride (XXIII).

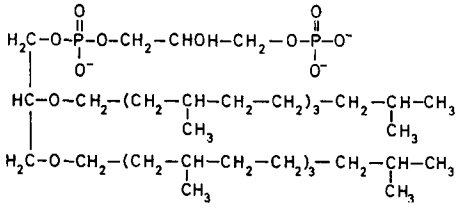
may be subject to change.[413] Undoubtedly, lipids play an essential part in chloroplast processes and a concept on the possible function of the galactolipids will be considered in Section IV.

(*d*) *Bacterial protoplast membrane.* It is not possible to deal in the present paper in an adequate manner with the phospholipids from bacterial origin and reference is made to more detailed reviews of this topic.[14, 15] Limiting our treatise to some recent developments, it may be recalled that lecithin has been detected in a relatively small number of bacteria only (e.g. *Azobacterium tumefaciens*,[258] *Lactobacillus acidophilus* and *Neisseria gonorrhocae*[15]. More recently Faure and Pillot[147] reported that *Reiter treponema* contains, in addition to polyglycerol phospholipids, a considerable quantity of phosphatidyl choline, and a similar observation was made in this laboratory by Houtsmuller. However, also with the modern analytical tools at hand, contradictory results have been obtained about the presence of lecithin in micro-organism, e.g. *Lactobacilli.*[247, 472] Related chromatographic properties of lecithin and more complex bacterial phospholipids and the unspecifity of certain reagents for the choline group easily can bring about misunderstanding. It seems not unlikely that part of the older

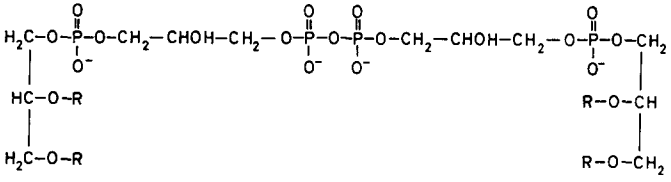
reports claiming evidence for the presence of choline-containing phosphoglycerides in bacteria will need a revision. On the other hand, large differences in the phospholipid pattern may result from variations in the conditions of the culture.

In *Clostridium butyricum*, which does not synthesize lecithin, Goldfine[178] detected the N-monomethyl derivative of phosphatidyl ethanolamine. Phosphatidyl ethanolamine is the amphiphatic lipid most frequently found in bacteria,[15] also when modern methods of separation and analysis are used.[237, 261, 267, 526] The serine analog has been encountered less frequently, but was detected in *E. coli* and demonstrated to act as a precursor of phosphatidyl ethanolamine.[261] The class of myo-inositol-containing phospholipids is present inba cteria as well. Apart from the simplest member, phosphatidyl inositol, more complex compounds with the general structure of phosphatidyl myo-inositol glycosides have been known for over 30 years to be present in *Mycobacteria*. The studies in the laboratory of Lederer recently led to a detailed structural definition of a phosphatidyl myo-inositol-dimannoside (Fig. 9; XV) and the pentamannoside; thereby establishing the stereochemical identity of the phosphatidyl inositols of these bacteria with those of plants and animals.[25] Carbohydrate-containing phospholipids and lipopolysaccharides of highly complex structure have been surveyed in detail by Asselineau and Lederer.[14, 15] The somewhat mysterious class of acidic phospholipids frequently reported to represent a significant part of the lipid fraction from several bacteria has been approached now by newer methods. Macfarlane reported that diphosphatidyl glycerol is present as a major component of the *M. lysodeikticus* protoplast membrane, and further work showed that this complex was a mixture of a phosphatidyl glycerol (G-P-G-lipid) and a glycolipid yielding a glyceryl mannoside.[331] Both diphosphatidyl glycerol and phosphatidyl glycerol were found by this investigator in *S. aureus*.[332] Cells of *Halobacterium cutirubrum* have been reported to contain a polyglycerol phospholipid containing ether-linked alkyl groups instead of the usual fatty acid ester bonds.[436] Quite recently Kates *et al.*[268] reported that the compound which behaved chromatographically very closely to diphosphatidyl glycerol is most likely a diether analog of phosphatidyl glycerophosphate (Fig. 13). At the same time Faure *et al.*[144] proposed an alternative structure (XXV) consisting of two glycerolphosphorylglycerolphosphoryl moieties linked together. The occurrence of the pyrophosphate linkage in phospholipids recently was also suggested by de Koning[300] in relation to the phosphate triester structures advocated by Collins.[85] Thus the family of polyglycerol phospholipids evidently is very complex and necessitates detailed investigation before the identity of the individual members in a given bacteria can be accepted with certainty. The observations of Macfarlane on the occurrence of phosphatidyl glycerol have been confirmed and extended to other bacteria, e.g. *B. cereus*[237, 267] and *E. coli*.[261] Apart from phosphatidyl ethanolamine, the phospholipid fraction of most eubacteria seems to contain a preponderance of negatively charged phospholipids. However, the occurrence of lipids which contain amino acids, including

basic ones, has been reported and recently one type of such compound has been identified. Studies carried out by Macfarlane[333] and independently in this laboratory[236, 238] showed that several bacteria contain amphiphatic phospholipids most likely to be structurally identical to O-amino acid esters of phosphatidyl glycerol (IX). These compounds, which include single members containing



XXIV



XXV

FIG. 13. Structures proposed for the diether phospholipid from *H. cutirubrum* by Kates *et al.*[268] (XXIV) and Faure *et al.*[144] (XXV).

ornithine, lysine appear to be different from the ornithine-containing lipid from *Mycobacterium*, which was found by Asselineau *et al.*[310] to behave as a chemical combination of fatty acids and ornithine. As regards the physiological significance of the phosphatidyl glycerol amino acids, many possibilities can be envisaged. The previously suggested role of lipo-amino acid complexes in protein synthesis,[242] or their involvement in transport processes across the membrane are two possibilities. Some recent observations made in the author's laboratory may be helpful for the further exploration of these problems. During an investigation on the influence of nutritional environment on the phospholipids of some bacteria, the addition of glucose was found to cause the most significant effects. A pronounced accumulation of the amino acid esters of phosphatidyl glycerol at the cost of the parent phospholipid molecule occurred in the bacteria when cultured in the presence of glucose. This shift in the phospholipid distribution could be attributed to a lowering of the pH of the medium as a result of the fermentation of the added glucose. As illustrated diagrammatically in Fig. 14, *S. aureus* could be governed by a simple change in pH to alter the proportions between the negatively charged phosphatidyl glycerol and the positively charged lysine derivative. It was speculated that this response to the acidity of

the surrounding medium might be connected with a charge balancing function of the lysine-containing phosphoglyceride in the membrane.[238] Another explanation may be that the pH change caused blockade of an enzymatically regulated transport process, perhaps connected with cell wall production. Comparable experiences resulted from a study on the phospholipids from *B. megaterium.*

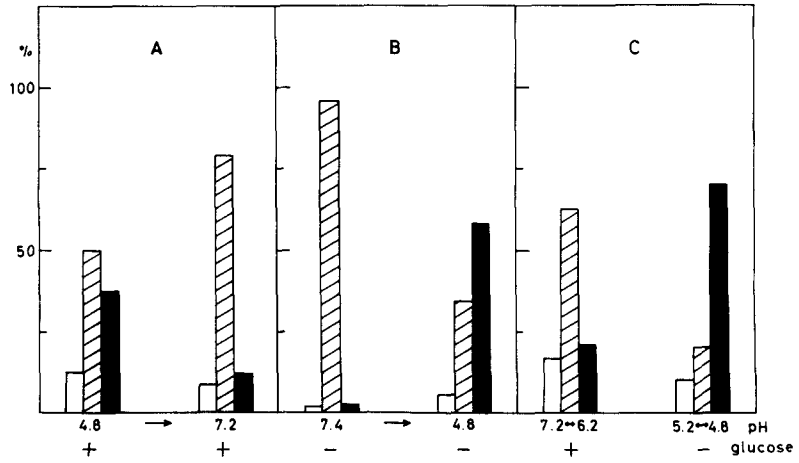


FIG. 14. The influence of the pH of the medium on the proportions of major phospholipids from *S. aureus.*[238] A. Left-hand columns represent the phospholipid distribution in a 16-hr culture (37°) grown in the presence of glucose (initial pH 7·2). At the time of harvesting the pH of the medium was 4·8. Right-hand columns give the phospholipid distribution of the same culture, which after adjusting the pH to 7·2 was incubated again for 3 hr. B. Left-hand columns indicate the situation in a 16-hr culture (37°) grown without glucose (initial pH 7·2). The pH at the end of the incubation was 7·4. Right-hand columns represent the composition of the phospholipids after the 16-hr culture was brought to pH 4·8 and incubated for a further 3 hr. C. Left-hand columns represent the phospholipid pattern of a 12-hr culture, grown with glucose. The pH was kept within the limits indicated. Right-hand columns concern a 12-hr culture grown at pH 5·2 without glucose. 1 hr before harvesting the pH was adjusted to 4·8. Open bars, unidentified compound (chromatographically related to diphosphatidylglycerol); shaded bars: phosphatidyl glycerol; solid bars: lysine ester of phosphatidyl glycerol.

The protoplast membrane in accordance with previous investigation was found to contain most, if not all, of the phospholipids of the whole cell. In addition to phosphatidyl ethanolamine, phosphatidyl glycerol, and a compound chromatographically behaving like phosphatidic acid or diphosphatidyl glycerol a phospholipid was present which behaved like a lysine ester of phosphatidyl glycerol. This composition applies, however, only to *B. megaterium* cultured in a medium of which the pH was prevented from falling below 7·2. When the bacterium was cultured in a broth containing glucose giving a change of pH to 5·4, another phospholipid was produced which was probably derived from phosphatidyl glycerol and reacted with ninhydrine. This compound which was

not detectable under the first mentioned conditions was found to account in various experiments for about 20 per cent of the total phospholipids. Further experiments are necessary to elucidate whether the compound is identical or related to the arginine-containing lipoamino complex from *B. megaterium* described by Hunter.[244] The accumulation of the positively charged phospholipid was proven also to be dependent on the pH of the medium, but several other factors, e.g. the age of the stock-culture, appear to effect its concentration. It will be of interest to investigate the properties of the bacterial membrane such as osmotic and pH resistance of protoplasts with different phospholipid compositions.

This approach may furnish further information about the significance of the chemical nature of the phospholipid headgroup for the membrane function.

3. Significance of the phospholipid headgroup

The diversity in the polar end-groups of the phospholipid molecules is evident. Phospholipids with certain headgroups are believed by various investigators to be involved in dynamic functions of the membrane, e.g. the transport of ions across these boundaries, but more in general these polar groups, being fit for ionic interactions, are considered to be responsible for holding together the lipid–protein network of these frameworks. Calculations about the long-range intermolecular forces which contribute to the stability of lipoprotein structures recently have been made by Salem.[420] The orders of magnitude derived for electrostatic forces, polarization forces and London–van der Waals' dispersion forces appear to support the view that charge–charge interactions between lipids and proteins are of paramount importance for the integrity of the biological interfaces. The Coulombic attraction between both charged groups of a amphiphatic phospholipid and two charged side-chain groups of a protein were considered by Salem to be at least equally apt for this purpose as interaction between an acidic phospholipid and one positively charged side group of the protein.

The tight binding of most phospholipids to other partners of membranous structure was recognized from the very onset of lipid extraction. Solvents like ether and chloroform, being very suitable to dissolve freed phospholipids, fail to extract adequately the phospholipids from (dehydrated) cellular structures. More polar solvent mixtures containing, for example, methanol or ethanol are required for this purpose. Parpart and Ballentine[386] differentiated the lipids from red cells by extracting lyophilized ghost with various solvents (dry ether, wet ether, alcohol-ether) into loosely, weakly and strongly bound. The predominant part of the phospholipids appeared to belong to the latter fractions. The principle of this procedure was reproducible in this laboratory and also, when applied to lyophilized cell organelles, the phospholipids were to be classified mainly as strongly bound as well. Another approach utilized by Lester and Fleischer[320] and Fleischer *et al.*[155] involves extraction with aqueous acetone. This solvent often has been used to dehydrate and remove neutral lipids from tissues prior to phospholipid extraction as well as for precipitation of phospholipids, but it is

well known now that such procedure can bring about considerable loss of phospholipids. Mitochondria, when treated with aqueous acetone, have been found to release a most significant part of their phospholipids, e.g. lecithin and phosphatidyl ethanolamine, the recovery being critically dependent on the water concentration.[320] The residual phospholipid of mitochondria extracted under these conditions was found to be predominantly cardiolipin.[155] The ease with which these amphiphatic lipids can be separated from the protein components and other evidence led to the conclusion that hydrophobic forces account for the interaction between the substances concerned, whereas ionic attractions were reserved mainly for the interaction between acidic phospholipids and basic proteins.[185] Studies carried out in 1957 in this laboratory and at that time considered not to be of sufficient interest to be reported in detail, involved a treatment of lyophilized mitochondria with dry ether and ether–ethanol (1:3 v/v). Since only the latter solvent system removed the bulk of the phospholipids, these components were considered to be rather firmly bound to the protein moiety. This approach certainly suffers of the drawback that the molecular organization in the lyophilized ghost may be different from that of the native membrane. Our experiences on the extraction of mitochondria by acetone–water mixtures confirmed the results of Fleischer *et al.*[155] It has to be noted that distinct conclusions about the tightness of binding of phospholipids to other membrane components as derived from extraction by ether–ethanol and acetone–water mixtures respectively, are rather a matter of terminology, since the dielectric constant of both types of solvent mixtures, supplying a release of the phospholipids from the membrane, is about the same. Further studies on the release of phospholipids by solvents of different, but defined, dielectric constant together with theoretical considerations as started by Salem[420] may be of help to evaluate the quantitative contribution of various types of bonding between phospholipids and proteins in membranes. With a view to the possible significance of the variety in polar headgroups, it is of interest that large differences have been observed in the extractability of different phospholipids. In mitochondria cardiolipin has been found to be very strongly bound and this feature has been explained by Strickland and Benson[462] on the basis of the paired charges and polar groups of this molecule (Fig. 15). The ionic interaction of the basic cytochrome C with acidic phospholipids was recently studied extensively in the laboratory of D. E. Green.[185] Studies of Folch[396] and co-workers emphasized the strong binding of a large part of the phospho-inositides of brain white matter to protein. The principal type of bonding in the extractable phosphatidopeptides as well as between these complexes and the insoluble protein appears to be electrostatic.[314] Dittmer and Dawson[130] also observed that the triphosphoinositide is tightly attached to brain protein.

Salem[420] calculated that two unit charges of opposite sign at a distance of 5 Å yield an attraction energy of 4·1 kcal per mole. The interaction of two negatively charged groups located at a certain distance apart in the phospholipid molecule, e.g. cardiolipin and diphospho-inositides, with two oppositely charged

side-groups of a protein consequently may account for the observed differences in binding.

The non-ionic interaction of proteins with phospholipid micelles was demonstrated recently by Green and co-workers,[185] who prepared complexes of structural protein from mitochondria with acidic as well as amphiphatic phospholipids. The basically hydrophobic character of the bonding was established by

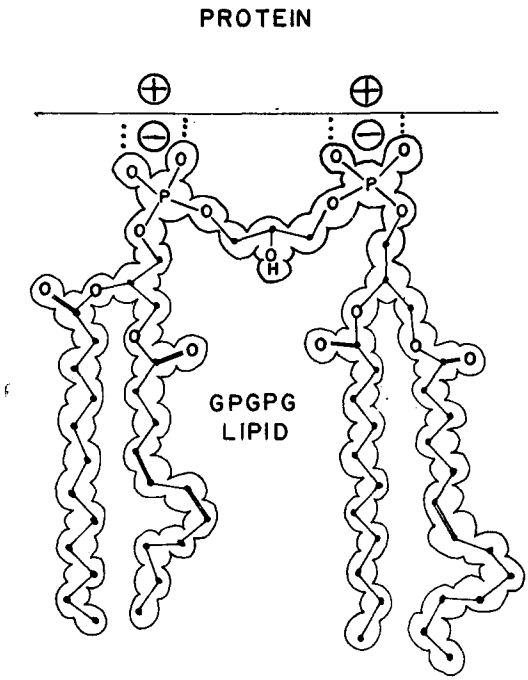


FIG. 15. Possible mode of ionic binding of diphosphatidyl glycerol at a protein surface.[462] [By courtesy of Dr. A. A. Benson.]

virtue of the failure of salt to dissociate or to prevent the formation of the complexes. The fact that the structural protein alone does not combine with cytochrome C, whereas the phospholipid–protein combines with the basic cytochrome C so as to form a ternary complex, added further support to the conclusion that the forces involved in the binary complex are hydrophobic in nature. The binding of alkylphosphates to the structural protein was found to be determined by the length of the side-chains.

In short, it appears that ionic binding is not exclusively involved in phospholipid–protein aggregation. However, the nature of the end-group of the phospholipid may well determine the magnitude of the electrostatic forces. Furthermore, it is recognized that a combination of phospholipids may behave quantitatively differently from the individual components, as well as that the phospholipid

fraction of many membranes studied exhibits a net negative charge. It is noteworthy that the red cell membranes of different animal species differ in their phospholipid pattern but that the sum of choline-containing lipids remains relatively constant. This possible limitation of too great an excess of negatively charged headgroups perhaps applies to many mammalian phospholipid in most tissues. On the other hand some tissue differences in the distribution of the polar end-groups of phospholipids are to be noted. When brain and liver are compared the membranes of the former tissue apparently contain a somewhat greater proportion of acidic phospholipids. With the exception of cardiolipin no conspicuous variations have been recorded so far in the phospholipid distribution of the intracellular membranes. This polyglycerol phospholipid has been found in very high concentrations in some of the enzymically active fragments of mitochondria, this already suggesting a possible specific role.

The lipid fraction of the chloroplast lamellae, having phosphatidyl glycerol as a major phospholipid and containing a sulpholipid, also exhibits a net negative charge, this being reduced, however, in its density by the appreciable amount of non-ionic galactolipids. The preponderance of acidic phosphoglycerides and negatively charged amphiphatic lipid molecules in the bacterial cytoplasmic membrane is apparent, but in a number of bacteria this negative charge is diluted by the presence of either lecithin or amino esters of phosphatidyl glycerol and perhaps by other complex amino acid-containing phospholipids.

Despite the recent progress, the alignment of proteins and in lipids in membranes viewed from the diversity of polar end-groups of the phospholipids is not disposed of all its mysteries. Fresh approaches made a start to a better understanding of the forces involved in phospholipid–protein interaction, but additional efforts are needed as well. Induction of a change in the phospholipid composition of a given membrane, when coupled with comparative investigations of the properties of the membranes, can be expected to reveal further information on the significance of the individual phospholipid types. Various possibilities recently became available for such studies. Schwarz *et al.*[434] reported that whole-body X-ray irradiation produces an increase of total phospholipids as well as a change of the phospholipid pattern of rat-liver mitochondria. The polyglycerol phospholipid, phosphatidyl glycerol, normally present only in very minor quantities, increased to over four times the control value, this being balanced by a decrease of phosphatidyl ethanolamine. It needs no argument that micro-organisms have not yet been sufficiently explored to elucidate the relationship between structure and function of phospholipids in the membrane. However, a beginning of such studies can be noticed. The application of mutants rendering unbelievable progress in many other fields of biochemistry will be fruitful for the lipid–membrane field as well. This is already demonstrated by the work of Hall and Nyc[193] on *Neurospora crassa* mutants which, because of a genetic abberation, lost the facility of making choline and accumulated the mono- and dimethyl ethanolamine phosphoglyceride analogs. Even simple variations in nutritional environment of the micro-organism may be helpful in this

respect. This is demonstrated, for example, by recent work of this laboratory on phosphatidyl-glycerol derivatives recorded above as well as by the studies of Kates *et al.*[269] on *M. halodenitrificans*. The proportion of phospholipids in the halophile bacterium appear to decrease with decreasing sodium chloride concentration in the medium, while the amount of unsaponifiable matter was found to be enhanced.

Perhaps the present treatise emphasizes too much the quantitative differences of the phospholipids with differently charged groups. Although having in common the phosphoryl-choline moiety, the known differences in chemical and physical properties between lecithins and sphingomyelins are likely to be of significance also in the biomembranes. However, much remains to be done before the meaning of variations in lecithin-sphingomyelin proportions can be interpreted in terms of membranous properties. This is true also for the variations between phosphoglycerides having the apolar chains linked either through a fatty acid ester bond, a saturated or an unsaturated ether bridge. It has been recorded in a survey by Rapport and Norton[399] that according to Schulman the proximity of an unsaturated bond to the polar moiety of the phosphoglyceride should produce important differences between the characteristic of monomolecular films of plasmalogens and diacyl analogs. With these considerations we are entering the domain of the apolar side-chains of phospholipids and their implications for the biological membrane.

D. Apolar side-chains

1. Variation and specificity of the paraffinic chains

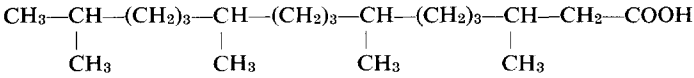
(*a*) *Chemical structures.* The analysis of the fatty acid and aldehydogenic constituents of lipids was greatly perfected by the introduction of gas–liquid chromatography by Martin and James. At present several hundreds of different fatty acids and aldehydes have been detected in nature. Though the distribution of the paraffinic chains is different between various classes of lipids, the phospholipids do not escape this overwhelming chemical variation and only a few aspects can be touched here. The subject has been reviewed in detail by Shorland.[438] Most phospholipids contain an appropriate amount of saturated fatty acids, having a chain length between 12 and 26 C-atoms, including some odd-

XXVI XXVII

FIG. 16. Branched chain fatty acids of the iso (XXVI) and anteiso type (XXVII).

numbered homologs. Traces of short-chain fatty acid constituents have been reported to be present in the phospholipids from bovine tissues.[207] In general stearic and palmitic acid serve as the major saturated fatty acid constituents of mammalian phospholipids. Branched-chain fatty acids are now known to be

more widespread than was earlier recognized. According to Shorland[438] both the odd series of the anteiso-(XXVI) and odd and even series of iso-acids (XXVII) have been detected in ruminant fats (Fig. 16). The excellent and careful work of Gray and Macfarlane[184] revealed the occurrence of small amounts of branched-chain fatty acids in various phospholipids from non-ruminants as well. The possibility was raised that these branched fatty acids are of bacterial origin.[184, 270] According to several investigators, polymethyl branched fatty acids also occur in ruminants, e.g. tetramethyl palmitic acid XXVIII which was



XXVIII

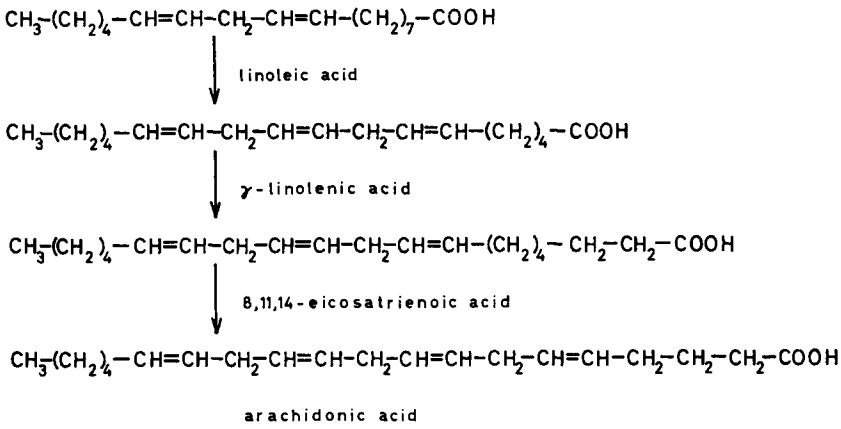
found in butter fat.[449] Quite recently a similar fatty acid has been isolated from the liver of a patient.[293]

Numerous variations in the physico-chemical properties of fatty acids arise by the introduction of unsaturated bonds. Monoenoic fatty acids can be distinguished by the location of the double bond and by the occurrence of a *cis* or a *trans* configuration. Many more variation possibilities accumulate by the presence of several double bonds which can be located at various positions to each other, e.g. non-conjugated and conjugated. A well-known dienoic fatty acid from animal phospholipids is linoleic acid; the trienoic acid and linolenic acid is found in quantity in many vegetable phospholipids. Among the poly-unsaturated fatty acid constituents of animal phospholipids, the essential fatty acid arachidonic acid is of particular interest. This subject, including the implications of the essential fatty acids for membranous structures and enzymic functions, has been thoroughly surveyed by Aaes-Jørgensen.[1] According to Thomasson[467, 468] the essentiality in a fatty acid depends on a specific structure of the terminal rather than the carboxyl end; the presence of double bonds between the 6th and 7th and 9th and 10th carbon atoms counted from the terminal methyl group was found to be a prerequisite for activity in nutritional experiments with rats. Studies from the laboratories of Mead[347] and Klenk[290, 294, 461] on the metabolism of poly-unsaturated fatty acids provided the biogenetical reasons for these findings. The poly-unsaturated fatty acids can be classified in four families:

$CH_3—CH_2—CH=CH$—linolenic acid family
$CH_3—(CH_2)_4—CH=CH$—linoleic acid family
$CH_3—(CH_2)_5—CH=CH$—palmitoleic acid family
$CH_3—(CH_2)_7—CH=CH$—oleic acid family

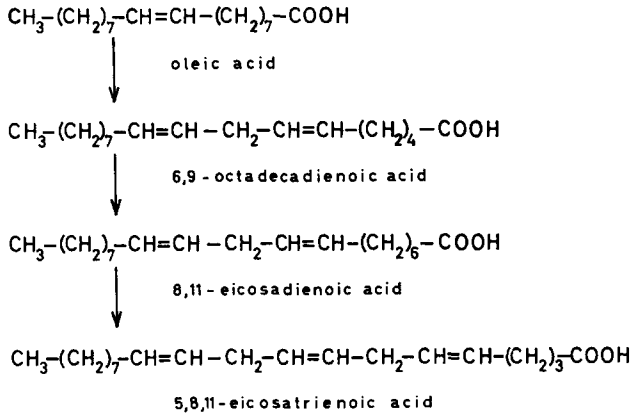
In the animal, two families of poly-unsaturated fatty acids, *viz.* linoleic and linolenic, are derived from the dietary acids,where as two families result from the mono-unsaturated acids which are themselves synthesized in the animal

tissue. The higher members of each family are produced by chain elongation and introduction of double bonds, e.g. the conversion of linoleic via γ-linolenic acid into arachidonic acid (Scheme 1). As demonstrated first by



SCHEME 1. Biogenesis of arachidonic acid.[290, 347, 461]

studies of Rieckehoff, Holman and Burr[404] and confirmed by many others, deprivation of linoleic acid results into an accumulation of 5,8,11-eicosatrienoic acid (dihydroarachidonate). This fatty acid was shown to arise from oleic acid[294, 347] (Scheme 2) by pathways similar to those for the conversion of linoleic



SCHEME 2. Pathway for the conversion of oleic acid into eicosatrienoic acid.[290, 347]

acid into arachidonic acid and linolenic acid into docosahexaenoic acid. The lipids of fat-deficient rats also appeared to contain 7,10,13-eicosatrienoate, a member of the palmitoleic family. While these eicosatrienoic acids in the deficient animals take the place of arachidonic acid, e.g. in the phospholipids,

apparently there is a failure to take over the physiological function of the latter fatty acid.

Among the unsaturated fatty acids occasionally acetylenic acids have been found, e.g. in the seed oils of a few species. Another rare class is formed by the acids containing cyclic systems, e.g. the cyclopropane-containing fatty acids abundant in *Lactobacilli.* The composition of the aldehydes from plasmalogens is nearly as complex as that of the fatty acid constituents. In a study of Gray Macfarlane[184] about 20 different aldehydes varying in chain-length from 12 to 20 C-atoms, including iso- and anteiso-branched homologs, mono-unsaturated and di-unsaturated constituents have been recorded. The principle ethers of the α-glyceryl ether-containing phospholipids are chimyl alcohol, batyl alcohol and selachyl alcohol,[201] but also small amounts of other derivatives have been detected in the total glyceryl ethers of fish liver oil,[194] bovine and human tissues.[195]

(*b*) *Fatty acid distribution.* Differences in the biosynthetic capacities between animals and higher and lower plants resulting in the formation of quite different fatty acids[438] will of course be reflected to a great extent by the fatty acid pattern of the phospholipids concerned. Limiting in this introduction the question about the specificity of the fatty acid composition of the phospholipid fraction to animal tissues, a number of generalizations appears to be possible. Comparing glycerides and phospholipids of the same mammalian tissue, the former are well known to be usually richer in palmitic acid, while the stearic acid concentration is higher in the phospholipids. The oleic acid level usually is higher in glycerides than in phospholipids. In fish, however, the fatty acid compositions of phospholipids and glycerides appears to be more similar.[438] Regarding the highly unsaturated acids, Klenk and Brockerhoff[289] observed in aquatic life a predominance of the linolenic family, whereas those of the higher land mammals are for the greater part of the linoleic type. In mammals the phospholipids appear to contain a much higher content of arachidonic acid, than do the glycerides. Furthermore, the fatty acid patterns of phospholipids are well known to reflect dietary influences, e.g. deprivation or overfeeding of the members of the essential fatty acid class. In addition to such dietary effects, the hydrogenation processes effected by ruminal micro-organisms appear to bring about significant differences between ruminants and non-ruminants in the fatty acid composition of the lipids including the phospholipids. The development of the living organism may also be accompanied by some changes in fatty acid composition.[131]

The overwhelming variations in chemical structure of the paraffinic acid connected with biogenetic, dietary and other factors challenge the investigator attempting to establish relations between the composition and function of phospholipids in membranes. The characterization of the apolar side-chains of membranous phospholipids forms an essential part of laborious studies required to enlighten the dark way leading to the recognitions of such relationships. Because of limits of space only a few examples can be communicated. One crucial

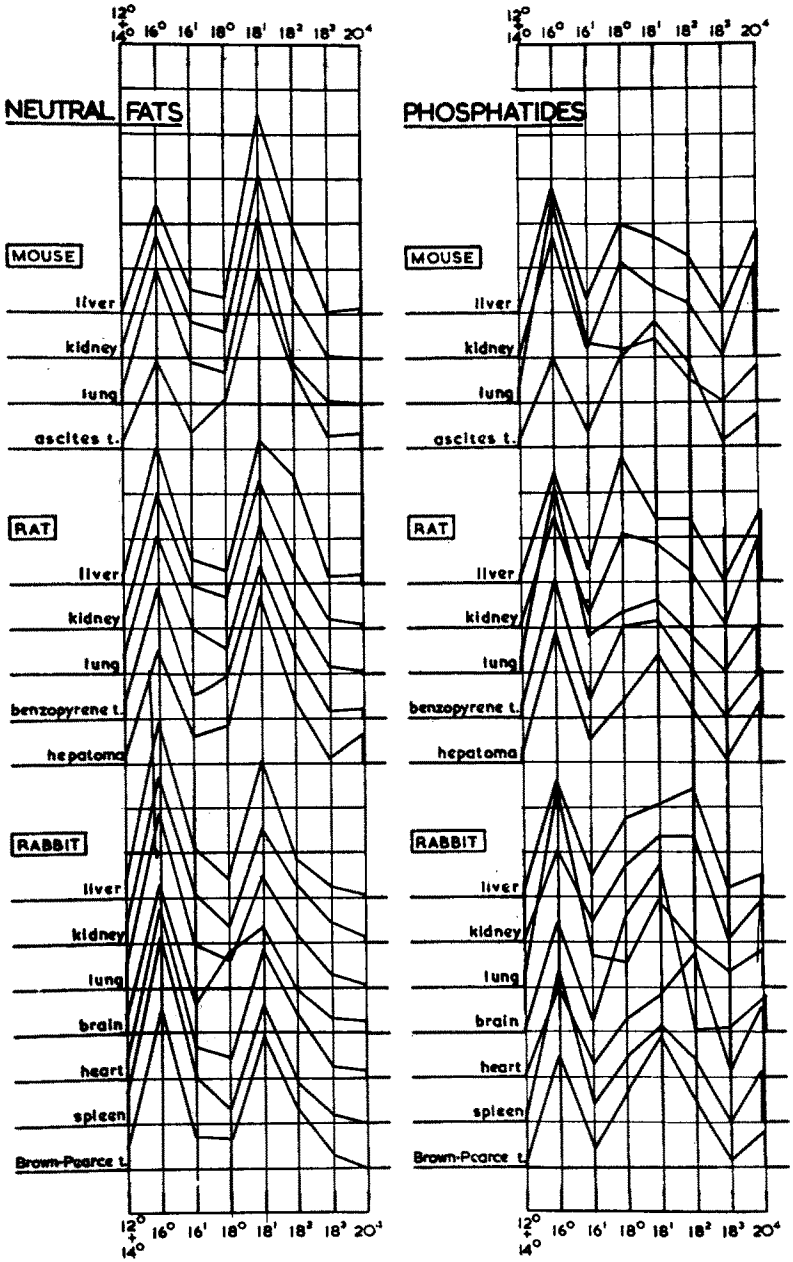


FIG. 17. Comparison of the fatty acid patterns of phospholipids and nonphospholipid fractions from several tissues of three animal species.[483] On the abscissa the fatty acids are indicated by means of the number of carbon atoms and the number of double bonds. On the ordinate weight percentages of fatty acid methyl esters are plotted. The distance between two horizontal lines corresponds with 10 per cent of fatty acid.

question may be: "To what extent is the fatty acid pattern of the phospholipid fraction specific for a given membrane?" Trying to find some answer to this question the many data available already in the literature[438] were somewhat extended in this laboratory by making comparative fatty acid analysis of the non-phospholipid and phospholipid fractions of several tissues from a number of mammals.[483] As illustrated diagrammatically, the neutral lipid fractions possess some degree of animal specificity, i.e. the fatty acid pattern may differ from animal to animal, but resembled one another for different tissues of one animal (Fig. 17). In the rat the fatty acid composition of neutral lipid fractions from several tissues appeared to be rather similar to that of the abdominal depot fat. The phospholipid fractions of the tissues studied were again found to be usually richer in stearic and poorer in palmitic acid when compared with the non-phospholipids. Furthermore, the phospholipids from several tissues of *one* animal species revealed a less characteristic fatty acid pattern than that given by the neutral lipids. By contrast, the fatty acids from phospholipids may exhibit a certain degree of similarity in homolog tissues of different animals. This type of specificity is demonstrated by comparing the fatty acid pattern of phospholipids from lung and brain tissue of a number of species (Fig. 18). The quantity of palmitic acid in the lung phospholipids is remarkable. One is tempted to suggest a relation with the function of the (rather saturated) lecithin, probably

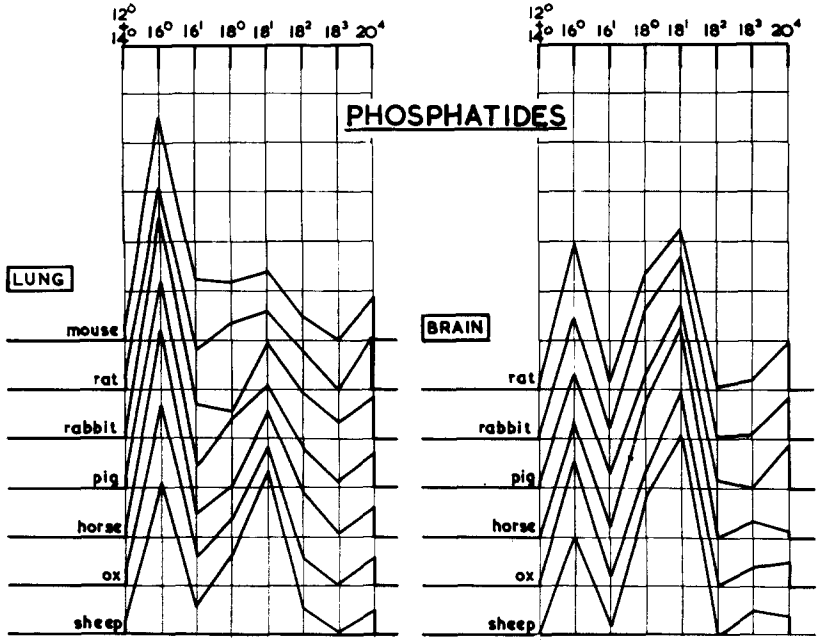


FIG. 18. Fatty acid patterns of the phospholipid fractions from lung and brain tissues from several mammalian species.[483] For explanation see Fig. 17.

produced by mitochondria,[288] which was found by Clements[81] to be responsible for the indispensable surface tension of lung lining. The low amount of linoleic acid in normal brain, well recognized by many investigators, is obvious for the brain phospholipids from all species investigated. Beside some similarities between the fatty acid patterns of phospholipids from the same tissues of different animals, there are also differences to be noted. In general the contents of 16:0 and 18:1 acids are lower and higher respectively in ruminants compared with non-ruminants. In spite of these and other differences which are to be attributed to the microbiological action of the rumen and dietary influences, the results appear to indicate subtle distinction, i.e. a species specificity in the neutral lipid fatty acids and a certain degree of a tissue specificity in the phospholipid fatty acids. The questions about the specificity of the apolar moiety of the phospholipids can be formulated also in a different manner, e.g. "Is a given type of phospholipid characterized by a specific fatty acid pattern?"

(*c*) *Fatty acid composition of individual phospholipids.* Earlier work on the fatty acid composition of specific phospholipids, involving a fractionation by precipitation and hydrolysis procedures, already revealed some significant differences, e.g. between phosphoglycerides and sphingomyelins. The methods applied restricted the comparison of closely related substances, e.g. lecithins and cephalins, because the isolated specimens were probably not fully representative in their fatty acids of the phosphoglycerides as occurring in the tissue. The chromatographic methods overcome this difficulty now and numerous reliable data are available on the fatty acid and aldehyde patterns of individual phospholipids from various tissues of different animal species. Instead of giving a cumbersome compilation of all pertinent data (rapidly increasing in their number), some selected examples believed to be rather representative for the present status may follow. An extensive study of Gray[182] and Gray and Macfarlane[184] on the phospholipids of beast, fish and bird revealed, apart from the mentioned differences between triglycerides and phosphoglycerides, significant and rather specific differences between lecithins and cephalins (Fig. 19). A preponderance of stearic acid as the saturated acid in cephalins (and the plasmalogen analogs) is evident, whereas palmitic acid dominates in the lecithins. An exception was made for the liver lecithin of both ox and rat which was found to contain more stearic acid than palmitic acid. It is of interest to note that Nelson[369] observed, however, a significant preponderance of palmitic acid in lecithin from mouse liver. Furthermore, the proportions of polyenoic fatty acids of the C_{20} series in general are higher in the cephalins than in the lecithins.

Cardiolipin from pig lung and kidney and pigeon breast was found to have a high proportion of linoleic acid (67·1, 74·0, and 84 per cent respectively), but in trout muscle cardiolipin was found to have a substantial amount of saturated fatty acids, oleic acid being the prevailing unsaturated fatty acid constituent. Cardiolipin from liver has been found to contain as much as 80 per cent of linoleic acid.[184] A generalization that cardiolipin of land animals is characterized by the preponderance of linoleate, however, is not allowed, since Biran and

Bartley[41] established that cardiolipin from brain mitochondria contains only about 10 per cent of this fatty acid. Inositol phospholipids appear to contain a conspicuously high amount of stearic acid. Phosphatidyl inositol from various origins revealed the following percentages of stearate: heart muscle[58, 358] about 50 per cent; liver,[146] 50 per cent; beef liver,[58] 78 per cent; rat liver,[58] 79 per cent.

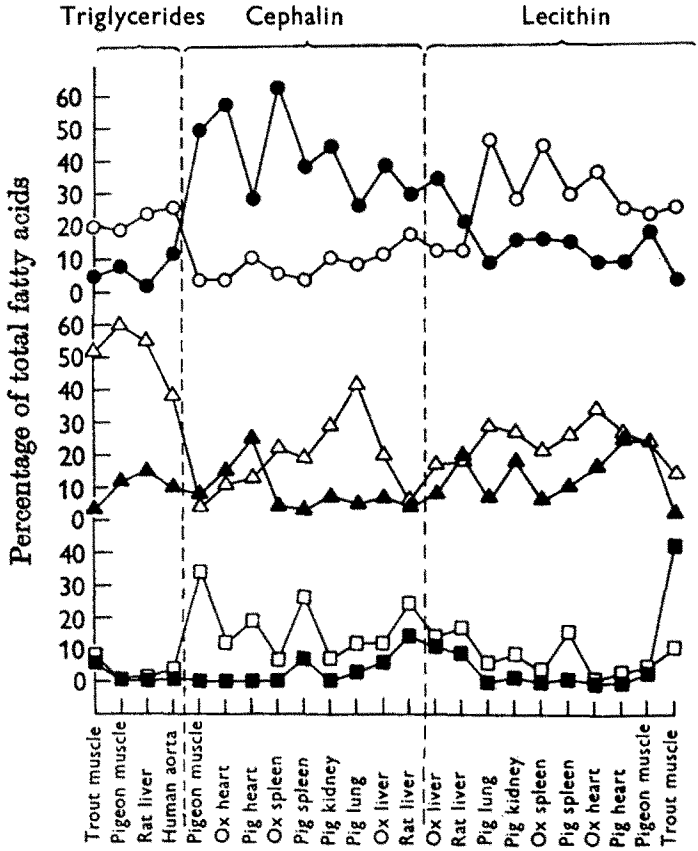


FIG. 19. Relative proportions of certain fatty acids occurring in the triglyceride, cephalin and lecithin fractions from different tissues.[184] ○, palmitic acid; ●, stearic acid; △, monoenoic C_{16} and C_{18} acids; ▲, dienoic C_{18} (including traces of trienoic C_{18} acid); □, polyenoic C_{20} acids; ■, polyenoic C_{22} acids. [By courtesy of Drs G. M. Gray and M. G. Macfarlane and the permission of the Biochemical Society.]

For mouse liver, a considerably lower value (about 15 per cent) has been reported.[369] The polyphosphoinositide fraction from calf brain (a mixture of di- and triphosphoinositides) was analysed recently by Kerr and Read[283] and found to contain 48·1 per cent of stearate.

Sphingomyelins from various sources contain quantities of palmitic and

stearic acid and are characterized by a high content of C_{24} acids, lignoceric acid (24:0) and nervonic acid (24:1). Appreciable amounts of hydroxy acids have been detected in sphingolipids as well.[69]

Evidently the individual phospholipids are distinguished by a characteristic fatty acid composition, being within certain limits irrespective of tissues or species. [184] This conclusion raises the question whether the differences noted in fatty acid composition of the total phospholipid fractions from different tissues can be accounted for by differences in the proportions of the individual phospholipids in the tissues concerned. That a distinction in phospholipid distribution does not always explain the characteristic differences in fatty acid composition of the total phospholipids between various tissues becomes readily clear by the exceptions noted in the specificity of fatty acid composition of the individual phospholipid classes. Whereas cardiolipin from mammalian origin usually is rich in linoleic acid, this polyglycerol phospholipid from brain mitochondria appeared to contain oleic acid as a major unsaturated constituent. Thus, brain cardiolipin reflects the typical low linoleic acid content of brain, but the preference of this phospholipid for this particular fatty acid is again demonstrated by the fact that cardiolipin possesses the highest linoleic acid content of all brain phospholipids. The phospholipid fraction from lung was demonstrated to contain a remarkable content of palmitic acid, and lecithin with its preference for this fatty acid constituent certainly is a major phospholipid in this tissue. On the other hand the lecithin of lung tissue was found to be extremely high in its palmitic acid content, e.g. nearly 50 per cent in pig lung.[184] Although tiresome work must provide more data before more definite conclusions about the subtle specificity of the fatty acid composition of membranous phospholipids can be made, the data at hand already demonstrate that oversimplifications have to be avoided. The specific pattern of fatty acids and aldehydes of the individual phospholipids and the tissue specific features appear to be interwoven. Apart from differences in the biogenetic capacities of fatty acid synthesis between species and tissues and the availability of certain dietary constituents, the enzymes regulating the proportions of phospholipids and the incorporation of the individual fatty acid constituents into the glyceride moiety determine the ultimate fatty acid composition of the phospholipid fraction. In this respect it is of particular interest to consider the distribution of fatty acids between the two ester positions available in the phosphoglyceride molecule.

(*d*) *Positional distribution of fatty acids.* The work of Hanahan[196, 197] clearly demonstrated that saturated and unsaturated fatty acids can be located in different position of the lecithin molecule. By virtue of the specific site of attack of snake-venom phospholipase A, recently re-evaluated to be directed exclusively towards the 2(or β)-fatty ester bond[190, 198, 466] it has been found that unsaturated fatty acids are located preferentially at the 2-position in lecithin from egg, plasma and liver. This feature is most pronounced for the polyunsaturated fatty acids, whereas oleic acid has been found in a significant proportion at the 1-position in lecithin from heart, liver and kidney.[127, 367] It

is highly interesting that in rats fed diets lacking in essential fatty acids, the 5,8,11-eicosatrienoic acid replaces the arachidonic acid at the 2(or β)-position.[127] This specific distribution of fatty acids was endorsed by the observation that in lecithins of marine animals the poly-unsaturated fatty acids of the linolenic family are located preferentially at the β-position. Brockerhoff *et al.*[59] found in cod, lobster and scallop the typical constituents of marine fats, *viz.* eicosapentaenoic and docosahexaenoic acids, which, as in terrestial animals, are derived from the diet, to have a clear-cut affinity for the β-position of lecithin. Comparing the members of the linoleic acid family in egg, rat and beef with the linolenic type in lecithin from tuna, salmon and menhaden muscle Menzel and Olcott[349] noticed a similar preference and "interchangeability" of the various poly-unsaturated fatty acids.

Phosphoglycerides containing a ratio of unsaturated to saturated fatty acids notably differing from one, will of course increasingly deviate in the positional specific location of the fatty acid constituents. However, also in phosphatidyl ethanolamine the poly-unsaturated fatty acids (though linoleic acid was not recorded) were found by De Tomás[127] to be located mainly at the 2-position thereby revealing again a similar location of 5,8,11-eicosatrienoic acid and arachidonic acid. Phosphatidyl serine from brain was demonstrated by Rathbone and Maroney[400] to exhibit a positional asymmetry of fatty acids as well.

Saturated and unsaturated fatty acids were reported[58] to be randomly distributed between the 1- and 2-position of phosphatidyl inositols, which contain over 50 per cent of stearic acid. However, Keenan and Hokin[273] observed a more specific fatty acid distribution in this phospholipid class. In the case of lecithin the specific distribution of fatty acids is to some extent a relative one, since by appropriate dietary conditions the level of poly-unsaturated fatty acids can be augmented to about 70–80 per cent. Questions about the enzymic mechanism responsible for the asymmetric fatty acid distribution and compelling problems about the specific differences in fatty acids composition between triglycerides and the various phospholipid classes will come into discussion in Section IV. Some further details of the paraffinic chains of the phospholipids from several distinct membranes will be considered now.

2. Fatty acid composition of membranous phospholipids

(*a*) *Erythrocyte membrane.* Analysis of the fatty acids of human red cell lipids have frequently been made. A representative fatty acid pattern of the total lipids from human erythrocytes is included in Fig. 20. Confining our present survey to some recent reports it can be stated that the results of several research groups are in good agreement. The data obtained by Kates *et al.*[265] on the total phospholipids revealed a somewhat higher palmitic acid content, being balanced by a lower stearic acid level. Furthermore, Kates *et al.*[265] and de Gier *et al.*[173] reported a higher linoleic acid content in the human erythrocytes than did Farquhar[124] and Hanahan and Ways.[493] Inasmuch as the fatty acid composition of erythrocytes reflects, also in man, certain differences in the ingested lipids, it

cannot be ruled out that the differences noted are due to dietary differences between the two continents. The various individual phospholipids from human erythrocytes[119, 142] revealed some of the common characteristics in their fatty acid pattern, e.g. a high content of palmitate in lecithin and sphingomyelin, an

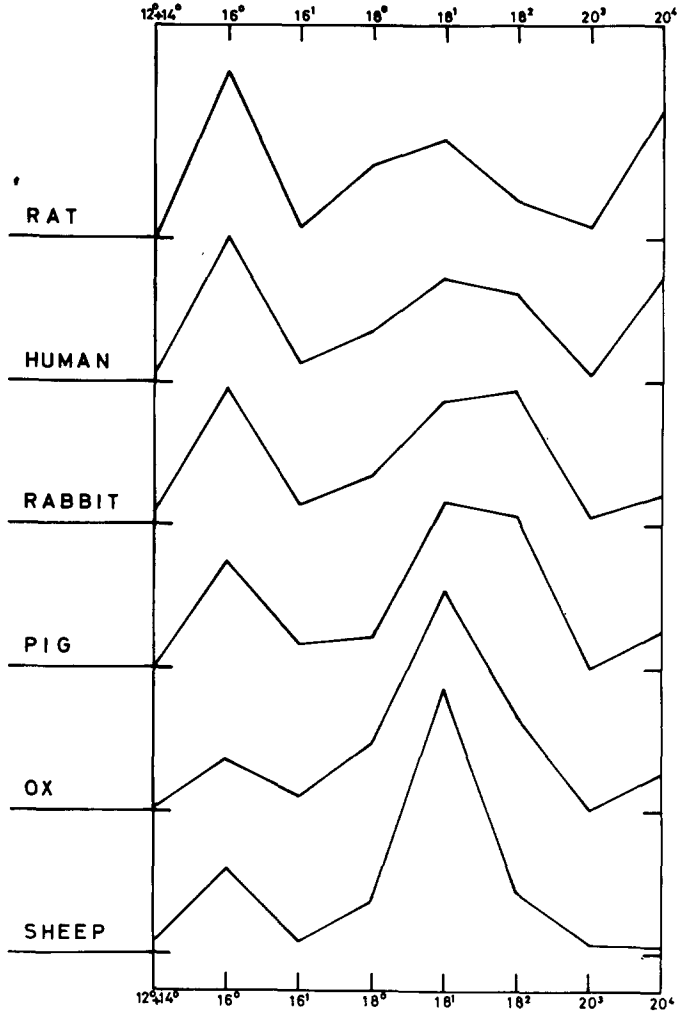


FIG. 20. Fatty acid patterns of the lipids from red cell membranes of different mammalian species. For explanation of abbreviations see Fig. 17.

accumulation of C_{24} fatty acids in the latter phospholipid, a preponderance of arachidonic acid in phosphatidyl ethanolamine; whereas phosphatidyl serine was rich in stearic acid as well.[142] Part of the human red cell phosphoglycerides belongs to the class of plasmalogens, and the aldehydogenic constituents have

Table 2. Fatty aldehyde composition of human erythrocyte plasmalogens

Fatty aldehyde	*Kates et al.*[265]	*Farquhar*[142]
14:0*	trace	—†
br. 15:0	0·8	1·6
15:0 iso or anteiso	—	0·1
15:0	0·6	0·5
Unknown	trace	0·2
cis 16:1	0·4	0·2
16:0	24·2	19·3
br. 17:?	—	0·1
br. 17:?	1·7	0·5
br. 17:0	7·5	4·4
17:0 iso or anteiso	—	2·5
17:0	1·3	4·4
cis, cis 18:2	—	1·3
cis 18:1	6·0	8·0
18:1 isomer	2·8	12·7
18:0	42·5	44·1
Unknown	2·9	—
br. 19:0	—	0·1
20:?	3·1	—
20:?	5·6	—

* This shorthand designation is analogous to that used for the fatty acids; br. 15:0 signifies a branched saturated fatty aldehyde with 15 carbon atoms.

† The data reported by Farquhar are recalculated for weight percentages.

been characterized.[142, 265] A comparison of the data for the total lipids and ethanolamine phospholipids, reveals little difference, since the latter class contain the majority of aldehydes (Table 2). The most abundant aldehydes are saturated straight chains 18:0 and 16:0, a branched 17:0 and the saturated 18:1. As demonstrated by Farquhar,[142] the 16:0 constituent is dominant in the choline-containing types. Similar observations have been made by Gray[182] on plasmalogens of other origins and apparently the difference in stearate and palmitate concentration between cephalins and lecithins is preserved fairly well in the aldehyde residues.

The fatty acid pattern of the erythrocyte lipids has been demonstrated in this laboratory[119, 297] to exhibit most significant differences between various mammalian species. In the series of animals: rat, man, rabbit, pig, ox and sheep there is a most conspicuous decrease of palmitic acid and arachidonic acid, accompanied by an increase in 18:1 fatty acids (Fig. 20). Apparently a tissue-specific fatty acid composition of the membranous phospholipids does not hold for this homologous membrane. As noted in this section (part B), in nearly the same order of animals, there are significant differences in the phospholipid content of the erythrocytes as well. However, the shift in sphingomyelin–lecithin ratio

appeared not to be linearly related with these variations in fatty acid composition. The latter differences were found to be most clearly reflected by the cephalin fraction which is relatively constant in the red cells of these species. Further work[174] showed that these differences in the fatty acid residues are attributable to at least three factors: (a) different dietary habits, (b) effects brought about by ruminal processes, (c) animal species differences in biosynthetic capacities. Studies in several laboratories conclusively established in the past few years the influence of dietary factors on the fatty acid pattern of red cell lipids. Most significant alterations were obtained by deprivation of essential fatty acids in monkey[186] and rat,[119, 355, 513] or by feeding an excess of certain fatty acids, e.g. oleic acid or linoleic acid, to man,[143, 235] rabbit[119] and rat.[119, 355, 357, 513] As will be outlined in Section IV, these alterations also occur in mature circulating red cells, which change their acid composition rather rapidly by various processes in response to dietary induced changes in serum lipids. An explanation of the formulation given above for the species differences is furnished by some dietary experiments on two mammals, *viz.* rat and sheep, which differ most extremely in the fatty acid pattern of the red cell phospholipids. A high caloric intake of hydrogenated fat (arachis oil) does cause a significant decrease of arachidonic acid and palmitic acid in the rat erythrocyte which is balanced by an increase in 18:1 acids and the appearance of eicosatrienoic acid(s) (Fig. 21). Thus this regimen makes the fatty acid pattern of rat erythrocytes nearly identical to that of normal sheep. In the sheep the ingested poly-unsaturates are well known to be subject to hydrogenation and isomerization reactions involving the ruminal micro-flora. Apparently, such alterations in the dietary fatty acids are reflected by the sheep erythrocyte and after supplementation of an appropriate diet a practically similar pattern can be induced in the rat erythrocyte. Conversely, it was of interest to investigate the fatty acid pattern of erythrocytes from sheep after exclusion of the ruminal action. For this purpose new-born animals where milk passes directly from the oesophagus to the omasum were given a diet of fat-free cow milk supplied with corn oil. After a period of one month the level of linoleic acid in the red cell phospholipids was increased about fivefold, while the amount of 18:1 acids was appreciably lower. The erythrocyte fatty acids of the experimental sheep, however, still differed significantly from the normal rat. In the latter animal the amount of arachidonate in the erythrocyte lipids is directly related to the concentration of linoleate in the diet as has been shown by a convincing study by Mohrhauer and Holman.[355] In the experimental sheep the linoleic acid supplied apparently was not readily converted into arachidonate, thus resulting in a significant difference in linoleate–arachidonate ratio of the red cell lipids between rat and sheep. Finally, it may be recorded that the dietary-induced alterations in fatty acid composition were not accompanied by notable alterations in the phospholipid distribution of the red cell, demonstrating again that these lipid characteristics can be independent of each other.

(*b*) *Mammalian cell organelles.* The results obtained by several research groups

on the fatty acid composition of nuclei, mitochondria and microsomes of rat liver show some differences, at least with respect to the quantitative distribution of individual fatty acid residues.[80, 170, 336, 340, 433, 483] Besides variations in the methods used, different dietary conditions may be responsible for these discrepancies. Even the fatty acid composition of brain lipids which is known to be relatively constant, recently was demonstrated by Mohrhauer and Holman[356]

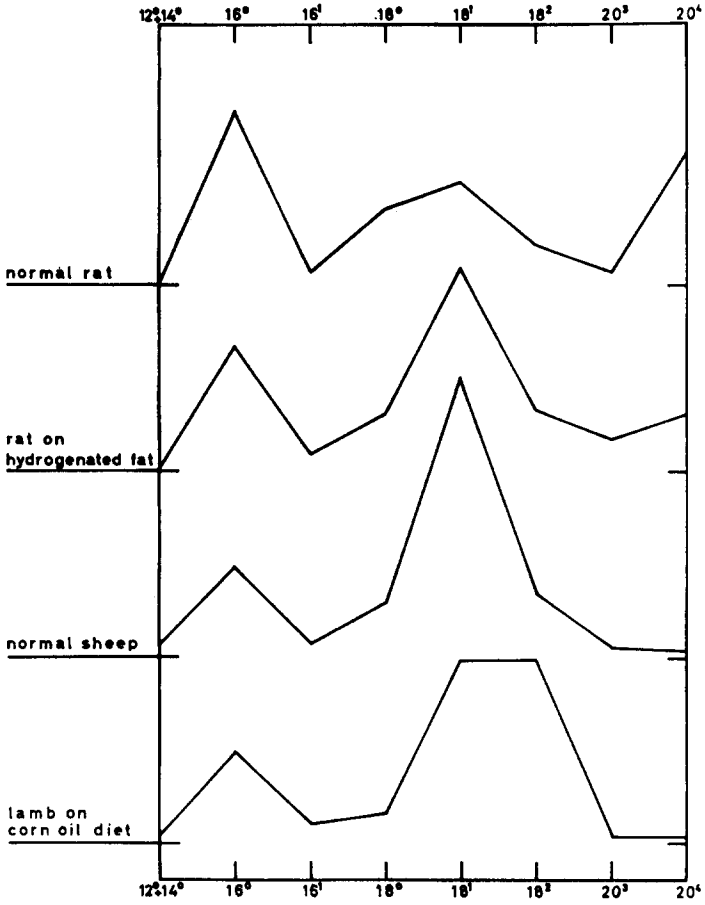


FIG. 21. Dietary effects on the fatty acid composition of mammalian erythrocytes.[174]

to be significantly altered, when either linoleate, linolenate or arachidonate were supplied to weanling rats on a fat-free diet. The dietary influence on the essential fatty acid content of liver is well established, e.g. by alteration of the diet, the level of the linoleate family in the phospholipids from liver mitochondria can be augmented by about 30 per cent in only a few days. Deleting therefore a detailed comparison of the data recorded in various communications, the general conclusion can be summarized briefly. The various subcellular fractions obtained

by ultracentrifugation did not reveal striking variations in the fatty acid composition of their lipid components. Thus the different biochemical activities of these particles are not specifically reflected by this lipid characteristic of their membranous structures, to which a more general structural function may be attributed. The phospholipids and neutral lipids in each cellular fraction revealed the characteristic differences in fatty acid pattern between both classes extracted from the cell as a whole.[80, 433, 483] The individual phospholipids and other classes from mitochondrial and microsomal fractions from rat liver have been characterized by Macfarlane *et al.*[336] and Getz *et al.*[170] In good agreement it was found that each lipid class had much the same fatty acid composition irrespective of the cell fraction. The cephalins again showed a higher proportion of arachidonic and docosahexaenoic acids than other lipids. Since according to Getz *et al.*[170] the relative amount of this phospholipid is higher in mitochondria, these particles contain a higher proportion of the poly-unsaturated fatty acids.

Holman and Widmer[232] analysed the poly-unsaturated fatty acids of enzymically active sub-mitochondrial particles from beef heart, and recorded no significant differences in either total content or in their distribution pattern. Mitochondria extracted by salt according to the method of Dallam[95] were found by Veerkamp *et al.*[483] to have a fatty acid composition which resembles very closely that of phospholipids isolated from the whole tissue. Accordingly, the differences existing in fatty acid constituents between various tissues are reflected by the mitochondrial membrane fragments. Biran and Bartley[41] observed only minor variations in the fatty acid composition of lecithins and cephalins in the mitochondrial and microsomal fractions from rat brain. For an understanding of the role of the phospholipid fatty acid constituents in membrane function a comparison of the data obtained on mammalian cell organelles with those of non-mammals and plants is of particular interest. Richardson *et al.*[403] demonstrated the fatty acid patterns from fish mitochondria to have a high level of the linolenate family, while intermediate levels of this class were detected in fish-eating birds and seals. In the mitochondria of sweet potato arachidonic acid was not detectable.

(*c*) *Chloroplasts.* Photosynthetic organisms provide the major biological source of poly-unsaturated fatty acids. As demonstrated by the work of Debuch[114, 115] not only high amounts of linolenic and linoleic acid are present in spinach leaves and chloroplasts but also unusual C_{16} unsaturated fatty acids. A C_{16}-trienoic acid and *trans*-3,4-hexadecenoic acid, were detected. Higher plants synthesize linoleic acid and (α)-linolenic acid, whereas by contrast the higher animals are able only to convert these fatty acids into poly-unsaturated members of the higher series. As demonstrated by Stumpf and James,[463] the major site of biosynthesis of saturated fatty acids and of oleic acid in leaves is in the chloroplast, and the synthesis is diminished in the dark. Under anaerobic conditions oleic acid synthesis was found to be suppressed as well. According to Ching Yuan and Bloch[75] and James,[250] by introduction of a system of methylene-interrupted double bonds approaching the terminal methyl the plant is able to convert oleic acid into linoleic acid and α-linolenic acid. The location

of the enzyme systems is not exactly known yet. Interesting pathways for the subsequent synthesis of poly-unsaturated C_{20} and C_{22} fatty acids in *Euglena* were quite recently outlined by Korn.[302]

Euglena which adapts its biochemical mechanism in dark and light either to a plant or animal mode of living turned out to be an excellent tool for exploring the differences in production of certain unsaturated fatty acids between plant and animals. Erwin and Bloch[139, 140] made the important discovery that in *Euglena gracilis* grown phototrophically, α-linolenic acid accounts for 20–30 per cent of the total cellular fatty acids. In dark-grown cells α-linolenic acid was found to be a minor component only, whereas the content of C_{20}, C_{22} and C_{24} poly-unsaturated acids was increased. It is intriguing that under such conditions fatty acids more typical of animal cells accumulated. Rosenberg[413] carrying out similar studies found that 18:3—although sometimes predominant—is not the only unsaturated fatty acid to undergo an increase during photosynthesis. This investigator observed a much higher content of saturated fatty acids of a chain length lower than 17 carbon atoms in the etiolated cells. The variation between these studies probably is attributable to strain differences and other experimental factors. The intimate connection of the production of α-linolenic acid with photosynthesis of higher and lower plants (with the exception of photosynthetic bacteria), is endorsed by the observation that α-linolenic acid is concentrated in the chloroplast of *Euglena*.[140] With a view to the possible significance of the fatty acid constituents in the function of the lipid in membranes of different intracellular structures, it is of interest to consider now the individual lipid types.

The galactolipids, which are known to be abundant in the chloroplast, are unique because of their high content of unsaturated fatty acids. The galactosyl glyceride fraction from red clover leaf was reported by Weenink[497] to have as much as 94 per cent of linolenic acid. The mono- and digalactosyl diglycerides isolated from runner beans by Sastry and Kates[422] appeared to contain almost exclusively linolenic acid (96 and 93 per cent respectively). Similarly Benson[34] detected in the monogalactolipid (95·0 per cent) and digalactolipid (82·0 per cent) a high content of this fatty acid, but in *Chlorella*, oleic acid appeared to dominate linolenate as a constituent of both glycolipids. The sulpholipid, on the other hand, possessed saturated fatty acids (palmitate) in quantity. This is true also for the class of phospholipids which like the sulpholipid are not exclusively concentrated in the chloroplast. A crude phospholipid fraction from red clover leaves was reported to contain 45 per cent of palmitic acid.[497] Wheeldon[505] observed that various phospholipid fractions from cabbage leaf phospholipid were fairly uniform in fatty acid composition and contained predominantly palmitic, linoleic and linolenic acids. Lecithin isolated in a pure form from runner beans[422] consisted of palmitic acid (27 per cent) and stearic acid (8 per cent), which were located at the 1-position, while linoleic acid (36 per cent) dominated linolenic acid (24 per cent) and oleic acid (5 per cent). In as much as phosphatidyl glycerol in contrast to the other phospholipids is believed to be concentrated mainly in the chloroplasts it is of interest to be informed about its

fatty acid composition. To the author's knowledge so far no pertinent data on this subject have been reported.* Recently, phosphatidyl glycerol was isolated from spinach leaves.[204] The crude lipid mixture from this source revealed a fatty acid pattern almost identical to that reported by Debuch[115] and Wolf *et al.*[517] A hexadecatrienoic acid and the *trans*-Δ^3-hexadecenoic acid, which latter structure was thoroughly investigated by Debuch, were found to be present.[206a] The separation into an acetone-soluble lipid fraction, containing a considerable part of the galactolipids, and an acetone-insoluble fraction containing together with other lipids all of the phospholipids, resulted in a fatty acid distribution (palmitate and linolenate) in agreement with previous observations on the distinct patterns of glyco- and phospholipids quoted above. However, an additional distinction became apparent, *viz.* the hexadecatrienoic acid accumulated in the acetone-soluble fraction, whereas some concentration of the C_{16} *trans* fatty acid occurred in the acetone-insoluble lipids (Table 3). After two column chromato-

Table 3. Fatty acid composition of lipids from spinach leaves (Spinacea oleracea)

	16:0	16:1	16:1 *trans*	16:3	18:0	18:1	18:2	18:3
Total lipids	17·0	tr*	2·4	7·4	0·5	6·0	14·6	51·4
Acetone-soluble lipids	6·7	tr	0·5	12·3	0·5	4·1	8·8	67·0
Monogalactosyl glyceride	9·7	—	—	25·3	—	—	—	65·1
Acetone-insoluble lipids	24·7	tr	4·8	tr	tr	9·2	25·0	36·3
Lecithin	21·2	—	—	—	1·3	15·8	31·6	30·1
Phosphatidyl ethanolamine	31·5	—	—	—	6·4	9·5	34·2	18·4
Phosphatidyl glycerol	20·0	tr	31·7	—	0·6	2·6	8·0	37·1
Lysophosphatidyl glycerol†	32·4	tr	0·5	—	1·2	2·4	2·1	61·4

* Less than 0·5 per cent.
† Prepared by enzymic breakdown with phospholipase A.

graphic separations phosphatidyl glycerol was obtained in a pure form. The gas–liquid chromatogram of the fatty acid revealed a considerable amount of this latter acid to be present. After its isolation by preparative GLC, IR spectrum and oxidative cleavage confirmed the nature and position of the double bond as established by Debuch. This 3-hexadecenoic acid, accounting for over 30 per cent of the fatty acids of phosphatidylglycerol from spinach, was found to be released by the action of phospholipase A[206a] (Table 3). Thus phosphatidyl glycerol from the chloroplast may deviate in having a significant amount of poly-unsaturated fatty acids located at the 1-ester position. Furthermore it is interesting to note that both the fatty acid composition and distribution differ

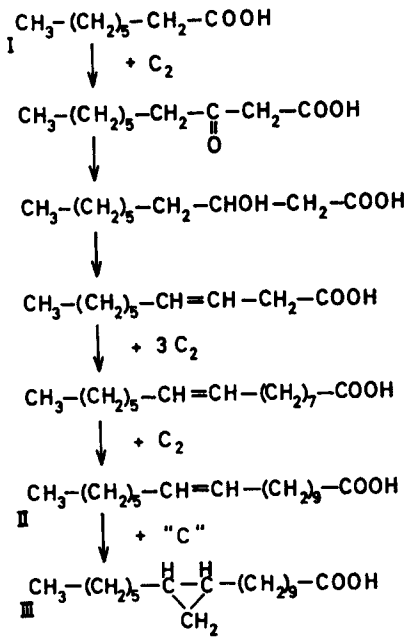
* Meanwhile C. F. Allen, P. Good, H. F. Davis and S. D. Fowler (*Biochem. Biophys. Res. Commun.* **15**, 424 (1964)) and R. O. Weenink and F. B. Shorland (*Biochim. Biophys. Acta*, **84**, 613 (1964)) reported similar findings about the fatty acid composition of phosphatidyl glycerol from leaves.

between lecithin and phosphatidyl glycerol from these green leaves. This observation may be relevant to the location of the two phosphoglycerides in different subcellular structures.

(*d*) *Bacterial protoplast membranes.* Although the number of bacteria studied specifically for their phospholipid fatty acids is still restricted a great variety in the paraffinic chains as well as striking qualitative and quantitative differences have been established. Considering first the saturated constituents, the major phospholipid of *H. cutirubrum* having a diether structure is an extreme example because both alkyl chains are saturated C_{20}-branched.[268] Among the straight-chain saturated fatty acids palmitic acid often appears to prevail, e.g. in *Lactobacillus casei, Serratia marcescens*,[42] *Clostridium butyricum*,[179] as well as in the phospholipids of *Salmonella typhimurium*,[332] *Azotobacter aglis*,[258] and *Agrobacterium tumefaciens*.[258] Lower homologs, i.e. myristic and lauric acid, as well as higher members including odd-numbered types occur as well. Several bacteria contain as major fatty acid constituents the saturated methyl branched fatty acids of the C_{15} and C_{17} series. Saito[419] reported that the isoacid 13-methyl tetradecanoic acid is the principal fatty acid present in *Bacillus subtilis*, and isoheptadecanoic acid was isolated as well. However, Kaneda[255] found apart from these iso-C_{15} and C_{17} that anteisohomologs were the major components of the total fatty acids in *B. subtilis*. Also Allison *et al.*[6] reported that an anteiso C_{15} fatty acid is abundant in this bacterium. Apart from these mentioned constituents 12-methyl tetradecanoic acid[5] (sarcinic acid), and the homologous anteiso acid 14-methyl hexadecanoic acid as well as the even-numbered iso-fatty acids isomyristic and isopalmitic acid have been isolated.[255] Marfarlane[331] has found that in the membrane phospholipids of *Micrococcus lysodeikticus* the anteiso-C_{15} acid dominates the iso-C_{15} acid, while both account for 80–90 per cent of the total fatty acid constituents. Also in *Staphylococcus aureus*[332] the anteiso- and iso-C_{15} and C_{17} were detected in quantity. According to Yudkin[524] branched-chain C_{15} acids accounted for some 80 per cent of the fatty acids of the membranous lipids from *Bacillus megaterium KM*, the iso-acid being twice as abundant as the anteiso-acid. Hunter and James[244] detected considerable quantities of branched fatty acids, and some straight-chain components (palmitate) in the phospholipids from *B. megaterium*. The fatty acid composition of a lipo-arginine complex, however, appeared to differ strikingly from other lipid fractions. Supporting the idea that isoleucine and leucine may serve as precursors of the two types of branched-fatty acids Lennarz[318] demonstrated the incorporation of α-methyl butyrate and isoleucine into C_{15} and C_{17} branched acids of *Micrococcus lysodeikticus*. Kaneda[256] accordingly reported that addition of short-chain fatty acid substrates caused an increased formation of the related branched long-chain fatty acids. The biosynthesis of branched-chain amino acids from branched-chain fatty acids by rumen bacteria was recently studied by Allison and Bryant.[7] Although the observations thus far cover only a limited number of examples, it seems likely that considerable quantities of saturated branched-chain fatty acids occur in bacteria having a low amount of

unsaturated constituents. It is of interest to note that ruminal bacteria have been found to produce fatty aldehydes, the major components being palmitaldehyde and branched-chain C_{15} aldehydes.[270]

The class of unsaturated fatty acids in bacteria is limited almost exclusively to monoenoic acids, but differences in chain-length and position of the double bond are responsible for some conspicuous distinctions. In an elegant series of investigations Bloch and coworkers[47] demonstrated that at least two different mechanisms occur, oxidative and anaerobic. The former pathway resembling that of yeast and involving an oxidation of the saturated fatty acid analog was established to be responsible for the formation of oleic acid in *Mycobacterium phlei*.[319] Apart from the direct aerobic conversion of stearic acid into oleic acid, the latter was found to be metabolized to 10-methyl stearic acid, methionine serving as methyl donor. Subsequent studies showed that *cis*-11-hexadecenoic acid, formed by desaturation of palmitic acid is more abundant than oleic acid.[426] The anaerobic pathway was first detected in *Clostridium*



SCHEME 3. Anaerobic pathway for the conversion of octanoate (I) into *cis*-vaccenic acid (II) and the formation of lactobacillic acid (III).

kluyveri and *Clostridium butyricum*, which unlike yeast, produce under anaerobic conditions unsaturated fatty acids.[179] This process does not involve a significant conversion of the saturated homologs, but short-chain fatty acids with a critical chain-length were found to be suitable precursors.[427] Octanoate was found to

give rise to unsaturated fatty acids with the double bond between carbon atoms 7 and 8 (Scheme 3), decanoate to the isomers with the double bond between 9 and 10 when counted from the terminal methyl group. This biosynthetic mechanism was found in other bacteria as well.[375] The products occur as such in bacterial lipids, e.g. in *Leuconostoc mesenteroides*, *cis*-vaccenic acid accounts for about 75 per cent of the total fatty acid,[248] but also serve as precursors for another type of bacterial fatty acid. By passing the hydroxy fatty acids which act as intermediated and according to Kaneshiro and Marr[259] represent half of the fatty acids from *Azotobacter agilis*, the cyclopropane fatty acids now deserve attention. The pioneering investigations of Hofmann and coworkers[220, 221, 324] on the lipids of *Lactobacillii* led to the discovery of a C_{19} fatty acid containing a cyclopropane ring. This acid was demonstrated to be identical to *cis*. 11,12 methylene octadecanoic acid, trivially denoted also as lactobacillic acid. A C_{17} isomer was detected in *E. coli* by Dauchy and Asselineau[101] and characterized as *cis*-9,10 methylene hexadecanoic acid by Kaneshiro and Marr.[257] Recently, Bishop and Still[43] detected both the C_{17} and C_{19} cyclopropane fatty acids in *Serratia marcescens*, and the former constituent was found to dominate. Various research groups agree in the conclusion that the synthesis of the cyclopropane ring involves addition of a one-carbon fragment across the double bond of *cis*-vaccenic acid.[71, 311, 324, 393] Recent studies of Zalkin *et al.*[526] revealed that a phospholipid is intimately involved in this process and the hypothesis was advanced that the enzymatic introduction of the cyclopropane ring may occur at the unsaturated fatty acid esterified in phosphatidyl ethanolamine.

3. Structure and function of the apolar side-chains

When comparing the composition of the paraffinic residues of phospholipids, the differences in biogenetic capacity for the synthesis of these chains between animals and higher and lower plants appear to be clearly reflected in the membrane. Apart from the apparent differences between marine and terrestrial animals, the influences of the rumen and the diet leave us with a bewildering variation in fatty acid and aldehyde composition, even within the class of mammals. On the other hand, some characteristic differences in the fatty acid composition between various phospholipid classes, interwoven with a certain degree of animal tissue specificity, point to a significance of a characteristic fatty acid composition for a given membrane. Furthermore the preference of saturated and poly-unsaturated constituents for the 1- and 2-position in the lecithin molecule respectively appears to be maintained within certain limits both in the animal and plant domain. One may consider such a feature merely as a remarkable result of the acylating enzymes concerned, but it cannot be ruled out that enzymes performed this special duty in order to fulfil an essential requirement for the membrane architecture.

Instead of arguing that the immense variation in fatty acids between homologous membranes indicates that structural specificity is not required at all for their function in the membrane one may attempt to establish whether an

alteration in fatty acid composition effects the properties of the membrane involved. The mammalian erythrocyte provides an example of a homologous membrane which differs extremely in the composition of the non-polar residues between various species. Apparently such differences do not interfere with the main function of these corpuscles, but in many respects the red cells of these animal species differ greatly, e.g. in their life span. More relevant to the membrane are the differences in permeability properties of these erythrocytes which appear to coincide to some extent with differences in the fatty acid composition. However, it would be an oversimplification to conclude that between both characteristics a strict relationship exists, since other variations in lipid composition could be established between these cell membranes as well. (See Section III.) Perhaps it is even possible that an environmentally forced fatty acid composition, being not completely proper for a given membrane is compensated by variations in other lipid characteristics or in protein composition. At this level variations in proportions of several lipid classes, revealing a different magnitude of lipid–lipid interactions (e.g. cholesterol-phospholipid ratio) as well as variations in the apolar region, e.g. diacyl, plasmalogen, glyceryl ether linkages or sphingosyl and glyceryl structures may participate in providing the membrane with a lipid core having desired properties. In spite of the facts that on one hand the possible relation between structural specificity and functions of lipids in membranes are complex, while on the other hand insufficient comparative data on lipid composition and membrane properties have been gathered, perhaps some pertinent observations can be interpreted to a certain limited extent. Although the situation in the biological membrane is quite different, a comparison of variations in chemical structure and interfacial properties of lipids may be helpful for this purpose.

Either by the presence of unsaturated bonds, by branching or by adapting their chain-length the fatty acids appear to prevent a crystalline or a condensed state of the lipid layer of the natural membrane. As known from La Mer's studies about the retarding effect of monolayers on water-evaporation only films of the close-packed condensed type formed by saturated fatty acids and alcohol of chain-length C_{16} and higher effectively lower the evaporation rate.[30] Close-packed monolayers of long-chain surfactants have been demonstrated by Blank[45] to retard the passage of gases, e.g. carbon dioxide and oxygen as well. The effectiveness of these films is dependent on the nature and length of the hydrocarbon chain, determining the magnitude of Van der Waals' attraction. As emphasized by Salem[420] the total attraction energy is proportional to the number of CH_2-groups in each of the interacting molecules and quite large forces may result between molecules containing long chains. The cohesion between the lipid molecules is significantly disturbed by the presence of unsaturated bonds, and there is general agreement that such effects will greatly influence the packing of the lipid molecule in the membrane. Thus it can be readily envisaged that the nature of the paraffinic chains of membranous phospholipids may be one of the most important factors involved in regulating the penetrability of the lipid barrier of

biomembranes. Although the effects of the nature of alkyl chains have been thoroughly established by the pioneers of surface chemistry: e.g. Langmuir, Harkins, Rideal and Adam,[3] comparisons with the living membrane are hampered by several factors. To exclude one difficulty, perhaps only a relatively minor one, *viz.* comparison of properties of soaps or alcohols with those of the more complex lipids present in membranes, work in this laboratory was concerned with the synthesis of phosphoglycerides with a great variety of fatty acids constituents.[124] The force area curves of monolayers of phosphatidic acid, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine all containing identical fatty acids showed only very small differences. The shifts of the film characteristics within one class of phosphoglycerides were more pronounced and are brought about by the apolar moiety. Shortening of the chain length and increasing unsaturation of the fatty acid constituents greatly expanded the films of the L-α-lecithins, thereby increasing the closest stable packing obtainable.[125] This decrease of the cohesive forces between the phospholipid molecules as measured at the air–water interface is illustrated diagrammatically by Fig. 22. Recent work by de Haas and Daemen [94, 189] resulted in the synthesis of well-defined phosphatidyl ethanolamines and lecithins with poly-unsaturated fatty acids, e.g. linoleic and linolenic acid. Measurements on monomolecular layers according to expectation again revealed the highly expanded character of these films. In the latter case some restrictions are still necessary because it was so far not sufficiently established whether oxidation of the polyene chains at the air–water interface was precluded in a satisfactory way. The variations in cross-section of different fatty acids appeared to be maintained in the phosphoglyceride organization. Nevertheless, the comparison of interfacial properties of phospholipids, rather than of single fatty acids is important for further understanding of the lipid–lipid interaction in the membrane. This became clear by recent experiments carried out by Mr. Demel in the laboratory of Dr. Pethica (Unilever, Port Sunlight), who showed that at the oil–water interface some unexpected, but pertinent, differences exist in the behavior between various synthetic phosphoglycerides containing different fatty acid residues. In general the conclusion appears to be that differences in fatty acid composition play a part in bringing about a distinction in molecular packing and thus may effect the properties of biomembranes. A possible relationship between the differences in chemical nature of paraffinic chains of red cell phospholipids and the obvious differences in permeability characteristics of red cells between various mammalian species finds support in these physico-chemical differences. On the other hand the abundance of cholesterol in the red cell membrane certainly will counterbalance these differences, in red cell fatty acid constituents, effected by different dietary habits, participation of ruminal processes and different biosynthetic capacities. The valuable approach of Blank[46] on the monolayer permeability to gases, also led this investigator to conclude that variations in the proportions of individual lipid constituents can account for differences in the permeability of various membranes of the same basic structures.

Recently Shanes[437] advanced an interesting model relating membrane permeability with lipid monolayers.

The fatty acid structures may play a critical role in making the lipid barrier of the membrane just loose enough to permit transport across the boundary.

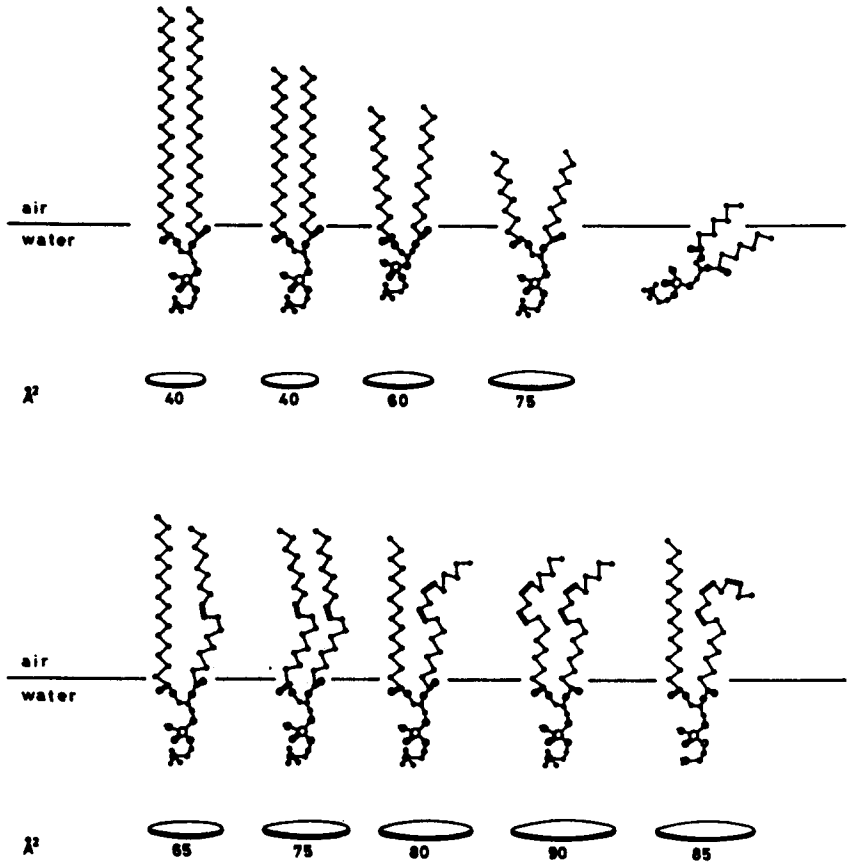


FIG. 22. Schematic comparison of the average molecular cross-section occupied at the air–water interface of synthetic phosphoglycerides containing different fatty acid constituents. At the arbitrary chosen surface pressure of 21 dynes/cm the molecular area exceeds that of the closest stable packing attainable.

The observations of Meyer and Bloch[351] on the fatty acid composition of the phospholipids from yeast which was grown anaerobically are highly interesting. Under these conditions the absence of unsaturation is compensated for by the appearance of saturated fatty acids with shorter chain length, *viz.* C_{10}, C_{12} and C_{14}. Remarkably these constituents replace the unsaturated fatty acid residues

by taking preferentially the 2- or β-ester position. Comparing the film characteristics of (stearoyl-oleoyl)-L-α-lecithin and (stearoyl-lauroyl)-L-α-lecithin (Fig. 23), it can be suggested that this organism made a successful attempt to produce a phospholipid with the same physico-chemical properties as synthesized under aerobic conditions. Another example is provided by bacteria which contain low amounts of mono-unsaturated fatty acids; and have in quantity methyl branched saturated acyl chains. The force–area curves of stearic and isostearic acids have been compared by Ries,[406] who established that the film of the latter com-

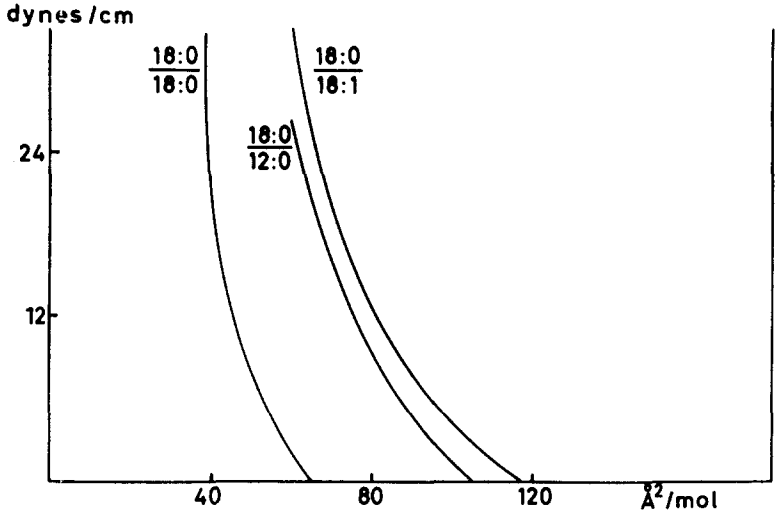


FIG. 23. Comparison of the force–area characteristics of monolayers from synthetic lecithins, *viz.* (distearoyl)-L-α-lecithin, 18:0/18:0; (γ-stearoyl-β-lauroyl)-L-α-lecithin, 18:0/12:0; (γ-stearoyl-β-oleoyl)-L-α-lecithin, 18 : 0/18:1.

ponent had a significantly more expanded character as well as a lower collapse pressure. Studies in this laboratory on synthetic substances showed that this difference between straight chain and branched fatty acids is preserved in the phosphoglyceride molecule. Salem[420] outlined that the presence of the isomethyl group in isostearic acid may reduce the total London–Van der Waals' dispersion energies in the monolayer by a factor of 3. Thus in membranes practically devoid of unsaturated fatty acid residues the branched fatty acids and aldehyde constituents may take over the function to regulate the distance between the paraffinic chains or the liquid crystalline state of lipids in the membrane. This is likely to be true also for the cyclopropane fatty acids abundant in several species of bacteria. Recently Dr. J. Law of Harvard University isolated in a pure state phosphatidyl ethanolamine from *E. coli*, and demonstrated that this phospholipid contained about 40 per cent of C_{17} and C_{19} cyclopropane fatty acids located together with about 10 per cent of unsaturated fatty acids at the 2-position, while the straight chain saturated ones occupied the 1-position. The

cyclopropane containing phosphatidyl ethanolamine preparation revealed a pressure–area curve, which was hardly distinguishable from the characteristics given by a film of (stearoyl-oleoyl)-L-α-phosphatidyl ethanolamine (Fig. 24).

The presence of more double bonds and their location in the acyl chains consequently will alter the forces of aggregation between lipid molecules in the

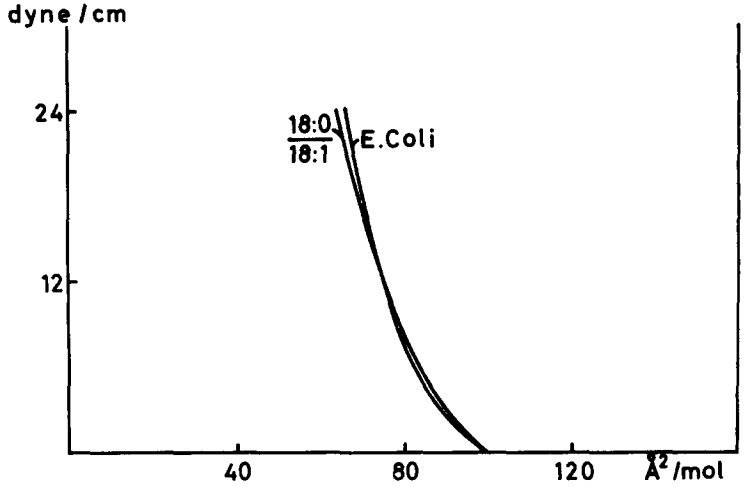


FIG. 24. Force–area characteristics of films from (γ-stearoyl-β-oleoyl)-L-α-phosphatidyl ethanolamine and pure phosphatidyl ethanolamine isolated by Dr. J. Law from *E. coli*. The latter compound contained 43 per cent of palmitic acid located at the γ-position and 44 per cent of C_{17} and C_{19} cyclopropane fatty acids which occupied the β-position.

membrane. The role of the double bonds of the fatty acids of the essential linoleic acid family still forms one of the intriguing problems of the membrane field. The abundance of these fatty acids in phospholipids from membranous structures, e.g. mammalian mitochondria did raise the question whether these constituents are involved actively in membrane processes or whether these structures participate more indirectly by donating a special alignment of the molecules in the membrane, because of a particular shape of the principle end-product of this family, i.e. arachidonic acid. Favoring a dynamic function of the essential fatty acids, it has been suggested that these constituents might act as coenzymes in oxidation-reduction reactions of the mitochondria,[231] but this concept has not been supported by other studies.[402]

Recent work in the laboratory of D. E. Green[156, 185] established the fundamental role of phospholipids in mitochondrial electron transfer and oxidative phosphorylation by proving that lipid-free mitochondria failed to carry out this process. Restoration of activity was achieved by adding back both coenzyme and phospholipid. A phospholipid requirement has been demonstrated for three segments of the electron transfer chain and the chemical nature of the active phospholipids was investigated (See also Section IV.)

As demonstrated by Fleischer and Klouwen[156] the capability to reactivate the enzymes appears to be a function of the extent to which the lipids can be solubilized in water. Hydrogenation of natural phospholipids strongly reduced the ability to form micelles. The importance of the physico-chemical state of phospholipids to interact with proteins has been demonstrated also in studies on the action of phospholipases. Apart from the requirement of phospholipases for a specific zeta-potential of the substrate micelles[26] the influence of the apolar chains for making the substrate particles accessible to the enzyme was recognized.[121, 122] Long-chain saturated lecithins are known to resist the action of the enzyme unless adequate solubilization was obtained by sonic vibration or addition of so-called activating lipid solvents. Statements that the substrate specificity of a given enzyme precluded interaction with saturated lecithins could easily be contradicted in this laboratory by demonstrating the ability of the phospholipase to attack saturated homologs with shorter-chains, e.g. (didecanoyl)-L-α-lecithin, resembling in physico-chemical properties closely the unsaturated homologs. The importance of the micellar state for this type of protein–lipid interaction was further endorsed by the observation that several (but not all) phospholipases revealed a diminished activity towards water-soluble substrates, *viz.* (diacetyl)-and (dibutyryl)-L-α-lecithin.[121, 122]

Regarding the implications of fatty acid unsaturation for the mitochondrial processes Jurtshuk *et al.*[253, 254] obtained very demonstrative results about the reactivation of isolated D(-) β-hydroxybutyric dehydrogenase, which enzyme possesses an absolute requirement for lecithin. The degree of unsaturation in the fatty acid constituents was of paramount importance for restoring the activity of the apo-enzyme. Whereas a synthetic saturated lecithin with two saturated acids is practically inactive, palmitoyl-oleoyl-L-α-lecithin induced a full activity, but higher concentrations were necessary than in the case of natural poly-unsaturated-L-α-lecithin. Recently Saunders *et al*[423] reported that clear micellar solutions could be obtained from dilinoleoyl-lecithin, but the analogs with one stearic and one oleoyl residue appeared to give less stable emulsions.

Although unsaturation of the phospholipids undoubtedly is an essential prerequisite for their function in mannalian membranes, the particular function of the essential linoleic acid family remains obscure. A structural function of these constituents in the molecular organization of membranes, first advanced in 1930 by Burr and Burr,[68] has found response in several studies, without rendering so far a precise formulation of this essentiality. The membranes of red blood cells were observed by Sinclair[339] to become more fragile in essential fatty acid deficiency, while Levine *et al.*[322] and Hayashida and Portman[209] concluded that the permeability properties of mitochondria are changed under such conditions. Brain microsomes of EFA-deficient rats were found to reveal an increased incorporation of ^{32}P into their phospholipids and Collins[86] suggested that arachidonic acid acts primarily as a modulator of phospholipid metabolism because molecules containing this fatty acid are more stable. In a subsequent paper[397] the conclusion was advanced, which we also incline to favor, that

the partial disorganization of the lipoprotein membrane may lead to an increased uptake of the radioactive phosphate. That the structural function of the membrane poly-unsaturated fatty acids is connected with their physico-chemical properties was recently championed also by Richardson *et al.*,[403] who found intriguing differences in the fatty acid constituents between mitochondria from quite different origins. For example, in fish mitochondria, there was no detectable linoleic or linolenic acid, while the higher unsaturated members of the *linolenate* family appeared to dominate, this in contrast to the significant levels of the *linoleate* family found in mammalian mitochondria. Mitochondria from sweet potato, which carry out identical biochemical processes appeared to contain a high amount of linoleic acid but turned out to be practically devoid of arachidonic acid. As argued by these investigators these results indicate that there is not a specific requirement for either one of the poly-unsaturated fatty acids to permit the mitochondrial metabolism in general. Further physico-chemical investigations must elucidate the exact relationship between the structure and the membrane function of the poly-unsaturated fatty acids in mitochondria which act under different conditions such as temperature.[403] Though various physical properties of several poly-unsaturated fatty acids are already known, e.g. the implications of *cis–cis* and *trans–trans* geometry,[488] a great deal of fundamental work still has to be done before we can hope to understand why the *linoleate* family is essential and other very similar fatty acids fail to take over its function.* Knowledge available now indicates model studies in this direction may be fruitful. Phosphoglycerides-containing arachidonic acid may be subjected to interaction experiments with the membrane protein, thus comparing them to analogue phospholipids containing physiologically inadequate replacant fatty acids. In this context the question arises whether it is of paramount importance that these polyenoic fatty acid constituents are located predominantly at the 2-position of the phosphoglyceride molecule. Monomolecular layers of structurally isomeric L-α-phosphoglycerides carrying dissimilar fatty acids in different positions gave at the air–water interface nearly identical pressure–area curves. However, quite recently when carrying out measurements at the oil–water interface two synthetic L-α-lecithins, containing oleic acid and butyric acid at different positions, were found to exhibit significantly different film characteristics. The preliminary explanation was afforded by Demel that the compound having the short-chain fatty acid in 2-position gave a bending of this chain into the water-phase. Remarkably, this compound was attacked also more rapidly by phospholipase A than the structural isomeric compound. Hence this combination of physico-chemical and enzymatic observations points to a possible involvement of the acyl chain linked in 2-position in the process of phospholipid–protein interaction.

* Recently D. A. van Dorp, R. K. Beerthuis, D. H. Nugteren and H. Vonkeman [*Biochim. Biophys. Acta*, **90**, 204 (1964)] and S. Bergström, H. Danielsson and B. Samuelsson [*Biochim. Biophys. Acta*, **90**, 208 (1964)] established a new metabolic pathway for the conversion of the essential fatty acids to the protaglandins, which finding is considered to explain, at least in part, the essentiality of these fatty acids.

Therefore it will be of interest to investigate whether arachidonate located mainly at the 2-position of phosphoglycerides contributes in a specific way by hydrophobic interaction with a protein side-chain and to the integrity of the membranous framework. It may be of interest to verify if the position occupied by the fatty acid is critical in this possible interaction, thus challenging the organic chemist to prepare these structural isomeric phospholipids.

III. Molecular Arrangements of Lipids in Biomembranes

A. Current Theories on Membrane Structure

1. *The bimolecular lipid leaflet*

When summarizing the conceptions on the molecular architecture of cellular membranes it has to be recalled that in the past 40 years considerable emphasis has been placed on the bimolecular lipid leaflet (Fig. 25). This arrangement of

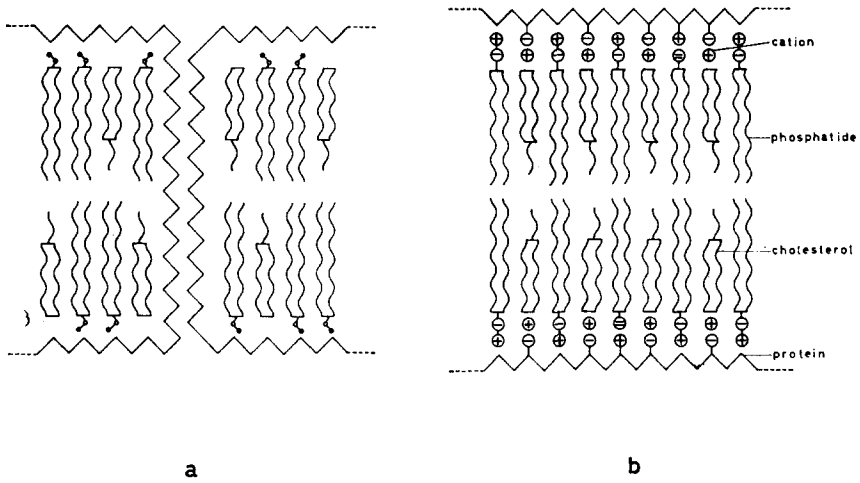


FIG. 25. Schematic diagrams of the conception of a bimolecular lipid leaflet in the plasma membrane. (a) Compare the model of Danielli and Davson.[104] (b) A double layer of cholesterol and phosphatides showing the tricomplex binding principle according to Bungenberg de Jong.[49]

the lipids, considered also by many contemporary scientists as the fundamental basis of membrane structure, was first formulated by Gorter and Grendel[180, 181] in 1925. As discussed already in Section II A3, this model was derived by measuring the surface occupied by a monomolecular film of the lipids extracted from erythrocytes. Unaware of the work[97] of Gorter and Grendel in 1934 Danielli and Davson[98] postulated their well-known membrane model consisting of a bimolecular lipid layer surrounded by protein. Based on the observation that the tension at the surface of many cells and at the surface of

oil drops in the interior of cells is very low,[99, 203] the presence of protein adsorbed to the lipid core was considered to be likely. Although the validity of this argument can be doubted, and it has been argued that the polar groups of the surfactant lipids abundant in the membranes can account for the low surface tension, the important role of protein in the cellular boundaries is generally accepted. From the X-ray studies on myelin by Schmitt[429] the participation of protein sheaths was deduced as well. The essential part played by proteins in the cellular membranes is also in agreement with the well-recognized function of enzymes in transport processes across the membranes. Support for the essentiality of proteins for the stability of the permeability barrier of the cell was furnished also by a long series of studies on biocolloids carried out by Bungenberg de Jong and co-workers.[49, 66, 67, 511] This work on artificial lipo-protein systems, better known among colloid chemists, led to the concept that an orderly arrangement of phospholipids involving London–van der Waals forces between the apolar chains and cholesterol units is stabilized by the formation of a tricomplex system held together by Coulombic forces between the ionized groups of phospholipids and proteins with cations (Fig. 25). Such a molecular arrangement of phospholipids was considered by Winkler and Bungenberg[511] to explain several properties of the red cell membrane. This fundamental work in the Leyden laboratory was extended later to a lipid double layer[49] and the models originating from this school gave more solid ground to the postulates made previously at that University by Gorter and Grendel. Recently Booy[48] emphasized the possibility that the double layer of lipids may be covered by other macromolecules, e.g. polysaccharides and nucleic acids as well, while the inside layer may be completely different from the outside. In this context it may be noted that part of the membranous lipids also may be located at the outside. The class of glycolipids, extensively studied by the schools of Klenk[291] and of Yamakawa[522] has been demonstrated to contribute to the surface charge of the red cell.[141]

Though the proposals made by Danielli and Bungenberg de Jong are basically the same, one important difference emerges. Whereas Danielli suggested that hydrophilic pores may enable the transport (diffusion) of certain molecules Bungenberg de Jong and Booy believed that the continuous mobility of the nonpolar lipid-chains will allow passage of small molecules with a polar character. It is clear from discussions at recent symposia[48, 97, 103, 153] that the problems about the existence of organized pores in the membrane have not been settled. Studies by Solomon *et al.*[447] on the penetration of the erythrocyte membrane have been interpreted in favor for the existence of statistical pores in a continuous membrane. As mentioned before, several workers[46, 437] utilizing monomolecular films endorsed the view that the characteristics of lipid layers provide an alternative to the rigid-pore theory for membrane permeability to polar molecules. On the other hand, Finean[153] advanced a hypothetical pore structure arising from a specific aggregation of phospholipid–cholesterol complexes.

Aside from these aspects the most crucial question is the problem whether

the lipids in the membrane are indeed organized in a bimolecular lipid leaflet. The attraction between the non-polar lipid residues certainly will contribute to the integrity of the lipid core, but this does not preclude that the molecules may be organized in the membranes in non-lamellar structures. The stimulating approach of Gorter and Grendel though being very suggestive, does not give any conclusive evidence at all about this point. A monolayer of a heterogenous lipid population can give a molecular alignment which may be quite different from that in the membrane and this mixed lipid film therefore can behave quite differently. Apart from this fact, which presents only a minor objection, several choices of area become possible, this depending on the surface pressure applied. The natural lipid mixture gives a film of the expanded type and by varying pressure one can bring its area to any desired area within certain limits. However, even when the pressure applied in the monolayer was equal to that in the membrane and the area occupied by the membrane lipids in the films turned out to be twice that of the available surface at the cell membrane, the decision that the cell membrane contains a bimolecular lipid leaflet still would be speculative.

Support for the conception of this pauci-molecular theory, however, has been provided by electron microscopic studies and X-ray analysis of membranous structures. It has been shown that a three-layered structure about 75 Å thick consisting of two dense lines (20–25 Å thick) bordering a light central zone, results at the surface of many dissimilar cells after fixation with $KMnO_4$ and OsO_4.[409] The interpretation of this image has been facilitated by electron microscopic studies on phospholipid–water model systems. Studies on myelin figures by Stoeckenius[455, 460] revealed alternating light and dark bands in the electron micrographs, which were interpreted as regularly spaced bimolecular layers of lipids. In the earlier experiments[455] it was assumed that the dark lines marked the middle of each fatty acid layer because of a deposit of osmium after reaction of OsO_4 with the double bonds, while the light bands were believed to represent the polar regions of two neighboring phospholipid layers. Further studies of Stoeckenius *et al.*[460] and Finean[151] cast doubt on this interpretation and is now accepted that the regions of highest density in the fixed preparations are located at the polar headgroup. This has been demonstrated very convincingly by experiments of Stoeckenius[456] on systems giving a hexagonal packing of lipid micelles, which can be arranged to have the ionic groups directed either towards or away from the micellar center. According to Riemersma *et al.*[284, 405] both the fatty acid double bonds and the polar groups are involved in the reaction of unsaturated lecithin with osmium tetroxide.

Another approach into the interpretation of the image of electron micrographs from lipid–water systems recently has been made by Elbers.[136] Using the principles of tricomplex formation a method was developed which enables the visualization of the polar headgroups of the phospholipids without any interference of the double bonds (Fig. 26). Assays with synthetic phospholipids having various saturated fatty acids demonstrated the distance between

dense lines to be dependent on the chain-length of the fatty acid constituents. Stoeckenius demonstrated that electron-micrographs made of bimolecular leaflets of phospholipid coated on both surfaces with protein, revealed after

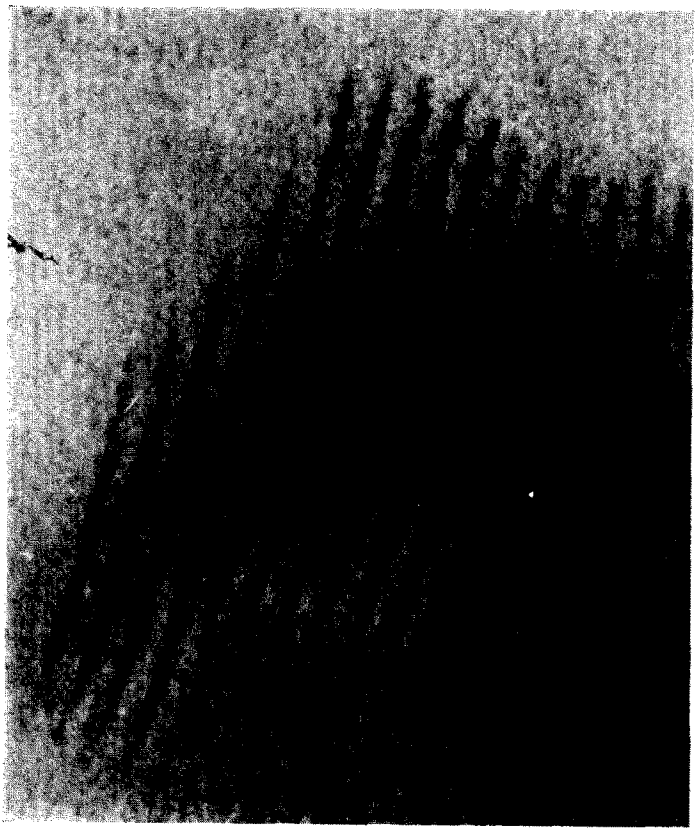


FIG. 26. Section of a tricomplex of L-α-(dipentadecanoyl)-lecithin with cobalt and molybdate ions embedded in methacrylate[136] (× 770,000). The dark bonds correspond to the polar region of the lipid layer, their width depending on the orientation of the layers with respect to the electron beam. [By courtesy of Dr. Elbers.]

fixation a triple-layer structure (Fig. 27), identical in appearance to the image of the plasma membrane obtained in many instances by the same techniques.[457] Osmium tetroxide, in contrast to permanganate, however, does not always reveal a double membrane, but can give one dense bond. Nevertheless the surfaces of many dissimilar cells, e.g. erythrocytes, bacteria and mammalian organelles, have been shown to exhibit a triple layered membrane structure. The ubiquitous occurrence of this entity led Robertson[409] to introduce the term "unit membrane"; and this structure is considered to be consistent with the molecular arrangement rendered by the model of Fig 25. Finean[152] has investigated

extensively the nerve myelin since this multilayered membrane system is highly favorable for a direct study of means of X-ray diffraction. The electron-density distribution as derived from Fourier analysis revealed two peaks

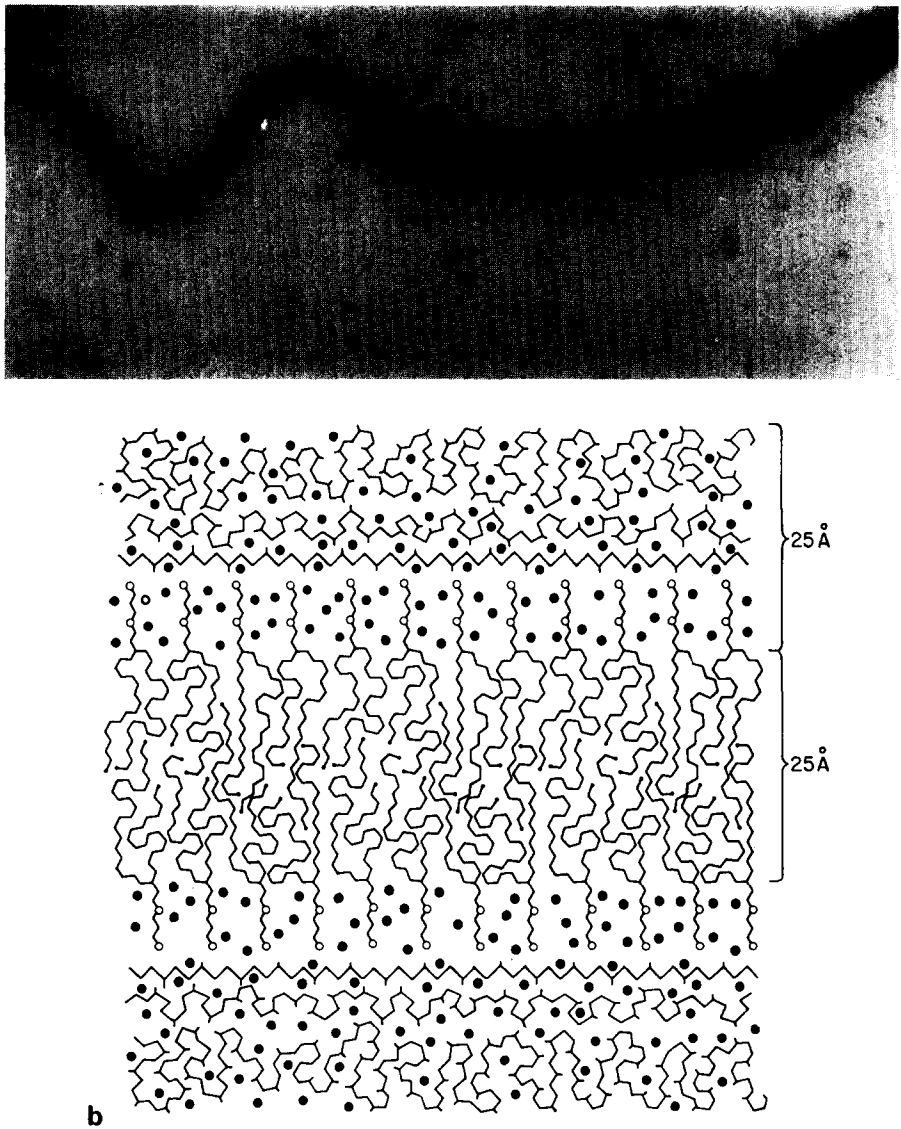


FIG. 27. Electron micrograph of bimolecular leaflets of phospholipids coated on both surfaces with protein (× 280,000; fixed with OsO_4 and contrasted by staining with lead hydroxide) and schematic drawing of the deduced molecular architecture.[457] The black dots represent the osmium deposited in the structure. [By courtesy of Dr. W. Stoeckenius; by permission of the American Heart Association.]

separated by deep troughs. On account of the low electron density of hydrocarbon chains and considering the peaks to arise mainly from the phosphate groups of the phospholipids, the distance between these polar groups could be established accurately. Correlation of the X-ray diffraction data and electron microscopy led Finean to careful interpretation of these structures, being in accordance with a bimolecular lipid leaflet. As discussed in Section II.B, Finean attributes a significant role to cholesterol in promoting the stability of this molecular arrangement. Recently Vandenheuvel[482] made molecular scale models and confirmed that the arrangements of the lipids in a bimolecular leaflet gives dimensions which correspond exactly to the data obtained by Finean with low angle X-ray diffraction of fresh myelin.

Thus the evidence that this pauci-molecular model correctly represents the molecular arrangement of lipds and proteins of many biological membranes is very strong. However, it is realized that neither osmium tetroxide nor permanganate are sufficiently specific agents to give complete information about the chemical nature of the membrane constituents visualized by electron microscopy. Furthermore the treatments of biological specimen involved in this technique may cause alterations in the molecular organization of the lipid molecules; thus giving a wrong impression. Therefore it has been argued that other arrangements of the lipids may exist in the protoplasma membrane of several cells, and an alternative theory will be considered now.

2. The mosaic structure

A model for the organization of lipids and proteins in the red cell membrane was proposed in 1952 by Parpart and Ballentine[386] to account for their observations on the differential extractability of lipids from this membrane. It has to be emphasized that this model was advanced as a rather specialized structure for the erythrocyte and was not intended to account for the lipid organization of, for example, the nerve myelin sheath. Parpart and Ballentine demonstrated that by treatment of bovine red cell ghosts with solvents of increasing polarity a distinction can be made into loosely, weakly and strongly bound lipids. Whereas cholesterol appeared to belong entirely to the loosely bound class the phospholipids exhibited a more complex extraction pattern. Treatment of the vacuum dried ghost with dry ether removed next to cholesterol a defined part of phospholipids (cephalins), while an additional part of cephalins was recovered by extraction with pyridine-ether. Distinct from these fractions of loosely and weakly bound lipids respectively, was the strongly bound portion which contained the major part of the phospholipids and was extracted only by solvents like ethanol–ether (3:1). These observations led to the conclusion that there are different sorts of combinations between the lipids and the protein of the plasma membrane and the hypothesis to account for such differences was visualized in a mosaic structure (Fig. 28). This model consists of a continuous network of protein, possibly two molecules thick, in which are interspersed islands of lipid molecules bound to the protein or to each other. The most numerous

phospholipids are strongly bound molecules being attached directly to the proteins. The loosely and weakly bound lipid units represent cholesterol and a small part of phospholipid molecules which are retained by interaction with the apolar moiety of the strongly bound phospholipids. Pores of different diameter were believed to result and for an explanation of various phenomena, e.g. haemolysis with this membrane model, the reader is referred to the original paper.[386]

The basis underlying this theory has been verified in the author's laboratory, confining the extraction of lyophilized red cell ghost to a subsequent treatment with ether and ethanol–ether (3:1). The former fraction denoted as weakly bound was shown to contain all of the erythrocyte cholesterol and a small but quantitatively defined part of the phospholipids, whereas the major amount of the latter lipid class was recovered in the strongly bound fraction. Although the quantitative data about the distribution of phospholipids among both fractions in the beef red cell differed from those reported by Parpart and Ballentine, the principle of their original observations could be confirmed.[410] A study on the red cells of various animal species showed that the amount of loosely bound phospholipids was characteristic for each species, but showed a decrease in the order of rabbit, man, pig, sheep and ox. In as much as both the phospholipid content and their fatty acid pattern had been demonstrated

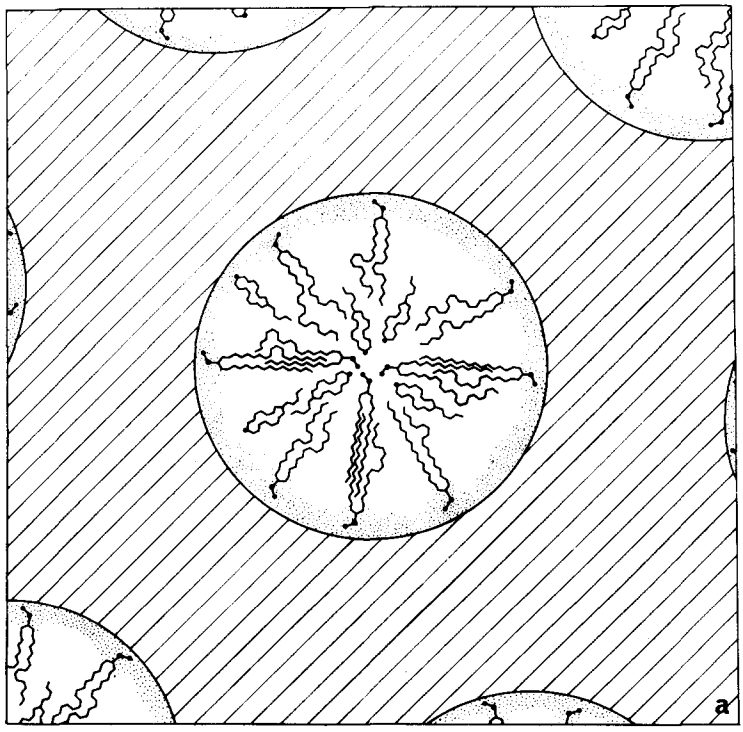


FIG. 28.

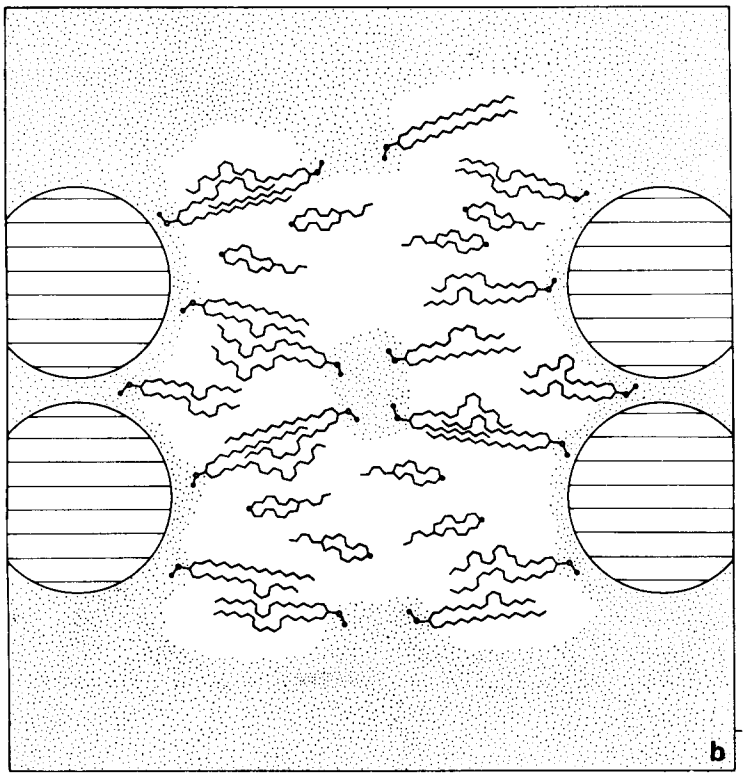


FIG. 28. (*cont.*) Schematic drawings of the molecular architecture of the red cell plasma membrane as proposed by Parpart and Ballentine.[386] (a) Section tangential to the surface. (b) Section perpendicular to the surface. Protein is designated by cross lines; water by stippling, nonaqueous phase clear.

to differ extremely from species to species a more detailed characterization of the phospholipids present in both fractions was desired. The phospholipid distribution in the loosely bound fraction of all red cell types investigated turned out to be nearly the same for the various erythrocytes, and exhibited a certain preponderance of ethanolamine phospholipids, whereas the strongly bound fraction reflected clearly the well-known species differences in phospholipid distribution. Furthermore it had to be proven that the distinction between both fractions is not to be attributed to a difference in solubility properties of the phospholipids, but to a different binding in the membrane. For instance, treatment of the red cell ghost with ether might preferentially solubilize those phospholipids containing poly-unsaturated fatty acids. Therefore analyses were made of the fatty acid constituents of both lipid fractions. Within one animal species, the composition of the fatty acid residues of loosely and strongly bound lipids appeared to be nearly identical.[410] This was true also for the

ethanolamine phosphoglycerides present in both fractions. Nor was there any difference apparent in the plasmalogen content of both lipid fractions. Hence, it was concluded that this distinction between loosely and strongly bound phospholipids as classified by a different degree in extractability is indeed to be attributed to a difference in the binding of the phospholipid molecules to other membranous constituents.

This experimental confirmation of the concept of different sorts of binding of phospholipid in the lyophilized membrane preparation does not approve the validity of the molecular arrangement as advanced by Parpart and Ballentine. Whereas these results are valid also for the native membrane they only indicate that part of the red cell phospholipds, *viz.* the loosely bound fraction, may be retained in the membrane by Van der Waals' attraction forces with other lipid constituents or by hydrophobic attraction with protein side-chains, whereas the strongly bound fraction is more firmly linked by additional charge–charge interactions. These facts must be taken into account when attempting to formulate a model of the red cell membrane, but it appears quite possible that a slight variation of the Danielli model as well as other hypotheses are equally apt to explain the phenomenon of loosely and strongly bound phospholipids.

The theory of Parpart and Ballentine received relatively little attention until 1962, when Dourmashkin *et al.*[133] obtained by using negative staining with phosphotungstate highly interesting electron micrographs demonstrating the presence of holes or pits in the surface membrane of *Rous sarcoma* viruses, erythrocytes and tissue cells after treatment with saponin. These "holes" and "pits" were considered to arise by removal of the cholesterol from the membrane, since prior treatment of the cell surfaces with digitonin inhibited the action of saponin. The "holes" or "pits" varying in size from 70 to 95 Å in diameter according to the membrane studied appeared to be arranged in a hexagonal array. The conclusion was reached that these results were in fair agreement with the type of structure proposed by Parpart and Ballentine. The pits were not seen in thin sections of saponin treated, osmium tetroxide-fixed preparations, thus leaving unsettled whether complete holes through the membrane had been effected by the saponin treatment. A study made by Muir[363] on the ultrastructures of intestinal epithelium showed discontinuities in traverse sections after saponin treatment, but it was not possible to conclude whether true holes were involved.

Very soon after the publication of Dourmashkin *et al.* the interpretation of these electron micrographs was re-evaluated by Bangham and Horne[27] and by Glauert *et al.*[176] These workers showed that patterns of hexagonal arrangements of lipid components can be induced in monolayers of cholesterol and mixed-films of cholesterol and lecithin by treatment with saponin (Fig. 29). Under these conditions the figures are formed by the insertion of saponin molecules into the lipid layer. Thus the conclusion was reached that the findings of Dourmashkin have to be interpreted by the addition of saponin to the cell membrane rather than by a loss of cholesterol from this structure. Hence, the observed molecular arrangement of the complexes formed after saponin treatment is now believed

not to correspond with the original lipid–protein orientation existing in the native cell membrane. For a discussion about the caution needed to interpret negatively stained lipoprotein membranes the reader is referred to recent notes.[154, 234] This approach was considered, however, to be of great value for the understanding of the process of haemolysis. Actually, Dourmashkin *et al.*[50a] carried out further studies on the haemolysis of sheep erythrocytes by various

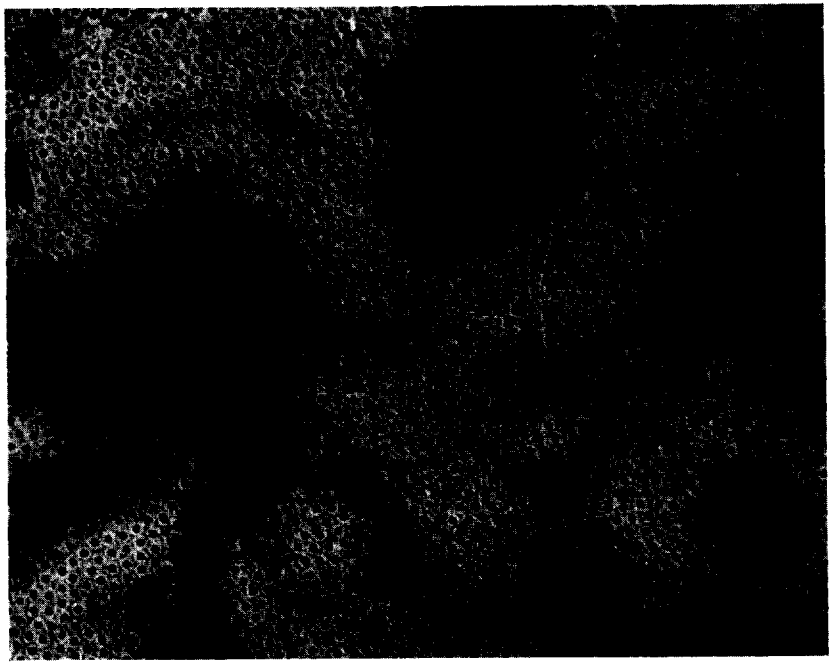


FIG. 29. Electron micrograph showing a negatively stained preparation of equimolar lecithin–cholesterol dispersions treated with saponin[25a] (×200,000). [By courtesy of Drs. A. D. Bangham and R. W. Horne.]

agents and observed that even the action of Forssman antibody and guinea-pig serum complement also produces holes in the membrane. However, in this case the shape and dimensions of the holes were unlike the pattern produced by saponin and were not arranged in a regular manner.

Regarding the saponin-induced patterns in addition to the hexagonal structure another arrangement, i.e. a helical structure, was observed by Dourmashkin *et al.*[133] and Bangham and Horne[25a] (Fig. 29). Though the solvation of saponin in the cholesterol–phospholipid layer of membranes is now believed to cause a re-arrangement of the lipids a further understanding of the molecular architecture of these artificial systems may give useful information about the potentialities of several lipid classes to form specific associations. Lucy and Glauert[327a] made systematic electron microscopic investigations on negatively stained

preparations of various mixtures of lecithin, cholesterol and saponin and gave an interpretation of lamellar, tubular, hexagonal and helical structures observed in terms of small globular lipid micelles, one containing primarily lecithin and cholesterol and the other containing mainly saponin and cholesterol. These

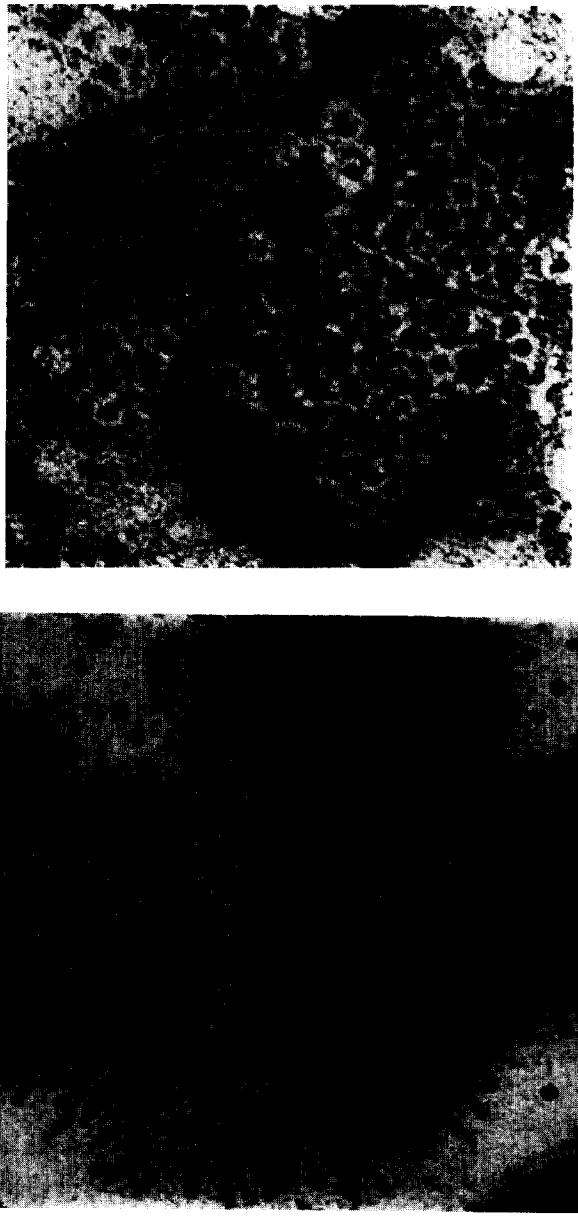


FIG. 30.

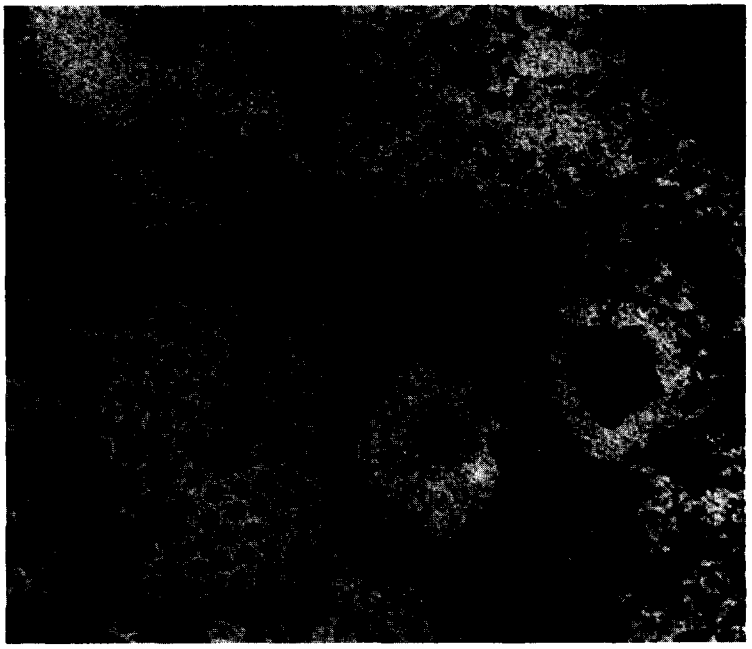


FIG. 30. (*cont.*) Hexagonal patterns of negatively stained preparations of aqueous lipid–saponin systems.[327a] (a) Preparation of saponin and cholesterol; the rings fuse and the "sides" between them are "shared" (× 300,000). (b) Preparation of ovolecithin–cholesterol–saponin; rings of lighter density surround dark holes and appear to consist of sub-units (× 300,000). (c) Like (b), but the complex contained synthetic L-α-(didecanoyl)-lecithin. The rings are now touching but have not fused (× 300,000). [By courtesy of Drs. J. A. Lucy and A. M. Glauert.]

micelles appear to aggregate spontaneously in aqueous dispersions so as to form the different complex structures observed under the electron microscope. The investigators considered hydrogen bonding and electrostatic interactions to be responsible for the complete assemblies.[327a] Regarding the hexagonal structure this interpretation was based on the striking difference in the electron micrographs obtained on cholesterol–saponin and cholesterol–lecithin–saponin systems. The hexagonal structure of the latter system (Fig. 30b) is composed of a fairly regular array of dark holes surrounded by rings of lighter density, which unlike the rings of a complex containing saponin and cholesterol only (Fig. 30a) are not fused. It was proposed that micelles of saponin–cholesterol are arranged in rings around aqueous holes (80 Å in diameter) and that in the system containing the lecithin the rings formed by these sub-units are separated in the hexagonal array by interstitial micelles of lecithin–cholesterol (Fig. 31). Support for this interpretation was obtained from the observation that a 3-component system in water containing a synthetic short-chain lecithin in place of ovo-lecithin gave as a result of the decreased length of the fatty acid chains a reduction in size of the interstitial micelles (Fig. 30c). The dimension of the

rings themselves were unchanged by the substitution of the short-chain lecithin for ovo-lecithin, and appeared to touch each other but were not fused as they are in the complex without lecithin present (Fig. 30a).

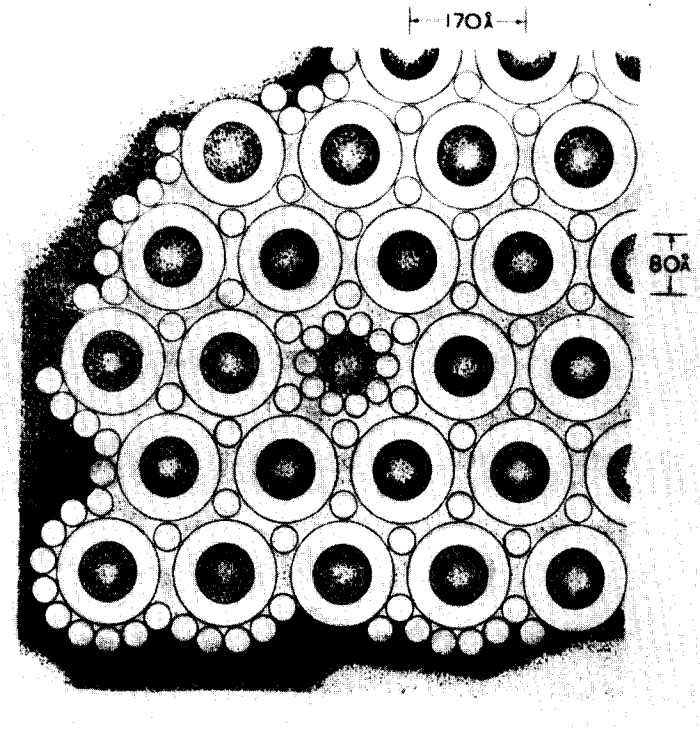


FIG. 31. A diagram of the structure proposed by Lucy and Glauert for the hexagonal phase of the lecithin–cholesterol–saponin complex.[327a] The rings are composed of micelles of cholesterol and saponin (as indicated in one of the rings) and are separated from one another by micelles of lecithin and cholesterol.

The structures shown in Fig. 32 also show a preparation of lecithin–cholesterol and saponin in water and are considered by Lucy and Glauert to be helical in nature and to resemble some of the helical viruses. In cross-section the helices are seen to be made up of two concentric circles of sub-units with a dark central hole of 70 Å to 80 Å in diameter. Also in this case both types of sub-units, *viz.* micelles containing primarily either lecithin–cholesterol and saponin–cholesterol, appear to be involved. There appears to be about 8 globular sub-units in the inner ring and 12 to 14 in the outer. A comparison of complexes composed of long-chain or short-chain lecithins enabled the suggestion that the outer ring is composed of lecithin–cholesterol micelles and the inner ring of cholesterol–saponin micelles. The helices containing short-chain lecithin were found to have outer sub-units that are smaller than those of long-chain

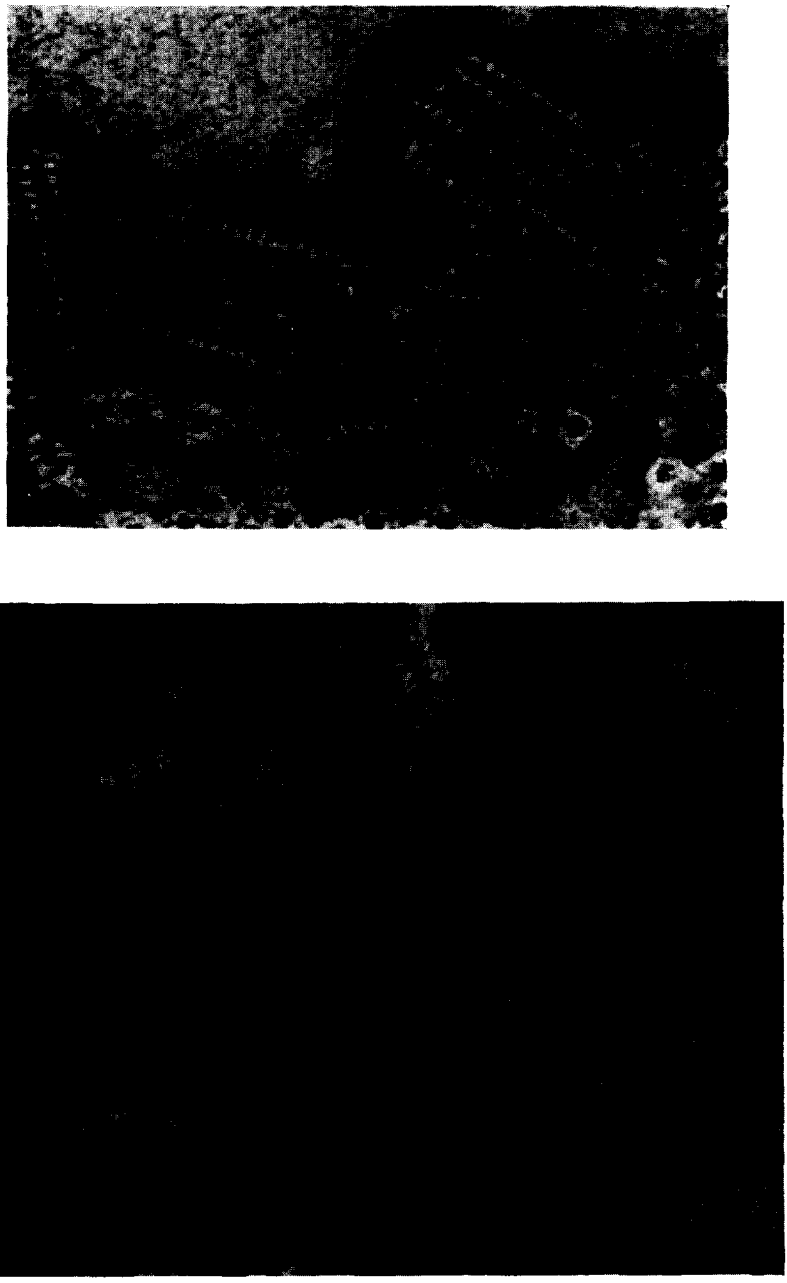


FIG. 32. Helical structures composed of ovo-lecithin, cholesterol and saponin.[327a] (a) Parallel arrays (×300,000). (b) End views of the helices showing two concentric rings (×300,000). [By courtesy of Drs. A. M. Glauert and J. A. Lucy.]

lecithins (Fig. 33a and b). Furthermore in the 3-component system containing the short-chain lecithin, the helices tended to extend and unwound and even to disrupt spontaneously (Fig. 33c). Lucy and Glauert gave a structure for the helix which is reproduced in Fig. 34.

FIG. 33.

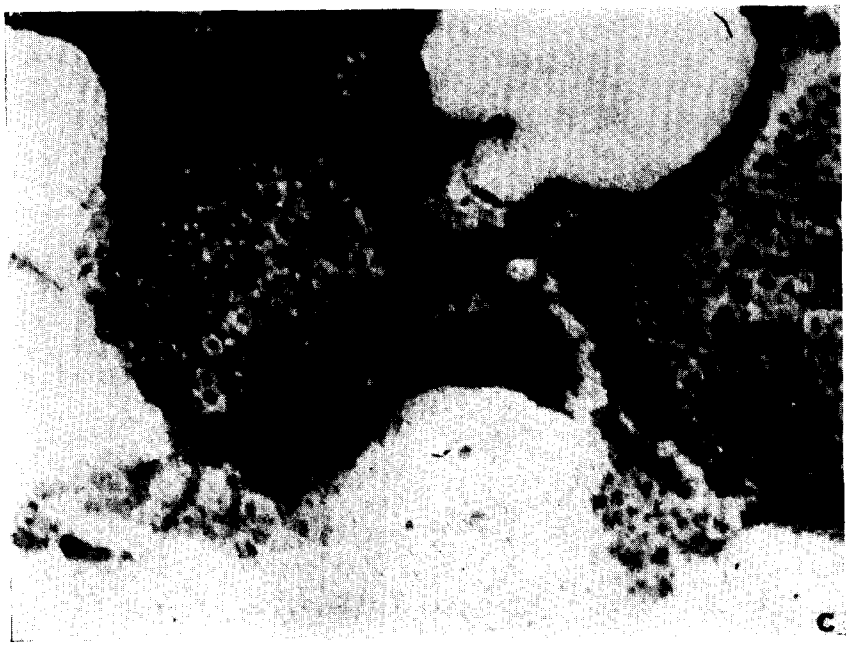


FIG. 33. (*cont.*) Helices containing synthetic lecithins, cholesterol and saponin. (a) Helices with (stearoyl-oleol)-L-α-lecithin show sub-units of the same size as in the case of ovo-lecithin (× 300,000). (b) Helices containing L-α-(didecanoyl)-lecithin have outer sub-units that are smaller and the helices tend to uncoil (× 300,000). (c) Spontaneously disrupted helices in a complex containing L-α-(didecanoyl)-lecithin (× 300,000). [By courtesy of Drs. A. M. Glauert and J. A. Lucy.]

Studying systems of cholesterol–lecithin without saponin present, these investigators observed layered structures which appeared to contain in certain regions individual globular sub-units. It was suggested that small globular micelles as well as bimolecular lipid leaflets may function as structural elements of biological systems.[327a]

The question whether the native cell membrane contains the lipid micelles arranged in a hexagonal pattern was tackled by Husson and Luzzati[245] by means of X-ray diffraction analysis. Previous studies of Luzzati and Husson[328] on lipid–water systems clearly demonstrated the presence of several liquid–crystalline phases, one of which is lamellar only. In the case of phospholipid from human brain in addition to the lamellar phase, involving an ordered sequence of lipid and water planar sheets, a hexagonal array of water cylinders covered by the hydrophilic groups of the lipid molecules was observed. On account of the phase diagrams thus established, Stoeckenius was able to confirm the existence of this hexagonal phase, in addition to the well-known lamellar structure, by electron microscopy, utilizing OsO_4 fixation.[458] Luzzati and Husson emphasized

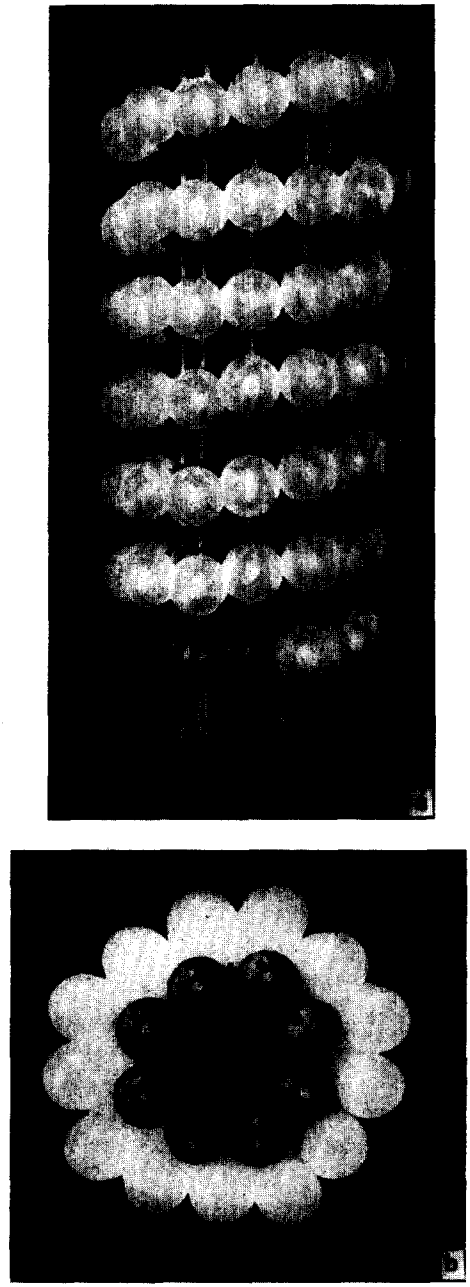


FIG. 34. Photographs of a scale model of the structure proposed for the helix by Lucy and Glauert.[327a] Grey balls represent cholesterol–saponin micelles and the white balls lecithin–cholesterol micelles. (a) Side view. (b) End view. [Reproduced with the kind permission of Dr. J. A. Lucy and Dr. A. M. Glauert.]

that the liquid-like structure of the liquid crystalline phases of lipids gives some short-range disorder which might be more relevant to the physiological properties of membranes than a rigid crystalline configuration. Phase transition of the lipids was speculated to have implications for the functioning of the membrane.[328] The original observations of Dourmashkin therefore were of particular interest when projected upon these basic properties of lipid–water systems. However, the X-ray diffraction method enabled Husson and Luzzati[245] to detect a hexagonal lattice only in the red cell ghosts previously treated with saponin, whereas in intact ghosts, lamellar structures were found to prevail. Therefore these preliminary experiments were again considered to indicate that the hexagonal structures are artefacts arising by the action of the detergent.

Though the independent approaches of several research groups do not support the existence of the hexagonal pattern observed after saponin treatment as a stable lipid modification in the membrane, it cannot be concluded that the bimolecular lipid leaflet represents the only possible orientation of lipids in biological interfaces.

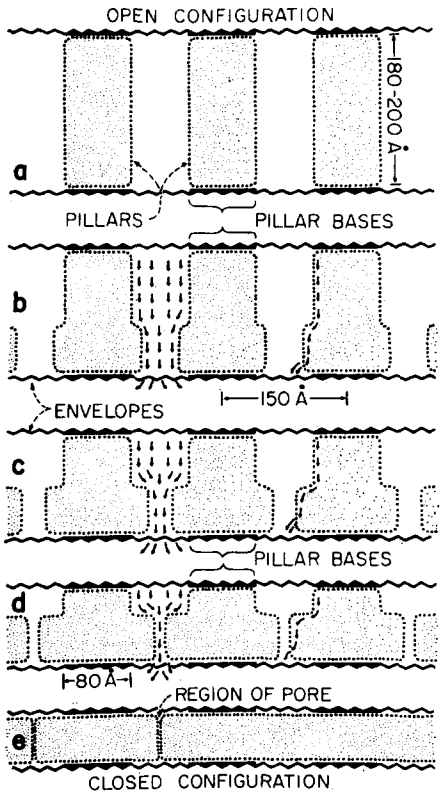
Recently Bertaud and Hedley[38] were unable to find in the marine protozoan *Gromia oviformis* a normal cell membrane, but detected a number of surrounding layers. The tangentially sectioned cell barrier revealed a hexagonal array of hollow cylinders, without any saponin treatment.

A hexagonal array of lipids in the membrane is not necessarily interpreted in terms of the Parpart–Ballentine model. Other structures may account for such lipid orientations as well and an alternative model which in addition forms a link with the bimolecular lipid leaflet must be considered now.

3. The flexible membrane

Searching for an explanation of protoplasmic streaming in terms of changes in the orientations and associations of the constituents of the biological membranes, Kavanau[271] developed a model for the membrane which includes some of the features of other concepts but is new because of its dynamic character. The clue of Kavanau's theory is that a protein–lipoprotein–lipid complex is capable of existing in, and readily transforming between several states (Fig. 35). The "open configuration" of the membrane contains a hexagonal arrangement of cylindrical lipid micelles, which are bound at their bases to the protein moiety both by charge–charge and hydrophobic interactions. The headgroups of the phospholipids at the walls of these pillars are spaced into a loose-latticework whereas the apolar chains extend into the interior and are held together by Van der Waals' forces, this including a stabilization by cholesterol. When viewed on the surface a mosaic structure results, which however is to be distinguished from the model of Parpart and Ballentine. In the model under discussion the lipid cores are not interspersed in a protein meshwork, but are sandwiched, also in the open configuration, between protein sheaths just as in the model of Fig. 25. The "closed-configuration" is very familiar to the latter membrane model and again consists of a bimolecular lipid leaflet

surrounded by proteins. The lipid molecules serving as anchors of the pillars to the protein remain firmly bound to the protein cores during this transformation, while the lipids newly neighboring the protein are considered by Kavanau to be linked in a less stable way. A detailed account of the thermodynamic basis of this interesting theory appears to be in preparation.[272] As outlined in Kavanau's paper, support for this concept is to be found in electron microscopic studies and physico-chemical studies on lipid–water systems, some of which have been dealt with above. An occurrence of hexagonal arrays in membrane structures may favor the possible existence of the open configuration; and the structure revealed by the protozoan membrane[38] was suggested to represent a "frozen state" of this configuration. The failure of several investigators to find the hexagonal configuration in various membranes (unless saponin treatment was involved) is explained by Kavanau as a transformation of membranes to the closed configuration, which is usually found by means of electron microscopy and X-ray analysis. Such a transformation was considered to be catalyzed by the removal of water and the exposure to multivalent cations. The principle of the transitions between phases is believed to find support in the



a

FIG. 35.

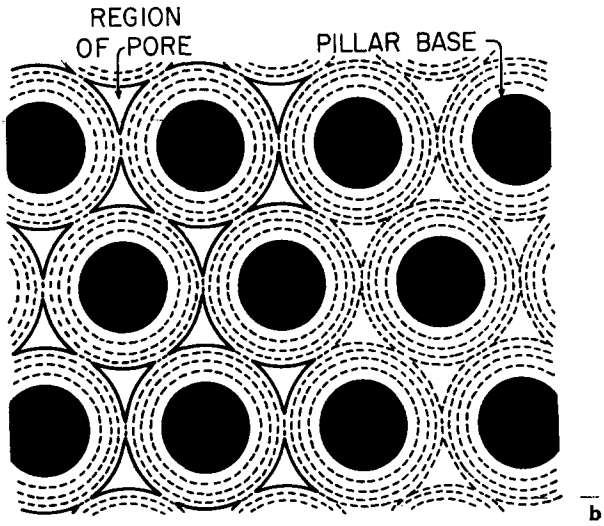


FIG. 35. (*cont.*) Molecular theory of the structure of biological membranes according to Kavanau.[271] (a) Cross-sectional diagrammatic representation of gross geometrical changes of micellar form in the transformation from the open to the closed configuration of a region of membrane. The heavy dots show roughly the regions of the lipid polar headgroups, the zigzag lines the positions of the protein envelopes. Arrows at the left show the movement of matrix "pumped" from between the cylindrical micelles and force filtered through the lower envelope (either luminal or cytoplasmic) as the cylinders collapse into "bimolecular" disks. Arrows at the right show the direction of flow of the polar lipid head groups of collapsing cylindrical micelles. The moving headgroups carry adjacent matrix molecules with them, the remaining matrix being impelled by viscous traction. Coalescence of the "bimolecular" disks into a continuous lamella is shown at the right in (e), incomplete coalescence (or merely close abutment) with pore formation, at the left in (e). Molecular components are not to scale and modifications of the protein envelopes and of the spacings of the lipid headgroups during the transformations (ref. 12) are not shown. Nor are conditions of, and associations between, lipophilic chains and weakly polar lipids within the micellar interiors depicted. (b) Surface view of region of membranous elements shown in (a). Dashed circles show the same successive positions of edges of "bimolecular" disks. The area to the left shows the outlines of pore regions on the assumption that "bimolecular" disks abut but do not obliterate these regions by compressional and dilational deformations, and do not coalesce into a continuous lamella. The area to the right shows a total absence of pore regions on the assumption of complete coalescence. [By courtesy of Dr. J. L. Kavanau.]

work and views of Luzzati and Husson on the phase-transformations in lipid–water systems. In this context reference can be made also to the recent work of Wolman and Wiener[518] on the influence of cations on the formation of oil/water and water/oil type emulsions in phospholipid–protein–water systems.

Apart from the provoking explanation given by Kavanau for the protoplasmic

streaming being effected by these membrane transformations, the theory includes many interesting but speculative viewpoints about fundamental membrane processes, e.g. active transport and permeability properties.

According to Kavanau a given membrane exists in a state of either a stable or a metastable equilibrium between both phases. It can readily be envisaged that for different membranes under the same conditions of pH, temperature, etc., the equilibrium can be different because of distinctions in the chemical composition of the membranous lipids. Thus differences in the fatty acid constituents may greatly influence the phase transitions and can provide a basis for differences in permeability behavior. Although it is too early to be factually critical of some of the views expressed by Kavanau, and the full issue of his theory has to be waited for, the emphasis for the possible importance of transformations of lipids in natural membranes is provoking.

4. Globular structures and mitochondria

Although a detailed discussion of specialized membranes cannot be included, the vast progress made on the structure of mitochondrial membranes requires particular attention. The pioneering work of Palade[381] made it clear that these cytoplasmic organelles are limited by double membranes, which are continuous with transverse membranes denoted as "cristae mitochondriales". The view of Palade that the cristae represent folds of the inner of the two limiting membranes has been confirmed by other investigators, and the mitochondrial membrane elements were included by Robertson[409] as part of the unit-membrane system. The two membranes of which mitochondria are composed both showed after potassium permanganate fixation the typical triple-layered structure, thus leading to the concept that two bimolecular lipid leaflets sandwiched between thin-layers of protein are separated by a gap substance about 100 Å thick. This mitochondrial double membrane was believed not to differ in principle from those structures of the endoplasmic reticulum and the cell-nucleus, and was accepted to be structurally related to the protoplasma membrane.[409]

Recently, however, Sjöstrand[442] reported that after potassium permanganate fixation the mitochondrial membrane and cytoplasmic membranes revealed a more complex structural pattern consisting of regularly arranged globular substructures with a diameter of about 50 Å. By contrast the plasma membrane gave a triple-layer pattern without these globular substructures. Regarding the fine-architecture of these sub-elements, the possibility was envisaged that lipid micelles are involved (see also Lucy and Glauert[327a]). According to Sjöstrand[442] the structural pattern observed by this method is not related to the globular structures which have been disclosed by the negative-staining technique. The latter approach applied by Fernández-Moran[148], Parsons[388] and Stoeckenius[459] revealed that the inner mitochondrial membrane carries globular components of a diameter of about 80 Å attached to the cristae by a stem which is 30 to 35 Å wide and 40 to 50 Å long (Fig. 36). These globular sub-units denoted

by Fernández-Moran[148] and Green [185] as elementary particles have been found to exhibit regular sub-structures of the order of 10 to 20 Å. In the laboratory of Green comparable particles have been isolated and these sub-structural units were found to contain about 25 per cent of the mitochondrial lipids. These globules devoid of a stalk were found to have a molecular weight of about 2×10^6. According to Green and Fleischer[185] the elementary particles carry all

FIG. 36. The sub-unit associated with the inner membranes or cristae.[388] (a) Part of a mitochondrion from a negatively stained preparation of mouse liver. A few of the cristae (c) are shown. The cristae consist of long filaments which branch at some points (j). The surfaces of the cristae are covered with projecting sub-units (× 192,000). (b) Negatively stained cristae (c) prepared by spreading isolated lysed rat liver mitochondria. The sub-units on the cristae appear similar to those of (a) (× 192,000). (c) Higher magnification—a few sub-units from the same preparation as (b). The spherical heads are 75 to 80 Å diameter and the stems 30 to 35 Å wide and 45 to 50 Å long. The centre-to-centre spacing is 100 Å. Reversed print (× 770,000). [By courtesy of Dr. D. F. Parsons.]

the functional components of the electron-transport chain consisting of four distinct enzymic complexes. Recently, however, Chance and Parsons[72] in discussing their results of a study of the cytochrome content and the size of these particles arrived at the conclusion that the sub-units do not contain the entire

cytochrome complement of the respiratory chain. Further studies of Chance *et al.*[73] on the cytochrome composition of mitochondrial fractions which were stripped off the inner membrane units also supported their view that not all the respiratory enzymes are located in the single sub-units.

Extensive studies have been carried out by Green *et al.*[185] also on the chemical compositon and the ultra-structure of the layer to which the elementary particles are attached, *viz.* the mesozone. This layer appears to be the locus of the structural protein, which amounts to some 50–60 per cent of the total protein of the mitochondrion. The mesolayer is considered to be a network of structural protein and lipid held together by forces which are believed to have a hydrophobic character. (See also Section IIC.) Each molecule of structural protein was calculated to contain about 7–8 *locii* available for interaction with amphiphatic phospholipids. Based upon chemical facts and electron microscopic observations models have been depicted for the arrangement of the building-stones in the mesolayer (Fig. 37). The orientation of the phospholipids in a bimolecular lipid leaflet being different from that vizualized by the paucimolecular theory, gives ample opportunities to hydrophobic interactions between the lipid micelles and the structural protein. It is of interest to note that extraction of mitochondria with acetone–water, which removes about 85 per cent of the phospholipids did not affect the trilaminar structure of the mitochondrial membranes. Therefore the disc model shown in Fig. 37b was considered by Green to account better for this phenomenon. The alignment of

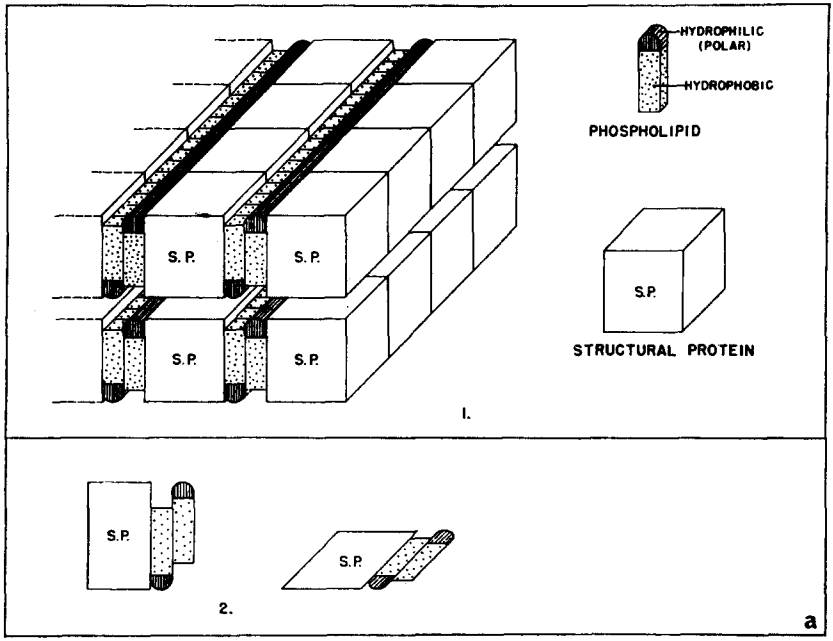


FIG. 37.

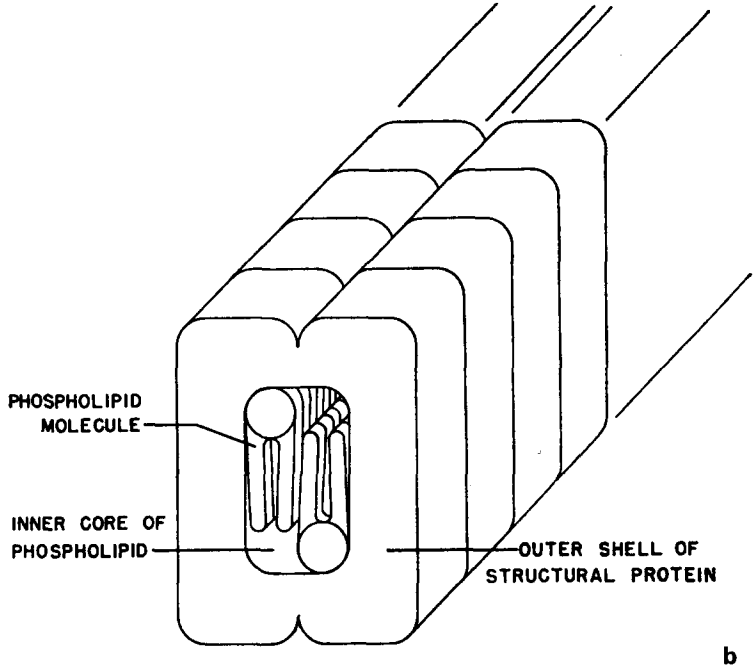


FIG. 37 (*cont.*). Diagrammatic representation of the structural protein-lipid network of the mitochondrial mesolayer according to Green and Fleischer.[185] (a) 1, three-dimensional representation of the network; 2, alternative modes of nesting of the structural protein (S.P.) with phospholipid. (b) Diagrammatic representation of the disk hypothesis of the structural protein–lipid network. [By courtesy of Dr. D. E. Green.]

the elementary particles and the nature of the attraction forces involved in the attachment of these sub-units to the mesolayer have been discussed extensively by Green and Fleischer.[185]

B. Phospholipids as essential constituents of enzymes

Circumstantial evidence indicates that the phospholipids are essential for the functioning of many enzymes located in or at the membrane. This important functional aspect of lipids has been surveyed in detail by several authors[8, 24, 185, 415] and only a few examples will be quoted here.

1. Mitochondrial enzymes

The intimate relations between phospholipids of membranous structures and enzymic activity are clearly demonstrated by the mitochondria. As early as 1938 Wooldridge and Higginbottom[519] observed that the toxin from *Cl. welchii* does cause an inhibition of the oxidation of succinate. The bacterial toxin later was demonstrated by Macfarlane and Knight[337] to contain a phospholipase C which catalyses the hydrolysis of a phosphoglyceride into a diglyceride and a

phosphomonoester (Section IVA). After the recognition of this fact and the localization of the succinate oxidation in the mitochondria, it became clear that a breakdown of mitochondrial phospholipids was responsible for this phenomenon.[135, 329] Not only phospholipase C but also other phospholipases[53, 368] subsequently were demonstrated to inhibit the action of succinic acid oxidase which enzyme contains an appreciable amount of phospholipids. In addition this type of approach showed that other enzymes of the energy-transducing machinery are inactivated, though to a different extent, by the action of phospholipases. In experiments with phospholipase A,[53] which produces the lytic monoacyl derivatives, an indirect effect is difficult to exclude. However, the essentiality of phospholipids for the mitochondrial function has also been demonstrated more directly. Whereas lipid-free mitochondria, still having their fine structure maintained, are unable to carry out the electron transfer, this capacity can be restored by adding back the mitochondrial lipid mixtures. This fundamental approach by Lester and Fleischer[320] was pursued by demonstrating a requirement for phospholipids in three segments of the electron transfer chain, *viz.* from succinate to coenzyme Q, reduced coenzyme Q to cytochrome C and reduced cytochrome C to oxygen.[185] As mentioned before, the micellar state of the phospholipids was found to be of paramount importance for the activity of the lipids. Das *et al.*[100] reported on the formation of complexes between purified phospholipids and cytochrome which were found to have properties similar to the cytochrome C bound in mitochondria. This type of study further endorsed the observations of Ball on the essentiality of intact phospholipids for succinate oxidase and Macfarlane's observations on the complete inhibition of succinate oxidase and cytochrome oxidase activities of liver mitochondria by phospholipase C.[329] The requirement of lipid for the electron flow has been interpreted by Green and Fleischer[185] in terms of providing a medium of low dielectric constant. The function of phospholipid also has been established for the the action of β-hydroxy butyrate dehydrogenase.[254] Apart from the essentiality of unsaturated fatty acid residues needed to provide the lipid in the right micellar state, this enzyme was found to have a specific requirement for lecithin. This is a quite unique situation in as much as in many other examples of protein–lipid interactions, e.g. in the field of blood coagulation and phospholipases, the surface charge of the phospholipid particles has been found to be more important than a particular type of phospholipid.[25a] It may be of interest to verify the lecithin requirement of the β-hydroxy butyrate dehydrogenase in terms of the zeta-potential of the phospholipid micelles.

2. ATP hydrolysing enzyme system

Increasing evidence supports the view that an ATP hydrolysing system, which is activated by $Na^+ + K^+$, is intimately involved in the active and linked transport of these ions. This enzyme system investigated by Skou[443] in peripheral crab nerves has been thoroughly characterized and a similar mechanism has been detected in many other biological membranes. Without discussing its full scope

it may be mentioned that this system has been shown to be located in submicroscopic particles which seems to be disintegrated in the cellular membranes.[444] The action of the specific enzyme system which catalyses the essential transfer of an energy-rich phosphate bond from ATP appears to depend upon the presence of lipids. Skou[444, 445] observed that incubation of the particles with phospholipase A effect a disappearance of the specific activating effect of sodium and potassium, while the addition of detergents also brought about a similar effect. The participation of membrane lipids in the ATP-ase system appears to concern also the red cell membrane. The involvement of this ouabain-sensitive enzyme system in the active transport of sodium and potassium across the barrier of the red cell was convincingly demonstrated by Post *et al.*[394] and confirmed by other research groups. Schatzmann[425] investigated the effects of phospholipase C from *Cl. welchii* upon the ATP-ase activity of red cells. This lipolytic enzyme which has been demonstrated to attack all the major phospholipids (lecithin, cephalin and sphingomyelins) present in the red cell,[118] appeared to reduce both the ouabain-sensitive ATP-ase involved in the active transport as well as the glycoside-insensitive enzyme. The essentiality of the intact phospholipids of the membrane may indicate that an organized lipoprotein structure is required but the possibility of more direct functions of the phospholipid in these processes has been envisaged as well. Recent studies of Charnock and Post[74] led them to suggest that in the kidney cortex phospholipids may participate in forming a phosphorylated complex being an intermediate in this active Na^+ transfer. The inhibition of the ATP-ase activity of skeletal muscle microsomes by phospholipase C was reported by Kielley and Meyerhof.[285] Recently Martonosi[344] found that this inactivation is due to a splitting of the microsomal lecithin and leads to an inhibition of the Ca^{++} uptake. Restoration of ATP-ase activity and Ca^{++} transport of the phospholipase C-treated microsomes was obtained after addition of natural and synthetic lecithin preparations as well as by lysolecithin. A relation between the soluble muscle-relaxing factor and phospholipids was reported[134] and Briggs[57] even suggested that this factor might be identical with a phospholipid.

Several other enzyme systems have been demonstrated to be intimately linked with membrane structures. An example of such an enzyme involved in phospholipid metabolism appears to be the phosphatidic acid phosphatase from microsomes which according to Coleman and Hübscher[83] is firmly bound to these lipoprotein structures.

C. Composition and properties of the cellular barrier

When comparing the information available on the chemical make-up of membranes with the models proposed for the molecular architecture of the membrane a general agreement about the participation of both phospholipids and proteins is evident. The essentiality of the phospholipids is not limited to the structural lipoprotein network forming the cellular boundary but applies

also to the various enzymes integrated in the membrane or attached in distinct sub-units to this framework. Although phospholipid molecules when brought in aqueous surrounding associate into micellar structures, the mechanical stability and specialized functions required by the biological interfaces make the presence of various proteins indispensable. In this respect the recent studies of Mueller and Rudin and co-workers,[360, 361, 362] who showed that macromolecules lend to artificial lipid systems physical properties which resemble those of cell membranes are of great interest. Stable lipid membranes being analogous to air–soap films were prepared in saline from crude brain lipids. These inert structures, shown by electron microscopy to be 60 to 90 Å thick, appeared to have a weak electrical polarization and poor ionic selectivity. However, the addition of unidentified water-soluble macromolecules extracted from bacterially fermented egg white, retina and white matter, being heat-stable and precipitable by ammonium sulphate, modified these structures by lowering their electrical resistance. The spontaneous adsorption of these macromolecules altered the inert lipid barrier into an electrically excitable membrane, and the d.c. stimulation pattern of this artificial membrane system was practically indistinguishable from the behavior of the excitable alga, *Valonia.*

The organization of lipids and proteins is depicted in several models quite differently, but in general the several possibilities considered for the membrane structure account fairly well for the specific physico-chemical properties of the phospholipids. The importance of ionic interactions between the phospholipid headgroups and charged side-groups of the structural protein is evident in all proposals, although the emphasis laid on this feature differs among the several theories. Evidently the importance of London–Van der Waals' forces for holdng together the lipid-chains, as well as the compacting function of cholesterol are recognized in all current theories attempting to formulate the molecular organization of the membrane. The apparent differences between the various hypotheses, when viewed from the aspect of binding between lipids and proteins, perhaps stems in part from a different appreciation of the magnitude and nature of hydrophobic interactions. Thus, the models advanced by Green and Fleischer (Fig. 37) though leaving open possibilities for ionic interaction offer ample opportunities for hydrophobic attraction between the apolar lipid residues with the structural protein. It may be recalled that these investigators arrived at the conclusion that the interaction between amphiphatic phospholipids and mitochondrial proteins is mainly hydrophobic in nature, whereas ionic interactions are considered to dominate in the case of negatively charged phospholipids. The observation that part of the phospholipids in the red cell membrane are not retained by ionic linkages led Parpart and Ballentine[386] to advance a mosaic structure having the major portion of phospholipids attached firmly to the protein meshwork, whereas the remaining part is linked by Van der Waals' forces to these strongly bound lipids. In the Kavanau model,[271] the lipids which are believed to be located between two protein sheets are also considered to be bound in a different way, *viz.* in the open state, only that part

of the lipids forming the base of the lipid pillars is firmly bound to the protein, while the remaining lipid molecules are held together by Van der Waals' forces, but when transferred into the closed state they also became subject to interaction with the proteins. It may be noticed that the appreciation for the magnitude of hydrophobic and ionic forces between lipids and proteins is reversed in this theory[271] when compared with the usual conception. The bimolecular lipid leaflet arrangement as interpreted by many investigators emphasized the charge–charge interaction, but interspersion of the non-polar side-chains of the structural protein between the chains of the lipids has been considered by Danielli and Davson to furnish an additional way of interaction. The latter conception was recently critically discussed by Haydon and Taylor;[210] taking into account the dimensions of protein side-chains and the polar end-groups of the phospholipids and the conclusion was reached that this arrangement is not likely to exist. An alternative but daring concept might be that in addition to the electrostatic aggregation, also hydrophobic interactions occur between a bimolecular lipid leaflet and protein sandwich-layers by an approach of a poly-unsaturated fatty acid on to the hydrophobic region of the structural protein. Such an arrangement is not attractive energetically, but the unknown three-dimensional structure of the protein concerned may facilitate such a construction. However, rather than by speculation, further experimental facts about the binding of the phospholipids to other membrane building-blocks may accelerate the resolution of various uncertainties about membrane structure. Such data also may be of great value supporting the interpretation of the membrane structure derived from electron microscopy. The evidence for the existence of a bimolecular lipid layer in cellular membranes obtained by this method is very strong indeed, but not yet conclusive. The picture derived from the myelin sheath by electron microscopy and X-ray analyses on fresh material probably is the most reliable one. However, it remains to be established whether the similar patterns obtained from various cell envelopes are not to be due to alterations in molecular orientation brought about by the various treatments involved. At a given lipid concentration and temperature the bimolecular lipid leaflet may be the energetically most favorable configuration. Disturbance of the original phospholipid–protein aggregation by the introduction of the fixation agents or by dehydrating procedures using polar organic solvents is not imaginary and may cause a transformation into this lipid orientation. Therefore the possiblity remains that in a certain cell membrane the lipid molecules are arranged primarily in a different way. Future progress in the knowledge of the molecular organization of the cell boundary will depend on a precise study of the interaction forces stabilizing the membranous lipoprotein structure.

It is a great challenge to account for the permeability properties of biological boundaries in terms of the chemical composition and interfacial properties of the lipid constituents. As outlined in Section IID, a great variation in the non-polar residues is evident and the nature of the acyl and alkyl chains appears to be adopted to preserve a liquid–crystalline phase of the membranous lipid

micelles. Though the information derived from monomolecular films can only partly be applied to the bimolecular lipid leaflet, it may be envisaged that variations in chemical structure of the fatty acid aldehydogenic and ether chains effect the penetrability of the lipid barrier. However, it can be argued also that the properties of a membrane structure switching between several lipid phases will depend greatly on the chemical composition of these membranous building-blocks. Several intriguing observations on lipid composition of membranes and the distinction in physico-chemical properties of the respective lipids already have been mentioned in Section II. A renewed discussion is deleted, since at our present stage tentative conclusions about structure and function of lipids can be made more or less compatible with several of the depicted membrane models. The complexity of the problems arising when attempting to relate some properties of membranes with lipid composition, however, may be demonstrated taking the red cell as a model.

The erythrocyte membrane of various mammals is characterized by a fairly constant ratio of phospholipids and unesterified cholesterol but differs greatly from species to species with respect to the phospholipid distribution and composition of the fatty acid constituents. (See Section II.) In addition, the red blood cells concerned have been demonstrated to exhibit significant differences in their properties intimately related with membrane structure or activity. Hence, the red blood offers unique opportunities for attempting to correlate lipid composition with membranous properties. As outlined in a recent survey, several characteristic differences between red cells do not coincide with variations in lipid composition. Differences in the sodium–potassium ratio between red cells of different animal species will depend on the presence of specialized functions in the membrane system, i.e. the participation of an enzyme system catalyzing the active transport and hence cannot be expected to be directly related with simple lipid characteristics. However, when a comparison is made between differences in lipid composition of a series of animal erythrocytes with data on the permeability behavior of these red cells for small compounds probably not transported by an enzymic mechanism a striking coincidence emerged (Fig. 38). In the red cells of the animal series, rat, rabbit, man, pig, sheep and ox the penetration velocity of glycol and glycerol has been established by Jacobs *et al.*[249] to decrease significantly. It is in nearly the same order as the decrease of the lecithin–spherigomyelin ratio of the red cell, and the decrease of the palmitic and arachidonic acid content balanced by an augmented level of aleic acid. The improved methodology of lipid analysis thus permitted some relations to be established which had been looked for already by Parpart and Dziemian.[327] Before attempting to explain the difference in penetrability of these membranes for the small poly-alcohols in terms of differences in size of the "statistical pores" as consequences of differences in fatty acids and alcoholic constituents of the phospholipids it has to be realized that other differences in phospholipid characteristics have also been noted among these membranes. The ethanolamine-containing lipids in human[142] and rat[120]

erythrocytes contain a high amount of aldehydogenic linkages, whereas in the cow erythrocyte, the saturated glyceryl ether structure is abundant.[201] Another distinction between these red cells, which may perhaps be very helpful to understand relations between lipid composition and membrane properties, become clear when investigating the binding of phospholipid in the red cell ghost.

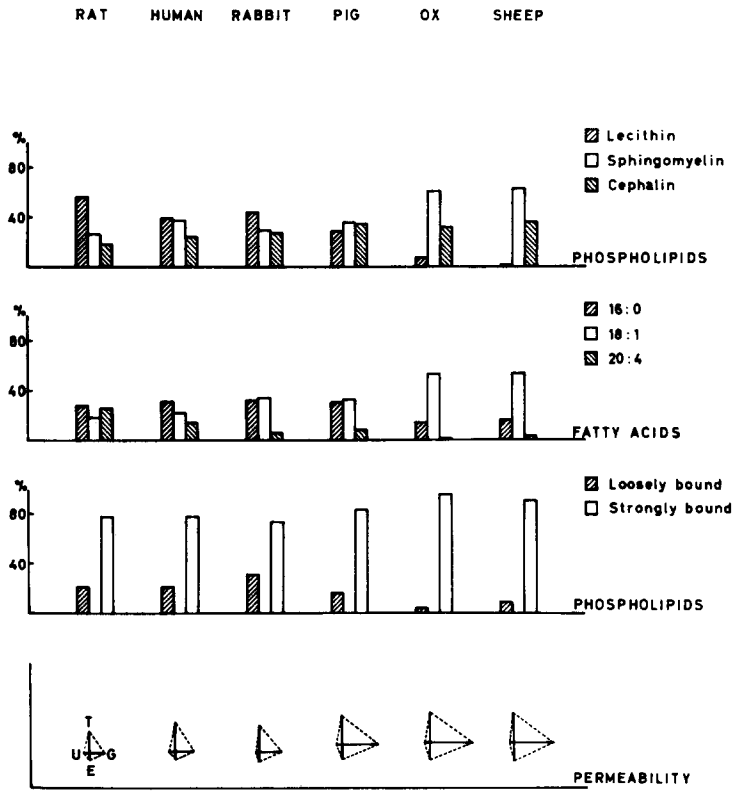


FIG. 38. Comparison of some lipid characteristics and permeability behavior of red cells of various species. Phospholipids are expressed as percentages of the total phospholipid fraction. Fatty acids are given as percentages of the fatty acid content of total lipids. The distinction between loosely and strongly bound phospholipids is based on differential extraction with ether and ethanol–ether (3:1 v/v) respectively. Figures on the permeability were taken from Jacobs *et al.*[249] and represent haemolysis times in isotonic solutions of urea (U), thiourea (T), glycerol (G) and ethylene glycol (E), on four perpendicular axes in a logarithmic scale.

The amount of loosely bound phospholipid, as classified above (III A2) appeared to decrease in the red cell in the order of rabbit, man, pig, ox and sheep. Therefore the suggestion is made that the variations in lipid composition result in a different cohesion between lipids and proteins in these red cells, thereby leading to a distinction in passive permeability behavior.[410]

This view appeared to find some support in the results obtained from an investigation on the action of phospholipase A from certain snake venoms on the phospholipids present in the red cell membrane of these animal species. This enzyme itself is known not to attack washed erythrocytes. The phospholipids of the red cell usually are not available to phospholipase A unless rupture of the membrane structure is effected by lytic agents or otherwise. Recent studies of Condrea *et al.*[87, 88] and Heemskerk and van Deenen[211] reaffirmed this fact already established some 50 years ago, but which is sometimes neglected and replaced by a belief subscribing a direct lytic action to this enzyme, Condrea *et al.*[88] now demonstrated that the direct lytic factor present in several but not all snake-venoms is identical to a protein clearly distinguishable from phospholipase A. The inaccessibility of phospholipids in the intact erythrocyte membrane for phospholipase A is not without implications from a structural point of view, i.e. either the phospholipid molecules are shielded by a protein or packed so closely that the active center of the enzyme cannot reach the fatty acid ester linkage to be hydrolyzed. It is intriguing that the presence of a sub-hemolytic concentration of deoxycholate enabled the phospholipase A to develop its action.[211] Probably this effect involves a reorientation of the lipid molecules, like that visualized in the case of saponins with the aid of the electron microscope.[133] After this introduction, the observation made on the rate of breakdown of phosphoglycerides in the red cell membrane fragments from different species deserves attention. The recent studies of Condrea *et al.*[87] and from this laboratory,[211] though performed in a different manner, were in good agreement, and showed that the susceptibility of the phospholipids in ruptured membrane is quite different for red cells of various species. Confining ourselves to the results obtained with the species under discussion, it can be recorded that a decrease of the hydrolysis of phosphoglycerides by *Crotalus adamanteus* venom was to be noted in red cell lysates in the order rabbit, man, pig, ox and sheep. The coincidence with the variations both in phospholipid composition, the binding of these constituents and the permeability behavior of these cells is believed too striking to be considered merely as fortuitous.

In short, these observations endorse the view that the chemical variations in phospholipids may contribute to the distinctions in properties of biomembranes. In addition to comparisons of differences in lipid composition with other characteristics of the respective membranes, still deeper insight into the structure function relationships of lipids may be obtained by comparing the properties of lipid species in model systems bearing some relations to biomembranes. Of particular interest are the membranes formed from lipids in a manner analogous to the generation of so-called "black" soap films, since the dimensions of these artificial membranes separating two aqueous phases and various physical properties appear to be very similar to the membranes of natural origin.[360] Recently, Drs. P. Mueller and D. O. Rudin succeeded in making secondary black membranes with synthetic components and sterols and upon adding the excitability inducing materials the membrane system showed complete action

potential. The preliminary experiments with synthetic phospholipids indicated it to be of interest to investigate various homologs to establish, e.g. the influence of fatty acid structure for the stability of the membranes. That studies of lipid membranes of this type may contribute to an understanding of structure and function of biomembranes was recently demonstrated also by the work of Thompson *et al.*[238a, 470a] According to this research group, the formation of these artificial membrane systems required the presence of both a phospholipid and a neutral lipid while the charge of the polar headgroup and the degree of unsaturation were found to be of paramount importance. It is intriguing that Thompson[470a] observed that only cholesterol when added to the phospholipid in a 1:1 mole ratio gave stable bilayer membranes.

IV. Dynamic Aspects of Membranous Phospholipids

A. Metabolic pathways of phospholipids

Depicting a membrane is hampered by the fact that the flexible character of the building stones is inadequately emphasized. Instead of being a static framework the membrane behaves as a structure which continuously replaces many of its essential components. The red cell, for example, has been shown to interchange quite rapidly unesterified cholesterol with that present in the serum lipoproteins. This dynamic character of the membrane lipids will not be without consequences for the permeability of the cellular barrier. The phospholipids also have a part in the dynamic events of the membranes. Although a proportion of them may be metabolically rather inert,[470] thus depending on the lipid species and the type of membrane, certain studies even led to the postulation of specialized dynamic functions of phospholipids in the basic processes of biological interfaces.

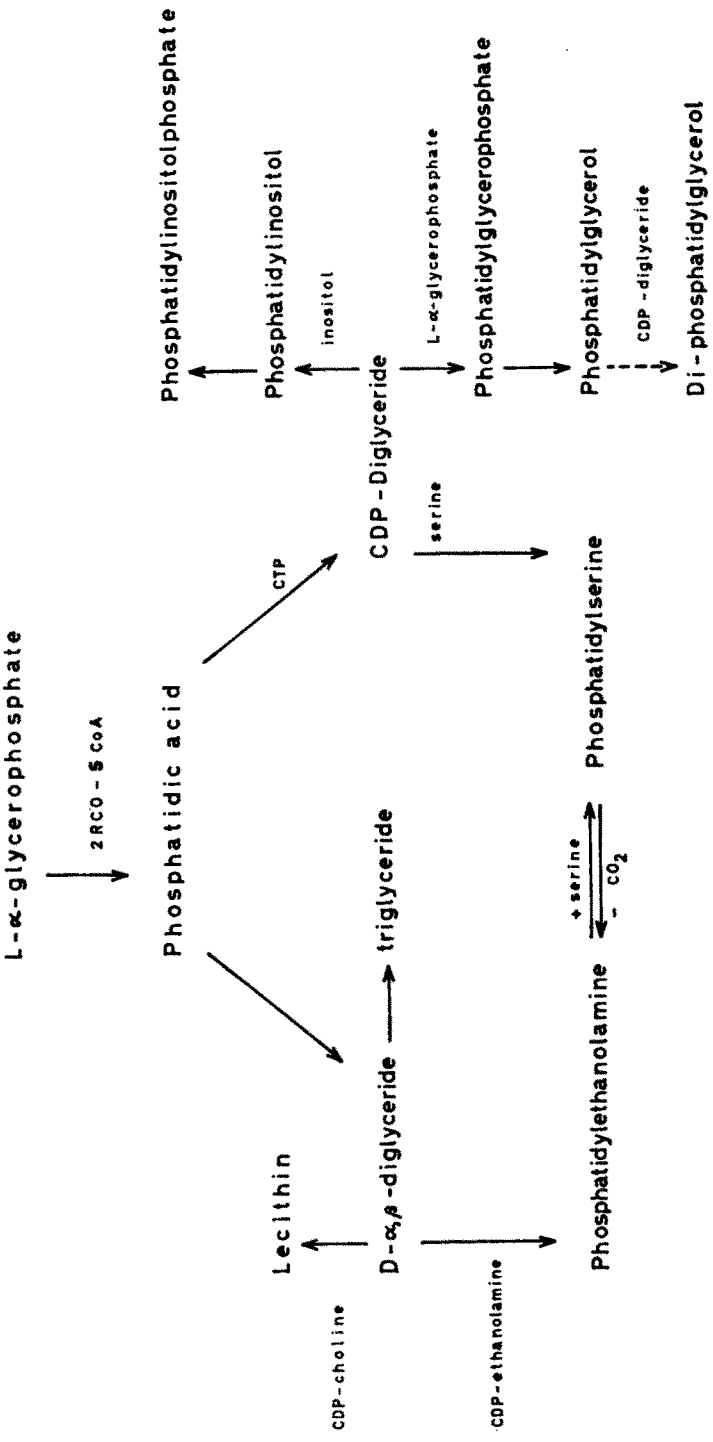
The metabolism of phospholipids in membranes may concern either a replacement of complete molecules, or a conversion of the polar headgroup giving a new phospholipid species or the introduction of fresh fatty acid constituents, both latter processes with maintenance of the major part of the molecular skeleton. Confining ourselves to the phosphoglycerides, some examples of such processes may follow in an abstracted form.

1. De novo *synthesis of phosphoglycerides*

Pathways for the biosynthesis of the more common phospholipids have been elucidated during the last decade and a large part of the credit for this important achievement goes to E. P. Kennedy and his co-workers. A detailed account of the biosynthesis of phospholipids has been given in several reviews.[8, 276, 415] A schematic diagram of the pathways involved (Scheme 4) was formulated by Kennedy[278] and clearly shows the key position of phosphatidic acid.[276] Kornberg and Pricer[303] demonstrated that CoA derivatives of higher fatty acids are enzymically esterified to glycerophosphate so as to form diacyl phosphatidic acid. In addition these investigators observed that phosphorylcholine doubly

labelled with ^{32}P and ^{14}C was incorporated as an entity by a liver enzyme into the phosphoglyceride concerned. Studies of Kennedy and Weiss[280] showed that phosphoryl choline was relatively ineffective as precursor for lecithin biosynthesis, when ATP was generated by oxidative phosphorylation, but the addition of a commercial ATP preparation resulted in an active incorporation. This effect was demonstrated to be due to a contaminant and additional experiments conclusively showed the specific requirement of cytosine-containing coenzymes for the biosynthesis of phospholipids. Kennedy[277, 280] and co-workers demonstrated that the biosynthesis of lecithin and phosphatidyl ethanolamines involves a condensation between D-α,β-diglycerides and cytidine diphosphate choline and cytidine diphosphate ethanolamine respectively (Scheme 4). The D-α,β-diglycerides precursors appeared to be formed by the removal of phosphate from phosphatidic acid by an enzyme denoted phosphatidic acid phosphatase.[446] The D-α,β-diglycerides produced have been demonstrated to act as precursors of both phosphoglycerides and triglycerides.[452, 454, 503] Apart from these pathways the formation of CDP-diglycerides from the phosphatidic acid precursor has been demonstrated (Scheme 4). These unusual nucleotides, displaying the properties of a lipid turned out to be involved in the biosynthesis of phosphatidyl inositol.[4, 389] Furthermore, a reaction with L-α-glycerophosphate was demonstrated by Kennedy and co-workers to give phosphatidyl glycerophosphate, which after enzymic cleavage of the terminal phosphate yields phosphatidyl glycerol[287] (Scheme 4). The latter compound is presumed to act as a precursor of diphosphatidyl glycerol or cardiolipin.

The formation of phosphatidyl serine in animal tissues has been reported to occur via a Ca^{++} stimulated exchange of free serine for the base of phosphatidyl ethanolamine.[50, 240] However, in *E. coli* Kanfer and Kennedy[260] discovered an enzyme catalysing a *de novo* synthesis of phosphatidyl serine from serine and CDP-diglyceride according to a mechanism analogous to that accounting for the formation of phosphatidyl inositol (Scheme 4). By contrast to mammalian tissues this bacterium did not reveal a direct biosynthesis of phosphatidyl ethanolamine and the formation of this phosphoglyceride appeared to involve phosphatidyl serine as an obligate intermediate. This example may illustrate that the pathways of phospholipid biosynthesis are very complex and may vary considerably among different living organisms. In addition to the reactions presented other conversions relevant to phospholipid metabolism have been established in mammalian tissues. Apart from the formation of phosphatidic acid through the esterification of L-α-glycerophosphate it has been demonstrated that phosphorylation of the free primary hydroxyl group of unsaturated D-α,β-diglycerides contributes to the dynamic character of this phospholipid.[229] A third anabolic pathway was reported by Pieringer and Hokin,[390, 391] who showed that monoglyceride and ATP give lysophosphatidic acid which is enzymically acylated so as to form phosphatidic acid. Numerous interconversions between phosphoglycerides have been discovered and will be discussed separately.



SCHEME 4. Biosyntnetic pathways of phosphoglycerides.

The complexity of the problems concerning the biosynthesis becomes clear when one attempts to understand on the basis of the well substantiated reactions (Scheme 4) the peculiar differences in fatty acid distribution among the biogenetically-related lipid species. As discussed in Section II, triglycerides, lecithins, phosphatidyl ethanolamines, phosphatidyl serines and phosphatidyl inositols from the same mammalian tissue have quite different fatty acid patterns. Recently Gray[183] reported that two poly-glycerol phospholipids, *viz.* phospha tidyl glycerol and cardiolipin from liver mitochondria, also revealed a significant different fatty acid composition. A great deal of work remains to be done before these differences can be explained with any certainty on the basis of a specificity of the enzymes[504] concerned in the conversions depicted by the scheme under discussion. Another intriguing problem is offered by the preferential location of poly-unsaturated fatty acids at the 2-position of many triglycerides[345] and phosphoglycerides. This coincidence in fatty acid position between both major lipid families is consistent with the pathways outlined in Kennedy's scheme. It has been suggested that the acylation of α-glycerophosphate involves the action of two enzymes and that the introduction of the unsaturated fatty acid (in plants) would be specifically located at the 2-position.[424] Quite recently, Lands and Hart[308] investigated the acylation of L-α-glycerophosphate in microsomal preparations of liver, but arrived at the conclusion that the specificity of the enzyme systems concerned was not adequate to account for the asymmetric distribution of saturated and poly-unsaturated fatty acids in this tissue. An alternative explanation, favored by Brockerhoff *et al.*[62] involves primarily a specific linkage of the poly-unsaturated fatty acids to the β-position of monoglycerides. A biochemical stability of such β-monoglycerides according to this proposal might give rise to diglycerides, triglycerides and phosphoglycerides retaining the poly-unsaturated fatty acids at the given ester position. In this context it is of interest to note that in the intestinal mucosa apart from the mechanism mentioned diglycerides have been demonstrated by Clark and Hübscher[78] to be formed also from monoglycerides. This pathway has been confirmed and Brown and Johnston[64] demonstrated that the reconstituted triglycerides possessed a distribution of fatty acids between the 2- and 1,3-positions strikingly similar to the triglycerides fed. The hypotheses of Brockerhoff includes a relation to a diacyl-monoacyl phosphoglyceride cycle which may account for the release of any excess of essential fatty acids. The acylation of the monoacyl phosphoglycerides or lysophosphoglycerides into the diacyl analogs[307] has been demonstrated in an elegant way by Lands[309, 350] to involve a preferential esterification of saturated and unsaturated fatty acids at the 1- and 2-position respectively of lecithin and phosphatidyl ethanolamine. Since this specificity was not observed during the acylation of glycerophosphate, it was concluded that a positionally specific redistribution of fatty acid constituents in tissue phosphoglycerides may arise after the introduction of the polar headgroup into the molecule.[308]

A location problem of another type provides the site of biosynthesis of

phosphoglycerides in the animal cell. According to a recent study of Wilgram and Kennedy[507] the enzymes catalysing the coupling between D-α,β-diglyceride and CDP-choline appeared to be located mainly in the microsomal fraction. The phosphatidic acid phosphatase, though present in the mitochondrial and microsomal fractions was found to exhibit the highest specific activity in an "intermediate fraction" identical or related to the lysosomes. The interesting conclusion was reached that the major phospholipid of rat liver mitochondria, lecithin is not synthesized by these cell particles. Thus both the phospholipids and protein of the mitochondrial membranes may originate at least in part from the endoplasmatic reticulum.[507] According to Sedgwick and Hübscher[435] the phosphatidic acid phosphatase is located predominantly in the mitochondrial and microsomal fractions though the lysosomal fraction also was found by these investigators to contain significant amounts of this enzyme.

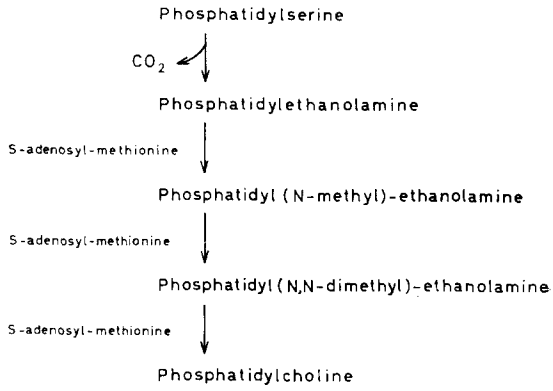
Schneider[430] reported that in rat liver the synthesis of lecithin from deoxycytidine diphosphate choline takes place mainly in the microsomal fraction. The synthesis *de novo* of phospholipids is, of course, essential for the production of newly formed membranes and also will play a part in generating repair molecules for existing membranes. Regarding the former process the experiments of Karnovsky and Wallach[262] are of interest. These investigators noted that when leucocytes ingest starch particles by phagocytosis, a process which may require the formation of much new cell membranes, the incorporation of ^{32}P into phosphatidic acid was increased about six-fold. Another example, probably relevant to the formation of membranes, is provided by the TSH-induced stimulation of phosphate incorporation into the phospholipids of the thyroid gland.[161, 296, 359] This effect, which can be noted *in vitro*, has been considered to be a first reflection of an enhanced biosynthesis of phospholipids needed for the construction of new cellular and intracellular membranes.[296] It is well-known that *in vivo* a considerable increase in weight of this gland can be brought about by this pituitary hormone. The rate of incorporation of phosphoryl choline into liver phospholipids was observed by Leal and Greenbaum[312] to be increased in rats treated with growth hormone.

Measurements of the rate of biosynthesis of phospholipids *in vivo* have been made with the use of isotopes. Measurements on the renewal of the phosphate moiety of phosphoglyceride showed that the replacement of the polar headgroup is a rather rapid process in the liver.[107] The turnover of phospholipids may differ greatly between various membranes. In the myelin sheath of normal adults, the various lipids laid down in early life according to Davison[16, 102] are not significantly metabolized.

2. Interconversions between phosphoglycerides

The phospholipid composition of membrane may not only depend on the balance of the activity of the enzymes involved in the synthesis *de novo*, but several additional reactions may govern the ultimate phosphoglyceride distribution. Several research groups demonstrated the existence of enzymes in the

microsomal fraction of rat liver which catalyse the incorporation of serine,[50, 240] choline,[128] ethanolamine[11, 239] and dimethyl ethanolamine[12] by an exchange reaction stimulated by Ca^{++}. Though the mechanism of these reactions may be very similar, Hübscher[239] outlined that specific enzymes are probably involved for the incorporation of each nitrogenous constituent. A comparable mechanism for inositol involving an activation by Mn^{++} has been observed as well.[389] Such reactions may greatly facilitate the adoption of the charge of the lipid core of the membranes. Of great interest is a sequence of reactions (Scheme 5) involving the decarboxylation of phosphatidyl serine, and a stepwise methylation



SCHEME 5. Interconversions of phosphoglycerides.

of the formed phosphatidyl ethanolamine which leads via the mono- and dimethyl analogs to phosphatidyl choline. The work of Udenfriend and coworkers[509, 510] and Bremer and Greenberg[54, 55] led these investigators to conclude that in liver serine is decarboxylated only after its conversion into the phospholipid. Similarly the successive methylation of ethanolamine to choline according to these investigators takes place not with the free components but in the phosphoglycerides exclusively. Regarding the methyl donor Artom and Lofland[13] observed that methionine stimulates the conversion of phosphatidyl dimethylethanolamine into lecithin. Also in the other steps S-adenosyl methionine appears to donate the methyl groups.[89] The successive transfer of methyl groups may involve more than one enzyme, since Goldfine [178] observed that in the lipids from *Cl. butyricum* the monomethyl ethanolamine derivative is present but the multi-methylated members are lacking. In the animal tissues the quantitative importance of this pathway in comparison to the lecithin production directly from diglycerides is not established. In *Neurospora crassa* Hall and Nyc[193] observed that the fatty acid pattern of the choline, dimethyl ethanolamine and monomethyl ethanolamine containing phosphoglycerides are identical, thus endorsing the quantitative importance of the

methylating reaction for the phosphoglyceride biosynthesis of this micro-organism.

An interesting combination of an exchange reaction and an interconversion between phosphoglycerides was provided by the work of Borkenhagen *et al.*[50] These investigators described two enzymes in liver catalysing the exchange of serine with the ethanolamine moiety of the respective phosphoglycerides and the decarboxylation of phosphatidyl serine to phosphatidyl ethanolamine. Thus a phospholipid cycle emerges which can be speculated to be relevant to the function of these differently charged phosphoglycerides in the membrane.

Another interesting pathway which was recently established concerns the phosphoinositides, well known to exhibit a high rate of incorporation of labelled phosphate. Experiments on the incorporation of labelled phosphate in mono-, di- and triphosphoinositides of rabbit brain slices led Brockerhoff and Ballou[61] to the conclusion that the following stepwise phosphorylation was involved:

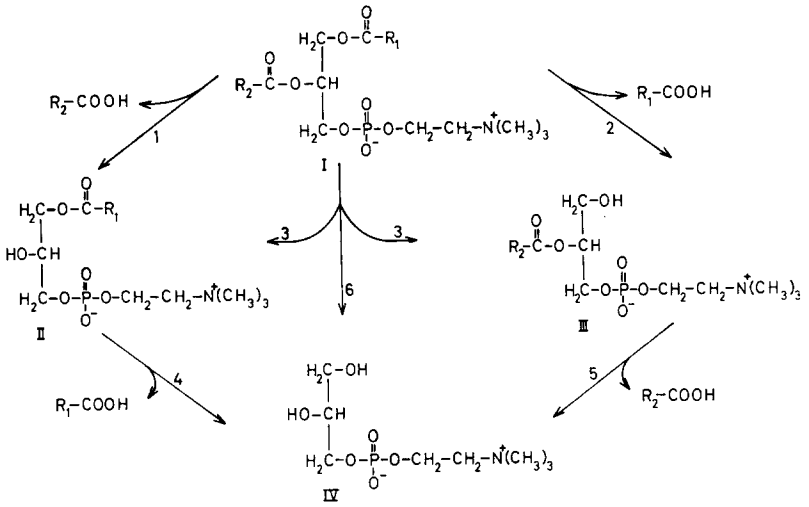
monophosphoinositide → diphosphoinostide → triphosphoinositide

In vivo studies of Wagner *et al.*[233, 490] on the ^{32}P-incorporation into di- and triphosphoinositides of various rat tissues appear to be in agreement with the proposal of Brockerhoff and Ballou. The reversed sequence of reactions was observed by Thompson and Dawson[471] to be catalysed by extracts of brain acetone powders. Some further implications of these metabolic reactions will come into discussion later.

3. Catabolism of phospholipids

As a counterpart of the anabolic pathways the dynamic phospholipids require a catabolic system which is provided by a number of enzymes denoted as phospholipases. Removal of one fatty acid residue from the phosphoglyceride molecule is catalysed by phospholipase A. The site of attack of this enzyme present in snake-venom is now generally considered to be the 2(or β)-fatty acid ester linkage exclusively (Scheme 6). The analogous phospholipase A from pancreas was demonstrated to exhibit the same mode of action.[123] Recent investigations made it clear that in mammalian tissues an enzyme(s) operates which also cleaves the 1(or γ)-ester position. Lloveras *et al.*[325, 326] observed that the nature of the fatty acids liberated and the fatty acid constituents of lysolecithin produced from egg lecithin by the action of an extract from rat spleen was compatible only with phospholipase attacking both ester positions. Recent experiments from this laboratory involving the use of doubly labelled substrates showed that homogenates of liver, lung and spleen from rat hydrolysed the two ester bonds so as to form two structurally isomeric lysolecithins[52] (Scheme 6). Indirect evidence for the existence of such isomers was recently provided by Tattrie and Cyr,[466] by making fatty acid analyses of lysolecithins from various origins. At present it is not known whether two phospholipases are involved, each attacking a distinct ester linkage or whether one enzyme acts on both positions (Scheme 6).

Removal of the second fatty acid is catalysed by a widely distributed lysophospholipase (or phospholipase B) furnishing the water-soluble phosphodiester which in turn may be degraded by phosphoesterases. Apart from the



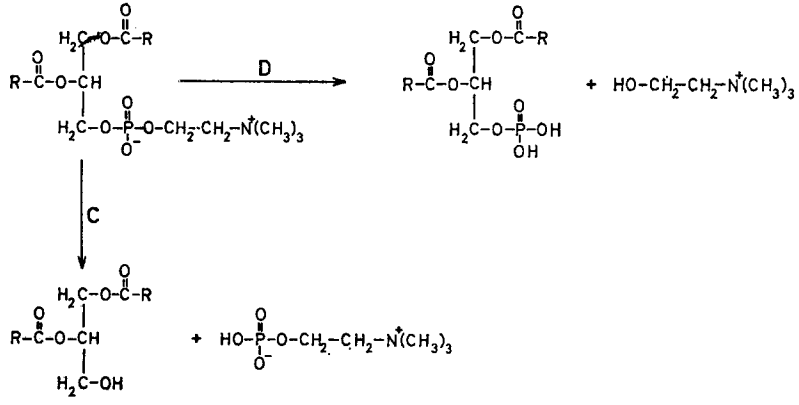
SCHEME 6. Possibilities for the enzymic hydrolysis of fatty acids from lecithin. Pancreatic- and snake-venom phospholipase A are known to catalyse exclusively reaction 1, while in mammalian cell organelles both isomeric lyso-derivatives II and III are formed, either by pathways 1 and 2 by a non-positionally specific enzyme (3). The formation of glycerylphosphoryl choline (IV) by the action of a lysophospholipase (reaction 4) or phospholipase B (reaction 6) has been ascertained.

combined action of two distinct types of phospholipases the possibility was envisaged that both fatty acids can be cleaved off by one enzyme[108] (Scheme 6). Furthermore, Erbland and Marinetti[138] and Kokke *et al.*[298] claimed evidence for a conversion of lysolecithin being of both catabolic and anabolic character, *viz.*:

$$2 \text{ lysolecithin} \rightarrow \text{lecithin} + \text{glycerylphosphorylcholine}.$$

Phospholipase C removes the polar headgroup with the formation of a D-α,β-diglyceride (Scheme 7). This enzyme has been found to act on sphingomyelin as well. As discussed in relevant reviews[110, 263] the enzyme is produced by several bacteria and is present in plants, but its importance in animal tissues still has to be assessed. A mechanism resembling that of phospholipase C, however, has been demonstrated by Kemp *et al.*[275] and Thompson and Dawson[471] to account for a breakdown of phosphoinositides. The enzyme trivially denoted

according to the recommendations of the Enzyme Commission as phospholipase D (Scheme 7) is known to be abundant in higher plants.[263] A related enzymic action may play part in the exchange reactions occurring in the microsomal fractions of animal tissues.[239] Two distinct enzymes concerned with the breakdown of the unsaturated ether linkage of the plasmalogens or their lysoderivatives have been detected recently.[10, 492]



SCHEME 7. Mode of action of phospholipases C and D.

The occurrence of various catabolic enzymes does not give sufficient information about their active involvement in the tissue concerned. The activity of these enzymes may be governed by many factors. In this respect the very able studies of Dawson and Bangham[25a, 26, 111] are of interest; they showed that several phospholipases require a certain net surface charge of the lipid micelles before activity develops. Studies on the catabolism of phospholipids by isotopic studies *in vivo* have been carried out only in a few cases. According to Dawson[105, 106, 110] in rat liver the breakdown of lecithin and phosphatidyl ethanolamine into the water-soluble phosphodiesters is the prevailing pathway. Similar observations have been made on the phospholipids from rat brain.[352]

4. Fatty acid renewal

From a chemical point of view the most susceptible part of the natural phospholipid molecule is formed by the poly-unsaturated fatty acid residues, which may be subject to peroxidation reactions. Removal of deteriorated molecules may involve a complete breakdown of the phospholipids concerned by the catabolic system and replacement by complete molecules freshly synthesized *de novo*. A more economic way might be provided by the replacement of the fatty acid constituents with maintenance of the molecular backbone of the phospholipid.

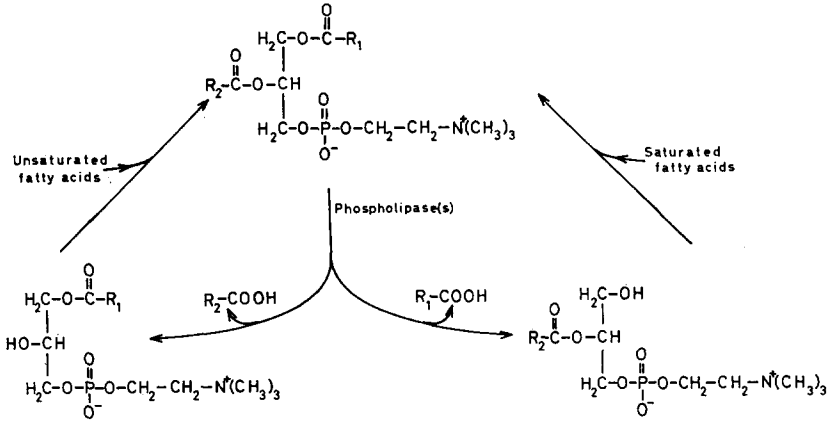
In addition to the distinct location of saturated and unsaturated fatty acid in the lecithin molecule, it is known from the work of Hanahan and co-workers[196, 197]

that the rate of incorporation of a given fatty acid varies depending upon its structure. The results of these investigations indicated that the unsaturated fatty acids bound at the 2(or β)-position of phosphoglycerides are much more metabolically active than the saturated ones located at the 1(or γ)-position. This independent turnover of both saturated and unsaturated fatty acid constituents of phosphoglycerides may occur by a pathway recently discovered by Lands.[309] An enzyme from rat liver microsomes was described that acylates lysolecithin in the presence of ATP and CoA. Further studies of this research group showed that these liver microsomal enzymes catalysing the acyl transfer, exhibit a strong positional specificity. Utilizing structural isomeric lysophosphoglycerides having attached the one fatty acid at the 1- or 2-position respectively it was demonstrated by Lands and Merkl[309, 350] that the enzyme system preferentially esterifies unsaturated fatty acids at the 2-position and saturated fatty acids at the 1-position so as to form the diacyl analogs (Scheme 8). Quite recently these workers observed that the acylation of glycerophosphate does not exhibit a positional specificity adequate to account for the non-random distribution of saturated and unsaturated fatty acids in natural phosphoglycerides.[308] This finding strongly endorses the view that the asymmetric distribution of the fatty acid constituents is brought about during the metabolic events of the completed phosphoglyceride molecules. Such a redistribution of the fatty acid constituents by the positional specific acylating enzymes implies that both monoacyl derivatives with fatty acids in different positions are furnished by the cell. The action of a specific phosphatidic monoacyl hydrolase resembling the action of the snake-venom or pancreatic phospholipase A may render one of the required lysoderivatives. Evidence for the formation of the 2(or β)-acyl isomer would strongly endorse this concept. A very able approach of Robertson and Lands[407] with lecithin substrates containing labelled fatty acids either in 1- or 2-position failed since no accumulation of lysolecithin was obtained. However, recent work of Lloveras *et al.*[326] on the phospholipase from spleen and studies from this laboratory with doubly labelled lecithins[52] proved that in various tissues of the rat both isomeric lysolecithins can be produced. Thus, the concept appears to be established that both fatty acid residues are separately exchangeable from the phosphoglyceride molecule. (Scheme 8). The difference in the turnover rate depends on the activities of the hydrolytic and acylating enzymes concerned with both ester positions.

The importance of this pathway is supported by the assessment that a diversity of phosphoglyceride species may participate in this diacylmonoacyl phosphoglyceride cycle. On one hand it is known that enzymes catalysing the removal of one fatty acid from the phosphoglyceride molecule may not require the presence of any specific polar end-group.[122] On the other hand the reacylation has been demonstrated now for the lysoderivatives of choline-,[309] ethanolamine-[350] and inositol-[273] containing phosphoglycerides. Of great interest is the observation of Pieringer and Hokin[390, 391] that also phosphatidic acid can be converted this way, namely via the sequence of reactions:

α(or β)-monoglyceride + ATP → α-lysophosphatidic acid + ADP
lysophosphatidic acid + fatty acyl-CoA → phosphatidic acid

The acylating enzyme(s) has been found to be present both in microsomal[307] and mitochondrial[117, 495] fractions from several tissues[451, 453, 495] so far studied. Regarding the competition between the re-acylating enzyme(s) with the lysophospholipase(s), it has been recorded that the activities of both reactions show a somewhat different intracellular distribution.[117] The mitochondrial and microsomal fractions from rat liver in the presence of ATP and CoA metabolized



SCHEME 8. Diacyl-monoacyl phosphoglyceride cycle.

lysolecithin for the greater part into lecithin. The supernatant fraction appeared to degrade lysolecithin rather than to utilize it for lecithin production. The formation of lecithin in the latter cellular fraction according to Erbland and Marinetti[138] may involve a distinct mechanism.

The viewpoint that the cycle under discussion is a general pathway involved in the renewal of fatty acids from membranous phospholipids is supported by recent investigations on the red blood cell membrane. Studies by Oliveira and Vaughan[379] and van Deenen and Mulder *et al.*[119, 366] demonstrated that fatty acids are incorporated in a selective way into the red cell phosphoglycerides, while in addition the capability of the red cell membrane to acylate lysolecithins and lysophosphatidyl ethanolamine was established.[119] Recently these results were confirmed by Robertson and Lands.[408] These *in vitro* studies are believed to bear some relation to the fatty acid renewal occurring in phospholipids from mature circulating cells.[119, 366] Dietary studies showed that maximal alterations can be brought about in the fatty acid of red cell phosphoglycerides from man,[143] rabbit[119] and sheep[174] in a period far shorter than the average life-span of these corpuscles. Furthermore a good correlation was found to exist between the *in vitro* and *in vivo* studies with respect to the selectivity concerning the nature and location of the fatty acid constituents. However, some differences between the

intracellular organelles and the red cell membrane have to be noted. So far it was not possible to detect any phospholipase A-like activity in the red cell,[117, 408] though rabbit erythrocytes were found to possess lysophospholipase-like activity which was presumed to be present as a safety enzyme.[211] Some evidence recently was obtained by Mulder[364] in favor of a participation of lyso-derivatives from the serum in the acylation reaction of the red cells. An exchange of complete phospholipid units between red cells and serum lipoproteins also contributes to the renewal of these membranous lipids.

Recently Erbland and Marinetti[138] proposed that in a liver preparation a fatty acid renewal of lecithin may proceed by a direct ester interchange with acyl CoA. At the present no detailed information is available about this interesting one-step conversion being identical to the overall reaction of the cycle discussed above.

B. Functions of phospholipids in membrane transport processes

The elucidation of the transport mechanism across the cellular boundaries forms one of the most challenging problems of biology. It will not be possible to refer here to the very great number of studies which led to the recognition of different types of biological transport.[76, 506] Confining ourselves to some current hypotheses on the involvement of phospholipids as carriers in transport, it is sufficient perhaps to recall the distinction between on one hand the active or "uphill" transport, the movement of a substance against a chemical or electrochemical gradient and on the other hand the "downhill" transport, in which the substance moves down an electrical or chemical gradient, by simple diffusion. The passive process also may involve the participation of a carrier facilitating the transport and in such cases the terms "facilitated diffusion" or "mediated transport" are frequently utilized.

The passage of the semipermeable structure of the bio-interfaces formed by the lipoprotein network may involve at several levels the participation or phospholipid. An indirect but crucial function was already illustrated by the essentiality of phospholipids for the enzyme system, e.g. the ATP-ase donating the energy required for the active transport (Section III). In addition the direct participation of phospholipids as carriers for the transport of ions and other substances has been envisaged.

1. Phospholipids as carriers of cations and anions

Several investigators favor the idea that phospholipids may transport ions across the membranous lipid barrier. Support for such a view was derived from the well-established properties that these membrane constituents are able to form lipid-soluble undissociated salts. Christensen and Hastings[77] concluded that crude cephalins can bind Na^+ and K^+ ions about equally. The acidic phospholipids from brain have been demonstrated to bind divalent and monovalent cations.[160] The acidic phospholipids, e.g. phosphatidic acid, have been

demonstrated to render a transfer of cation from an aqueous into a lipophilic phase.[491] Also the amphiphatic phospholipids carrying a net negative charge have been considered as apt for such a carrier function. In a model system Hoffman *et al.*[218] and Schulman and Rosano[412, 432] studied the passage of ions under the influence of membrane constituents. The investigation of the passage of ions through a liquid immiscible with water indicated that phosphatidyl ethanolamine could serve as a carrier between two aqueous phases effecting a counterflow of potassium and sodium ions. Solomon *et al.*[448] investigated the specificity of phospholipids for the binding of these ions and observed a preferential uptake of radioactive potassium by blood lipids. Also purified phospholipids, e.g. phosphatidyl serine preferred K^+ to Na^+ ion. On the other hand Kirschner[286] extracted a phosphatidyl serine-containing fraction from swine erythrocytes and observed a relatively greater binding of sodium and suggested that phospholipids may play a part in the extrusion of sodium from the red cell. Vogt[487] reported that a substance "Darmstoff" which is highly potent in stimulating the intestimal contraction and is chemically related to a lysophosphatidic acid, may act as a sodium carrier.

Regarding the active transport of anions, reference can be made to a recent study of Vilkki[486] on the binding of iodide by phospholipids. Lecithin from the thyroid gland was observed to be particularly active in concentrating iodide into the apolar phase of a chloroform–water system, thus leading this investigator to suggest that this phospholipid plays part in the iodide trap of the thyroid gland. The higher activity of thyroidal lecithin in this system when compared with lecithins from other origins was not yet explained in chemical terms.

Various investigations uniformly showed the ability of phospholipids, particularly the acidic types to bind cations into a lipid-soluble form. However, much work still is to be done on the chemical nature of the carrier involved in the membrane before it can be concluded with any certainty that phospholipids are directly implicated in the selective processes of ion transport. Another problem relates the carrier function with the coupling between Na^+ and K^+ transport and the energy requiring enzyme system. A few years ago an interesting theory involving phosphatidic acid as a transducer between ATP and the sodium carrier mechanism was advances by Hokin and Hokin.

2. The phosphatidic acid cycle

In an extensive series of investigations, Hokin and Hokin demonstrated that acetylcholine caused a stimulation of the incorporation of radioactive phosphate into the phospholipids of tissue slices from exocrine and endocrine glands. The increase in the amount of ^{32}P was found to be located mainly in phosphatidic acid and phosphatidyl inositol of the microsomal fraction and this phenomenon was correlated with the stimulation of the secretory process. This work was extended to the nasal gland of certain marine birds, e.g. the albatros, which was demonstrated by Schmidt-Nielsen *et al.*[428] to provide an extrarenal route for the secretion of concentrated solutions of sodium chloride. Slices of this

salt gland revealed *in vitro* a marked response to the addition of low concentration acetylcholine, as measured by the oxygen consumption. Again labelling of the phospholipids was greatly stimulated by acetylcholine, the amount of ^{32}P incorporated into phosphatidic acid being increased by a factor of about 15.[222, 223, 230]

As mentioned before, three pathways have been established by which phosphatidic acid can be formed: *viz.* starting from glycerophosphate, monoglyceride and α,β-diglyceride. The acetylcholine-stimulated process according to Hokin and Hokin is identical to the reaction of ATP with a diglyceride catalysed by the diglyceride kinase. As outlined recently by these investigators in the human red cell membrane the rate of formation of phosphatidic acid by this pathway is 40 times greater than that from monoglycerides and even 3000 times greater than the biosynthesis from α-glycerophosphate.[224, 227] A cyclic reaction mechanism in which the energy-requiring phosphorylation is rapidly followed by a dephosphorylation catalysed by the enzyme phosphatidic acid-phosphatase was related with the active transport of sodium. The latter enzyme which is known to be widely distributed was found to be present in various membranes giving an increase of ^{32}P incorporation into phosphatidic acid in response to the addition of acetylcholine. The activities of both diglyceride kinase and phosphatidic acid phosphatase were found to be of a sufficient order to relate this cyclic process with the ATP-ase system previously shown to regulate the coupled active Na^+ and K^+ transport across the human red cell membrane.

Thus evidence accumulated that in membranes the following phosphatidic acid cycle may be connected with the uphill transport.

$$\text{ATP} + \text{D-}\alpha,\beta\text{-diglyceride} \xrightarrow{\text{diglyceride kinase}} \text{L-}\alpha\text{-phosphatidic acid} + \text{ADP}$$

$$\text{L-}\alpha\text{-phosphatidic acid} \xrightarrow[\text{phosphatase}]{\text{phosphatidic acid}} \text{D-}\alpha,\beta\text{-diglyceride} + \text{phosphate}$$

Originally Hokin and Hokin postulated that phosphatidic acid functions itself as sodium carrier, by transporting two sodium ions across the lipid barrier. It was presumed that at the inner surface of the membrane the phosphorylation reaction proceeded and that the anionic phospholipid specifically combined with this cation. After a diffusion or rearrangement of the lipid the sodium ion might be released at the outer surface of the membrane during the hydrolytic action of the phosphatidic acid phosphatase.

This concept has deservedly obtained wide attention and several principal features of this hypothesis have been the subject of much discussion.[251, 279, 372, 523] It will not be possible to cover the various relevant papers here and reference is made to recent surveys of Hokin and Hokin.[226, 228] Several considerations led these investigators to modify their original working hypothesis, with maintenance of the metabolic cycle of phosphatidic acid, which reactions are well

substantiated at present.[228] In short, this cycle is now believed to produce a series of conformational changes in a lipoprotein-containing phosphatidic acid or its split product and these changes would induce the transfer of Na ions. These conformational changes in the lipoprotein are presumed to produce and to abolish specific cationic-binding sites, the nature of which is still unknown.[228] The borderline work on the conformational changes in macromolecules in the membrane of mitochondria recently led to the recognition that lipids may play an essential role in the contractile processes.[378]

3. Phospholipid involvement in swelling and contraction of mitochondria

The complex field of the uptake and extrusion of water in isolated mitochondria and the relations with oxidative phosphorylation have been comprehensively reviewed by Lehninger.[315] Recent work from several laboratories demonstrated relationships between these membrane processes and phospholipid metabolism.

(*a*) *Phosphatidyl inositol and mitochondrial contraction.* The mitochondrion is known to be capable of cyclical contraction or swelling which has been interpreted by Lehninger[315] in terms of the action of a mechano-enzyme system. Ohnishi and Ohnishi[377, 378] isolated from liver mitochondria a protein fraction having ATP-ase activity and capable of an ATP-induced conformational change similar to that of actomyosin from muscle. Further work in Lehningers laboratory revealed that contractile proteins from mitochondria possessed some but not all of the properties of actomyosin and the proteins were found to restore ATP-induced contraction in mitochondria which had lost this property.[484] The ability to promote mitochondrial contraction disappeared when the lipid was extracted from these complexes. Subsequently Vignais *et al.*[485] showed that phosphatidyl inositol was the only component able to restore contraction of aged mitochondria in the presence of ATP and Mg^{++}. Other phospholipids including the anionic phosphatidic acid and cardiolipin were uneffective, thus indicating a specific role of phosphatidyl inositol in this mitochondrial membrane process. Perhaps relevant to these observations is the work of Garbus *et al.*,[167] who showed that an inositol phospholipid was very rapidly labelled when liver mitochondria were incubated with ^{32}P-phosphate. The labelled compound was identified by Galliard and Hawthorne[166] as diphosphoinositide. In accordance with the previous observation of Brockerhoff and Ballou[61] on the formation of diphosphoinositides in brain, the mitochondrial diphosphoinositide was found to arise by a phosphorylation of phosphatidyl inositol, ATP donating the phosphate.[352a] The active transport of bivalent ions across the mitochondrial membrane has been demonstrated to involve an entrance of ion pairs of these cations with phosphate in definite molar ratio.[56, 317, 414] Hawthorne and Kemp[208] suggested that this accumulation of bivalent cations might depend on the alternate phosphorylation and dephosphorylation of the phosphoinositides, but more recently an alternative mechanism was considered to be more likely.

(*b*) *Fatty acids and mitochondrial swelling.* Fatty acids have been demonstrated

by Pressmann and Lardy[395] and Hülsmann *et al.*[241] to uncouple oxidative phosphorylation, while the swelling of mitochondria effected by fatty acids became clear from the studies by Lehninger and Remmert[316] and Avi-Dor.[18] It is well established that the magnitude of these effects depends on the chain length and the unsaturation of the fatty acids.[18, 51, 527] Studies of Wojtczak and Lehninger[515] indicated that the swelling of rat-liver mitochondria induced by Ca^{++} (or thyroxine) has to be ascribed to the enzyme liberation of fatty acids from a lipid precursor. Contraction of mitochondria by means of ATP caused a disappearance of the so-termed U-factor and under the same conditions an incorporation of fatty acids into phosphatidic acid and the cephalin fraction could be observed. Wojtczak and Wlodawer[514, 516] reported that a close relationship exists between the synthesis of phospholipids and the contraction process. The formation of ^{32}P-phosphatidic acid from labelled glycerophosphate was found to parallel the contraction process. Relevant to this phenomenon are the studies of Siliprandi[441] who observed that phosphorylcholine exerts a protective action on mitochondrial oxidative phosphorylation. Originally the explanation was forwarded that phosphorylcholine aids in the removal of freed fatty acids by their conversion into phospholipids, but more recently the action of phosphorylcholine was attributed to an inhibitory effect on the mitochondrial phospholipases.

These studies point to an intimate relation between phospholipid metabolism and the swelling–contraction cycle of the mitochondrion. Some of the possibilities include a mono- and diacyl phosphoglyceride cycle (Scheme 8) and a complete breakdown by phospholipases combined with a synthesis *de novo*. Recent observations in this laboratory showed that the formation of lysophosphoglycerides and their reacylation are highly active in liver mitochondrial fractions, at least with exogenous substrates. The difference in physico-chemical properties of a mixture of a lysoderivative and fatty acid, when compared with the corresponding diacyl phosphoglyceride, are compatible with an alteration in membrane permeability. The process of mitochondrial swelling and contraction, however, is very complex, and it has to be recalled that phosphatidyl inositol was found to restore the ATP-induced contraction of mitochondria swollen in the presence of oleate as well.

Of great interest are the observations on the variations in uncoupling and swelling effected by different fatty acids.[18, 51, 527] As outlined by Zborowski and Wojtczak[527] in the saturated series, the acids with a chain length of C_{12} and C_{14} were the most active swelling agents, while among the longer-chain fatty acids only the *cis*-unsaturated components were potent. It may be noted that the swelling effect of higher fatty acids correlates fairly well with the known properties of their monomolecular films. The fatty acids, which must have a certain minimal chain-length so as to be inserted in the membranous lipid layer, in general evoke a significant swelling action when their monomolecular films have an expanded character. The observations of Hoffsten *et al.*[219] on the formation of "lipid peroxides" under conditions which give swelling and

lysis of mitochondria have been tentatively related by these investigators to a change in structure and or permeability and this discovery certainly deserves further exploration.

4. Functions of phospholipids in amino-acid transport and protein synthesis

Several research groups favor the view that phospholipids present in membrane structures are directly and dynamically involved in protein metabolism. The basis of these hypotheses is often formed by observations that in bacteria or tissue preparations, when incubated with radioactive amino acids, a significant part of the radioactivity is recovered in a lipid soluble fraction. This work certainly deserves attention here, but a detailed discussion is greatly hampered by the fact that the "lipopeptides", "lipo-amino acids" or "phosphatido-peptides" involved are not yet adequately defined in terms of chemical structure.

The ability to transport amino acids against a concentration gradient is present in many cells and various highly specific mechanisms have been detected.[76] Regarding the nature of the carrier involved Tria and Barnabei[28, 29, 476] advanced the hypothesis that a metabolically active phosphatido-peptide fraction of the cell membrane incorporates amino acids on one side of the membranes with the expense of ATP and delivers the amino acid on the other side after a cathepsin catalysed hydrolysis. Experimental investigations concerned the transport of amino acids across the liver cell membrane[476] and amino acid transport from maternal to fetal blood across the placental membranes.[29]

Functions of phospholipids in the biosynthesis of proteins have been suggested by Gaby and co-workers,[163, 164, 165, 440] by Hunter *et al.*[177, 242, 243] and by Hendler.[212, 213, 214, 215] The first mentioned group reported the formation of lipo-amino acid complexes in liver slices[165] a bacterium[440] and *Penicillium chrysogenum.*[164] In a recent study on this mold Gaby *et al.*[163] detected a great number of amino acids in the hydrolysates of phospholipid fractions obtained by chromatography on silica columns.

Hunter and Goodsall[243] observed that protoplasts of *Bacillus megaterium*-bound ^{14}C-amino acids from the medium very tightly in a lipid-soluble complex, the amino acid being recovered unchanged from such components after hydrolysis. The labelled complexes were separated into a phospholipid and phosphorus-free fraction. The formation of such complexes was found to be inhibited by chloroamphenicol, while control experiments with killed preparations or with the addition of the amino acid to the lipid extract showed that the complex formation was not an artefact. These results led Hunter and Goodsall to suggest that the phospholipid may play a specific role in the later stages of protein synthesis. However, new developments recently led Hunter and James[244] to consider it less likely that these complexes are intermediates acting in the transfer of amino acids during the biosynthesis of lipoproteins.

Hendler[214] studied the formation of complexes of amino acids with lipids in the hen oviduct and also favored the idea that their formation is related to protein biosynthesis. Recently, an isolation of two lipopeptides was des-

cribed[215] and considered to confirm the covalent binding of amino and carboxyl groups of the amino acid constituents. The amino acids were found to be both incorporated and hydrolysed rapidly from the complexes and the findings were related to an entrance of amino acids into a lipid phase during the early stages of protein synthesis.[215]

At present the various interesting hypotheses still require an adequate chemical characterization of the complexes concerned before the specific function of phospholipids or other complex lipids in the processes of active amino acid transport or protein synthesis can be regarded as unequivocally established. However, it is of interest to note that recently one class of amino acid-carrying phospholipids was chemically disclosed after the discovery of O-amino acid esters of phosphatidyl glycerol as bacterial constituents (compare Section IIC). It will be of interest to elucidate whether these compounds are connected to the chemical entities involved in the studies discussed above. As mentioned before, the amino acid derivatives of phosphatidyl glycerol may play a part in transport processes and perhaps their occurrence is not confined to the bacterial membrane, although Gray[183] was not able to detect this phospholipid class in rat-liver mitochondria. A further search for these compounds in other cellular organelles and cell membranes still is desirable.

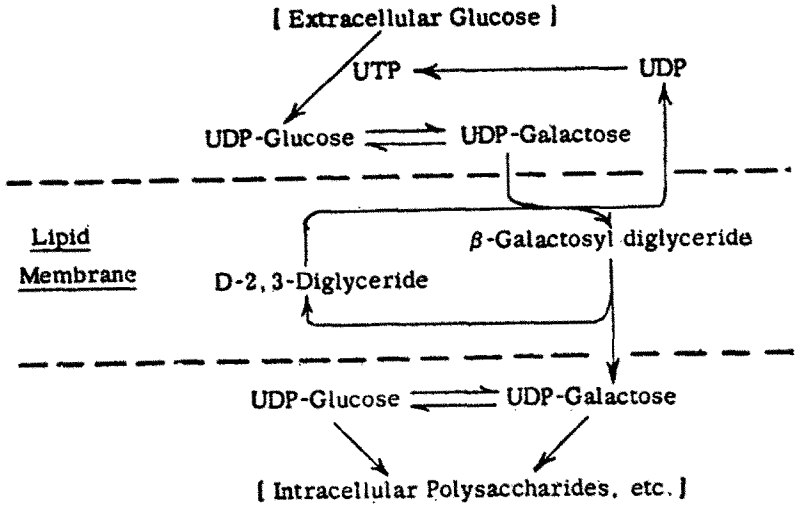
5. Lipids and sugar transport

The movement of sugars across the membranes of most cells appears to be facilitated by a carrier, while in a few cases the transport appears to involve an uphill process. A complex between the carrier and the sugar to be transported is believed to be formed at the outer surface of the cell membrane and after its translocation by either diffusion or by a conformational change in the membrane the sugar might be released at the inner side of the cell barrier. Enzymes may catalyse the formation and disrupture of the complex and the transport system may be specific for various sugars. Of great interest are the studies of Cohen and Monod[82] on the control of the stereospecific transport of β-galactosides across the bacterial cell membrane. The active transport of β-galactosides was proved to be coupled to energy production.[281] Recently Nikaido[374] reported that the phospholipid fraction from *E. coli* revealed a specific augmentation of phosphate incorporation in cells induced for β-galactosides transport.*

A very interesting proposal for the nature of the carrier and the mechanism for transport of sugars through the membranes was advanced by Benson[32] (Scheme 9). As in Section IIC the galactosyl diglycerides are the major lipids of the photosynthetic unit. The incorporation of galactose in these lipids is extremely rapid and exceeds that of the fatty acid or glycerol moieties. These lipids are believed to be orientated with the polar headgroup readily available

* Although the finding that the accumulation of β-galactosides is accompanied by an increase labeling of glycerophosphatides by ^{32}Pi was confirmed, the effect was not found to represent a specific stimulation of a phosphatidic acid cycle (E. P. Kennedy, 6th International Congress Biochemistry, 1964, Abstracts VII, p. 556).

for the enzymes of carbohydrate synthesis. The galactolipids appear always to have a β-glycosidic linkage; which fact points to a relationship with the established specificity of carbohydrate transport across cell membranes. According to Benson the photo-synthesis and transport of hexoses within the chloroplast may be related to the rapid labelling of the galactosyl glycerides. These compounds are widely distributed in nature and occur in mammalian membranes as



SCHEME 9. Mechanism for glucose transport through a membrane proposed by Benson.[32]

well, thus a general function in the glucose transfer through the lipid barrier perhaps is to be attributed to β-galactosyl diglycerides. Recently LeFevre *et al.*[314a] investigated the ability of phospholipids to solubilize monosaccharides in lipid solvents, and observed that the formation of these complexes showed some resemblance to the glucose transport in the red cell.

V. CONCLUDING COMMENTS

Though the imperfections in the isolation of pure natural membranes make it difficult in many cases to evaluate precisely their phospholipid content, the essentiality of these surface active lipids for cellular and intracellular interfaces is beyond doubt. The integrity of the lipid barrier from natural membranes generally is accepted to originate from the ability of phospholipids to participate both in lipid–lipid and lipid–protein interactions. In regard to the closely related problems of the nature and magnitudes of the forces involved in the various types of associations and the exact alignment of lipids, proteins and other components in the membrane, however, quite different opinions have been forwarded. Whereas there is not much argument about the tight ionic binding of negatively charged phospholipids to the protein partners, the question appears

to remain whether charge–charge or hydrophobic interactions are the major forces bringing about the binding of certain amphiphatic phospholipids, e.g. lecithin to protein. In the last decade impressive advance has been made on the electron microscopy and X-ray analysis of membrane structures, and detailed considerations on surface-chemical and physical properties of the lipids from the living cell have been made in relation to the behavior of lipids in relatively simple model systems. The recurring pattern that has been demonstrated in many biological membranes, has been interpreted as a continuous bimolecular lipid leaflet, sandwiched between protein layers and this concept found support by observations on certain phospholipid–(protein)–water systems. However, both in natural lipid–protein structures and in artificial systems other sub-units and arrangements have been detected as well and various investigators favor the idea that biological structures containing a high proportion of phospholipid need not necessarily be considered solely as bi-molecular lipid leaflets. Lamellar and non-lamellar structures have been regarded to be formed by associations of globular lipid micelles. In addition the possibility has been envisaged that transitions may occur between different lipid phases, being at a dynamic equilibrium. The possibility that not all membranes are present in one unit-structure perhaps does better agree with their non-uniformity in lipid composition and it may be rewarding to try to correlate the preference for certain molecular associations with the chemical make-up of the lipids concerned.

The natural membranes are known not to be static structures but act as metabolically active entities. In several ways phospholipids contribute to this dynamic character. Various enzyme systems located in or at the membrane involved, e.g. in energy-transducing reactions or the active transport of cations across these barriers have been demonstrated to depend on the presence of phospholipids. The near future probably will uncover in detail the mechanism underlying this phospholipid requirement. Furthermore, many intriguing observations have been made on possible active functions of phospholipids in various membrane processes, but a strict role of these lipids themselves as carriers in the active or facilitated membrane transport still is highly speculative. On the other hand, phospholipids certainly have to be classified among the metabolically active constituents of membranes. Both catabolic reactions combined with a synthesis *de novo*, as well as conversions concerned with the polar end-group, the renewal of the respective fatty acid constituents and other cycles have been recognized in membrane structures. Significant differences appear to exist not only in the metabolic activities between various types of phospholipids, but also among the respective membranes. The possible specialized functions of certain highly dynamic phospholipid species, however, are very poorly understood.

The basic lipid structures present in biological systems are extremely flexible and from a chemical point of view allow for a wide variation in composition between different membranes. Distinctions in chemical make-up of the lipid

part of membranes include significant variations in proportions between various lipid classes, e.g. sterol–phospholipid ratio, a multiplicity of polar groups of phospholipids and an unbelieved assortment of apolar side-chains. When one tries to evaluate these structural variations in terms of biological functions, it becomes clear that the role played by the distinct lipid species is only partly understood. Comparison of physico-chemical properties of defined phospholipids with lipid data of membranes originating from different sources reveals an apparent tendency of these structures to maintain a lipid core having a liquid-expanded character or liquid-crystalline state. Depending on the biosynthetic abilities of the cells concerned, such properties appear to be mediated by the length, the degree of branching or unsaturation of the fatty acid and aldehydogenic residues of the phospholipids. In addition, variations in the phospholipid–cholesterol ratio may control the physico-chemical state and integrity of the flexible lipid arrangement in the biomembranes. However, the essentiality of the linoleate family for the mammalian membranes and their positionally specific location in the phospholipid molecule remain obscure. The biological implications of variations in the alcoholic moiety of the phospholipids, or the replacement of fatty acid ester linkages by saturated or unsaturated ether bonds are unknown. An enzymatic conversion of phosphatidyl serine into phosphatidyl choline or another intermediate of the cycle concerned apparently will be accompanied by an alteration in charge, but the consequences for the membrane function are hardly recognized. On the other hand, several phospholipid species are very similar to each other with respect to their charge distribution, e.g. lecithin and sphingomyelin, but it is not clear whether a replacement of each by the other causes certain disturbances in the membrane. One is tempted to speculate whether the metabolically active members of the phosphatidyl inositol and phosphatidyl glycerol families share some of their functions.

The composition of the lipid core in a given membrane to a certain extent is biogenetically determined, but also a number of environmental factors are known to influence the lipid composition of normal interfaces. Fresh approaches in this direction have been made and by the use of mutants and variations in environmental conditions striking variations can be induced in membranous lipids. This type of investigation combined with a search for alterations in membrane properties and structure, when projected upon the results of physico-chemical investigations on relevant model systems may be very rewarding for improving our insight into the relations between structure and functions of the numerous lipid species present in natural membranes. In the vast field of interdisciplinary research on the molecular architecture and functioning of biomembranes many basic biological, chemical and physical problems on lipids still challenge the investigator.

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