Pure & Appl.Chem., Vol.54, No.12, pp.2443-2454, 1982.
Printed in Great Britain.
0033-4545/82/122443-12$03.00/0
Pergamon Press Ltd.

ON MEMBRANE PHOSPHOLIPIDS AND PROTEIN-LIPID ASSOCIATION

L.L.M. van Deenen, J.A.F. Op den Kamp, B. Roelofsen
and K.W.A. Wirtz

Department of Biochemistry, University of Utrecht,
The Netherlands

Abstract - Membranes of blood cells have an asymmetric bilayer distribution of phospholipid classes. In both erythrocytes and platelet plasma membranes the choline containing phospholipids (sphingomyelin and phosphatidylcholine) are preferentially located in the outer half of the membrane, whereas a large fraction of phosphatidylethanolamine and nearly all phosphatidylserine are confined to the cytoplasmic surface. The rationale of this phospholipid topology appears to be related to the function of phospholipids in blood coagulation. An abnormal phospholipid distribution in sickle cells appears to be accompanied by a clot-promoting activity.

Native phosphatidylcholine molecules of erythrocytes can be replaced by defined synthetic species during incubation of intact cells with artificial phospholipid bilayers in the presence of a specific phosphatidylcholine transfer protein. All of the phosphatidylcholine present in the outer layer can be replaced by (1-palmitoyl-2-oleoyl)phosphatidylcholine without causing significant changes in membrane stability and permeability. However, an induced increase of disaturated species or introduction of dipoly-unsaturated phosphatidylcholine molecules modified drastically various membrane properties. This approach appears to give new information about the relation between chemical structure and membrane function of phospholipids.

Earlier studies indicated that a phosphatidylcholine transfer protein contains a specific phospholipid binding site. The primary structure of the protein was elucidated and based on the predicted secondary structural elements a tentative folding model of the protein was proposed. Experiments with photoactivable phospholipid analogs allowed to identify the amino acid residues of the lipid binding site and gave information about the localization of the phosphatidylcholine molecule within the transfer protein.

PHOSPHOLIPID ASYMMETRY OF BLOOD CELLS

The erythrocyte membrane is probably one of the most widely studied biological membranes and has served in many respects as a stimulating model (Ref. 1). The total lipid content of the erythrocyte constitutes about 40% of its dry weight, and in molecular ratios consists of about equal parts of cholesterol (40%) and polar lipids, e.g. phospholipids (50%) and glycolipids (10%). The major phospholipids are sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (Fig. 1). The sum of the two choline-containing phospholipids (sphingomyelin and phosphatidylcholine) is always 50-60% of the total phospholipid fraction, but their relative contents vary considerably in erythrocytes from different animal species (Ref. 2). The most extreme situation is found in erythrocytes from ruminants in which most, if not all, of the choline-phospholipids appears to be sphingomyelin.
During the past decade several laboratories have established the topological distribution of these phospholipid classes in the erythrocyte membrane. It is generally accepted that in this membrane the lipids are arranged in a bimolecular leaflet to which peripheral proteins are attached via polar interactions and which is interrupted to allow hydrophobic interactions with integral membrane proteins embedded in or spanning the bilayer. The distribution

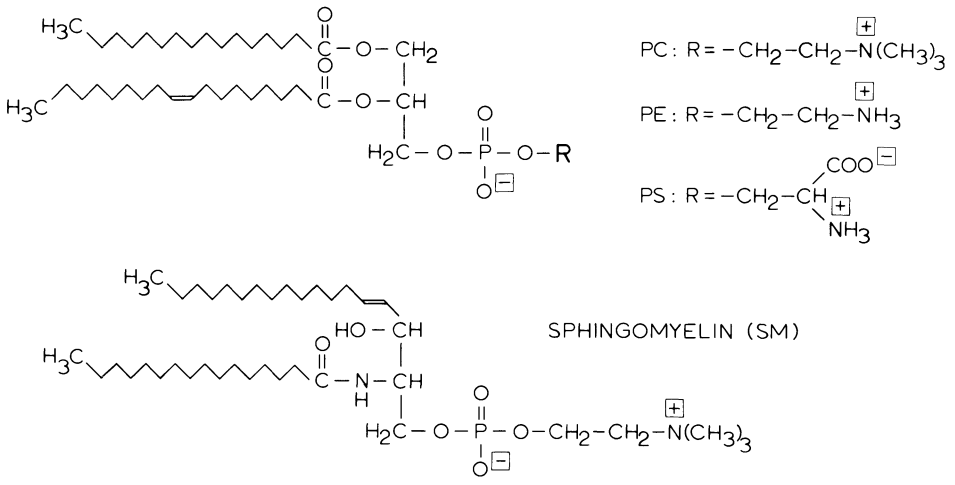


Fig. 1. Structure of four major phospholipids of the erythrocyte membrane. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

of phospholipids over the outer and inner layer of the membrane has been studied mainly by chemical labeling, enzymatic modification with phospholipases and through the use of exchange (or transfer) proteins (Fig. 2). In principle the approach is simple, namely a comparison of the action of these tools on intact cells and open membranes (ghosts), but many pitfalls can be made. In order to obtain unambiguous results the complete action of the impermeable probe should not disrupt the membrane structure and not cause phospholipid redistribution. Furthermore, the intrinsic transbilayer movement of phospholipids has to be a low order of magnitude. Because it is difficult to meet all prerequisites the information about phospholipid topology of many biomembranes is either incomplete or contradictory. However, the sidedness of erythrocyte membrane phospholipids has been detected in independent studies in several laboratories involving chemical labels, pure phospholipases and phospholipid exchange proteins, and the overall view is consistent (Ref. 3). Studies performed in this laboratory used the combined action of a phospholipase A_2, which enzyme catalyzes the hydrolysis of the fatty acid ester linkage at the *sn*-2-position of the phosphoglycerides, and a sphingomyelinase which cleaves its substrate into ceramide and phosphorylcholine. Treatment of open membranes with these enzymes gives a complete hydrolysis of all phospholipid present. When intact cells are reacted with the particular phospholipases no lysis of the cells occurs and only those phospholipids present in the exterior half of the bilayer are available for reaction. In the human erythrocyte membrane 75% of the phosphatidylcholine and 80% of the sphingomyelin is found in the outer monolayer whereas 80% of the phosphatidylethanolamine and all of the phosphatidylserine is located in the inner monolayer of the membrane (Fig. 3). In the erythrocyte membrane transbilayer movement of phospholipids (flip-flop) is a rather slow process and does not interfere with the enzymatic determination of phospholipid topology as can happen in other membranes (Ref. 4). Furthermore, it was demonstrated that the gross membrane organization is preserved after phospholipase action on erythrocytes. ^{31}P NMR studies on model systems of pure lipids have demonstrated the possibility to detect the occurrence of bilayer, hexagonal and micellar orientations of phospholipids and this technique could be applied successfully to biological membranes (Ref. 5). The erythrocyte membrane exhibits ^{31}P NMR spectra which are consistent with the vast majority of the phospholipid being in the bilayer configuration (Fig. 4). After treatment of intact cells with phospholipase A_2 and sphingomyelinase the spectra revealed that the residual phospholipids and monoacyl-phosphoglycerides remain organized in a bilayer arrangement (Ref. 6). Even after complete enzymatic degradation of the phospholipids in erythrocyte ghosts no signal was observed indicating isotropic ^{31}P motion despite the fact that the hydrolysis products (monoacyl-phosphoglycerides and fatty acids) individually associate in solution in micelles. In this context it is important to note that in combination equimolar amounts

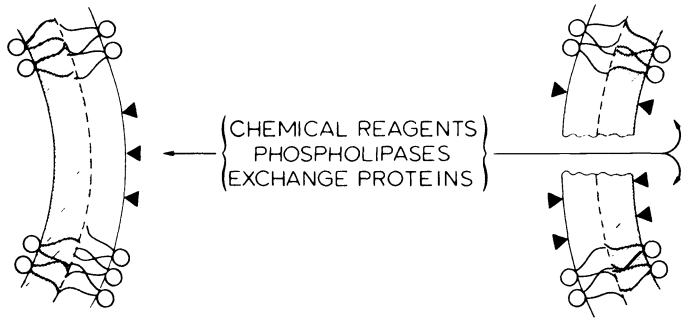


Fig. 2. Determination of the topology of phospholipids in biomembranes. Experiments are performed on intact erythrocytes and on open ghosts.

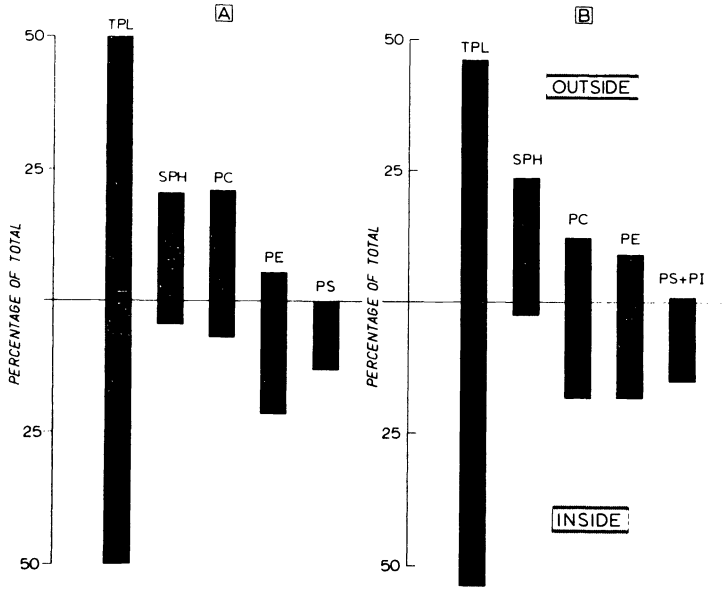


Fig. 3. Asymmetric orientation of phospholipids in plasma membranes of human erythrocytes (A) and platelets (B). Abbreviations: TPL, total phospholipid; SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

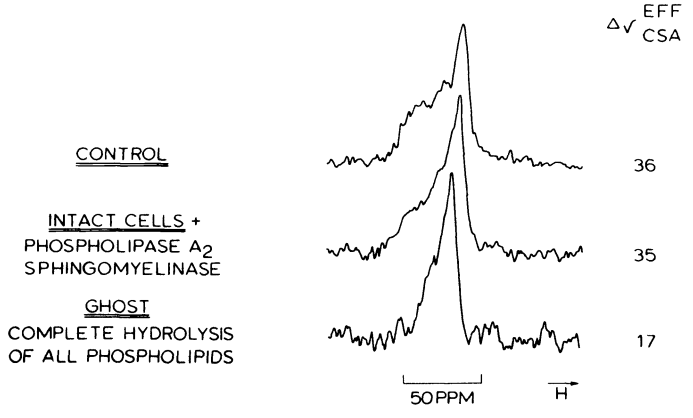


Fig. 4. ^{31}P NMR spectra of phospholipase-treated erythrocytes and ghosts (Ref. 6).

of monoacyl-phosphoglycerides (lysolecithin) and free fatty acids tend to assemble in a bilayer-type of organization (Ref. 7). In addition cholesterol which is abundant in erythrocytes can direct lysolecithin in a bilayer orientation (Ref. 8) and it appears likely that the protein network of the membrane contributes to the maintenance of a lipid-bilayer structure after phospholipid hydrolysis. However, the action of sphingomyelinase appears to produce small spheres (75 Å and 200 Å diameter) consisting of ceramides without causing a gross disorganization of the bilayer membrane (Ref. 9). Although cells treated with these phospholipases revealed morphological changes and an increased osmotic fragility relative to control cells the framework of the erythrocyte membrane is sufficiently stable to allow a detection of phospholipid topology by means of phospholipase hydrolysis.

With regard to the biological significance of the phospholipid asymmetry it is of interest to note that in the plasma membrane of platelets a pattern was detected which is comparable to the distribution found in red cells (Fig. 3). The choline-containing phospholipids, particularly sphingomyelin, are located in the outer layer whereas a large fraction of phosphatidylethanolamine and practically all negatively charged phospholipids - phosphatidylserine and phosphatidylinositol - are confined to the cytoplasmic surface of the membrane (Ref. 10). It appears likely that the asymmetric arrangement of phospholipids in blood cells is related to blood coagulation. The participation of phospholipids in blood coagulation has been appreciated for many years and in the past two decades their involvement in the coagulation cascade has been defined rather precisely (for a review compare Ref. 11). The main function of phospholipids in blood coagulation is to provide an interface on which various protein factors interact, thus increasing their local interaction. For example, the conversion of prothrombin into thrombin which requires two other proteins (factor Xa and V) and Ca^{++} is strongly catalyzed by a phospholipid interface (Fig. 5). The clot-promoting activity of the phospholipid layer is dependent on a particular negative surface charge, which can be provided by certain concentrations of negatively charged phospholipids such as phosphatidylserine. Taking into consideration the localization of phosphatidylserine at the cytoplasmic side of the erythrocyte membrane (Fig. 3), it is not surprising that intact erythrocytes lack proagulant activity (Ref. 12). Therefore it is interesting to note that as early as 1886 Wooldridge (13) reported that membrane fragments of erythrocytes produced intravascular clotting, an observation confirmed later by other investigators (Ref. 12). Zwaal *et al.* (14) showed that

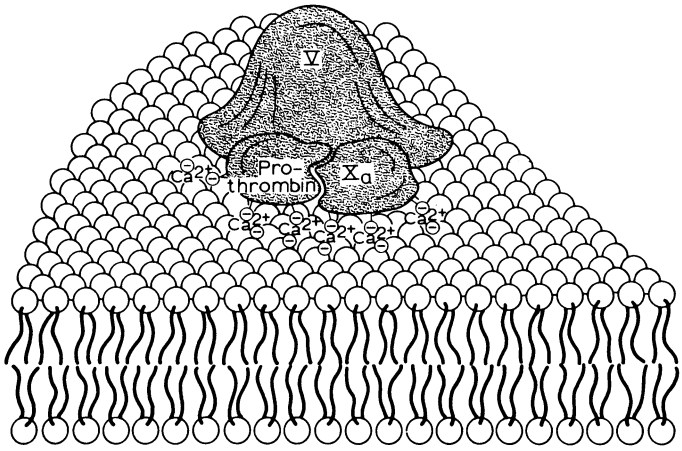
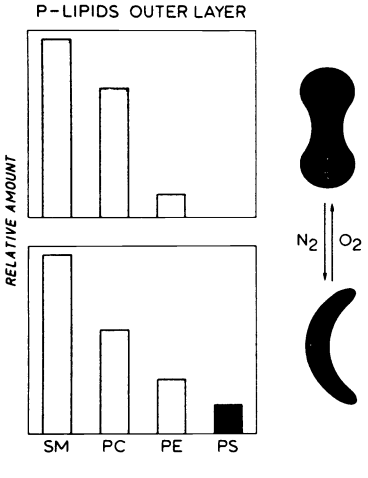


Fig. 5. Schematic representation of the prothrombin-prothrombinase complex (Ref. 11).

the clotting time of plasma is not reduced when the outside of the erythrocyte membrane is exposed, but exposure of the cytoplasmic surface catalyzes blood coagulation. Lipid vesicles prepared from a phospholipid mixture of which the composition is similar to that of the inner layer of the erythrocyte or platelet membrane appeared to be clot-promoting, whereas vesicles simulating the lipid composition of the outer surface of these cells were remarkably inert in coagulation systems. Apparently, the phospholipid asymmetry in blood cell membranes is constructed so as to avoid thrombosis. However, platelets can be activated by a number of stimulants to become more active in coagulation. Recently, Bevers *et al.* (15) demonstrated that a generation of prothrombin-converting activity at the outer surface of platelets by the combined action of collagen and thrombin is accompanied by an exposure of a significant fraction of phosphatidylserine at the outside of the membrane. This increase in the concentration of the negatively charged phospholipid in the outer membrane



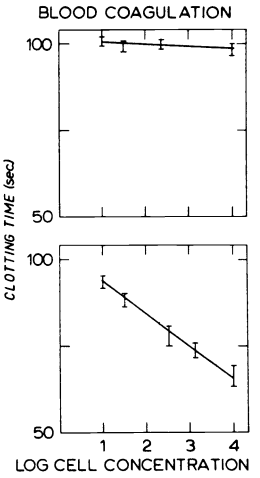


Fig. 6

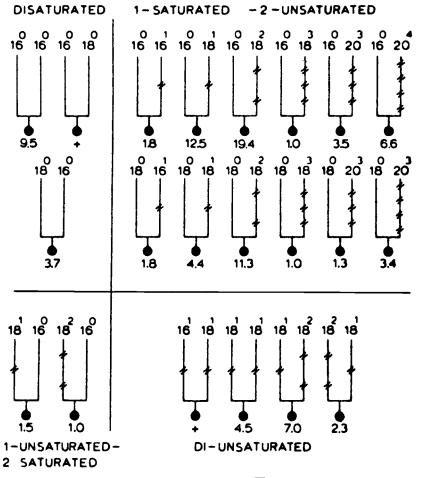


Fig. 7

Fig. 6. Comparison between normal and sickled erythrocytes of their phospholipid composition in the outer half of the lipid bilayer and their activity in a prothrombinase assay (Ref. 17). Abbreviations as in Figs. 1 and 3.

Fig. 7. Major molecular species of phosphatidylcholine from human erythrocyte membranes. The fatty acid constituents are indicated by the number of carbon atoms and unsaturated bonds (Ref. 1).

layer probably involves a redistribution of phospholipid possibly caused by a transbilayer movement of phospholipid induced during the activation process. In this respect it is of interest that transbilayer movement of phospholipid has been triggered in lipid-bilayer vesicles in different ways, some including the involvement of proteins (Ref. 3).

Recently, observations were made on an abnormal phospholipid asymmetry in sickle cell disease which may be related to the painful crises in these patients caused by blockage of blood flow. Using phospholipases to determine the phospholipid topology Lubin *et al.* (16) found that oxygenated reversibly sickled cells have a phospholipid asymmetry identical to that in normal erythrocytes with all of the phosphatidylserine located on the cytoplasmic layer (Fig. 6). In contrast the distribution of phosphoglycerides within the membrane of irreversibly sickled cells and deoxygenated reversibly sickled cells appears to be quite different from that in non-sickled cells. Compared with the biconcave shaped erythrocyte the outer membrane leaflet of the sickle cells is enriched in phosphatidylethanolamine and contains substantial quantities of phosphatidylserine which is normally confined to the inner lipid membrane layer. The distribution of sphingomyelin over the two halves of the bilayer appears not to be affected significantly by sickling. In irreversibly sickled cells the phospholipid asymmetry was abnormal under both oxy- and deoxy conditions but reoxygenation of the reversible sickled cells restored the transbilayer distribution of phospholipid to the normal pattern (Fig. 6). Because phosphatidylserine plays an important role in the catalytic surface for the binding of blood coagulation factors Chiu *et al.* (17) studied the effect of sickling on *in vitro* coagulation. Sickled cells appeared to shorten the clotting time, whereas no effect on coagulation is observed when reversibly sickled cells attain the normal shape after reoxygenation (Fig. 6). Inasmuch as liposomes with identical composition to the outer leaflet of either biconcave or sickled cells produced similar effects it was concluded that reduction of the clotting time is caused by the appearance of the negatively charged phosphatidylserine at the cell surface. Therefore, it has been argued that not only the shape of sickle cells may block blood flow in capillaries, but that also the abnormal phospholipid asymmetry may have a thrombotic effect and thus contribute to the pathogenesis of the vaso-occlusive episode in sickle cell anaemia.

REPLACEMENT OF PHOSPHATIDYLCHOLINE SPECIES IN THE ERYTHROCYTE MEMBRANE

Each of the individual phospholipid classes of the erythrocyte membrane represents in chemical terms a group of components containing different fatty acid constituents which vary in chain length and number of unsaturated bonds. A phospholipid such as phosphatidylcholine contains a number of fatty acid constituents which are not randomly combined (Fig. 7). Most abundant are the

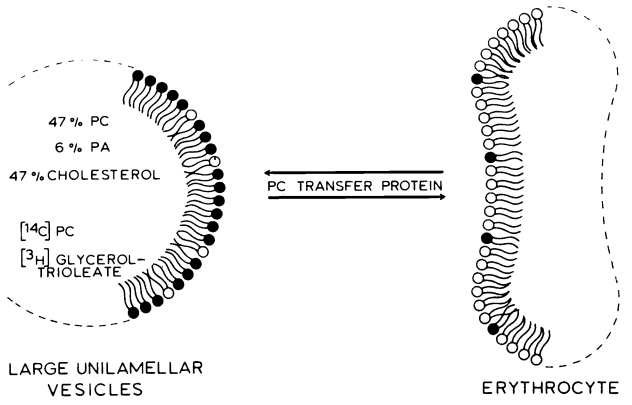


Fig. 8. Replacement of phosphatidylcholine in the outer membrane layer of erythrocytes.

species of the mixed-acid type containing a saturated and an unsaturated chain at the *sn*-1- and 2-positions, respectively. Among the disaturated species the components containing at least one mono-unsaturated chain are present, but species containing two poly-unsaturated fatty acyl residues are not detectable. Despite the highly asymmetric localization of phosphatidylcholine in this membrane the molecular species appear to be randomly distributed over both parts of the lipid bilayer (Ref. 18). Many of these species have been obtained in a pure form by chemical synthesis and were investigated with several physical techniques (Ref. 1). In order to validate extrapolations from experiments on model systems to biological membranes it is desirable to establish the relationship between chemical make-up of lipids and properties of biomembranes by introducing structural variations in one single phospholipid species in a systematic way. In this respect significant progress was made by alteration of the fatty acid composition in microorganisms, but in intact mammalian membranes such studies have been less feasible. Although it is possible to alter by dietary means the fatty acid composition of membrane phospholipids, including those of the erythrocyte (Ref. 2), homeostatic mechanisms operating *in vivo* will limit and balance the chemical changes so as to keep the overall physical properties of the biological interfaces between certain limits. Phospholipid exchange- or transfer proteins now offer good opportunities to modify in a well-controlled manner the membrane lipid composition. One of these proteins has been shown to exchange phosphatidylcholine molecules between various donor-acceptor pairs, and some properties of this particular protein are discussed in a subsequent section. Recently van Meer *et al.* (19, 20) demonstrated an exchange of phosphatidylcholine between intact erythrocytes and various donor systems such as microsomes, liposomes and unilamellar vesicles. The transfer protein introduces phosphatidylcholine molecules from the donor system into the outer lipid layer of the erythrocyte membrane (Fig. 8). In experiments of Lange *et al.* (21) phosphatidylcholine from intact rat erythrocytes was replaced by a number of synthetic species and under the chosen conditions neither the total lipid content nor the phospholipid-cholesterol ratio or the ratio of different phospholipid classes was found to be altered. In this respect it is important that the donor system has a phosphatidylcholine-cholesterol ratio which is equal to that of the erythrocyte membrane. Under the chosen conditions the only variation induced in the erythrocyte membrane concerned the increase of the relative content of the one particular phosphatidylcholine species provided by the donor system. The increase in the content of this species appears to be compensated for by a loss at random of other molecular species with different fatty acid combinations. It was observed that with progression of phosphatidylcholine exchange considerable morphological changes could occur, leading finally to cell lysis. Furthermore, it appeared that the onset of hemolysis occurred at different degrees of exchange characteristic for the type of phosphatidylcholine species employed. Using human erythrocytes current experiments of Kuypers confirmed and extended the earlier observations.

a. Incorporation of disaturated phosphatidylcholine. Incubation of human erythrocytes at 37°C with phosphatidylcholine-exchange protein and vesicles containing cholesterol and dipalmitoylglycerophosphocholine (Fig. 8) resulted in a progressive incorporation of this saturated phosphatidylcholine. This species

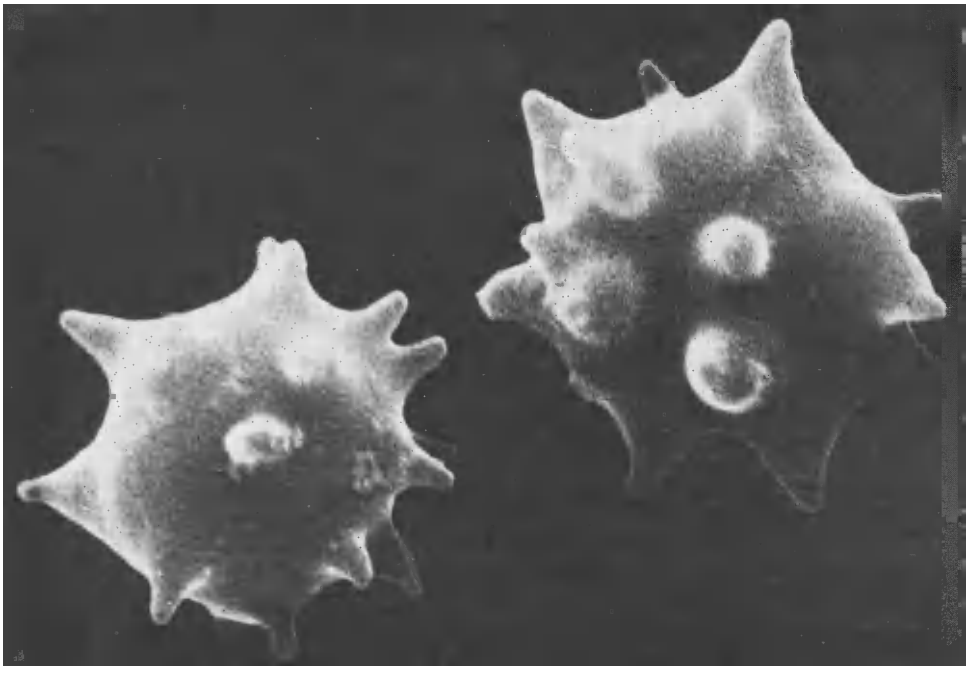


Fig. 9. Scanning electron micrograph of erythrocytes in which 40% of the native phosphatidylcholine has been replaced by diplamitoylglycerophosphocholine.

represents in the normal human erythrocyte about 10% of the total phosphatidylcholine fraction (Fig. 7). When this percentage has increased to about 25% the cells displayed an increased osmotic fragility, but under isotonic conditions cell lysis did not start before some 40% of the native phosphatidylcholine was replaced by the disaturated species. Despite cell lysis an exchange between vesicles and the remaining part of intact erythrocytes continued and it appeared possible to obtain a red cell population in which some 70% of the native phosphatidylcholine was replaced by dipalmitoylglycerophosphocholine. Taking into account the slow transbilayer movement of phospholipid in the intact human erythrocyte, most of the phosphatidylcholine of the outer half of the bilayer now consisted of the disaturated species. Although no significant leakage of potassium ions was detected, the modified cells revealed a strongly increased osmotic sensitivity and gross echinocyte formation was visible (Fig. 9). Because a red cell population is heterogenous with respect to cell age (1-120 days) it will be of interest to investigate whether the progressive lysis induced by an increased incorporation of the saturated phospholipid is in some ways related to cell age.

b. Incorporation of 1-saturated-2-unsaturated phosphatidylcholine. Similar experiments were performed with a synthetic 1-palmitoyl-2-oleoylglycerophosphocholine, a component which normally amounts to some 12% of the total phosphatidylcholine of the human red cell membrane. This species is readily incorporated into the erythrocyte and it was possible to exchange some 75% of the total phosphatidylcholine of the membrane, indicating that the outer layer phosphatidylcholine has been completely replaced by this species. In contrast to the results observed with the disaturated phosphatidylcholine the incorporation of this analog-containing one saturated and one unsaturated fatty acid constituent-did not cause any appreciable cell lysis and the osmotic fragility test appeared to give a practically normal pattern. Apparently, the chemical structure of this phospholipid molecule does not interfere with the maintenance of the gross membrane integrity of the human erythrocyte.

c. Incorporation of di-unsaturated phosphatidylcholine. Experiments with dioleoylglycerophosphocholine, a species which is present in relatively small quantities in the human erythrocyte membrane (Fig. 7), gave results which were identical to those obtained with the palmitoyl-oleoyl containing analog. However, incorporation of dilinoleoylglycerophosphocholine gave quite drastic alterations of the properties of red cell membrane. Poly-unsaturated fatty acid constituents of native phosphatidylcholine are normally paired with a saturated fatty acyl chain (Fig. 7), because phosphatidylcholine species containing two poly-unsaturated acyl chains are not synthesized by mammalian tissues. The dipoly-unsaturated species, obtained by chemical synthesis, was readily incorporated into the red cell membrane, but already after introduc-

tion of some 5% of this component the red cells displayed both an increase in osmotic fragility and a leakage of potassium ions. Replacement of more phosphatidylcholine induced a further increase in potassium leak, and osmotic fragility; after introduction of some 40% of the dipoly-unsaturated species a complete lysis of the entire cell population resulted. These results demonstrate that the properties of this unnatural species are disruptive for the barrier function of the biomembrane. These observations vindicate previous conclusions made from studies on artificial bilayers; liposomes of dilinoleoylglycerophosphocholine appeared to give very leaky barriers compared to bilayers of phosphatidylcholines of the mixed-acid type (Ref. 1). The approach outlined above can be extended in various directions so as to include measurements of enzymatic activities and various physical parameters in relation to lipid structure. Very recently the phosphatidylcholine transfer protein was used to introduce phosphatidylcholine with perdeuterated acyl chains into human erythrocyte membranes and the NMR spectra indicate that the normal membrane is fluid down to -5°C (Ref. 22).

LIPID BINDING SITE OF A TRANSFER PROTEIN

The cytosol of eukaryotic cells contains a number of proteins which are capable to catalyze the transport of phospholipids between two distinct membranes. The current knowledge on these phospholipid exchange or phospholipid transfer proteins has been reviewed in detail (Ref. 23,24). The present discussion will focus upon one protein isolated from beef liver which specifically stimulates transfer of phosphatidylcholine between both natural and model membranes. First some major properties are summarized:

a. Experiments with phospholipid monolayers demonstrated that this protein acts as a true carrier and forms a one-to-one protein-phospholipid complex (Ref. 25).
b. The protein catalyzes a one-for-one molecular exchange process, but under certain conditions a net transfer of phosphatidylcholine was demonstrated (Ref. 26,27).
c. The protein is highly specific and no transfer of e.g. phosphatidylethanolamino or sphingomyelin was observed. Experiments with synthetic phospholipid analogs demonstrated that transport is inhibited when (i) the distance between the phosphoryl and nitrogenous moieties is altered, and (ii) a methyl group on the quarternary nitrogen is removed or substituted by an ethyl or propyl group (Ref. 28).

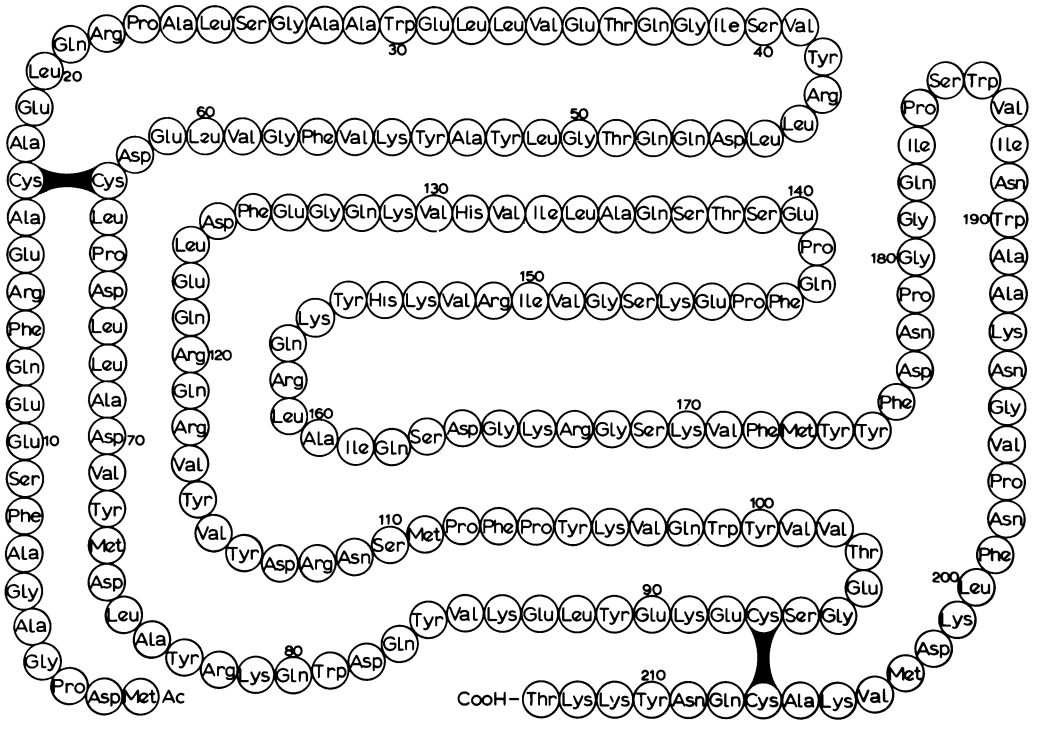


Fig. 10. The primary structure of the phosphatidylcholine transfer protein from bovine liver.

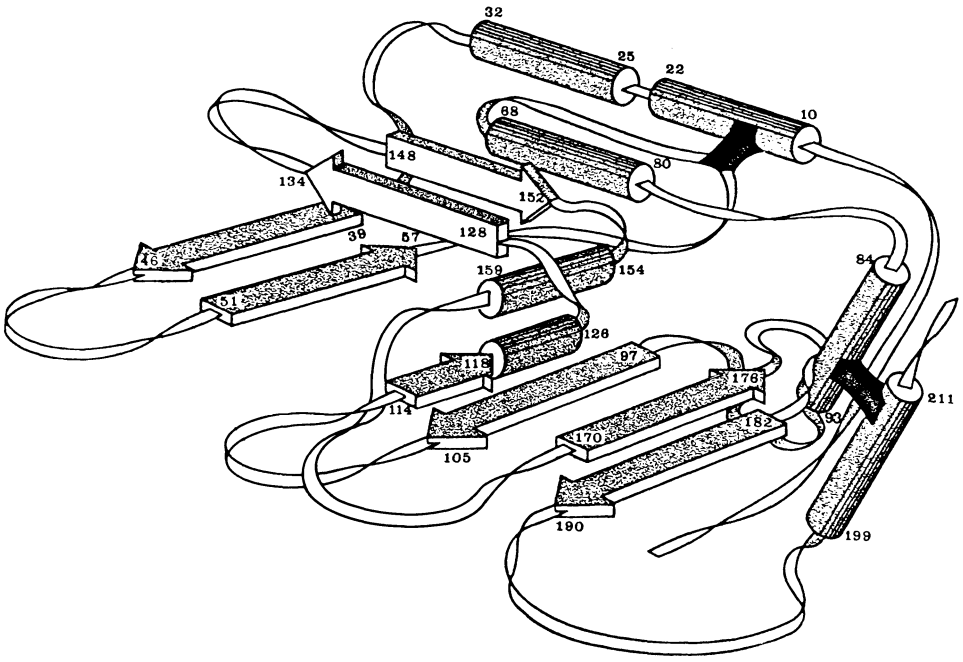


Fig. 11. A tentative model of the phosphatidylcholine transfer protein based on recent concepts of prediction and folding of secondary structure elements. Cylinders indicate α helix and arrows represent β strand.

d. The phosphatidylcholine molecule bound to the protein appears to be well shielded from the environment. Phospholipases acting on the ester bonds binding fatty acyl residues and the polar group to the glycerol moiety produced negligible hydrolysis only (Ref. 29). ESR spectroscopy demonstrated that a complex of protein with a 2-acyl spin-labeled phosphatidylcholine reveals a spectrum typical of a strongly immobilized probe (Ref. 30,31). Addition of ascorbate did not affect the spectrum of the complex.

These observations indicate that the transfer protein contains a specific binding site for phosphatidylcholine which involves both electrostatic and hydrophobic association. In order to obtain more information on the lipid binding site and the mode of action of the protein the amino acid sequence was determined, and the locus of interaction between protein and photosensitive precursors of phosphatidylcholine was investigated.
Moonen *et al.* (32) and Åkeroyd *et al.* (33) elucidated the primary structure of the phosphatidylcholine transfer protein from bovine liver. This protein consists of a single polypeptide chain of 213 amino acid residues, contains two disulfide bonds at Cys^{17}-Cys^{63} and Cys^{93}-Cys^{207} and has an acetyl methonine residue as a blocked N-terminus (Fig. 10). Examination of the structure reveals that a number of hydrophobic peptide regions are present, of which the most prominent are Val^{98}-Val-Tyr-Trp-Gln-Val^{103}, Val^{171}-Phe-Met-Tyr-Tyr-Phe^{176} and Trp^{180}-Val-Ile-Asn-Trp-Ala-Ala^{192}. Particular peptide segments may be involved in the interaction of the protein with the membrane and the incorporation of a phosphatidylcholine molecule. The binding of the lipid molecule implies that a transient protein-membrane complex is formed in which it is energetically favourable for phosphatidylcholine to leave the hydrophobic environment of the bilayer. The transfer protein may have a tertiary structure with some unique features but to date X-ray crystallography was not feasible. A speculative model of the folding of the protein was developed which is based on the prediction of the secondary structural elements and the hydrophobicity profile. The α helix, β strand and β turn structures in the phosphatidylcholine transfer protein were derived by the method of Chou and Fasmann (34) and the method of Lim (35); both approaches yielded results that agreed rather well. The hydrophobicity profile was derived from the primary structure by the methods of Rose (36). Details of these predictions have been recently presented (Ref. 37). Furthermore, the method of Manavalan and Ponnuswamy (38) was applied and gave confirmative and additional information about the distribution of hydrophobic maxima and minima along the peptide chain. Using this information a working model was proposed which is illustrated in Fig. 11. Following several general concepts about folding of proteins, the tentative model of the transfer protein incorporates a close packing by the

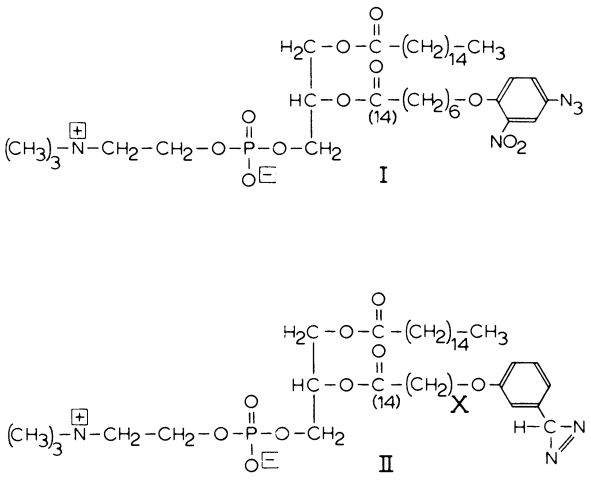


Fig. 12

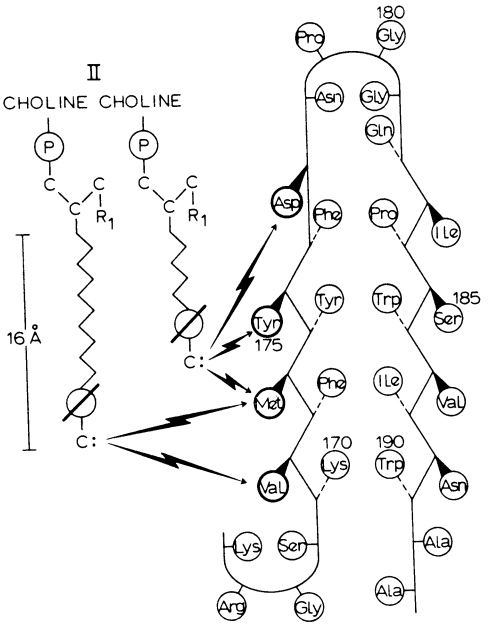


Fig. 13

Fig. 12. Structure of 1-palmitoyl-2-[7-(4-azido-2-nitrophenoxy) [1-^{14}C]heptanoyl]-*sn*-glycero-3-phosphocholine (I) and 1-palmitoyl-2-[w-(m-diazirino-phenoxy)[1-^{14}C]acyl]-*sn*-glycero-3-phosphocholine (II).

Fig. 13. Sites of labeling of phospholipase transfer protein after reaction with the *sn*-2-[1-^{14}C]hexanoyl and *sn*-2-[1-^{14}C] undecanoyl analog of II (Fig. 12).

occurrence of antiparallel β sheets and the occurrence of helix-helix interactions. Hydrophobic structures are primarily located in the core of the model but a number of segments are tentatively placed at the surface of the protein because of their amphiphilic nature. Inasmuch as reduction of the disulfide bridges abolishes transfer activity it is important to note that the bond at Cys^{17}-Cys^{63} restricts the arrangement of the secondary structure at the N-terminus; the bond at Cys^{93}-Cys^{297} may be important for the alignment of the hydrophobic and amphiphilic strand at position 97-105 close to the β sheets residues 170-190.

In order to localize the lipid binding site of the transfer protein, two types of photosensitive phospatidylcholine derivatives have been used (Fig. 12). Moonen *et al.* (39) synthesized 1-palmitoyl-2-{7-(4-azido-2-nitrophenoxy)-[1-^{14}C]-heptanoyl}-*sn*-glycero-3-phosphocholine. In this component the bulky azidonitrophenoxy group is attached to the w-terminal position of a short-chain fatty acyl constituent in order to obtain a configuration that resembles phosphatidylcholine with a normal acyl chain. During incubation of protein with vesicles of the [^{14}C]phosphatidylcholine analog the protein exchanges its endogenous phosphatidylcholine for the photosensitive phospholipid. Photolysis of the complex generated the reactive nitrene and about 30% of the incorporated [^{14}C]phosphatidylcholine was covalently linked to the protein. In order to detect which site on the protein has interacted the photolyzed complex-after reduction, carboxymethylation and citraconylation-was subjected to proteolytic hydrolysis. Prior to chromatographic separation of the peptides a mild alkaline hydrolysis of the cross-linked 1-acyl-2{7(4-N-2-nitrophenoxy)[1-^{14}C]heptanoyl}-*sn*-glycero-3-phosphocholine moiety decreases the hydrophobic character of the ^{14}C-labeled peptides, leaving the N-nitrophenoxy-[1-^{14}C]heptanoic acid covalently linked. The radioactive 2-acyl chain of the phosphatidylcholine was recovered in a peptide segment Gly-Ser-Lys-Val-Phe-Met-Tyr-Tyr (Ref. 39). Similar experiments were performed with the carbene precursor 1-palmitoyl-2(w(m-diazirinophenoxy)-[1-^{14}C]-undecanoyl)-*sn*-glycero-3-phosphocholine, a compound synthesized by the group of Khorana (Ref. 40). It appeared that photolysis of the protein-phospholipid complex caused a reaction with the same peptide segment of the transfer protein (Ref. 41). These preliminary studies were carried out at a time that the determination of the primary structure of the protein had not been fully

completed. It was obvious, however, that a region of attachment of the photosensitive nitrene and carbene precursor of phosphatidylcholine was located at or near a hydrophobic cluster of amino acids Val-Phe-Met-Tyr-Tyr-Phe, now known to occupy position 171-176 (Fig. 10).
Recently, a more detailed study on the lipid binding site was made in collaboration with H.G. Khorana and R. Radhakrishnan using two phosphatidylcholine analogs which carry a diazirinophenoxy group linked to the w-carbon of either the *sn*-2-[1-^{14}C]hexanoyl or *sn*-2-[1-^{14}C]undecanoyl chain (Fig. 12). About 30-40% of the incorporated carbene-generating phospholipids appeared to be covalently bound to the protein after photolysis. After chemical and enzymatic degradation of the complex the ^{14}C-labeled peptides were sequenced by automated Edman degradation (Ref. 42). Major sites of coupling shown by release of radioactivity were identified as Tyr^{54} and the peptide segment Val^{171}-Phe-Met-Tyr-Tyr-Phe-Asp^{177} (Fig. 13). This peptide segment has a high content of aromatic amino acid residues and presents the most apolar region of the transfer protein (average hydrophobicity of 2440 calories per residue). The analysis of the secondary structure predicted a β strand structure for the segment Lys^{170}-Phe^{176} with β turns on either side (Ref. 37). The heptapeptide Lys^{170}-Phe^{176} has an estimated length of 14 Å which could accommodate the 2-fatty acyl chain of the endogenous phosphatidylcholine molecule located within the transfer protein. Interesting differences were observed in the pattern of coupling of the segment Val^{171}-Asp^{177} depending on the chain length of the photoreactive acyl constituent of the phospholipid prbe. It turned out that coupling occurred preferentially to Tyr^{175} and Asp^{177} with the *sn*-2-[1-^{14}C]hexanoyl-containing analog, while Val^{171} and Met^{173} were labeled preferentially with the derivative-containing the *sn*-2- 1-^{14}C undecanoyl chain (Fig. 13). This shift in coupling is compatible with an increase in the length of the *sn*-2-fatty acyl chain with 5 carbon atoms (i.e. 6 Å), assuming that the segment Val^{171}-Asp^{177} has adopted the β strand configuration. These results suggest that the phosphatidylcholine molecule has a particular orientation at the lipid binding site with a preference of the 2-*sn*-fatty acyl chain for one phase of the β strand. Future experiments with phosphatidylcholine probes carrying the photosensitive group at the *sn*-1-fatty acyl chain are needed to identify possible other segments involved in the lipid binding.
In addition to the sites of coupling within the Val^{171}-Asp^{177} segment both photoactivable carbene precursors caused extensive coupling to Tyr^{54}. In spite of the variation in acyl chain length of the phospholipid analogs only Tyr^{54} and none of its neighbouring amino acid residues was labeled. On the other hand the analog with a *sn*-2-hexanoyl chain was coupled to Tyr^{54} more extensively than the derivative with a *sn*-2-undecanoyl chain, viz. 90% and 50% of the total label, respectively. This leaves the question whether Tyr^{54} is indeed part of the actual binding site. In this respect it is of interest to note that Akeroyd (43) observed that a lactoperoxidase-catalyzed iodination of the transfer protein does not iodinate Tyr^{174} and Tyr^{175} in the binding site but gives a reaction with Tyr^{54}. This observation endorsed again the view that part of the binding site (Val^{171}-Asp^{177}) is well-shielded from the environment and that Tyr^{54} is probably more oriented at the surface of the protein.
Although further investigations are needed to elucidate more details of the lipid binding site of the transfer protein, the available results illustrate that photosensitive derivatives of phospholipids are useful tools to study protein-lipid interaction at the molecular level.

REFERENCES

1. L.L.M. van Deenen, Pure and Applied Chem. 25, 25-56 (1971).
2. L.L.M. van Deenen and J. de Gier, in: The Red Blood Cell (D.M. Surgenor, ed.) Vol. 1, 148-205, Academic Press, New York (1974).
3. L.L.M. van Deenen, FEBS Letters 123, 1-16 (1981).
4. J.A.F. Op den Kamp, Annu. Rev. Biochem. 48, 47-71 (1979).
5. P.R. Cullis and B. de Kruijff, Biochim. Biophys. Acta 559, 399-420 (1979).
6. G. van Meer, B. de Kruijff, J.A.F. Op den Kamp and L.L.M. van Deenen, Biochim. Biophys. Acta 590, 1-9 (1980).
7. M.K.Jain, C.J.A. van Echteld, F. Ramirez, J. de Gier, G.H. de Haas and L.L.M. van Deenen, Nature 284, 486-487 (1980).
8. C.J.A. van Echteld, B. de Kruijff, J.G. Mandersloot and J. de Gier, Biochim. Biophys. Acta 649, 211-220 (1981).
9. A.J. Verkleij, R.F.A. Zwaal, B. Roelofsen, P. Comfurius, D. Kastelijn and L.L.M. van Deenen, Biochim. Biophys. Acta 323, 178-193 (1973).
10. H.J. Chap, R.F.A. Zwaal and L.L.M. van Deenen, Biochim. Biophys. Acta 467, 146-164 (1977).

11. R.F.A. Zwaal, Biochim. Biophys. Acta 515, 163-205 (1978).
12. D.M. Surgenor, in: The Red Blood Cell (D.M. Surgenor, ed.) Vol. 1, 362-386, Academic Press, New York (1974).
13. L. Wooldridge, Practitioner 36, 187-198 (1886).
14. R.F.A. Zwaal, P. Comfurius and L.L.M. van Deenen, Nature 268, 358-360 (1977).
15. E.M. Bevers, P. Comfurius, J.L.M.L. van Rijn, H.C. Hemker and R.F.A. Zwaal, Eur. J. Biochem. 122, 429-436 (1982).
16. B. Lubin, D. Chiu, J. Bastacky, B. Roelofsen and L.L.M. van Deenen, J. Clin. Invest. 67, 1643-1649 (1981).
17. D. Chiu, B. Lubin, B. Roelofsen and L.L.M. van Deenen, Blood 58, 398-401 (1981).
18. W. Renooy, L.M.G. van Golde, R.F.A. Zwaal, B. Roelofsen and L.L.M. van Deenen, Biochim. Biophys. Acta 363, 287-292 (1974).
19. G. van Meer, B.J.H.M. Poorthuis, K.W.A. Wirtz, J.A.F. Op den Kamp and L.L.M. van Deenen, Eur. J. Biochem. 103, 283-288 (1980).
20. G. van Meer, L.G. Lange, J.A.F. Op den Kamp and L.L.M. van Deenen, Biochim. Biophys. Acta 598, 173-177 (1980).
21. L.G. Lange, G. van Meer, J.A.F. Op den Kamp and L.L.M. van Deenen, Eur. J. Biochem. 110, 115-121 (1980).
22. B. Maraviglia, J.H. Davis, M. Bloom, J. Westerman and K.W.A. Wirtz, Biochim. Biophys. Acta 686, 137-140 (1982).
23. J.C. Kader, in: Dynamic Aspects of Cell Surface Organization (G. Poste and G.L. Nicolson, eds.) Vol. 3, 127-204, Elsevier/North-Holland, Amsterdam (1977).
24. K.W.A. Wirtz, in: Lipid-Protein Interactions (P.C. Jost and O.H. Griffith, eds.) Vol. 1, 151-233, Wiley-Interscience, New York (1982).
25. R.A. Demel, K.W.A. Wirtz, H.H. Kamp, W.S.M. Geurts van Kessel and L.L.M. van Deenen, Nature 246, 102-105 (1973).
26. K.W.A. Wirtz, P.F. Devaux and A. Bienvenue, Biochemistry 19, 3395-3399 (1980).
27. D.B. Wilson, J.L. Ellsworth and R.L. Jackson, Biochim. Biophys. Acta 620, 550-561 (1980).
28. H.H. Kamp, K.W.A. Wirtz, P.R. Baer, A.J. Slotboom, A.F. Rosenthal, E. Paltauf and L.L.M. van Deenen, Biochemistry 16, 1310-1316 (1977).
29. H.H. Kamp, E.D. Sprengers, J. Westerman, K.W.A. Wirtz and L.L.M. van Deenen, Biochim. Biophys. Acta 398, 415-423 (1975).
30. P.F. Devaux, P. Moonen, A. Bienvenue and K.W.A. Wirtz, Proc. Natl. Acad. Sci. USA 74, 1807-1810 (1977).
31. K. Machida and S.I. Ohnishi, Biochim. Biophys. Acta 507, 156-164 (1978).
32. P. Moonen, R. Akeroyd, J. Westerman, W.C. Puyk, P. Smits and K.W.A. Wirtz, Eur. J. Biochem. 106, 279-290 (1980).
33. R. Akeroyd, P. Moonen, J. Westerman, W.C. Puyk and K.W.A. Wirtz, Eur. J. Biochem. 114, 385-391 (1981).
34. P.Y. Chou and G.D. Fasmann, Adv. Enzymol. 47, 45-148 (1978).
35. V.I. Lim, J. Mol. Biol. 88, 857-894 (1974).
36. G.D. Rose, Nature 272, 586-590 (1978).
37. R. Akeroyd, J.A. Lenstra, J. Westerman, G. Vriend, K.W.A. Wirtz and L.L.M. van Deenen, Eur. J. Biochem. 121, 391-394 (1982).
38. P. Manavalan and P.K. Ponnuswamy, Nature 275, 673-674 (1978).
39. P. Moonen, H.P. Haagsman, L.L.M. van Deenen and K.W.A. Wirtz, Eur. J. Biochem. 99, 439-445 (1979).
40. C.M. Gupta, R. Radhakrishnan and H.G. Khorana, Proc. Natl. Acad. Sci. USA 74, 4315-4319 (1979).
41. K.W.A. Wirtz, P. Moonen, L.L.M. van Deenen, R. Radhakrishnan and H.G. Khorana, Ann. N.Y. Acad. Sci. 348, 244-255 (1980).
42. J. Westerman, K.W.A. Wirtz, T. Berkhout, L.L.M. van Deenen and H.G. Khorana, paper submitted (1982).
43. R. Akeroyd, Thesis University of Utrecht (1981).