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SEVENTH COLLEGE ON BIOPHYSICS:

Structure and Function of Biopolymers: Experimental and Theoretical Techniques.

4 - 29 March 1996

Membrane Asymmetry - General Aspects & Lipid - Protein Interactions

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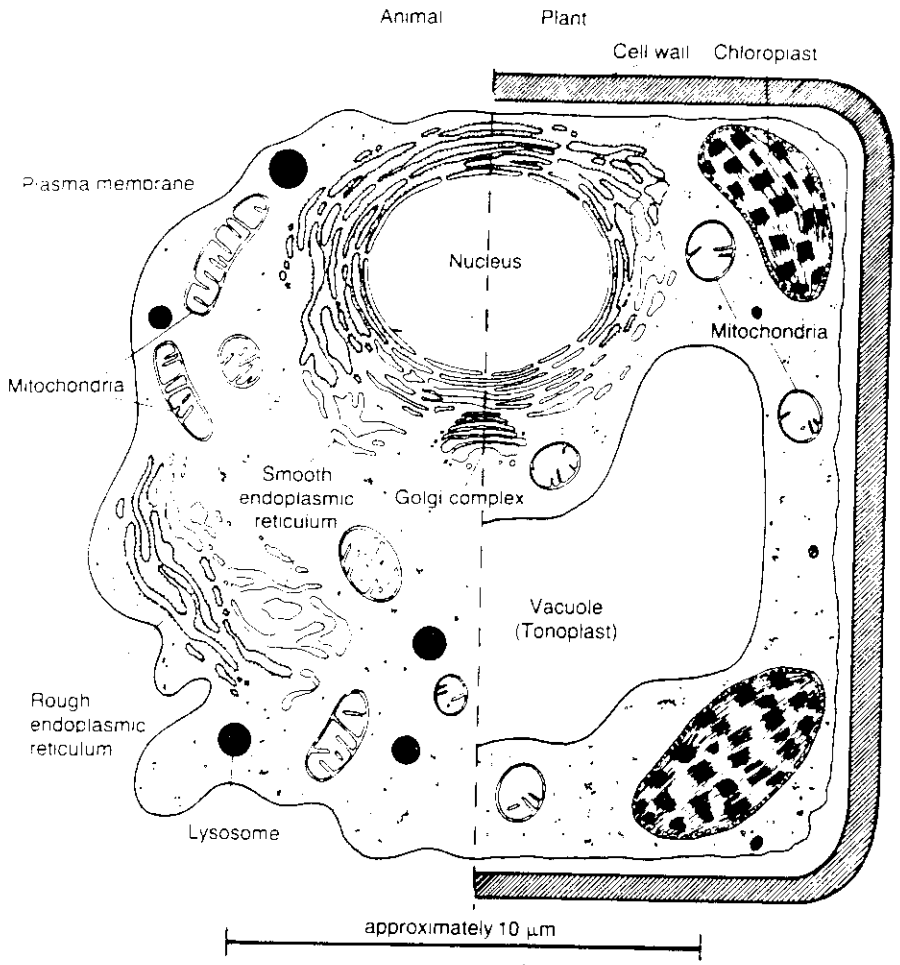
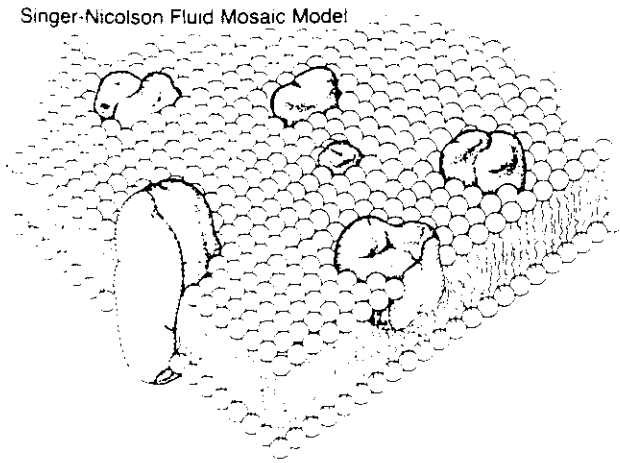
Membranes are essential in the function of all cells and define the compartments. Membranes also define the communication between cell-inside and -outside. This may involve passage of ions and molecules or conformational changes of membrane compounds. Furthermore, many enzymes are attached to membranes. Eukaryotic animal and plant cells show a large number of membranous organelles which comprise a large part of the cellular volume.
There is a remarkable diversity in membrane function related to different protein functions and differences in composition and organization of membrane proteins and lipids.



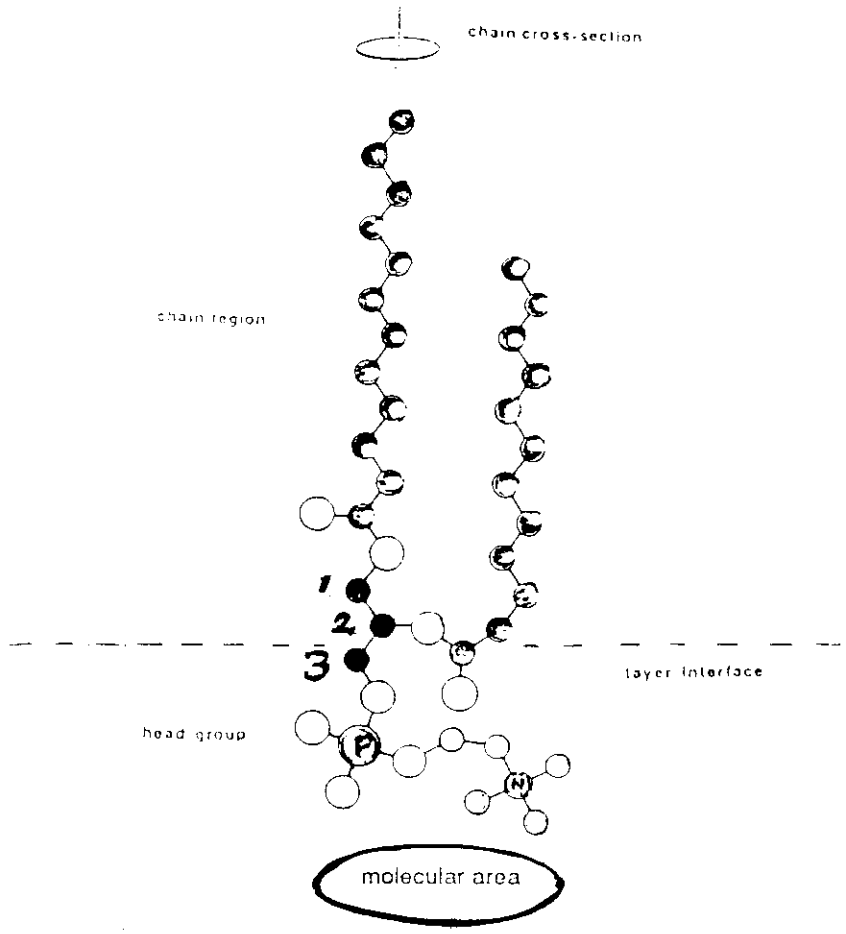
Schematic showing organelles of eukaryotic animal and plant cells as revealed by electron microscopy.

A number of figures are derived from Robert B. Gennis : Biomembranes Molecular Structure and Function Springer advanced texts in chemistry (1989)

The barrier properties of the membrane are largely determined by the lipid bilayer. The lipid constituents ought to have amphiphatic properties which lead to the formation of a bilayer membrane.

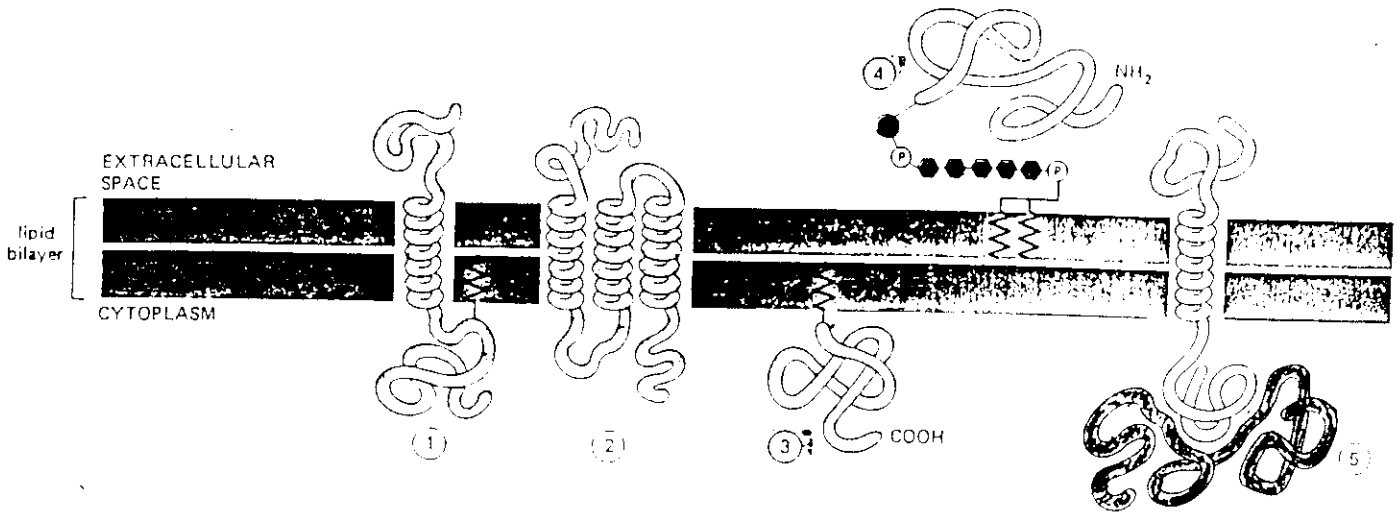


The membrane can be ascribed as a 2 dimensional, semipermeable, liquid surface structure with high dynamic properties.
Differentiation in membrane properties is achieved by differences in composition and organization of lipid and protein building blocks.
The lipid constituents can be classified in phospholipids, glycolipids and neutral lipids.
Lipids can vary in fatty and acyl chains, the type of linkage, and the polar head group.



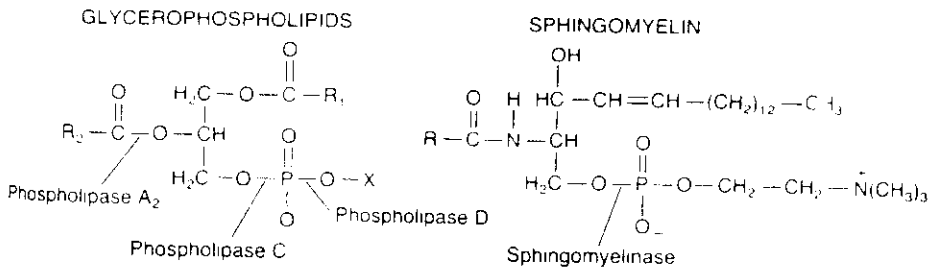
Structure of 1,2-diacylphosphatidylcholine molecule emphasizing the relative orientation and conformation of the head group and of the acyl chain.

The physico-chemical properties of the membrane lipids can be related to their membrane organization, distribution and mobility.
Membrane proteins are generally bound to the membrane by non covalent forces as hydrophobic and electrostatic.
Some are covantly bound to fatty acid residues or by a glycosidic link to phosphatidylinositol.
The carbohydrate residues of glycoproteins are always located on the extracytoplasmic side and therefore are asymmetrically distributed.



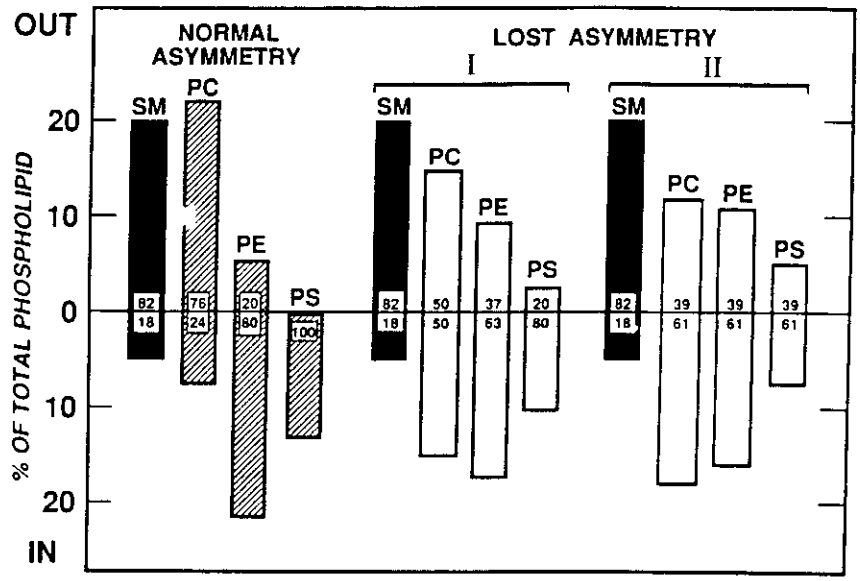
Schematic illustration of a variety of ways by which membrane proteins can be attached to the membrane.
Example of integral (1, 2) and peripheral (3-5) membrane proteins.

Biomembranes are asymmetric since membranes face two different environments, cytoplasmic and extracytoplasmic. Therefore, differentiation of the two monolayers is sensible. Membrane protein asymmetry is a consequence of the way they are originally inserted. Lipids are also asymmetrically disposed as was very convincingly demonstrated for erythrocytes. It is yet not completely clear how lipid asymmetry originates and how it is maintained.
The concept of asymmetric distribution of the four major phospholipid classes in the human red cell membrane could be completed by results of studies involving phospholipases. These enzymes will interact only at the outer surface of the bilayer.



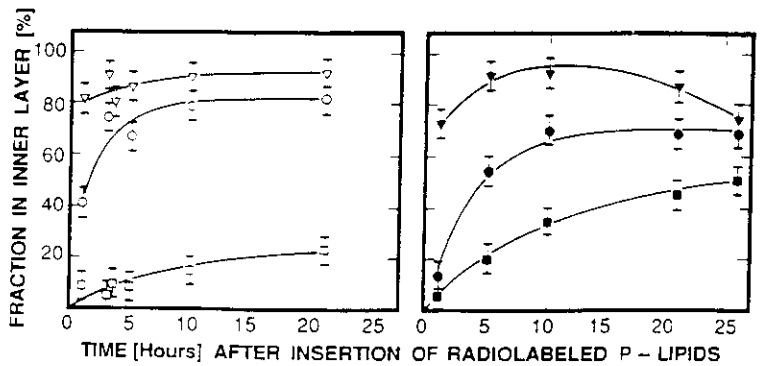
Action of phospholipases, showing the bonds cleaved by the several classes which have been well characterized

The data are not conclusive for subcellular membranes, as membranes become instable before the lipids at the outerface are completely degraded.
It is believed that the membrane skeleton makes the major contribution to maintain membrane asymmetry. This would involve mainly an interaction with spectrin. No change in asymmetric distribution of endogenous phosphatidylethanolamine can be observed in ATP depleted cells. This indicates that an active aminophospholipid translocase is not required for maintenance of asymmetry once it has been established.



Ultimate transbilayer redistributions of the three glycerophospholipids in the human erythrocyte membrane that may be expected to occur under complete failure of the mechanisms that normally maintain their asymmetric distribution. In either of the two possibilities shown, sphingomyelin (SM) is not subject to transbilayer redistributions (see text). (I) Proportional outward migration of phosphatidylethanolamine (PE) and phosphatidylserine (PS), which is limited by a compensatory inward migration of phosphatidylcholine (PC) to reach an equal distribution over both leaflets. (II) Identical transbilayer distributions of the three glycerophospholipids that will be established when the system does not discriminate among the different subclasses.

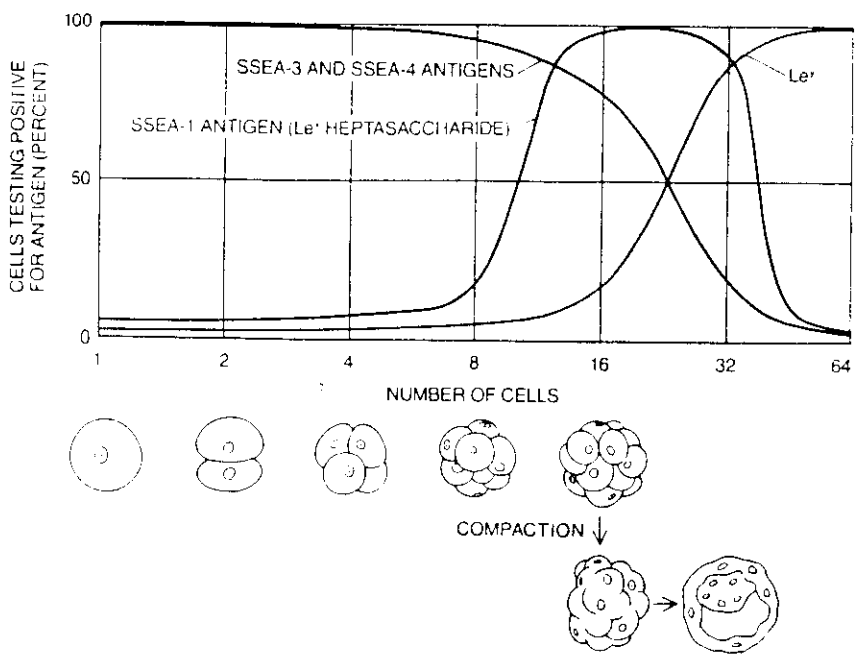
The choline-containing phospholipids dominate the outer leaflet in which we find 76% of the phosphatidylcholine (PC) and 82% of the sphingomyelin (SM). In the inner leaflet we find 80% of the phosphatidylethanolamine (PE) and phosphatidylserine (PS) is exclusively located at the cytoplasmic side. Approximately 20% of the following phospholipids is present the outer leaflet of the human red cell membrane, phosphatidicacid (PA), phosphatidylinositol (PI), and phosphatidylinositol 4,5-bisphosphate (PIP_2).
Interestingly phosphatidylinositol-4-monophospate (PIP) failed to be detected, suggesting that it is exclusively located in the cytoplasmic leaflet. A feature if would share with phosphatidylserine.
The role of spectrin interaction in red cell membrane lipid distribution is illustrated in the next figure.



Transbilayer migration of radiolabeled glycerophospholipids in control (*left*) and diamide-treated (*right*) human erythrocytes at 37°C. Trace amounts of radiolabeled phospholipids were inserted into the outer membrane leaflet of intact cells using nonspecific lipid transfer protein from bovine liver. After a 30-min incubation, which started at zero time, cells were washed thoroughly and subsequently incubated at 37°C to facilitate a re-equilibration of the probe molecules. At the indicated time points, cells were treated with Pal-116-AMPA . The fractions of the radiolabeled phospholipids that could be degraded this way were assumed to be present in the exofacial membrane leaflet. The results shown are the means of independent experiments; bars indicate corresponding SD values. PS, Phosphatidylserine (▽, ▼); PE, phosphatidylethanolamine (○, ●); PC, phosphatidylcholine (□, ■).

Introduction of trace amounts of phosphatidylserine and phosphatidylethanolamine in the outer membrane led to an accumulation in the inner leaflet. However, phosphatidylcholine remained mainly in the outer leaflet in accordance with the control distribution of the lipids. In the presence of diamide which destroys the spectrin interaction (and partially the flipase) the distribution becomes more random over both membrane faces.

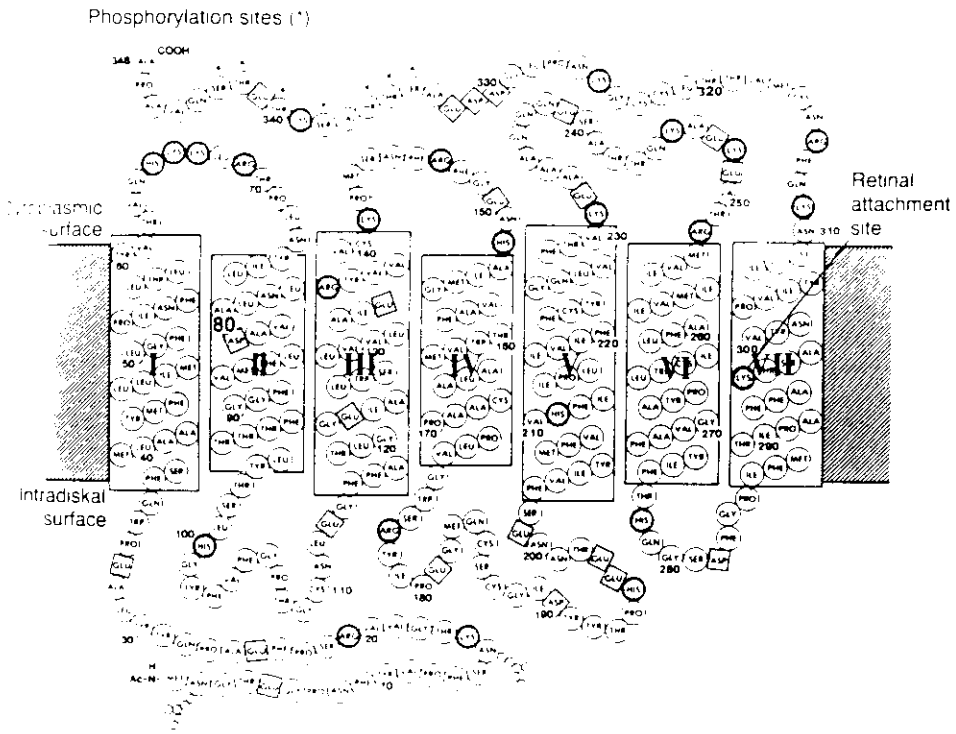
Under conditions that give rise to changes in transbilayer distribution, the transbilayer distribution of SM, characteristic of the normal red cell membrane is maintained. Therefore, a shift of aminophospholipid to the outer membrane can only be compensated by an opposite movement of PC (fig. 6). This means that, considering SM, a complete los of asymmetry may not occur easily. This implies that maximally ~20% PS will appear in the exofacial leaflet. From a physiological point there are strong reasons to keep PS in the inner leaflet. Exposure of PS on the outer surface of erythrocytes render them increasingly adherent to endothelial cells and susceptible to recognition by macrophages.
As already mentioned glycosphingolipids are facing the outside and mark the cell surface. Glycosphingolipids are also the point of attack of different virus and bacterial infections. The appearance and concentration varies with the different stages of development.



STAGE-DEPENDENT APPEARANCE and disappearance of glycosphingolipid antigens is plotted for the preimplantation mouse embryo. The *SSEA-1* antigen (the acronym stands for stage-specific embryonic antigen) appears in the eight-to-32-cell stage and declines greatly after the cells cluster tightly together in a stage called compaction (*colored curve*). When *SSEA-1* declines, the *Le*^{y} antigen appears (*gray curve*); it is chemically similar to *SSEA-1* in that it has an additional fucose sugar at the terminal galactose. The *SSEA-3* and *SSEA-4* antigens are highly expressed until the early stage of compaction, and they disappear almost completely by the 32-cell stage (*black curve*). Such glycosphingolipids may be closely associated with the regulation of cell-cell recognition and the growth of tissues.

membrane protein topography

It can be expected that many membrane proteins will require an asymmetric orientation for its function which is unidirectional.

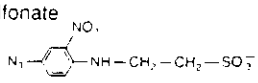
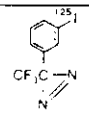
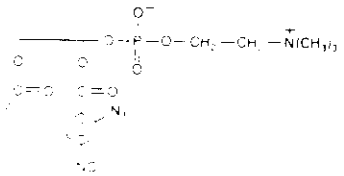


Topographic model of bovine rhodopsin indicating the seven transmembrane helices. Phosphorylation sites (asterisks) and N-linked glycosylation sites are indicated, as is the lysine in helix 7 which is the retinal attachment site. The aspartate in helix 2 is suspected to function as a counterion for the Schiff base formed by retinal. Cysteines 110 and 187 are suspected to form a disulfide bond. Note the presence of proline in five of the seven membrane-spanning helices.

Protein topology can be determined with proteases, immunological methods, chemical labeling and genetic approaches.

Some reagents used to probe the topography of membrane proteins.

Reagent	Target
Diazotized sulfanilic acid (DSA, DABS)	Surface-accessible amino groups, tyrosine, histidine, cysteine.
Trinitrobenzene sulfonic acid (TNBS)	Surface-accessible protein or phospholipid amino groups

N-(4-Azido-2-nitrophenyl)-2-aminoethane sulfonate (NAP-Taurine) 	Non-specific label for surface-accessible groups. Photoactivated to reactive nitrene.
3-(trifluoromethyl)-3-*m*-[^{125}I]iodophenyl diazirine (TID) 	Non-specific label for membrane-buried groups. Forms reactive carbene.
1-palmitoyl-2-(2 azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H] phospho-choline. 	Non-specific label for membrane-buried groups near polar headgroup.

Lateral heterogeneity

The high lateral mobility of membrane components (for lipids $D=10^{-8}$ cm^2/sec, corresponds to a net distance traversed of about 2µm in 1sec) would suggest a homogeneous distribution in the plane of the bilayer. There is however direct and indirect evidence for domains with a district composition.

- macroscopic domains which are separated by barriers, as the apical and basolateral domains of epithelial cells and thylakoids.
- protein aggregation can result in domain formation, enriched in a particular protein as is the case for bacteriorhodopsin in purple membranes.

Lipids can undergo lateral phase separations yielding coexisting liquid and crystalline lamellar domains, which can be induced by changes in temperature, pressure, pH, ions or proteins.

The transition from a lamellar gel phase ($L\beta$) to a lamellar liquid crystalline phase ($L\alpha$) is accompagnied by the formation of rotational isomers in a very cooperative manner. As a consequence of the phase transition there is a reduction in bilayer thickness of 0,4 nm and a lateral expansion. The molecular area will increase from 0,48 nm^2 to 0,65 nm^2.
The transition temperature (T_c) and the transition enthalpy (ΔH) (the heat required for the entire transition normalized per mole) is influenced by length and unsaturation of the acyl chains and the lipid polar head group, e.g. The transition temperature of phosphatidylethanol amines is 29°C higher than of the respective phosphatidylcholines.
The coexistence of the gel and liquid crystalline phase can be visualized with fluorescence microscopy.

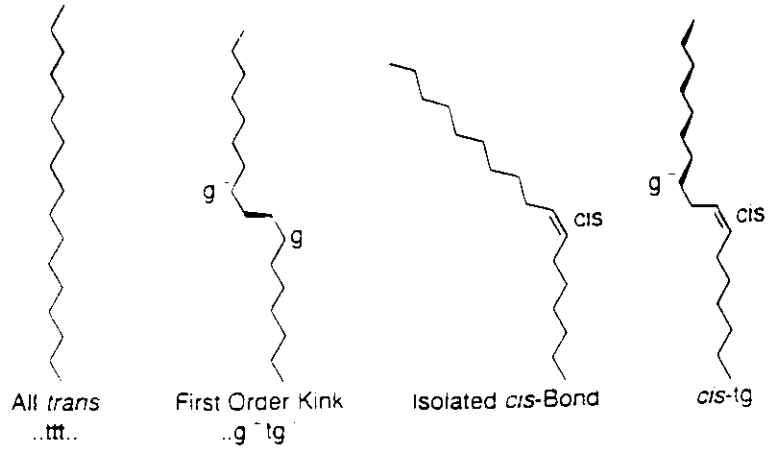
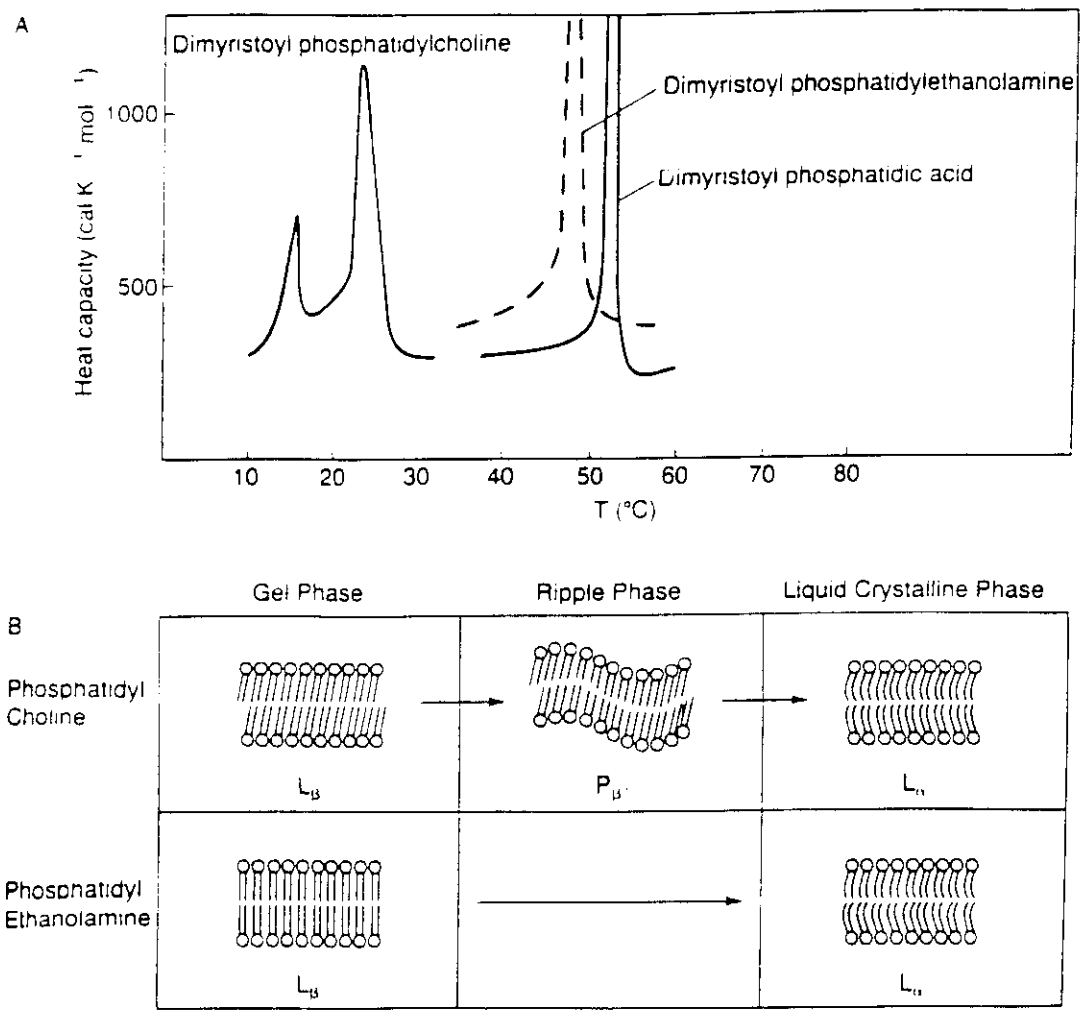


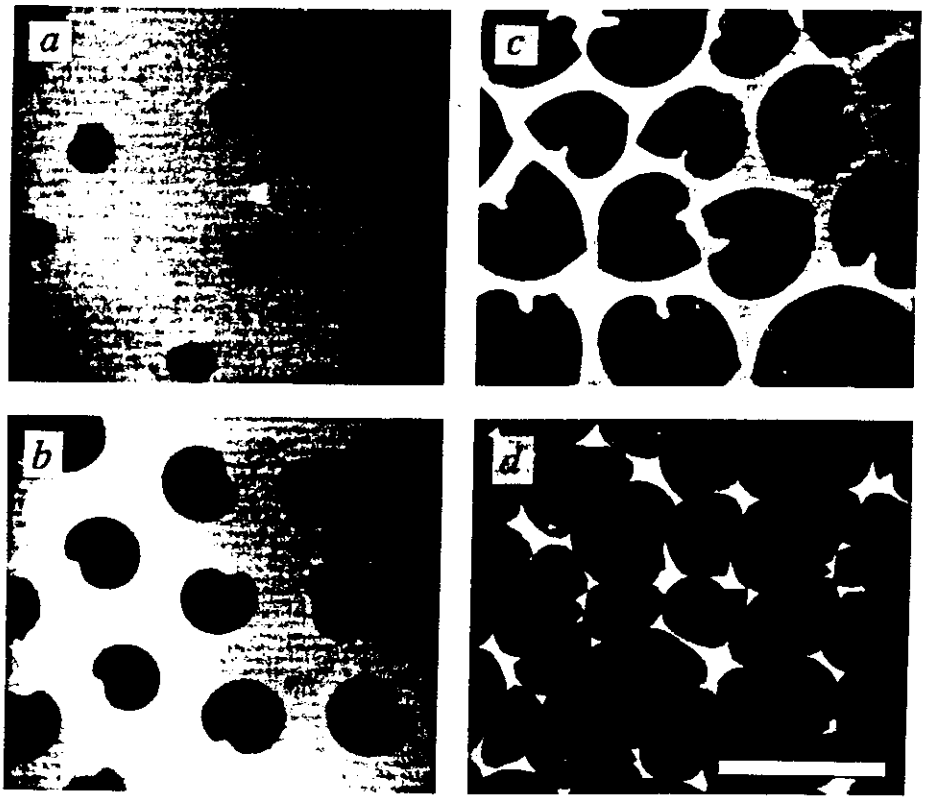
Illustration of several alkyl chain configurations.



(A) Differential scanning calorimetry profiles of three phospholipids.

(B) Schematic showing the molecular organization of phosphatidylcholine and phosphatidylethanolamine as a function of temperature

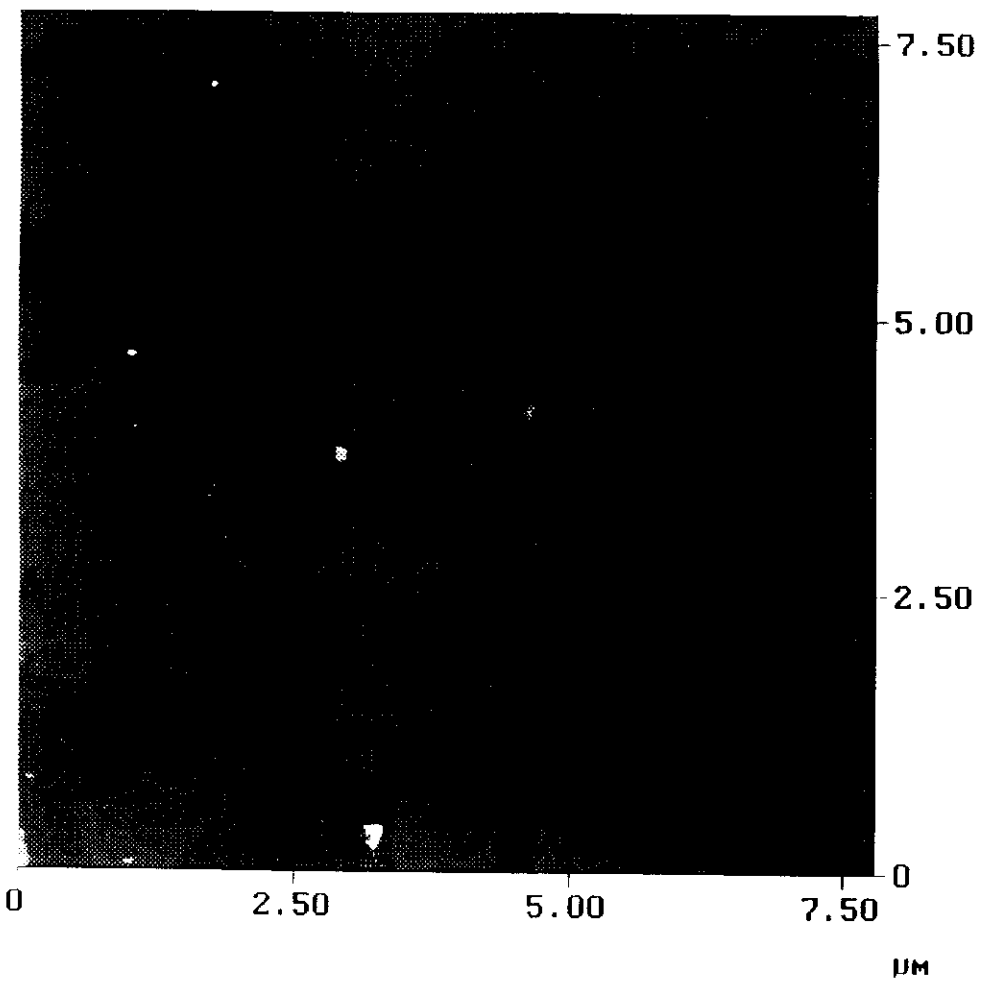
Lipid domains which are dynamically maintained may be seen as either lateral density fluctuations or compositional fluctuations. The macroscopic fluctuations can be important for passive and active membrane functions such as transmembrane permeability enzymatic activity and protein complex formation.
The effects of a variety of compounds interacting with cholesterol or anesthetics may be explained by their ability to modulate dynamic membrane heterogenety.

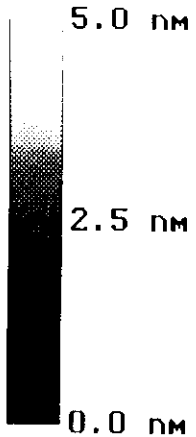


Fluorescence micrographs of a monolayer consisting of dipalmitoyl-phosphatidylcholine and 2 mol% NBD-phosphatidylcholine on a distilled water subphase. The photographs record the appearance of the monolayer at various points in the solid- fluid coexistence region, with values of A and π, respectively, of: (a) 0.814 nm^2, 2.4 mN/m; (b) 0.674 nm^2, 4.7 mN/m; (c) 0.515 nm^2, 12.4 mN/m; (d) 0.415 nm^2, 24.1 mN/m. Subphase temperature 19.5°C; scale bar = 50 μm (Weis, 1991)

The organization of the bilayer is highly cooperative and as a consequence will lead to differentiated regions of the membrane. Results from atomic force microscopic studies of (fluid) binary lipid monolayers transferred to an Si surface indicate domain formation on a scale of 10-20 nm. Also high sensitivity differential scanning calorimetry experiments present evidence in this direction.

selected reading: B. Roelofsen and J. A. F. Op den Kamp. Plasma membrane phospholipid asymmetry and its maintenance: The human red cell as a model (1994) Current topics in membranes **40,** 7-45.



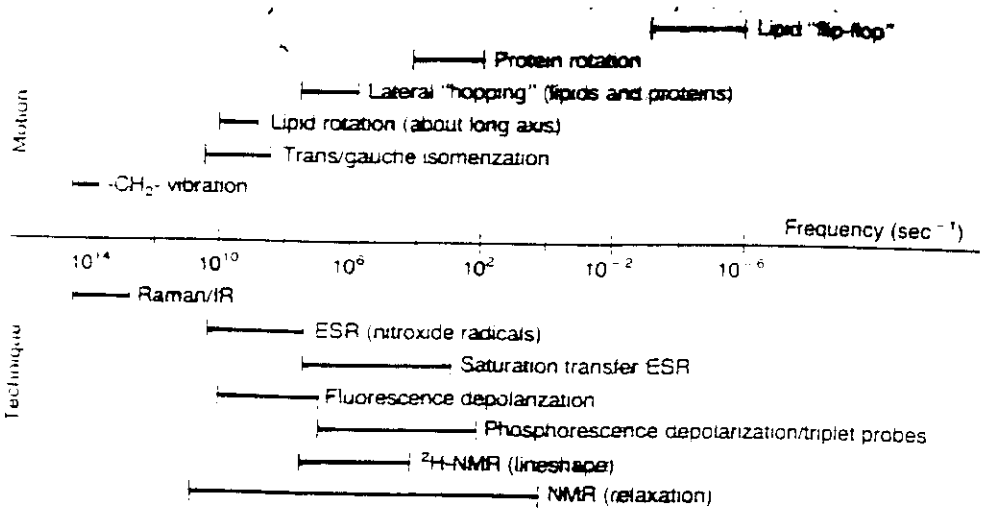


Microscope	NSIII AFM
Scan size	7.786 µm
Setpoint	-2.350 V
Scan rate	1.489 Hz
Number of samples	512

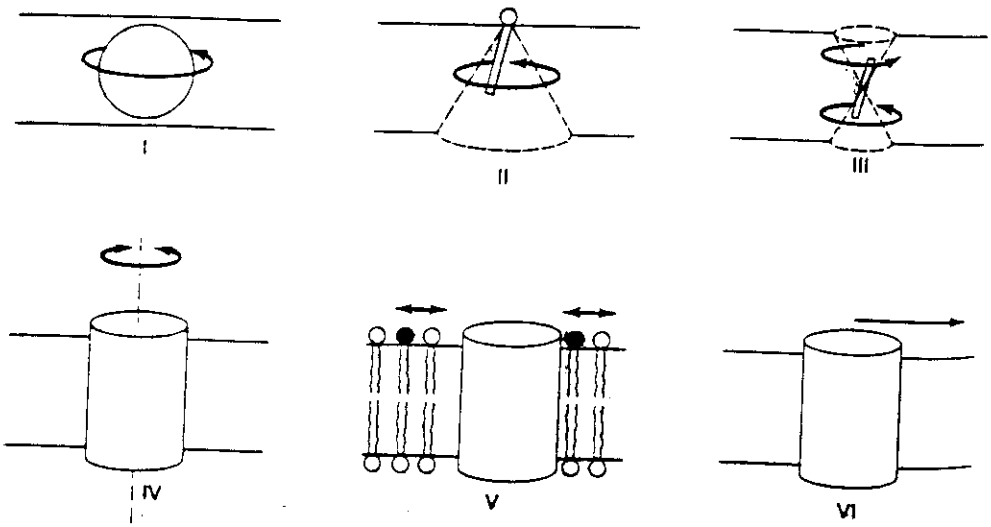
AFM picture of a mixed monomolecular layer of ceramide and cholesterol (molar ratio 1: 0.2)

Lipid-protein interactions

In membranes lipid-protein interactions are important for the specific properties.
It is important to realize the wide range of motions that have been examined in membranes. Which range from molecular vibrations at 10^{-14} sec to transbilayer motion which can take days. Some of these motions and the time range in which different biophysical techniques can be used are indicated



The characteristic frequencies of molecular motions of membrane proteins and lipids compared with the frequency ranges in which various spectroscopic techniques are sensitive to molecular motion. The characteristic times are obtained by taking the reciprocal of the indicated frequencies. Boundaries are very approximate.



Some models used to analyze rotational and translational motion within membranes. (I) Isotropic rotation of a spherical molecule within the hydrophobic core of the membrane. (II) Wobbling-in-cone model for a "rod-like" molecule tethered at the surface and able to move rapidly within a cone-shaped region defined by an angle with respect to the bilayer normal. (III) Wobbling-in-cone model for rod-like molecule within the hydrophobic core. (IV) Rotation of a cylindrical transmembrane protein. (V) Lateral motion of lipids within the bulk (left side) and exchanging between bulk lipid and lipid adjacent to protein (right side). (VI) Lateral motion of a cylindrical transmembrane protein.

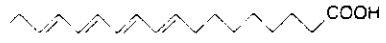
The fluidity of membranes can be measured by the use of ESR-spin probes and fluorescent probes. These methods can also be used to examine lipid-protein interactions and lateral diffusion of membrane proteins or lipids. Since molecular interactions will affect the dynamics of the membrane compounds.

Some probes used to study the dynamics of membranes.[1]

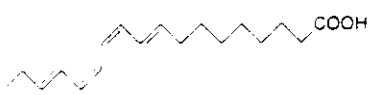
A. Fluorescent Probes

(1) diphenylhexatriene (DPH)

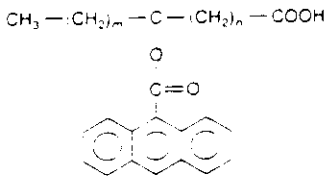
(2) (a) *trans*-parinaric acid



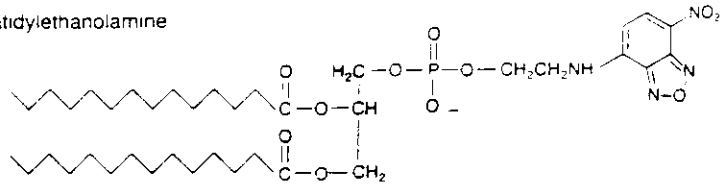
(b) *cis*-parinaric acid



(3) (*m,n*) anthroylstearic acid

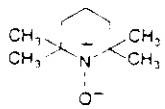


(4) NBD - phosphatidylethanolamine (NBD-PE)

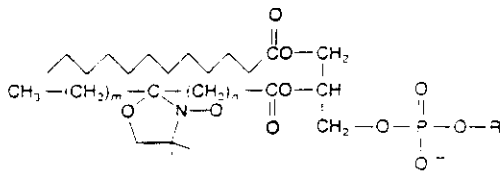


C. Spin-probes

(1) TEMPO

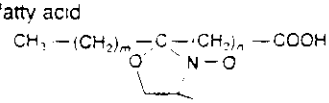


(2) spin-labeled phospholipids

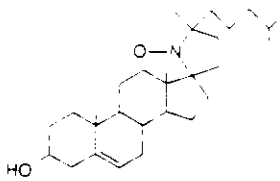


eg R = $(CH_3)_2—N^+(CH_3)_3$ (*m,n*)PC

(3) spin-labeled fatty acids (*m,n*) fatty acid



(4) spin-labeled cholesterol (pyrrolidine cholesterol)



Perturbations that result in decreasing the area per lipid molecule such as increased hydrostatic pressure, lower temperature, or the addition of cholesterol to phospholipids in the liquid crystalline state, all result in a decrease in fluidity.

Biomembranes are generally in a fluid crystalline phase and it appears that maintenance of membrane fluidity is critical to function. The properties of a gel phase is in general incompatible with the organization and function of membrane proteins. An exception is the semicrystalline structure of purple membranes containing bacteriorhodopsin. The relevance of membrane fluidity is evidenced by the adaptation to environmental conditions. Various organisms can adapt to low temperatures by increasing the degree of lipid unsaturation or decreasing the average acyl chain length such that lipid packing density is decreased and results in maintenance of membrane fluidity.

Based on the membrane composition a large fraction of membrane lipids is in contact with membrane proteins.

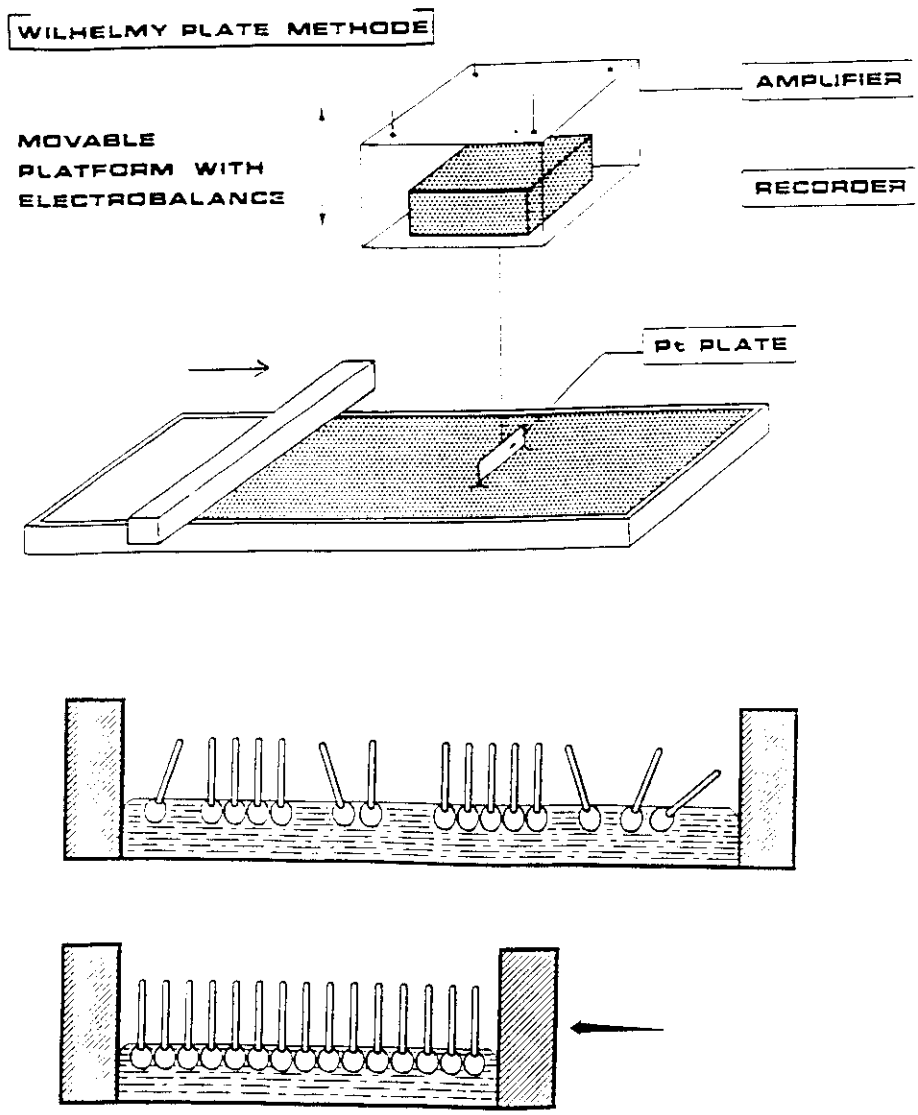
The following questions are relevant to understand membrane structure and function.

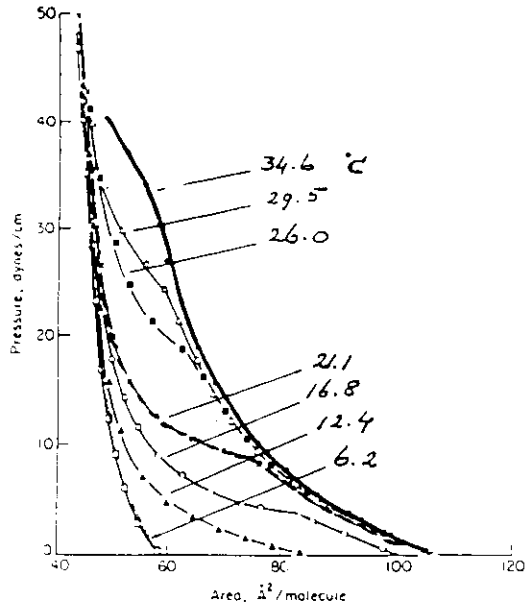
(1) Is there a tight and specific interaction between lipid and protein and what is the nature of the interaction?

(2) Do lipids influence the structure or function of membrane proteins?

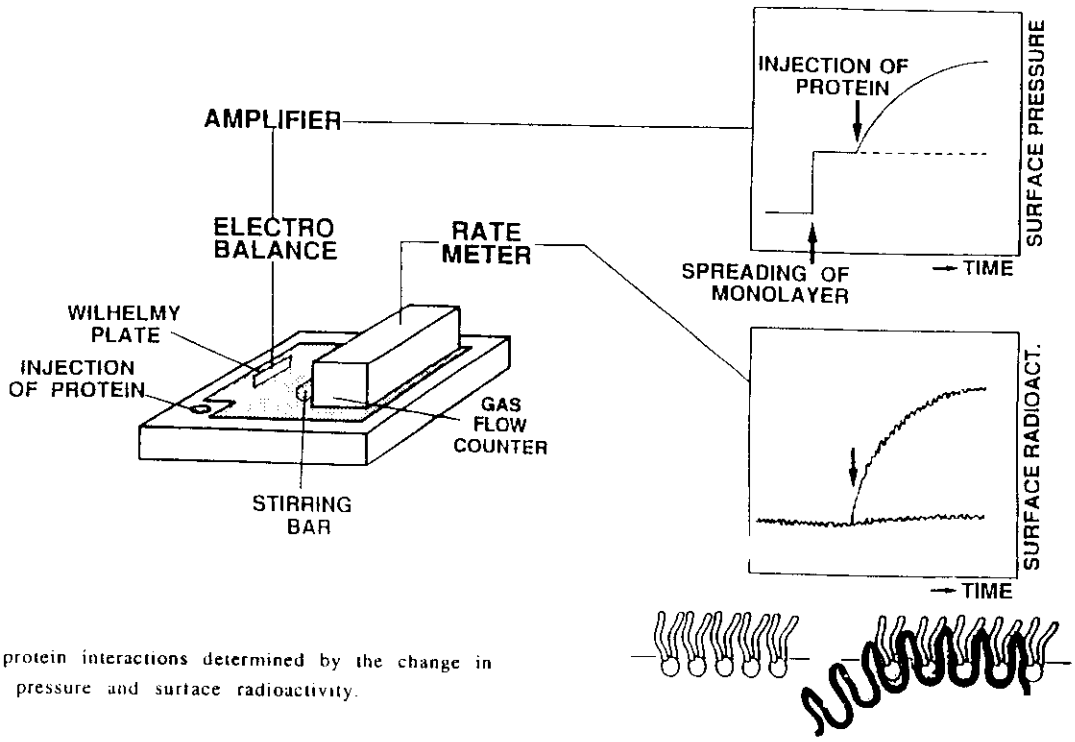
Monomolecular layers at the air-water interface is a membrane model system that has proven to be very useful to address these questions.

Compression of monomolecular layers at the air-water interface



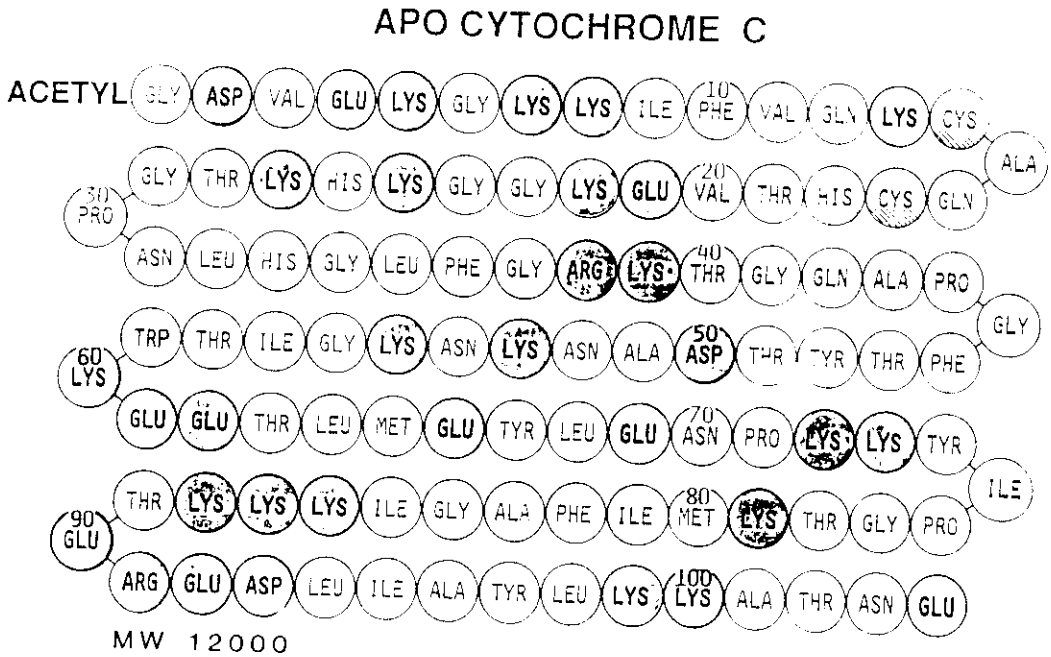


Pressure-area curves of di-palmitoyl phosphatidylcholine at different temperatures

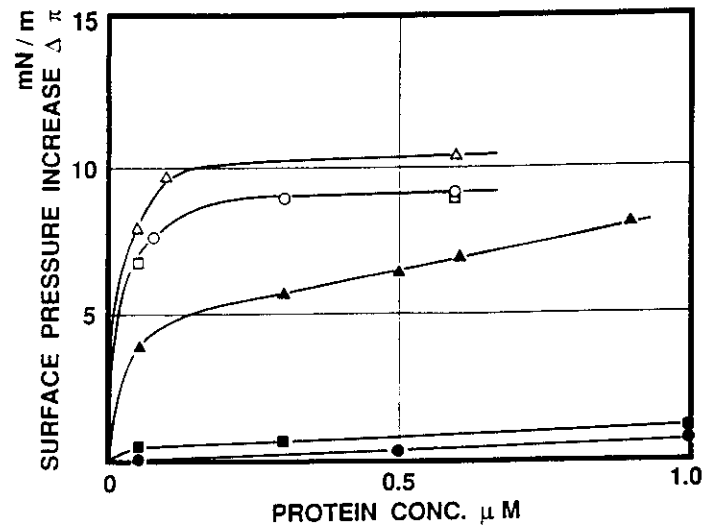


Lipid -protein interactions determined by the change in surface pressure and surface radioactivity.

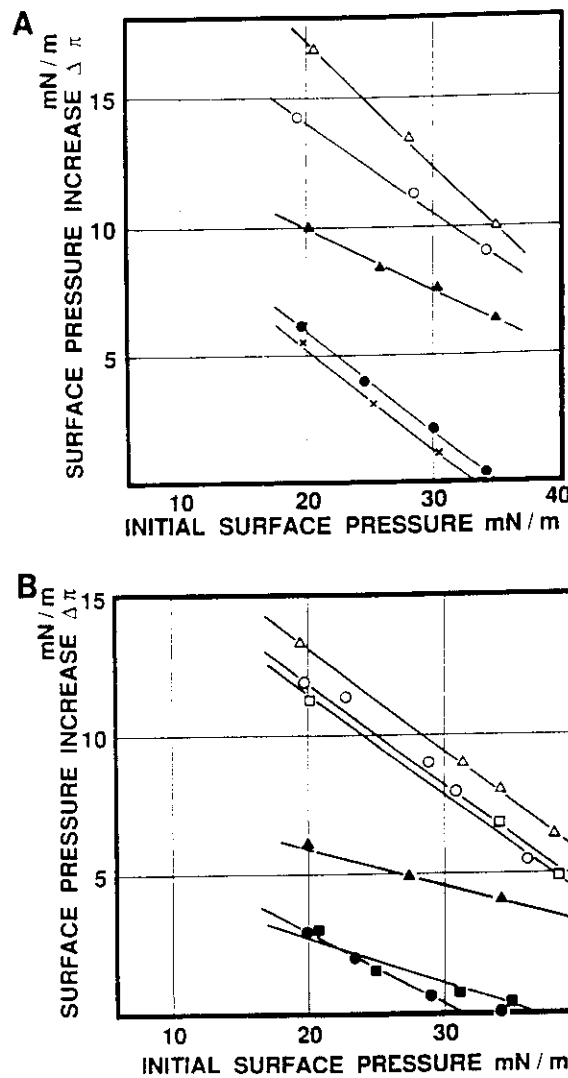
An example of the specificity of lipid protein interactions studied by monomolecular layers is the differential interaction of apo- and holocytochrome c with acidic membrane lipids.
Apocytochrome c and cytochrome c from horse heart have identical polypeptide chains of 104 amino acids but differ in secondary and tertiary structure.
Apocytochrome c has virtually no structure whereas cytochrome c is a highly structured nearly spherical protein. The final localization of cytochrome c is at the outside of the inner mitochondrial membrane.
Large differences were observed both with respect to the nature and the lipid specificity of the interaction. The initial electrostatic interaction of the heme-free precursor apocytochrome c with anionic phospholipids is followed by penetration of the protein in-between the acyl chains. Apocytochrome c shows similar interactions for all anionic lipids tested. In strong contrast the holoprotein discriminates between cardiolipin for which it has a high affinity and phosphatidylserine and phosphatidylinositol for which it has a much lower affinity. For these latter lipids the interaction with cytochrome c is primarily electrostatic. The cytochrome c-cardiolipin interaction shows several unique features which suggest the formation of a specific complex. These properties account for the large preference of cytochrome c for the inner over that of the outer mitochondrial membrane lipid extract. Only apocytochrome c was able to induce close contacts between monolayers of the mitochondrial outer membrane lipids and vesicles of mitochondrial inner membrane lipids.



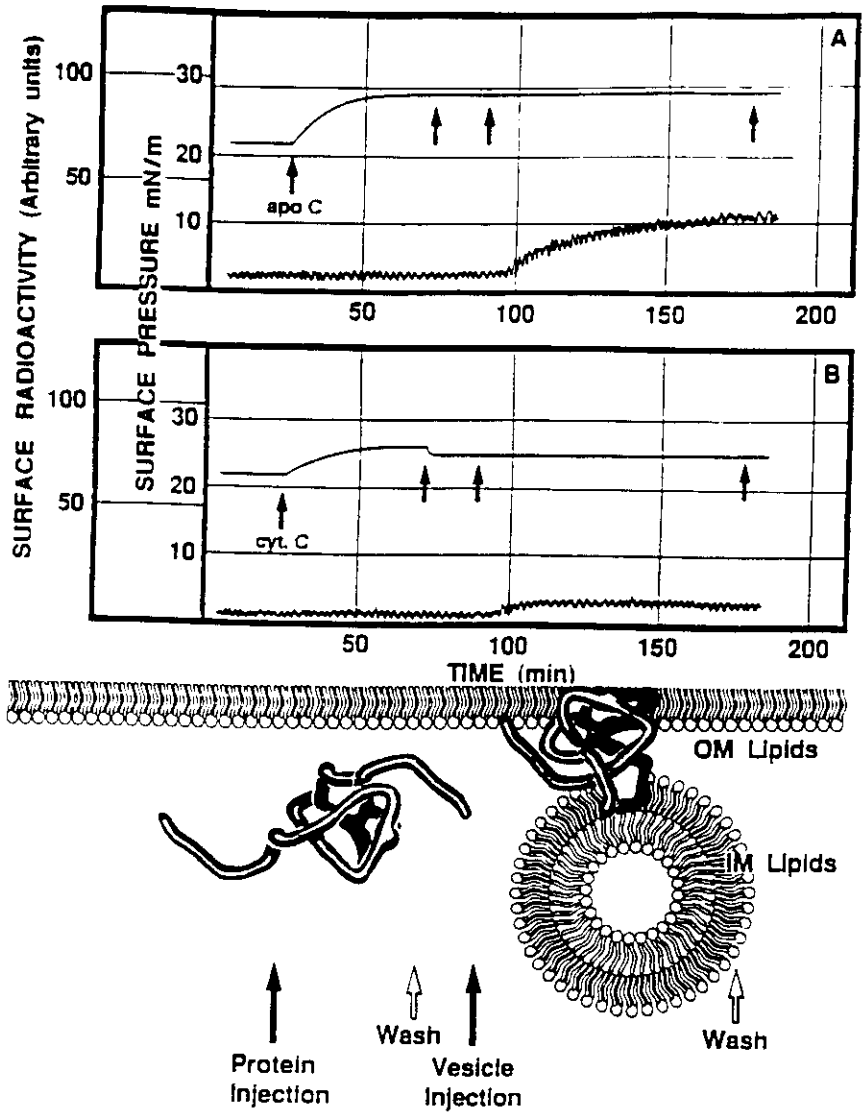
Primary structure of horse heart (apo)cytochrome c.
Characteristics: 1 tryptophan risidue no. 59 ;19 lysine residues; 2 arginine residues; 12 Glu and Asp residues; heme group coupling to Cys-14 and 17



Surface pressure increase of CL, PS, and PI monomolecular layers as a function of apocytochrome *c* and cytochrome *c* concentration at an initial surface pressure of 34 mN/m. Apocytochrome *c*: △, CL; ○, PS; □, PI. Cytochrome *c*: ▲, CL; ●, PS; ■, PI. The subphase contained 10 mM Pipes, pH 7.0, 50 mM NaCl.



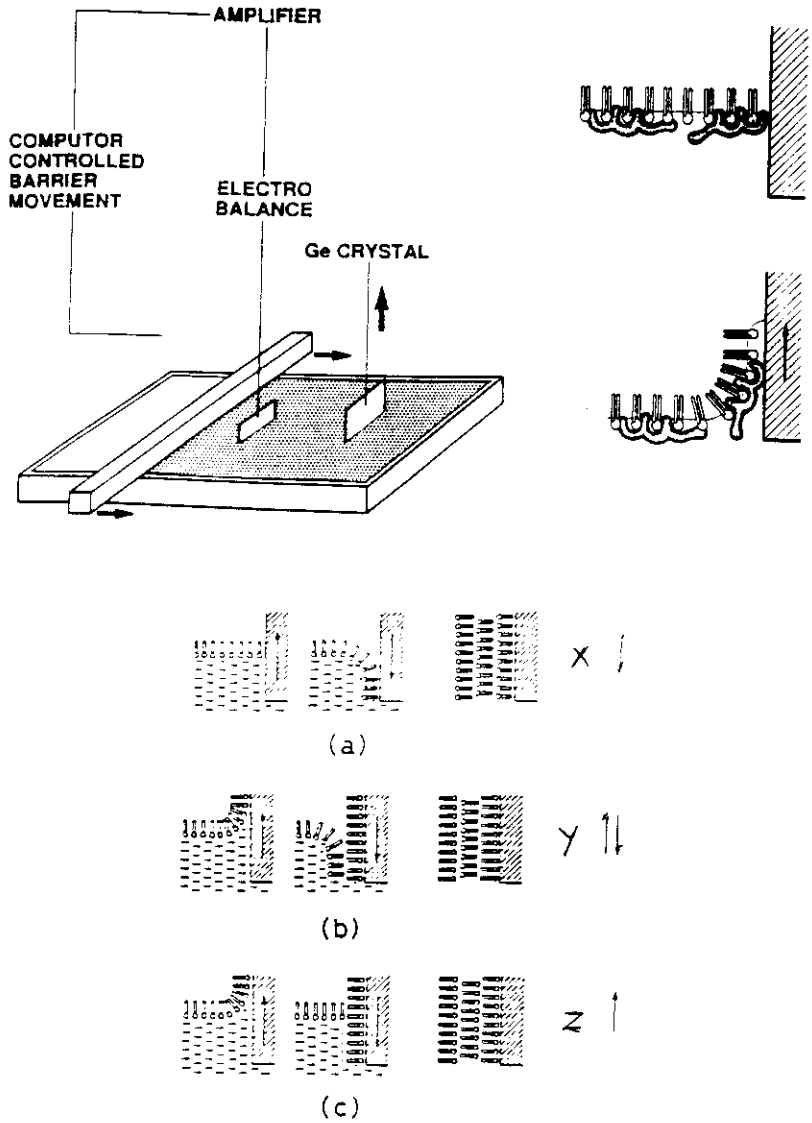
Surface pressure increase after injection of apocytochrome *c* and cytochrome *c* underneath monomolecular layers of CL and PS at different initial pressures. *A*, protein concentration 0.5 μM. *B*, protein concentration 0.05 μM. Apocytochrome *c*: △, CL; ○, DOPS; □, PI. Cytochrome *c*: ▲, CL; ●, DOPS; ×, beef heart PS; ■, PI. The subphase contained 10 mM Pipes, pH 7.0, 50 mM NaCl.



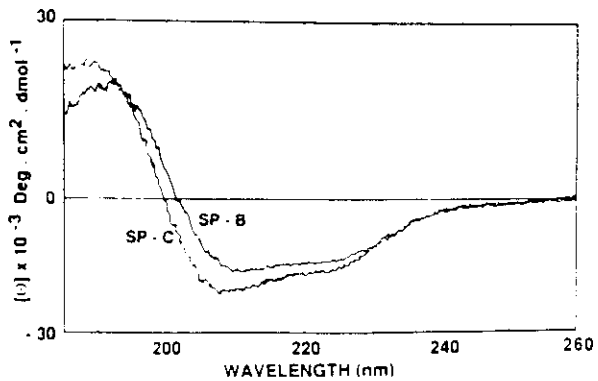
A, binding of ^{14}C LUVs of inner mitochondrial membrane lipids to a monomolecular layer of outer mitochondrial membrane lipids containing apocytochrome *c*. *B*, binding of ^{14}C LUVs of inner mitochondrial membrane lipids to a monomolecular layer of PS containing cytochrome *c*. The monomolecular layer was formed on a subphase of 10 mM Pipes, pH 7.0, the protein concentration at the injection point was 0.05 μM. The subphase was refreshed with 10 volumes of the same buffer containing 50 mM NaCl at the indicated points. LUVs concentration at the injection point was 0.5 μM.

After depositing one (or more) monolayers on a Si, Ge or mica support spectroscopic methods can be used to determine the secondary structure of the protein after interaction with the lipid monolayer. Quantification of the amount of protein involved in the interaction and measurement of the pressure change induced by the protein can be used to determine the surface area of the membrane penetrating part of the protein.
Atomic force microscopy of deposited mono- or bilayers can give information on the distribution of membrane compounds and eventually on the organization of the interacting proteins at a molecular level.

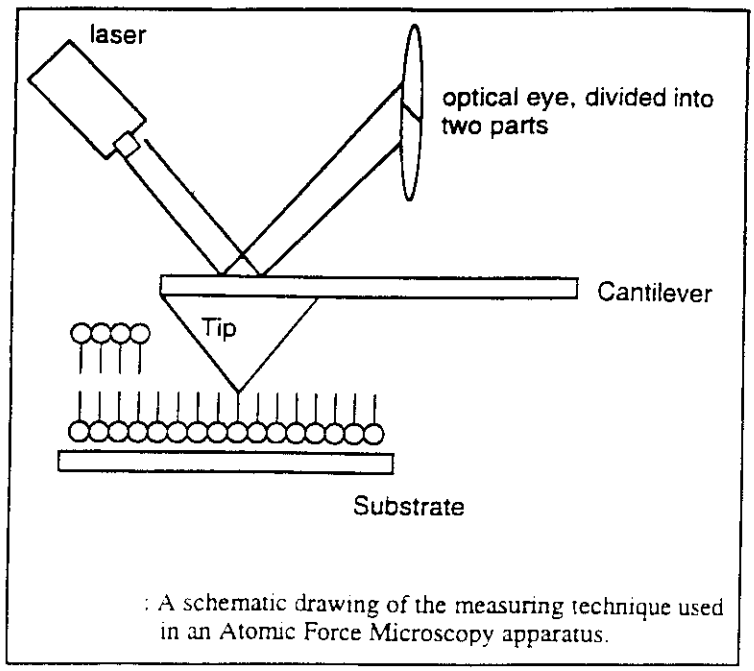
selected reading: R. A. Demel. Physicochemical methods in the study of biomembranes, Monomolecular layers in the study of biomembranes. (1994) Subcellular Biochemistry **23**, 83-120



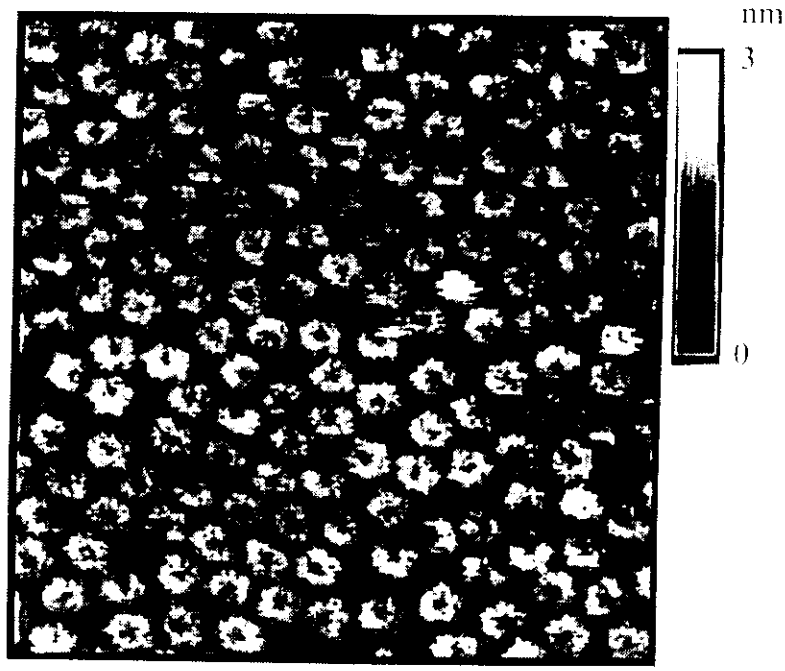
Langmuir-Blodgett method for depositing monolayers on a solid support Vertical dipping method. There are three types, according to the manner of deposition: (a) X-type; (b) Y-type; (c) Z-type



. Circular dichroism spectra of SP-B and SP-C monolayers collected at a surface pressure of 25 mN/m and measured at room temperature.



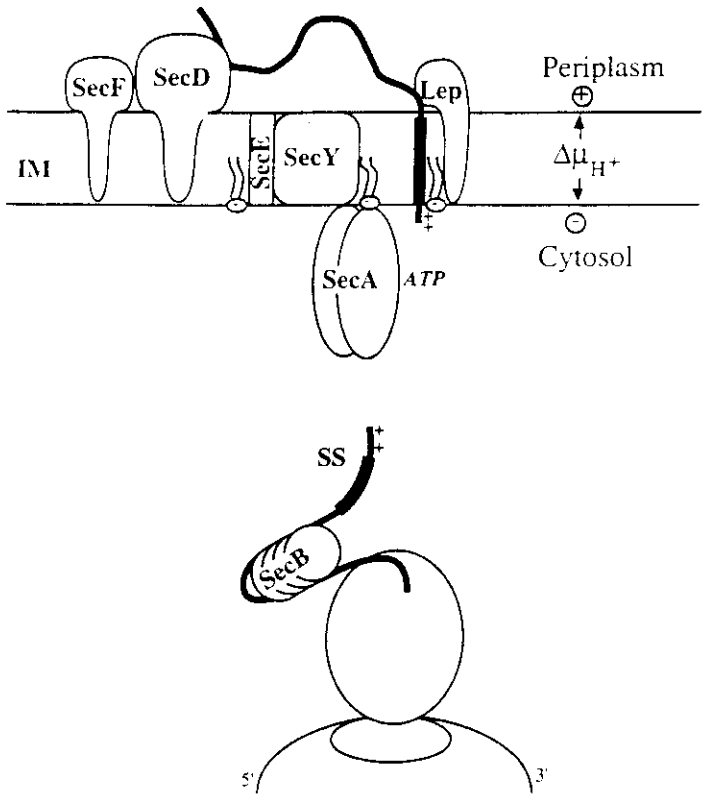
: A schematic drawing of the measuring technique used in an Atomic Force Microscopy apparatus.



. Cholera toxin B-oligomer on egg-PC bilayer. Note that, beyond the five fold symmetry, finer details are resolved which are reproducible at different scales. This image is quite similar to the calculated image from the X-ray structure by assuming a 0·7 nm AFM tip without compression. At larger probe forces, the subunits are seen much farther apart, because the tip can press into the area between subunits where the connection is much weaker. See text for further discussions. Image size: 80 nm.

Lipid involvement in protein translocation.

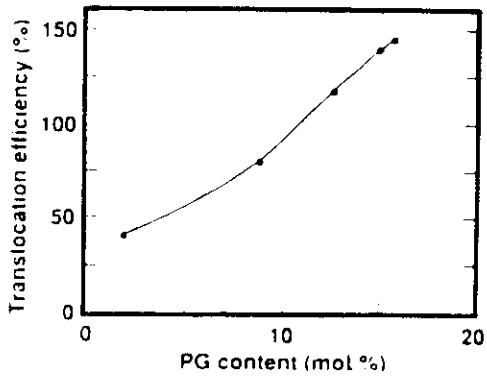
In membrane biogenesis and protein targeting polypeptide chains often insert or translocate biological membranes. Correct membrane assembly will depend on the interaction of the main membrane components, lipids and proteins. It can be reasoned that protein-lipid interactions must be involved in membrane insertion and translocation of newly synthesized proteins. Such an involvement can be of a general nature such as the correctly structured proteinaceous translocation complex and to provide the membrane barrier function, membrane lipids could also be more directly involved in providing a translocation pathway.



Components involved in protein translocation across the *E.coli* inner membrane.

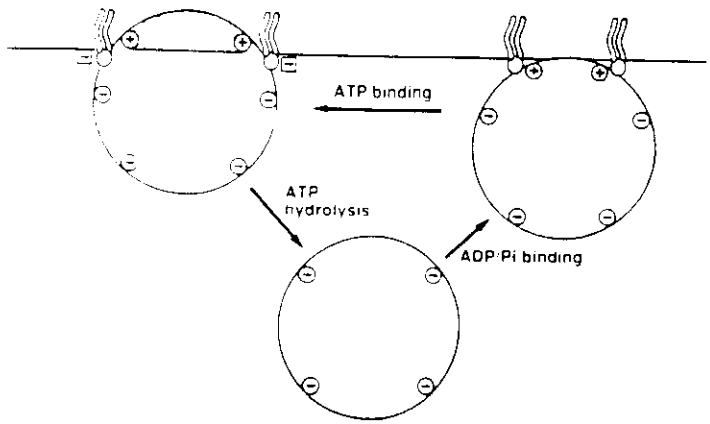
Prokaryotic protein secretion

The translocation route of the outer membrane pore-protein PhoE across the *Escherichia coli* inner membrane is shared by many periplasmic and outer membrane proteins. The protein is synthesized in the cytosol as precursor carrying a signal sequence. The signal sequence and the Sec proteins A, B, E, Y, F and D are essential for membrane translocation, which is driven by ATP and facilitated by the proton motif force. During or after translocation leader peptidase (Lep) removes the signal sequence and the mature protein is assembled as a trimeric pore in the outer membrane. Proof for the involvement of a particular class of lipids in the protein translocation came from studies using an *E.coli* mutant strain with a low phosphatidylglycerol (PG) and cardiolipin (CL) content. Translocation was decreased *in vivo* and *in vitro* using the inverted inner membrane vesicles.



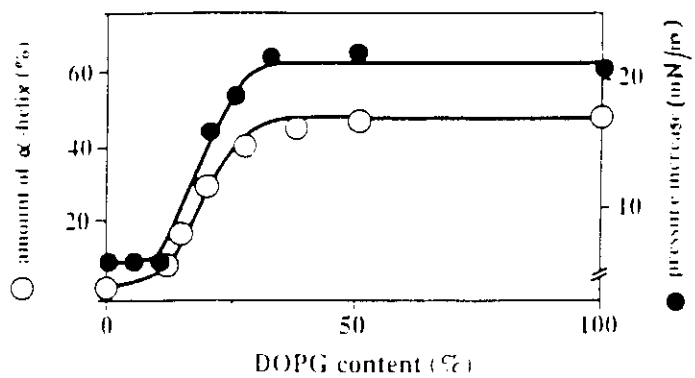
prePhoE translocation depends on the PG content of the inner membrane. Translocation efficiency, expressed as the percentage of protein translocated across wild-type SD12 inner membrane vesicles is presented as a function of the PG content of inner membranes of strain HDL11 grown at different concentrations of IPTG.

A suggested possibility for the PG involvement is the PG requirement of SecA which is a membrane associated ATPase and interacts both with SecB and SecY. Negatively charged lipids stimulate ATPase activity and induce a conformational change. Indeed it could be demonstrated that SecA can penetrate into a lipid monolayer only when PG is present. From the modulation of the penetration of SecA into anionic lipids by various nucleotides, a cyclic model of SecA membrane penetration can be proposed.



Modulation of SecA-lipid interaction by nucleotides. The model is based on data presented by Breukink et al. [11]), in which binding and penetration of SecA was measured in the presence of different nucleotides. Upon ATP binding, the overall anionic secA is proposed to expose a positively charged domain which preferentially interacts with anionic phospholipids resulting in membrane penetration. Upon ATP hydrolysis, the protein undergoes a conformational change resulting in a decreased affinity for the interface. In the presence of ADP and P_i the SecA has an efficient binding with the lipids but penetration is restricted. Upon ATP binding, the SecA again inserts deeper in the lipid layer.

In early translocation models an interaction of the signal sequence with membrane lipids was proposed as an obligatory step in the translocation process. This view was based on the overall chemical structure of the signal sequence, with a positive N-terminus and hydrophobic central region. The consequences of this interaction on the structure have been studied by different biophysical techniques. Membrane interaction and α-helix formation is observed only above a certain threshold concentration of PG.

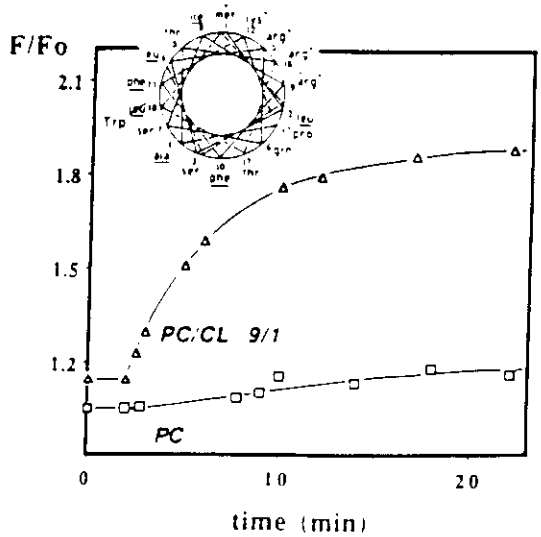


Penetration and α-helix formation of a signal peptide in a lipid/water interface requires a threshold amount of anionic phospholipids. The synthetic signal peptide of prePhoE (MKKSTLALVVMGIVA-SASVQA) was injected at room temperature underneath a lipid monolayer (initial surface pressure 20 mN m) or was added to a solution of small unilamellar vesicles (9 mM lipid), whereafter the increase in surface pressure or the amount of α-helix formation was determined via the Wilhelmy plate method or circular dichroism, respectively

Mitochondrial protein import

Most mitochondrial proteins are synthesized as precursor proteins in the cytosol. The N-terminal temporal sequences play an essential role in import into mitochondria. The presequences share a basic and amphipathic character with signal sequences but differ by a more uniform distribution of the positive charges and a high content of hydroxylated amino acids such that an amphiphatic α-helix can be formed.

Studies with precursor proteins pointed to a specific role of the unique mitochondrial lipid cardiolipin. The membrane potential ($\Delta\psi$) across the mitochondrial membrane is essential to initiate translocation. As was shown in studies with a synthetic peptide corresponding to the presequence of yeast cytochrome c oxydase subunit IV in which Leu 18 was replaced by tryptophan to use its fluorescence properties.

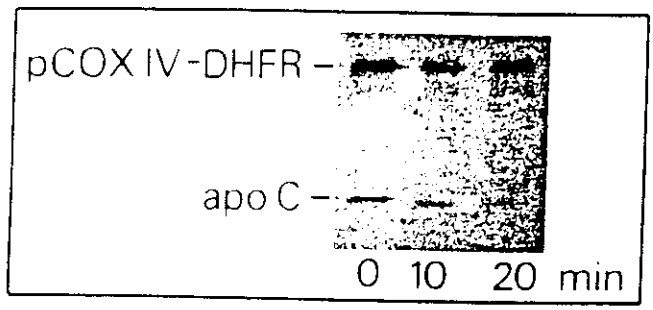


Cardiolipin renders a mitochondrial presequence susceptible to a membrane potential across a lipid bilayer. PreCOX IV 1-25 W^{18} (2 μM) was added to large unilamellar vesicles (300 μM lipid) experiencing a K^+_{in} Na^+_{out} gradient. The tryptophan fluorescence change at 340 nm was followed in time. At t = 2 min valinomycin was added to generate a membrane potential (negative inside). The fluorescence measured (F) is related to F_0, the fluorescence intensity in the absence of vesicles. The 9:1 ratio of PC CL reflects the ratio of lipid phosphorus

The increase in tryptophan fluorescence intensity demonstrates that the peptide is entering a more hydrophobic environment.

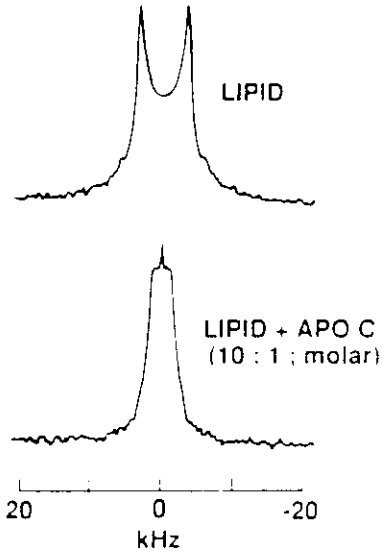
Cytochrome c follows a unique pathway, it does not contain a cleavable presequence and does not require a proton motif force or ATP.

In bilayer vesicles containing negatively charged lipids, the precursor is able to reach spontaneously the opposite membrane-water interface. A precursor following the general pathway, as (pCOX IV-DHFR) the fusion protein of yeast cytochrome c oxidase subunit IV and mouse dihyrofolate reductase, does not show this behavior despite a high affinity for anionic lipids.



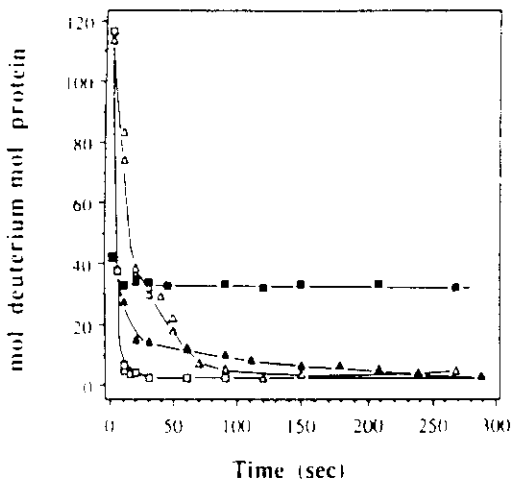
Digestion of in vitro synthesized pCOX IV-DHFR and apocytochrome c by trypsin enclosed in vesicles composed of an outer mitochondrial membrane lipid mixture. A mixture of in vitro synthesized pCOX IV-DHFR and *Neurospora crassa* apocytochrome c was added to large unilamellar vesicles composed of an outer membrane lipid mixture with entrapped trypsin and incubated for various times. Outside protease activity was fully inhibited. The samples were analyzed on a polyacrylamide gel and visualized by autoradiography

Insertion of apocytochrome c into a bilayer affects lipid organization as is illustrated by ^2H NMR. The effect is not seen for the holoprotein.



^2H NMR spectra (46.1 MHz) at 30°C of 1,2-[11,11-$^2H_2$]-dioleoylphosphatidylserine dispersions in the absence or presence of apocytochrome c

Anionic detergents and phospholipids greatly increase the dynamics of the secundary structure of cytochrome c. ^2H-^1H exchange experiments demonstrated the dynamics of the lipid associated α-helices. The rate of amide ^2H-^1H exchange of the unfolded (amide deuterated) precursor is extremely rapid. In contrast the interior of the tightly folded cytochrome c is slow. However, in the presence of detergent micelles a rapid exchange is also observed for the holoprotein.



$^2H \rightarrow {}^1H$ amide exchange of apocytochrome c (□, △) and cytochrome c (■, ▲) in 10 mM acetate (pH 4.75) at 0°C in the absence (□, ■) and presence (△, ▲) of dodecylphosphoglycol dodecylphosphocholine (9:1, molar) micelles (lipid to protein molar ratio of 120). The deuterated proteins were diluted in H_2O. At the indicated time points the samples were quenched in liquid N_2, whereafter the residual deuterium content of the samples was analyzed after freeze drying and unfolding

selected reading: B de Kruijff, E. Breukink, R. A. Demel, R. van 't Hof, H. H. J. de Jongh, W. Jordi, R. C. A. Keller, J. A. Killian, A. I. P. M. de Kroon, R. Kusters and M. Pilon.(1992)Membrane Biogenesis and Protein Targeting, Lipid involvement in protein translocation. Elsevier Science Publishers p 85-101