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WORKSHOP ON THE STRUCTURE OF BIOLOGICAL MACROMOLECULES

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"Lipid Microstructures and Structures of Cationic Lipid-DNA Complexes"

presented by:

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Lipid Microstructures and Structures of Cationic Lipid-DNA Complexes

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One of the important goals of modern biology is to understand the cell functions in terms of the structure and interactions of its constituents, namely molecules of life, which would possibly lead biologists a step closer to understanding the essential features that constitutes life. Our understanding of cell function in molecular terms-gene organization, cell growth and differentiation, biology of various diseases etc. to mention a few has grown to an unprecedented level in recent years. This advancement has been possible due to the development of powerful highly sophisticated biochemical and biophysical techniques for structural elucidation.

In my lectures, I will deal with the structural aspects of one of the components of biological membranes, namely lipids. The two topics which I will cover are (i) Self-assembled lipid microstructures, in particular, lipid tubules - their structures and application (ii) Cationic lipid - DNA complexes and their use in gene delivery.

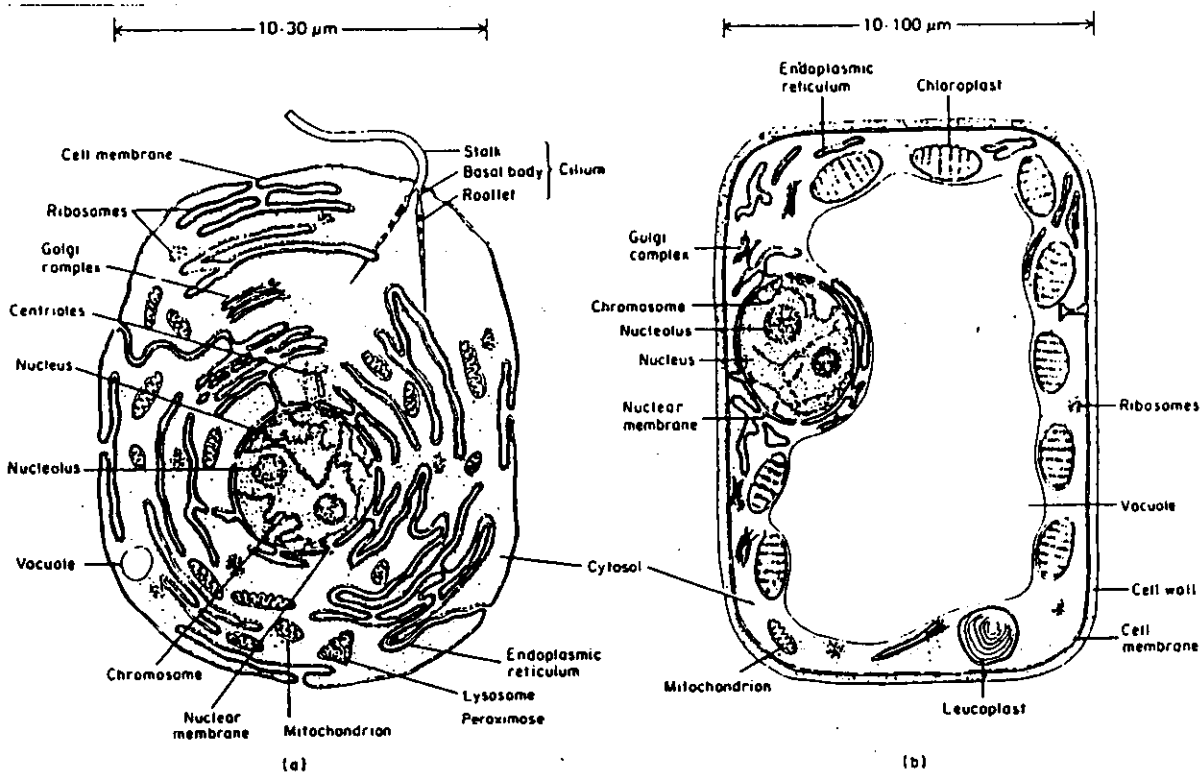
Biomembranes: Biomembranes are essential in the function of all cells and define boundaries of the intracellular compartments. Protein which catalyze most of the activities in a cell would be unable to co-ordinate their activities without membranes. The essential functions of biomembranes are:

- Compartmentalization, which provides morphological identity to the cell and organelles.

- Selective barrier property which help in controlling the internal cell and organelles.

- Communication and stimulus response coupling which provides a basis for functions such as excitability, adhesion, immune response and hormone action.

- transmembrane transport of ions and solute.



Caricature of the thin section of a generalized animal (a) and of a higher plant cell (b) illustrating relationships between different compartments created by the plasma membrane and the membranes of organelles.

- Major structural elements of biological membranes are lipids and proteins. The composition and type of lipids and proteins in membranes from different sources vary. A variety of membrane lipids such as glycerophospholipid, phosphonolipids, plasmogen, glycolipid, cardiolipins, sphingolipids and gangliosides are found to occur in membranes.

Chemical composition of some purified membranes (in percentages)

Membrane	Protein	Lipid	Carbohydrate	Membrane	Protein	Lipid	Carbohydrate
Myelin	18	79	3	*Halobacterium* purple membrane	75	25	0
Plasma membrane				Mitochondrial			
Human erythrocyte	49	43	8	inner membrane	76	24	0
Mouse liver	44	52	4	Chloroplast			
Ameba	54	42	4	Spinach lamellae	70	30	0

SOURCE: G. Guidotti, 1972, *Ann. Rev. Biochem.* 41:731.

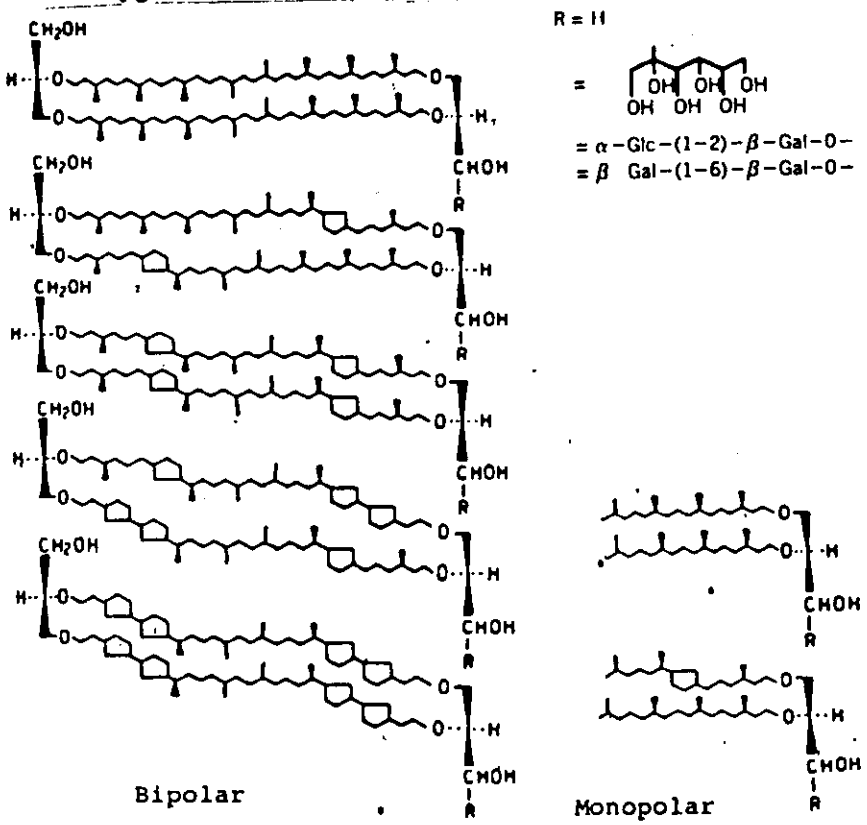
Lipid composition of membrane preparations (in percentages)*

Source	Cholesterol	PC	SM	PE	PI	PS	PG	DPG	PA	Glycolipids
Rat Liver										
Plasma membrane	30.0	18	14.0	11	4.0	9.0	—	—	1	—
Endoplasmic reticulum (rough)	6.0	55	3.0	16	8.0	3.0	—	—	—	—
Endoplasmic reticulum (smooth)	10.0	55	12.0	21	6.7	—	—	1.9	—	—
Mitochondria (inner)	3.0	45	2.5	24	6.0	1.0	2.0	18.0	0.7	—
Mitochondria (outer)	5.0	50	5.0	23	13.0	2.0	2.5	3.5	1.3	—
Nuclear membrane	10.0	55	3.0	20	7.0	3.0	—	—	1.0	—
Golgi	7.5	40	10.0	15	6.0	3.5	—	—	—	—
Lysosomes	14.0	25	24.0	13	7.0	—	—	5.0	—	—
Myelin	22.0	11	6.0	14	—	7.0	—	—	—	12
Rat erythrocyte	24.0	31	8.5	15	2.2	7.0	—	—	0.1	3
E. coli cytoplasmic membrane	0	0	—	80	—	—	15.0	5.0	—	—

*PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol (cardiolipin); PA phosphatidic acid.

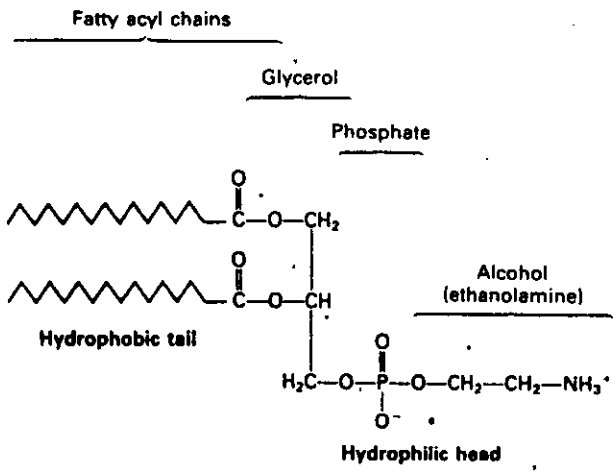
SOURCE: M. K. Jain and R. C. Wagner, 1980, *Introduction to Biological Membranes*, Wiley.

In addition, unusual membrane lipids occur as major components in Archaebacteria which encounter and thrive under unusual conditions of pH, high salt concentration and absence of oxygen.



Structures of monopolar and dipolar lipids with isoprenyl ether chains. These are major components of membranes from *Archaebacteria*. Note that the stereochemistry of the glycerol backbone is *sn*-2,3-dialkyl- rather than 1,2-dialkyl.

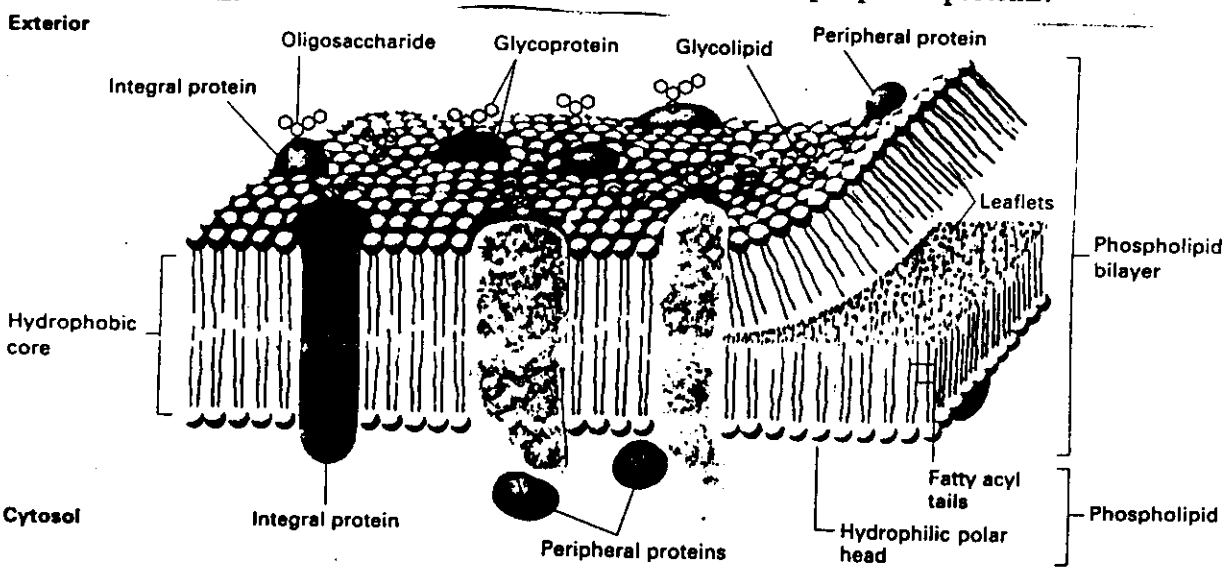
The diverse class of lipids vary in fatty acid chains, the type of linkage and polar head group. They are amphipathic- the polar and non-polar regions of the molecule are separate. Common features of major classes of lipids are (a) the hydrophobic region containing two polymethylene chains with one or more double bonds with branching towards the methyl end (b) the backbone made of glycerol, propanediol, glycol or carbohydrates like nonitol, ceramide or ornithine (c) a hydrophilic group incorporating phosphate amino acids, sugar or amine.



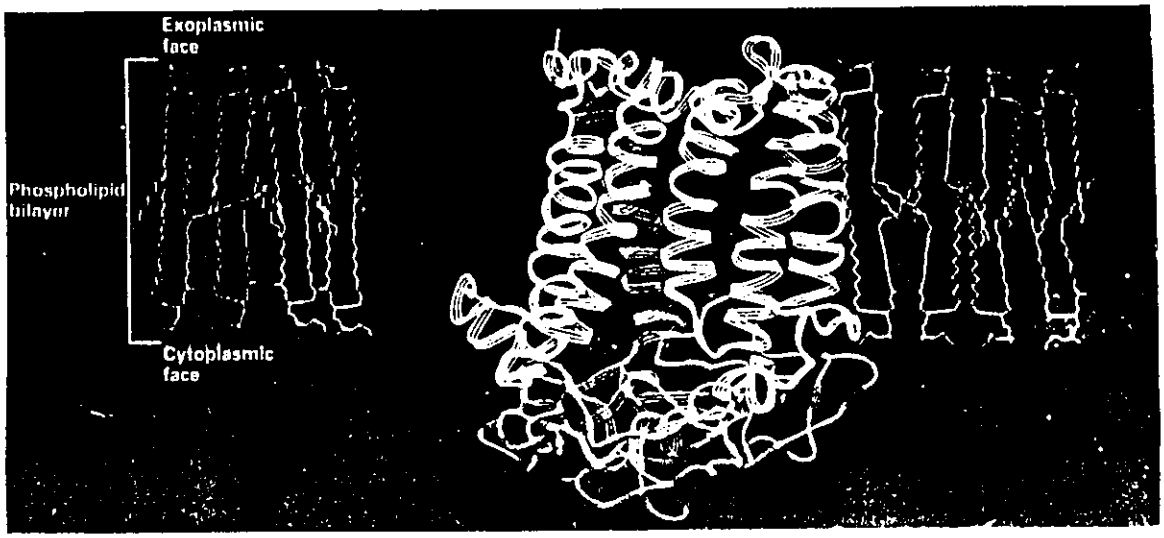
The structure of phosphatidylethanolamine, a typical phospholipid.

Basic structure of biological membranes:

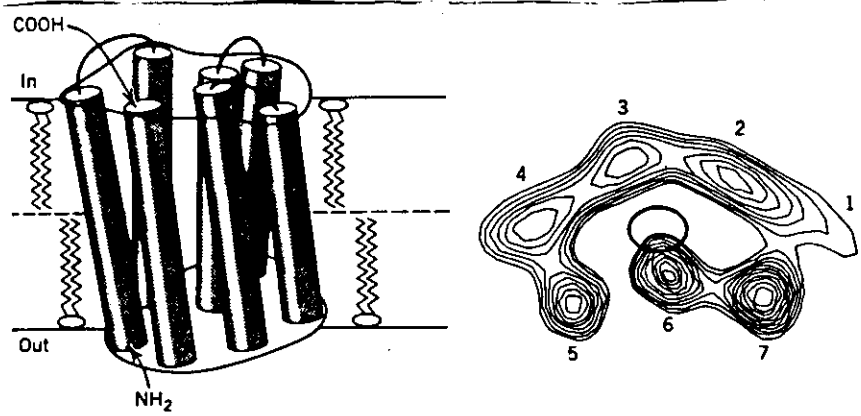
In spite of the varied functions of the biological membranes, they all have a basic structure having a phospholipid bilayer with integral proteins imbedded in the lipid bilayer (Singer & Nicholson fluid mosaic model). Although every membrane has the same basic structure of a phospholipid bilayer, a different set of membrane proteins enables each subcellular membrane to carry out distinctive functions. The protein-lipid ratio varies. For ex.: The inner mitochondrial membrane is 76% protein whereas the myelin membrane has only 18% protein. The lipid composition also varies greatly among different membranes. The membrane integral proteins interact directly with the phospholipid bilayer and usually contains one or more helical sequences in the hydrophobic part of the bilayer. Examples of integral membrane proteins are bacterial photosynthetic reaction center, bacteriorhodopsin, major erythocyte glycoprotein-glycophorin etc. Very few structural data are available for the membrane integral proteins. Peripheral proteins are bound to the membrane directly by interactions with membrane integral proteins or directly by interactions with polar head groups. The cytoskeletal proteins, spectrin and actin which are bound to the cytoplasmic face of the erythrocyte cell membrane are some examples of peripheral proteins.



A phospholipid bilayer constitutes the basic structure of biological membranes. The hydrophobic fatty acyl tails of the phospholipids form the middle of the bilayer; the polar, hydrophilic heads of the phospholipids line both surfaces. Integral proteins have one or more regions embedded in the lipid bilayer. Peripheral proteins are primarily associated with the membrane by specific protein-protein interactions. Oligosaccharides bind mainly to membrane proteins; however, some bind to lipids, forming glycolipids.



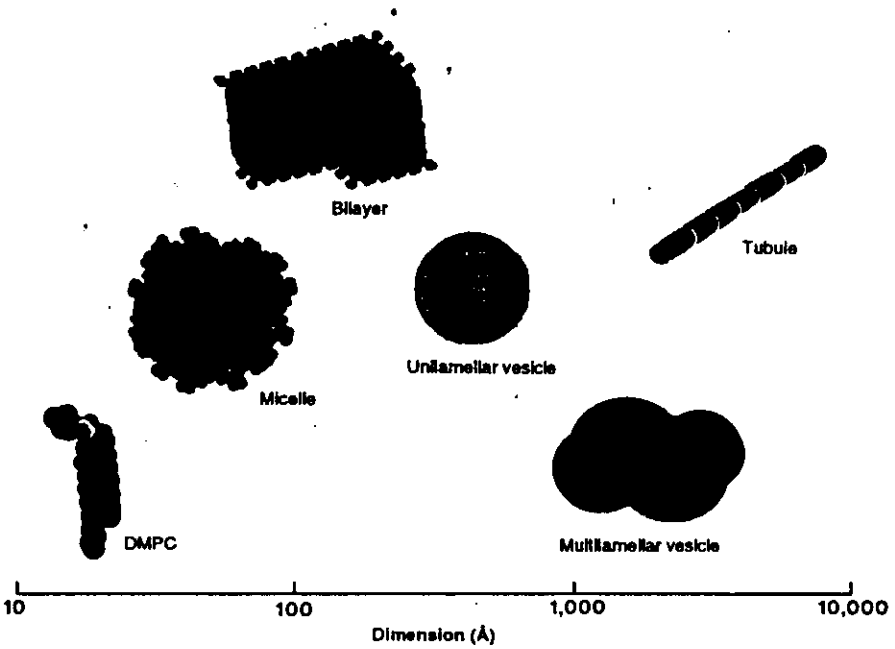
a: The structure of the bacterial photosynthetic reaction center as determined by x-ray crystallography. Only the polypeptide backbone of the three membrane spanning proteins is shown: the L subunit (yellow) and the M subunit (dark blue) form five transmembrane α helices and have a very similar structure overall; the H subunit (light blue) is anchored to the membrane only by a single transmembrane α helix. (b) A cross-sectional view through the middle of the membrane, showing the locations of the 11 transmembrane α helices. The center of the protein (orange) is occupied by prosthetic groups such as free (nonheme) iron and the chlorophylls. *Part (a) courtesy of D. Rees;* part (*b*) *after R. Henderson, 1985,* Nature *318:598–599.*



(Left) Topography of the seven helices of bacteriorhodopsin in relation to the lipid bilayer. (Right) The position of retinal residue and the topography of the seven helices obtained from neutron diffraction. (From W. Stoeckenius, *Trends Biochem. Sci.* 483–486, 1985.)

- **Lipid microstructures that self-assemble from lipid molecules:**

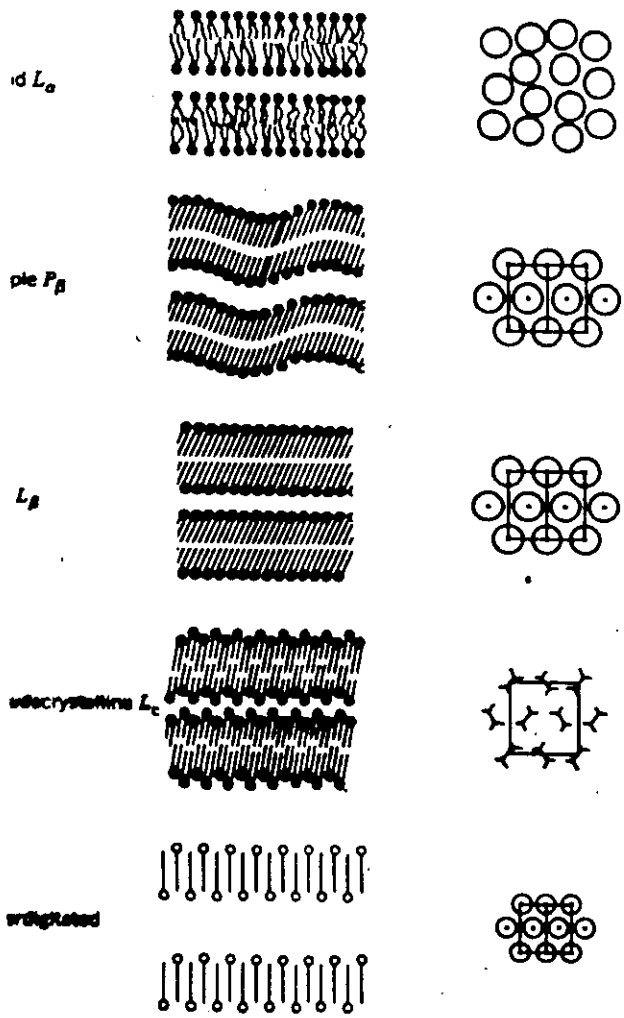
In aqueous solution phospholipids assume different structural forms: micelles, bilayers, vesicles/liposomes, tubules etc. The type of structure formed depends on the length and degree of saturation of the fatty acid acyl chains, substitution in the acyl chains and head group and ionic composition of the medium. A great deal of theoretical and experimental studies are in the literature to understand the various interactions of the amphipathic molecules leading to the fascinating self-assembled microstructures. The factors that determine the shape of the aggregate and the long-range order is the generalized shape of the phospholipid molecule, molecular organization and dynamics, and conformational transitions in the aggregate. For ex: cylindrical molecules organize into bilayers, cone-shaped molecules into micelles, wedge-shaped molecules into inverted micelles and hexagonal rods etc.



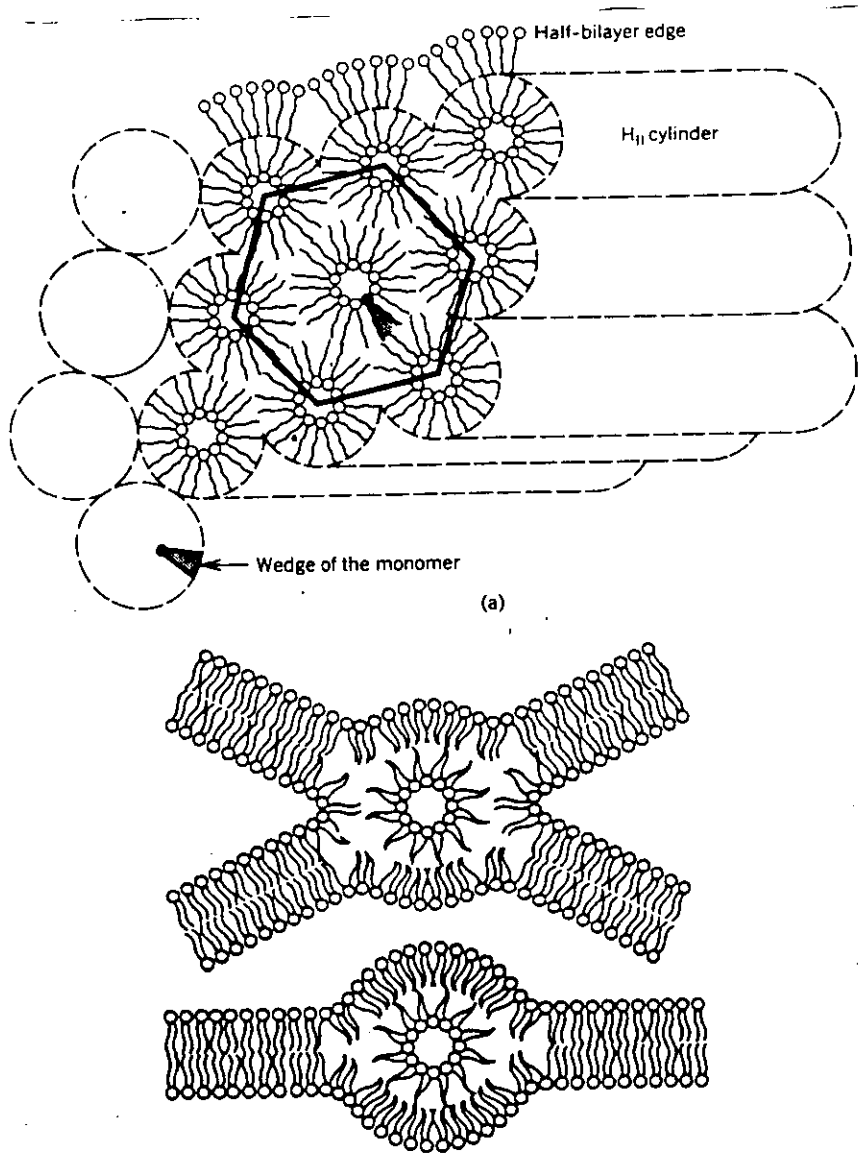
Schematic representation of some lipid-based microstructures. [Courtesy of R. W. Light and B. Gaber]

- **Phase characteristics of lipids in aqueous dispersions:**

Amphipathic molecules such as phospholipids aggregate in the aqueous phase to form a variety of phases which includes, monolayer, bilayer lamellar, micelles, hexagonal and cubic. Which phase predominates in a given system depends on the structure and conformation of the amphipath, the composition and water content of the mixture and environmental factors such as ionic strength, pH and temperature. Transitions from one phase to the other can be induced by changing the conditions. Also within the overall phases different states are also observed. For example, hexagonal phase can exist as hexagonal I or the hexagonal II depending on the organization of the molecules with in the phases. The lamellar bilayer phase organizes in the fluid, ripple, gel, pseudocrystalline or interdigitated states.



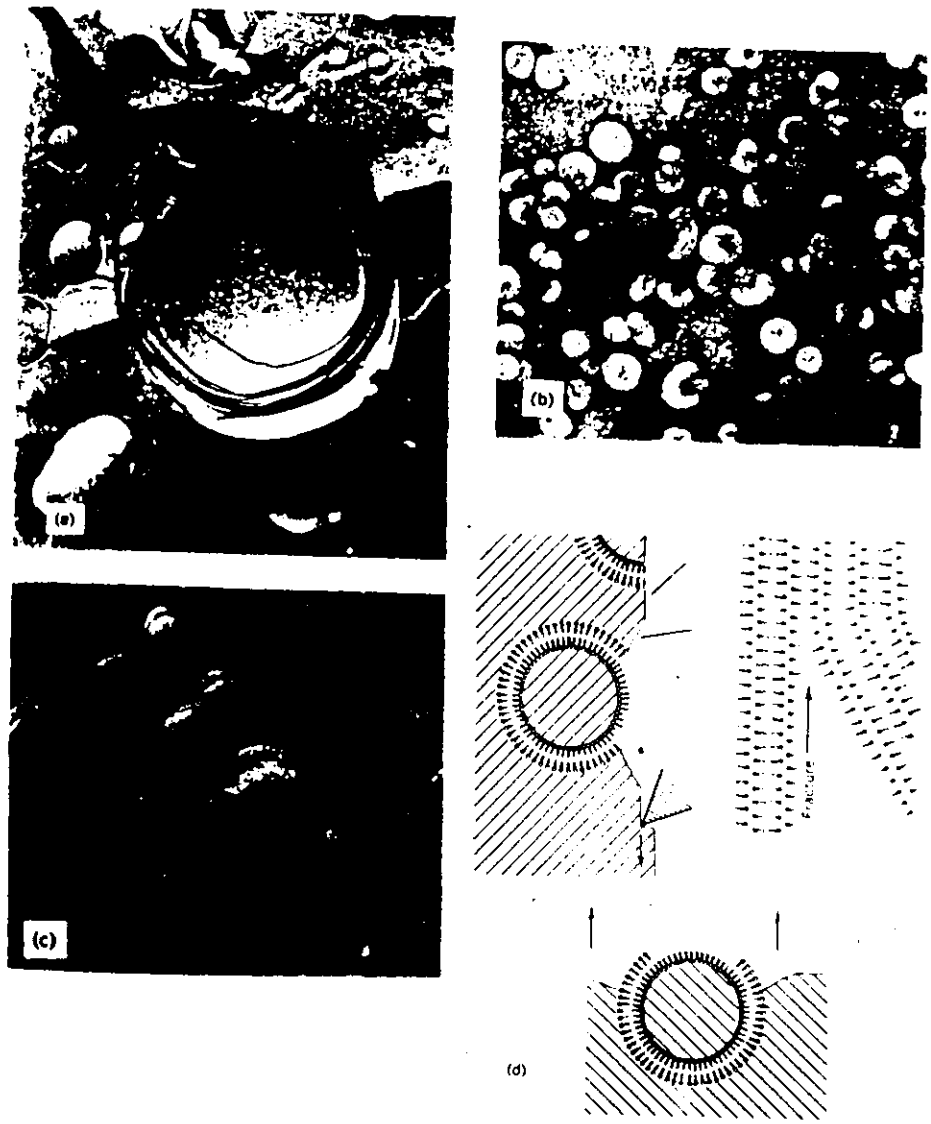
Organization of the lamellar bilayer phase in the fluid, ripple, gel, pseudo-crystalline, and interdigitated states (top to bottom). The top view is shown in the last column.



(a) A cross-sectional view of a bundle of water-filled tubes in hexagonal II symmetry. Note that the individual molecules in a tube are wedge-shaped ($s > 1$), that the interaction between cylinders is hydrophobic, and that the bundle of cylinders is separated from excess water by a bilayer. (b) In a nonpolar solvent some lipids can form inverted micelles. Two possible modes of integration of inverted micelles into bilayers are shown. (From Cullis et al., 1985.)

- **Liposomes:**

Edges of lamellar dispersion seal readily to form enclosed structures so that acyl chains are not exposed to the aqueous phase. Due to low interfacial pressure of the bilayer, such structures are generally spherical and their characters range from 50 nm to well over 5000 nm depending on the method of preparation and the structure and composition of phospholipid.



Electron micrographs of (a) multilamellar vesicles (MLV), (b) sonicated unilamellar vesicles (SUV), and (c) large unilamellar vesicles (LUV). Micrographs of MLV and LUV are obtained by freeze-fracture as shown in (d). When the sample is frozen the fracture plane passes through the middle of the bilayer. In c a pair of vesicles is captured just after fusion. The images in b are obtained by negative staining with uranyl nitrate on grids coated with Pioloform FN50 and then made hydrophilic by coating with carbon. (c is from Bearer et al., *Biochim. Biophys. Acta* 693, 93, 1982; a and b are through the courtesy of P. Kaufman.)

- **Experimental methods used for the study of lipid microstructures and phase properties:**

  -Nuclear magnetic resonance (1H, 2H, 31P & 13C NMR)

  -Differential scanning calorimetry (DSC)

  -Fluorescence spectroscopy (using fluorescent lipids and probes)

  -X-ray diffraction

  -Electron microscopy

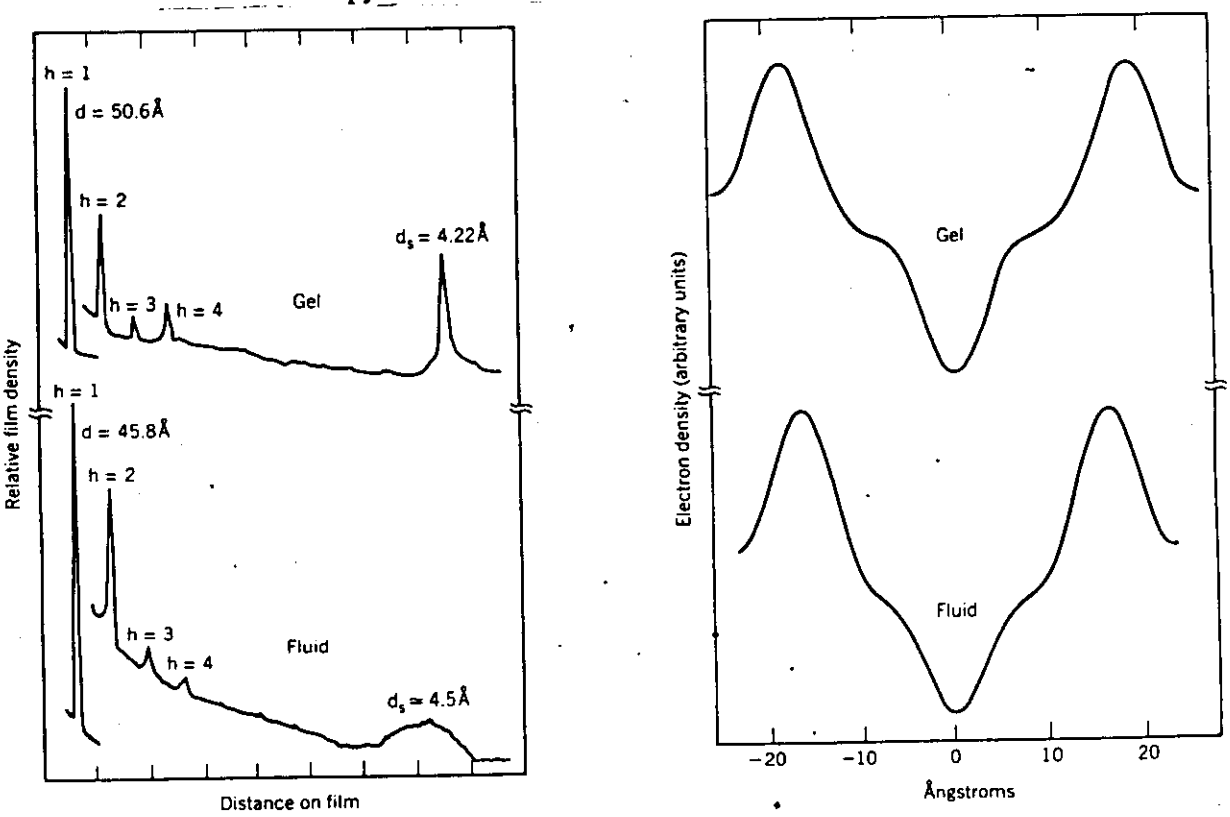
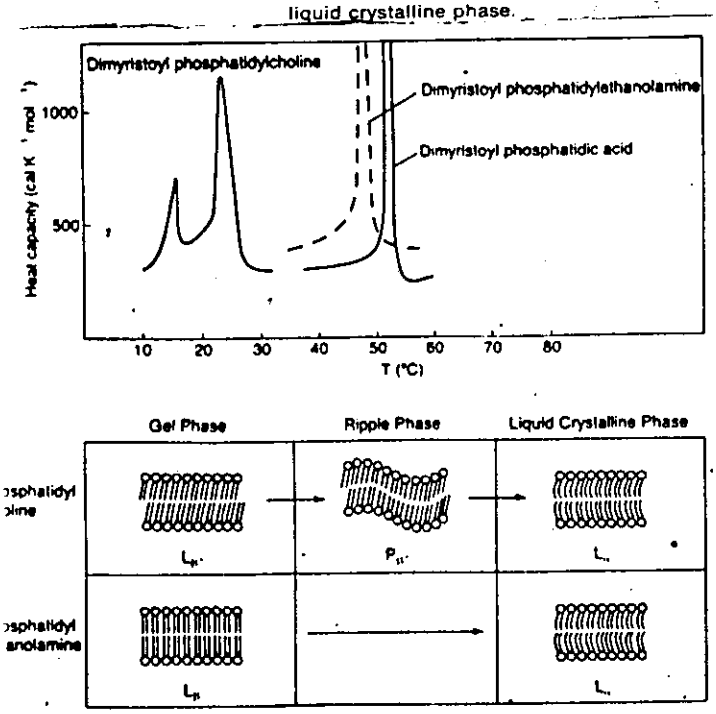
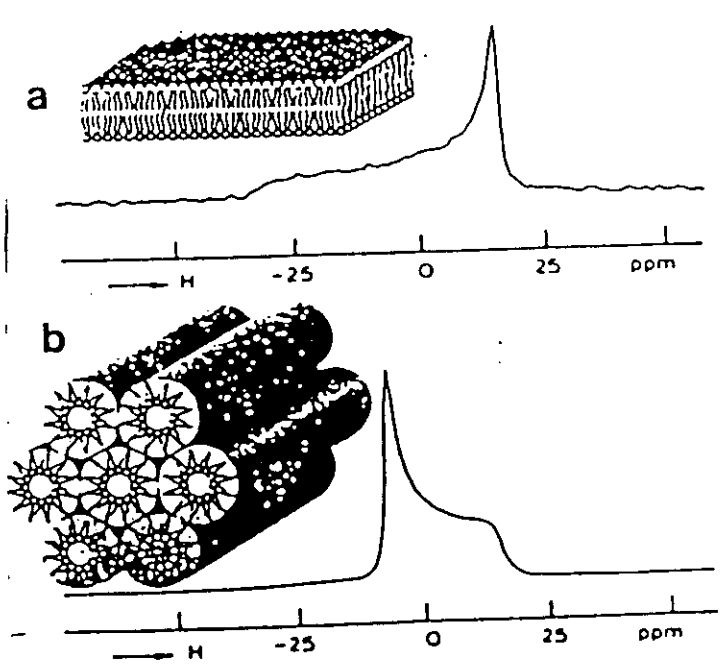


Fig. 3-15. (Left) Densitomer traces of X-ray diffraction profiles of hydrated dilauroylphosphatidyl ethanolamine in the gel and the fluid phases. For both phases, four orders (h = 1–4) of a lamellar repeat period are observed. (Right) Electron density profiles of DLPE in the gel and the liquid crystalline phase.



(A) Differential scanning calorimetry profiles of three phospholipids. Adapted from ref. 112. Reprinted with permission from "Apparent Molar Heat Capacities of Phospholipids in Aqueous Dispersion," by A. Blume, Biochemistry 22, pp. 5437-5438. Copyright 1983, American Chemical Society.(B) Schematic showing the molecular



31p NMR Spectra a) lamellar phase
b) hexagonal II phase

References:

1. J. Darnell, H. Lodish and D. Baltimore "Molecular Biology" : Scientific American books, W.H. Freeman & Co. New York, 1990.

2. M.K. Jain, "Introduction to Biological Membranes: : John Wiley & Sons, New York, 1988.

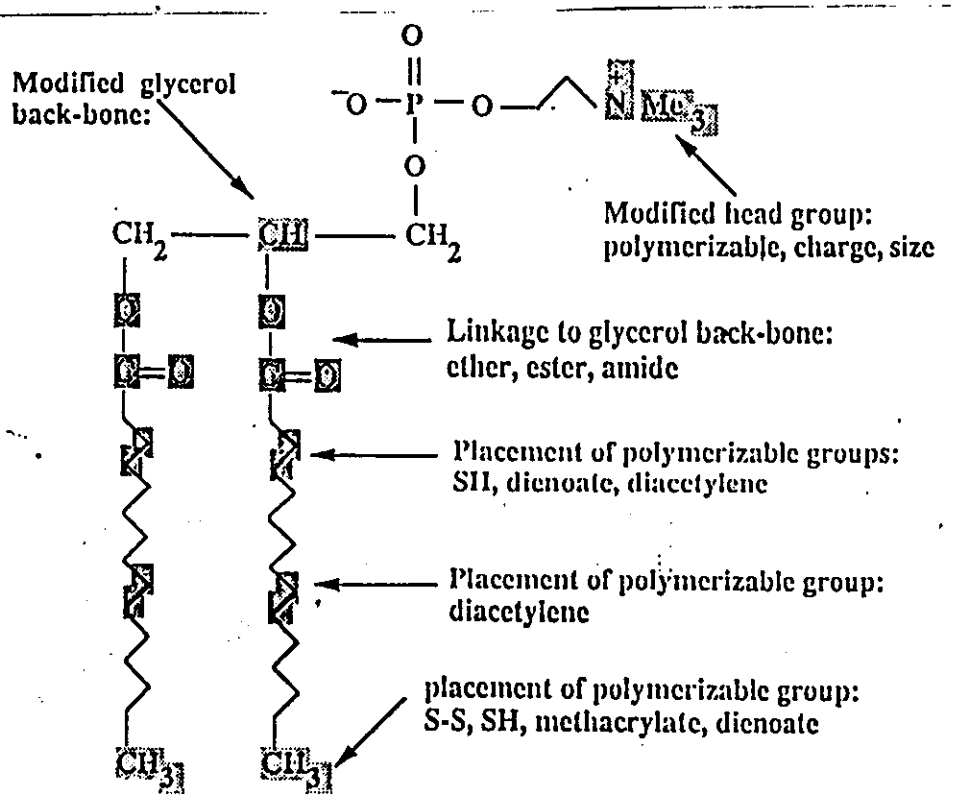
3. D. Lasic, "Liposomes", American Scientist **80**, 1992, 20-31.

4. M.J. Ostro, "Liposomes", Scientific American **256**, 1992, 103-111.

Structures of Lipid Tubules:

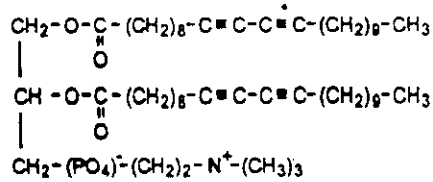
The self assembly of biologically based lipids into unusual microstructures has been the subject of intense study in recent years both for basic research and for potential applications (Schnur, J.M. Science **262**, 1669, 1993 and references therein). While most long-chain phsopholipids self-assemble into spherical bilayer aggregates, known as liposomes (American Scientist **80**, 20, 1992) certain synthetic phospholipids with modified head groups or acyl chains self assemble into novel microstructures under some conditions (A. Blume, Chem. & Phys. of Lipids **57**, 253, 1991; A. Singh and J.M. Schnur, Phospholipid Handbook, ed. G.Cevc Marcel Dekker Inc. 233-291, 1993). These lipids have been observed to self-assemble into hollow, cylindrical structures, known as tubules (Yager, P. and Schoen, P.E., Mol. Cryst. Liq. Crystal **106**, 371, 1984).

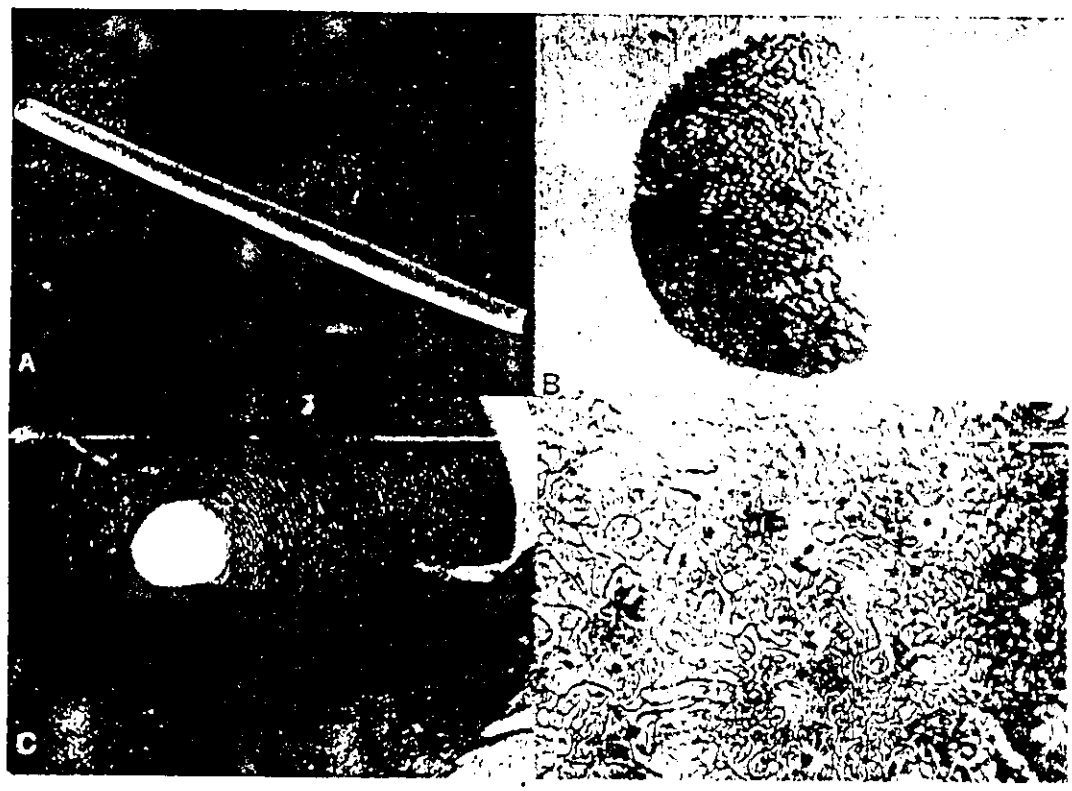
In this talk I shall present the details of the structures of the lipid tubules, conditions of formation, experimental studies on the chiral molecular architecture of these structures and potential technological and bio-medical applications.



Possible sites for the placement of polymerizable moieties and possible modifications in a phospholipid.

Diacetylenic Phospholipid ($DC_{8,9}PC$)





Three electron micrographs and one optical micrograph of four microstructures fabricated from diacetylinic lipids. (**A**) Tubule: The diameter of the tubule is ~0.5 μm. The wall thickness is ~200 Å. The length is ~30 μm. (**B**) Liposome with bacteriorhodopsin incorporated in membrane (dark). The diameter is ~0.7 μm. [Reprinted from (*65*) with permission © Elsevier] (**C**) A ring-like microstructure prepared by A. Singh and M. Markowitz. The inner diameter of the ring is ~1 μm. (**D**) Optical micrograph of fibrils prepared by cooling a diacetylenic lipid (*13*). Typical diameter of fibril is ~0.3 μm. [Reprinted from (*13*) with permission © Macmillan]

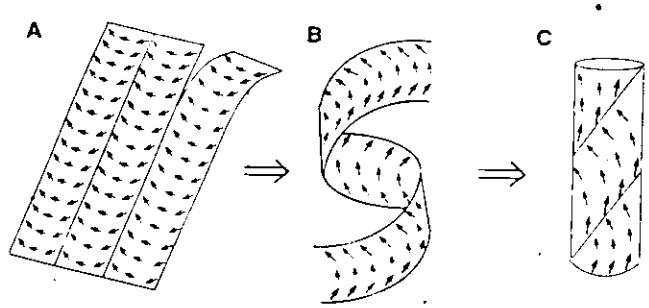


Fig. 2. Schematic illustration of a chiral bilayer (**A**), with the molecules tilted with respect to the local layer normal. (The arrows indicate the direction of the molecular tilt, projected into the layer plane.) The favored twist between chiral molecules leads the whole membrane to curve into (**B**) a wound ribbon, then fuse into (**C**) a cylindrical tubule. The observed CD spectra come from the chiral molecular packing common to all three figures, not from the micrometer-scale helical structure in (B) and (C).

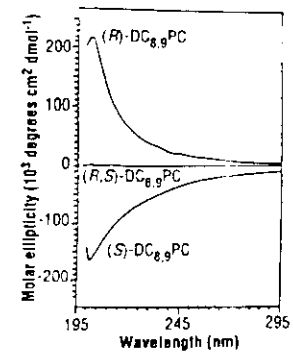


Fig. 4. CD spectra of (*R*)-$DC_{8,9}PC$, (*S*)-$DC_{8,9}PC$, and a racemic mixture in methanol containing 20% water. Concentration of the lipid was 0.33 mg/ml.

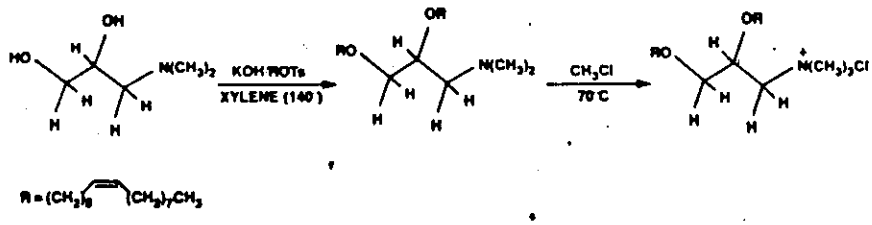
Cationic lipid-DNA complexes and their implications in Gene transfer:

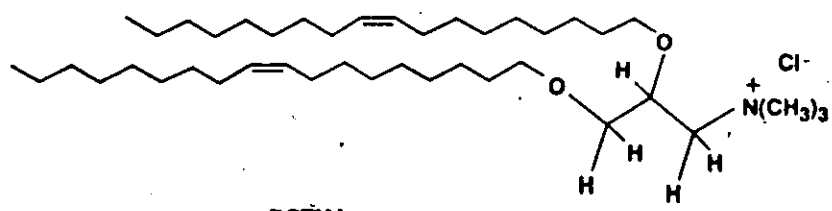
The basic processes in recombinant DNA technology involves isolation of specific genes, manipulation of the resulting nucleic acid sequence and the transfer of the DNA constructs into living cells. Reliable and efficient systems for introductions of DNA into Target cells has been the subject of intense study over the last a decade (Anderson W.F., Science **226**, 401,1984; **256**, 808, 1992. Due to the large size and charge of DNA and to the number of enzymatic and membrane barriers imposed by the cell, the spontaneous entry of intact DNA into the cell and subsequent expression is very inefficient. A number of methods have been developed for optimal gene delivery. The most frequently used delivery systems *in vivo* are viral constucts (ie.) genes inserted into viral genes and reconstituted into noninfective viruses (R.W. Old and S.B. Primorse, Principles of Gene Manipulation, 3rd Ed., Blackwell Sci. Publications, Oxford 1985, Chapter 4). Other applications include electroporation, calcium phosphate precipitation and use of polycations (Jose C. Perales et. al J. Biol. Chem. **272**, 7398, 1997; A. Loyter et al. Proc. Natl. Acad. Sci. **79**, 422, 1982; M. Maccarrone et al. Chapter 5, Vol. IV, Handbook on non-medical applications of liposome Ed. D.D. Lasic, CRC Press, 1995). However, none of the methods have been shown to be efficient as they suffer from one or more problems related to cellular toxicity, poor reproducibility and inefficiency of DNA delivery. The use of cationic liposomes to facilitate delivery of DNA first proposed by Felgner et al. (D.L. Felgner et. al, Proc. Natl. Acad. Sci. **84**, 7413, 1987 are one of the most promising on-viral systems for gene transfer. The possible use of cationic liposomes in DNA transfection has been the subject of numerous study in recent years (See Handbook of non-medical applications of Liposomes, Vol. IV, Ed. D.D. Lasie, CRC Press, 1995, Vol. IV Chapter 1-5). A large number of cationic lipids have been synthesized, complexed with DNA and cationic liposome mediated DNA transfection in many cell types have been demonstrated. However, different cationic-lipid formulation showed different levels of gene expression in a variety of cells, tissues and organs. The efficiency for DNA delivery depended on several properties of the complexes such as :

-cationic lipid species
-cationic lipid/DNA ratio and net charge
-DNA (and cationic lipid) dose concentration
-amount and identity of co-lipid
-vesicle composition and method of preparation
-cationic liposome size
-temperature during complex assembly
-cell type
-extracellular matrix elements
-DNA purity

In spite of the successful use of various cationic DNA complexes and continuous development of novel and higly efficient cationic liposomes, their mode of formation, structural and biophysical characteristics of the various complexes and mechanism of their action and DNA release from the complex are not well understood. One of the recent papers on the structure of DNA-cationic liposome compex using synchrotron X-ray diffraction and optical microscopy has thrown some light on the important structural parameters relevant to transfection efficiencies (J.O. Radler et. al Science **275**, 810, 1997). A mechanism to explain DNA release from cationic liposome/DNA complexes in cells have been proposed recently (Y. Xu and F.C. Szoka, Biochemistry **35**, 5616, 1996).

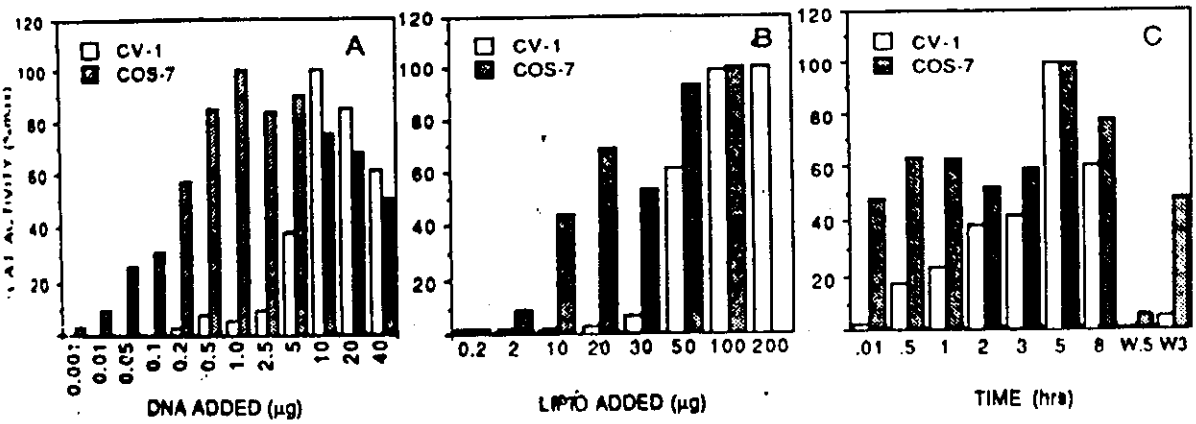
In this lecture I shall present an overview on the structural features of DNA-cationic liposome complexes and their implication for DNA delivery.





DOTMA

Chemical synthesis of DOTMA. ROTs, oleyl *p*-toluenesulfonate.



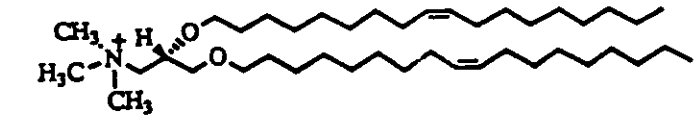
CV-1 (stippled bars) and COS-7 (open bars) cells were transfected with pSV2cat complexed with PtdEtn/DOTMA (1:1). The standard transfection technique described in *Methods* was used, with the variations noted below. Cells were harvested after 2 days and CAT activity (ng of cell protein) was determined. (*A*) Various concentrations of DNA with 100 μg of lipid were used in parallel transfections. (*B*) Various concentrations of lipid were used in parallel transfections with either 10 μg (CV-1) or 1 μg (COS-7) of DNA. (*C*) Cells were transfected using 30 μg of lipid and either 10 μg (CV-1) or 1 μg (COS-7) of DNA. After the time indicated, 10 ml of growth medium was added. W.5 and W3 represent parallel incubations where at the indicated time (0.5 and 3 hr, respectively), the lipid–DNA mixture was removed and replaced with 10 ml of fresh medium.

Stable transfection: Lipid compared with calcium phosphate

Cell line	Plasmid	Method	Frequency × 10^4
L-M(TK⁻)	pSV2neo	Calcium phosphate	3
		Lipid	45
ψ-2	pZIPSVX	Calcium phosphate	0.6
		Lipid	>49
ψ-2	pZIPC5a	Calcium phosphate	1.8
		Lipid	>68
MSN610.2	pSV2neo	Calcium phosphate	1.3
		Lipid	8.2
TA1	pSV2neo	Calcium phosphate	2
		Lipid	14
TA1	pZIPSVX	Calcium phosphate	0.7
		Lipid	17
TA1	pMSGC5a	Calcium phosphate	1.3
		Lipid	19

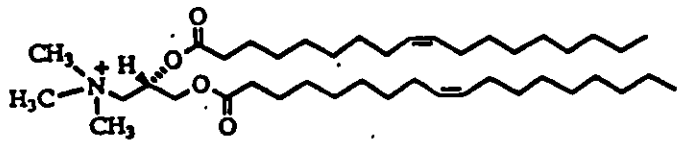
Cells were transfected with 7 μg of the indicated plasmid with no carrier DNA, except for pSV2neo where 1 μg of plasmid and 10 μg of carrier DNA were used. The transfection frequency is the number of drug-resistant colonies expressed as a fraction of the total number of cells plated.

a) **DOTMA**



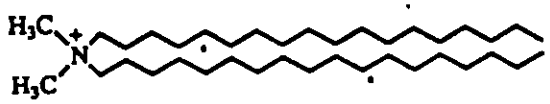
N-(1-(2,3-Dioleoyloxy) propyl)- *N,N,N*- trimethyl ammonium chloride

b) **DOTAP**



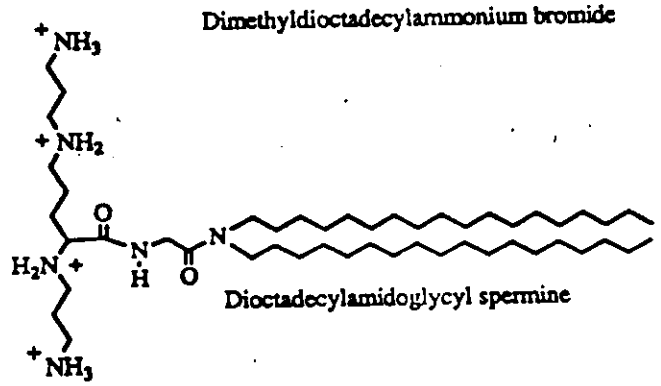
1,2-Dioleoyl-3-trimethylammonium-propane

c) **DDAB**



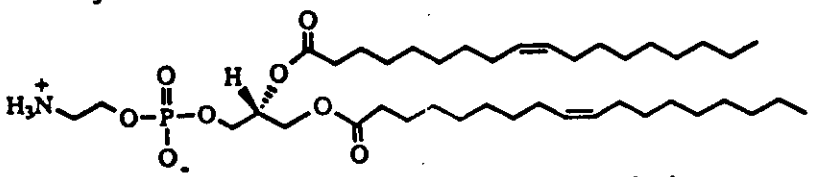
Dimethyldioctadecylammonium bromide

d) **DOGS**



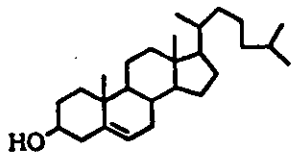
Dioctadecylamidoglycyl spermine

e) **DOPE**



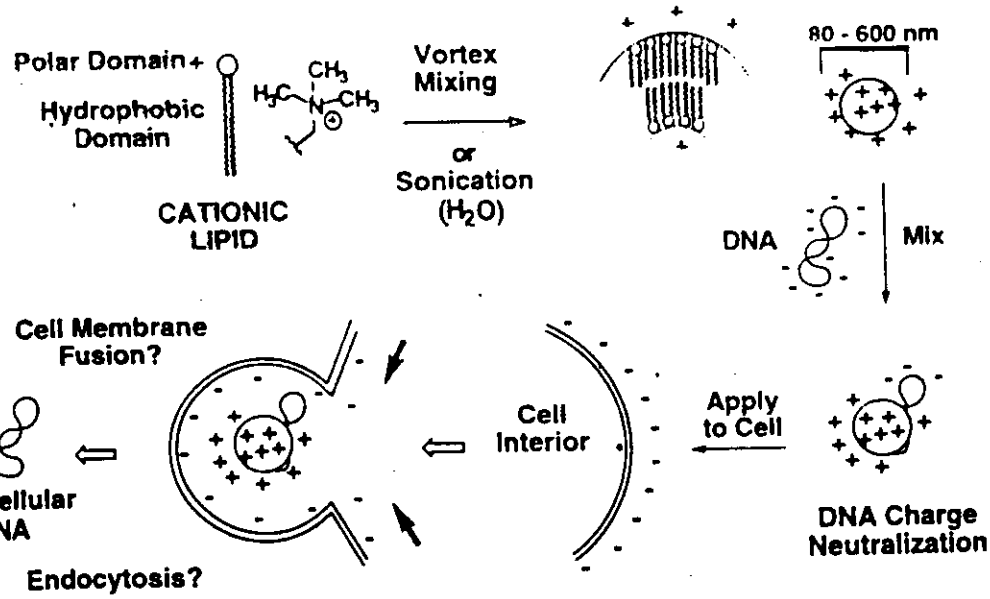
1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine

f) **Cholesterol**



a) to d) are cationic lipids e) and f) neutral lipids

Proposed mechanism of transfection



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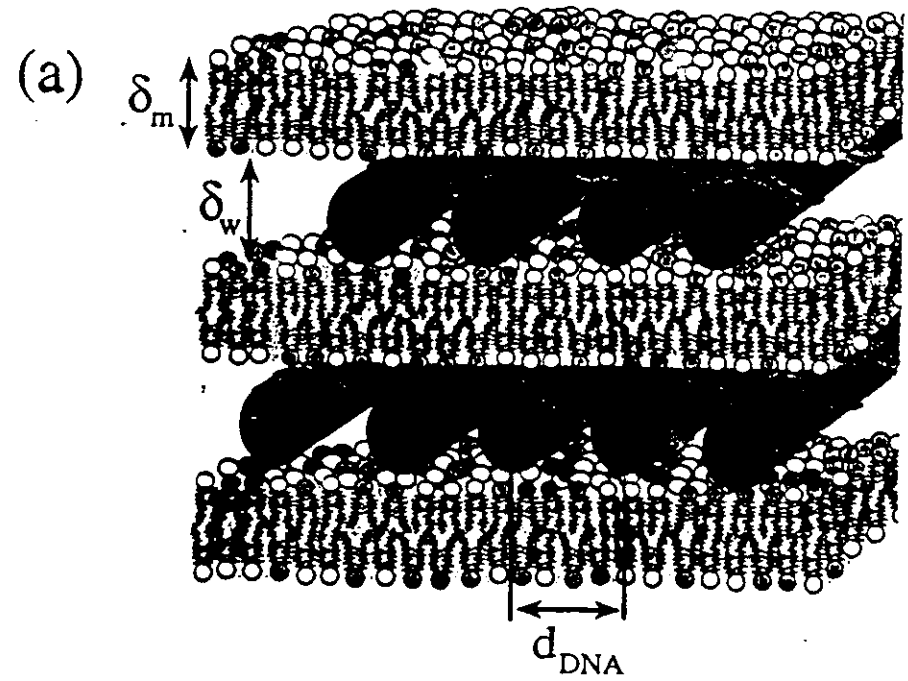
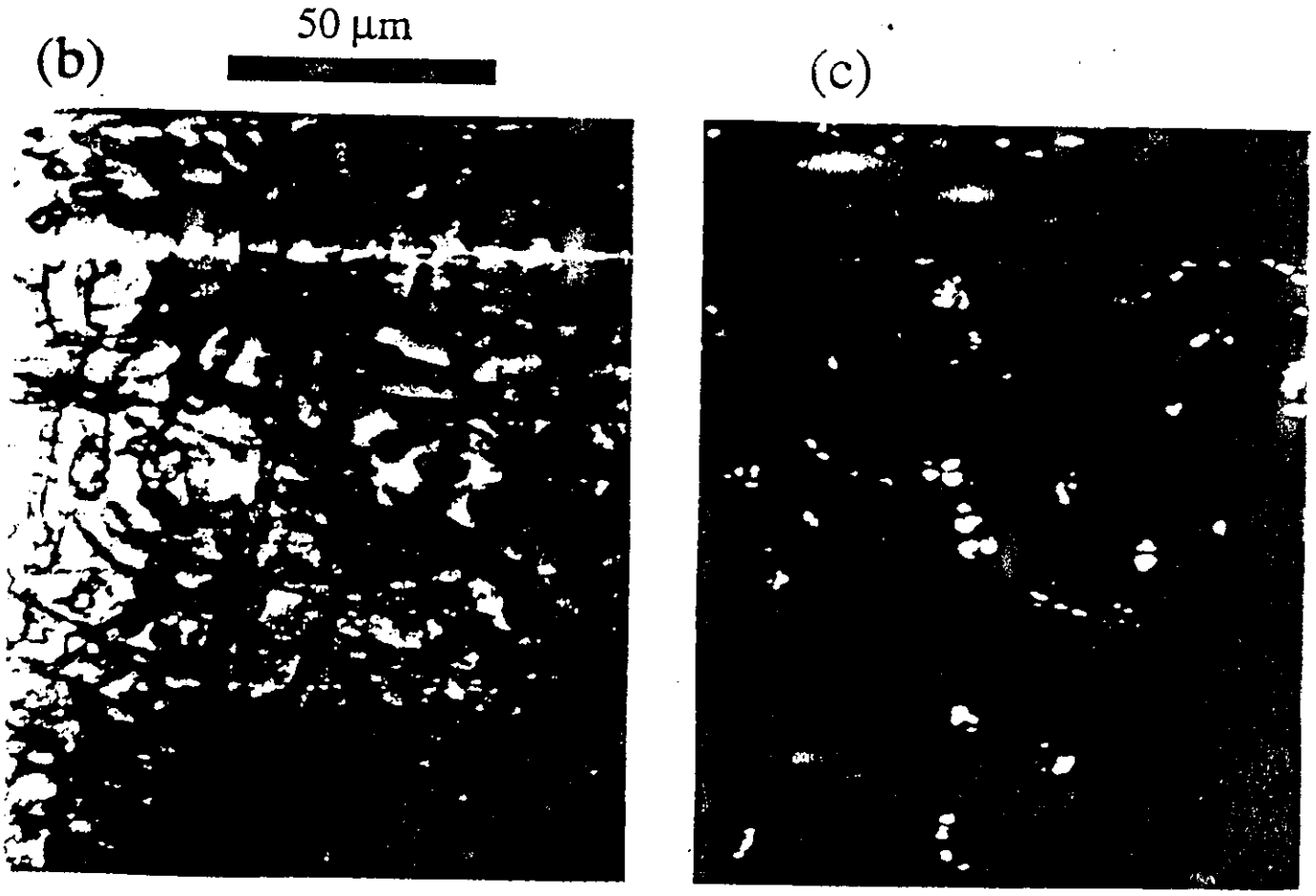
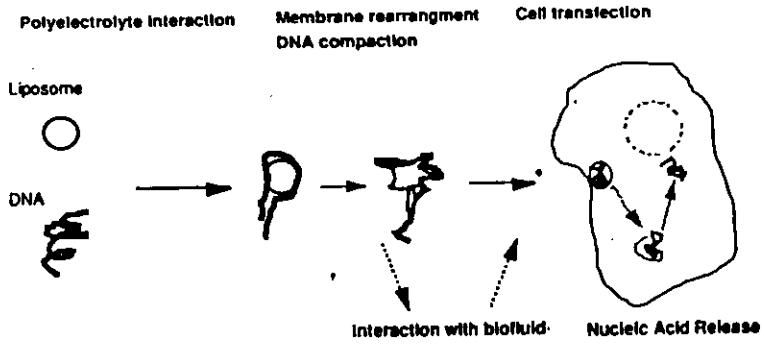
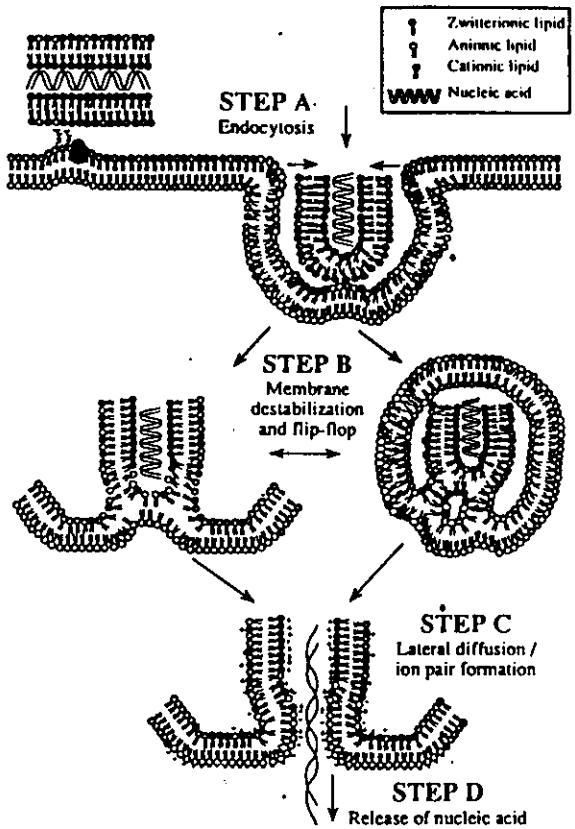




Schematic picture of the local arrangement in the interior of lipi[illegible]JA complexes (shown at two different concentrations in Fig. 1A and in (**B**) below. The semiflexible DNA [illegible]lecules are represented by rods on this molecular scale. The neutral and cationic lipids comprising the membrane are expected to locally demix with the cationic lipids (red) more concentrated near the DNA. Micrographs of DNA-lipid condensates under (B) bright light and (**C**) crossed polarizers showing LC-like defects.



Mechanisms Involved in Liposome/DNA Complex Nucleic Acid Delivery.



Schematic Representation of the Cationic Liposome/Nucleic Acid Complex Uptake Pathway and Mechanism of Nucleic Acid Release from the Complex. **Step A:** After electrostatic interaction with cell membrane, cationic liposome/nucleic acid complexes are endocytosed. **Step B:** In the early endosome, membrane destabilization results in anionic phospholipid flip-flop. Step C: The anionic lipids diffuse into the complex and form charge neutral ion-pair with cationic lipids. Step D: The nucleic acid dissociates from the complex and is released into the cytoplasm.

F. C. Szoka, Y. Xu and O. Zelphati
Jour. Liposome Res. 6, 567 (1996)