Thin Lipid Membranes with Aqueous Interfaces: Apparatus Designs and Methods of Study

ROBERT E. HOWARD[1] and ROBERT M. BURTON, Departments of Pathology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract

Thin membranes can be formed in aqueous media from amphiphilic lipids, and will spontaneously approach a limiting thickness of bimolecular dimensions (bilayers). This paper describes apparatus and methods for studying such thin lipid membranes, and illustrates their use in determining some of the basic properties of the membranes, especially bilayers.

Several methods of forming thin lipid membranes are described. The early stages in apparatus development are traced, and the theoretical variables and operational parameters relating to apparatus and system design are discussed.

Designs for two basic types of apparatus are presented in detail: one is a cylindrical chamber especially constructed to permit optical investigation of the membrane; the second is a multiple chamber system designed for the study of several different membranes either simultaneously or in rapid succession. Interchangeable chamber units are held in a thermostat block, and assemblies of electrodes and provisions for perfusion or sampling of aqueous medium are placed in the chambers as required. Methods are described which enable simultaneous mechanical, electrical, optical, and chemical operations and studies to be performed on the same membrane with either type of apparatus.

Membranes were formed from several purified amphiphilic lipids and from mixed-lipid extracts from a variety of biological membranes. The types and mechanisms of drainage of thin lipid membranes with aqueous interfaces are analogous to those previously described for aqueous soap films in air. The limiting bilayer thickness is confirmed by electrical measurements. The resistivity of the bilayers is ca. 10^{12} to 10^{14} ohm-cm, their capacity is ca. 0.4 μfd-cm^{-2} and their dielectric breakdown voltage is ca. 3×10^5 V-cm^{-1}. Other physical properties of the bilayers are described. Permeability of the bilayers to various substances was determined by diffusion flux, osmotic flux, and electrochemical potential methods using the apparatus described. Substances studied included water, small monovalent ions, glucose, acetylcholine, salicylamide and synaptic vesicles.

The chemical, physical, electrical, and permeability properties of the experimentally formed lipid bilayer membranes are similar to those of biological membranes. These similarities strongly support the Danielli-Davson hypothesis, which proposes that a lipid bilayer is the basic structure of biological membranes. The apparatus, methods, and information presented in this paper provide tools for further investigation of lipid bilayer membrane properties and for further testing of hypotheses relating to membranes.

Introduction

Biological Membranes

THE TERM "MEMBRANE" is derived from the Latin for a thin sheet of animal or vegetable tissue. Membranes may be many cells thick (multicellular membranes), a single cell thick (cellular membranes), or may be thin relative to the size of the cell (subcellular membranes). The plasma membrane, a cell surface layer functionally and morphologically distinct from the cytoplasm and from extracellular structures, has generally been considered as the prototype for subcellular membranes.

The ultrastructure, function, and chemistry of membranes are highly significant in all biological systems. The major ultrastructural cell components (organelles) are the plasma membrane, the nucleus, the endoplasmic reticulum, the Golgi apparatus, and the mitochondria. Membranes similar to the plasma membrane are an ultrastructural feature common to all of these cell organelles.

The functional integrity of the plasma membrane and of the membranes of cell organelles is essential to the living cell. The passive, facilitated, and active transport functions of absorption and secretion directly involve these membranes, primarily through selective permeability mechanisms which compartmentalize substances and regulate their concentration. Membranes play a central role in amoeboid movement, excitability phenomena, and synaptic transmission, and contain receptor sites for drugs and hormones. They provide structural order for enzyme arrays functioning in the mitochondrion, chloroplast, and endoplasmic reticulum, and participate in cell repair.

Chemically, all biological membranes contain amphiphilic phospholipid, that is, phospholipid containing a water-soluble polar group and a nonpolar hydrocarbon portion with a very low affinity for water. When hydrated, the favored conformation of these amphiphilic lipids is a lamellar liquid crystalline phase in which the lipid molecules are arranged in layers (lamellae) with their polar groups in a regular array, as in a crystal, while their hydrocarbon portions are in a liquid state.

Danielli-Davson Concept

Danielli and Davson in 1935 postulated that the basic chemical structure of the plasma membrane is a lipid bilayer, that is, an amphiphilic lipid lamella of bimolecular thickness, with protein adsorbed to it in some fashion (1). This concept was based primarily on three lines of evidence (2,3): first were the analyses of fatty substances extracted from cells and the assumption that these substances were concentrated at the cell surface. The classic study by Gorter and Grendel demonstrated sufficient lipid in red blood cells to form a monolayer film with twice the surface

[1] Portions of this material are from a dissertation presented in partial fulfillment of requirements for the PhD degree, Graduate School of Arts and Sciences, Washington University. Present address: Department of Pathology, University of Colorado Medical Center, 4200 East Ninth Avenue, Denver, Colorado 80220.

area of the extracted cells (4). Second, studies of interfacial tension (and other factors) suggested that protein was present on the surface of the lipid (5,6). Third, the movement of substances into and out of cells suggested that the primary permeability barrier was lipid (7), and that specialized regions enabled the passage of ions and large molecules (8). Much additional evidence supports the Danielli-Davson hypothesis.

Previous Models of Biological Membranes

The varied ultrastructural relations, multiple functions, and complex chemical composition of natural membranes make difficult the assessment of the relative contributions of specific molecules and atomic groups to various functional properties and structural details. Such correlations would be facilitated by an adequate model of biological membranes.

Many different systems have previously been studied as models of biological membranes, and have been extensively reviewed (3,9–11). These include, for instance, interfaces between bulk phases of aqueous solutions and lipid (12); micellar and colloidal suspensions (13); monolayers of lipid, protein, or synthetic polymers at the air-water interface (14); filter-supported lipid in aqueous media (15); and aqueous soap films in air (16).

Unfortunately, all of the above models fail to meet some of the criteria of analogy to natural membranes which a fully adequate model should possess. These criteria are examined in the Discussion. A major fault with all of these models is the inability to use them to adequately study permeability properties of the interface per se. The study of such properties is especially important since permeability mechanisms play a major role in natural membrane functions.

Thin Lipid Membranes with Aqueous Interfaces

Thin membranes can be formed in aqueous media from amphiphilic lipids, and will spontaneously thin and approach a limiting thickness of bimolecular dimensions (bilayers). Thin (bilayer) lipid membranes with aqueous interfaces theoretically fulfill the criteria of analogy to biological membranes which will be discussed, and should be usable in studies of membrane permeability. Therefore, development of apparatus and methods for studying such thin lipid membranes separating two aqueous phases was undertaken. Determination of some of the basic properties of these membranes was sought, and development was especially oriented toward determination of their permeability properties. This paper presents in detail the apparatus designs and methods which were developed, and illustrates their use with data on membrane properties.

The initial approach in this work was based upon the 1962 report of Mueller et al. (17), although thin lipid membranes in aqueous media had been described previously (2,18,19). During the five year period encompassed by this work, a number of other scientists from various disciplines have also studied the properties of thin lipid membranes. Their findings are related to this work in the Discussion.

Materials and General Methods

Reagents and Materials

The lipids, solvents, and radioisotopes of special importance to this work are given below. All other reagents were of the highest purity available and were used as obtained.

L-α-Phosphatidyl choline (L-α-lecithin) was satisfactory for membrane formation only when chromatographically purified. Commercially chromatographed egg yolk lecithin (Sigma or Pierce, Type III, Grade I) was used as obtained. Impure commercial lecithins extracted from various sources (Sigma Types II-E from egg yolk, II-S from soybean, and animal) and synthetic lecithin (Sigma Type II, β-γ-dipalmitoyl) were further purified by column chromatography as described in a subsequent section. Phospholipid preparations were analyzed for purity by paper or thin-layer chromatography (TLC), also described in a subsequent section.

Cholesterol was incorporated into some solutions used for membrane formation, and was satisfactory only when extensively purified. Cholesterol (Fisher or Sigma, USP) was twice dissolved in boiling 95% ethanol, mixed with activated charcoal, filtered, and allowed to crystallize. It was then oven dried and recrystallized from boiling absolute ethanol. Cholesterol (Sigma, standard for chromatography, 99+%) was used in preliminary studies as obtained.

Chloroform, methanol, and all inorganic salts (Mallinckrodt, AR) and *dl*-α-tocopherol (Merck, NF) were used as obtained from freshly opened bottles. The even-chain normal alkanes from *n*-hexane through *n*-octadecane (Distillation Products, practical grade) were passed through an alumina column to remove polar impurities and then fractionally distilled. L-Histidine (Calbiochem, NRC, A grade) and Tris (hydroxymethyl) aminomethane (Sigma, Trizma Reagent) were obtained as the hydrochloride and the free base, and Trizma Reagent as defined mixtures. Each of these types was used at various times in the preparation of buffers. Salicylamide (Distillation Products) was twice dissolved in boiling water, mixed with activated carbon, filtered, and recrystallized.

Radioisotope-labeled compounds were obtained from New England Nuclear Corporation and were used without further purification. They included tritiated water (THO,NET-1, 1 mc/mg), D-glucose-1-^{14}C (NEC-043P, 1 mc/mM), and acetylcholine methyl-^{14}C iodide (NEC-265, 17.2 μc/mg).

Water was distilled from a commercial tin-lined metal still into glass, then passed through a mixed-bed ion exchange column (Culligan Co., Northbrook, Ill.). The water thus produced had a resistivity greater than 1 megohm-cm (<0.1 ppm as NaCl).

Preparative Methods

Phosphatidyl Choline Purification by Alumina Column Chromatography. Phosphatidyl choline can be rapidly separated from crude phosphatide extracts and from aged, oxidized, or commercial preparations by column chromatography on alumina using a modification of the method of Singleton et al. (20). A slurry of 100 g of 80–200 mesh alumina (Fisher A-540) in 110 ml chloroform is poured into a 2.5 cm I.D. × 100 cm column (this results in an adsorbent height of ca. 93 cm), and washed with 50 ml chloroform.

Crude phosphatides (1–4 g, depending on the fraction of phosphatidyl choline present as determined by TLC) are dissolved in sufficient chloroform to make a 5% (w/v) solution and applied to the column. The effluent is collected beginning with this application. Neutral lipids are eluted from the column with 100 ml of chloroform, and phosphatidyl choline is eluted with 250 ml of a chloroform-methanol mixture (9:1, v/v, successive 25 ml fractions are collected).

The major phosphatidyl choline fraction (usually the third to fifth 25 ml fractions) can be recognized by the appearance of opalescence in the eluate. Ten μl samples of each fraction are analyzed by TLC on silica gel along with standard lipids. The phosphatides are developed in chloroform-methanol-water (65:25:4, v/v/v) (21), and detected with iodine vapor. Trace contamination with sphingomyelin may be present in some preparations from animal phosphatides.

Preparation of Fractions of Natural Origin. Mixed lipid extracts from whole tissues (e.g., rat brain) or isolated membranous fractions (e.g., synaptic vesicles) were obtained by a modification of the phase partition method of Folch (22). Fresh tissue or tissue fraction was homogenized for 2 min in 20 parts (v/w) of a nitrogen-saturated chloroform-methanol mixture (2:1, v/v) in a conical glass homogenizer at 4C. The homogenate was filtered through a Whatman No. 1 filter paper or a coarse sintered glass funnel under a nitrogen atmosphere. Two volumes of distilled water were added to the supernatant fluid and the mixture was emulsified by shaking. The lower organic phase containing a mixture of extracted lipids was recovered after brief centrifugation to break the emulsion.

Purified bovine and rat brain lipids were prepared from mixed lipid extracts by TLC on silica gel, using a solvent mixture of chloroform-methanol-water (70:30:5 or 65:25:4, v/v/v) (21). Bovine brain proteolipids were isolated and purified by the method of Folch-Pi (23).

Rat liver mitochondria and microsomes were prepared by homogenizing freshly dissected rat liver for 40 seconds in 10 volumes of 0.32 M sucrose containing 5×10^{-4} M eserine and 1×10^{-5} M $CaCl_2$ in a Waring Blendor. Nuclei and cell debris were removed by centrifugation at 3,000 rpm ($1,084 \times g$) for 10 min in a Servall Model RC-2 refrigerated centrifuge. Rat liver mitochondria were sedimented by centrifugation at 8,500 rpm ($8,700 \times g$) for 10 min, and rat liver microsomes were obtained from the supernatant fluid by centrifugation at 17,000 rpm ($34,800 \times g$) for 30 min.

Bovine brain myelin purified by the method of Laatsch et al. (24), human erythrocyte ghosts prepared by the method of Dodge et al. (25), and a mycelial suspension of *Neurospora crassa* were also used.

Rat brain synaptic vesicle fractions (M_2) were prepared by De Robertis' method (26) of differential centrifugation, hypo-osmotic shock, and separation by ultracentrifugation on a continuous sucrose density gradient in a Beckman Spinco ultracentrifuge (Model L or L-2).

Preparation of Solutions. Solutions used to form membranes were prepared from a variety of materials which are listed in Table IV. Mixed lipid extracts were used directly or dried under reduced pressure under N_2 and dissolved in 5.2 volumes (relative to the original tissue or tissue fraction weight) of chloroform-methanol (2:1, v/v), chloroform-methanol-α-tocopherol (2.2:2.8:1.0, v/v/v) or chloroform-methanol-*n*-alkane (2.2:2.8:1.0, v/v/v). The resulting solutions were approximately 2% in mixed lipids. In some cases cholesterol was added to portions of these solutions to give a final cholesterol concentration of 0.5–5.0% (w/v).

Membrane-forming solutions were also prepared from purified lipids in the same solvent mixtures at 0.5–2.0% (w/v) concentrations, or in pure hydrocarbon at 1×10^{-2} M phosphatide with or without 2×10^{-2} M cholesterol. Membrane-forming solutions were still usable after storage under nitrogen at 4C in screwcap vials for periods up to two months, or in sealed ampules for over one year.

The composition of the aqueous media was varied widely depending on the experiments. Two solutions frequently used were histidine-buffered Tyrode solution for mixed lipid extract membranes, and Tris-buffered saline for pure phosphatide membranes (Table I). Mercaptoethanol or Dithiothreitol (threo-2, 3-dihydroxy-1, 4-dithiobutane) (27) were added to some saline solutions at 0.001 M concentration to retard lipid oxidation.

General Analytical Methods

Thin Layer Chromatography. Thin-layer chromatography on silica gel was used to analyze mixed lipid extracts, to monitor column effluents, and for small scale preparations. Thin (250 μ) layers of silica gel (Adsorbosil-2, Applied Science Laboratories) were spread on glass plates by standard techniques (28) and activated by heating in an oven at 110C for 1 hour. Samples and standard mixtures (Applied Science Laboratories) were spotted at the origin and separated by ascending chromatography with appropriate solvent mixtures. Total lipid extracts and crude phospholipids were separated with chloroform-methanol-water (65:25:4, v/v/v) (21) or with diisobutylketone-glacial acetic acid-water (8:5:1, v/v/v) (29). Glycolipids were separated with *n*-butanol-pyridine-water (6:4:3, v/v/v) (30). Neutral lipids were separated with *n*-heptane-diethyl ether-glacial acetic acid (73:25:2, v/v/v) (31) or *n*-heptane-diethyl ether-ethyl acetate (80:20:3, v/v/v) (32). After separation lipids were visualized by exposure to iodine vapor, charring with sulfuric acid and heat, or illumination with ultraviolet light after staining with alcoholic Rhodamine 6 G.

Paper Chromatography. Paper chromatography was used for analysis of neutral lipids and phospholipids. Commercial silica gel impregnated filter paper (Whatman grade SG-81) was used with solvent systems suggested by Marinetti (33). Neutral lipids were separated with *n*-heptane-diisobutyl ketone-acetic acid (85:15:1, v/v/v). Phospholipids were

TABLE I
Composition of Aqueous Media

Aqueous media	Components	Concentration mM/l
Histidine-buffered Tyrode solution	NaCl	129
	$NaHCO_3$	6
	Na_2PO_4	2
	KCl	4.2
	$CaCl_2$	5
Adjust to pH 7.3 at 37C with HCl or NaOH	$MgCl_2$	1.6
	Histidine	5
Tris-buffered saline	NaCl	100
	Tris (hydroxymethyl) aminomethane	50
Adjust to pH 7.4 at 25C with HCl or NaOH	Mercaptoethanol (when present)	1
	Dithiothreitol (when present)	1

separated by diisobutyl ketone-acetic acid-water (40:25:5, v/v/v) or chloroform-methanol-water (65:25:4, v/v/v). Phospholipids were detected by the tricomplex stain of Hooghwinkel and van Niekerk (34).

Radioisotope Determination. Radioisotope-labeled compounds containing ^{3}H or ^{14}C in aqueous solution were measured by adding aliquots (up to 0.1 ml) to 4.0 ml of Bray's solution [5.0 g naphthalene, 0.7 g 2,5-diphenyloxazole (PPO), and 0.005 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in one liter *p*-dioxane] (35) and 0.75 ml toluene-fluor solution (4.0 g PPO and 0.1 g POPOP in one liter toluene), mixing, and counting in a Packard Tri-Carb Model 314EX Liquid Scintillation Counting System.

Fluorometric Salicylate Determination. Salicylic acid and other salicylate derivatives, including salicylamide and methyl salicylate, were assayed by a modification of the fluorometric procedures of Saltzman (36), Udenfriend et al. (37,38), and Chirigos and Udenfriend (39). Aqueous samples (25–100 μl) were mixed with 0.5 ml of 0.5 M borate buffer (pH 10.0) at room temperature. Fluorescence was measured on a Farrand Model A Photoelectric Fluorometer using a Corning Glass No. 5860 primary filter and Corning Glass No. 4308 and No. 3389 secondary filters. Appropriate standards were run with each experimental group.

Photographic Methods. Black and white, perforated 35 mm Kodak Tri X film was used for photographically recording the tracings on the oscilloscope screen with a Grass Kymograph Camera. The appearance of the membranes was recorded with color photomicrographs made on Kodak High Speed Ektachrome EH 135, Daylight Type (ASA Speed 160).

Experimental Procedures: Apparatus Development System Design, and Specific Methods

Methods of Membrane Formation

Basic Approach. The formation of a lamellar lipid membrane separating aqueous phases is conceptually simple (Fig. 1): two compartments are separated by a thin partition and communicate through an aperture in the partition. The compartments are filled with aqueous medium and a (thick) membrane of amphiphilic lipid is formed in the aperture separating the aqueous phases. The membrane spontaneously thins by draining centrifugally to form a ring or annulus of bulk phase lipid around the margin of the aperture. At equilibrium a metastable lipid membrane of bimolecular thickness continues to separate the aqueous phases.

"Painting" Method. A number of methods have been developed to form thin lipid membranes separating two aqueous phases. The "painting" method used in this work was originally described by Mueller et al. (17,40). A 2 mm diameter aperture was drilled in the thinned side of a polyethylene pH cup (5 ml, Beckman). The pH cup was filled with saline and was placed in a Petri dish (ca. 50 ml capacity) which was also filled with saline. A trimmed number 3 sable hair artist's bright (brush) was dipped in a mixed lipid extract and a film of lipid was spread over the aperture.

Since the sable hair brushes contaminated the membrane-forming materials and disintegrated in a few weeks, they were replaced by polyethylene or Teflon spatulas, which improved the method of spreading the lipid film over the aperture. Polyethylene stirring devices called "Plumpers" (California Corporation for Biochemical Research) were heated at their broad end over a Bunsen burner, and the softened polyethylene was spread and thinned by squeezing it with smooth-jawed metal pliers, forming a tapered blade with the major axis at approximately 45 degrees to the long axis of the handle. The sides and end of the tapered blade were further shaped and trimmed with a razor blade. Similar spatulas were formed from 1/8 in. sheet Teflon with a razor blade. These spatulas could be cleaned thoroughly after each use, did not contaminate the membrane-forming solutions, and lasted for years.

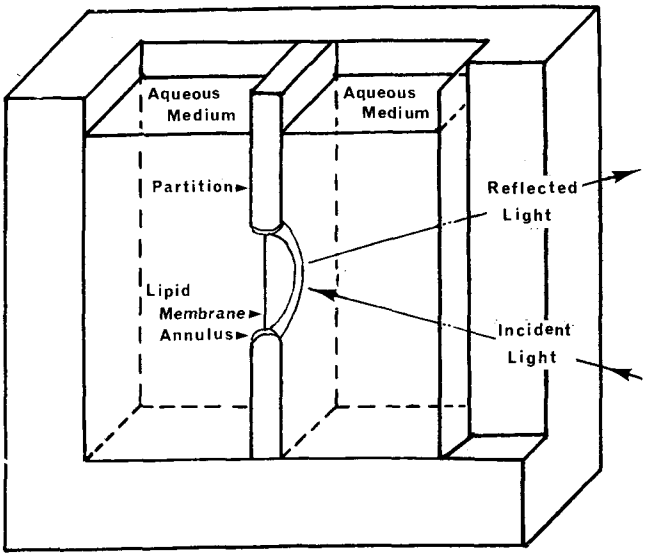


FIG. 1. Cutaway schematic diagram of a thin lipid membrane separating two aqueous phases, and the chamber in which it is formed.

A technique using a short length of Teflon tubing on the end of a syringe was tried, as recently reported (41), but the spatulas proved more satisfactory with the larger holes used in this work.

Dipping Method. A dipping method was also used to form membranes for simple visual observation or for testing the efficacy of membrane-forming solutions. Thin glass or polyethylene loops or Teflon O rings were dipped into the lipid solution and then transferred through the air to a beaker containing aqueous medium.

Bubble-Blowing Method. A method of blowing nonsupported membranes was tried early in the course of this work. A 1 ml tuberculin syringe tipped with a blunt-ended 22 gauge needle was filled with aqueous medium, the needle was dipped into and coated with membrane-forming solution, and a bubble "blown" in a beaker of aqueous medium. This method of membrane formation was not further pursued because: optical and electrical characterization of the spherical membranes so formed was difficult; light was reflected only from a small area of the surface; and the available electrical apparatus was not adequate. Mueller and Rudin (42) have reported using the same method, and a recent report by Pagano and Thompson (43) describes in detail the formation of spherical lipid bilayer membranes and their electrical characterization after impaling them with glass microelectrodes. A related but somewhat different method has been described by some Russian workers (44).

Marginal Suction Method. Preliminary experiments showed that supported membranes could also be formed by a marginal suction method similar to that reported for soap films (45). A Teflon O ring (7 mm

O.D. × 3 mm I.D.) was drilled through from the outside to the lumen side and supported by the hypodermic needle of a Hamilton microliter syringe (No. 1705). The syringe was filled with membrane-forming solution and the needle and O ring were immersed in thermostated aqueous medium. Membranes were formed by ejecting solution from the syringe until the O ring lumen was completely filled with a drop of lipid. The bulk phase lipid solution was then withdrawn back into the syringe, causing the drop of lipid to become a biconcave disc. As the lipid solution was further withdrawn by suction on the margin of the disc, the concave surfaces met and formed a planar membrane, supported by an annulus of bulk phase lipid as in the case of "painted" membranes.

Early Stages of Apparatus Development

Simple Chambers. The initial steps in development of the system were the design and construction of several simple chambers consisting of glass or plastic vessels containing some form of partition for supporting the lipid membrane. Each chamber was filled with aqueous medium, a lipid film was painted over an aperture in the partition and reflection of light from the film was observed by the naked eye or with low power magnification. Many supporting materials, methods of making apertures, and methods of fixing the supporting partition in place were evaluated, but details will not be presented here.

All-Glass Chamber. An all-glass chamber was constructed with a fire-polished aperture in the glass partition for film support. A water jacket for temperature regulation and a transpartition valve for equalization of hydrostatic pressure were added. The latter prevented bulging and rupture of the lipid film by the hydrostatic pressure gradient which was created by displaced aqueous medium as the brush was withdrawn. The glass chamber was easy to clean and was chemically inert, but the glass partition did not support membranes as well as polyethylene, and the inner surfaces of the water jacket became dirty with use, could not be adequately cleaned, and thus prevented visualization of the membrane.

Early Lucite Chamber. A simple design utilizing two Lucite half-chambers, between which a separate partition could be firmly clamped, allowed interchangeable partition materials and aperture sizes, easy disassembly for cleaning, and ready access for further modification. Fig. 2A is a schematic diagram of a design with cylindrical compartments for the aqueous media, wells for tiny magnetic stirring bars, and access for a thermometer, for stainless steel or platinum conductivity electrodes, and for pH electrodes. Small capillary tubes connected to each compartment enabled the addition of reagents or withdrawal of samples by micropipettes. A chamber of this design was constructed from clear Lucite (Fig. 2B), with a flattened "optical" surface for viewing the light reflected from the membrane. The chamber was not water-jacketed and this prevented precise control of temperature, although the chamber proved generally quite useful.

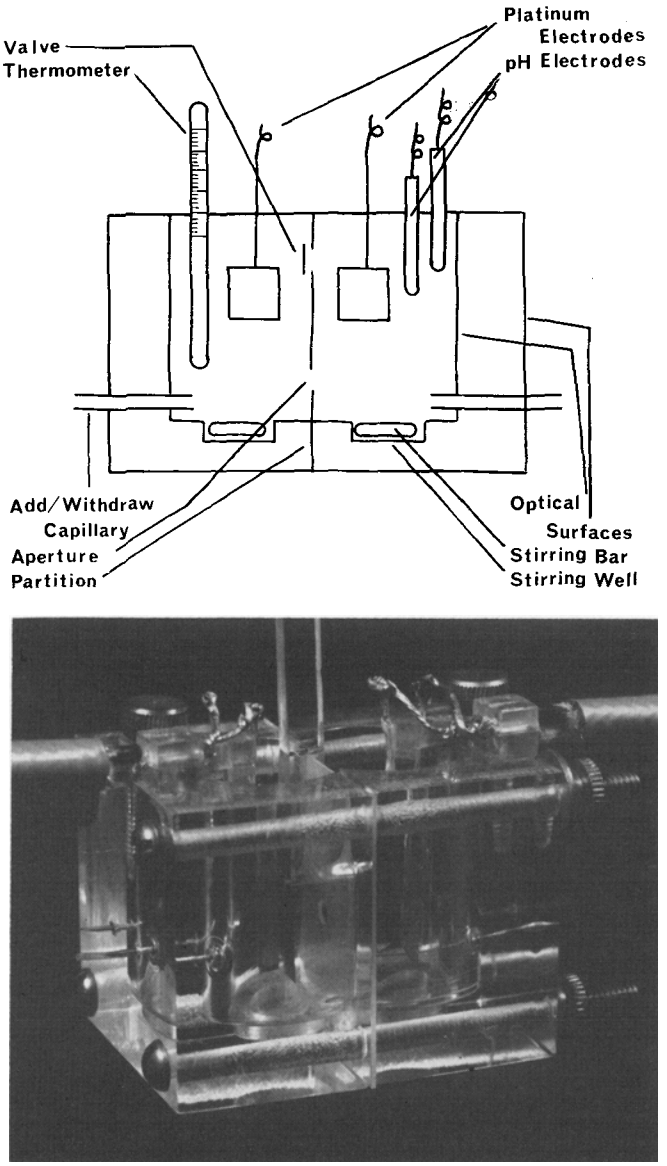


FIG. 2. Early design Lucite chamber. *A*, above schematic diagram indicating components used for the study of thin-lipid membranes; *B*, assembled chamber.

TABLE II

Outline of Theoretical Variables for Thin Lipid Membranes in Aqueous Media

- Annulus
  - Quantity
  - Composition
- Aqueous medium (in each compartment)
  - Quantity
  - Composition
    - Molar chemical composition (molarity)
    - Molar ionic composition (ionic strength)
      - Hydrogen ion concentration (pH)
    - Osmolar concentration
  - Homogeneity
- Gas phase (when present)
  - Quantity
  - Composition
- Membrane
  - Quantity
    - Thickness
    - Area
  - Composition
  - Homogeneity
  - Symmetry
- Pressure (environmental)
- Temperature
- Transmembrane gradients
  - Molecular concentration
  - Ionic concentration
  - Osmolar concentration
  - Electromotive force
  - Pressure

Theoretical Variables in Apparatus and System Design

Introduction. The multiple variables in any system must be controlled, nulled, or quantitatively measured to derive valid and meaningful information from that system. Experience with early chambers contributed to a theoretical analysis of the lipid membrane model, which yielded the variables discussed in this section and outlined in Table II.

The variables which may readily be controlled are the aqueous media, the gas phase (when it is present), the environmental pressure, and the temperature. The various transmembrane gradients may be nulled in more simple cases, and may be controlled or measured in more elaborate situations. The bulk phase lipid annulus and the membrane are variables which can-

not be so well controlled.

Annulus. Ideally, in the complete thin lipid membrane system, the total quantity of lipid present in the system will be distributed between the annulus and the membrane. However, the major portion of the lipid is present as the annulus which surrounds and directly supports the membrane, and the quantity in the membrane is insignificant relative to the quantity in the annulus. There may also be certain portions of the total lipid present as a thin film on the partition, as droplets or a film on the surface of the aqueous medium, in free solution, in micellar solution, or as a fine suspension in the aqueous medium. Although it is theoretically possible to control the amount of lipid initially introduced into the system, this is not possible with the "painting" method. However, the marginal suction method enables approximate measurement, with a microsyringe, of the total amount of lipid (membrane-forming solution) introduced into the system.

Theoretically also, the composition of the lipid in the annulus may be controlled, at least in the case of a membrane-forming solution containing only one component. However, multicomponent solutions are required to form the membranes at present, creating the problem of the relative distribution of a given component between the annulus and the membrane. For substances with significant solubility in the aqueous medium, the problem is further compounded. Such solubility introduces the possibility of a gradual but significant change in both annulus composition and in the total quantity of material present in the annulus. Conversely, material from the aqueous medium may become incorporated into the annulus. Although sufficiently sensitive methods of analysis are available to determine the general composition of the annulus, they have not been utilized since they can provide no information about membrane composition.

Aqueous Medium. The quantity (volume) of aqueous medium in each compartment adjacent to the membrane can be controlled at the stage of chamber design and again in actual use. However, gradual changes in volume may be caused by hydrostatic or osmotic gradients. The composition of the aqueous medium can be controlled prior to forming the entire system. The major aspects of composition are the molar concentration (molarity) of the various constituent substances, the pH, and when salts are present, ionic strength. Because osmotic pressure gradients can cause membrane deformation and volume flow of water, osmolar concentrations should generally be the same in each aqueous compartment.

The homogeneity of the aqueous medium is a variable of considerable importance in this system, because many of the theoretical treatments of similar systems assume homogeneity. It is also important operationally since in determinations of membrane permeability the composition of the aqueous medium in the entire compartment is assumed to represent the composition at the aqueous-lipid interface. When differences in composition of the two aqueous media exist across the membrane, the movements of solute and solvent along their respective concentration gradients across the membrane will cause local inhomogeneities in the aqueous medium. In addition, the membrane may cause a local ordering of water, creating a boundary layer which will cause the true transmembrane concentration gradient to differ from that in the bulk phases. Theoretically, all but the last of these factors may be overcome by sufficiently vigorous stirring.

Gas Phase. The model system as described contains a gas phase over each aqueous medium which is usually contiguous with the atmosphere. However, the gas phase can be part of a closed system or can even be absent. When it is part of a closed system, its quantity, composition, and pressure must be considered. In the usual situation the quantity of gas phase is infinite, its pressure is atmospheric, and its composition is either that of air or of some superfusing gas, such as nitrogen.

Membrane. The membrane is the most important variable in the system, and the most difficult to control. Thick, newly-formed lipid membranes and thinning membranes contain a continuously decreasing quantity of lipid. The quantity of membrane lipid can be estimated from the membrane volume by taking the product of membrane area and membrane thickness. Available optical methods can determine these parameters, but they require an inordinate amount of time relative to the significance of the information obtained on thinning membranes. In the case of bilayers, thickness and area measurements are more straightforward. For instance, a bilayer of 1.0 mm^2 area and 50 Å thickness would have a volume of 5×10^{-12} liters. If a reasonable value of 50 $Å^2$ surface area per lipid molecule is assumed, a bilayer of 1.0 mm^2 area would contain 4×10^{12} molecules or 6.7×10^{-12} moles of lipid.

The composition of the membrane is of paramount interest. However, the quantity of lipid is minute and since multicomponent solutions must presently be used to form membranes, the problems of composition discussed above for the annulus become greatly magnified. The membrane area is great relative to its volume even for thick membranes and the dissolution of substances from the membrane phase into either aqueous medium is therefore hastened. The absorption of substances from the aqueous phase by the membrane is likewise hastened. Because there is a very great difference between the volumes of the membrane and of the annulus, a large change in the composition of the membrane due to altered distribution of components would result in only a minute change in the composition of the annulus. Methods of sufficient sensitivity to directly analyze the membrane composition are available, but no method has been developed to obtain a sample of the membrane distinct from the annulus.

There are two aspects of membrane composition which deserve attention—membrane homogeneity and membrane symmetry. In thick and thinning membranes the composition of the interfacial surface may be different from that of the bulk phase lipid in the interior. Theoretically, composition may also vary in the plane of the membrane, even at the bilayer level. No methods have been developed to detect or analyze homogeneity at this level. Thin lipid membranes in aqueous media contain two interfaces, which generally are symmetrical in their composition, but it is theoretically possible for the two interfaces to be of different composition (asymmetric), even at the bilayer level.

Pressure. The environmental pressure on the membrane and the aqueous medium is the atmospheric pressure of the contiguous gas phase plus the hydrostatic pressure of the aqueous medium. It is possible to increase or reduce the pressure on the entire system, and if one or both aqueous compartments is

made a closed system, with the gas phase absent, the pressure may still be varied in the system.

Temperature. Temperature is an important variable in this system because the various transitional forms of liquid crystalline phase lipid are usually quite temperature-dependent. The formation, thinning, and stability of thin lipid membranes are also markedly affected by small temperature changes. In addition, such changes can result in significant changes in the solubility and diffusion rates of all components, and in the viscosity of the liquid phases.

Transmembrane Gradients. Several of the most interesting variables to consider are transmembrane gradients of molecular, ionic, and osmolar concentration, electromotive force, and hydrostatic pressure. The first three gradients are closely related. The concentration gradient for any diffusable molecule or ion provides the driving force for its transmembrane flux, and for the osmotic flux of water in the opposite direction. Ionic concentration gradients may give rise to a transmembrane electromotive force (either mono-ionic, bi-ionic, or multi-ionic). Additional electromotive force can be derived from an applied transmembrane voltage. Finally, the hydrostatic pressure may differ on the sides of the membrane, although only minute gradients can be resisted by the membrane.

Operational Parameters in Apparatus and System Design

General. Although many practical, operational design parameters were recognized at the outset, operational design requirements were established by empirical, trial-and-error testing of theoretical considerations with construction and use of many simple chambers.

The bilayer chamber must be easy to thoroughly clean in order to prevent chemical contamination. It should be relatively simple to construct and modify. Although the bilayer system requires many functional components, any component should be removable for repair, substitution, modification, or calibration without disrupting and disassembling the entire system. When necessary, disassembly and reassembly of the entire system should be reasonably simple. Optical and electrical methods have been used to analyze the thickness and area of the lamellar membranes, and methods of addition and withdrawal of material from the aqueous medium have been used in determination of membrane permeability properties. These methods are described in detail below, but it is important to note here that their use imposes further requirements on the design of apparatus and system.

Optical. The system should provide an optical path for visualization of the membrane, such that the incident and reflected light rays lie in a plane which is perpendicular to the plane of the membrane. Brewster angle measurements require that the angles of incidence and reflection be variable around an axis in the plane of the membrane. Since the membrane is vertical and the axis of rotation of most spectrometers and ellipsometers is vertical, the vertical axis through the center of the membrane was chosen as the axis of rotation in chamber design to facilitate the use of the chamber with a spectrometer or ellipsometer. This is one significant advantage of the cylindrical chamber design described in a following section.

Shielding should be provided to eliminate extraneous light, and stray or transmitted light from the light source should be trapped. Where possible, optical surfaces should be inclined to prevent spurious reflections.

Thermal. The system must be thermostated, preferably with provision for both heating and cooling. Temperature measurement and regulation should ideally be achieved with the thermometer or thermistor placed directly in the aqueous medium.

Electrical. Electrical measurements are necessary for determination of several experimental variables, such as the pH and ionic strength of each aqueous medium, and the transmembrane electrochemical potential, resistance, capacity, and dielectric breakdown voltage. This requires that no ion or electron conductive pathways exist between the two aqueous media except via the membrane. It also requires that the entire membrane and the membrane-supporting partition be of nonconducting material, and that all electrodes, valves, thermistors, alignment pins, stirring provisions, etc., be insulated from each other, from ground, and from their counterpart on the opposite side of the membrane. In addition, the design should permit electromagnetic shielding of all conducting leads and electrodes from extraneous and uncontrolled electromagnetic fields and from each other, by a common ground or a driven shield.

Materials. A marked limitation on materials usable for chamber construction is imposed by the requisites of chemical inertness to both aqueous and organic solvents, electrical nonconductance, and optical transparency. Additional properties were considered in selection of materials for specific portions of the system.

Because glass possesses these requisites, it was utilized in many early, simple chambers. Difficulties in precise glass-blowing, in machining, and in cementing glass parts with an adhesive insoluble in both aqueous and organic solvent types, and the expense of commercial glasswork led to its rejection as a suitable construction material for the entire system.

Teflon, polyethylene, and polypropylene have the requisite chemical and electrical properties and may be machined or cast with precision, but are not transparent, are not easily cemented, and are only semi-rigid. They may nonetheless be used for the supporting partition and other components not in the optical path, if held in place by pressure fit, clamping, and/or alignment pins. Nylon, Dacron, and Bakelite were also used for some chamber components but were generally less satisfactory.

Cast Lucite is electrically nonconductive, transparent, resistant to many organic solvents, and easily machined, and was therefore chosen as the material most suitable for constructing the more complex chambers. Stainless steel or brass was used throughout for metal components.

Cylindrical Chambers

Cylindrical Chamber 1. The developmental stages and considerations presented in the previous two sections culminated in the design, construction and use of two basic types of apparatus. The type described in this section is a cylindrical chamber (46) designed especially for optical investigations of membrane properties. The second type of apparatus is a multiple chamber system designed for the study of several different membranes, and is described in the next section.

Cylindrical Chamber 1 (Fig. 3A) is fully water-jacketed and cylindrical around a vertical axis through the membrane partition hole. A solid, clear Lucite half disc was laminated between two hollow,

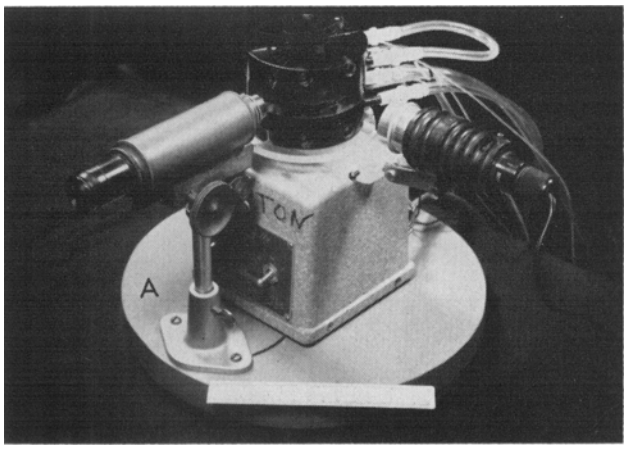


FIG. 3A. Cylindrical Chamber 1. Assembled chamber mounted on magnetic stirrer. The chamber and light source can be rotated around a vertical axis but the microscope is stationary. Attachments for thermostated water, perfusion capillaries, electrodes, and nitrogen enter at the rear of the chamber (top right).

opaque black Lucite half discs to form one half chamber. Various control and measurement assemblies enter from one edge of the half chamber. The two half chambers and an intervening Teflon partition are assembled with bolts to form the complete chamber, and mounted on a magnetic stirrer. Both the chamber and the light source can be rotated around the vertical axis, whereas the viewing microscope is stationary.

The design of the chamber (Fig. 3B,C,D) incorporates essentially all of the previous considerations. The optical path is the clear Lucite center piece, a thick-walled cylinder which allows visualization of the membrane by reflected or transmitted light over a 110° arc. The opaque black Lucite top and bottom pieces provide shielding from extraneous light. Channels machined in the top and bottom pieces for water circulating from a thermostated water bath communicate through a hole in the clear center piece.

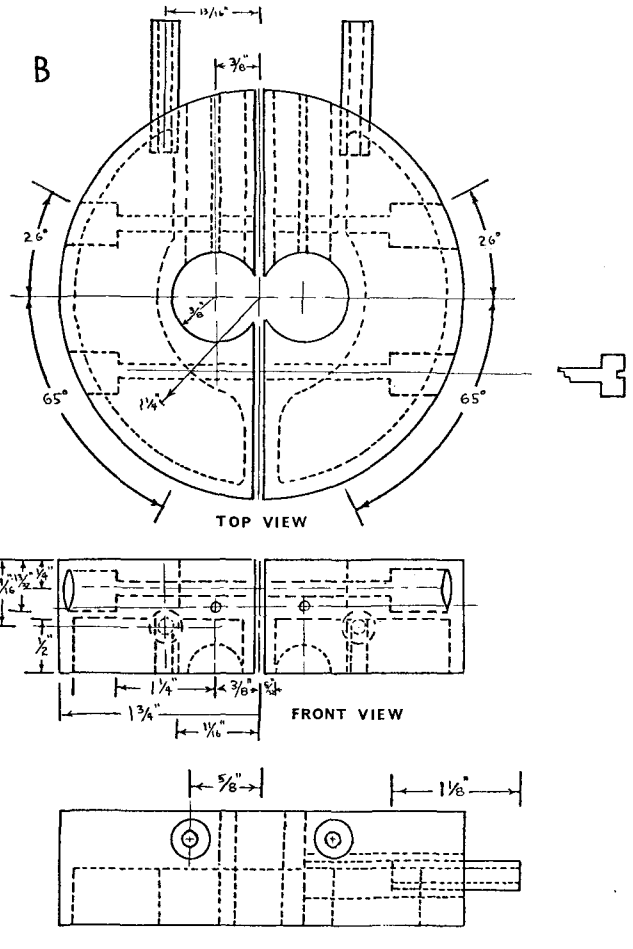


FIG. 3B. Cylindrical Chamber 1. Mechanical drawing for the top pieces of the chamber.

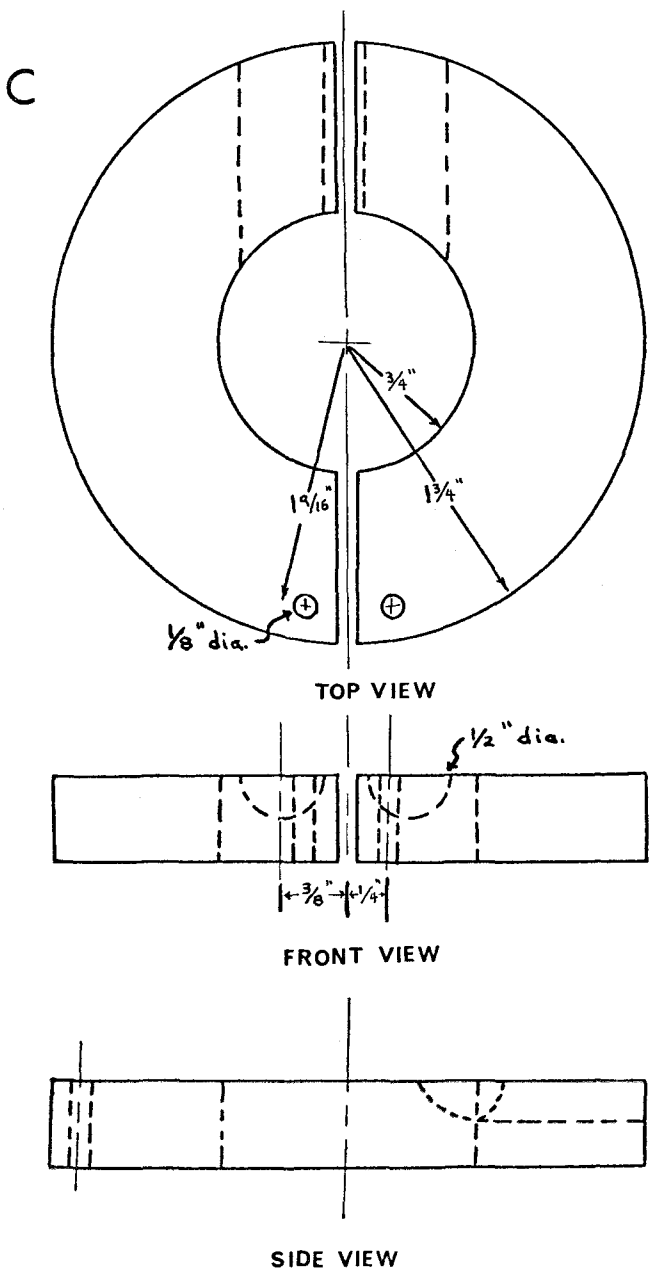


FIG. 3C. Cylindrical Chamber 1. Mechanical drawing for the center pieces of the chamber.

The compartment which contains the aqueous medium is nearly cylindrical to enable better mixing. The bottom of this compartment is a Teflon plug containing a well to hold the magnetic stirring bar. Beneath the plug is a space for a small plastic-encased stirring device which provides a rotating magnetic field to drive the magnetic stirring bars.

Cylindrical ports for electrode and valve assemblies enter each half chamber above and below the optical half disc, respectively. An inlet capillary for nitrogen and connectors for the water-jacket also enter each half chamber. By designing the entrance of all con-

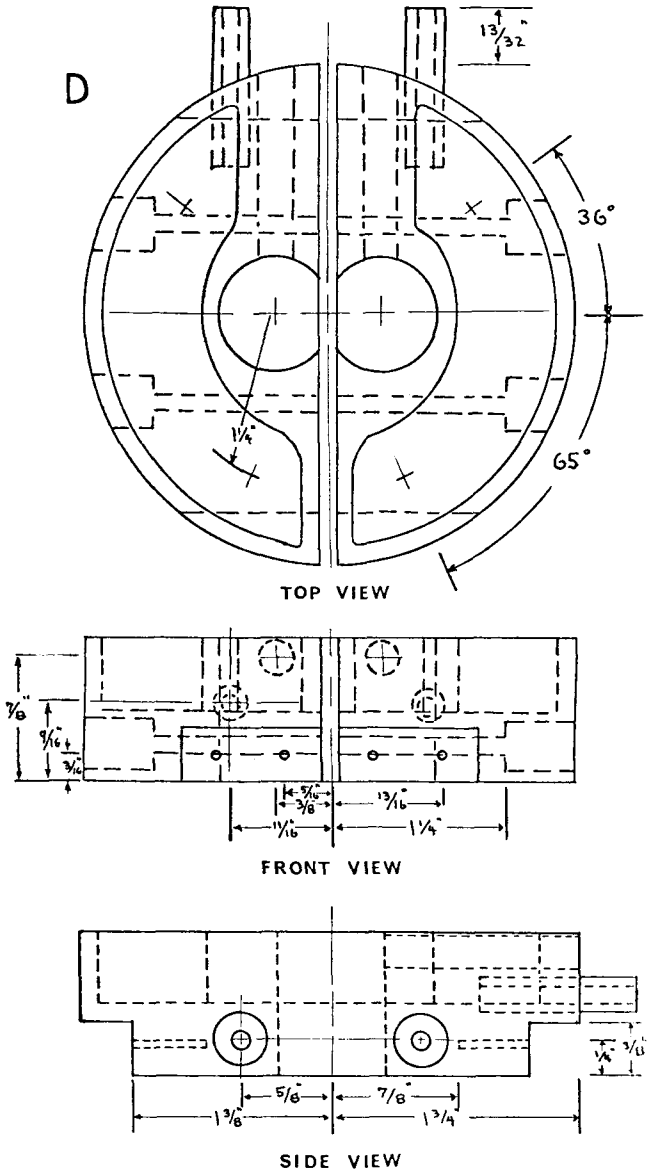


FIG. 3D. Cylindrical Chamber 1. Mechanical drawing for the bottom pieces of the chamber.

nections from the rear edge of each half chamber, it is possible to disassemble the two halves and partition for cleaning without removing all the accessory equipment. In order that the geometric relationships of membrane partition aperture and half chambers remain constant, the assembly bolts were machined to function as alignment pins.

One cylindrical Teflon plug in the rear of each compartment provides access for a pair of conductivity electrodes. Another smaller cylindrical Teflon plug contains two pieces of 22 gauge stainless steel tubing which serve to connect the compartment with provisions for fluid addition and withdrawal.

A Beckman model H2 pH meter, No. 39290 glass electrodes, and No. 39270 fiber junction calomel electrodes were modified and used for pH measurement. Conductivity electrodes were made of nickel-stainless steel or bright platinum. On some occasions the electrodes were coated with fine platinum black. Intercompartment and intracompartment conductivity measurements were made on an Industrial Instruments Model 16B2 Conductivity Bridge.

Cylindrical Chamber 2. Another cylindrical chamber was designed and built (Cylindrical Chamber 2, Fig. 4) after a great deal of experience with the previous design, including problems of leakage around virtually all Teflon-Lucite junctions. Opaque black Lucite top and bottom pieces containing water channels for temperature regulation were laminated to form each half chamber. A small cylindrical compartment for aqueous media was machined in each top piece.

The assembled chamber is mounted on a vibration-damping base over a magnetic stirrer. Both the chamber and light source can be rotated around a vertical axis through the center of the membrane partition aperture. Optical paths normal and oblique to the membrane are permanently mounted in the half chambers. Electrodes, thermistor probes, and inlet and outlet tubes enter from the top of each compartment.

Partitions. The membrane-supporting partition may be made of different materials and may vary in design. Simple chambers were constructed with glass, Bakelite, Teflon, and polyethylene partitions. Routine subsequent work has used 0.020 inch polyethylene sheet with a heat-polished aperture of approximately 2 mm diameter, or 0.010 in. Teflon sheet with a drilled 3.1 mm diameter aperture (0.075 cm^2 area). Other survey studies have used thinner and thicker partitions (0.002–0.125 inch) with conical, square, rectangular and triangular holes, multiple circular apertures, and Nylon and Teflon mesh (47), with individual aperture diameters ranging from 0.5 mm to 6.0 mm.

Multiple Chamber Systems

Rationale. Experience with all of the previous chambers demonstrated that much time was expended in assembling the chamber and preparing it for use, and in disassembling and thoroughly cleaning it at the end of an experiment. Furthermore, in many types of experiments such as those using radioisotopes, rupture of the membrane before sufficient data had been obtained would necessitate a delay for disassembly, cleaning, reassembly, and readjustment before the experiment could be restarted.

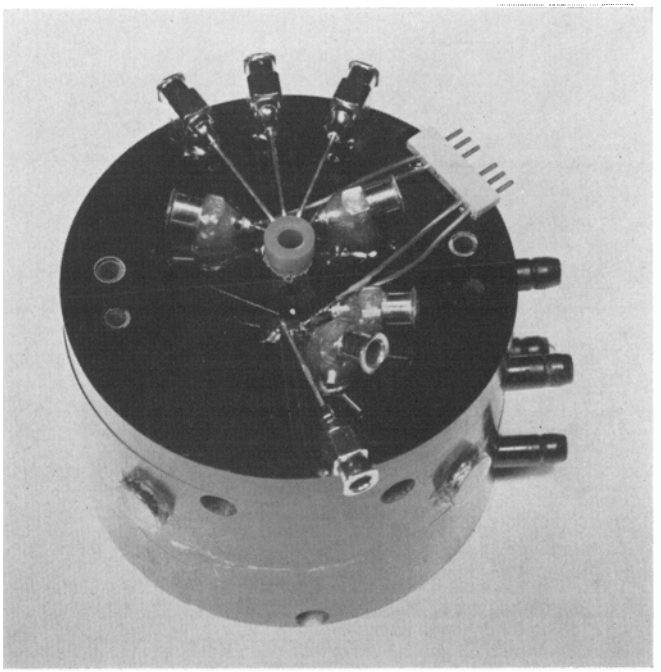


FIG. 4A. Cylindrical Chamber 2. Assembled chamber with top. The platinum hypodermic needles serve as electrodes and also provide for addition and withdrawal of fluid from the aqueous compartments.

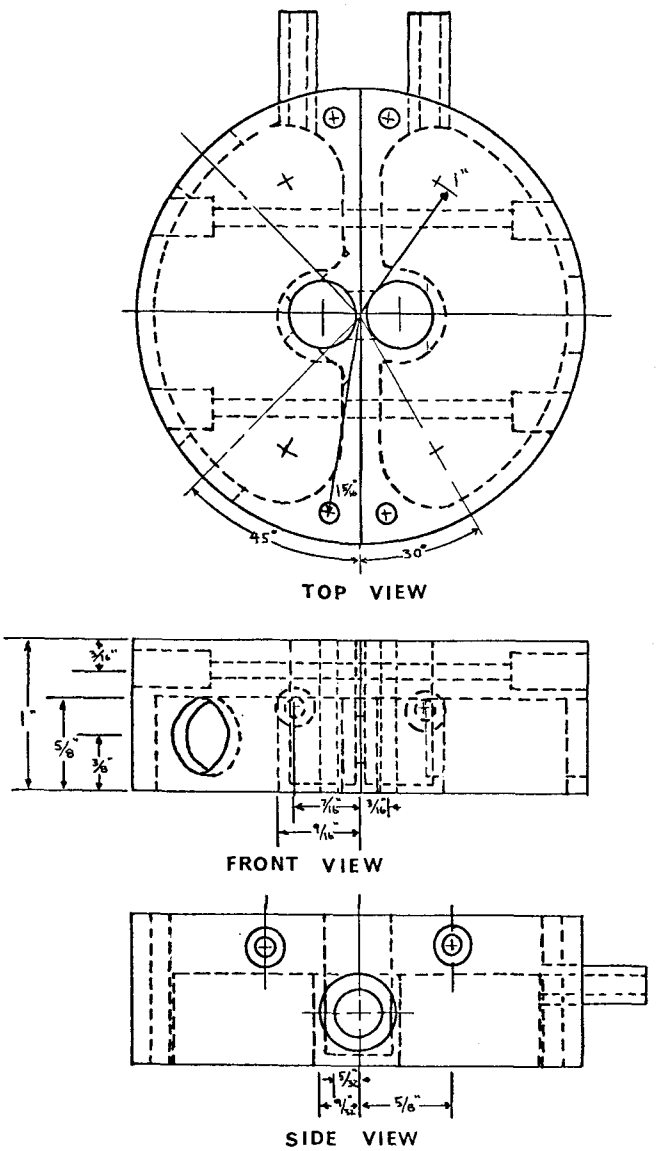


FIG. 4B. Cylindrical Chamber 2. Mechanical drawing for the top pieces of the chamber.

The early Lucite chamber (Fig. 2B) had proven highly serviceable. It was easy to clean, could be assembled and disassembled rapidly, and did not tend to leak. The cylindrical chambers had shown the advantages of an optical path normal or nearly normal to the membrane, and the top for Cylindrical Chamber 2 had proven the utility of mounting all electrodes, thermistors, addition and withdrawal provisions, etc. as a separate unit.

These considerations led to the design and construction of a multiple chamber system in which several different membranes can be studied either simultaneously or in rapid succession. Small interchangeable chamber units (similar to the early Lucite chamber) are held in four wells in a thermostat block, and assemblies of electrodes and provisions for perfusion or sampling of aqueous medium are placed in the chambers as required.

System Design. The overall design of the multiple chamber system integrates the chamber units, thermostat block, and electrode/perfusion assemblies with appropriate mechanical, optical, and electrical apparatus. This design is indicated as a block diagram in Fig. 5. The various components of the system fall into four overlapping subsystems: the basic chamber apparatus, the optical apparatus, the electrical apparatus and the perfusion apparatus. These subsystems are outlined in Table III and coded to the block diagram.

Chamber Units. Each chamber unit consisted of a clear cast Lucite front section, an intervening Teflon partition, and a Lucite rear section (Fig. 6). The front and rear sections of the unit were designed to serve slightly different functions. The rear sections contained a single cylindrical aqueous compartment with a volume of 2.4 ml. A hole was machined through and perpendicular to the side of the section adjacent to the partition, and was continued through the aqueous compartment and a portion of the outer wall. The bottom of the hole was then polished to form a flat optical viewing port. The four assembly screw holes were countersunk to take ¾ inch long cap screws. Each front section contained a cylindrical compartment for aqueous medium with a volume of 2.6 ml. A hole was machined in the side adjacent to the partition and an optical port formed as above. The upper portion of the medial wall of the section was then machined away to permit ready use of the spatula (or brush) in membrane formation.

This design permits easy disassembly of the chamber unit for thorough cleaning of the chamber sections and Teflon partition. Partitions of various thicknesses and with different aperture sizes may be used.

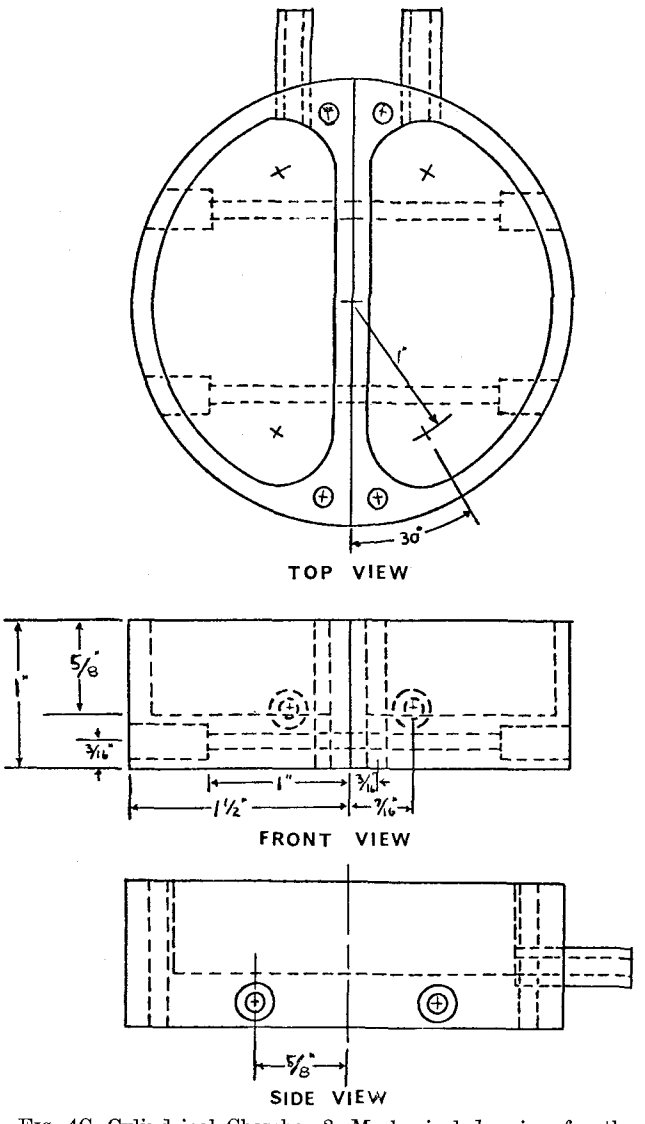


FIG. 4C. Cylindrical Chamber 2. Mechanical drawing for the bottom pieces of the chamber.

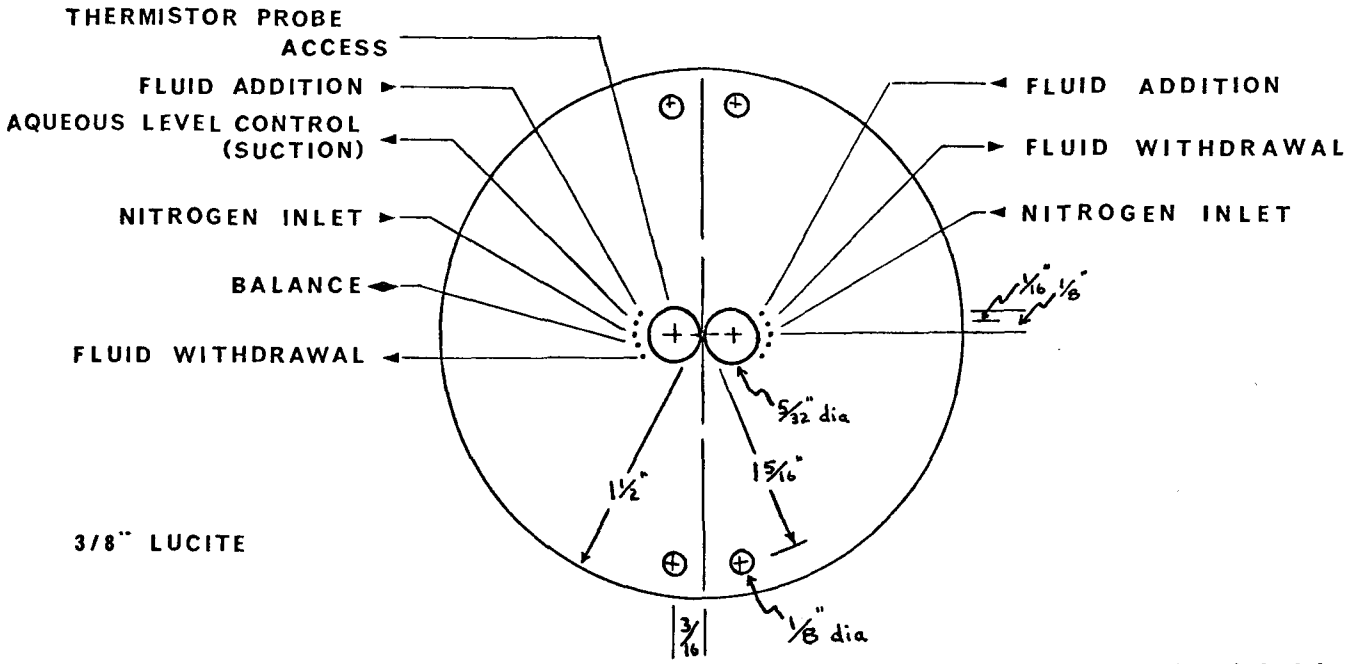


FIG. 4D. Cylindrical Chamber 2. Mechanical drawing for the chamber top indicating the location and function of the holes.

It also permits complete closure of the rear chamber by an electrode/perfusion assembly (see below) thus stabilizing the membrane against bowing due to hydrostatic pressure differences.

Normally, several chamber units are assembled, their partitions preconditioned, the compartments filled with aqueous media, and the chambers placed in the thermostat block to reach thermal equilibrium. This technique enables several membranes to be studied simultaneously, or enables the chambers to be used in rapid sequence. For instance, if the membrane breaks during the course of a permeability experiment, the entire electrode/perfusion assembly may be rinsed and transferred to another chamber, a new membrane formed, and the experiment rapidly restarted.

The cylindrical shape of the bottom of each compartment facilitates stirring of the aqueous media by a small Teflon-coated miniature magnetic stirring bar (3/8 in. × 1/8 in., No. 9235-U8, Arthur H. Thomas Co.). The thin, flat bottom and the relatively small size of the chamber units permit fairly rapid (0.75C/min) attainment of thermostatic equilibrium, and maintenance of the equilibrium temperature.

Chamber Modified for Perfusion. One modified rear section for a chamber unit was made with a very small volume, which could be perfused with rapid total exchange of the aqueous media (Fig. 7). This section is designed for permeability studies so that the very small amounts of permeant substance crossing the bilayer will be minimally diluted and can be collected in toto. The rear aqueous compartment is closed except for the addition and withdrawal capillaries, and has a total volume of 50 μl. Chlorided 18 gauge silver wire electrodes, and 22 gauge stainless steel tubing addition and withdrawal capillaries for both front and rear aqueous compartments are permanently mounted with epoxy cement in the modified section, which is used with a regular front section and a partition with a 3 to 5 mm diameter aperture. The entire volume of the rear compartment can be exchanged easily in less than 30 seconds using coupled 2.0 ml micrometer syringes. A well to hold a thermistor probe is also provided.

Thermostat Block. The assembled thermostat block (Fig. 8A) contains four wells, each holding one chamber unit. The component sections were machined from block aluminum. The base section (Fig. 8B) contains machined channels for water pumped from a thermoregulated water bath. Entering water may pass through the assembled block via an unimpeded channel bordering the edge of the base, or pass through a series of four stirring wells (each containing a small magnetic bar in a Teflon propeller) and through a common exit channel. The rate of flow and route of the entering water may be controlled by stopcocks, which permit control of the rate of stirring in the chambers. Temperature is monitored directly, at the chamber unit or thermostat block, with a single circuit thermistor thermometer (Model 43TD, range 0–52C, with No. 404 probe, Yellow Springs Instrument Co.).

TABLE III
Components of the Multiple Chamber System

Subsystem 1, basic chamber apparatus
- Membrane chamber, F
  - Chamber units
  - Thermostat block
  - Electrode/Perfusion assemblies
- Thermoregulated bath, B

Subsystem 2, optical apparatus
- Light source, I
- Chamber units, F
- Microscope, K
- Photomicrograph camera, O
- Photometer photomultiplier, P

Subsystem 3, electrical apparatus
- DC signal source, A
- Electrometer amplifier, C
- Oscilloscope camera, D
- AC signal source, E
- Electrode/Perfusion assemblies, F
- Operational amplifier, G
- Oscilloscope and differential amplifier, H
- Multichannel recorder, L
- Electronic switching unit, M

Subsystem 4, perfusion apparatus
- Electrode/Perfusion assemblies, F
- Perfusion pump, J
- Injection and sampling syringes, N

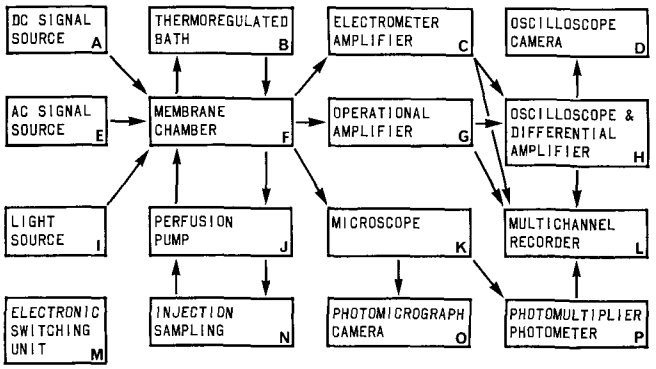
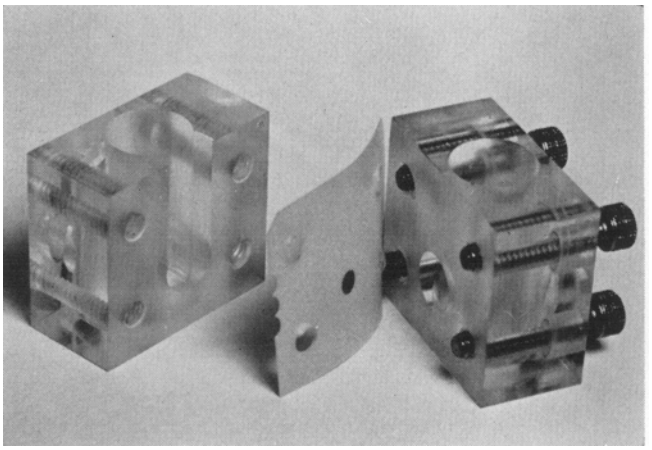


FIG. 5. Block diagram of the multiple chamber system. The letters in each block code that component to the various subsystems outlined in Table III.

A Teflon gasket separates and electrically insulates the intermediate (Fig. 8C) and top (Fig. 8D) sections of the block from the base. Similarly, Teflon tape separates and electrically insulates the top sections at the front from those at the back. The entire thermostat block is assembled with nonconductive Nylon screws and fastened with a Lucite insulator to a lead brick (see below). The top and intermediate sections are electrically connected to the circuit ground in the front, and to the driven shield from the Bak amplifier in the rear (see below). The base of the thermostat block is grounded separately. The design



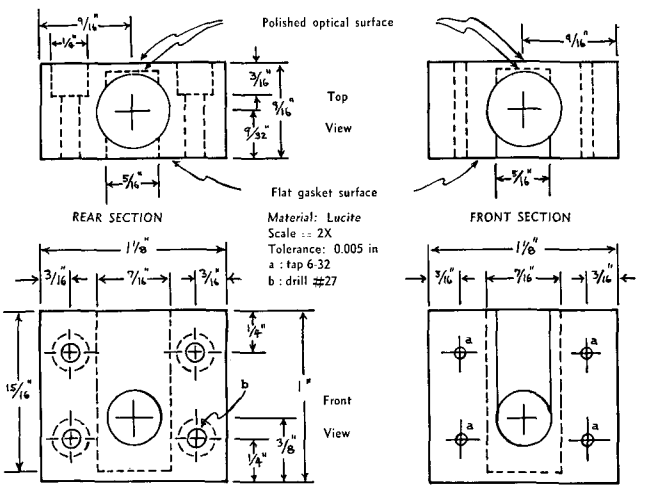
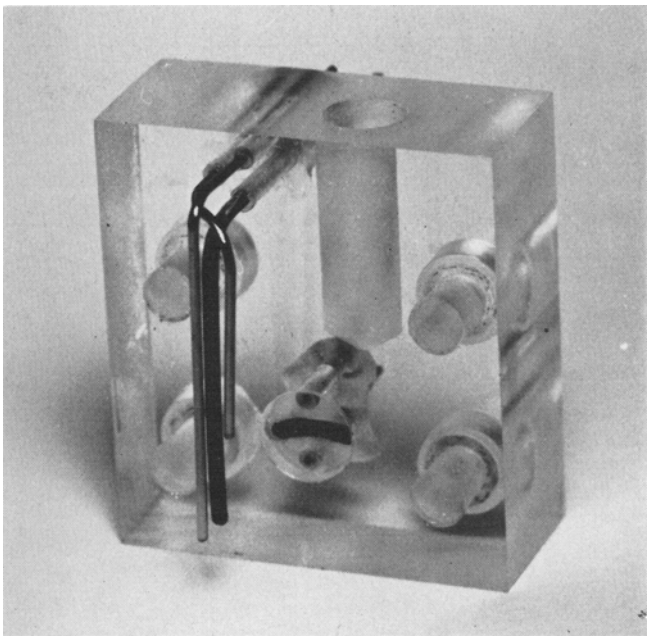


FIG. 6. Chamber unit normally used in multiple chamber system. *A*, exploded view showing the front section (left), Teflon partition with aperture (center), and rear section (right). *B*, Mechanical drawing for both sections of chamber unit.



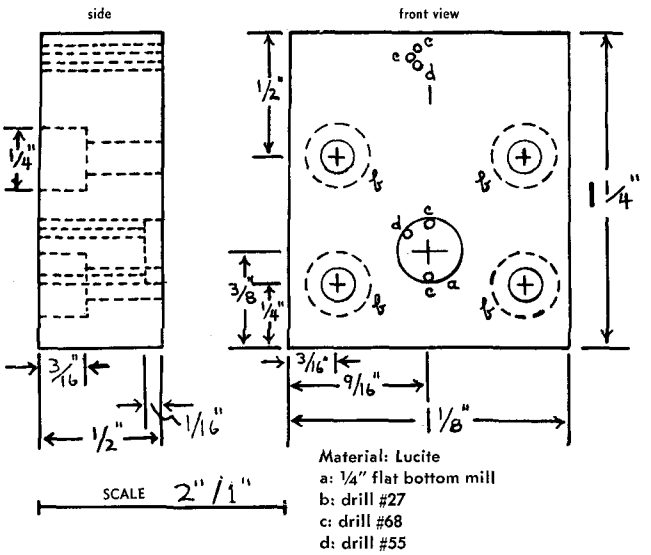


FIG. 7. Modified rear section of a chamber unit for perfusion of the rear aqueous compartment. *A*, front view of section showing the shallow 50 μl compartment. Black Ag/AgCl electrodes, and capillary tubing for addition and withdrawal of fluid in both compartments are permanently mounted in the section. *B*, mechanical drawing.

of the chamber wells in the thermostat block provides a vertical slit in both the front and rear of the well, permitting optical viewing of the membrane aperture in either reflected or transmitted light.

Electrode/Perfusion Assemblies. The electrode/perfusion assemblies (Fig. 9) were designed to fit into and completely close the rear compartments of the chamber. They contain both Ag/AgCl electrodes and perfusion inlet and outlet capillaries. The Ag/AgCl electrodes are made by plating polished 18 gauge silver wire in 0.1 M HCl with 1 mA for 1½ to 2 hr. The perfusion inlets and outlets are made from 22 gauge stainless steel hypodermic needle tubing. The assemblies are cast with General Electric Silicon Rubber Monomer RTV-41 (white) using approximately 0.05% dibutyltin dilaurate as catalyst and curing at room temperature for at least 4 hr. A No. 13 polyethylene stopper is shortened by cutting off the bottom, closed with a No. 8 Caplug, and used as a mold. The electrodes and perfusion inlets and

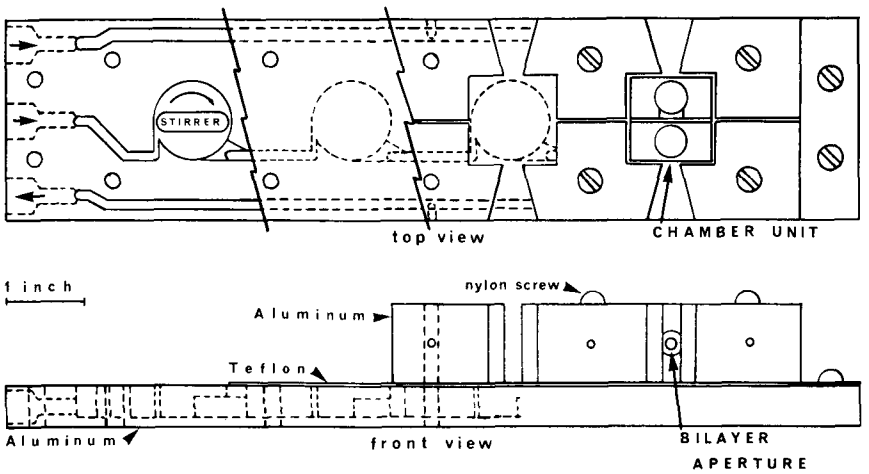


FIG. 8A. Thermostat block used in multiple chamber system. Stepped cutaway schematic diagram of the assembled thermostat block, showing the water connections in the base on the left and an assembled chamber unit in the well on the right.

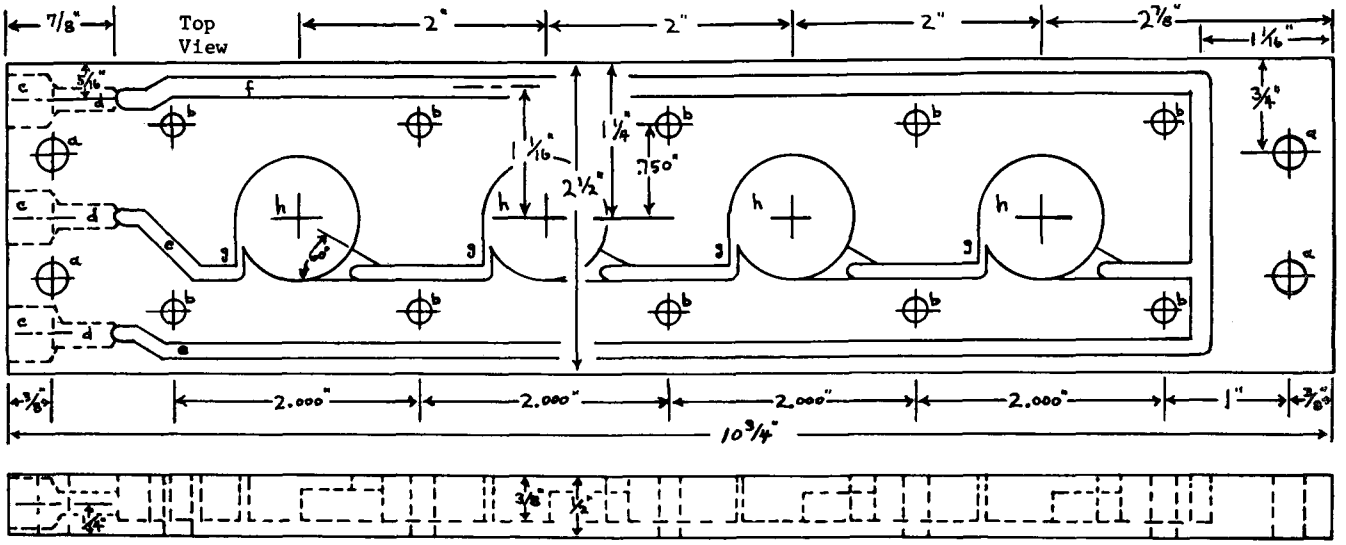
outlets are so designed that they do not extend below the upper edge of the membrane aperture and thus do not obscure the view of the membrane in either reflected or transmitted light.

Optical Apparatus. The light source is an AO Universal Microscope Lamp. The lamp was powered by a transformer in early work, but the AC current to the light was a major source of 60 cycle interference in electrical measurements and the lamp subsequently has been powered by an 8 volt DD Marine Storage Battery (Delco) using a Powerstat (Type 20, The Superior Electric Co.) to control intensity.

The polished surfaces of the optical ports in the Lucite chamber units provide adequate visualization of the membranes, even though they are not optically flat. Because Lucite is totally opaque to light of less than ca. 365 mμ, the Lucite chambers are not usable in the short ultraviolet range.

A 40 mm focal length 3.5× objective, and a 10× ocular fitted with a cross-hatched reticle (1.0 mm rulings) were used in a monocular Leitz microscope. Photomicrographs were taken with a Leitz microattachment and Leica M 1 Camera back mounted on the microscope. The optical magnification and reticle rulings were calibrated on the photomicrographs directly by photographing a hemacytometer with 1.0, 0.2 and 0.05 mm rulings. Apertures in the partitions were also calibrated directly by superimposing them on the hemacytometer and viewing them under low power magnification. A Leica Micro-Six Exposure Meter attachment was used with the Leitz photomicrographic apparatus.

Electrical Apparatus. The overall electrical circuit design is shown in Fig. 10. The circuit applied 0.01–20,000 cps sine wave input signals from a Hewlett-Packard Model 202 Frequency Oscillator, and/or long



Front View
a : drill 1/4″
b : tap 10-24
c : 1/8″ N.P.T.
d : drill 3/16″
e : 1/8″ flat bottom mill
f : 3/16″ flat bottom mill
g : 1/16″ flat bottom mill
h : 1″ flat bottom mill
Material : stress relieved aluminum
Tolerance : fraction - 0.010″
decimal - 0.002″
errors noncumulative

FIG. 8B. Mechanical drawing for base section of thermostat block.

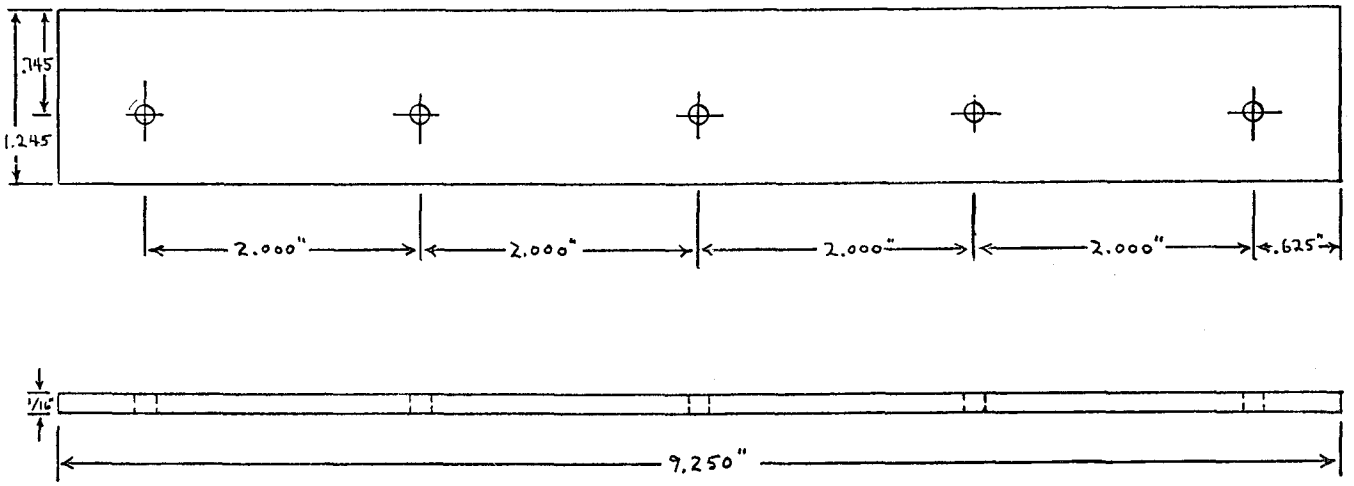


FIG. 8C. Mechanical drawing for the intermediate section of the thermostat block.

rectangular pulse or DC input signals from a Grass Model S8B Stimulator, across the membrane through a series of high precision current-limiting resistors (Victoreen Instrument Co.). A two or four electrode system detected the transmembrane potential difference with a high input impedance ($>10^{10}$ ohm) unity gain preamplifier (Bak Standard Wide Band Electrometer, Electronics for Life Sciences). The transmembrane current was detected with a solid state operational amplifier (Philbrick P65QU Differential Operational Amplifier) whose summing point was connected to one current electrode. Detected transmembrane voltage and current signals were respectively displayed on the A and B beams of a Tektronix Type 502 Dual Beam Oscilloscope. Sinusoidal transmembrane voltage signals were also rectified at the electrometer amplifier output, amplified with a Grass Model 5D Polygraph DC Driver Amplifier, and recorded on a Recti/Riter strip chart recorder (Texas Instrument Co.).

Electrical noise and 60 cycle interference were minimized by shielding all leads, perfusion tubing, electronic equipment, and chambers, and by enclosing the entire chamber assembly, limiting resistor circuit, electrometer head, and operational amplifier circuit in a grounded screen cage. The oscilloscope was calibrated with an accurate vacuum tube voltmeter.

Perfusion Apparatus. The primary perfusion apparatus was a Harvard Model 600–000 multispeed transmission dual peristaltic pump. In some cases, however, 2.0 or 0.2 ml capacity RGI micrometer syringes were used to balance transmembrane pressure,

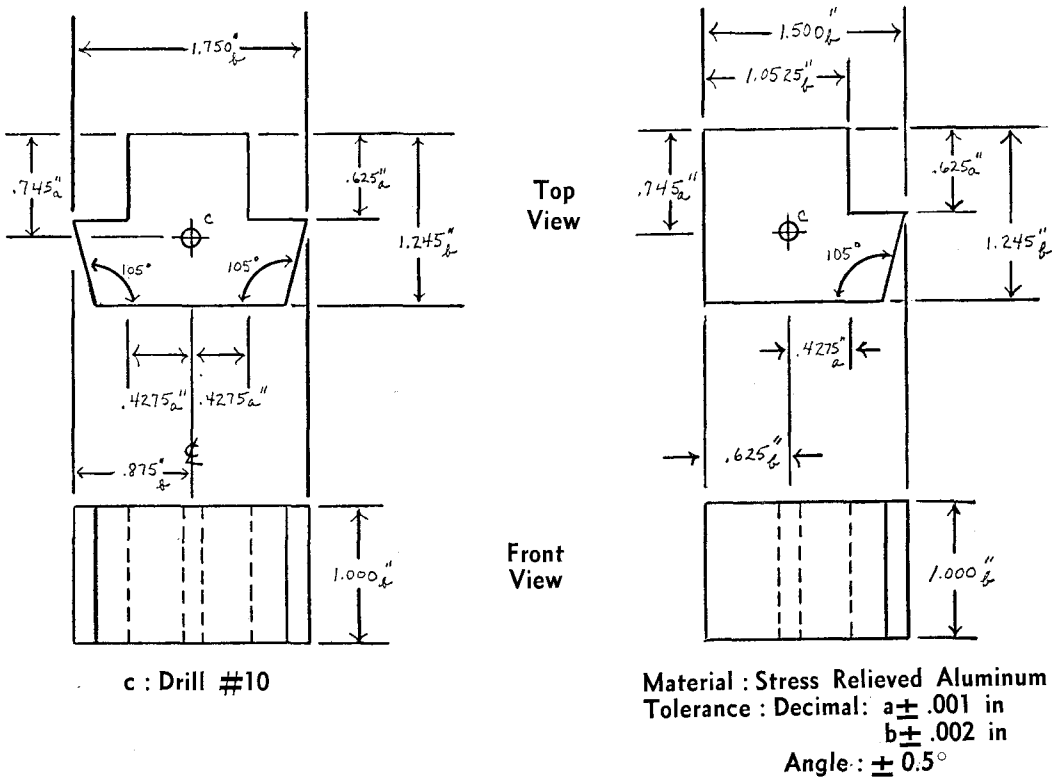


FIG. 8D. Mechanical drawing for top sections of the thermostat block.

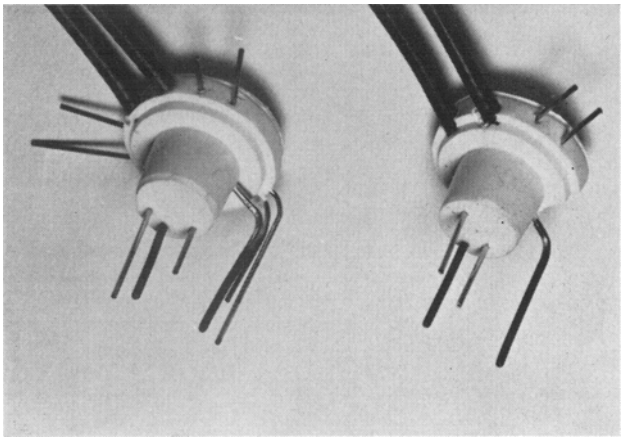


FIG. 9. Electrode/perfusion assemblies used in multiple chamber system. Shielded and insulated electrical leads enter each molded silicon rubber assembly from the upper left, and connect within the assembly to the Ag/AgCl electrodes (larger, dark wires). Smaller stainless steel capillary tubing provides for fluid addition and withdrawal from one (right assembly) or both (left assembly) aqueous compartments.

and/or directly coupled to add and withdraw samples simultaneously. Hamilton microliter syringes were used to add small amounts of solution (1.0 to 50 μl) to the front compartment. The peristaltic pump and tubing were calibrated with the micrometer syringes.

Vibration Damping Apparatus. Ambient vibrations, which tended to rupture thinning membranes, were damped by a $2 \times 4 \times 10\frac{3}{4}$ in. lead brick resting on a $6 \times 12 \times 1\frac{1}{4}$ in. Float-on-air base (Ralmike's Tool-A-Rama, 245 Broad St., Summit, N. J. 07901). The entire membrane chamber-lead brick assembly was positioned on a slab of polished marble, and "floated" with clean, dry, compressed air at approximately 2 psi. Because there was essentially no friction to prevent horizontal movement when the air was turned on, the assembly was moored with rubber bands attached to each of the four corners of the thermostat block.

The table on which the apparatus and marble rested was effectively insulated from vibrations transmitted from the floor by tennis balls under each of the four legs. The tennis balls were held in place and the table prevented from rolling by inverted soft rubber coaster pads fastened to the bottom of each leg.

Optical Methods of Membrane Analysis

Introduction. Direct optical methods were used to observe the formation of the thick membrane, to follow subsequent thinning, to determine planarity and continuity, and to measure the area of the bilayer at equilibrium. When the membrane is illuminated with white light and the light reflected from the membrane is viewed through a low power microscope, three distinct stages are observed during the thinning process. These stages are schematically represented in Fig. 13. The thick lipid membrane which is initially formed appears gray (Fig. 13A). As the membrane thins, interference color patterns appear and pass through several series of faint, higher-order bands of color to the brilliant colors of first-order interference and finally to a "silvery" blue appearance (Fig. 13B). The final stage is a completely transparent or "black" appearance which develops with a sharply demarcated border from the "silvery" membrane as the membrane thins to a bilayer (Fig. 13C).

Geometrical Optics. According to the most simple assumptions for geometrical optics (48), light is reflected from a thin lipid membrane in an aqueous medium at both the first aqueous-lipid and the second lipid-aqueous interface (Fig. 14). The refractive index increases at the first interface from n_0 to n_1, and decreases at the second interface from n_1 to n_0. Because the increment of change in refractive index is equal at both interfaces, the reflection factors are equal for beams reflected at both the first and second interface. However, the sign of the refractive index change is opposite for the two interfaces, resulting in a 180° phase difference between the wave fronts of the first and second reflected beams. Additional phase difference betweeen the two beams is introduced by the increased optical path traveled by the second beam. This increased path length is a function of the angle of incidence, the refractive indices involved, and the thickness of the membrane.

When the membrane thickness is equal to a quarter wavelength ($\lambda/4$) of normally-incident monochromatic light of wavelength λ, the optical path difference of one-half wavelength ($\lambda/2$) produces an additional 180° phase shift in the second reflected beam. Under

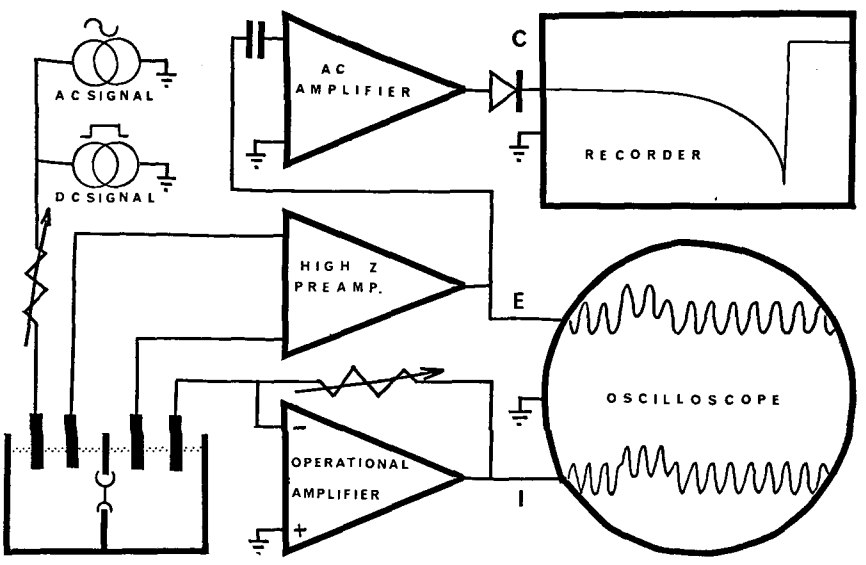


FIG. 10. Electrical circuit diagram for multiple chamber system.

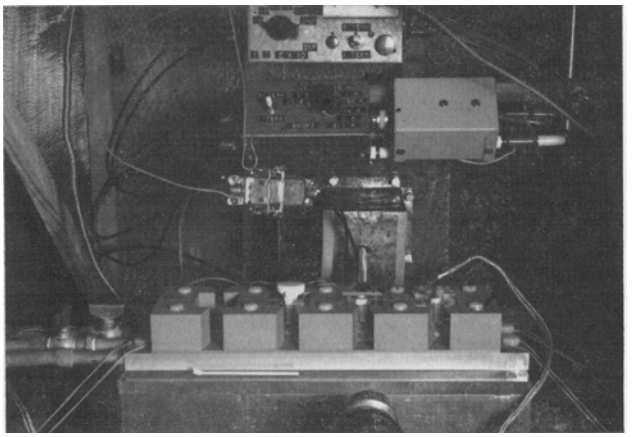


FIG. 11. Multiple chamber system, close-up view showing several types of chamber units in place in the thermostat block. An electrode perfusion assembly is in place in the left hand chamber, and a small spatula used to "paint" membranes is shown immediately in front of the thermostat block. The detection circuit box and operational amplifier are at the extreme top. Below this is the source circuit box containing the current-limiting resistors, connected on the right to the electrometer probe housing.

these conditions constructive interference occurs, and maximum intensity of reflection is present. Thus, a maximum of reflection and minimum of transmission can be observed whenever the membrane thickness is such that the optical path difference is $\lambda/4$, $3\lambda/4$, $5\lambda/4$. . . .

In a thinning membrane illuminated with white light (an achromatic mixture of wavelengths) both constructive and destructive interference operate to

FIG. 12. Multiple chamber system, wide-angle view showing all of the components of the system as used. The electronic signal source and detection equipment is on the left. In the upper left, AC and DC signal sources are on top of a moveable panel-mount rack. The rack contains, in order from the top: the Bak electrometer amplifier; the thermistor thermometer gauge and electronic switching unit; a device for plating Ag/AgCl electrodes; a function generator which can serve as a sine, triangular, or square wave signal source; the AC amplifier, rectifier, and DC driver amplifier for strip chart recording; and the power supply for the Bak amplifier. The seated investigator can operate all equipment, observe the membrane, and collect all necessary data. The photomicroscope is in front of the membrane chamber assembly and other equipment shown in Fig. 11. The light source is directly under the photomicroscope and hidden by it. White micrometer syringes are mounted on either side of the screen cage just above the table. Above the screen cage, on the shelf, is the peristaltic perfusion pump. Visual observation, electrical data, and event marks are recorded on the right. The oscilloscope is in the upper right with its recording camera in right center. The strip chart recorder with event markers is positioned so that notes may be made directly on the chart while recording. The thermoregulated bath is on the floor at right.

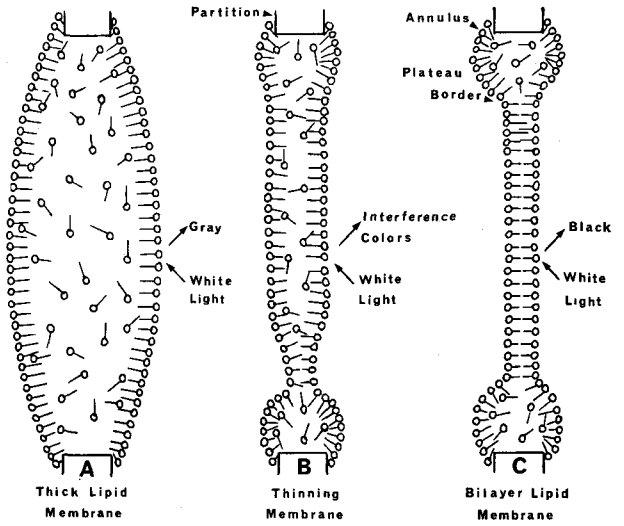


FIG. 13. Diagram illustrating the three stages observed during thinning of a lipid membrane in aqueous media, and indicating the patterns of reflected light.

produce a sequence of chromatic bands of reflected light. When the optical path difference in a membrane illuminated with white light is equal to or slightly less than one quarter of the average wavelength, constructive interference of the reflected light is maximal and the membrane appears yellow to "silvery" blue.

Because of the complete destructive interference between reflected light wave fronts 180° out of phase, a membrane which is very thin relative to a quarter wavelength of the incident light exhibits essentially no reflection. Such a transparent, non-reflecting membrane appears black rather than colored or "silvery" when viewed against a dark background. Under optimal conditions a faint gray shimmer of specular reflection from the "black" membrane may be seen.

Tien has recently published an excellent paper which presents a more complex geometrical analog of the thin lipid membrane in aqueous media (49).

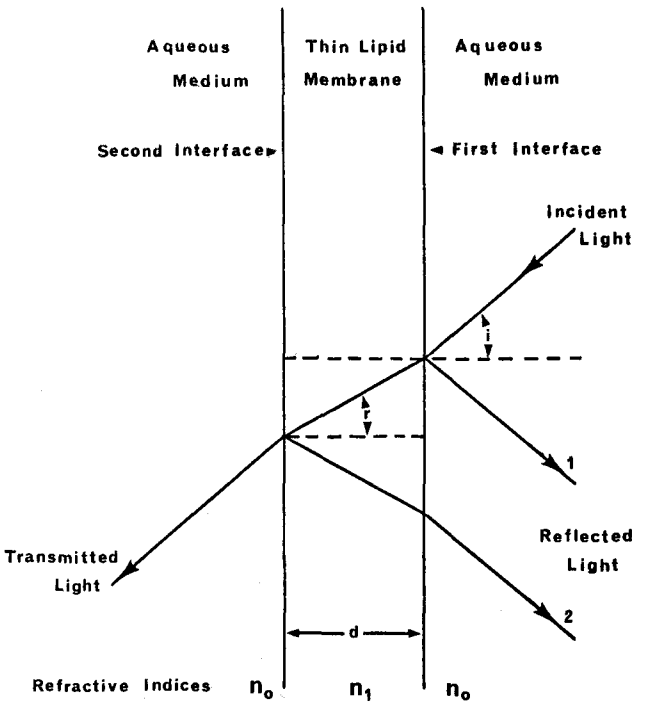


FIG. 14. Diagram illustrating the geometrical optics of reflection from a thin lipid membrane in aqueous media.

This analog provides for reflection of light at the interfaces of free solution and the bound water around the polar head groups as well as at the interfaces between (bound) aqueous phase and hydrocarbon. Tien's proposal more exactly represents the optical nature of membranes, and correlates better with experimentally determined reflection intensity measurements.

Measurement of Membrane Area. Measurement of the aperture size of the partition is not an adequate method of determining membrane area because the amount and distribution of lipid in the annulus may vary widely. The border at the junction of the membrane and the bulk phase annulus (Plateau border) reflects light strongly. Membrane area can thus be determined by direct visual measurement of the Plateau border diameter, using a calibrated reticle at known magnification. Measurements of total membrane area, or of the area of black or of a particular color can also be made directly from color photomicrographs. Photomicrographs taken at known time intervals can be used to determine the types of drainage and mechanism of thinning of the membrane and to calculate the rate of bilayer formation. The thickness range for an area of any specific color in a thinning membrane can be determined by reference to standard works (50).

Electrical Methods of Membrane Analysis. Electrical methods used for membrane analysis included DC transient and AC steady state techniques at both constant current and constant voltage (51,52). Constant current DC transient analysis was used most often, because it was the simplest technique and the least susceptible to errors in measurement. DC pulses of varying duration were passed through the current-limiting resistor network to the transmembrane electrodes. The reversible Ag/AgCl electrodes or reversible calomel electrodes which were used introduced electromotive forces of less than 2 mv into the circuit. The transmembrane voltage drop E across the membrane resistance was detected with the cathode follower input of the Bak amplifier, displayed on the upper beam of the oscilloscope, and recorded with the oscilloscope camera.

The transmembrane current I was measured as the output voltage of a differential operational amplifier and displayed on the lower beam of the oscilloscope. The current electrode was connected to the summing point of the operational amplifier and was held at ground potential by feedback from the output through a high precision resistor. The output voltage was thus proportional to the current into the summing point. Control measurements were made on all chambers with Teflon and polyethylene partitions not containing apertures. No current could be detected with applied voltages up to 150 volts.

The transmembrane resistance R was calculated from the simultaneously measured E and I values using Ohm's law,

$$R = E/I, \qquad [1]$$

normalized by multiplying by membrane area A, and expressed in ohm-cm^2. The resistivity ρ (specific resistance) was calculated from the measured values of R and A using the equation

$$\rho = RA/\delta, \qquad [2]$$

where δ is an assumed membrane thickness of 50 Å.

The time constant τ was determined from the E trace as the time in seconds to reach 0.632 E_{max} with a pulse duration of $\geqq 5\tau$. The transmembrane capacity C was calculated from

$$C = \tau/R, \qquad [3]$$

normalized by dividing by A, and expressed in μfd-cm^{-2}.

Resistance and capacity of membranes with black areas comprising greater than ten per cent of the total area were calculated on the basis of the area of black. For other membranes, calculations were based on total membrane area. This treatment is justified by the much lower resistance and higher capacity of the black membrane than thicker, colored membranes.

Electrical measurements at constant voltage were made by connecting the low output impedance signal from the DC or AC source directly to the current electrode rather than through a current limiting resistor.

The technique of applying a sinusoidal (e.g., 1000 cps) transmembrane voltage, rectifying it, and recording it on a strip chart proved highly valuable. It provided a continuous, automatic record of membrane formation, thinning, bilayer plateau, and rupture (Fig. 15). The ordinate of the record is a nonlinear function of the membrane impedance and reflects primarily the changes in capacitive reactance of the membrane.

Methods of Determining Membrane Permeability

The permeability of lipid bilayer membranes to various substances has been determined by diffusion flux, osmotic flux, and electrochemical potential methods. With the diffusion flux method a known amount of a substance for which the permeability of the membrane was to be determined was added to one aqueous compartment. Sequential aliquots were then removed from both compartments at various time intervals, and the concentration of the substance determined, e.g., by radioactivity analysis of radioisotope-labeled substances. With the osmotic flux method the volume flow of water under an osmotic pressure gradient was directly measured. Electrochemical potential methods were used to determine the relative and absolute permeability of the membranes to small ions. A more detailed account of the permeability methods and experiments will appear elsewhere (53).

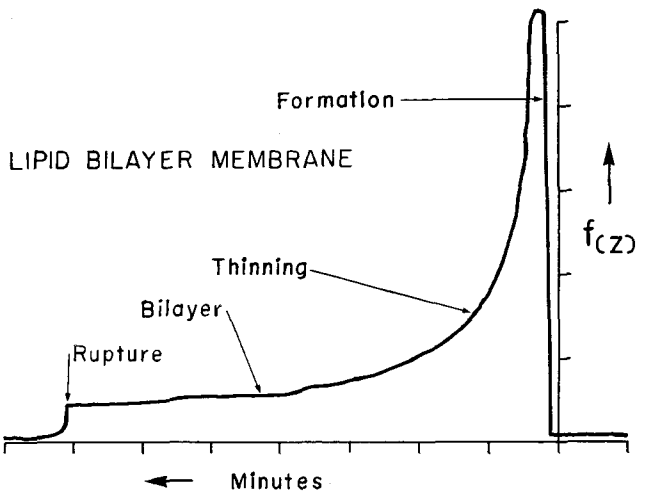


FIG. 15. Strip chart recorder tracing indicating the formation of a lipid membrane, its thinning to bilayer thickness, and its rupture. The ordinate is a nonlinear function of the membrane impedance Z.

Experimental Procedures: Membrane Properties

Membrane Composition

Criteria Used for Selection of Membrane-Forming Materials. In the initial stages of this investigation, a variety of lipid substances and biological materials were surveyed to determine which ones would form thin lipid membranes in aqueous media. Some materials spread on the partition and formed membranes easily with a single brush stroke, or with a few strokes. Other materials required many strokes and would not form membranes easily, although when a membrane was obtained it occasionally lasted for several minutes. Still others would not form membranes at all. Several operational criteria were used to evaluate the materials which did form membranes for their usefulness in further studies.

Because one goal of the project was to study bilayer membranes, rapid thinning of the lipid was desired, i.e., thinning to a completely black state within five or ten minutes. However, this rapidity was considered less important in initial studies, provided that the membrane shortly thinned enough to exhibit interference color formation and eventually developed black areas.

The duration of the membrane is dependent upon its stability to an assortment of mechanical, chemical, and electrical factors. It was important that the membrane duration be sufficient to enable electrical and permeability studies to be made—generally twenty minutes or longer.

Another criterion was that membranes formed from solutions of identical composition have the same properties. Reproducibility was primarily sought in resistance and capacity values and in a black appearance of the membrane at the final stage of thinning, because the principal methods used in this study were electrical and optical.

Survey of Materials. Thin lipid membranes were formed from the lipid and proteolipid materials listed in Table IV. The first material tried was the lower (organic) phase from an unmodified Folch extraction of bovine brain. The membranes formed were thick and rigid and often would not thin. The addition of α-tocopherol (40), *n*-tetradecane (54), or *n*-dodecane enabled thinning to proceed more regularly. The Folch procedure was then modified (as described above) in order to decrease the number of preparatory steps and therefore the likelihood of lipid oxidation, and was used in preparing all of the mixed lipid extracts listed in Table IV.

Many materials did not form membranes satisfactorily. These included commercially available lecithin, cephalin, inositide, and mixed phospholipid preparations as obtained. They also included mixed lipid extracts which had been oxidized by exposure to air, and previously purified phospholipid or glycolipid which had aged for over six months.

Mueller et al. (40) described the addition of α-tocopherol to their membrane-forming solutions in order to facilitate the thinning and increase the stability of their membranes. A variety of substances were tested in chloroform-methanol-lipid solutions in attempts to find a less readily oxidized substitute for α-tocopherol. These included cholesterol, paraffin oil, long chain fatty acids, long chain fatty alcohols, and several *n*-alkanes. Only purified cholesterol and the *n*-alkanes significantly increased membrane stability. Unpurified cholesterol and nonredistilled, practical grade alkanes were unsuitable.

Membranes obtained from solutions of each of the mixed lipid extracts in chloroform-methanol-α-tocopherol or in *n*-tetradecane would thin to complete black. Purified bovine brain phosphatidyl choline and proteolipid in similar solutions also formed black membranes. The composition of several membrane-forming solutions is given in Table V.

The electrical methods employed in these preliminary studies consisted of measurements with an AC conductivity bridge which was relatively insensitive, and with which the membrane capacity could not be satisfactorily balanced. The resistance values of the membranes were generally greater than the upper limit of detection sensitivity, which was ca. 1×10^6 ohms. The high resistance of the membranes and their black appearance strongly suggest that the membranes indeed thinned to bilayer thickness. (The reasons for this conclusion are discussed in more detail in a subsequent section.) Membrane-forming solutions made with freshly prepared mixed lipid extracts formed membranes as stable as those formed from purified lipids. However, the goal visualized in this work was to study membranes of fully defined composition, and therefore, mixed lipid extracts were not further utilized.

Solutions of Defined Composition. In subsequent experiments with phosphatidyl choline the chloroform and methanol were omitted from membrane-forming solutions and replaced by the liquid hydrocarbons *n*-decane, *n*-dodecane, or *n*-tetradecane. This was done because the chloroform-methanol solutions evaporated and deteriorated rapidly, and also because they attacked the Lucite chambers. Solutions of phosphatidyl choline in *n*-hexane would form membranes but the membranes were unstable, and membranes of phosphatidyl choline in *n*-hexadecane would not thin rapidly enough. Solutions of phosphatidyl choline in *n*-decane or *n*-dodecane were most satisfactory. Sphingomyelin and cerebroside in *n*-decane formed stable bilayers either with or without added cholesterol, although temperatures of 38–45C were required to obtain satisfactory thinning. The most

TABLE IV

Material Which Formed Thin Lipid Membranes in Aqueous Media

Mixed lipid extracts from:	Purified substances:
Whole bovine brain	Soybean phosphatidyl choline
Whole rat brain	Egg yolk phosphatidyl choline
Bovine brain white matter	Bovine brain phosphatidyl choline
Purified bovine brain myelin	Synthetic β,γ-dipalmitoyl-L-α-phosphatidyl choline
Rat liver mitochondria	Bovine brain proteolipid
Rat liver microsomes	Bovine brain sphingomyelin
Purified human red blood cell ghosts	Bovine brain cerebroside
Neurospora crassa	

TABLE V

Composition of Solutions Used To Form Membranes[a]

Components	Composition			Approximate mole fraction
	mg/ml	μl/ml	M/l	
I. Mixed lipid extract	20		2.7×10^{-2}	0.002
Cholesterol	20		5.2×10^{-2}	0.003
Chloroform		352		0.276
Methanol		448		0.695
α-Tocopherol		160	35×10^{-2}	0.022
II. Mixed lipid extract	20			0.002
Chloroform		480		0.413
Methanol		320		0.544
n-Tetradecane		180		0.041
III. Purified egg phosphatidyl choline[b]	7.52		1×10^{-2}	0.002
Purified cholesterol	7.74		2×10^{-2}	0.004
n-Decane		985		0.994

[a] The compositions shown are representative types of the various solutions used.
[b] Or other purified lipid.

stable and generally useful membranes by the criteria previously cited were formed from highly purified egg phosphatidyl choline in *n*-decane with cholesterol added. The electrical and permeability data subsequently presented are from such membranes.

Experimental Problems

Cleaning. Several experimental problems were encountered initially in forming lipid membranes in an aqueous medium, despite the apparent simplicity of the technique. Traces of surface active agents from a variety of sources impaired or prevented membrane formation. These sources include plasticizers, curing agents, catalysts and mold release agents contained in the plastic portions of a newly-constructed system, body oils or other contaminants from handling, and residual oxidized lipids or minute traces of detergent due to incomplete washing and rinsing of the chambers. Surface active agents could be completely removed from new Lucite by washing it and exposing it to ultrasound in hot aqueous detergent, rinsing it for several hours in hot running tap water, and then boiling it in distilled water. Teflon and polyethylene pieces were washed, extracted with chloroform-methanol (2:1, v/v) overnight, and boiled in distilled water. Subsequently the Lucite, Teflon, or polyethylene pieces were briefly washed with detergent, rinsed carefully with hot tap water and distilled water, and air dried before use. Precautions such as routinely storing the Lucite under distilled water, or storing Teflon or polyethylene under petroleum ether were found to be unnecessary. However, plastic pieces exposed to the atmosphere for several days became contaminated and had to be rewashed before use.

Preconditioning. Thick membranes and bilayers could be formed on clean untreated membrane partitions, but their formation and their stability, especially during early stages of thinning, were improved by "preconditioning" the partitions. This was done by air-drying a small annular ring of the membrane-forming solution around the aperture on both sides of each partition.

Amount of Lipid. Lipid is less dense than water and thus, if too much lipid were on the brush, it would frequently flow upward and rupture newly-formed membranes. This upward flow with rupture of the membrane was particularly a problem when sable brushes were used, and with apertures greater than 4 or 5 mm in diameter. The Teflon or polyethylene spatulas held less lipid and generally did not create this problem. Conversely, too little lipid on the spatula, especially with the larger apertures, did not permit ready membrane formation because a sufficient supporting annulus could not be formed.

Oxidation. Oxidation of the lipids in the membrane-forming solutions by prolonged exposure to air made the formation of membranes difficult, led to premature rupture of the membrane, or prevented membrane formation altogether. Freshly-prepared lipid solutions in *n*-decane were stable for several months when stored under nitrogen in a refrigerator and maintained their membrane-forming capacity even if exposed to air at room temperature for a working day. Lipid solutions made with chloroform-methanol were less stable, becoming unusable after 4–6 weeks in the refrigerator or after a few hours of exposure to air at room temperature.

Bubbles. Bubbles created optical and electrical interference, distorted the membrane from planarity, and frequently caused the rupture of the membrane. Bubbles arose from dissolved gases in the aqueous media when its temperature was increased, and from air carried into the aqueous media on the brush or spatula. Bubbles from the media could be removed with a microsyringe or small spatula before the membrane was formed, and bubbles carried in by the brush or spatula could be avoided with sufficient care.

Temperature. Temperature greatly affected the initial formation of the membrane. In many cases a particular membrane-forming solution would form gray-appearing membranes which broke in a fraction of a second. When the temperature was increased as little as 1C, the same solution would often form stable membranes which would thin and exhibit interference colors. Except where specified otherwise, experiments were carried out at 37C.

Types of Drainage

After a lipid membrane was formed, it thinned by drainage into the surrounding bulk phase annulus. Each of the types of drainage described by Mysels et al. (16) for aqueous soap films in air was observed during the thinning of thin lipid membranes in aqueous media. The major types which were observed are simple mobile, irregular mobile, and rigid drainage. A complete description of the observations on thinning lipid membranes will be presented elsewhere (55).

Bilayer Stability

The black bilayer membranes were remarkably stable once formed. Membranes were occasionally permitted to persist overnight, but more typically they lasted for one to three hours. The bilayers were much more stable to vibration, transmembrane hydrostatic pressure changes, and the effects of stirring than were thicker membranes. They were liquid in the plane of the membrane and generally showed low surface viscosity. This was indicated by the fluid motion of particulate material or islands of thicker lipid under the influence of convection, vibration, or stirring. Some bilayers would permit puncture with a very fine glass or metal needle without rupture, and would reseal themselves wheen the needle was withdrawn. In addition, some bilayers could be slightly deformed with a blunt glass rod without rupturing.

The bilayers were stable to a wide range of pH in the aqueous media, from approximately pH 4 to pH 10, depending upon the lipids used. While bilayers formed and persisted for short periods in distilled water, they were markedly stabilized by aqueous media with monovalent ion concentrations as low as 1×10^{-4} M. They were stable to concentration gradients as great as one hundred-fold with nonelectrolytes such as urea, provided the lower concentration of nonelectrolytes was greater than a certain minimum (ca. 1×10^{-3} M). They were also stable to concentration gradients of electrolytes, although they usually ruptured before a fifty-fold gradient was reached. Note that the osmotic pressure gradient across the membrane is similar in these two cases.

Bilayer Electrical Properties

Resistance. The DC resistance of completely black membranes (bilayers) measured in 0.1 M NaCl or KCl solutions was generally in the range $R = 1 \times 10^6$ to 1×10^8 ohm-cm^2 (Table VI). However, there was some variation in the resistance even of membranes formed from the same membrane-forming solution on

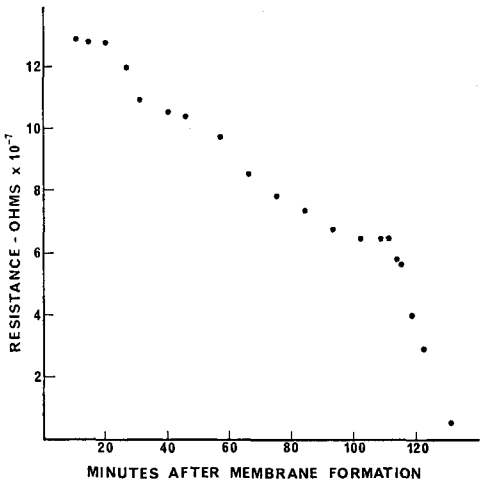


FIG. 16. Graph showing resistance changes with time for a thin lipid membrane. The membrane became completely black at 12.4 min and ruptured at 150 min after formation.

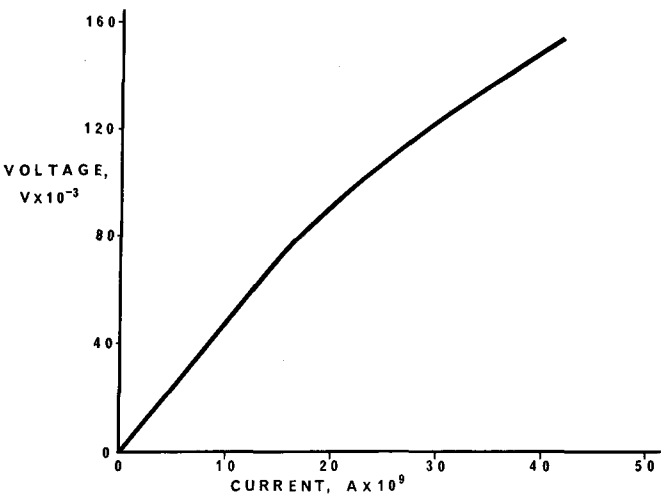


FIG. 17. Graph of voltage vs. current for a lipid bilayer membrane. The voltage-current relationship is linear to ca. 60 mv, then exhibits a gradual decrease in the voltage developed with further increase in current until dielectric breakdown at 155 mv.

the same aperture. Since the highest measured resistance values for any particular solution are similar when normalized for aperture size, the lower resistance measurements are thought to be due to surfactant contamination, oxidation, capillary leakage pathways, or other high-resistance short circuits. A planar high resistance membrane generally showed slow variation in resistance with time from the time the bilayer state was reached until a few minutes before rupture, when the resistance decreased sharply (Fig. 16). Assuming a dielectric thickness of 50 Å, the resistivity calculated for these membranes is $\rho = 2 \times 10^{12}$ to 2×10^{14} ohm-cm.

The resistance of the membranes was linear (Ohmic) with applied DC potentials up to ca. 60 mv, but gradually became nonlinear with higher potentials (Fig. 17). The nonlinearity was always in the direction of decreasing resistance. Black membranes formed from phosphatidyl choline or phosphatidyl choline and cholesterol did not show rectification or more than one stable resistance state.

Dielectric Breakdown. The bilayers were stable to large current flux (up to 2×10^{-6} A-cm^2) but ruptured at applied potentials ranging between 80 and 200 mv, usually at about 150 mv. For an assumed dielectric thickness of 50 Å this would correspond to a dielectric breakdown voltage range of 1.6×10^5 V-cm^{-1} to 4×10^5 V-cm^{-1}, with a value of 3×10^5 V-cm^{-1} for the usual membrane.

Capacity. Capacity was determined from the time constant of the DC charging curve for the membrane. Unlike the resistance values, which varied over three decades, the capacity of the black membrane was relatively constant at ca. 0.4 μfd-cm^{-2} (Table VI).

Membrane Potentials. Membrane potentials were determined for known concentration gradients of NaCl and KCl in an effort to determine the relative permeability of the bilayer to the various ionic species. Using highly purified egg phosphatidyl choline-cholesterol-*n*-decane and electrolyte concentration gradients up to one hundred-fold, no reproducible potentials could be obtained. Small potentials of ca. +20 mv were obtained with a tenfold concentration gradient of NaCl in one experiment. The developed transmembrane potential was also determined for 0.1 M NaCl on one side of the membrane and 0.1 M KCl on the other side. Any existing (biionic) potential was not discernible over the approximately 2 mv baseline drift.

Bilayer Permeability Properties

Preliminary experiments were undertaken to determine some of the permeability properties of lipid bilayer membranes and to evaluate the design and function of the apparatus which had been constructed. Only a brief report of these findings is made here, a more complete description will appear elsewhere (53). A summary of the calculated permeability coefficients is given in Table VII.

Water Flux Under an Osmotic Gradient. The permeability of lipid bilayers to water was determined both by volume flux of water under an osmotic (urea concentration) gradient and by diffusion of tritiated water. A bilayer membrane was formed in Tris-buffered saline and successive additions of a concentrated urea solution were made to the front aqueous compartment. The bilayer was maintained in a planar state by addition of Tris-buffered saline to the rear compartment with a micrometer syringe, to replace the water lost through the membrane into the compartment containing the urea. Micrometer syringe volumes were read as a function of time, and the volume flux of water determined for each urea concentration.

TABLE VI
Representative Electrical Properties of Lipid Bilayers

Property	Symbol	Units	Experiment number		
			72911	72921	823110
Voltage	E	mV	28	55	37
Current	I	A $\times 10^9$	2.0	1.0	.35
Time constant	τ	sec	0.4	1.7	3.2
Bilayer area	A	mm^2	7.1	7.1	7.3
Resistance	R	ohm-cm^2	1.0×10^6	3.9×10^6	7.7×10^6
Resistivity	ρ	ohm-cm	2.0×10^{12}	7.8×10^{12}	1.5×10^{13}
Capacity	C	μfd-cm^{-2}	0.40	0.44	0.41

TABLE VII
Permeability Coefficients of Lipid Bilayers and Biological Membranes

Permeant substance	Permeability-Coefficient μ-sec^{-1} Lipid bilayers	Biological membranes	Reference
Water	3.9	.....	Present work
(Isotopic	3.1,4.1	53	85
methods)	4.4	0.23–63	86
	2.3	.....	61
	1.06	.....	91
(Osmotic	3.3–24	.....	Present work
methods)	17.3–104	0.37–400	86
	8.3–14.4	.....	61
	1.14	.....	91
Glucose	<2	.....	Present work
	$5–6 \times 10^{-4}$	$1–3 \times 10^{-4}$	85
Acetylcholine	<6	.....	Present work
Salicylamide	29	.....	Present work

Water Flux by Diffusion. The diffusion of tritiated water through lipid bilayers was determined by its injection into one aqueous compartment with vigorous stirring, and removal and analysis of 10 μl samples from the opposite compartment at various time intervals.

Permeability to Glucose. In early studies of bilayer permeability, glucose-1-^{14}C was injected into one aqueous compartment to give 3.06×10^5 cpm/ml. Fifty-microliter aliquots were removed from the other aqueous compartment and analyzed. No radioactivity could be detected up to 20 min with an intact bilayer, placing an upper limit on the permeability coefficient for glucose of 2 μ-sec^{-1}.

Permeability to Acetylcholine. In similar early studies, acetylcholine methyl-^{14}C iodide was injected into one aqueous compartment to give 4×10^5 cpm/ml and 50 μl aliquots taken from the opposite compartment were analyzed. Radioactivity was not detectable with intact membranes up to 32 min, placing a calculated upper limit of the permeability coefficient for acetylcholine of 6 μ-sec^{-1}.

Permeability to Salicylamide. The permeability of bilayers to salicylamide was determined using the multiple chamber system with the rear section modified for perfusion. After the bilayer was formed, salicylamide was added to the front compartment, making a final concentration of 2.1×10^{-3} M salicylamide. Twenty-five microliter aliquots were removed from the rear compartment at 3 min intervals with simultaneous replacement of 25 μl of fresh media. The aliquots were assayed fluorometrically and the calculated total number of micromoles of salicylamide having diffused through the bilayer was plotted against time.

Permeability to Synaptic Vesicle-Bound ^{14}C-Acetylcholine. Synaptic vesicle fractions from rat brain were radiolabeled by incubation with acetylcholine methyl-^{14}C iodide and injected into one aqueous compartment. Injection of the synaptic vesicle fraction did not cause immediate bilayer rupture, although the bilayer duration was relatively short, less than 10 min, in all experiments. The synaptic vesicle fraction caused marked turbulence and birefringence in the compartment into which it was injected, but caused no visible alteration in the opposite compartment. No movement of radioisotope across the bilayer was detected during the period in which the membrane was intact.

Discussion

Lipid Membrane Thinning and Bilayer Formation

Drainage Types. Three different types of drainage have been observed during the thinning of lipid membranes in aqueous media, corresponding to the types previously described for soap films (16). Membrane-forming solutions of a particular lipid composition generally show the same type of drainage. Furthermore, the same solution can show more than one type of drainage and many show intermediate gradations betweeen types, depending on the influence of physical factors such as vibration or changes in temperature. These observations suggest that drainage is influenced by the character of the lipid used, by the composition of the membrane-forming solution, and by external physical factors. Future studies of thin lipid membranes formed from a series of chemically synthesized lipids will be necessary to clarify these relationships although the effects of temperature and composition observed in the present work are in accord with it.

Rates of Thinning. The methods developed in this work provide accurate means of determining rates of thinning based on either optical or electrical measurements. The direct optical interference methods used here are based on the changes of bilayer membrane area with time. The apparatus is also capable of measuring the rate of thinning by indirect optical methods, such as reflection intensity ratio determination (49,56).

The electrical method developed here provides a record of all stages of membrane presence. The formation of a membrane, even for a brief moment, is recorded with this method, and for stable membranes a complete record of thinning to the bilayer state and of the time of rupture is obtained. It also provides a convenient means of recording when during the membrane lifetime various operations were performed on the membrane, photographs taken, samples withdrawn, etc.

The results from both methods indicate that thinning is a continuous process, that the rate of thinning is essentially constant for a given membrane, and that under a given set of conditions, it is a characteristic of the membrane-forming solution.

Relationship to Biological Membranes. Little is known about the mechanisms of biological membrane formation, regeneration, growth, or movement. However, living cells exhibit processes similar to, and perhaps explainable by, the types and mechanisms of drainage observed in experimental thin lipid membranes. Examples of such processes are the movements of oil droplets on the inner surface of marine eggs, the formation of new membrane over a site of injury, and the fluid alterations of the plasma membrane in amoeboid movement. Thin lipid membranes in aqueous media provide an excellent model for further study of such mechanisms.

Electrical Properties of Lipid Bilayer Membranes

Equivalent Electrical Circuit. Consideration of interference colors, as described in the section on

optical methods, indicates that black lipid membranes are much less than 1000 Å thick. However, the evidence that they are indeed bilayers rests upon the electrical data.

The electrical properties of the thin membrane system may be represented as an equivalent electrical circuit. The control measurements show that the resistance and capacity terms from the electrodes, the aqueous media, the assembled chamber itself, and from stray effects are sufficiently small to be ignored. The resulting effective equivalent circuit thus consists only of a resistance R in parallel with a capacity C due to the membrane itself.

Dielectric Properties. The electrical capacity of the aqueous-bounded membrane is due to its properties as a dielectric core between two conductors, i.e., as a parallel plate capacitor. The significant dielectric core of the membrane theoretically is the liquid-state mixture of *n*-decane and the hydrocarbon chains of the phospholipid fatty acids. This concept is supported by the exceedingly high values of resistivity (up to $\rho = 2 \times 10^{14}$ ohm-cm), and dielectric breakdown voltage (up to $V_B = 4 \times 10^5$ V-cm^{-1}) for an assumed thickness of 50 Å. This dielectric strength compares with that of solid paraffin (1×10^5 V-cm^{-1}), zircon porcelain (1.6×10^5 V-cm^{-1}) and unfilled flexible polyvinyl chloride (4×10^5 V-cm^{-1}), which are all among the best insulators known (57).

The experimentally determined, normalized values for the capacity of the black membranes is $C = 0.4$ μfd-cm^{-2}. Slight variations in the measured capacity of individual membranes probably results from errors in measuring membrane area and in determining the time constant τ.

Thickness. The overall thickness δ of the black membrane can be estimated from the thickness δ_ϵ of the dielectric portion of the membrane. This can be determined in turn from the measured capacity C and the parallel-plate capacitor formula

$$\epsilon = C\,\delta_\epsilon/A, \qquad [4]$$

where ϵ is the permittivity (dielectric constant) of the membrane and A is its area. Two approaches to this determination are possible: 1) to assume a value for ϵ and calculate δ_ϵ, or 2) to assume a value for δ_ϵ and compare the calculated permittivity δ to known values for similar dielectrics. The latter approach is used here.

The length of an extended molecule of phosphatidyl choline calculated from models is 34.5 Å, and the length of the hydrocarbon chains of the fatty acids is about 25 Å (58,59). A bilayer of phosphatidyl choline molecules should thus have an overall thickness of almost 70 Å, and the hydrocarbon region should have a thickness of about 50 Å, assuming no interdigitation.

If a value of 50 Å is assumed for the dielectric thickness δ_ϵ and the experimental value of $C/A = 0.4$ μfd-cm^{-2} is used, the value calculated for $\epsilon = 2.1$. This value compares with measured values for ϵ of 2.00 for *n*-decane, 2.07 for *n*-octadecane, and 2.14 for *n*-octadecene (60).

If a 5–14 Å allowance is made for the polar head groups on each side, depending on their relative orientation, the overall thickness of the black lipid membrane is 60–78 Å. This is consistent with the interpretation of the black membrane as a bilayer and is in general agreement with the values obtained by electrical determinations of bilayer thickness made by several other investigators (19,41,42,60–65). It is also in agreement with bilayer thickness determinations made by optical methods (49,56,66–69) and by electron microscopy (42,70,71), and with thickness determinations of biological membranes (9,72).

Sources of Error. The most probable source of error in such an analysis is the assumption that the permittivity of the bilayer is analogous to the permittivity of the bulk liquid phase hydrocarbon. The true value for dielectric thickness is unlikely to be greater than twice that of the assumed value, since the true value for ϵ is unlikely to be greater than 4. Other possible sources of error are the assumptions that the polar groups contribute insignificantly to the transmembrane capacitance, and that the measured capacitance is identical to the geometrical capacitance of equation 4. Evidence is available that these are valid assumptions (41).

Voltage-Current Relationships. Although the bilayers show a linear voltage-current relationship below ca. 60 mv, their resistance decreases slightly at higher applied voltages (Fig. 17), although it is still high. This decrease indicates that some type of reversible resistance changes are taking place in the membrane itself. Since the current through the membrane is ionic, the resistance change must reflect an increased permeability (conductivity) to small ions at the higher applied voltage.

Induced Electrical Changes. Mueller et al., in both their original paper (17) and in subsequent papers (42,73–75), have described changes in the electrical properties of lipid membranes induced by adding certain proteins or polyelectrolytes to the system. These reports have been confirmed by Bean and co-workers (76,77). Other reports have described changes in electrical properties due to addition of surfactants (78), 2,4-dinitrophenol (79) and enzyme-substrate or antigen-antibody sequences (80). The apparatus and methods described here are fully capable of extending such studies, or of testing hypotheses such as that proposed by Tobias for excitation (81).

Permeability Properties of Lipid Bilayer Membranes

Water. The results obtained by both volume flow and isotopic tracer diffusion measurements show a high permeability of the lipid bilayer to water. The values for both osmotic and diffusion permeability coefficients are within the range reported in the literature for natural membranes and for other bilayers (82–91, see also Table VII).

Small ions. The high resistivity of the bilayer ($\rho = 2 \times 10^{12}$ to 2×10^{14} ohm-cm in 0.1 M NaCl or KCl solutions) demonstrates an extremely limited permeability of the bilayer to small ions. This is particularly striking in contrast to the rather high water permeability discussed above, since the molecular sizes of water and of the hydrated ions are similar (H_2O effective radius ~2 Å; Na^+, 3.4 Å; K^+, 2.2 Å; Cl^-, 2.2 Å). The current through such a bilayer must be ionic in nature, and corresponds to ca. 1×10^{-8} ampere per volt applied across the membrane. Using Faraday's laws to convert this to an ionic flux, a value of ca. 1×10^{-13} *g* equiv-sec^{-1}-volt^{-1} is obtained.

The lack of a developed transmembrane potential due to concentration gradients shows that with the phosphatidyl choline-cholesterol-*n*-decane membranes the transference numbers for Na^+, K^+ and Cl^- are all essentially 0.5. This means the membrane shows no ionic selectivity and that the current is carried equally by monovalent cations and anions. Similar findings

have recently been reported by two other laboratories (41,92,93).

Since the measured specific conductance κ of the membrane is 5×10^{-13} ohm^{-1}-cm^{-1}, and the equivalent conductance Λ of a 0.1 M NaCl solution at 25C is 107 ohm^{-1}-cm^{-2}, a calculation of the equivalent pore area A was made using

$$A = \frac{1000\,\kappa m\delta}{\Lambda c}, \qquad [5]$$

where $\delta = 5 \times 10^{-7}$ cm is the thickness of the bilayer and c = 0.1 is the NaCl concentration in equivalents per liter. The calculated value for the equivalent pore area is 2.5×10^{-19} cm^2 (per cm^2 bilayer). A significant conclusion drawn from this analysis is that the "lipoid-sieve" hypothesis of Collander and Barlund (8) appears invalid for lipid bilayer membranes. This hypothesis postulates small aqueous channels or pores through the membrane through which small molecules and ions may rapidly pass.

The results would appear to correlate better with the concept of small ion permeation of a site-free, low dielectric membrane (94) rather than via carriers or through aqueous pores, but more must be learned about both the basic mechanisms of membrane permeation and about thin lipid membrane properties.

Glucose. Glucose (MW 180) is readily transported across many plasma membranes with apparent diffusion coefficients up to 1 μ-sec^{-1}. In the experimental study of bilayer permeability to ^{14}C-glucose reported here, the overall sensitivity of the methods used was sufficient to detect levels of this order of magnitude, but no positive values for ^{14}C-glucose appearance at this level were obtained. Wood and Morgan have reported a significant refinement of sensitivity and a permeability coefficient of 6×10^{-4} μ-sec^{-1} for glucose using a bilayer formed from human erythrocyte phospholipid (85) .

Acetylcholine. Acetylcholine (FW 163.5) is a small organic ester with an ionic quaternary nitrogen similar to the polar groups of phosphatidyl choline. It is considered to be a biological "transmitter" agent acting across synapses and neuromuscular junctions. The data obtained for the permeability of the bilayer to free acetylcholine is useful only in indicating the upper limits of acetylcholine permeability. No permeation was seen at sensitivities which would have detected permeability to ^{14}C-acetylcholine with a permeability coefficient of 6 μ-sec^{-1}.

Salicylamide. Salicylamide (MW 137) is a nonionic member of the salicylate family of analgesics, and is an organic ring compound of smaller size than that of the cholesterol and phospholipid composing the bilayer. The solubility of salicylamide in water (0.2% at 30C) is much lower than its ready solubility in nonpolar organic solvents.

The results of the experiments with salicylamide show a marked permeability of the bilayer to such a large molecule. This finding is consistent with the Overton hypothesis of lipoid solubility for lipid bilayer membranes (7), which postulates that the rate of permeation of a biological membrane is proportional to the ratio of the permeant substance's solubility in the lipid phase to its solubility in the aqueous phase. The hypothesis has been substantiated by many years of extensive experimentation on tissue, cells, and various other models (3,11), but has not been tested previously on the most adequate model of all, the experimental lipid bilayer membrane.

Synaptic Vesicles. An additional type of permeability surveyed was the transport across the plasma membrane of substances contained within membranous organelles, or the transport of the organelles themselves. An example of considerable neurophysiological interest is the release of bound acetylcholine from the membranous synaptic vesicle across the presynaptic membrane and into the synaptic cleft as free acetylcholine during synaptic transmission.

Burton, Howard, Baer, and Balfour have proposed a hypothetical mechanism for this acetylcholine transport which also suggests a functional role for the gangliosides (95,96). The hypothesis is based on the parallel distribution of acetylcholine and gangliosides in different areas of the brain (97) and in subcellular fractions of brain, the marked concentration of both in the synaptic vesicle fraction (95), and on the physical chemical properties of gangliosides (96).

According to this hypothesis acetylcholine is contained in synaptic vesicles which have diffusion-limiting membranes stabilized ("plugged") with ganglioside molecules. The gangliosides are viewed as being attached to the (protein?) matrix and/or membrane by nonionic, noncovalent bonds such as van der Waals and "hydrophobic" bonds. Such noncovalent bonding in gangliosides has previously been shown to occur in aqueous media but to be rapidly broken in nonpolar solvents (98).

As the action potential reaches and depolarizes the nerve terminal, the negatively charged synaptic vesicle is electrophoretically impelled towards a lipid region of the presynaptic membrane. When the synaptic vesicle reaches the lipid membrane, the gangliosides are dissociated by the nonpolar environment from each other and from the vesicle, locally removing the diffusion barrier to the contained acetylcholine. The highly polar acetylcholine is expelled from the nonpolar lipid phase and the interior of the vesicle into the aqueous synaptic cleft space as free acetylcholine.

Back flow of acetylcholine into the nerve terminal cytoplasm is prevented by that part of the vesicle still in contact with the aqueous cytoplasm and therefore with the gangliosides still associated. With repolarization the vesicle fragments are repulsed away from the cell membrane, and the dissociated gangliosides in the lipid phase partition into the cytoplasm and reassociate with and reconstitute the synaptic vesicle. The energy for the transport of acetylcholine through the presynaptic membrane would thus be derived from the depolarization-repolarization of the nerve membrane rather than being directly linked to a metabolic chemical energy source such as adenosine triphosphate.

The preliminary experiments to test this hypothesis have been limited due to the current levels of sensitivity of the methods used, and to the technical complexity of the experiments. It is significant that free acetylcholine does not appear to cross the bilayer rapidly, in spite of its organic nature and small size, and that synaptic vesicles do not rupture the bilayer or appear to pass through it within the limits of current experimental methods. This indicates that the bilayer itself is sufficient to constrain cell organelles and similar structures. Similar stability of the membrane to virus has recently been reported (99).

Relationship of Lipid Bilayers to Biological Membranes

Criteria of Analogy to Biological Membranes for Experimental Models. One (scientifically) useful ap-

proach to the study of biological membranes has been the construction and study of experimental models. The criteria of analogy between any such model and natural, biological membranes must be established, and it must be determined to what degree the experimental model fulfills these criteria. The criteria which an adequate experimental model to biological membranes should fulfill may be outlined as follows:

Chemical	(composition)
Dimensional	(thickness)
Interfacial	(aqueous-lipid-aqueous)
Thermodynamic	(stability)
Operational	(dissimilar aqueous phases separated)
	(asymmetric structure possible)
	(vectoral function assayable)

It is obvious that the chemical composition of any adequate model should be similar to, or chemically related to, the composition of biological membranes. It is perhaps less obvious that the model must also possess similar dimensions to its natural counterpart, i.e., a thickness of about 70 Å. This has been the least frequently met criterion in previous models, yet is of ultimate importance in studying the permeability properties of the membrane since flux is inversely proportional to membrane thickness.

Biological membranes generally exist as liquid structures bounded on either side by an aqueous phase. Thus there are two liquid-liquid interfaces associated with each membranous structure. From the viewpoint of interface science, biological membranes and lipid bilayers are the limiting case of a three component liquid system, aqueous phase — liquid phase — aqueous phase, in which the central lipid phase has been reduced to molecular dimensions.

Biological membranes are remarkedly stable structures, maintaining their integrity for many hours or even days, even when separated from their parent cell. The thermodynamic stability of biological membranes (and bilayers) is surprising, because the interfacial free energy of most lipid-water interfaces is quite high (e.g., surface tension $\gamma = 50$ erg-cm^{-2} for a hydrocarbon-water interface) (100). The expected configuration of lowest free energy for undissolved lipid in a lipid-saturated aqueous phase would be a sphere of separate bulk phase lipid. Nonetheless, an adequate model must possess sufficient stability to enable it to be studied.

The aqueous phases separated by biological membranes are characteristically dissimilar, e.g., the cytoplasm and extracellular fluid which are separated by the plasma membrane. Electron microscopy of biological membranes has shown frequent structural asymmetry of the two sides of the membrane. Another characteristic of biological membranes is their ability to function in a vectoral or directional manner, e.g., glucose accumulation in red blood cells by carrier-facilitated or active transport. These three characteristics impose corresponding requirements on a model: the model should be capable of separating dissimilar aqueous phases, should enable the formation of structural asymmetry and should provide conditions such that unidirectional, vectoral functions, such as transport, are assayable.

The Bilayer as a Model Membrane. It is instructive to examine thin lipid membranes in aqueous media as models of biological membranes. The chemical composition of the solutions used to form such membranes, especially the mixed lipid extracts, is closely similar to the composition of natural membranes. In fact, amphiphilic lipids similar to those in natural membranes appear virtually essential for bilayer formation, although other substances have been used (101–103). However, the composition of the bilayer itself is not well characterized. Since large amounts of solvent or liquid additives such as α-tocopherol or n-decane have been necessary for adequate thinning and stabilization of the membranes, it must be assumed that they constitute a significant and possibly large proportion of the thick and thinning membranes.

The thickness of biological membranes has been well established in the range of 50–120 Å, with an average value of about 70 Å, as determined by electron microscopy. The lipid bilayer is the only model with a similar thickness which also fulfills the other criteria discussed above.

The liquid-liquid nature of the membrane interfaces in biological systems generally has not been present in previous models. For instance, a monolayer of amphiphilic lipid present at the air-water interface or at a water-metal interface exists between two different phase states. An aqueous soap film in air may be a bilayer membrane, but exists as a liquid phase bounded by two gas phases. Models such as bulk phase phospholipid in contact with an aqueous phase, or a lipid monolayer at an oil-water interface are liquid-liquid phases, but contain only one interface. The lipid bilayer separating two aqueous media is the only membrane model with two liquid-liquid interfaces which is an adequate model in other respects also.

A thin lipid membrane between aqueous media and in contact with bulk lipid might be expected to have virtually no stability if its surface tension is large. However, it has long been known that biological phospholipids have much lower interfacial tension than the neutral lipids (104). Historically, early studies of Harvey and Danielli were interpreted as requiring the presence of protein on the lipid membrane in order to lower the interfacial tension and stabilize the membrane (5,6). It is now known that protein is not necessary either to lower the interfacial tension or to stabilize the bilayers.

Danielli has qualitatively considered the theoretical stability of bilayer membranes in contact with bulk lipid, and calculated that they represent a membrane thickness with minimum surface free energy (ca. 10 ergs-cm^{-2} less for bilayer than for bulk) (105). The experimentally determined stability of lipid bilayers to mechanical and electrochemical insults supports the theoretically predicted stability, and is analogous to the stability properties of cell membranes.

Bilayers and Membrane Structure. The Danielli-Davson hypothesis has received sustained interest and investigation by its author, Danielli (1–3,5,105), but has been challenged in publications proposing that the cell membranes consist of protein-lipid subunits rather than a lipid bilayer (106–108). The subunit concept has received support through the isolation from natural membranes of fractions, considered to be subunits, by several investigators (109–111). The question of membrane structure has been reexamined by Korn (112), who suggested that in biological membrane formation, the protein may aid in organizing the lipid, perhaps as a bilayer, with resulting associations which may be isolated as "subunits." A different interpretation of the morphologic evidence for subunits was suggested earlier by Lucy (113). This interpretation considers the subunits (in plasma membranes visualized by electron microscopy) as micelles

arising by a reversible transformation from the lamellar phase lipid. Whether biological membranes are constituted of subunits or bilayers will not be further discussed here. However, it is important to note that neither concept excludes the other.

Bilayers and Membrane Function. The major importance of lipid bilayers as models is in the study of analogs of biological function, even though the analogy may be incomplete. The use and implications of thin lipid membranes and bilayers in studying excitability, mobility, and permeability has already been discussed. Two major areas of membrane function have not been discussed: transport and drug action.

Some types of membrane transport, in contrast with simple diffusion, appear to involve "carriers" which somehow facilitate the passage of materials through the membrane. In the broadest sense the area of transport also includes the movement of structures such as micelles and organelles through membranes, and the processes of pinocytosis and phagocytosis. Many theories have been proposed for both the mathematical relations followed and the actual mechanisms of transport for a range of substances from water to micellar lipid. The bilayer system provides an adequate experimental model for testing these theories.

Membrane lipids and drugs action have been the subject of an excellent review by Cuthbert (11). Although the study of bilayers is still new, three reports of drug action on bilayers have been published. Andreoli (114), and Mueller and Rudin (115), have reported marked alteration in ion selectivity after treatment of the bilayer with macrocyclic antibiotics such as Valinomycin. Zutphen and van Deenen have reported decreased survival of cholesterol-containing bilayers when exposed to the polyene antibiotics Filipin and Nystatin (116). Hypotheses have been made for the interaction of membrane phospholipids with local anesthetics (117), and for the role of membrane phospholipids as pharmacological receptors (118). Obviously, the bilayer system provides one excellent means of testing these hypotheses.

ACKNOWLEDGMENTS

This work was supported in part by United States Public Health Service grant NB-01575 from the National Institute of Neurological Diseases and Blindness, and grants GM-00096 and GM-00897 from the National Institute of General Medical Sciences. A Medical Scientist Fellowship (R.E.H.) from the Life Insurance Medical Research Fund was received during a portion of this work. Aid in machining much of the early apparatus was provided by S. Michael Orgel. Aid in electrophysiological techniques and informative discussions were provided by Alfred L. Ochs. Purified bovine brain myelin and human erythrocyte ghosts were provided by R. H. Laatsch and a mycelial suspension of *Neurospora crassa* by S. C. Kinsky.

REFERENCES

1. Danielli, J. F. and H. Davson, J. Cell. Comp. Physiol. *5*, 495–508 (1935).
2. Danielli, J. F., J. Cell. Comp. Physiol. *7*, 393–408 (1936).
3. Davson, H. and J. F. Danielli, "The Permeability of Natural Membranes," 2nd ed., Cambridge University Press, Cambridge, England, 1952.
4. Gorter, E. and F. Grendel, J. Exptl. Med. *4*, 439–443 (1925).
5. Danielli, J. F. and E. N. Harvey, J. Cell. Comp. Physiol. *5*, 483–494 (1935).
6. Harvey, E. N. and H. Shapiro, J. Cell. Comp. Physiol. *5*, 255–267 (1934).
7. Overton, E., V. Naturforsch. Ges. Zuerich. *40*, 159 (1895).
8. Collander, R. and R. Bärlund, Acta Botan. Fennica *11*, 1 (1933).
9. Kavanau, J. L., "Structure and Function in Biological Membranes," 2 Vol., Holden-Day, Inc., San Francisco, 1965.
10. Lakshminarayanaiah, N., Chem. Rev. *65*, 492–565 (1965).
11. Cuthbert, A. W., Pharmacol. Rev. *19*, 59–106 (1967).
12. Dean, R. B., Nature *144*, 32 (1939).
13. Saunders, L., J. Pharm. Pharmacol. *15*, 155–156 (1963).
14. Tobias, J. M., D. P. Agin and R. Pawlowski, J. Gen. Physiol. *45*, 989–1001 (1962).
15. Gaines, G. L., Jr., "Insoluble Monolayers at Liquid-Gas Interfaces," Interscience Publishers, New York, 1966.
16. Mysels, K. J., K. Shinoda and S. Frankel, "Soap Films, Studies of Their Thinning and a Bibliography," Pergamon Press, New York, 1959.
17. Mueller, P., D. O. Rudin, H. Ti Tien and W. C. Wescott, Nature *194*, 979–980 (1962).
18. Langmuir, I. aand D. F. Waugh, J. Gen. Physiol. *21*, 745–755 (1938).
19. Dean, R. B., H. J. Curtis and K. S. Cole, Science *91*, 50–51 (1940).
20. Singleton, W. S., M. S. Gray, M. L. Brown and J. L. White, JAOCS *42*, 53–56 (1965).
21. Wagner, H., L. Hörhammer and P. Wolff, Biochem. Z. *334*, 175–184 (1961).
22. Folch, J., M. Lees and G. H. Sloane-Stanley, J. Biol. Chem. *226*, 497–509 (1957).
23. Folch-Pi, J., in "Brain Lipids and Lipoproteins and the Leucodystrophies," J. Folch-Pi, ed., Elsevier Publishing Company, Amsterdam, 1963, pp. 18–30.
24. Laatsch, R. H., M. W. Kies, S. Gordon and E. C. Alvord, Jr., J. Exptl. Med. *115*, 777–788 (1962).
25. Dodge, J. T., C. Mitchell and D. J. Hanahan, Arch. Biochem. Biophys. *100*, 119–130 (1963).
26. DeRobertis, E., G. Rodriquez de Lores Arnaiz, L. Salganicoff, A. Pellegrino de Iraldi and L. M. Zieher, J. Neurochem. *10*, 225–235 (1963).
27. Cleland, W. W., Biochemistry *3*, 480–482 (1964).
28. Stahl, E., "Thin-Layer Chromatography. A Laboratory Handbook," Academic Press, Inc., New York, 1965.
29. Marinetti, G. V., J. Lipid Res. *3*, 1–20 (1962).
30. Burton, R. M., L. Garcia-Bunuel, M. Golden and Y. M. Balfour, Biochemistry *2*, 580–585 (1963).
31. Krell, K. and S. A. Hashim, J. Lipid Res. *4*, 407–412 (1963).
32. Dobiášová, M., J. Lipid Res. *4*, 481–482 (1963).
33. Marinetti, G. V., J. Lipid Res. *6*, 315–317 (1965).
34. Hooghwinkel, G. J. M. and H. P. G. A. van Niekerk, Koninkl. Ned. Akad. van Wetenschap. Proc., B, *63*, 258–276 (1960).
35. Bray, G. E., Anal. Biochem. *1*, 279–285 (1960).
36. Saltzman, A., J. Biol. Chem. *174*, 399–404 (1948).
37. Udenfriend, S., "Fluorescence Assay in Biology and Medicine," Academic Press, New York, 1962, p 19, 421–424, 471.
38. Udenfriend, S., D. E. Duggan, B. M. Vasta and B. B. Brodie, J. Pharmacol. Exptl. Therap. *120*, 26–32 (1957).
39. Chirigos, M. A. and S. Udenfriend, J. Lab. Clin. Med. *54*, 769–772 (1959).
40. Mueller, P., D. O. Rudin, H. Ti Tien and W. C. Wescott, J. Phys. Chem. *67*, 534–535 (1963).
41. Läuger, P., W. Lesslauer, E. Marti and J. Richter, Biochim. Biophys. Acta *135*, 20–32 (1967).
42. Mueller, P., D. O. Rudin, H. Ti Tien, W. C. Wescott, in "Recent Progress in Surface Science," Vol. I, J. F. Danelli, K. G. A. Pankhurst, and A. C. Riddiford, eds., Academic Press, New York, 1964, p 379–393.
43. Pagano, R. and T. E. Thompson, Abstracts of the Biophysical Society Eleventh Annual Meeting, Houston, Texas, Feb. 22–24, 1967, 62.
44. Tsofina, L. M., E. A. Liberman and A. V. Babakov, Nature *212*, 681–683 (1966).
45. Overbeek, J. T. G., J. Phys. Chem. *64*, 1178–1183 (1960).
46. Howard, R. E. and R. M. Burton, Federation Proc. *26*, 403 (1967).
47. Berg, H. J. van den, J. Mol. Biol. *12*, 290–291 (1965).
48. Kingslake, R., in "Applied Optics and Optical Engineering," Vol. I, R. Kingslake, ed., Academic Press, New York, 1965, p 201–244.
49. Tien, H. T., J. Theoret. Biol. *16*, 97–110 (1967).
50. Chamot, E. M. and C. W. Mason, "Handbook of Chemical Microscopy," 3rd ed., Vol. I, John Wiley & Sons, Inc., New York, 1958.
51. Nastuk, W. L., ed., "Physical Techniques in Biological Research. VI. Electrophysiological Methods, Part B," Academic Press, New York, 1963.
52. Nastuk, W. L., ed., "Physical Techniques in Biological Research. V. Electrophysiological Methods, Part A," Academic Press, New York, 1964.
53. Howard, R. E., manuscript in preparation.
54. Huang, C., L. Wheeldon and T. E. Thompson, J. Mol. Biol. *8*, 148–160 (1964).
55. Howard, R. E., manuscript in preparation.
56. Huang, C. and T. E. Thompson, J. Mol. Biol. *13*, 183–193 (1965).
57. Hodgman, C. D., ed., "Handbook of Chemistry and Physics," 37th ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1955.
58. Vandenheuvel, F. A., JAOCS *40*, 455–471 (1963).
59. Vandenheuvel, F. A., JAOCS *42*, 481–492 (1965).
60. Hanai, T., D. A. Haydon and J. Taylor, Proc. Royal Soc. (London), Ser. A., *281*, 377–391 (1964).
61. Hanai, T., D. A. Haydon and J. Taylor, J. Gen. Physiol. *48*, (5, Pt. 2) 59–63 (1965).
62. Hanai, T., D. A. Haydon and J. Taylor, J. Theoret. Biol. *9*, 278–296 (1965).
63. Hanai, T., D. A. Haydon and J. Taylor, Ibid. *9*, 422–432 (1965).
64. Hanai, T., D. A. Haydon and J. Taylor, Ibid. *9*, 433–443 (1965).
65. Schwan, H. P., C. Huang and T. E. Thompson, Abstracts of the Biophysical Society Ninth Annual Meeting, Boston, Mass., Feb. 23–25, 1966, 151.
66. Thompson, T. E. and C. H. Huang, J. Mol. Biol. *16*, 576 (1966).
67. Ti Tien, H., J. Mol. Biol. *16*, 577–580 (1966).
68. Tien, H. T. and E. A. Dawidowicz, J. Colloid Interface Sci. *22*, 438–453 (1966).
69. Thompson, T. E., in "Cellular Membranes in Development," M. Locke, ed., Academic Press, New York, 1964, p 83–96.
70. Henn, F. A., G. L. Decker, J. W. Greenawalt and T. E. Thompson, Abstracts of the Biophysical Society Eleventh Annual Meeting, Houston, Texas, Feb. 22–24, 1967, 63.
71. Henn, F. A., G. L. Decker, J. W. Greenawalt and T. E. Thompson, J. Mol. Biol. *24*, 51–58 (1967).
72. Robertson, J. D., Biochem. Soc. Symp., Cambridge, England, *16*, 1–43 (1959).
73. Mueller, P., D. O. Rudin, H. Ti Tien and W. C. Wescott, Circulation *26*, 1167–1170 (1962).
74. Mueller, P. and D. O. Rudin, J. Theoret. Biol. *4*, 268–280 (1963).
75. Mueller, P. and D. O. Rudin, Nature *213*, 603–604 (1967).
76. Bean, R. C. and W. C. Shepherd, American Chemical Society Abstracts of Papers, 150th Meeting, 1965, 19C.
77. Bean, R. C., W. C. Shepherd, C. D'Agostino and L. Smith, Federation Proc. *25*, 656 (1966).
78. Seufert, W. D., Nature *207*, 174–176 (1965).
79. Bielawski, J., T. E. Thompson and A. L. Lehninger, Biochem. Biophys. Res. Commun. *24*, 948–954 (1966).

80. Del Castello, J., A. Rodriquez, C. A. Romero and V. Sanchez, Science *153*, 185–188 (1966).
81. Tobias, J. M., Nature *203*, 13–17 (1964).
82. Harris, E. J., "Transport and Accumulation in Biological Systems," Academic Press, Inc., New York, 1960.
83. Paganelli, C. V. and A. K. Solomon, J. Gen. Physiol. *41*, 259–277 (1957).
84. Wirth, F. P., H. E. Morgan and C. R. Park, Federation Proc. *24*, 588 (1965).
85. Wood, R. E. and H. E. Morgan, Abstracts of the Biophysical Society Eleventh Annual Meeting, Houston, Texas, Feb. 22–24, 1967, 63.
86. Huang, C. and T. E. Thompson, J. Mol. Biol. *15*, 539–554 (1966).
87. Maddy, A. H., C. Huang and T. E. Thompson, Federation Proc. *25*, 933–936 (1966).
88. Thompson, T. E., and C. Huang, Ann. N. Y. Acad. Sci. *137*, 740–744 (1966).
89. Hanai, T., and D. A. Haydon, J. Theoret. Biol. *11*, 370–382 (1966).
90. Hanai, T., D. A. Haydon and W. R. Redwood, Ann. N. Y. Acad. Sci. *137*, 731–739 (1966).
91. Cass, A. and A. Finkelstein, J. Gen. Physiol. *50*, 1765–1784 (1967).
92. Andreoli, T., Science *154*, 417 (1966).
93. Andreoli, T. E., J. A. Bangham and D. C. Tosteson, J. Gen. Physiol. *50*, 1729–1749 (1967).
94. Eisenman, G., J. P. Sandblom and J. L. Walker, Jr., Science *155*, 965–974 (1967).
95. Burton, R. M., R. E. Howard, S. Baer and Y. M. Balfour, Biochim. Biophys. Acta *84*, 441–447 (1964).
96. Burton, R. M. and R. E. Howard, Ann. N. Y. Acad. Sci. *144*, 411–432 (1967).
97. Howard, R. E. and R. M. Burton, Biochem. Pharmacol. *13*, 1677–1678 (1964).
98. Howard, R. E. and R. M. Burton, Biochim. Biophys. Acta *84*, 435–440 (1964).
99. Petkau, A., W. S. Chelack, W. L. Parker and W. Stackiw, Abstracts of the Biophysical Society Ninth Annual Meeting, Boston, Mass., Feb. 23–25, *1966*, 148.
100. Haydon, D. A. and J. Taylor, J. Theoret. Biol. *4*, 281–296, 1963.
101. Ti Tien, H. T., S. Carbone and E. A. Dawidowicz, Nature *212*, 718–719 (1966).
102. Tien, H. T., Abstracts of the Biophysical Society Eleventh Annual Meeting, Houston, Texas, Feb. 22–24, 1967, 62.
103. Leslie, R. B. and D. Chapman, Chem. Phys. Lipids *1*, 143–156 (1967).
104. Adam, N. K., "The Physics and Chemistry of Surfaces," Oxford University Press, Oxford, England, 1930.
105. Danielli, J. F., J. Theoret. Biol. *12*, 439–441 (1966).
106. Green, D. E. and J. F. Perdue, Proc. Nat. Acad. Sci. *55*, 1295–1302 (1966).
107. Green, D. E. and A. Tzagoloff, J. Lipid Res. *7*, 587–602 (1966).
108. Green, D. E., D. W. Allman, E. Bachmann, H. Baum, K. Kopaczyk, E. F. Korman, S. Lipton, D. H. MacLennan, D. G. McConnell, J. F. Perdue, J. S. Rieske and A. Tzagoloff, Arch. Biochem. Biophys. *119*, 312–335 (1967).
109. Blasie, J. K., M. M. Dewey, A. E. Blaurock and C. R. Worthington, J. Mol. Biol. *14*, 143–152 (1965).
110. Gent, W. L. G. and N. A. Gregson, Nature *204*, 553–555 (1964).
111. Razin, S., H. J. Morowitz and T. M. Terry, Proc. Nat. Acad. Sci. *54*, 219–225 (1965).
112. Korn, E. D., Science *153*, 1491–1498 (1966).
113. Lucy, J. A., J. Theoret. Biol. *7*, 360–373 (1964).
114. Andreoli, T., P. Cook and D. C. Tosteson, Abstracts of the Biophysical Society Eleventh Annual Meeting, Houston, Texas, Feb. 22–24, 1967, 9.
115. Mueller, P. and D. O. Rudin, Biochem. Biophys. Res. Commun. *26*, 398–404 (1967).
116. Zutphen, H. van, and L. L. M. van Deenen, Biochem. Biophys. Res. Commun. *22*, 393–398 (1966).
117. Feinstein, M. B., J. Gen. Physiol. *48*, 357–374 (1964).
118. Watkins, J. C., J. Theoret. Biol. *9*, 37–50 (1965).

Discussion

DR. ALAN GOLDUP (B.P. Health Service, State University of New York at Buffalo, Buffalo, New York): I have recently been attempting to measure the transport of lower alcohols across lecithin bilayers and have had considerable difficulties due to the fragility of these membranes. Did you notice in your investigations whether the support material had any significant effect on membrane stability. I notice that you have used glass but get the impression that you have abandoned its use recently. Silanized glass may perhaps be better.

DR. HOWARD: We had very little success with glass partitions and abandoned them, also. We found that either polyethylene which has been die cut or melted with a hot needle, or Teflon which has been sharply punched, make very adequate partitions. Teflon makes a much more rigid septum than does polyethylene, however it is somewhat more difficult to punch holes in Teflon and obtain a very sharp edge.

DR. GOLDUP: You mentioned in your presentation that stirring did not affect the rate of black film formation. In my experience I find the rate of stirring is important. If the aqueous medium is stirred just sufficiently to increase the length of black border between the black and thick regions then the rate of black film formation is increased. On the other hand, if the stirring is sufficiently fast to create isolated islands of thick film then these sometimes take an appreciable time to disperse.

DR. HOWARD: I did not go into that in detail, but I would agree with your comment.

DR. GOLDUP: It has been reported in the literature (Babakov, A. V., et al., Nature *210*, 953 (1966)) that thinning occurs more rapidly under an applied potential. Did you observe this?

DR. HOWARD: No, I can't say that we have although I have read of this. Since we have had the system adequate to monitor thinning, we have continually had a potential across the membrane because we have been monitoring thinning electrically.

DR. GOLDUP: You did mention that some absorption spectra of these lipid bilayers had recently been made. Is this work that you are currently doing or has somebody else reported this?

DR. HOWARD: The published report is a recent paper by R. B. Leslie and D. Chapman, Artificial Phospholipid Membranes and Bioenergetics in Chem. Phys. Lipids *1*, 143–156 (1967), in which they examined bimolecular phospholipid membranes containing either β-carotene or all-*trans* retinene.

DR. TIEN: I should like to make a brief comment on the question of thinning. In our studies we have frequently encountered a thick lipid film (showing interference colors) that would not thin readily to the black state. We have found that there are a number of ways which would speed up the BLM formation. For instance, one can change the solvent to one of a lower viscosity. The other effective method in our experience is to speed up the border suction by brushing the area around the hole with the brush used to introduce the lipid solution. The other equally effective method is to poke the thick membrane with a fine object such as a wire or a micro-pipette, thus initiating the so-called "zipper" mechanism.

DR. T. E. THOMPSON (University of Virginia, Charlottesville, Virginia): I would like to mention a recent effort made in my laboratory by Dr. F. Henn to determine the actual composition of a bilayer membrane (F. A. Henn and T. E. Thompson, "Properties of Lipid Bilayer Membranes Separating Two Aqueous Phases Composition Studies," J. Mol. Biol, *31*, 227–235, 1968. Although knowledge of the composition of the bilayer is a prerequisite to the solution of the structure-function problem, this information has proven difficult to obtain. It is apparent that the composition of the bulk phase from which the bilayer forms can easily be determined. While this composition clearly sets a limit on the chemical species present in the bilayer, it is not necessary that the relative composition of the bilayer membrane be the same as that of the parent bulk phase.

Using the fixation technique described by Henn et al. (F. A. Henn, G. L. Decker, J. W. Greenawalt and T. E. Thompson, Properties of Lipid Bilayer Membranes Separating Two Aqueous Phases: Electron Microscope Studies in J. Mol. Biol. *24*, 51–58, 1967) and radioactively labeled components of high specific activity it has been possible to examine the composition of bilayers composed of phosphatidylethanolamine and

decane with or without added cholesterol. The results show that under the conditions of study the molar ratio of decane to phospholipid in cholesterol, free membranes is 11.8 ± 3.4 and is 10.5 ± 1.8 in membranes containing a cholesterol to phospholipid ratio of 1.5 ± 0.2. A consideration of the calculated and measured surface area per phospholipid molecule and the electron micrographs of the fixed membranes leads to the conclusion that these structures are bilayers of phospholipid which contain appreciable amounts of decane. At least some of this decane is trapped in the bilayer in the form of micro-lenses.

DR. TIEN: Dr. Howard mentioned that perhaps ellipsometry can be used to determine the thickness of these membranes.

DR. HOWARD: I have considered ellipsometry briefly in an attempt to find whether it would be sensitive enough. It seems that the theoretical resolving power of ellipsometry can be in the Angstrom level and also it can give information about small portions of the membrane surface. In terms of actual surface structure this information has been primarily derived from the work with thin metal films in air. The problem which arises is related to the refractive index of the lipid film, in that a thin metal film in air is quite different from a thin lipid film in aqueous media.

DR. THOMPSON: I think the only use you could have for ellipsometry would be to study a membrane which has been made asymmetrical. For example, by the addition of protein to one side.

DR. HOWARD: The approach that we had in mind was to try to alter the refractive index in one aqueous media relative to the other media.

DR. MYSELS: Now, I wonder whether I could be permitted to take about five minutes not to ask a question but to present something that is rather new and that explains, I think, the reduced thinning by mixing and also some effects that Professor Tien described to me which he observed upon expanding a membrane when measuring its surface tension.

We call this behavior "juvenescence," and I believe that it must occur in invert films as well as in soap films and others. A full description and explanation has been submitted (M. N. Jones and K. J. Mysels, J. Am. Oil Chem. Soc. *44*, 284 (1967)). Very briefly, it involves the fact that sometimes the kinetics of thinning of a film which normally take minutes slow down and even appear to stop completely, although a considerable amount of motion in the film can still be seen. We have tracked down this anomaly to the presence of volatile surface-active minor components which distill out of the film. In case of invert films, exactly the same should be happening when such components can dissolve in the aqueous phase. The loss of such a component from the film tends to increase the surface tension, therefore causing a local contraction and thickening of the film. This local loss of area causes the pulling of fresh thin film out of the border. Gravity (or buoyancy) may then cause movement of the thickened film into the border and the process may become cyclic. It is important to note that any trace of such behavior should be absent when one wishes to measure the true equilibrium thickness of any of these films.

I would expect that in an invert system the water layers immediately adjacent to the film would soon become saturated in a quiescent system, and the effect would be very greatly reduced. Any displacement of the membrane or any convection in the liquid should, however, bring fresh unsaturated water near the membrane and cause the film to thicken and perhaps to become juvenescent if such soluble surface-active minor components are present. Therefore, it should be quite important to equilibrate the two phases thoroughly before making a membrane if one desires equilibrium conditions to prevail.

DR. THOMAS E. ANDREOLI (Department of Physiology and Medicine, Duke University, Durham, North Carolina): I would like to comment on the membrane chamber design. We used roughly the same kind of arrangement. However, we sealed the rear chamber. Under these conditions, we could easily change solutions in one of the chambers bathing the membrane in brief time intervals. Your design also raises a technical point. You mentioned that the measured resistances are in the range of 10^6 to 10^8 ohm-cm^2. Many workers agree that membrane resistances, for a lipid-decane system are in the range of 10^8 ohm-cm^2. It may be that significantly lower electrical resistances, in the absence of other modifying factors, are due to electrical "leakage" pathways. In your apparatus, there are many metal inlets to the chamber itself, and these may produce "leakage" pathways, which can be eliminated by inert plastic connections.

DR. HOWARD: I would agree with that. Also, though, I think that a somewhat lower resistance can also reflect very low levels of surfactants or oxidized lipid. I think that in the cases where we have obtained lower values for unmodified membranes, that what we were seeing was either an unclean chamber or an impure lipid preparation.

DR. CHARLES PAK: I would like to turn the trend of the discussion to proteins again. Protein monolayers and long-chain lipid films have collapse pressures generally exceeding 24 dyne/cm at both the air-water and oil-water interface. Below this pressure, the protein and lipid will form a mixed monolayer. In other words, if a lipid monolayer is formed over a solution of protein, the protein may penetrate as well as adsorb onto the film. Thus, in speaking about the adsorption of protein over lipid bilayers, one ought to consider the possibility of penetration by protein.

DR. D. A. HAYDON: I have two comments on topics that have already been mentioned. First, on the question of producing thinning that Dr. Tien has just commented on. One can instigate thinning by an electric pulse. But, one can do it equally well by tapping the bench. However, I think the presence of a field across the membrane certainly does produce further thinning. Secondly, we recently reported some work on films which were made with hydrocarbon solvents of chain lengths varying from hexadecane down to hexane. Below decane, for instance for hexane and heptane, the solubility in water of the hydrocarbon is still small, but is nevertheless sufficiently great that quite often the films become abnormally thin due to the diffusion of the solvent hydrocarbon from the film more rapidly than it is replenished from the edges. This can be avoided by presaturating the aqueous phase and, of course, the vapors above it with the lipid solution from which the films are made. It is no use saturating with pure solvent because then the film tends to thicken again, owing to an osmosis of solvent back into the film.

DR. ANDREOLI: I wish to ask Dr. Haydon if the films that he makes in hexane or heptane are stable. The films that we can make in decane are quite stable for longer than three hours, but the films in

hexane or heptane have been very unstable, generally not lasting longer than about 15 to 30 minutes. Additionally, they rupture quite easily if solutions are flowed by them.

DR. HAYDON: I would say ours did not last as long as that.

DR. MYSELS: I would like again to bring in an analogy between soap films and invert films. The main cause of bursting of soap films is evaporation of water. If this is prevented, many films will last practically indefinitely. The same must occur in invert systems if the water phase can dissolve any of the components of the membrane unless it has been previously thoroughly saturated, and this would explain the observation that apparently the lower molecular weight solvents which are also more soluble in water tend to give less stable films.

DR. WOLF D. SEUFERT (The Rockefeller University, New York, N.Y.): I would like to comment on the question of lipid-protein interaction in bilayer membranes. I looked into the effects of surfactants on the Mueller-Rudin film some time ago and found the following (Nature *207,* 174 (1965)): 1. The high resistance of the membrane can be lowered by a factor of 10^3 to 10^4 when an anionic detergent is added to the aqueous phase on one side of the membrane. 2. If a salt gradient exists across the membrane, this decrease of R on addition of a detergent is accompanied by the development of a cationic potential. The potential is controlled by the salt gradient.

These effects led me to the conclusion that the detergent-lipid interaction might well be a model case for the protein-lipid interaction. Proteins could behave like detergents do and penetrate with their hydrocarbon residues into the lipid phase and have their polar groups still anchored in the water. That proteins are surface-active had been shown by Danielli in 1938.

I would like to believe that the detergent-lipid interaction brings about a change in the configuration of the lipid layer from the palisade array to some other micellar arrangement. Charged pathways would be provided which would only permit cations to pass. The development of a potential by detergents and the sign of it would find the simplest explanation in such an hypothesis.

DR. GOLDUP: I am not very happy with the suggestion that films formed from solution in lower hydrocarbons are less stable because of diffusion or solubility of the solvent into the surrounding aqueous phase. I believe their low viscosity is a more important factor. As is well known when these solutions thin in vertically orientated orifices the torus is appreciably thicker at the top than the bottom. In fact it is often hardly discernible on the lower circumference. This surrounding reservoir fulfills a very important function in enabling the delicate membrane, which remember has an aspect ratio often around 500,000:1, to withstand the small vibrations present in most laboratory setups. Once part of this torus becomes very thin a weak point is created. Drainage of the border will occur more rapidly with hydrocarbons of lower viscosity and the life expectancy of films formed from these solutions would be less than from solutions of higher viscosity. Further, the overall viscosity of the bilayer itself is, of course, important and this will be enhanced by the presence of more viscous filler molecules. Indeed, the probable reason that bilayers have not yet been formed from molecules that form condensed monolayers at interfaces is that the corresponding bilayers would tend to be brittle and unable to tolerate the small vibrations that experimentally are difficult to avoid.

DR. MYSELS: Another important condition for the stability of a film is that its border must not detach itself from the support. Soap films are generally supported by glass, and when very clean, this is sufficiently well wetted to give a zero contact angle and thus satisfy this condition. In the oil-in-water systems, one has to depend on a finite contact angle, and it should be possible to improve the situation by providing a very narrow groove at the point where the film is attached. This would make it more difficult for the oil to de-wet the Teflon and also would provide a reservoir of solution for equilibrating with the film.

DR. THOMPSON: We did something very similar to what you have just suggested. We made a septum out of two pieces and put a spacer between them with a hole in the spacer bigger by about half a millimeter than the hole in the two pieces. Using this system you can make membranes which have diameters of up to one centimeter.

DR. TIEN: We have used two very thin Teflon sleeves stacked together with a hole punched through both for BLM support. The space between the sleeves serves as a reservoir for the lipid solution. We believe that the thick region of the membrane, known as the Plateau-Gibbs border, is crucial for BLM stability. This Plateau-Gibbs border is thought to regenerate and absorb BLM continually in response to stretching caused by fluctuations in hydrostatic pressure across the membrane. This border may be gradually depleted to a critical level as a result of the dissolution of lipid solution or some other causes. Therefore, it would be advantageous in long-term studies to construct a membrane support which has a lipid reservoir around the periphery of the opening.

DR. SHINPEI OHKI (State University of New York at Buffalo, Buffalo, New York): I would like to ask about determination of bilayer thickness. Since the bilayer consists of oriented hydrocarbon molecules and oriented polar groups, the refractive index for oriented molecular layers must be used. As far as I know there is no expression giving the refractive index for an oriented molecular assembly, and I would like to ask you how you estimate the refractive index for bilayer membranes.

DR. HOWARD: As I said, we have not measured the refractive index of the lipid bilayer directly, so I would have to refer that question to Dr. Tien who has made such determinations.

DR. TIEN: In the previous determinations of BLM thickness, the membrane has been treated as a homogeneous and isotropic system. Since the substances used such as phospholipids are not isotropic, I think that a three-layered model is a better approach. The bilayer membrane may be pictured as composed of a "polar group/hydrocarbon core/polar group" type of arrangement [for a detailed discussion, see J. Theoret. Biol., *16,* 97 (1967)]. The refractive index of BLM may be estimated with the aid of Brewster's law. It involves essentially a measurement of BLM reflectance with polarized light.

DR. NORTON: One can't help but wonder if you couldn't take the refractive index of single crystals of lipids and extrapolate this value to at least a first approximation, especially when experimental information is impossible to get.