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Phospholipid Cell-Membrane Models

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Although the general electrical and lipid characters of biological membranes were recognized by the beginning of the present century (5), one of the ultimate objectives of work on cell membranes—the production of an *in vitro* model to enable study of the properties, and of factors affecting transport of substances across them without the complications found with the *in vivo* preparations—has not been realized completely. Recently these investigations have been facilitated by the progress that has been made in the isolation and characterization of the phospholipids (1) and during the last few years reports of some of the most successful model membranes have been published.

Phospholipids are present in most biological tissues and it is now accepted that they form part of the basic structure of the natural cell membrane, but beyond this it is difficult to make a more specific statement. This chemical group of compounds contains numerous members which are usually found as mixtures together with proteins and other substances such as cholesterol. Thus, the properties of membranes can vary considerably with the organ and species of animal, depending on the proportions of the constituents and the arrangement of the molecules (1–3, 20). Examples are given in Table I to illustrate this diversity of composition.

Before discussing the membrane models in which these substances have been used, some of the more important work on the structure of the cell membrane is mentioned in the following paragraphs, but as it has been published in greater detail elsewhere (4–7) only a brief survey is given here to explain what the workers, who have suggested models, are attempting to reproduce.

In 1925, Gorter and Grendel (8, 9) compared the area of a layer of lipid extracted from red blood cell ghosts with the dimensions of the cell and postulated the bimolecular lipid layer structure (see Fig. 1). About the same time other workers, calculating the thickness of films from capacitance data (10) and from measurements of reflected light (11) obtained evidence for one and possibly four layers of lipid molecules in membranes, respectively. Without knowledge of the work of Gorter and Grendel, Danielli *et al.* (12, 15) suggested the paucimolecular theory. They proposed that the cell

Table I—*Lipid Content and Composition of Cell Membranes (19)*[a]

	Human CNS Myelin	Beef PNS Myelin	Human Erythrocyte	Rat Liver Mitochondria	Guinea Pig Brain Mitochondria	Spinach Leaf Chloroplasts
Lipid content, % of dry weight	78.7	75.9	40–50	27–29		51.5
			Molar % of Total Lipid			
Cholesterol	40.1	39.0	41.9	5.9	13.7	—
Ethanolamine glycerophosphatides	13.4	12.7	13.9	27.4	28.7	0.6
Serine glycerophosphatides	5.0	6.8	4.8	tr.	4.8	—
Choline glycerophosphatides	10.8	10.2	15.0	43.4	33.8	4.1
Inositol glycerophosphatides	2.0	2.0	—	6.7	4.5	1.4
Cardiolipin	tr.	tr.	—	8.5	7.3	0.6
Phosphatidyl glycerol	tr.	tr.	—	2.6	—	5.3
Sphingomyelin	4.6	13.0	9.4	4.4	3.4	—
Cerebroside	15.4	11.5	tr.	—	2.6	—
Cerebroside sulfate	4.0	1.8	—	—	—	—
Other sphingolipids	1.6	—	6.0	—	—	—
Galactosyl diglyceride	—	—	—	—	—	14.7
Digalactosyl diglyceride	—	—	—	—	—	35.3
Sulfoquinovosyl diglyceride	—	—	—	—	—	4.9
Chlorophyll	—	—	—	—	—	23.5
Carotenoids	—	—	—	—	—	4.9
Other minor lipids	3.1	3.0	5.2	1.1	1.2	4.7
Total glycerolipids	34.4	34.7	42.7	89.7	80.3	66.3
Total sphingolipids	25.6	26.3	15.4	4.4	6.0	—

[a] [Reproduced with permission from *J. Theoret. Biol.*, **15**, 307(1967).]

membrane consists of a bimolecular lipid leaflet with a layer of protein molecules on either side (see Fig. 2). These are probably unfolded and are covered by a second layer which is not denatured. Patches of this membrane could be modified to give selective activity (16). It was suggested that electrostatic and Van der Waal's forces hold the protein and lipid molecules together.

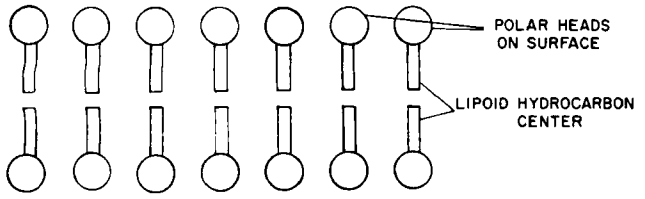

Figure 1—*Diagram of bimolecular lipid layer structure of cell membrane postulated by Gorter and Grendel.*

The recent, and more direct, evidence for the structure of these membranes has come by the use of the electromicroscope with which Robertson (4–6) and others (7, 13, 14) have been able to photograph cell preparations in detail (Figs. 3 and 4). Much of this work has been concerned with the structure of myelin, in which Robertson has demonstrated the parallel bimolecular layers of lipid. It may be that most membranes are formed basically of bilayers and the differences in thickness are due to the arrangement of the molecules. Thus, the degree of unsaturation of the hydrocarbon chain of the lipid molecule may be important, as this would affect the compressibility of the film and, therefore, its thickness; myelin is thought to be a condensed film while the lipids of the chloroplast and mitochondrial membranes form an expanded film. O'Brien (19) gave interesting data on the area per molecule of lipids forming these structures. On the outside surfaces of the biological bilayer, a layer of mucopolysaccharide or mucoprotein may be found. Finean (17, 18) has also studied similar preparations with X-rays but difficulties have been encountered in dehydration of the tissues. Until a few years ago, this concept of the unit membrane was the only postulate. However, Korn (21) has shown that the data were not conclusive and recently alternative theories have been proposed.

Studies of the physical and structural properties of lipids in water by Luzzatti and Husson (22) showed that,

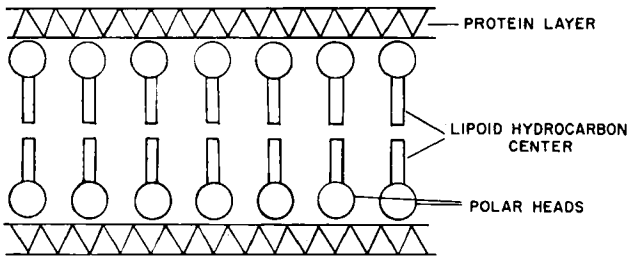

Figure 2—*Diagram of structure for cell membrane (after Danielli and Davson).*

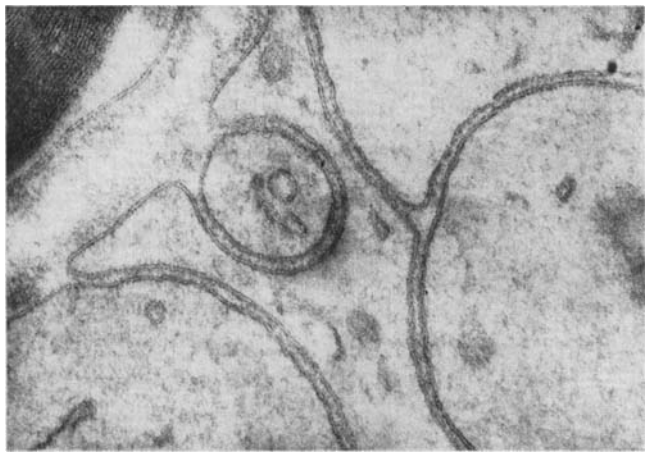

Figure 3—*Portion of a nonmyelinated nerve fiber in mouse sciatic nerve showing the unit membrane of the axon and of the Schwann cell. Note the gap between the two unit membranes. Mag. × 128,000. [Reproduced with permission from* Protoplasma, **63**, *218(1967).]*

depending on the temperature and degree of hydration, the molecules were arranged in lamellae or in hexagonally arrayed columns. This forms the basis of a second theory which was postulated by Sjöstrand and his colleagues (23) and seems to be particularly applicable to nonmyelin membranes. From electron microscopic data, it appears that the lipid molecules are arranged in globular form in the membranes. This would allow for additional structures, such as enzymes, to be incorporated, and gives the impression that the bimolecular layer need not be continuous, but it is in opposition to the theory of Robertson and there is some discussion of its validity.

Spectroscopic and optical rotatory dispersion data have made it necessary to consider another membrane structure. Wallach and Zahler (24) thought that there was a more intimate interaction between the protein and phospholipids of the plasma membrane of the Ehrlich ascites carcinoma cells. It was proposed that the ionic portions of both types of molecules lay on the surface of the membrane, and that the nonpolar side chains of the protein and the hydrocarbon of the lipid were hydrophobically bonded in the interior of the membrane. Lenard and Singer (25) have proposed a similar structure for red blood cell and *B. subtilis* membranes, as shown in the diagram which is reproduced in Fig. 5. Additional evidence has been published this year (26). By hydrolyses of the phospholipids in the membrane, 68 to 74% of the phosphorus was obtained, but the average conformation of the protein remained the same and the membrane did not disintegrate. Again this seemed to indicate that the polar groups of the phospholipid were on the surface in contact with the phospholipase.

As knowledge of the structure of cell membranes has advanced, parallel attempts have been made to construct models. The transport and partitioning of substances between lipid and aqueous layers, as in the techniques of Doluisio and Swintosky (27), Perrin (28), and Martin and Khalil (29), were intended for study of the kinetics of transport and no attempt was made to produce a replica of the cell surface. Since, until recently, cell membranes appeared to be a bimolecular leaflet formed of phospholipids it seemed obvious to many

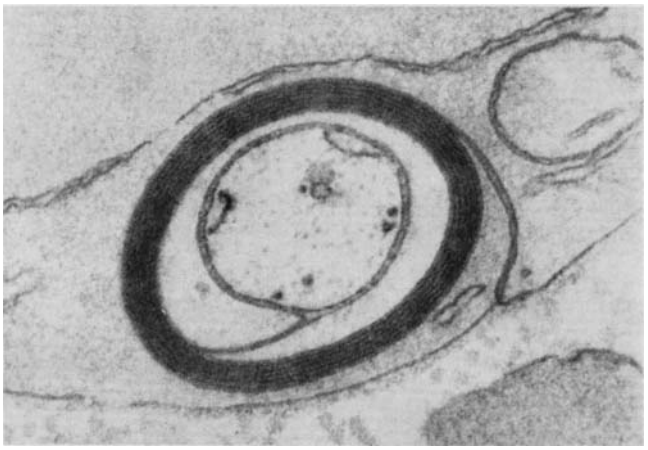

Figure 4—*Young mouse sciatic nerve fiber showing developing myelin sheath. Note the relationships of the unit membranes of the mesaxon to the compact myelin. Mag.* ×*160,000.* [*Reproduced with permission from* Protoplasma, **63,** *218(1967).*]

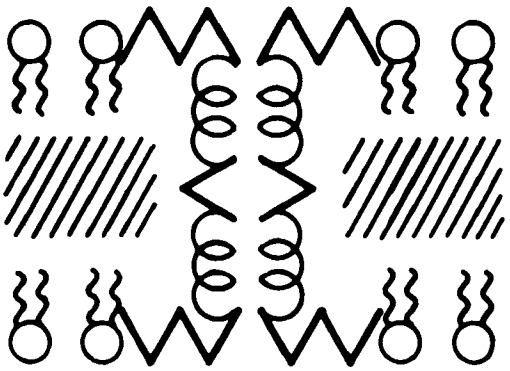

Figure 5—*Generalized membrane as described in the text. The Proteins consist of helical (⌇⌇⌇) and random-coil (∿∿) portions. The Polar lipids (♀) are orientated with their polar heads (○) facing out. The cross-hatched areas are assumed to be occupied by relatively nonpolar constituents hydrophobic amino-acid residues or lipids). Single polypeptide chains are drawn to transverse the entire membrane, but there is no evidence bearing on this point.* [*Reproduced with permission from* Proc. Natl. Acad. Sci., **56,** *1828(1966).*]

workers to try to adapt methods used for the study of lipids and soap films. The work was pioneered by Langmuir (30) who showed that a monomolecular film could be formed at the liquid-air surfaces and suggested possible biological applications. This review is concerned chiefly with models of the last decade and for convenience of discussion it has been divided into five sections: *Recent monolayer studies; Phospholipid membranes formed on a mechanical support; Bimolecular lipid membranes; Liquid crystal systems; Phospholipid-protein systems in aqueous media.*

RECENT MONOLAYER STUDIES

At first monolayers of proteins and lipids on aqueous phases at interfaces were examined (31–36). Lipoprotein monolayers were usually formed by injection of protein under a lipid layer which had been previously formed at the interface. Basically, the methods of Langmuir are still used but these have been refined and studies made at both the oil-water and air-water interfaces were, at one time, the best models available. Although the workers realized that as far as reproduction of the cell membrane was concerned, the monolayers were not very satisfactory, they found that if the monolayer was assumed to reproduce one side of the bilayer it was particularly useful for investigating reactions at surfaces, and for the detailed study of the orientation of the molecules in a layer. The presence of electrolytes in the solutions under the films caused a redistribution of ions and a change in the potential energy of the film. In 1941 Cassie and Palmer (37) developed a theory which agreed quantitatively with the data available at the time and showed that the ions of opposite charge to the film had most influence. The discrete ion effect may account for various phenomena, if it is assumed that binding between counterions and head groups on the film is small (38). Haydon and Taylor (39) proposed a theory of ionized monolayers but Levine *et al.* (40) have shown that it was incorrect. Rogas *et al.* used monolayers to investigate the ion-exchange properties of

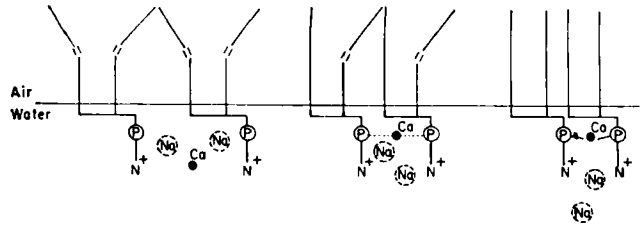

Figure 6—*Schematic representation of interaction of calcium ion with lecithins of different degrees of unsaturation. The dotted line represents weak and the continuous line, strong Ca^{++} interaction. [Reproduced with permission from* J. Lipid Res., **6**, *341(1965).]*

phospholipids which probably provided the negative ionic sites in the cell membrane (41, 42).

Since 1965 there have been some interesting studies made by Shah and Schulman (43–47) on the binding of metal ions. They confirmed that the limiting area of the lecithin molecule in a layer depended on the degree of unsaturation, such that dipalmotoyl lecithin had a smaller area than egg lecithin which was, in turn, smaller than yeast lecithin. They found that the binding ions increased the surface potential, which also depended on the degree of unsaturation of the fatty acid portion of the lipid molecule. The authors suggested that the results could be due to stearic effects and their diagrams of the lecithin molecules have been reproduced in Fig. 6. The compounds containing unsaturated hydrocarbon chains permitted water and large hydrated ions, such as sodium, to be attached to the phosphate groups whereas, when the hydrocarbon was saturated, there was close alignment and the smaller calcium ion fitted well, forming a strong bond between two phosphate groups. The figure also shows egg lecithin intermediate between the yeast and dipalmotoyl lecithins. In cardiolipin, unsaturation has little effect since the structure of the molecule prevented the hydrocarbon chains from coming close (see Fig. 7). Further work (44, 45) with sphingomyelin showed that there was less interaction of ions with this molecule than with dipalmotoyl lecithin. With reference to Fig. 8 of sphingomyelin, it can be seen that ion dipole of the hydroxyl and phosphate was possible, reducing the net charge of the phosphate oxygen, while the effect of the ammonium group increased resulting in the observed decrease of action with metal ions. If higher concentrations of the metal chloride were used, the chloride swamped the quaternary ammonium and there was a greater reaction between the phosphate and the metal ions. The change in the measured surface potential could be due to an increase in the thickness of the Gouy layer around the ionic groups in the case of the sphingomyelin, whereas with the lecithin there was a change in the Stern layer.

Measurements of surface potential were more reliable than surface pressure in studies of the incorporation of cholesterol. Combinations of cholesterol and dicetylphosphate follow the area per molecule additivity rule but the lecithin-cholesterol mixture did not (46). From surface-potential data there did not appear to be any interaction between the lecithin and cholesterol, so that when condensation between these compounds occurred it must have been due to occupation of spaces between the molecules. Maximum effect occurred when the dipalmotoyl lecithin–cholesterol ratio was one since this gave the geometry for optimum packing. Reactions with calcium depended on concentration and on the degree of unsaturation of the lecithin, as with an increase of unsaturation there was an increase in shrinkage (47). Calcium solidified the layer and may have formed an internal salt linkage.

The inclusion of cholesterol on the layers has been investigated recently by Demel *et al.* (48, 49). Again the force area curves depended on the unsaturation of the fatty acid and on the temperature, but the characteristic change in properties with egg lecithin–cholesterol ratios of 3:1 and 1:3 observed by the previous workers (46) was not noted. Cholesterol had the condensing effect previously shown by Bernard (50) but it was dependent on the fatty acid in the lecithin molecule. If the acid was oleic then condensation occurred, but if the lecithin molecule contained linolenic or linoleic acid there was no condensation. There did not appear to be a simple explanation of the problem. Recent experiments by Demel and Joos (51) at the oil-water interface showed ideal behavior and therefore they concluded that interactions at the air-water interface were mainly due to Van der Waal's forces. Surface pressure and potential measurements of synthetic phosphatides mixed with cholesterol showed that only dimyristoyl DL-phosphatidyl choline-cholesterol monolayers deviated greatly from the ideal (52); surface potential indicated

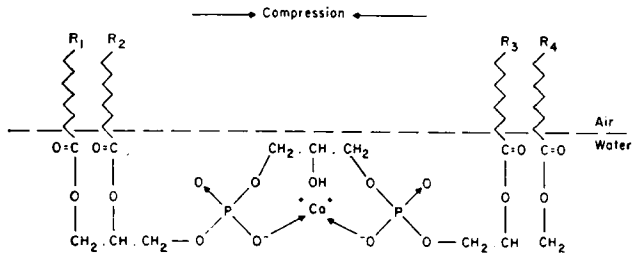

Figure 7—*Orientation of cardiolipin molecule at the air-water interface and calcium ion binding by its two phosphate groups. [Reproduced with permission from* J. Lipid Res., **6**, *341(1965).]*

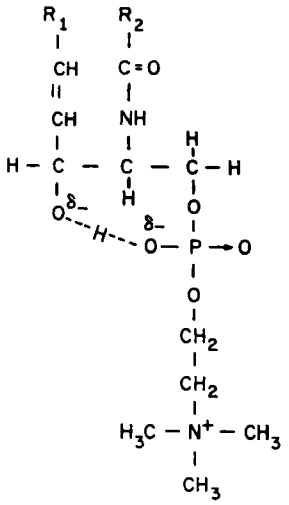

Figure 8—*The ion dipole association between the hydroxyl group and the ionic oxygen of the phosphate group of sphingomyelin. [Reproduced with permission from* Biochim. Biophys. Acta, **135**, *184 (1967).]*

dipole shifts. Since it appeared that unsaturation was not the only determining factor, chain length and temperature also had to be considered. Vilallonga *et al.* (53) evaluated the free energy of mixing at the air-water interface for lecithin-cholesterol mixtures. Postulating the existence of specific interactions was not sufficient to explain the results and a geometrical arrangement was necessary.

Addition of fatty acids and glycerol esters in lecithin monolayers caused negative derivations from ideality when oleic acid was used. Results with di- and triglycerides were not conclusive (54). Monolayer studies were also being performed with ^{35}P-labeled lecithins (55) and were being used to investigate reactions at surfaces. Shah and Schulman (56) carried out the enzymic hydrolysis of various lecithin monolayers and obtained the corresponding lysolecithin and fatty acids. They were able to examine the reaction in the presence of spacers and chelating agents. Colacicco *et al.* (57) have used monolayers of glycosphingolipids to study immunological reaction with such proteins as globulins and albumins. The preliminary experiments of Schaubman and Felmeister (58) have indicated that monolayers can be useful in studying photosensitizing reactions. Lecithin films were formed on subphases containing chlorpromazine and then subjected to UV irradiation, and to changes in pH and surface pressure, all of which were shown to have effects on the penetration of the film by the chlorpromazine molecule.

PHOSPHOLIPID MEMBRANES FORMED ON A MECHANICAL SUPPORT

General—Since the films are fragile, often an inert mechanical support is provided on which the phospholipid can be deposited over large areas. Collodion is suitable but more recently Millipore has been used. Collodion membranes were impregnated with lecithin by Weatherby in 1942 and 1943 (59) and were shown to have some of the properties of natural membranes. In a recent review of transport phenomena Lakshminarayanaiah (60) discussed the formation of artificial membranes simulating the behavior of cell membranes. He used parlodion membranes incorporating stearic acid and phospholipids in some of them (61). Some workers have made membranes from synthetic polymers and alginates, and nonaqueous membranes have been formed from short-chain alkyl alcohols (60). Monnier *et al.* (62), in 1962, suggested that a thin leaf of porous polyethylene with a gel of phosphorylated fatty alcohols and amines was suitable. A resistance developed when this membrane was used to separate two identical salt solutions.

Use of Millipore—Following work on the excitation processes of living cells, Tobias *et al.* (63, 64) decided to use a model of the cell surface made by dipping a Millipore filter into a solution of a lipid in benzene and then clamping this membrane between two plastic 15-ml. vessels. The latter were filled with electrolyte solutions of varying composition and the electrical properties of the membrane measured with platinum electrodes and the usual associated electrical circuit. The membranes contained 1 to 1.8 mg. lipid/sq. cm. (see Table II), but as the distribution could not be determined accurately, the membranes were not exactly reproducible and the results were only semiquantitative. Initially, they investigated the resistance of the membranes (63) but later examined hydration (65), conduction and uptake of ions, and finally compared the model with the human red blood cell (66).

Table II—Average Resistance of the Millipore Membrane as a Function of its Composition and as a Function of the Salt in the Adjacent Compartments (63)

Material on Millipore—Name	mg. cm.^{2}	Ambient Salt	Resistance, ohms cm.^{2}
Cephalin alone	1.2	KCl	12
	0.9	NaCl	9
	1.1	$CaCl_2$	853
Cholesterol alone	—	KCl	1×10^5
	—	NaCl	1×10^5
	—	$CaCl_2$	1×10^5
Equimolar cephalin and cholesterol	1.6	KCl	21
	1.4	NaCl	26
	1.5	$CaCl_2$	1,075
	1.7	$MgCl_2$	425
	1.5	$BaCl_2$	260
	—	$AlCl_3$	1,290
	—	Protamine sulfate KCl tris	412[a]

[a] From Leitch and Tobias (65).

Table II shows values that Tobias *et al.* (63) obtained for the resistance of the membrane and illustrates the effects due to composition of the membranes and of the surrounding medium. The high resistance of the cholesterol membrane was very prominent and may have been due to the well-known nonwettability of cholesterol surfaces. A steady state was reached quickly in solutions of monovalent salts but slowly in calcium chloride. In general, the phospholipid membranes had a low resistance in solutions of monovalent cations and a high resistance in the presence of polyvalent cations and of protamine sulfate. There did not appear to be much difference between the action of potassium and sodium but calcium had pronounced effects; an increased calcium-ion concentration in an otherwise constant medium caused a rise in the resistance of the barrier, and calcium was taken up in preference to the monovalent ions leading to a decrease in permeability to the latter (66). Response to a current flow across a membrane with potassium chloride solution on one side and calcium chloride on the other depended on which electrode was made positive; if the potassium side was positive the resistance fell, presumably because the potassium ions displaced the calcium in the membrane. The opposite was found when the polarity was reversed.

Studies of the water content of the membranes were made by Leitch and Tobias (65) employing three experimental methods:

(*a*) Soaking the membranes in aqueous electrolyte solutions and drying them to determine the water content by loss of weight. Table III shows that the amount of water varied with the concentration of salt but in the case of calcium chloride the results were not statistically convincing. However, it is interesting to note that water uptake was greater in potassium chloride than in calcium chloride particularly at low concentrations. When protamine was present the uptake was less than that of the controls.

Table III—Water Content of Membrane Model After Soaking in KCl, $CaCl_2$, or Protamine Solution (65)

No. Samples	Solution Used	mg. H_2O/mg. Lipid	No. Samples	Solution Used	mg. H_2O/mg. Lipid	Difference Diff.	Difference p^a
12	100 meq. KCl	1.54 ± 0.02	12	100 meq. $CaCl_2$	1.41 ± 0.04	0.13	0.01
12	300 meq. KCl	1.44 ± 0.02	12	300 meq. $CaCl_2$	1.36 ± 0.02	0.08	0.01
12	500 meq. KCl	1.36 ± 0.02	12	500 meq. $CaCl_2$	1.32 ± 0.03	0.04	0.28
12	100 meq. KCl, 0.05 *M* tris	1.64 ± 0.04	12	100 meq. KCl, 0.05 *M* tris, protamine, 1.0 mg./cm.[3]	1.42 ± 0.05	0.22	0.01

[a] Statistical evaluations given as *SE*.

(*b*) Equilibrium studies showed that the potassium salts of cephalin absorbed more water vapor in the membrane form than calcium cephalinate (see Fig. 9) but although protamine mimicked calcium in the first technique, it had no significant effect here.

(*c*) Qualitative demonstration of hydro- and oleophobicity of pieces of membrane was according to the method published in the previous paper (63). It was found earlier that pieces treated with calcium remained in the oil phase while those treated with potassium descended into the aqueous phase.

The effects of calcium and potassium ions on the osmotic flux through the membrane were in opposition, as was illustrated by the 61% reduction of the basal rate by calcium ions which was restored in potassium chloride solution and *vice versa*. The water flux varied inversely with the Ca:K ratio and was near zero when the Ca:K ratio was the same as that for maximum resistance of the membrane. Protamine also decreased the osmotic flow. In all of the electroosmotic experiments water traveled toward the cathode indicating that there was a net negative charge on the channel walls.

These biologically interesting effects seemed to depend on the phospholipid. The authors conjectured that the main role of the phospholipid was to provide a path of finite and modifiable ion mobility. The resistance rise in the presence of calcium could have been due to the acid groups and also to dehydration of the membrane whereas potassium caused increased hydration. It is possible to account for the results by postulating that the lipid is basically an anionic-exchange material. In a study of the Ca:K reaction and the cation exchanger characteristics of the membrane, Mikulecky and Tobias (66) suggested two models for describing the transport mechanism, based on (*a*) Donnan equilibrium and (*b*) the mass action equilibrium between membrane, counterions, and the membrane-counterion complex. Either explanation seemed possible; but on application of the Teorell-Meyer-Sievers theory as modified by Schögl (67, 68) the experimental results were not predicted by the first model. This could have been due to various factors and Mikulecky and Tobias suggested that a weak salt was formed between the membrane anions and calcium. Application of the law of mass action showed a preference for calcium over potassium. In experiments with red blood cell ghosts selectivity was also demonstrated for calcium when in the presence of potassium ions, but it decreased with increasing concentration of total salt, and as the calcium concentration increased, the water content decreased.

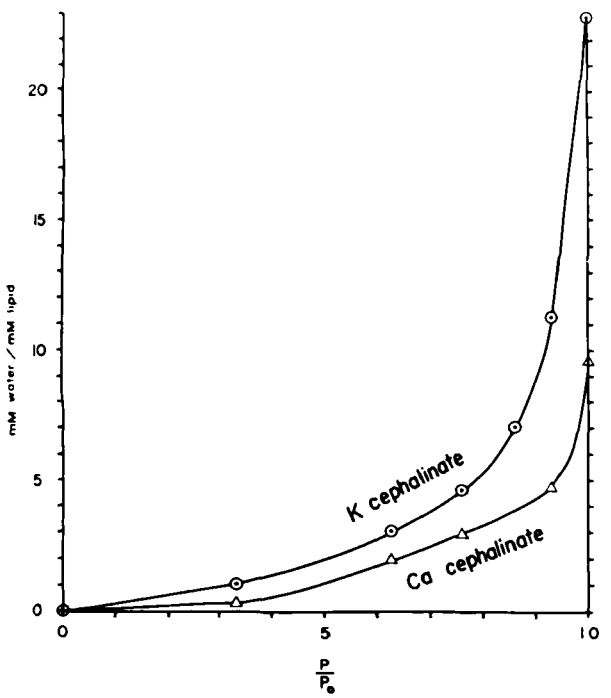

Figure 9—*Vapor sorption isotherms of K cephalinate and Ca cephalinate.* [*Reproduced with permission from* J. Cell Comp. Physiol., **63**, *225(1964).*]

Some correlation of results with axonal function was made. This was speculative but seemed to fit with previous data. When potassium passed to the axonal surface from the axoplasm, it competed with the calcium in the membrane and caused an increase in hydration, thus more aqueous channels were made through the membrane leading to a decrease in the resistance. The fixed-charge theory may be applicable as the channels are probably lined with phosphate, carboxyl, and amino groups and have an overall negative charge particularly at a pH of 7. When potassium dominates there are secondary structural changes since potassium is monovalent while calcium is divalent. Protein may be important by causing reorientation and displacement of the ionic groups and much evidence supports the view that excitation is an unbonding of calcium by potassium.

Millipore has also been used as an inert base by O'Neill and Goddard (69) who impregnated it with oils and oil-solid mixtures and then added cholesterol and lecithin. They likened the matrix to the keratin of the stratum corneum and investigated the action of oils. Free cholesterol and crude soya lecithin reduced the permeability to water vapor of cottonseed oil irregularly.

The development of methods from the technique of Hooke and Newton for the preparation of secondary black soap films in air was pioneered by Mueller *et al.* (70–73) and has been rapidly adopted by many workers. The films were prepared by spreading an organic solution of the lipid over a small hole in an aqueous medium and allowing diffusion or evaporation of the solvent to take place so that the transition to the lowest stable state, the bilayer, could occur. This change was followed by observing the interference colors of light reflected off the surface which finally became a nonreflecting bilayer; thus giving rise to the term secondary black films (see photographs in Fig. 10). Work on these membranes can be most easily classified under the names of the authors who have carried out the research, and has not therefore been reviewed in a strictly chronological order. In general only preparations using phospholipids are discussed in detail in the following paragraphs with a brief mention of the use of certain other compounds. A discussion of the work up to 1967 has been published by Tien and Diana (80).

Mueller, Rudin, Ti Tien, and Wescott (70–73)—These authors dissolved the phospholipids in a chloroform-methanol solvent and spread them over a wire loop. They gave a detailed description of the extraction procedures for obtaining phospholipid (72) which were later modified to avoid the necessity of removing proteins (73). The membranes were manipulable, resilient, self-sealing to puncture, and liquid in the plane of the bilayer. They stained with osmium tetroxide and were observable with the electron microscope. Experimental conditions and physical properties are listed in Tables IV and V, respectively, where they can be compared with preparations from other laboratories. In ionic gradients the film is weakly polarized. It has poor selectivity for sodium and potassium ions and the resistance decreases from a maximum with current change when hydrogen and calcium ions pass into the membrane (73). Magnesium and monovalent ions had to be present to render the membrane excitable when an unidentified protein was added. Certain additives such as tetradecane and α-tocopherol prevented solidification of the membrane by allowing the formation of secondary black to go to completion. Of the compounds listed in Table VI, some are absorbed onto the membrane but do not lower the resistance while others cause a reduction to 10^3 ohms/cm.^{2} or less. The authors thought that these substances probably penetrated the membranes to form channels thus allowing the passage of ions.

After this preliminary work, these workers have divided into two groups: one led by Ti Tien and the other by Mueller and Rudin. The first membranes had been made from phospholipids, but Ti Tien *et al.* (74, 75) were interested to know if other materials were equally suitable. Initially they used the techniques described above (61), then modified the method considerably (75) devising an apparatus to enable rapid generation of new films. Fresh cholesterol did not form stable films but aging was beneficial (74). Presumably this was due to oxidation of the material since they found that films were also made successfully with oxidized cholesterol and 7-dehydrocholesterol. In the second paper (75) they

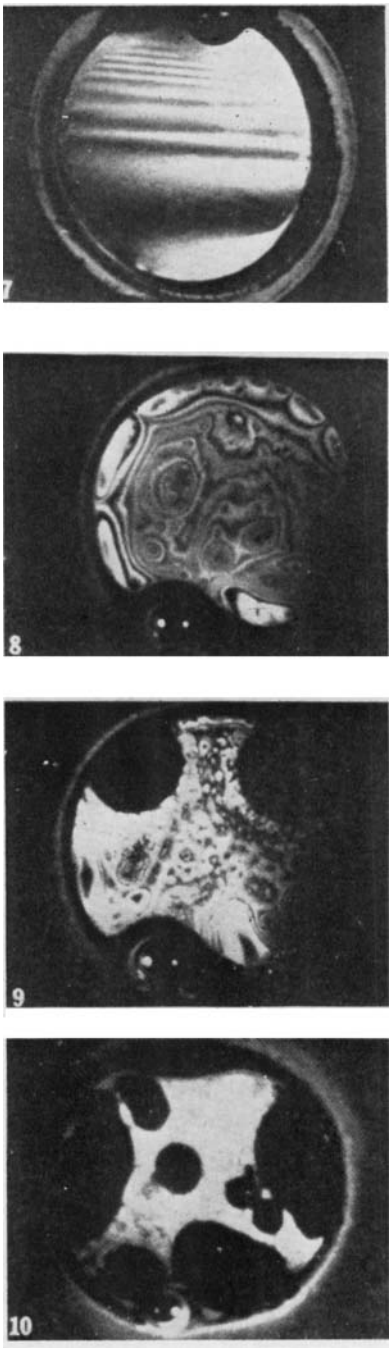

Figure 10—*Selected frames taken from a color movie first shown at the Symposium on Plasma Membrane, American Heart Association, New York City, December 1961. The growth of black film at various stages. (Permission from Drs. Tien and McGarth, Academic Press.)*

described the formation and properties of membranes formed with long-chain fatty acids, glyceryl distearate, and some surfactants of which sorbitan tristearate was one. The films were mobile when formed in weak sodium chloride solutions and rigid (plastic) in saturated solutions. The later method offered greater opportunity for the characterization of factors governing the formation of the films and contamination was less easy, but the first technique was still necessary for examining the electrical properties. Even more recently Ti Tien *et al.* have designed an apparatus to measure interfacial tension and electrical properties (76–78). Cholesterol membranes were stabilized with various surfactants and the

Table IV—Experimental Conditions for Formation of Bimolecular Lipid Membranes

Experimental Factor	Mueller *et al.* (70–73)	Thompson *et al.* (93–94)	Hanai *et al.* (107)	Writh *et al.* (117)	Finkelstein and Cass (91–92)	Andreoli *et al.* (103–104)	Lgäuer *et al.* (120–122)
Source of material	Ox white matter	Egg	Egg	Calf brain	Ox white matter	Erythrocyte	Egg and synthetic
Organic solvent	Chloroform-methanol	Chloroform-methanol	*n*-heptane *n*-decane	Methanol	Chloroform-methanol	Decane	*n*-Decane
Solutes other than phospholipids	Tetradecane, α-tocopherol	*n*-Tetradecane	—	α-Tocopherol, cholesterol	α-Tocopherol, cholesterol	Cholesterol	Cholesterol
Salts	NaCl, KCl, histidine	NaCl	NaCl, KCl, $CaCl_2$	Buffered saline	NaCl, $MgCl_2$, histadine	NaCl, KCl	NaCl, KCl, iodides
pH solutions	7	—	7.5–1	—	7	5–7	—
Time of formation, min.	Few	4	1–2	—	—	6–10	>1
Time persisted, hr.	24	1	5	—	—	1	Several
Temperature	30–47	36	20	33	35–37	22–24	35

interfacial free energy measured (76). Although phospholipids were not used these experiments are of interest as the same type of membrane is formed, and the materials used are generally found in association with phospholipids. Recently Ti Tien (79) proposed a theory of the structure of the black lipid membranes. He believed that the original model of one bilayer was too simple and that there could be a triple-layer structure: polar/oil/polar. He also thought that the phospholipid could have been fully extended and perpendicularly aligned to the membrane-water surface, which did not agree with the theory of Hanai and Haydon (see later).

In 1967, a paper by Mueller and Rudin (81) reported that derivatives of the antibiotics, valinomycin, actin, and enniatin increased the membrane conductance to a magnitude of 10^{-3} ohms cm.$^{-2}$ and were active on a variety of membranes. The single-ion conductances differed by as much as 300 times and were in the following order: Li < Na < Ca < K < Rb. If there was a concentration gradient across the membranes, resting potentials were induced, which tended to 150 mv. when 0.1 *M* sodium and potassium chlorides were on opposite sides of the membranes. Hydrogen bonding of the carbonyl oxygen atom in place of the water molecules of the hydration shell around the cations may have occurred. There seems to be some relationship between the size of the ring compounds and that of the cation. This is supported by the result that the four- and two-unit valinomycin rings show little activity, but the enniatins are smaller and are active. There is a possibility that the ring compounds form both pores and carriers. The membrane permeability to thiourea increased from $4.51 \pm 0.52 \times 10^{-6}$ cm. sec.$^{-1}$ to 12.10 ± 2.30 cm. sec.$^{-1}$ by enniatin B, thus showing that the latter substances can affect the permeabilities of unionized and ionized substances (83). At the same time the membrane resistance decreased.

These experiments have developed in an interesting fashion, as Mueller and Rudin have now formed membranes that show electrokinetic phenomena similar to the action potential (82, 84–86). The bimolecular leaflet was formed in a solution containing histidine hydrochloride at a pH of 6.8 (82). Phosphate or sulfate ions were then added and an unidentified protein, which they have termed excitation-inducing material (EIM) was introduced into one compartment. The membrane under these conditions had a resistance of 2×10^5 ohms cm.^{2} and a negative resting potential of approximately 50 mv. on the inside. After titration of protamine to an approximate concentration of 10^{-4} g./ml. the membrane responded to applied pulses and rhythmic firing. It was necessary to carefully control the composition of the medium since no effect was obtained with histones or cholesterase substituted in place of the protamine. Salts also had a controlling action; calcium ions blocked the rhythmic firing and then changed the thresholds; monovalent anions caused bistable kinetics, while anions such as phosphate and sulfate produced unstable and rhythmic action potentials. The responses were reversibly blocked by local anesthetics of the cocaine group at a concentration of 2% which was interesting as it was the physiologically active concentration, and acridine and phenothiazine derivatives modified the responses (1 mcg./ml. required). The authors suggested that EIM develops cation-conducting channels and protamine converted some of these to conduct anions. This postulate fits with the Hodgkin-Huxley theory of the production of action potentials in the squid nerve.

Table V—Properties of Bimolecular Lipid Membranes

Property[a]	Mueller *et al.* (70–73)	Thompson *et al.* (93–94)	Hanai *et al.* (107)	Writh *et al.* (117)	Finkelstein and Cass (91–92)	Andreoli *et al.* (103–104)	Läuger *et al.* (120–122)
Diameter, mm.	1	2	1.4	0.4	—	1–2.5	3.5
Thickness, Å.	60–90	72–105	48	—	<100	46–132	70 ± 10
Resistance, Ω cm.^{2}	11×10^7 1×10^8	1×10^6	1×10^7	—	—	1×10^8	1×10^8
d.c. Conductance, mhos cm.$^{-2}$	—	—	$<1 \times 10^8$	1×10^{-6}	—	—	—
Capacitance, μ f. cm.$^{-2}$	1.0	—	0.38	0.5–17	—	0.38–4	0.3
Breakdown, mv.	150–200	200	150	—	—	—	—
Water permeability, μsec.$^{-1}$	—	4.4–17.3	8.3–14.4	1.8 μmin.$^{-1}$	in order of 10^{-3}	—	—

[a] A range of numerical values of these properties were often given in the original papers which should be consulted if more detail is required.

Table VI—The Effect of Adding Certain Compounds to Black Lipid Membranes

Compound Added	Effect
Cholinesterase	No change resistance-increase rate of formation
Ricinoleic acid	Decrease resistance-titratable
Digitonin	Rupture of film
Sodium oleate	Rupture of film
Saponin	No effect
Unidentifiable molecule from egg white	Decrease resistance

Recently Mueller and Rudin have published (85) a description of the use of alamethicin to develop action potentials in lipid membranes in the presence of proteins. Membrane potential can be controlled with very small concentrations of antibiotics. As for the other antibiotics, the authors thought that six or more molecules formed channels or carriers for the passage of ions, and it was suggested that they form sites bridging the bilayer. They may have a proton-acceptor function or cause a reverse micellization of the bilayer. The work was discussed in detail in a recent publication (86).

After the early publications of Mueller and Rudin, Seufert (87), experimenting with surfactants, found there was a lowering of the membrane resistance, and that a resting potential existed under the influence of a salt gradient. However, there were no electrokinetic phenomena comparable with those produced by EIM. The surfactants were placed on one side of the membrane and all had qualitatively the same effects. The anionic compounds were the only ones producing steady results. As expected, they produced small anionic potentials which were reversed in the salt gradient. Seufert suggested that the lipid molecules of the membrane were rearranged by the surfactant into micellar structures which had a negative charge thus making a cation selective pore.

The technique of Mueller *et al.* has been employed by a number of workers (88–92) whose results are discussed under *Other Work*.

Thompson et al.—These authors used a technique similar to that of Mueller *et al.* but they formed the membranes from highly purified egg phosphatidyl choline and *n*-tetradecane in an aqueous medium of 0.1 *M* NaCl at 36° (93). Not all the samples of egg phosphatidyl choline would form membranes, but if the latter material was treated with hexane an unidentified substance was removed and the membranes could then be formed. When they tried to vary the composition of the organic solution it was found that both egg phosphatidyl choline and *n*-tetradecane must be present and that the concentration of the constituents was critical. The egg phosphatidyl choline could be replaced by yeast phospholipid but samples of cardiolipin, beef heart phosphatidyl choline, and synthetic phospholipids containing saturated fatty acids were of no use. The authors, therefore, concluded that the molecule of phospholipid must contain a certain amount of unsaturated acid if a stable membrane was to be formed. The tetradecane could be replaced by methyl oleate, and cholesterol and its derivatives were incorporated in equimolar quantities with phospholipid. The proportion of methanol to chloroform in the solvent was also critical and influenced both the time for the formation and the stability of the membrane; the time decreased as the concentration of chloroform increased, but when chloroform was used alone the membrane was unstable; with less than 55% chloroform the time of formation was long; an optimum ratio was 3:2 v/v. As the temperature of the surrounding medium was increased so the time for formation decreased (see Table VII).

The workers had no reliable information on the composition of the membranes but they inferred that phospholipid and water were present—the former by oxidation and refractive index studies and the latter from permeability experiments.

In two later publications Huang and Thompson reported the thickness (94) and the water permeability (99) of their membranes. Thickness was determined by the reflection of light from the surfaces using white and green light and results were 88 ± 10 Å. and 61 ± 10 Å., respectively. The authors thought the latter result more reliable as it compared with Hanai *et al.*'s value of 48 Å. (107) while the depth of a theoretical layer was 74 Å., but since it was likely that the molecules were not fully extended the membrane would be less than 74 Å. Ti Tien (96) drew attention to an error in the calculation and the membrane thickness was corrected to 72 ± 10 Å. in green light and 105 ± 10 Å. in white light (95). Electronmicroscopy of thin sections and shadowed preparations suggested a mean thickness of 73.4 – 21.8 Å. and there appeared to be multilayer structures (97). Andrews and Haydon (98) thought that one could infer the presence of such layers from the appearance of spots in the membrane which depended on the organic solvent used for the phospholipid.

Water permeability was studied by two methods (99): diffusion exchange of tritiated water (THO) and measurement of an osmotic permeability coefficient. The results, 4.4 ± 0.5 μ/sec. and 17.3 μ/sec., respectively, did not agree but since the divergence could not be attributed to stagnant layers or to chemical isotope effects, it remained unexplained.

In a more recent preparation, Miyamoto and Thompson (102) showed that the conductance of the membrane varied with the sodium chloride concentration in the aqueous phase (see Fig. 11), and that the transport number of the cations, except the hydrogen ion, was higher in the membranes than in the solution (see Table VIII). In presence of monovalent alkali cations, the resistance was approximately 0.5×10^6 ohms cm.^{2}, except for lithium when a value of 0.8×10^6 ohms cm.^{2} was recorded. If cadmium, manganese, or copper were added the resistance increased to a new stable value in about 20 min.; calcium, magnesium, and strontium had no effect while iron caused a decrease in resistance. They discussed the conduction path and whether or not there could be leaks around the membranes, but believed conduction took place mainly through the bilayer. The authors suggested that conductance was probably electrolytic, but that hydrogen and hydroxyl ions were not the most important ones. By calculation, the number of ions in the bilayer should be 5.6×10^{-12} *M* if conduction was in the aqueous phase only, but as the actual

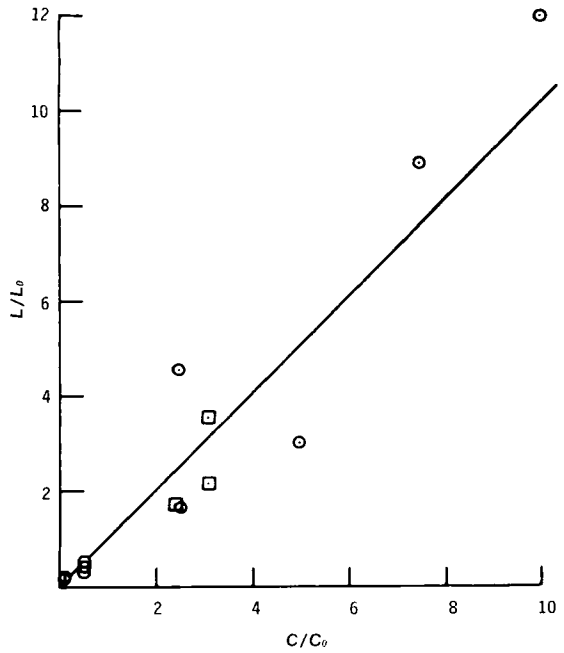

Figure 11—*Membrane conductance as a function of the concentration of NaCl in the aqueous phase* [*Reproduced with permission from* J. Coll. Interface Sci., **25**, *16(1967).*]

figure was approximately 5.6×10^{-7} *M*, they concluded that other ions in the membrane must have played a part.

Transference numbers of ions through membranes formed of extracts of H ˂ and L ˊ sheep red cells were obtained by Andreoli *et al.* (103, 104) indicating a high degree of selectivity as the values of sodium and potassium were approximately 0.8 and that for chloride 0.2. The numbers were independent of temperatures ranging from 21 to 38°, hydrocarbon solvent, osmolarity of the solutions, and cholesterol content.

Spherical lipid membranes were made by Pagano and Thompson (105). The system consisted of a spherical bilayer membrane with a cap of bulk lipid phase over not more than 10% of the surface. Aqueous phase was both inside and outside the sphere. The structure was freely suspended in a density gradient and had the following properties: area of 1 cm.^{2}; thickness 90 Å.; specific resistance 0.35–0.68 $\times$ 10^5 ohms cm.^{2}; capacitance 0.5 μ f.cm.$^{-2}$; breakdown voltage 200 mv. and permeability of water 10 μ sec.$^{-1}$. Resistance was ohmic and conductance was a linear function of area. Thus, it was similar to the planar membranes but had the advantages that there was no mechanical support, the surface area per unit volume was large, and electrophoretic experiments were possible to determine the surface charge.

Hanai, Haydon, and Taylor (106, 107)—These workers realized that in the early work of Mueller *et al.* (70, 72) no data were obtained on the thickness or electrical properties of the films. Bilayers were formed in aqueous solutions of potassium, sodium, and calcium chlorides on a hole in the Teflon screen, and there was a dielectric dispersion characterized by one realization time which was a function of the conductivity of the solution. The films behaved as simple parallel-plate condensers. Numerical values for the capacitance and other properties are given in Table IV. The capacitance of the film was reproducible, independent of the frequency and of the nature and concentration of the electrolyte. It was shown to be due mostly to the hydrocarbon portion of the lipid molecule with a negligible contribution from the polar region (108). The film capacitance was lower than that quoted by Mueller *et al.* (71), but since these workers did not determine the thickness of their membranes this may account for the difference. From further work (108, 109) by Hanai *et al.*, when measurements of potential were made, it was deduced that the quaternary ammonium groups of the lipid molecules were effectively coplanar in the film. This was supported by electrophoresis (108).

When the membranes were bulged (111) under hydrostatic pressure, some showed a direct relationship between conductance and area at low conductances but this became erratic at high conductances when, they concluded, leaks developed. They suggested that the current was carried by the ions alone or in combination with the phospholipid. Capacitance also varied linearly with area after neglecting origin effects. Some experiments with a mixture of cholesterol and phospholipid were not too successful owing to the development of leaks.

It is to be noted (110) that the measured capacitance (0.38 μ f.cm.^{2}) of these films was lower than that of the cell membrane (0.5 μ f.cm.^{2}). This may have been due to the nature of the lipid or to the effect of adsorbed proteins on the natural biological membrane. Addition of various amounts of cholesterol caused the capacitance to rise to 0.6 μ f.cm.^{2} when a cholesterol–lecithin ratio of 0.8 was used. One would expect cholesterol to be absorbed into the film tending to decrease the thickness to 35 Å., and it was inferred that the dielectric constant of the hydrocarbon region of the membrane was increased. The problem was also examined by measuring the effect of other polycyclic ring compounds on the dielectric constant of liquid aliphatic hydrocarbons, and estimating the capacitance of a bimolecular layer. This would probably have a value of 0.56 μ f.cm.^{2} when equimolar

Table VII—Effect of Temperature on Time of Formation of Membranes (93)

Temperature	Time, min.
<20	No membrane
20	30
36	4
>39	<1 (unstable)

Table VIII—Cationic Transference Numbers (102)

Concn.,[a] moles/l.	t^+, Membrane at 36°	t^+, Free Solution at Temp. T	T, °C.
0.005 HCl	0.365 ± 0.050	0.814	35
0.01 HCl	0.502 ± 0.073	0.816	35
0.10 HCl	0.293 ± 0.050	0.823	35
0.50 HCl	0.657 ± 0.137	0.831	35
0.10 LiCl	0.519 ± 0.132	0.317	25
0.10 NaCl	0.698 ± 0.153	0.389	35
0.10 KCl	0.719 ± 0.043	0.489	35
0.10 RbCl	0.527 ± 0.066	0.494	18
0.10 CaCl	0.706 ± 0.005	0.500	18

[a] Concentration of the aqueous phase in which the membrane was formed.

quantities of phospholipid and cholesterol were present. Everitt and Haydon (112) derived an expression for the capacitance of the membrane and showed that it was simpler than the one suggested by Läuger *et al.* (120) for low concentrations of salt. Using the concentrations usually encountered the predictions were less accurate. It was difficult to test the theory practically since surface charge densities were very sensitive to trace materials.

Proteins (110) had no effect within the limitations of the work and electrophoresis showed that the membrane surface was probably covered. When they produced such membranes, there was no permanent tendency to a higher capacitance and there was no noticeable change in resistance. Therefore, to explain the difference in capacitance of the model and natural cell membranes, it was suggested that either the average number of carbon atoms in the hydrocarbon portion of the layers was less than eighteen and therefore the cell membranes were thinner, or that a small number (perhaps 1%) of polar pores existed in the natural cell membrane. The first explanation did not fit the data and the second could not be demonstrated experimentally (109).

Mention should be made that Haydon and Taylor (113) have attempted to measure the contact angles between the two-sided film as Mysels *et al.* (114) obtained data for soap films in air. The method used the appearance of Newton's rings in the membrane. They made certain assumptions and proposed to carry out a further investigation.

Other Work—Membranes made by the above methods were small and had a low breakdown voltage. Van den Berg (116) found that the size could be increased by passing a hole in a Teflon screen through the oil-water interface. He used holes up to 9 mm. in diameter and slits of various widths. He usually applied voltages of about 10 mv. and found the resistance of the membrane was 10^7 ohms cm.^{2}. There was often a sharp fall in resistance and the film was not markedly affected by salts, temperature, or pH.

Writh *et al.* (117) have modified the technique to enable the formation of films across holes in polyethylene sheets in a buffered saline medium. The properties of the membranes are given in Table IV and the exact composition of the phospholipids did not affect them.

Lev *et al.* have prepared membranes, but since these showed a higher selectivity to sodium than to potassium ions, the model was not therefore very suitable for comparison with the living cell (88). Valinomycin (89) caused a decrease in resistance of the membrane and an increase in specificity for potassium ions. The value depended on the ionic strength of the ions in the surrounding medium and monovalent ions were specific in the order: $H > Rb > K > Cs > Na \approx Li$; which is similar for natural membranes except that the positions of Rb and Cs are interchanged.

Protein absorbed onto the surface of films made with brain phospholipids conferred excitability in a medium of 0.1 *M* NaCl at pH. 7.3 (90). There were no significant changes in excitability when magnesium and potassium ions replaced the sodium. Small concentration differences (0.005 *M*) across the film of magnesium, calcium, and manganese ions caused a decrease in resistance and

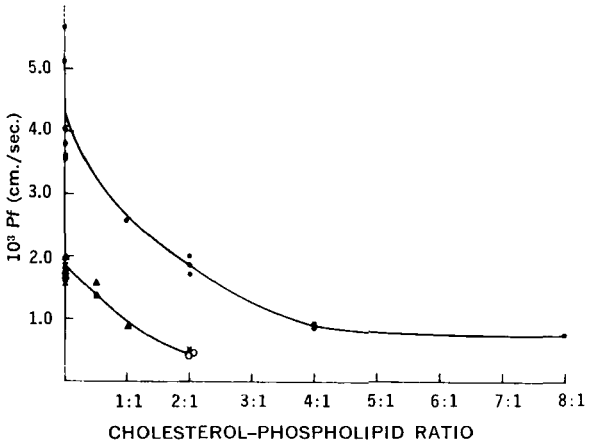

Figure 12—P_f *as a function of molar ratio cholesterol–phospholipid.* [*Reproduced with permission from* Nature, **216,** *717(1967).*]

an increase in the excitation threshold of potential. Zinc, cadmium, and aluminum caused a large membrane potential (20–100 mv.) when the film was made of lipid only, but this disappeared when adsorbed protein and aluminum ions prevented excitation.

Finkelstein and Cass (91) examined the effects of adding various amounts of cholesterol to the lecithin solution which was used to make membranes in buffered and unbuffered systems. The results of osmotic experiments are diagrammed in Fig. 12, which shows that the coefficient decreased as cholesterol concentration increased in preparations of single and mixed phospholipids, but was greater in the latter case. To try to simulate the lipid phase of the membrane, cholestane and hexadecane were mixed together to replace the cholesterol and hydrocarbon region of the phospholipid molecule, respectively. If the ratio of the concentration of hexadecane–cholestane was equal to phospholipid–cholesterol, the effect on the permeability could be attributed to an increase in viscosity of the hydrocarbon region of the film and a corresponding decrease in the diffusion rate. However, this concept of viscosity of a film has certain drawbacks and may not be a very satisfactory analogy.

The results of Cass and Finkelstein (92) did not agree with reports that the osmotic permeability coefficient was greater than the tagged water permeability coefficient [see above (99)]. They obtained values of approximately 1×10^{-3} cm. sec.$^{-1}$ which were comparable with values for plasma membranes. Glucose and sucrose affected the permeability in a complicated manner and this was discussed in detail in their paper. Urea was comparable to the effect of sodium chloride and EIM caused no change.

The disagreement in the values of the refractive index of the membranes has led to some discussion. Results from Cherry and Chapman (115) on the refractive index of egg lecithin films formed with tetradecane disagreed with Huang and Thompson (94). Water may partly account for the difference and they are making further investigation to discover if there are several layers, each characterized by different refractive indexes, together with solvent in the film.

Leslie and Chapman (118) found that β-carotene could be incorporated into the films and that *n*-tetrade-

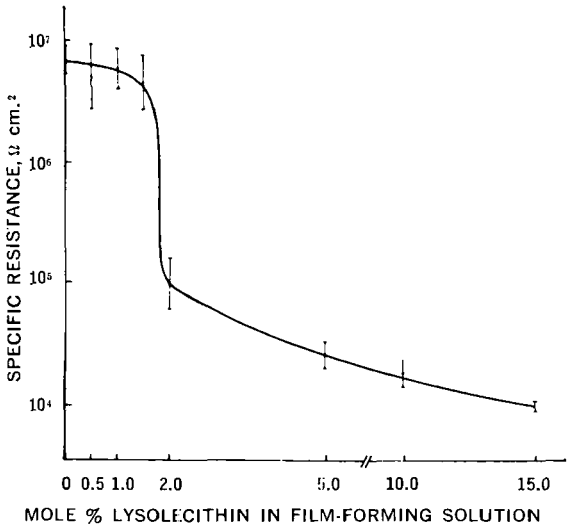

Figure 13—*Electrical resistances of lipid bilayers generated with various mole percentage of lysolecithin.* [*Reproduced with permission from* Chem. Phys. Lipids, **1**, *389(1967).*]

cane, cholesterol, and α-tocopherol were not necessary. In these experiments they demonstrated that spectroscopic measurements were useful to show the incorporation of the pigment in the film. Retinene could also be used in place of the carotene but the rate of formation of the membrane was longer than with the lecithin-tetradecane solution. From the spectral analysis, the films did not appear to be homogeneous and there may have been patches several layers thick. This work opened the field for the application of spectroscopy and to the study of more specialized membranes mimicking a particular biological function, in this case, sight. Suggestions for further studies employing polarized light and membranes incorporating chlorophyll were made.

To determine whether or not the local accumulation of lysolecithin in the membranes may influence the permeability, Van Zutphen and van Deenen (119) prepared a film from egg lecithin containing various amounts of lysolecithin. They could generate stable membranes containing up to 15% lysolecithin but with 20% the films were no longer stable. With 0.5 to 1.5 mole % lysolecithin-lecithin there was no appreciable change in electrical resistance compared with the control lecithin membranes (see Fig. 13), but 2 mole % or more gave significant changes in the electrical resistance.

Membranes have also been prepared by Läuger *et al.* (120–122) (see Table IV). Recently these workers showed that the resistance in iodide solutions compared to chloride solutions was lower by 10^3; in other words, the membrane appeared to be more permeable to iodide. When iodine itself was present there was a decrease in the membrane resistance, which returned to a value as high as that in the sodium or potassium chloride solution if the free iodine was removed with sodium thiosulfate, thus indicating that there is no exceptionally high permeability for iodide. The conclusion was supported by experiments with ^{131}I. In discussing the possible mechanism of charge transport, which could be by ionic or electronic conduction, no conclusion was reached. Lecithin formed a strong charge-transfer complex with iodine and the following two mechanisms could be operative: (*a*) lecithin · I_2 complex → lecithin · I^+ + I^- and the iodide ions moved across the membrane; (*b*) because of the presence of the lecithin · I_2 complex the bilayer became an electronic conductor. Under the influence of a current the reaction

$$I^- \rightarrow {}^1/_2\, I_2 + e$$

occurred on one side of the membrane and the electrons passed to the other side. Rosenberg and Jendrasiak (124) thought that the increase in conductivity was due to a donor-acceptor complex which indicated the electronic conductance mechanism.

Lesslauer *et al.* (123) showed that bimolecular lipid membranes of normal lecithins were not formed in solutions of phospholipase A and prephospholipase A due to hydrolyses of the fatty acid ester linkage. Analogs of lecithin would form the membranes and the lowering of the resistance was comparable to the effect of protein, but there was no significant difference in the capacitance, indicating little penetration of the membrane by protein. A small number of pores may be formed but the data were not sufficient for more than tentative conclusions.

An interesting application of the black lipid membrane was made by Skulachev *et al.* (125). These people made a study of the effect of uncoupling agents on the respiratory chain by comparing the action of the compounds on mitochondria, artificial phospholipid membranes, and phospholipid dissolved in heptane. The compounds which uncoupled oxidative and photosynthetic phosphorylation in natural preparations, have qualitatively the same effects—first causing an inhibition as the concentration is increased. The authors confirmed the results by Bielawski *et al.* (126) that small concentrations of 2,4-DNP decreased the electrical resistance of the artificial membranes. When more than 1 m*M* was used there was an increase. The suggestion, that these compounds facilitate the passage of an electrical current, fits in well with the theory of Mitchell who thought that they collected in the lipid phase and caused transfer of protons (126). There is also the possibility that the decrease could be due to an increase in transport of sodium or of chloride ions as these were also present in the media.

Carbonylcyanide phenylhydrazones also increased the specific conductance of the membranes sufficiently to account for the uncoupling action (127). The pH determined the maximum effect which was dependent on the concentration of undissociated molecules and anions in the lipid phase and of hydrogen ions in the aqueous phase, influencing the transport of hydrogen and hydroxyl ions. At a low pH the anion concentration was the rate-determining step and at high pH the hydrogen-ion concentration in the aqueous phase was controlling. Dicoumarol had qualitatively the same effect to a lesser degree.

It is interesting to note another application using viruses. The permeability of thin lipid membranes to tobacco mosaic virus (128) and *Coxsackie* B2 (129) were studied by Petkau *et al.* The membranes were permeable to tobacco mosaic virus in the presence of magnesium ions, whereas when the permeabilility of the membrane was modified with a protein extracted from the broth in which *Aerobacter cloacae* has been grown, and under the

influence of magnesium ions and an applied electric field, *Coxsackie* B2 virus passed through.

LIQUID CRYSTAL SYSTEMS

As the concentration of soap or soap-like material is increased in a solution, it becomes first cloudy in appearance due to the formation of micelles and then viscous. This latter phase exhibits anisotropy when viewed under a microscope with crossed polaroids, and is called the liquid crystalline phase. The soap molecules are aggregated in various structures which are influenced by the disperse medium, the presence of electrolytes and nonelectrolytes, and by temperature. Much interest is now being shown in these structures as it is thought that they are intimately connected with many biological systems. Osipow (130) gave a good introductory description; a comprehensive review of the physical properties has been published by Winsor (131) and both Stewart (132) and Chapman (133) have discussed the biological functions. Chapman *et al.* (134) have proposed three crystalline phases for phospholipids which were influenced by temperature and water content. They used saturated phosphatides and examined the preparations with X-rays, IR, or NMR spectroscopy and other techniques. They thought that additional forms might be present depending on the purity of the phosphatide and on the solvents. Recently Chapman *et al.* have published a paper on the d.c. electrical conductance properties of these structures (135). A detailed investigation of the structural characteristics of hydrated liquid crystals was also made by Papahadopoulos and Miller (136). They characterized various phospholipids and studied the permeability.

Bangham *et al.* (137, 138) criticized the preparations described in the previous section because (*a*) the composition of the films was unknown; (*b*) it was necessary to have a filler such as tetradecane; and (*c*) the use of organic solvents was undesirable since as little as 1% chloroform remaining in the membrane was sufficient to anesthetize it. In the development of membrane models, they were first to use a preparation of liquid crystals by allowing the lecithin to swell in aqueous electrolyte solution. The product was a milky white color and did not froth. Microscopic examination showed that particles (spherulites and myelins) of various sizes and shapes were present. The commonest arrangement of the phospholipid molecules was a lattice in which there were concentric bimolecular lipid layers separated by aqueous layers so arranged that, at equilibrium, each lipid layer acted as a membrane. On separation of the lipid fraction by dialysis it was possible to determine the ion content. The more hydrated the anion, the more it associated with the phospholipid; for example, there was less chloride than sulfate. Bangham *et al.* thought that the capture of ions was due to a physical rather than to a chemical process. During the initial dialysis, no significant diffusion of the captured cation out of the phospholipid phase was noticed. When, on further dialysis, they measured the rate of appearance of radioactive ions, it was found that the diffusion-limiting process was a property of the water-lipid interface. Water was exchanging as fast or faster than chloride ion and the membrane could have been susceptible to osmotic lysis. The permeability to water was analogous to that of natural membranes, and the differential barrier to cations varied in several orders of magnitude from anions. They suggested the mosaic arrangement of coplanar zwitterions, in which the ionic groups could form time averaged positively charged surface clusters or pores to allow the passage of anions in a negatively charged membrane.

Other analogies to cell membranes were found in the action of steroids and streptolysin S (139) which changed the permeability in a manner similar to the change seen in the biological tissues, and in the effects of narcotic drugs (140). Organic compounds in the latter class (chloroform, ether) caused an increase in the rate of movement of potassium as the chain length decreased, which was interesting as there was an increase in the potassium permeability of the cell *in vivo* when narcotics were used. Local anesthetics led to a decrease of cation penetration and the authors were able to show that the concentration necessary to make the zeta potential less negative was comparable to the amount which would cause nerve shock, thus supporting the theory that the biologically active form is ionized.

Recently Bangham *et al.* (141) have published a paper on osmotic properties. Absorption and centrifugation techniques were used, and volume and surface area measurements were made. In alkali metal salts, glucose, and other sugars the liquid crystal was found to be an almost perfect osmometer, *i.e.*, it obeyed the Boyle-van't Hoff law. Some compounds were graded in order of the permeability and ranged between 0.8 and 0.16 μ/sec. The results were discussed in relation to the results in the literature and may depend on the ionization of the polar groups and the ionic strength of the solution. The permeability to solutes such as ethylene glycol and methylurea was comparable to water but urea, glycerol, *etc.*, moved more slowly. This is analogous to that which has been reported for biological membranes.

If polyene antibiotics were added to phospholipid spherules, the gross permeabilities were altered (142). Sessa and Weissman, using filipin, nystatin, and amphotericin B showed a direct interaction with phospholipids which was independent of the overall charge. The latter two antibiotics preferentially disrupted cholesterol-containing systems.

PHOSPHOLIPID-PROTEIN SYSTEMS IN AQUEOUS MEDIA

A precipitate was formed in reactions between proteins and phospholipids, and the exact nature depended on the pH, the presence of electrolytes, and proportions of the constituents (142, 143). The complex was probably in the form of laminar micelles of alternate layers of lipid and protein with the ionic surfaces toward each other. Electrostatic and Van der Waals' forces appeared to hold the aggregates together.

Attempts by Langmuir and Waugh (144) to form lipoprotein membranes on wire loops containing protein films were not very successful. The wire loops were lowered through a benzene-water interface to prepare protein films. If a solution of lecithin in the benzene was used the stability of the protein film improved and the membrane lasted several minutes. If they prepared

folded protein films there was less tendency to collapse, indicating an increased stabilization. Membranes were obtained in distilled water and probably consisted of two layers of protein separated by one or two of lipid.

It is to be expected that there would be a better resemblance to biological conditions if only aqueous phases are used. Saunders (145–150) has reported the formation of films between phospholipid sols and protein solutions. Lipid films formed at a sol-water interface were stable for a few days and were strengthened by albumin. In conductivity studies of the rate of diffusion of salts through the lipoprotein interface formed at the boundary between a lecithin-cholesterol sol and a solution of albumin, Saunders (148, 149) found that potassium and sodium chlorides moved more slowly than in aqueous solutions, that potassium was quicker than sodium, and that the rate of diffusion was reduced further when calcium chloride was present (150). Calcium increased the rate of formation and the film was more elastic. Choline chloride diffused rapidly and increased the rate of diffusion of sodium chloride, as also occurred when carbachol was added. These studies were extended recently (151, 152) by employing radiochemical techniques which have the advantage that they are not limited to ionic substances, and that compounds can be distinguished in an otherwise homogeneous medium. The open-ended capillary technique was modified and the rate of diffusion of sodium-22 in albumin solutions and lecithin sols was followed. It was shown to be reduced in these solutions and was attributed to a binding of the ions to the macromolecules. When the solutions were layered one on top of another preliminary investigations indicated that the interfacial layer at the boundary between the two media did not affect the diffusion. This was not supported by later experiments in which a permeability coefficient could be calculated (152).

DISCUSSION

The availability of these preparations has opened a new and stimulating field not only because many of them are interesting from the point of structure and physical chemistry. They appear to be applicable to the study of drug action, particularly so in the case of compounds which influence the permeability of the cell wall, and for the study of the passage of substances into cells; a step which is necessary at some stage in the absorption and transport of drugs. Some beginnings have been made in this direction, but more work is necessary. In the future, comparisons such as those of Watkins (153) between pure preparations and drug extracts, will be important.

Once the technique has been mastered most of the preparations are easy to reproduce—difficulty sometimes being encountered in the isolation and purification of the necessary compounds. It must be obvious that no preparation will be an exact replica of the cell membrane but one can hope that by incorporating the more important components and by simplifying the structure the important characteristic properties can be obtained. In addition, it means that the possibility of studying different factors isolated from one another exists.

Many of the preparations have obvious disadvantages. The presence of an inert support in the membrane formed on Millipore is unfortunate since no such structure occurs in the natural condition. This preparation also has the disadvantage that it is not exactly reproducible and results are therefore only qualitative. In the case of the black lipid films, there is always the possibility of organic solvent remaining in the membrane together with the fact that there are certain stabilizing additives—as, for example, α-tocopherol in the lecithin membranes and hexadecyltrimethylammonium bromide for cholesterol membranes. It is worth noticing that each preparation varies with the source and the batch of phospholipid, and also seems to depend on the apparatus and the workers themselves. The liquid crystal preparations have the disadvantage that the bimolecular lipid layers are arranged close together while the natural membrane is, presumably in many cases, a single bilayer. The unimolecular film studies help elucidate the detailed behavior of molecules and analogy then allows one to infer what takes place in the bimolecular layer.

When looking at the properties of the membranes, particularly the black lipid membranes, an important criticism is that many have a higher capacitance than the natural membrane. Although it is possible to mimic some of the transport properties of ions, difficulties have been encountered as there is usually some selectivity for calcium but none for sodium or potassium unless another substance is added. In addition, Lev *et al.* (88) have made membranes with reverse selectivities to those of Mueller *et al.* (81). Finally, it would be preferable if the use of antibiotics could be avoided, and yet a membrane produced with the responses obtained by Mueller and Rudin (86). It is to be hoped that further research will indicate a method.

Since the only theory, until recently, was the one proposed by Davson and Danielli (12) most workers have attempted to form a bimolecular lipid layer with protein on both outer surfaces. As explained in the introduction this does not seem to be the true structure in all cases and it will now probably be necessary to prepare alternative *in vitro* models.

In this review emphasis has been given to the physical properties of the membranes but the theories concerning the forces which hold the lipid molecules together are interesting. Vandenheuval (154, 155) has examined the structure of the myelin sheath. The surface free energy of a system was calculated by Danielli (156) to be at a minimum when the membrane was a bilayer, which explains why so many of the membranes were two molecules thick. Also of interest are the theoretical calculations of Parsegian (157, 158) who has applied statistical thermodynamics to examine the forces between lecithin bimolecular leaflets of liquid crystals. He suggested considering the surface as a diffuse charge layer (157). When the leaflets approached one another changes occurred in the arrangement of the polar groups and the electrostatic potential was a function of the charge density and of the thickness of the water layer. As water was withdrawn the leaflet became thicker because there was repulsion between the layers. The bimolecular leaflets repelled each other to a distance of less than, or equal to, 25 Å. in the liquid crystal state (158). The interaction was strong enough to alter the leaflet size and consequently the possibilities in orientation of the polar groups were

greater than had been previously thought. This led to some dispute between various authors (*loc. cit.*), and Parsegian also suggested that the polar groups were not coplanar to the surface but protrude from it.

The mechanisms underlying the various phenomena of the transport of water, electrolytes, and nonelectrolytes in both physical and biological applications have been investigated (160–162), and studies of the physicochemical properties and of the permeabilities of membranes have led to numerous hypotheses. It seems probable that more than one mechanism is operative depending on the substance to be transported. In many biological tissues movement can be described by diffusion processes but where transport occurs against an electrochemical potential gradient, active transport or carrier mechanisms may be operative. There is some evidence for the transport of ions, *etc.*, in pores through the cell membrane while other workers suggest diffusion and solution within the membrane phase. Exchange reactions could possibly play an important part since the selective movement of ions depends upon the ionic groups of the membranes (163, 164). Recent studies (159, 163) with phospholipids show that they have defined ion-exchange properties which may depend upon the orientation and spacing of the molecules. The preparation of mosaic membranes with cation and anion exchangers adjacent to one another was developed by De Körosy *et al.* (165) and Botré *et al.* (166). There was enhanced diffusion between the borderline which may be compared with nerve conduction (165). In the model the neutralization of charges was due to the adjacent cations and anions. In the nerve the wave potential could be due to the esterification of the acidic sites with choline, or to hydrolysis with the corresponding change of charge.

However, most theories are still speculative. With an improved understanding of the composition and function of natural membranes and of the physical processes involved, it is to be hoped that knowledge of the transport of substances will be increased.

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RESEARCH ARTICLES

Interfacial Barriers in Interphase Transport: Retardation of the Transport of Diethylphthalate Across the Hexadecane-Water Interface by an Adsorbed Gelatin Film

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Abstract □ In order to investigate the possible influence of an adsorbing substance upon the oil-water interphase transport rate of a solute, experiments were conducted on the rate of release of diethylphthalate from hexadecane droplets dispersed in an aqueous sodium sulfate solution. It was found that when an adsorbed layer of gelatin is presented, the rates of solute release are the order of 1×10^4 times slower than diffusion controlled. The permeability coefficient for the interfacial barrier was estimated to be $1.5 \times 10^{-5} \pm 0.3$. These findings are, to the authors' knowledge, the first of their kind, and suggest the possible importance of such nonspecific barriers in biopharmaceutics. The data have been critically analyzed by several physical models that relate the interphase transport rate to the partition coefficient, the diffusion coefficient in the aqueous phase, the particle-size distribution of the droplets, the interfacial resistance of the gelatin layer, and the adsorption of the solute to the interface. The analysis has shown that under certain conditions adsorption of diethylphthalate at the interface must be accounted for to provide quantitative agreement of the theory with the data.

Keyphrases □ Interphase transport—diethylphthalate □ Interfacial barrier—hexadecane-water □ Gelatin, adsorbed layer—interfacial barrier □ Particle-size distribution—emulsion systems □ Partition coefficients (o/w)—diethylphthalate □ UV spectrophotometry—analysis

An understanding of the influence of interfacially adsorbing agents on the transport-rate behavior of drugs across oil-water interfaces is basic to the understanding of drug dynamics in man. When meaningful mechanistic analyses are sought, there are many instances where the interphase transport of a drug must be considered after its administration to the patient. Cases in point are, for example, drug-release situations involving availability, drug absorption, distribution in tissues and in various body fluids, metabolism, or excretion. Hence, the question of the influence of substances adsorbed at the interface becomes an important one.

A survey of the literature has shown that very little is known about the oil-water situation. The classic work of LaMer and his collaborators (1) has given an excellent example of the air-water case. These workers found that long-chain aliphatic alcohols significantly reduced the evaporation rate of water by providing a condensed monolayer. The adsorbed layers, in these instances, create sufficiently large barriers against the movement of water molecules through them. Another case of transport-rate reduction by interfacial adsorption is the marked reduction in the dissolution rate of hydroxyapatite by adsorbed long-chain amine hydrochlorides (2). To the authors' knowledge, however, such effects have not been clearly demonstrated for the oil-water interface case.

Recently (3) a well-defined method for measuring the oil-to-water interphase transport rate was described. It involves the use of dispersed oil droplets as a sink or source for the solute in the aqueous external phase. As was pointed out (3), this technique is generally much more sensitive and more reliable than most methods for studying interfacial barrier to interphase transport.

In this communication, this technique has been applied to the investigation of the influence of gelatin adsorbed at the oil-water interface upon the transport rate of an organic solute. The present findings, to the writers' knowledge, represent the first definitive demonstration of a significant retardation of the oil-water interphase transport rate by a reversibly adsorbed substance. These results should be generally important to the eventual understanding of the role of proteins and other polymeric substances on the transport of drugs and other physiologically important substances *in vitro* and *in vivo*.