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Red Blood Cell Lipids and the Plasma Membrane

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Abstract

The red-blood cell occupies a unique position both in the historical development of membrane theory and current experimentation on membrane properties. The cell is readily accessible and has been studied in many animal species and disease conditions. Differences in composition, structure, and properties have been described, and a number of highly specific cell types are now available for investigation.

Several techniques are used to study the membrane of the intact cell. The membrane surface may be labeled in specific areas by the selective exchange of lipids such as cholesterol. Exchange studies provide information about lipid-lipid and lipid-protein interactions in the intact structure. Membrane lipids are hydrolyzed by phospholipases, and enzymatic activity is modified by the action of penetrating hemolytic agents. Permeability is readily measured by hemolysis and correlated with chemical structure, partition coefficient, and biological activity of different metabolites and drugs. The red cells of Vitamin E-deficient animals are particularly susceptible to hyperoxia. Hemolysis in these cells is correlated with alterations in membrane structure through the formation of lipid peroxides.

Gorter and Grendel first used monolayers prepared from red-cell lipids to show that sufficient lipid was present in the membrane to form a bimolecular layer. Lipids extracts from the red cells of different animal species and disease states have been used in recent monolayer studies. The surface properties of these lipids and their purified neutral lipid and phospholipid fractions yield additional information about lipid-lipid interactions which may exist in the membrane. Enzyme hydrolysis and penetration studies indicate that lipids in monolayers and membranes have similar properties.

Introduction

THE CONCEPT OF A PLASMA membrane developed slowly from an intuitive explanation for the osmotic properties of plant cells to the unequivocal demonstration of a distinct phase boundary which surrounds all living cells. The early osmotic history of the plasma membrane has been reviewed by Jacobs (1). He outlines three distinct periods in the development and final acceptance of the membrane hypothesis. The first period was initiated in 1877 by Pfeffer, who described similarities between plant cells and osmometers with copper ferrocyanide membranes. Pfeffer's postulate that the membrane is a universal diffusion barrier for water and all solutes was recently disproved (2). Nevertheless his initial suggestions were responsible for the controversy which apparently occupied the research efforts of many biologists during the late 19th century and early decades of this century. The most important generalization in the period from 1877 to 1900 was made by Overton (1), who investigated the penetration of a large number of solutes into living cells and found that their penetration rates depended on lipid solubility. A quantitative explanation for this correlation is still being developed.

Suggestions about a phase boundary were generally criticized by colloid chemists, who preferred to interpret osmotic behavior as a reflection of the colloidal nature of protoplasm. This second critical phase was followed by a third period, in which experimental verification was obtained for the phase boundary. Höber (3) showed that erythrocytes have a high electrical resistance even though the interior of the cell is a good conductor, and Chambers (4) developed micromanipulation techniques which showed that solutions diffused rapidly when they were introduced into the interior of a cell.

Although conductivity and micromanipulation established the existence of a phase boundary, a model for this boundary was not accepted until publication of the classic paper, "A Contribution to the Theory

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TABLE I
Cholesterol and Phospholipid Content of Erythrocytes from Several Animal Species

Species	Chol./Cell mg × 10^{-10}	P. Lipid/Cell mg × 10^{-10}	Chol./P. Lipid Molar	Chol./P. Lipid Weight	Ref.
Human	1.39	3.15	0.89	0.44	This study[a]
	1.13	2.88	0.79	0.39	15
	1.26	3.18	0.79	0.40	16
	1.12	3.00	0.74	0.37	18
Rat	0.89	2.03	0.87	0.44	This study[a]
Bovine	0.82	1.63	1.01	0.50	This study[b]
Sheep	0.56	1.30	0.86	0.43	This study[a]
	0.64	1.42	0.91	0.45	This study[b]

[a] Chloroform-isopropanol extraction.
[b] Chloroform-methanol extraction.

of Permeability of Thin Films," by Danielli and Davson (5). These investigators suggested that permeability was explained by a lipid interface, conductivity was explained by a paucimolecular membrane or bilayer, and surface tension was explained by a protein adsorbed to the lipid bilayer. This model, a lipid bilayer surrounded by protein, remains the most important generalization in membrane research even though the initial interfacial tension studies were misinterpreted. Danielli and Harvey (6) had shown that egg oil, a supernatant fraction isolated by centrifuging ruptured cells, gave a high oil-water tension of approximately 9 dynes cm^{-1}. Interfacial tension was lowered to 1 dyne cm^{-1} when protein was adsorbed at the oil-water interface. Danielli and Davson therefore postulated a protein layer to explain the 1-dyne cm^{-1} tension which was measured at the cell surface. However the egg oil fraction probably contained appreciable amounts of neutral lipid and did not approximate membrane lipid in composition. Recent measurements with phospholipid bilayers (7) have yielded tensions near 1 dyne cm^{-1} for the lipid-water interface. Some protein undoubtedly exists at the cell surface, but it was unnecessary to postulate a protein layer from tension measurements with egg oil.

Support for the Danielli-Davson model was found in a brilliant investigation of the surface properties of membrane lipids by Gorter and Grendel (8). These investigators extracted lipids from erythrocytes, spread the lipid as a monolayer, and determined the ratio of film area to cell area. Since a 2:1 ratio was obtained for erythrocytes from a number of species, Gorter and Grendel suggested that the cell membrane was formed by a lipid bilayer. Although this was published in 1925, 10 years before the Danielli-Davson model, Danielli and Davson were apparently unaware of its existence and did not cite it in their initial paper. The Gorter-Grendel experiments have been criticized in great detail by a number of investigators since they

TABLE II
Lecithin Content of Human Erythrocyte Phospholipids (% of Total Phospholipid)

Date	TLC[a]	CC + TLC[b]	PC[c]	CC[d]	H + PC[e]	Ref.
1964	30.5					21
1964		29.5				16
1963			32.1			22
1961			36.0			23
1961			29.6			24
1963				27.0		14
1962				36.0		25
1962				32.7		26
1959				32.9		27
1962					38.3	28
1962					34.7	29
1960					42.1	30

[a] Thin-layer chromatography.
[b] Column chromatography and thin-layer chromatography.
[c] Silicic acid-impregnated paper chromatography.
[d] Column chromatography.
[e] Selective hydrolysis and paper chromatography.

TABLE III
The Percentage Composition of Human Erythrocyte Phospholipids

Fraction	TLC-WC	PC-G	CC-WC	CC + TLC-WC	H + PC-G
Lysolecithin	2.9	2		.....	
Phosphatidyl choline	30.5	36	32.4	29.5	33.5
PC-plasmalogen			3.6		1.2
Sphingomyelin	24.6	32	21	23.8	20.1
Phosphatidyl ethanolamine	27.5	28	9.7	25.7	17.6
PE-plasmalogen			19.3		10.4
Phosphatidyl serine	13.3	2	9.2	15.0	14.3
PS-plasmalogen			0.8		.....
Phosphatidic acid	.....	....	.....	.....	2.2
Phosphatidyl inositol	.....	....	.....	2.2	.....
Other	1.2	....	3	3.3	1.5
Ref.	21	23	25	16	29

[a] See Table II for abbreviations: analyses were performed on whole cell (WC) and ghost (G) extracts.

appear to offer direct support for the bilayer model (9,10).

A recent generalization, the unit membrane, has been proposed as a model for many different membranes (11). The unit membrane is similar to the Danielli-Davson model; however it is more detailed and specifies a fully spread protein monolayer and membrane asymmetry. The unit membrane is based largely on data obtained by electron microscopy and x-ray diffraction. These data may be interpreted in several ways, and a vigorous debate has developed about fixation artifacts, and generalizations from specific structures such as myelin (9,11,12). A freeze-etching technique which does not require a fixative has been used to examine plant membranes (12). The photomicrographs suggest that membranes are organized in part as extended bilayers (nuclear membranes) and in part as globular subunits (chloroplast membranes).

The erythrocyte has been studied extensively by many investigators interested in the plasma membrane. The cell is readily available from a number of species and in several diseases where membrane properties are modified. The mammalian erythrocyte contains only one membrane, and lipids are concentrated almost exclusively in this phase boundary. The erythrocyte was used in early studies on solute penetration and surface area and continues to be used in recent studies of these membrane properties. Our discussion of the plasma membrane has been limited therefore to the isolation and composition of erythrocyte lipids, the properties of these lipids in monolayers, and the penetration of solutes through this phase boundary.

Isolation of Erythrocyte Lipids

Erythrocyte lipids may be extracted from washed cells or erythrocyte membranes which are called ghosts. Ghosts were originally isolated by adjusting a cell hemolysate to pH 4.5–5.5 and sedimenting the membrane material which agglutinates in this pH

TABLE IV
The Percentage Composition of Erythrocyte Phospholipids from Several Animal Species

Species	Lecithin	Sphingomyelin	Cephalins	Other	Ref.
Rat	56	26	18	..	23
Human	39	37	24	..	23
Bovine	7	61	32	..	23
	5	60	28	7	31
	19	44	29	8	30
Sheep	1	63	36	..	23
	19	49	24	8	30
	3	56	41	..	32
	5	50	38	7	33
	1	52	41	6	34

range (13). Hemoglobin-free ghosts have been prepared in 20 imOsm (ideal milliosmolar) phosphate buffer at pH 7.4 and found to contain essentially all the erythrocyte lipid (14). The extraction of a ghost preparation is more complex than the direct extraction of washed cells; however heme pigments are often extracted together with lipid by direct methods.

A number of direct extraction procedures have been published. In several procedures the suspension is precipitated with methanol and extracted with chloroform. The pigmented chloroform-methanol extract is then evaporated to dryness and extracted with chloroform (15) or chloroform-methanol (16). Contamination with heme pigments is largely eliminated when isopropanol is substituted for methanol in the extraction procedure (17). Chloroform-methanol, chloroform-isopropanol extractions, and published analysis for several animal species are compared in Table I.

The composition of erythrocyte neutral lipids and phospholipids has been reviewed extensively by van Deenen and de Gier (19), also by van Deenen alone (20). Data compiled in their review and other studies show the wide variations that have been reported even for the lipid composition of normal erythrocytes. Twelve studies on the phospholipid composition of normal human erythrocytes, published between 1959 and 1964 (Table II), give a lecithin content which varies from 27 to 42% of the total phospholipid fraction. Similar variations are found in other phospholipid fractions (Table III), and it is apparent that methodology must be improved before composition is unequivocally established. Variations in the published data on the phospholipid content of erythrocytes from other animal species are even larger than the variations reported for the human being (Table IV). Nevertheless there are significant differences between species in the relative concentrations of specific phospholipids. The rat erythrocyte membrane, for example, contains 56% lecithin whereas recent data for the sheep erythrocyte indicate that this membrane contains less than 1% of its phospholipid as lecithin. These cells differ not only in composition but also in other properties, such as their hemolysis time in isosmotic glycerol (19,20,35), and some attempts have been made to correlate composition with other cellular properties.

The composition and properties of erythrocyte membranes are different in young and old cells and are altered by diet and in certain disease states (Table V). Human erythrocyte lipids are difficult to modify by diet (51); however variations may be induced in experimental animals, and these variations should yield some insight into the structural requirements for specific membrane properties. Gross analytical studies

TABLE V

The Effect of Age, Diet, and Disease on Erythrocyte Phospholipids

Condition	Phospholipids	Ref.
Young cells	Saturated F. A. increased 18:1, 18:2, 20:4 decreased	36,37
Old cells	Total decreased Distribution normal	38
Fat-Free diet	"Cephalins" decreased 18:1 and 20:3 increased 18:2 and 20:4 decreased	39–45
Acanthocytosis	Lecithin decreased Sphingomyelin increased 18:1 increased 18:2 and 20:4 decreased	46,47
Tay-Sachs disease	Total decreased Sphingomyelin decreased	48
Sickle-Cell anemia	Total increased	18
Spherocytosis	Normal	49,50
Paroxysmal nocturnal hemoglobinuria	Normal	50

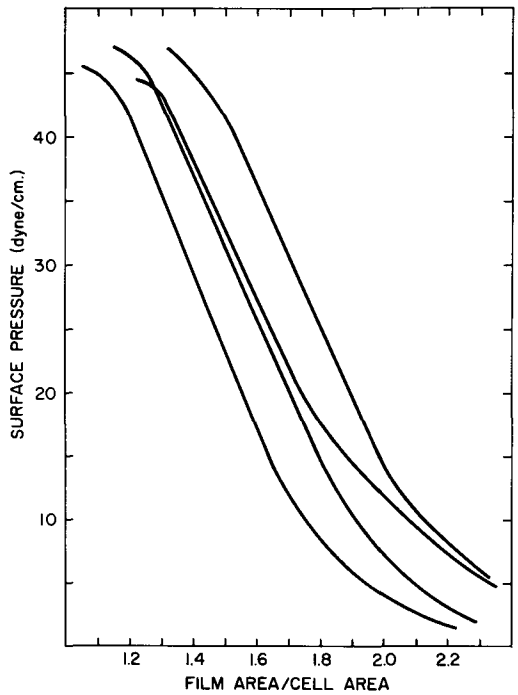

FIG. 1. Ratio of film area to erythrocyte area at increasing surface pressures (10). Copyright 1966 by the American Association for the Advancement of Science.

with several disease states have not been particularly rewarding. Several investigators reported differences in spherocytosis and paroxysmal nocturnal hemoglobinuria, but these initial reports were not substantiated (49,50) and may have resulted from the analysis of autoxidation artifacts (52). It is unnecessary to postulate a membrane abnormality as the primary defect in all functionally abnormal erythrocytes. For example, the essential abnormality in paroxysmal nocturnal hemoglobinuria may be related to protein synthesis instead of membrane structure (53). Hyperoxia induces hemolysis in the erythrocytes of Vitamin E-deficient animals (54). The Vitamin E-deficient cell may develop as one of the more interesting models for relating structure and function.

The neutral lipids of the erythrocyte membrane have been studied less extensively than the phospholipids. From 80 to 100% of this fraction is free cholesterol (19,20). Significant variations in composition have not been reported. In fact, the cholesterol/phospholipid ratio is remarkably constant in different animal species and disease states. The erythrocyte membrane contains a number of interesting glycolipids, which have been discussed recently by Yamakawa (55). Glycolipids are important in determining serological properties, and quantitative glycolipid studies may contribute important information about structural-functional relationships.

Correlations for Cell and Surface Area

The quantitative measurement of erythrocyte dimensions has been attempted by a number of investigators. The volume distribution in a cell population is readily obtained by electronic sizing with a Coulter counter. The cell area of the biconcave disc is more difficult to calculate. The area is usually calculated by measuring the diameter and thickness and then treating the average cell as a spheroid (56–58). Surface area data for human erythrocytes, calculated by this method, vary from 134 to 167 μ^2 (56–58).

Few surface area data or cell dimension measurements for other animal species are available in the

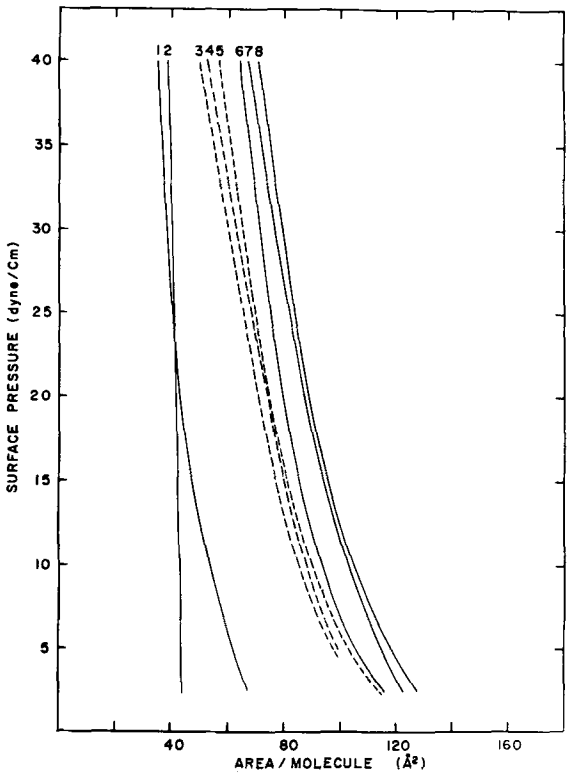

FIG. 2. Molecular areas for cholesterol and erythrocyte lipids at increasing surface pressures: 1) erythrocyte neutral lipid; 2) cholesterol; 3) mean A'_{PL} of four subjects; 4) A'_{PL} one subject; 5) mean A'_{PL} one subject, five tracings; 6) A_{PL} corresponding to A'_{PL} in tracing 4; 7) A_{PL}, one subject; 8) mean A_{PL} one subject, seven tracings from three extractions. Unpublished observations.

recent literature. Danon and Perk (59) measured cell diameters of ghosts prepared from young and old populations of a number of animal species; however the diameter of the ghost appears to be somewhat smaller than the diameter of the whole cell (56). Cell diameter measurements are important since Ponder (56) has shown that surface area may be approximated from the empirical equation:

$$\text{Area} = 2.1\,D^2$$

where D is the cell diameter. This equation has been used in correlating the surface area of membrane lipids and the area of the cell.

Gorter and Grendel (8) extracted erythrocyte lipids from a number of animal species and compared the surface area of these lipids with the surface area of the erythrocyte. Their experiments were based on several questionable assumptions. For example, lipids were extracted with acetone and the surface area of the human erythrocyte, 99 μ^2, was calculated from measurements obtained on dried cells (9,10). Therefore the Gorter-Grendel experiment has been repeated with lipids extracted by chloroform-methanol and chloroform-isopropanol, and the surface area of these lipids correlated with a recent estimate, 145 μ^2, of cell surface (58). The relationship of the film area/cell area ratio to surface pressure is presented in Fig. 1. It is apparent that the ratio is 2 at low surface pressures. Incomplete lipid extraction with acetone was compensated by the low cell area estimated from a dried cell. The conclusions of Gorter and Grendel are indeed valid.

At the time of the Gorter-Grendel experiment, Leathes (60) described an interesting effect of cholesterol on the surface area of phospholipid in mixed cholesterol-phospholipid films. The surface areas of

TABLE VI

The Apparent Condensing Effect of Cholesterol on Total Erythrocyte Phospholipids

cyne/cm	A_{PL} (Å²)[a]	A'_{PL} (Å²)[b]	(Å²)[c]
40	68	53	15
35	73	58	15
30	77	64	13
25	82	68	14
20	87	74	13
15	94	80	14
10	102	88	14
5	114	101	13

[a] Pure phospholipids.

[b] Calculated from $A'_{PL} = \frac{A_f}{m_{PL}} - \frac{A_{Chol}\,M_{Chol}}{m_{PL}}$.

[c] $A_{PL} - A'_{PL}$.

components in a mixed film are additive and should be expressed by the relationship:

$$A_F = A'_{PL}\,m_{PL} + A_{Chol}\,m_{Chol}$$

where A_F is the area of the film at a specified surface pressure, A'_{PL} and A_{Chol} are the surface areas of phospholipid and cholesterol, and m_{PL} and m_{Chol} are the molecules of phospholipid and cholesterol in the film. Since cholesterol forms a condensed film with a cross-sectional area near 38 Å², the area if this molecule was assumed to be constant and the area of the phospholipid was calculated from the relationship:

$$A'_{PL} = \frac{A_F}{m_{PL}} - \frac{A_{Chol}\,m_{Chol}}{m_{PL}}$$

Leathes (60) and later de Bernard (61) found that cholesterol had a "condensing effect" on the film. The A'_{PL} calculated from a mixed film was lower than the A_{PL} measured for a pure phospholipid film. This "condensing effect" is obtained when the A'_{PL} calculated from the surface area of total erythrocyte lipids is compared with the A_{PL} of the phospholipid fraction separated from total lipids by silicic acid chromatography (Fig. 2).

The assumption that neutral lipids may be represented by the cross-sectional area of cholesterol is valid since the neutral lipid fraction gives the same force/area curve as cholesterol except at low surface pressures (Fig. 2). The condensing effect calculated

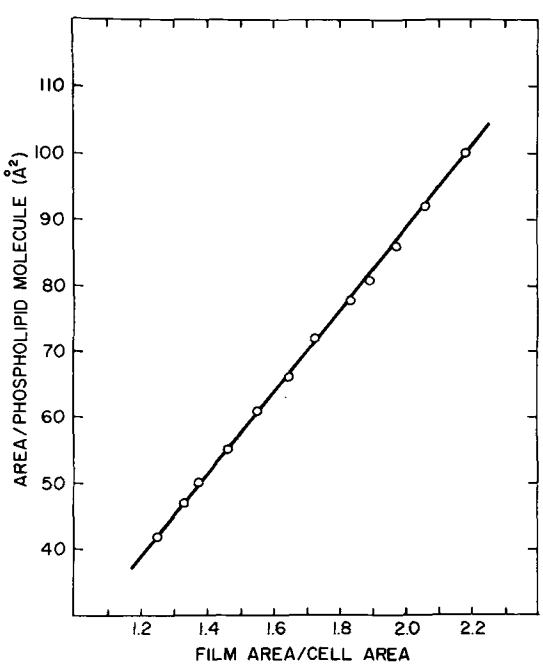

FIG. 3. Relationship between the ratio of film area to erythrocyte area and the molecular area of erythrocyte phospholipid (10). Copyright 1966 by the American Association for the Advancement of Science.

TABLE VII
Relationship Between Surface Area of Erythrocyte and Phospholipid Content

Species	D(Å)[a]	A_{Cell} (μ^2)[b]	$m_{PL} \times 10^{7}$[c]	A_{Cell} (μ^2)[d]
Human	8.5	152	24.4	145
Rat	7.5	118	15.8	93.7
Bovine	6.0	75.6	12.6	74.6
Sheep	5.2	56.8	10.6	62.9

[a] Ponder (56).
[b] A_{Cell} calculated at 2.1 D^2.
[c] Unpublished observations (Table I).
[d] A_{Cell} calculated as 59.3 m_{PL}, where 59.3 is the slope of the regression line.

in this manner (Table VI) is similar to the condensing effect obtained by Leathes (60), de Bernard (61), and van Deenen (35) for several synthetic phospholipids. It is difficult to see how cholesterol decreases the surface of phospholipid to a value below the area of the phospholipid at the collapse pressure. The effect may be explained if it is assumed that cholesterol and phospholipid fit together and occupy portions of the same statistical space. The idea of molecular cavities was proposed recently by Shah and Schulman (62), who investigated both the surface area and surface potential of mixed monolayers.

The apparent area of membrane phospholipid, A'_{PL}, is directly proportional to the A_F/A_{Cell} ratio and may be related by the equation:

$$A'_{PL} = \frac{A_{Cell}}{m_{PL}} R - \frac{A_{Chol}\, m_{Chol}}{m_{PL}}$$

This equation yields a straight line (Fig. 3). The relative bilayer content of an erythrocyte membrane is readily estimated from this figure and the molecular area assumed for the membrane phospholipid. The intercept, $\frac{A_{Chol}\, m_{Chol}}{m_{PL}}$, depends on the cholesterol/phospholipid ratio, which is similar for all mammalian species. Preliminary experiments give similar force/area curves for purified phospholipids from several species. It is therefore assumed that this equation may be used to calculate the surface area of any mammalian erythrocyte from the slope and m_{PL} or the molecules of phospholipid/cell. Areas calculated in this manner for several species are in close agreement with cell areas calculated from the cell diameter (Table VII) even though the phospholipids are different in composition.

Lipid Solubility and Penetration

In 1899 Overton demonstrated that the penetration of a solute through the phase boundary which surrounds the cell depended on the lipid solubility of the solute molecule (63). Since the cell is surrounded by an aqueous extracellular medium, he suggested that the partition coefficient for a solute between water and lipid was related to the penetration rate. Overton (64) and Meyer (65) were able to correlate partition coefficients with the biological activities of hypnotic and anesthetic drugs. Other investigators found that the partition coefficient did not correlate with the penetration of inorganic ions and organic compounds of endogenous origin. However pharmacologists, such as Ferguson (66), Brodie and Hogben (67), later established the importance of lipid solubility or the partition coefficient on the absorption, transport, distribution, and metabolism of drugs. They studied the relationships between the pKa of the drug, pH of the biological fluid, and penetration through a membrane and found that unionized or lipid-soluble drugs penetrated the membrane at a rapid rate.

Danielli (68) related the penetration rate for solute molecules, dm/dt, to a permeability constant, ϕ, by the equation:

$$\frac{dm}{dt} = \phi\,(A)\,(\Delta C)$$

where A is the membrane area and Δ C is the concentration gradient for solute molecules across the membrane. The permeability constant is approximately proportional to the partition coefficient. This correlation is improved if it is assumed that the solute passes through a partially vaporized state during its transport from the aqueous medium to the membrane. Coe and Coe (69) derived a linear equation which related the permeability constant, the partition coefficient, a, and the vapor pressure, P, of the solute:

$$\log \frac{\phi}{a} = \gamma \log \frac{P}{a} + \text{constant}$$

where the slope, γ, represents the fraction of solute surface in the vapor state. Experimental data yield a straight line for the penetration of a number of different solutes through the erythrocyte membrane; however vapor pressure data are not readily available for many biologically active compounds. Other investigators have compared permeability constants for solutes through the erythrocyte membrane with aqueous diffusion constants and partition coefficients and found that penetration only correlates with the partition coefficient (70).

Molecular structure and biological activity in a homologous series have been related through a substituent constant, π, which is defined by the equation:

$$\pi = \log a_B - \log a_A$$

where a_A and a_B are the partition coefficients of a parent compound A and its derivative B, which differ by a group X. The substituent constant thus represents the change in the logarithm of the partition

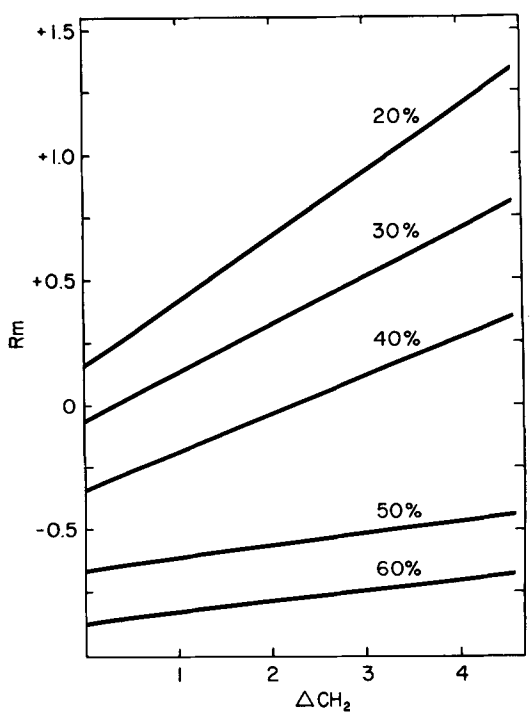

FIG. 4. Relationship between R_m and Δ CH_2 for N,N'-bis(dichloroacetyl)-1,5-pentamethylenediamine and its homologues through the nonamethylene derivative. R_m values were measured in silicone/acetone-water reversed phase TLC systems. The acetone-water varied from 20 to 60% (v/v). Unpublished observations (82,83).

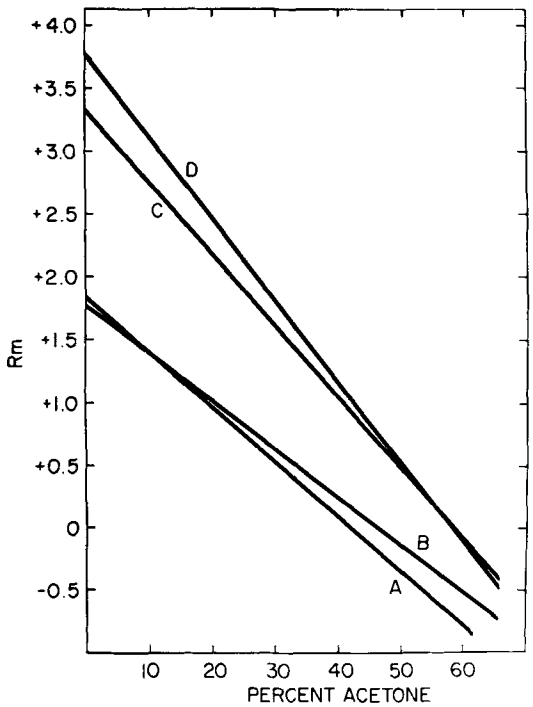

FIG. 5. Relationship between R_m and the acetone concentration in silicone/acetone-water reversed phase TLC systems. (A) N,N'-bis(dichloroacetyl)-1,8-octamethylene diamine; (B) N,N'-diethyl-N,N'-bis(dichloroacetyl)-hexamethylenediamine; (C) N,N'-4-xylylene-bis-(N-butyl-dichloroacetamide); (D) N,N'-dibutyl-N,N'-bis(dichloroacetyl)-1,6-hexamethylenediamine. Unpublished observations (82,83).

coefficient by the introduction of group X and is a characteristic constant for this group within a homologous series. Substituent constants have been used to investigate a number of compounds and biological systems including the action of penicillins on bacteria (71), the relative sweetness of aromatic nitroamines (72), the binding of organic compounds by proteins (73,74), the inhibition of photosynthesis by amides (75), the inhibition of cholinesterase by phosphate esters (76), the microsomal demethylation of tertiary amines (77), and substrate enzyme interactions (78).

The partition coefficient is difficult to measure accurately with solutes that are slightly soluble in either phase. Furthermore, partition coefficient data are usually limited to olive oil-water or chloroform-water, and it is unlikely that these nonpolar phases resemble membrane lipid. In 1941 Martin and Synge (79) showed that the partition coefficient was related to the R_f value which was obtained in liquid-liquid partition chromatography by the equation:

$$a = K\left(\frac{1}{R_f} - 1\right)$$

where the constant K is the ratio between the volumes of the stationary and mobile phases. Bate-Smith and Westall (80) later introduced the logarithmic function, R_m, of the partition coefficient:

$$R_m = \log a = \log K + \log\left(\frac{1}{R_f} - 1\right).$$

It is apparent that ΔR_m and the substituent constant described previously are identical. The R_m values of N-n-alkyltritylamines were recently correlated with their biological activity by Boyce and Milborrow (81).

The R_m values for several homologous series of N,N'-bis-dichloroacetamides have been determined by reversed phase thin-layer chromatography (82,83). The plates were impregnated with silicone as the stationary lipid phase, and the compounds were partitioned between this phase and a mobile acetone-water phase. Regression lines calculated from data obtained with one homologous series (Fig. 4) show that the addition of a CH_2 group gives a linear increment in the R_m for a number of different acetone-water phases. The ΔR_m value is different for every partition system, and this demonstrates that the structure of the acetone-water phase is an important aspect of the partition system. When the R_m value is plotted as a function of the acetone concentration in the mobile phase, a regression line indicates that a linear relationship is obtained (Fig. 5). Statistical analysis shows that linear regression lines give the best fit for the experimental data. Linear regression lines are also obtained with more complex ternary mixed solvent/pure solvent partition systems (84).

TABLE VIII
Relationship between Biological Activity and R_m[a]

Compound[b]	Biological activity[c]	R_m Acetone (%) 0	60
A	4.390	1.764	−0.505
B	4.425	1.826	−0.796
C	4.523	3.321	−0.099
D	5.160	3.753	−0.099

[a] Biagi, G. L., A. J. Merola and D. G. Cornwell, unpublished observations.
[b] Bis-dichloroacetamides from different homologous series. See figures for compounds.
[c] Biological activity is estimated as −log concentration necessary for 30% inhibition.

The R_m-acetone concentration regression line may be used to extrapolate the R_m or partition coefficient to a simple nonpolar/water system, and it is interesting that R_m values obtained from this extrapolation for several homologous series correlate with biological activity. The inhibition of pyridine nucleotide-linked electron transport in heart mitochondria is compared with R_m values obtained for several bis-dichloroacetamides from different homologous series in Table VIII. Compound A has a higher R_m than Compound B when these compounds are partitioned in 60% acetone even though Compound B has a greater biological activity. The expected correlation is obtained when the R_m is extrapolated to a simple nonpolar/water system. Compounds C and D have the same R_m in 60% acetone even though Compound D has a greater biological activity. The expected correlation is obtained when the R_m values are extrapolated to the nonpolar/water system.

Reversed phase thin-layer chromatography has several advantages for partition studies. Very small differences in solubility may be measured accurately. Membrane lipids, such as cholesterol or phospholipid, may be substituted for the nonpolar stationary phase. Binary mobile phases which give significant R_f values may be used and extrapolated to a simple nonpolar/water system. It is apparent that this technique should yield new data on correlations between the partition coefficient and solute penetration through the plasma membrane.

Several other models are available for correlating membrane structure and solute penetration. Cuthbert (85) recently discussed monolayer, bilayer, and spherulite models which have been used in addition to binding and partition systems. Surface chemists have investigated the penetration of spread films by dissolved solutes for a number of years. Indeed correlations between chain length of fatty acids, their penetration of mixed protein-lipid films, and their hemolytic activity provided early evidence for the nature of the plasma membrane (86). For example,

palmitic acid was more effective than pelargonic acid in both penetration and hemolysis studies. In later studies Bangham et al. (87) were unable to correlate the penetration of Vitamin A_1 alcohol and Vitamin A_1 aldehyde with their hemolytic activity. Hemolysis may be less specific than penetration. For example, Hooghwinkel et al. (88) found that alkyl trimethylammonium bromides had the same hemolytic properties with human, bovine, and sheep erythrocytes.

Penetration is a complex phenomenon (89). Both kinetics and equilibrium conditions may be investigated. The experimental conditions, constant surface pressure or constant surface area, modify the data. Nevertheless several recent observations on the structural specificity of membrane components have been reported with penetrating solutes. Polyene antibiotics penetrate cholesterol, erythrocyte neutral lipid, and erythrocyte total lipid monolayers but do not penetrate erythrocyte phospholipid monolayers (90). Psychoactive drugs penetrate ganglioside monolayers more effectively than cholesterol or phospholipid monolayers (91). Vitamin A_1 alcohol however penetrates a lecithin monolayer more effectively than cholesterol or mixed lecithin-cholesterol monolayers (87). It will be interesting to correlate monolayer penetration and partition behavior for other solutes with their penetration of the plasma membrane.

Although monolayers are interesting models for the plasma membrane, it must be emphasized that simple monolayer systems often have unique properties and bear little resemblance to the plasma membrane. For example, a number of investigators have used stearic acid monolayers as membrane models to test theories of calcium and drug interaction at ionic sites (92–96). Calcium stearate monolayers have specific properties which depend on a unique lattice structure (97,98). It is not possible to extrapolate from the properties of a saturated fatty acid monolayer and its interaction with calcium to the cell membrane. Other monolayers and indeed many model systems undoubtedly have similar limitations.

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Discussion

DR. CORNWELL: Gorter and Grendel reasoned from x-ray diffraction data on soap and fatty acid crystals where the unit cell was described by a bilayer with the carboxylic acid groups oriented toward each other. I do not think that a specific structure is the significant point in their paper. Gorter and Grendel did show that sufficient lipid was available to yield a bilayer if the area of the lipid film was measured at a low surface pressure.

DR. GARY J. NELSON (Lawrence Radiation Laboratory, Livermore, California): I would like to make some comments. The red cell contains three classes of lipids in its membrane; cholesterol, phospholipids and glycolipids. If one measures only phospholipid and cholesterol and then computes the ratio of the two while ignoring the glycolipids, it is difficult to interpret the significance of such ratios. It is perhaps better to consider the total cell lipid content and then calculate a cholesterol to polar lipid (phospholipid plus glycolipid) ratio. Even then, I am not convinced that cholesterol to phospholipid or polar lipid ratios have any particular biochemical or physiological significance.

DR. CORNWELL: I agree that glycolipids are present and will affect the structure of the membrane. Erythrocyte glycolipids were recently discussed by Yamakawa (see my reference 55). Quantitative analyses are difficult to find. Does some one here have good data on glycolipid composition?

DR. NELSON: I have some glycolipid data here for a number of mammalian erythrocytes which were obtained recently in our laboratory. Rat erythrocytes have about 2% ceramide polyhexosides and 6% gangliosides. Dog has about 10% ceramide polyhexosides and 12% gangliosides while the horse yielded 11% ceramide polyhexosides and 16% gangliosides. The goat has 18% ceramide polyhexosides and 6% gangliosides. Sheep erythrocytes have only 2.5% ceramide polyhexosides and 8% gangliosides. All of these data are given as weight percent of the total lipid of the erythrocyte extracted with 2 to 1 chloroform-methanol. Thus, the total glycolipid content of the red cell in different species varies considerably. On the other hand, the cholesterol content in the same species is fairly constant. Rat erythrocyte had 25% cholesterol which was the lowest value obtained in our series of ten common mammalian species while the highest value was 27.5% cholesterol found in cells from cows. The phospholipid content showed greater variation, from a low of 50% in goat erythrocytes to a high of 67% in rat. I did not calculate any cholesterol ratios from the data. However, it seems obvious that the glycolipids are a major component of the mammalian erythrocyte membrane and should not be neglected when making molecular models of the membrane.

DR. CORNWELL: Quantitative glycolipid data may indeed contribute important information about structural-functional relationships.

DR. THOMPSON: There is a lipid that is found in higher plants, in the chloroplast membrane, which is a glycolipid. We have studied several preparations of this kind of material. One can make very good bilayer-type structures out of this material and a neutral lipid.

DR. NELSON: Another point which I would like to consider is whether the lipid bilayer idea does, in fact, have any validity. My personal opinion is that it may not. There is a considerable body of biochemical and physical evidence to support my opinion, as Korn has recently pointed out in his article in Science [*153*, 1491–1498 (1966)]. One facet which is difficult to reconcile with a continuous lipid bilayer surrounding the cell is the ability of the membrane to dissociate into soluble lipoprotein subunits having a constant molecular weight. I do not see how such subunits could be obtained from a continuous lipid bilayer. I would like to hear other opinions on this subject.

DR. THOMPSON: I think one simple answer is that if one has a cake and cuts in into pieces, it is ridiculous to imagine that when the cake was made it was made out of the pieces that you cut it into. I think that this does not argue for the existance of a phospholipid bilayer in biological membrane but it does argue that subunits derived from a nonbonded system such as the cell membranes may not have any structural or functional significance in the intact membrane.

DR. NELSON: Yes, I agree that you have a valid point. On the other, hand, if the membrane can be dissociated using mild conditions, then one would suspect that the subunits have some special relationship to the original membrane.

DR. THOMPSON: Well, of course, but mildness is but a relative term.

DR. NELSON: Mild conditions, in my opinion, are such that no covalent bonds are broken during the experimental procedure.

DR. THOMPSON: Well, let's hear somebody else on the point. I disagree with you.

DR. NELSON: So do many others.

DR. CORNWELL: There is one problem with this data as Dr. Nelson pointed out. The glycolipid component will contribute to the area.

DR. SMALL: Two or three percent glycolipid would not make any difference.

DR. CORNWELL: No, but there is more glycolipid in some species.

DR. NELSON: Possibly, but the ceramide polyhexosides are large molecules containing several carbohydrate moieties that may spread over the surface of the membrane. Perhaps, only a single fatty acid or acyl chain from each molecule penetrates into the membrane leaving the remainder of the molecule spread over the surface. With this arrangement the glycolipids would not increase the total surface area of the membrane appreciably.

DR. SMALL: The next paper is one which deals with phospholipid mono- and bilayers, and it is especially important because the early work on phospholipids, carried out by Leathes, Schulman and Rideal, Dervichian, and others, were all done with impure extracts of egg or plant phospholipids. Dr. Demel, coming from the laboratory of Dr. van Deenen in Utrecht, had the great advantage of using synthetic phospholipids in which the chains could be varied in a known fashion.