WHAT COMPONENT OF THE LIVING CELL IS RESPONSIBLE FOR ITS SEMIPERMEABLE PROPERTIES? POLARIZED WATER OR LIPIDS?

GILBERT N. LING

From the Department of Molecular Biology, Pennsylvania Hospital, Philadelphia, Pennsylvania 19107

ABSTRACT A close correlation ($r = +0.96$) exists between the permeability (at 0°, 4°, and 25°C) of H_2O and nine other hydroxylic nonelectrolytes through reversed frog skin and through synthetic cellulose-acetate sheets. By the method of least squares, the data yield the following relation: log ($P_{frog\ skin}$) = 0.9900 log ($P_{cellulose\ acetate}$) −0.1659. Both the reversed frog skin and the cellulose-acetate sheets are semipermeable (while the lipoid membrane is not), showing higher permeability to water than to any other solute used in this series. The data offer support for the theory that it is not lipid, but water polarized in multilayers by cellular proteins, that provides the living cell with its selective surface barrier.

INTRODUCTION

The ability of living cells to lose or gain water on exposure to various solutions of different strengths led Pfeffer to postulate that all living cells are surrounded by a "semipermeable" membrane, that is, a membrane with a high permeability to water and a much lower permeability to alcohols, sugars, etc. (1, 2). The models often used to demonstrate semipermeability are animal bladder, parchment, frog skin, and above all, copper-ferrocyanide gel membrane introduced by Traube (3).

Overton studied the permeability of living cells to many compounds (4, 5). He found that, as a rule, compounds with high solubility in lipid enter the cell faster than compounds with low lipid solubility. Based on these observations, he suggested that the semipermeable membrane barrier of living cells was lipoid in nature. His lipoid membrane theory received support from the careful work of Collander who demonstrated a good correlation between the oil/water distribution coefficients of many nonelectrolytes and the rates of permeation of those nonelectrolytes into the alga, *Nitella mucronata* (6).

Impressive as these correlation studies were, this model has a serious flaw: that is, a lipid membrane per se is not a bona fide semipermeable membrane. Thus, the oil/water distribution coefficient of ethyl alcohol is some 50 times higher than that

of water (6). Therefore, according to the theory of Overton and Collander, the living cell membrane should be many times more permeable to ethyl alcohol than to water. Yet the first recorded observation of the osmotic phenomenon was that of Abbé Nollet (1748), who demonstrated that whereas water passed through an animal bladder membrane to the side containing alcohol, conversely, alcohol cannot pass to the side containing water (2, 7).

Perhaps it was partly to reconcile this contradiction of the simple lipoid membrane theory that Collander and Bärlund introduced the "mosaic membrane" concept, in which the continuous lipid membrane is pierced with small holes just large enough to allow the passage of the water molecule but too small to admit the large alcohol and sugar molecules (6). Indeed, this concept represented a revival of Traube's original "atomic sieve" idea, which was introduced to explain the semipermeability of copper-ferrocyanide gel membrane but ultimately rejected because of experimental contradictions, i.e., the holes are too big to act as molecular sieves (see reference 2).

The simultaneous reports in 1967 of Napolitano et al. (8) and Fleischer et al. (9) that the "unit membrane" structure is not materially altered after the removal of 95% of the lipoid materials, casts doubts on the theory that the unit membrane is primarily a continuous sheet of lipoid. Indeed, both groups concluded that the unit membrane structure is proteinaceous. However, without a continuous lipid barrier, how can we explain the vast differences in the permeability of living cells to various substances (4, 5, 10–12)? What could prevent the nonselective "leakage" of all substances alike through the interstices between the protein molecules?

As part of a more general theory called the association-induction hypothesis, I suggested briefly in 1962 and in greater detail in 1965 (13–15) that the continuous phase responsible for the semipermeable property of the cell surface is water, not ordinary water, but water existing in the state of polarized multilayers. In the present paper, I shall present additional evidence that now supports this theory. A brief mention of preliminary work appeared in 1970 (15).

MATERIALS AND METHODS

We used the abdominal skin of well-fed Northern leopard frogs (*Rana pipiens pipiens*, Schreber). Untreated cellulose-acetate membrane was obtained from Eastman Organic Chemicals Div. (Eastman Kodak Co., Rochester, N. Y.). All data reported below are from a single roll of type HTOO membrane received early in 1969 and kept in water at room temperature. Before use, a piece was cut out and kept heated in water maintained at 90°C for ½ h. (This heat treatment "activates" the membrane.)

For these permeability studies, we used essentially two types of apparatus. In the first type, shown in Fig. 1 A, the frog skin or cellulose-acetate membranes are placed vertically between chambers carried on two pieces of ½-inch Lucite and sealed with Vaseline (Fig. 1 A). Solutions on both sides were stirred with a pendulum composed of a lead shot enclosed in silicon rubber and suspended on a piece of string. The pendulum effectively stirred the solution when the Lucite chamber was placed on a shaker and shaken at the rate of 1 excursion/s, the am-

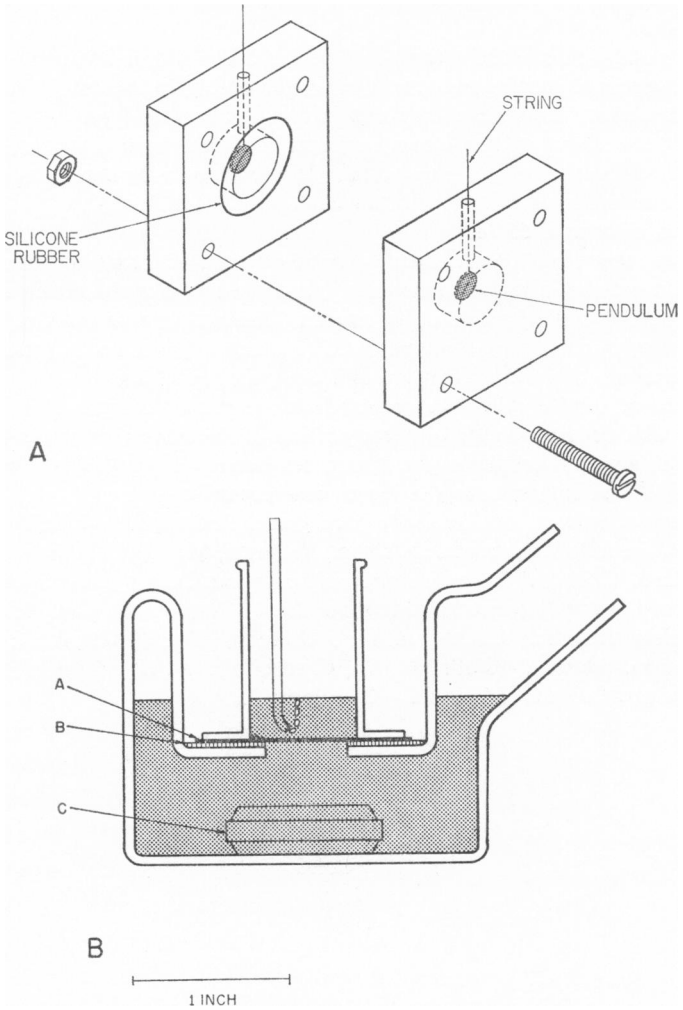

FIGURE 1 Diagrams of two types of vessels for permeability studies. (A) Lucite chamber: larger surface area made this the choice for studies on slowly diffusing substances. Can be submerged in water bath. Circular groove filled with silicon rubber acts as gasket to prevent leakage. (B) Glass permeability vessel: 50 ml sink compartment makes this the choice for fast diffusing substances through membranes that depend on a proper oxygen supply. Larger samples can be taken out without affecting the size of the diffusing surface. Cellulose-acetate membrane on frog skin (A) sealed with Vaseline to frosted glass surfaces (B). Magnetic stirring bar (C).

plitude of excursion being about 1 inch. The vessel was placed either in a constant temperature room maintained at $\pm 1.0°C$ or submerged in a water bath maintained at $\pm 0.05°C$.

A second type of chamber used is shown in Fig. 1 B. In this case, the membrane is placed horizontally between the top and bottom parts of the vessel and also sealed with Vaseline. The solution in the top compartment is stirred with a stream of moistened air and that in the

bottom compartment with a magnetic stirring bar. Results from the two types of chambers are quite similar.

In the case of frog skin, the serosal surface faced the source solution. This "reversal" eliminated any possible active transport that might occur. In the case of cellulose-acetate membrane, the active "skin" faced the "sink" compartment.

In all cases, the source and sink compartments contain solutions that are chemically the same, the only difference being the radioactive label added to the source solution at 0 time. As a rule, the concentration of the substance under study is 20 mM, with the exception of water which is, of course, 55 M.

Samples from the "sink" compartment were taken out at different intervals, depending on the permeability of the substance under study, and the radioactivity of the samples was assayed in a Packard 314E β-scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Bray's scintillation fluid was used (16) and adjusted so that the water and salt contents were nearly the same, to ensure uniformity in quenching.

The amount of labeled substance collected on the "sink" compartment is then plotted against time. The slope of the initial linear portion of the curve then yields the rate of flux, expressed in terms of micromoles per square centimeter of membrane area per hour per millimolar of labeled substance in the source compartment.

All chemicals used were of chemically pure grade. Radioactively labeled chemicals were obtained from the following sources: Nichem., Bethesda, Md. ([^{14}C]methanol; [1,3-^{14}C]glycerol); ICN Corp., Chemical & Radioisotopes Div., Irvine, Calif. ([UL-^{14}C]sucrose; [1,2-^{14}C] ethylene glycol; [1,3-^{14}C]glycerol); Amersham-Searle Corp., Arlington Heights, Ill. ([^{14}C] xylitol); Nuclear Research Chemicals, Inc., Orlando, Fla. ([^{14}C]xylitol); New England Nuclear, Boston, Mass. ([^{14}C]ethanol; D-[^{14}C]sorbitol); L-[^{3}H]glucose; [^{3}H]water); Mallinckrodt Nuclear, Orlando, Fla. ([UL-^{14}C]erythritol).

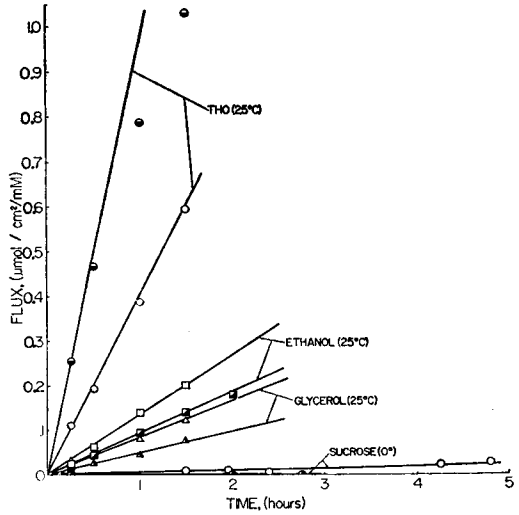

FIGURE 2 Time courses of flux of THO, ethyl alcohol, glycerol at 25°C, and sucrose at 0°C through reversed frog skin (empty symbols) and through cellulose-acetate membrane (half-filled symbols). Scale of ordinate and abscissa same as illustrated for first three. For sucrose, the ordinate should be multiplied by 0.1 and the abscissa by 10.

RESULTS

Fig. 2 shows the flux rates through both frog skin (empty symbols) and cellulose-acetate membranes (half-filled symbols) of the most permeable and least permeable substances (respectively, water at 25°C and sucrose at 0°C), in addition to ethanol and glycerol, which have intermediate permeabilities. The vast difference between the permeability of water and that of sucrose can be appreciated if one realizes that the data for sucrose were plotted on a different scale. The ordinate for sucrose is to be multiplied by 0.1 and the abscissa by 10. In other words, the permeability of sucrose is 100 times less than it appears to be on the graph. The constancy of the flux rates over the period of observation shows no deterioration occurred in either type of membrane.

TABLE I

DETAILS OF EXPERIMENTAL DATA ON THE PERMEABILITY OF REVERSED FROG SKIN AND HEAT-TREATED CELLULOSE-ACETATE MEMBRANE

Compound	Temperature	Permeability Cellulose-Acetate	Frog skin
	°C		μmol/cm² per h per mM
THO	25°	0.75 ± 0.041 (12)	0.447 ± 0.049 (13)
	4°	0.29 (2)	0.152 ± 0.014 (3)
	0°	0.280 ± 0.011 (5)	0.185 ± 0.021 (12)
Methanol	25°	0.28 ± 0.057 (5)	0.283 ± 0.026 (3)
	0°	0.069 (2)	0.0403 ± 0.0015 (3)
Ethanol	25°	0.106 ± 0.012 (4)	0.208 ± 0.047 (4)
n-Propanol	25°	0.102 ± 0.0061 (3)	0.132 ± 0.018 (3)
Ethylene glycol	25°	0.127 ± 0.025 (12)	0.0150 ± 0.0031 (7)
	0°	0.0181 ± 0.0009 (4)	0.0063 ± 0.0018 (3)
Glycerol	25°	0.0539 ± 0.0042 (4)	0.082 (2)
	4°	0.0050 (2)	0.0035 ± 0.0014 (4)
	0°	0.0022 (2)	0.00040 ± 0.000029 (3)
Erythritol	0°	0.00049 ± 0.000024 (5)	0.000071 ± 0.0000087 (3)
Xylitol	0°	0.000057 ± 0.000005 (4)	0.00014 ± 0.000046 (6)
Sorbitol	0°	0.00033 ± 0.00018 (5)	0.000075 ± 0.000032 (4)
L-Glucose	0°	0.0000465 ± 0.0000091 (6)	0.000049 ± 0.000011
Sucrose	25°	0.00098 ± 0.00054 (5)	0.000020
	0°	0.000012 (2)	0.000038 ± 0.0000092 (7)

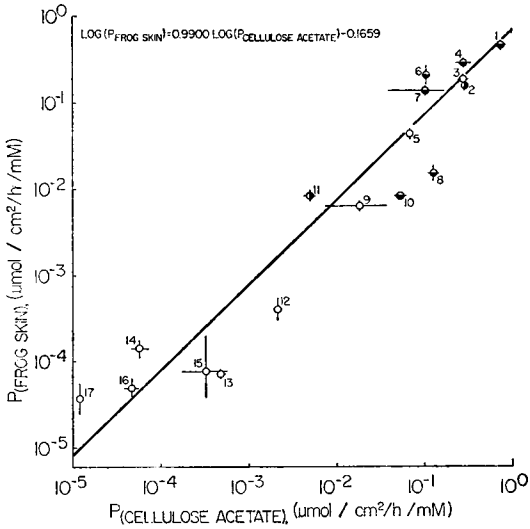

FIGURE 3 Plot of the permeability of reversed living frog skin against the permeability of heat-treated cellulose-acetate membrane. Complete data are given in Table I. Horizontal and vertical lines indicate standard error, except in the few cases when only two determinations were made. Straight line described by equation shown in graph was obtained by the method of least square. Number in graph refers to different compounds: *1*, THO, 25°C; *2*, THO, 4°C; *3*, THO, 0°C; *4*, methanol, 25°C; *5*, methanol, 0°C; *6*, ethanol, 25°C; *7*, *n*-propanol, 25°C; *8*, ethylene glycol, 25°C; *9*, ethylene glycol, 0°C; *10*, glycerol, 25°C; *11*, glycerol, 4°C; *12*, glycerol, 0°C; *13*, erythritol, 0°C; *14*, xylitol, 0°C; *15*, sorbitol, 0°C; *16*, L-glucose, 0°C; *17*, sucrose, 0°C. The empty circle (○) represents experiments at 0°C; circle with the right half filled (◑), 4°C; circle with the bottom half filled (◒), 25°C.

There is little question that the reversed frog skin and the cellulose-acetate membrane are both semipermeable, showing a higher permeability to water than to any other substance we investigated, including alcohol.

The results of all successful experiments are tabulated in Table I and graphed in Fig. 3. The linear correlation coefficient r is +0.961. The straight line through the points, obtained by the method of least square, is described as follows:

$$\log (P_{\text{frog skin}}) = 0.990 \log (P_{\text{cellulose acetate}}) - 0.1659. \quad (1)$$

The level of significance is far beyond the 0.001 level. The data also show that for THO, methanol, ethylene glycol, and glycerol, roughly similar activation energies for permeation were observed in the frog skin and in the cellulose-acetate membrane.

DISCUSSION

Collander demonstrated a close correlation between the permeability of *Nitella mucronata* cell protoplasm and the oil/water distribution coefficients of 69 compounds comprising alcohols, ethers, amides, ureas, etc. The present series of studies

is much more limited in scale, involving only 10 hydroxylic compounds. However, it should be noted that whereas Collander's studies were all conducted at one temperature, ours involved three: 0°, 4°, and 25°C.

There are two other major differences between our studies and those of Collander (10).

(*a*) *Correlation vs. Correspondence.* Although in both sets of studies the correlation covers permeability spanning five decades, Collander was plotting two different parameters in totally different dimensions. Thus, the permeability P of a solute through a thin sheet is related to q, the equilibrium (oil water) distribution coefficient of the solute in the diffusion medium, by the relation $P = Dq$, where D is the diffusion coefficient through the sheet (see reference 17). The linear correlation between P and q has been considered as evidence in favor of the lipoid membrane theory: that is, a lipoid layer on the cell surface which determines the permeability rate of all solutes in and out of the living cells. The validity of this argument rests upon the assumption that D either remains constant for all nonelectrolytes or decreases monotonically with a decrease of q. Neither of these postulations has been experimentally verified.

The data presented in Fig. 2 are quite different. Here frog skin permeability is plotted against cellulose-acetate membrane permeability, both in the same dimension, indeed in the same units. The method of least square yields the straight line with a slope of 0.99, which indicates not simple correlation but also correspondence. However, it would probably be unwise at this time to overemphasize this exact correspondence, because the thickness of the rate-limiting barrier in the frog skin is not known with certainty. In the cellulose-acetate sheet, the active "skin" is about 2500 Å thick (18) although the whole sheet is much thicker (approximately 100 μm).

(*b*) *Semipermeability.* The postulations of the membrane theory are based on the semipermeable behavior of living cells. However, an examination of Collander's data shows that out of 69 compounds studied, 62 have a higher oil/water distribution coefficient than water (DHO), and are, therefore, predicted to have higher permeability through the lipid barrier than water. Membranes of this nature are not bona fide semipermeable membranes; their use as models of the living cell membrane or protoplasm creates a serious internal conflict with the original reason for postulating the membrane theory in the first place.

Our model of oriented water as the permeability barrier, on the other hand, is perfectly semipermeable. That is, water is more permeable than any other substance studied including methyl, ethyl alcohols.

Polarized Water as Semipermeable-Selective Barrier

Traube's original "atomic sieve" idea failed because of experimental findings that holes in copper-ferrocyanide gel are much larger than molecules to which the gel is

highly impermeable (2). The recent work of Schultz and Asunmaa (18) beautifully verified those earlier findings with the aid of electron microscopy. They found that the active skin of cellulose-acetate membrane similar to those I studied is made up of spherical subunits of cellulose acetate in a random orientation interspersed by pores of 44 Å effective diameters, which are four times bigger than the diameter of the extremely impermeant sucrose molecules (approximately 9.4 Å, assuming a spherical shape). It is also worth noting that Schultz and Asunmaa concluded that the water filling these 44-Å pores is highly structured, with a viscosity 37 times higher than that of ordinary water.

Now each water molecule is 2.7 Å in diameter; pores 44 Å in diameter are filled with no less than 15 water molecules from one side to the other. Clearly, this is not a single layer of water molecules. It is water in multilayers.

Elsewhere we have presented the theoretical basis for the conclusion that deep layers of water molecules can be brought under the polarizing influence of surfaces if these surfaces have a mosaic structure of alternatingly positive and negative sites (19). Is there any basis for this kind of structure with cellulose-acetate sheets? The answer is definitely yes. In the commercial preparation of cellulose acetate, the D-glucose units of the cellulose are only partially acetylated (20). In the cellulose-acetate molecules there is an abundance of "positive" OH groups and "negative" C=O groups at close intervals, thus fulfilling the need for producing deep polarized multilayers of water.

Frog skin does not possess CO, OH groups, but being proteinaceous, it contains the counterparts, CO, NH groups in the form of the stretched polypeptide chains. Experimental evidence exists in support of the view that under suitable conditions these backbone NHCO groups can polarize more than one layer of water (15, 19). While the location of the active surface of the cellulose acetate is well-defined, that of the "active" surface in the frog skin is far less so and must await future investigation.

Historical Evidence in Favor of the Present Theory

Not the least important evidence in favor of the concept of polarized-multilayered water as the semipermeable barrier is to be found in the history of osmotic studies. The long list of models with semipermeable properties developed over the ages includes dead animal bladder, parchment, gelatin, copper-ferrocyanide gel, Prussian blue, various tannates, and silicates (2, 3), to which we now can add porous glass (21) and cellulose-acetate sheet. This list comprises a truly heterogeneous group of substances; all, however, possess water and a matrix containing both positive and negative charges.

The demonstration of the low surface tension of living cells by Harvey et al. led to the postulation that cell surfaces are covered with proteins with hydrophilic groups (22–24). Quite independently, Ling and Ochsenfeld have demonstrated that

the (hydrophilic) anionic groups on muscle cell surfaces have pK values of 4.6, which strongly suggests that they are indeed the β- and γ-carboxyl groups belonging to glutamic and aspartic side chains on proteins (25). It has been shown recently (19) that all known proteins hydrate, that is, they adsorb water. Globular proteins, however, as a rule hydrate only a single layer of water around their polar side chain, a fact attributed to the internal saturation of the bulk of backbone NHCO groups in the globular conformation. Fibrous proteins, like gelatin, adsorb much more water than can be accounted for by their polar side chains. It is most interesting to note that gelatin-water membranes are also semipermeable (although nowhere nearly as perfect as the other models such as the copper-ferrocyanide gel membrane) (2, 26). One can thus surmise that proteins on the resting living cell surface must exist primarily in an extended conformation, and participate in intense interaction with deep layers of polarized water, which then serve as the seat of selective permeability seen in many living cells.

Such a model permits the all-or-none type of permeability change during excitation when the proteins cooperatively alter their electronic conformation, thereby liberating the adsorbed water in a reversible manner (27, 28). Such a cooperative shift of proteins may follow the adsorption or desorption of certain key agents called cardinal adsorbents, including ATP, Ca^{++}, and acetyl choline (for evidence see references 29–31).

The author wishes to thank Miss Sandy Will, Miss Ellen Ferguson, and Mrs. Janice Malseed for their invaluable contributions.

This investigation was supported by National Institutes of Health research grant GM 11422-08, 09 and by contract nonr N00014-71-C-0178 (NR 105-327) between the Office of Naval Research and the Pennsylvania Hospital. The senior investigator was supported by U. S. Public Health Service research career development award, K3-GM-19, 032. The John A. Hartford Foundation provided most of the basic facilities.

Received for publication 1 February 1973.

REFERENCES

1. Pfeffer, W. 1921. Osmotische Untersuchungen. Engelmann, Leipzig. 2nd edition.
2. Findlay, A. 1919. Osmotic Pressure. Longmans, Green, New York. 2nd edition.
3. Traube, M. 1867. *Arch. Anat. Physiol. Wissenschaft Med.*, 87.
4. Overton, E. 1899. *Vierteljahresschr. Naturforsch. Gesamte Zur.* **44**:88.
5. Overton, E. 1902. *Arch. Gesamte Physiol. Menschen Tiere (Pfluegers).* **92**:115.
6. Collander, R., and H. Bärlund. 1933. *Acta. Bot. Fenn.* **11**:1.
7. Glasstone, S. 1946. Textbook of Physical Chemistry. van Nostrand Reinhold Company, New York. 2nd edition.
8. Napolitano, L., F. Lebaron, and J. Scaletti. 1967. *J. Cell Biol.* **34**:817.
9. Fleischer, S., B. Fleischer, and W. Stoeckenius. 1967. *J. Cell Biol.* **32**:193.
10. Collander, R. 1960. *Plant Physiol.* **2**:1.
11. Wright, E. A., and J. M. Diamond. 1969. *Proc. R. Soc. Lond. B Biol. Sci.* **172**:227.
12. Diamond, J. M., and E. A. Wright. 1969. *Proc. R. Soc. Lond. B Biol. Sci.* **172**:273.
13. Ling, G. N. 1962. A Physical Theory of the Living State: The Association-Induction Hypothesis. Blaisdell Publishing Co., Waltham, Mass.

14. LING, G. N. 1965. *Ann. N. Y. Acad. Sci.* **125**:401.
15. LING, G. N. 1970. *Int. J. Neurosci.* **1**:129.
16. BRAY, G. A. 1960. *Anal. Biochem.* **1**:279.
17. CRANK, J. 1956. The Mathematics of Diffusion. Clarendon Press, Oxford.
18. SCHULTZ, R. D., and S. K. ASUNMAA. 1969. *Recent Prog. Surf. Sci.* **3**:291.
19. LING, G. N. 1972. *In* Water and Aqueous Solutions. John Wiley & Sons, Inc., New York. 663.
20. PIGMAN, W. 1957. The Carbohydrates. Academic Press, Inc., New York.
21. KRANS, K. A., A. E. MARCINKOWSKY, J. S. JOHNSON, and A. J. SHOR. 1966. *Science (Wash. D.C.).* **151**:194.
22. COLE, K. S. 1932. *J. Cell. Comp. Physiol.* **1**:1.
23. HARVEY, E., and R. COLLANDER. 1932. *J. Franklin Inst.* **1**:214.
24. DAVSON, H., and J. F. DANIELLI. 1943. The Permeability of Natural Membranes. Cambridge University Press, London. 2nd edition.
25. LING, G. N., and M. M. OCHSENFELD. 1965. *Biophys. J.* **5**:777.
26. TINKER, F. 1916. *Proc. R. Soc. Lond. A Math. Phys. Sci.* **92A**:357.
27. LING, G. N. 1969. *Int. Rev. Cytol.* **26**:1.
28. LING, G. N. 1971. Die Zell. H. Metzner, editor. Wissenschaftliche Verlags gesellschaft mbH., Stuttgart. 2nd edition.
29. LING, G. N., and M. M. OCHSENFELD. 1973. *Ann. N. Y. Acad. Sci.* **204**:325.
30. GULATI, J. 1973. *Ann. N. Y. Acad. Sci.* **204**:337.
31. JONES, A. W. 1973. *Ann. N. Y. Acad. Sci.* **204**:379.