

THE FUNCTIONS OF POLARIZED WATER AND MEMBRANE LIPIDS: A REBUTTAL

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- *In 1973, experimental evidence was reported in support of the view that water polarized in multilayers rather than simple lipid is responsible for the semipermeable properties of living cell membranes. The present article presents a full rebuttal to a subsequent attack on that view.*

INTRODUCTION

In 1973 I reported evidence to the effect that water polarized in **multilayers** by proteins may account for certain semipermeable properties of living **cells**.¹ A subsequently published paper vigorously criticized the report and my science in **general**.² In the following pages I present detailed rebuttal to the criticisms offered.

For adequate background, I first describe briefly both the membrane-pump model of the cell, to which the author of the critical paper subscribes, and the directly opposed association-induction model, which informs my own work and in my view more accurately explains observed cell phenomena. Secondly, I present a condensed but fundamental critique of the membrane-pump construct.

The Competing Approaches

As a broad base for cell physiology, two kinds of theories remain candidates, a third having long since been **disproven**.^{3,4} The contending survivors may be characterized as follows:

Membrane-pump theory. In this approach, the existence of living cells as entities separate from their environment is ascribed to enclosing membranes containing a battery of continually operative energy-consuming pumps. The water in living cells is considered essentially the same as liquid water. The high level of K^+ in cells is attributed to inward pumping and the low level of Na^+ to outward pumping.

The association-induction hypothesis. Alternatively, the association-induction hypothesis **holds** that the entire cell, rather than solely the membrane, is the seat of the cell's separateness from its **environment**.⁵⁻⁷ The cell membrane is viewed as an integral part of the cell structure and the locus of a variety of important physiological functions (but not "pumping"). The resting cell is seen as existing in a metastable equilibrium state. Maintenance per se of that state, as in the case of any

equilibrium state, consumes no energy. The asymmetry between concentrations of ions, nonelectrolytes, etc. in the cell and concentrations in its immediate environment reflects not **membrane pumps** but a high degree of association among the cell constituents. That is, certain affinities exist between macromolecules and macromolecules, between macromolecules and the bulk of cell water, between macromolecules and ions as well as other solutes. The diversity of solute distribution patterns is regarded as reflecting two opposing mechanisms: (*a*) selective adsorption, which raises the concentration of a solute in the cell to above that in the environment, and (*b*) selective exclusion from the cell water, which diminishes the concentration of a solute in the cell to a level below that found in the environment. Thus the relatively high level of K^+ in cells can be ascribed to selective adsorption on β - and γ -carboxyl groups of cell proteins and, under certain circumstances, to the carbonyl groups of the **backbone**.⁸⁻¹⁰ Na^+ , excluded from most of these adsorption sites due to less favorable adsorption energy, is to a large extent confined to the cell water which, existing in a state of polarized **multilayers**, dissolves less Na^+ ; hence the low level found in most **cells**.¹¹⁻¹⁵

Insufficiencies of Membrane-Pump Theory

Some of the more serious weaknesses of the membrane-pump approach are as follows:

I. Energy. The published work of R. McElhaney, author of the paper² so critical of mine,¹ has been careful and often ingenious in dealing with nonelectrolyte permeability problems. But to my knowledge he has never directly addressed the question of how a given nonelectrolyte, once inside the cell, may fail to reach the same level of concentration as that found in the external medium while others may reach a level higher than that in the external medium. Instead, he and other membrane biologists simply accept the convenience of postulating more and more pumps, apparently little bothered by the excessive energy needs of the ever-lengthening list.

Yet it has been nearly fifteen years since it was shown⁵ that even one **pump**—the Na **pump**—under specified conditions would consume at least 15 to 30 times the energy a resting tissue has at its command (see also refs. 6, 7). No serious challenge to that conclusion has been made. (A call by Hazlewood in Science for public debate of the issue, which should have provoked refutation if any existed, was ignored by pump-theory **advocates**.^{16,17}) On the other hand, the finding of excessive energy need has been confirmed independently by Jones¹⁸ and by Minkoff and Damadian.^{19,20} In artificially isolating permeability from the inseparable problem of distribution, the criticizing author² and other membrane-pump theorists have not contributed to the coherent understanding to which science aspires.

2. Inheritance. A variety of major physiological properties of living cells reflects properties and activities of cell membranes. It follows that since cells differ greatly in their physiological traits, clearly the cell membrane must be **cor-**

respondingly different and characteristic. Yet there are data in existence, including those provided by **McElhane**,² supposed to show that the membrane lipid composition can be varied merely by adding and removing lipids from the cell's environment. Thus if the cell membrane were indeed primarily lipid, logic would demand that the cell's characteristic properties be determined by its environment rather than by its genes—hardly an acceptable proposition in this day and age. It needs no reiteration that genes determine cell character, and by specifying protein composition rather than lipid composition.

3. **Ubiquity.** All living cells are semipermeable. Therefore if a continuous lipid layer is actually the seat of semipermeability, then all living cells must possess enough lipids in their membranes to provide a continuous layer. In truth, however, the lipid content of cell membranes varies greatly and except in myelinated nerves and human red blood cells tends to be low. To cite a few cases: the lipid content of rat muscle membrane is only **15%** (**65%** proteins); rat liver membrane, **10%** (**85%** proteins); and avian erythrocytes, **4%** (**89%** proteins).²¹ However, even these quoted figures are higher than actual lipid content because the chemical composition of membranes is conventionally based on dry weight. The water associated with proteins and phospholipids is conventionally ignored, apparently because of the sheer technical difficulty in isolating pure wet membranes (see below). In summary, not all cells have enough lipids in their membranes to form a continuous covering layer is depicted by the lipid bilayer model. Therefore a continuous bilayer cannot be a universal component of living cell membranes.

4. **The disproven sieve concept.** J. H. van't Hoff in **1886** first suggested the adjective "semipermeable" to describe certain membrane types that "allow free passage of water but not of the dissolved substances."²² This usage of "semipermeable" is now conventional in the scientific community. Thus if the membrane-pump school is correct in believing—and our criticizing author is correct in titularly stating—that "membrane lipid, not polarized water, is responsible for the semipermeable properties of living cells,"²² then lipid membrane *per se* should allow free passage of water but not of the dissolved substances. But water, which has very low solubility in lipids, cannot possibly pass freely through lipid membranes. Indeed, it was in recognition of this contradiction that Collander and Barlund introduced the "mosaic membrane" theory—postulating the existence of small water-filled pores in the lipid layer to provide rapid passageways for water, other **nonelectrolytes** being barred due to their larger **sizes**.²³

Thus Collander and Barlund willy-nilly "revived" the age-old "sieve" theory as the basis of semipermeability. That theory, however, was falsified by X-ray and electron-diffraction studies as long ago as the **nineteen-thirties**,²² studies establishing that water-filled pores in semipermeable membranes are too large to account for semipermeability. The modern concept of water in polarized multilayers with demonstrable semipermeable properties resolves the difficulty.

REBUTTAL: THE OBSERVATIONAL EVIDENCE

Having established essential background, I proceed now to point-by-point refutation of the paper² assailing my previously published report.'

Existence of Lipid Bilayers in Biological Membranes

The critical paper misrepresented my view by stating I had concluded that "... the unit membrane structure is in fact entirely proteinaceous." (ref. 2, p. 779).

What I actually had written was "... the reports of Napolitano et al.²⁴ and Fleisher et al.²⁵ cast doubts on the theory that the unit membrane is primarily a continuous sheet of **lipoid**." By insertion of the word "entirely," my view was distorted and an element of unreasonableness thrust into my argument.

Both Napolitano et al. and Fleisher et al. found that the extraction of the **bulk** of lipid materials from the "unit membrane" fails to produce a drastic change in its trilaminar structure. It is true that Napolitano et al. observed this lack of response to lipid extraction in membrane preparations prefixed in glutaraldehyde. On the basis of that observation alone, the critical paper argued that prefixation was responsible for the maintenance of the unit membrane structure after lipid removal. However, the paper ignored the work of Fleisher et al.,²⁵ who observed a similar lack of drastic change in the trilaminar structure of rat liver mitochondria membranes even though these mitochondria had not been prefixed but on the contrary had been subjected to acetone extraction before fixation. The findings of Fleischer et al. were later confirmed by Morowitz and Terry,²⁶ who also showed retention of the unit membrane structure in purified membranes of *Mycoplasma laidlawii*, after 95% or more of the lipid had been removed from the membranes before fixation. In addition, they observed that digestion of pronase, which destroys proteins, led to "pronounced loss in electromicroscope contrast, decrease in membrane thickness, and loss of prominent triple layered structure." These observations showed that retention of unit membrane structure after lipid extraction is not dependent on prior fixation in glutaraldehyde as contended in the critical paper.

In any case, and contrary to what that paper implied, whether all unit membrane structures can be completely preserved after lipid extraction is of no particular significance. The reason is that if all cell membranes were indeed primarily lipid layers with proteins dissolved in those lipid layers, then all the membranes should have dissolved away in the lipid solvents.

Just as informative in this context (though not mentioned in my report) was the finding of S Fox²⁷ that in a pure protein-water system, which he manufactured from pure amino acids plus pure water and called "proteinoid microspheres," a similar trilaminar membrane structure was seen when these microspheres were stained with osmic acid, imbedded in methacrylate, and sectioned. In this case, the electron-sparse layer between the two heavily stained layers could hardly be due to lipid since no lipid existed in Fox's preparation.

The statement in the critical paper that "every author of a major review article on membrane structure and function since 1970 has found the evidence for the existence of lipid bilayers in cellular membrane to be compelling" is not difficult to understand. One can hardly expect journal editors to accept "major" reviews of lipid membranes from those who do not subscribe to the conventional view of lipid membranes. To be fair and scientific, the critical paper also should have mentioned that other reviewers (including **Korn**^{28,29} and **Richardson**³⁰) have published considerably different views about the structural role of lipids in cell membranes.

Semipermeable Properties of Lipid Bilayer Membranes

As clearly stated in my report of 1973,¹ the question I posed was whether it is water existing as polarized multilayers or lipid that constitutes the continuous semipermeable barrier. Here lipid is meant as lipid, pure and uncomplicated as used in Collander's famous study quoted widely in **textbooks**.³¹ The model for this lipid was olive oil (and in the study of Wright and Diamond, **ether**³²). The **distribution** coefficients of some 60 compounds between olive oil and water were correlated with permeability through *Nitelta micronata*, thereby furnishing, in my opinion, what seems the most convincing evidence in favor of the lipid membrane model.

But in my 1973 paper¹ I pointed out that this simple lipid layer is not in fact semipermeable. Actually the **oil/water** distribution coefficient for water is **some** 50 times less than for ethanol, manifesting precisely the reverse of semipermeability; **i.e.**, much higher water permeability to water than to ethanol.

My question was never directed at the phospholipid bilayers made much of in the criticizing paper. Such bilayers are not pure lipid membranes in the original **Overton** sense since they are equipped with water-filled pores (ref. 21, p. 119). The high permeability to water of the lipid bilayer is primarily attributable to the **water-filled** pores rather than the lipid phase itself. Lipid as such is not a *bona fide* semipermeable membrane, as Collander's data have shown clearly and unequivocally.

In the report at issue¹ my argument was that the major seat of semipermeability is water itself as it is polarized in multilayers by charged surfaces lining large pores. I pointed out that insofar as semipermeable properties themselves are concerned, the specific nature of the ionic or H-bonding polar groups that polarize the water molecules is of relatively lesser importance. Thus cellulose acetate, copper **ferrocyanide**, parchment, gelatin, Prussian blue, various tannates, silicates, and porous glass, all of which present bipolar sites and contain water (but not lipid), are semipermeable. It would seem that with their high charge density, phospholipid bilayers may well add still another item to this long list of models of water-containing, polarizing matrices possessing semipermeable properties.

Interpretation of Epithelial Membrane Permeability Coefficients

To substantiate the view that polarized water can serve as a selective semipermeable barrier, I demonstrated that reversed frog skin and cellulose acetate sheets have permeability coefficients to **H₂O** and 10 other hydroxy compounds at 3 **dif-**

ferent temperatures, spanning over 4 log scales, which are closely correlated ($\gamma = +0.96$). Indeed the relation between the two permeabilities, P's, can be described by the following equation:

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

The near unity slope demonstrates close correspondence, not merely good correlation as was the case in the work of **Collander**³¹ and of **Wright and Diamond**.³²

The critical paper claimed that this close correspondence I reported was a mere coincidence, having nothing to do with either frog skin or cellulose acetate but only with the presence in both cases of an unstirred layer of water. The paper also claimed that frog skin is too complex so that the data are "essentially **uninterpretable**." Solutes may leak through extracellular space, for example.

The purity of material yearned for in the paper does not exist, unfortunately, in the real world—except perhaps in absolute vacuum. Even pure water itself is not homogeneous but a mixture. Biologists must be satisfied with compromises. My choice of reversed frog skin was just such a compromise. Similarly, of course, in all other systematic studies of nonelectrolyte permeabilities, investigators have had to resort to complex living preparations. For example, when **Collander** studied membrane permeability of *Nitella* cells his data on the permeation rates of various chemical compounds were not obtained using a simple homogeneous plasma membrane, as undoubtedly he would have preferred. He was obliged to use a complex system including (1) cell wall, (2) plasma membrane, (3)-cytoplasm, and (4) tonoplast.

The critical paper cited favorably and repeatedly the data of **Diamond and Wright**³³ (presented as supporting the lipid layer as barrier) but not our data for frog skin (presented as contradicting lipid barrier). Yet **Diamond and Wright's** data were derived from studies of gall bladder wall which, like frog skin, is a complex sheet of cells with the intracellular and extracellular passageways stated in the paper to make the frog skin study particularly "uninterpretable."

For the measurement of the relative steady-state permeability coefficients of water and solutes, the number of layers of cells in the skin offers no serious problem since only the rate of permeation through the most resistant step would be measured. **MacRobbie and Ussing**³⁴ have shown that the barrier to osmotic water transport in the multilayers of cells is located near the outer surface of the frog skin.

A more serious possible source of error is intercellular space. If that space were entirely open, and if the cross-sectional area of the space were, say, 10% of the total skin surface, then the observed permeability values for the least permeable solutes would be higher than the true values. However, in that case the permeability for water would be less than 50 times that of sucrose. Actually, the observed ratio is 20,000 to 1. This **reaffirms** the well-known consideration that in frog skin, the intercellular space is not open but sealed by a "tight junction" which, under similar conditions to those employed (**i.e.**, identical chemical composition of

solutions in both the "source" and "sink" compartment in the permeability apparatus), is an **effective** barrier to water and solute movement (see ref. 35, p. 35).

In speculating on an unstirred layer of water as the basis for all the data I collected from studies of frog skin and of **cellulose** acetate membrane, **McElhane**y chose to ignore the fact that in the experiments both the "sink" solution and the "source" solution were vigorously stirred and so described under MATERIALS and METHODS.'

His paper's daring speculation that water, merely by being unstirred, can be transformed into a semipermeable barrier with selective reduction of the **permeability** to sucrose by four orders of magnitude can hardly be seriously considered.

Indeed, if one pushes the theory of the unstirred layer one step further, one might conclude that all the permeability data on living cells from efflux studies would have nothing at all to do with cell membrane barriers because it is rare that anyone had adequately stirred the inside of a cell while studying the efflux. However, efflux data obtained from rapidly **perfused** squid axons agree with those obtained from **axons** with intact and unstirred cytoplasm, contrary to what the paper's "unstirred" theory predicts.

Membrane Lipids and Semipermeable Properties of Living Cells

The critical paper cited its author's work on the effect of varying the fatty acid and cholesterol contents in plasma membrane of the procaryote *Acholeplasma laidlawii*, and in liposomes prepared from the total lipids extracted from the cells, on altering the nonelectrolyte permeability.

Once more I emphasize that when I referred in my report to the lipid layer, I did so in the original meaning put forth by **Overton**: lipids as represented by the olive oil model. The quantitative agreement between *Nitella* permeability and the **oil/water** distribution coefficient was based on the distribution coefficient between water and olive oil, a mixed glyceride of oleic acid (83.5%), **palmitic** acid (9.4%), **linoleic** acid (4.0%), stearic acid (2.0%), and arachidic acid (0.9%).

Now, "lipids" actually isolated from cell membranes have properties quite different from those of the original Overton-Collander model. Phospholipids **are** as different from pure lipid as ATP is from adenosine. There is no justification for assuming that the nonelectrolyte **oil/water** distribution coefficient between olive oil and water could be the same as between phospholipid-cholesterol and water. If the coefficients were the same, the phospholipid-cholesterol-water system should be anti-semipermeable as in the case of olive oil.

If the original theory of Overton-Collander is correct and these lipid bilayer models have the properties predicted on the basis of olive oil studies, then such models should have, for example, equal permeability coefficients for water and for acetamide. In truth, the data of Andreoli et al.³⁶ established that in phospholipids from sheep and red blood cells, the permeability coefficient for water is

more than ten times higher than **that** of acetamide. Clearly phospholipid membranes do not behave like pure olive oil membrane.

Indeed, this departure from the predicted relative permeability based on the **oil/water** distribution coefficient is one long known to exist for living cells. The remedial answer is that the lipid membrane has water-filled pores. A simple interfacial energy consideration would naturally lead one to suspect that the charged groups may in part be instrumental in forming the water-filled pores. In that case, an increase in the percentage of non-charged cholesterol molecules should naturally cause a diminution in the water-filled pores and a corresponding reduction in the rate of permeations through the membrane of ethylene glycol, glycerol, and erythritol which travel through these aqueous channels.

The critical paper also stated: "The extensive treatment of intact cells with proteases which remove most of the protein molecules exposed on the outer surface of the cell membrane does not destroy or markedly alter the cellular permeability barrier. . . ." In support, three references **were** cited. One of these, however, Zwaal et al., put the matter quite differently, as follows: "Proteolytic release of these glycopeptides (in intact cells) is accompanied by alteration of the permeability properties of the cell membrane . . ." (ref. 37, p. 175). The critical paper then went on to say that "treatment of susceptible cells with purified phospholipase **A₂**, which cleaves fatty acyl groups from membrane phospholipids, destroys the semipermeable properties of living cells and leads to cell lysis. . . ." Again the cited reference was in contradiction rather than support, as follows: "So far, no pure phospholipase **A₂** (from any sources) has been shown to have lytic activity toward erythrocytes." (ref. 37, p. 165).

FUNCTIONS OF MEMBRANE LIPIDS AS SEEN IN THE CONTEXT OF THE ASSOCIATION-INDUCTION HYPOTHESIS

In my view, the primary structure of the cell membrane, like that of other parts of the protoplasm, consists of the proteins specified by the genome of the living organism. It is the extended "backbone" of part of the membrane protein system that polarizes deep layers of water which then act as a semipermeable barrier without having to rely on pore-sizes as the discriminatory mechanism permitting passage of one molecule and barring that of another. Evidence favoring this idea has been presented for two model systems: cellulose acetate¹ and ion exchange **resin**.¹⁵ In each **case**, it was shown that low permeability to sugars is **due** not to pore size but to the different physical state of water in the systems and different (often reduced) solubility and diffusion rates of these nonelectrolytes and other solutes in and through the water. The polar and non-polar groups on these and other membrane proteins also provide the basis for adsorption in single and perhaps even multilayers of lipids. The presence, specificity, and quantity of the lipid in the membrane are dictated by the **nature** of the proteins.

What then is the role of lipids in the association-induction context? I can see at least two major functions: first, stabilization of the structure of the protein-water-ion system through hydrophobic bonding, and second, service as an insulating barrier. The insulating barrier par excellence, of course, is **myelin**. In this light, the strikingly large quantity of membrane lipids in a variety of intensely studied microbes, such as those investigated by **McElhane**y, is not difficult to interpret; many such microbes must survive dry conditions, and lipids slow down evaporation. Why then should human erythrocytes show such high lipid content? I suggest that this generous lipid quotient may occur in order to insure that **intracellular** proteins be conserved; lacking a nucleus, human erythrocytes do not manufacture proteins but must make do with what they have.

The association-induction model readily accounts for the confirmed observation that in frog ovarian **egg**³⁸ and in giant barnacle single muscle **fibers**,³⁹ the cell membrane offers no higher resistance to the diffusion of labeled water than does the cytoplasm. These findings have been verified by spin echo NMR studies⁴⁰ and pulse gradient spin echo NMR **studies**.⁴¹ Assuming that all muscle membranes are alike and that frog ovarian eggs—like rat muscle, avian erythrocytes, and rat liver—are poor in lipid, then with the entire bulk of cytoplasmic water being in a similar state of polarized **multilayers**,⁷ the uniformity of diffusion from the cell interior out is reasonable. Pulse-gradient spin-echo NMR studies of Cooper et al.⁴¹ and of Finch (personal communication) have also shown that human red cell membrane, in contrast, is a barrier to the movement of water, in harmony with the high lipid content and the interpretation here presented of the role of lipid.

Finally, because in this model the membrane permeability property is primarily that of the membrane water, such permeability is readily under control of proteins. These in turn are under the control of low concentrations of the physiologically active compounds called "cardinal adsorbents," chief among which are Ca^{2+} and **ATP**.^{2,42} Rapid and reversible alteration of membrane permeability could thus be achieved as it is well known to occur in living cells for both electrolytes and non-electrolytes during excitation!"

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