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Review

Do sterols reduce proton and sodium leaks through lipid bilayers?

Thomas H. Haines*

Department of Chemistry, City College of the City University of New York and Biochemistry, City University of New York Medical School, New York, NY 10031, USA

Abstract

Proton and/or sodium electrochemical gradients are critical to energy handling at the plasma membranes of all living cells. Sodium gradients are used for animal plasma membranes, all other living organisms use proton gradients. These chemical and electrical gradients are either created by a cation pumping ATPase or are created by photons or redox, used to make ATP. It has been established that both hydrogen and sodium ions leak through lipid bilayers at approximately the same rate at the concentration they occur in living organisms. Although the gradients are achieved by pumping the cations out of the cell, the plasma membrane potential enhances the leakage rate of these cations into the cell because of the orientation of the potential. This review proposes that cells use certain lipids to inhibit cation leakage through the membrane bilayers. It assumes that Na^+ leaks through the bilayer by a defect mechanism. For Na^+ leakage in animal plasma membranes, the evidence suggests that cholesterol is a key inhibitor of Na^+ leakage. Here I put forth a novel mechanism for proton leakage through lipid bilayers. The mechanism assumes water forms protonated and deprotonated clusters in the lipid bilayer. The model suggests how two features of lipid structures may inhibit H^+ leakage. One feature is the fused ring structure of sterols, hopanoids and tetrahymenol which extrude water and therefore clusters from the bilayer. The second feature is lipid structures that crowd the center of the bilayer with hydrocarbon. This can be accomplished either by separating the two monolayers with hydrocarbons such as isoprenes or isopranes in the bilayer's cleavage plane or by branching the lipid chains in the center of the bilayers with hydrocarbon. The natural distribution of lipids that contain these features are examined. Data in the literature shows that plasma membranes exposed to extreme concentrations of cations are particularly rich in the lipids containing the predicted qualities. Prokaryote plasma membranes that reside in extreme acids (acidophiles) contain both hopanoids and iso/anteiso- terminal lipid branching. Plasma membranes that reside in extreme base (alkaliphiles) contain both squalene and iso/anteiso- lipids. The mole fraction of squalene in alkaliphile bilayers increases, as they are cultured at higher pH. In eukaryotes, cation leak inhibition is here attributed to sterols and certain isoprenes, dolichol for lysosomes and peroxysomes, ubiquinone for these in addition to mitochondrion, and plastoquinone for the chloroplast. Phytosterols differ from cholesterol because they

* Tel.: +1-212-650-6988; fax: +1-212-721-9556.
E-mail address: thaines@prdi.org (T.H. Haines).

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contain methyl and ethyl branches on the side chain. The proposal provides a structure-function rationale for distinguishing the structures of the phytosterols as inhibitors of proton leaks from that of cholesterol which is proposed to inhibit leaks of Na^+. The most extensively studied of sterols, cholesterol, occurs only in animal cells where there is a sodium gradient across the plasma membrane. In mammals, nearly 100 proteins participate in cholesterol's biosynthetic and degradation pathway, its regulatory mechanisms and cell-delivery system. Although a fat, cholesterol yields no energy on degradation. Experiments have shown that it reduces Na^+ and K^+ leakage through lipid bilayers to approximately one third of bilayers that lack the sterol. If sterols significantly inhibit cation leakage through the lipids of the plasma membrane, then the general role of all sterols is to save metabolic ATP energy, which is the penalty for cation leaks into the cytosol. The regulation of cholesterol's appearance in the plasma membrane and the evolution of sterols is discussed in light of this proposed role.

Contents

1. Introduction

This review addresses the structure of certain lipids in those biological membranes that maintain a cationic (H^+, Na^+) electrochemical gradient. These membranes are central to energy

transduction and cellular function. To the extent that the cations leak through the lipid bilayer domains of plasma membranes, to that extent energy stored in the gradient is lost, i.e. ATP must be consumed to pump out the cations. Furthermore, the leakage is always enhanced by the electrochemical gradient because the cations are pumped to the positive side of the gradient and the membrane potential is so oriented as to enhance cation leakage into the cell. Lipid structures such as cholesterol have been shown to decrease the permeability of these membranes to cations.

For the handling of life's energy, living cells have invested in two fundamental mechanisms: chemical energy (primarily ATP), and cation electrochemical gradients (principally H^+ and Na^+) across membrane bilayers. These two mechanisms are intimately, and largely reversibly, entwined. The two principal sources of energy for life, photons and redox, produce electrochemical gradients. These in turn produce proton gradients that subsequently produce ATP. This occurs at mitochondrial, chloroplast and bacterial plasma membranes. Thus the source of energy increases the cation concentration on one side (positive) of the membrane bilayer whereas the ATP synthesis occurs on the other side (negative). In these membranes, the stored cations pass down the gradient through the ATPase's proton pore. Thus the electrochemical cation gradient is used to synthesize ATP. However, this is only one use of the electrochemical cation gradient. It is used for a myriad of activities such as transport of nutrients, excretion, bacterial flagellar motion, rejection of toxins from the cell and many other membrane functions. (Under some circumstances, i.e. anaerobic conditions, prokaryotes or eukaryotes produce ATP metabolically, and use it to create an electrochemical gradient in the plasma membrane.) Proton leakage though the bacterial plasma membrane is especially important for those organisms that live in hostile pH environments, the acidophiles and the alkaliphiles. These organisms appear to have unique lipid structures that may inhibit the proton leakage.

Eukaryote cells contain "prokaryote organelles" that produce ATP, much of which is used to generate an electrochemical gradient across the eukaryote plasma membrane. In animals, the cation gradient uses sodium; in prokaryotes and in all other eukaryotes, plants, yeast, fungi, etc., a proton gradient is used. All cells use K^+ as the counterion to adjust the osmolality and the overall potential across the cell membrane. Cells maintain a relatively high and relatively constant internal $[K^+]$. The extracellular $[Na^+]$ together with the sodium channels permits controlled lateral signals along the membrane, and therefore motion in animals. Nature does not contain gated H^+ channels that permit signaling along membranes. Gated channels, which permit such signaling, are exclusively for metal cations, principally Na^+, K^+, and Ca^{++} or anions, principally Cl^-. The latter are used by plants. These channels permit signaling and therefore neural activity [1]. The switch to sodium taken together with the evolution of the sodium-gated channel served animals by granting them motion. Meanwhile, the use of the Na^+ electrochemical gradient, with its energy supplied by mitochondrial ATP also served all the purposes in the animal eukaryote plasma membrane for which microbes and plants use the H^+ gradient.

The first measurements of K^+, Na^+ leakage across defined lipid bilayers were conducted in 1972 by Papahadjopoulos [2,3]. His group examined the diffusion of many cations across a variety of defined lipid bilayers without a membrane potential. They found that the permeability of all of the phospholipid bilayers they tested to either Na^+ or K^+ was approximately 10^{-12} cm/s. They, and most workers at the time, considered this too low to be of any biological significance. In those experiments where the cholesterol concentration was in the range of that found in living

animal membranes the Na^+ leakage was reduced to one third that of the control cholesterol-free bilayers.

Following up Nicholls' [4,5] early studies on proton leakage in brown fat mitochondrial inner membrane (which turned out to be due to uncoupling proteins), Hinkle [6,7], measured the H^+ leakage through the lipids of heart mitochondrial inner membrane. He found that the rate of proton leakage was such that, in order to get accurate measurements of H^+ utilization for ATP synthesis (the P/O ratio), he had to measure the proton leakage through the lipid bilayer. He was also motivated by the then recent measurements [8,9] of the proton permeability of vesicular phospholipid bilayers (10^{-5} cm/s), Hinkle made another important observation, that mitochondrial membranes display about the same permeability for K^+ as they do for H^+. He noted that the $[K^+]$ is 10^{-1} M, seven orders of magnitude greater than $[H^+]$, which is about 10^{-7} M. Because measurements [2,3] of lipid bilayers display virtually the same permeabilities (10^{-12}) to Na^+ and K^+, and because that permeability is seven orders of magnitude smaller than the observed H^+ leakage, the leakage of the two cations is approximately the same in plasma membranes. In summary, the lipid bilayer domains of living *plasma* membranes have approximately the same permeabilities to H^+ and Na^+. In all plasma membranes, the electrochemical gradient is so oriented to enhance the leakage of these cations into the cell. Potassium leakage need not be considered since it is on the negative side of the gradient.

There are four factors that affect the leakage rate of the cations, H^+ or Na^+, across lipid bilayers. These are

1. The structures of the lipids in the membrane.
2. The relative concentration of the cations on the two sides the bilayer.
3. The temperature.
4. The electrochemical potential of the membrane, the magnitude and direction of which provide a driving force for leakage in membranes containing it.

This review focuses on a hypothesis that explains how protons leak across lipid bilayers. The hypothesis explains a wide variety of lipid structures including the distinction between the molecular structures of cholesterol and that of the phytosterols. It provides a rational additional role for the ubiquitous occurrence in cells of isopranes and isoprenes, hopanoids and iso/anteiso lipids in bacteria. But perhaps most important, it provides a rational role for the sterol requirements by eukaryotes.

Included in the discussion is the lipid structures and lipid content of those membranes that, according to the above factors would be expected to protect the cell from lost energy. Some of the membranes discussed are resident in high (or low) cation concentrations. Such membranes would be rich in lipids that inhibit cation leaks. The permeability of lipid bilayers to H^+ is discussed in the context of a proposed mechanism for proton leakage. We examine the lipids found in the cell membranes of organisms exposed to extreme of pHs in the context of that model. A change in the cation concentration outside the plasma membrane, a change in the temperature, or a change in the membrane potential, must trigger a signal to regulate those lipids that inhibit the cation leakage. The proposal suggests many experiments. It also suggests why many of the unique and unusual lipids in natural membranes are designed the way they are.

2. Cation (Na^+, H^+) leaks across lipid bilayers

Interest in the permeability of phospholipid bilayers to cations began in the late 1960s and early 1970s as chemically defined bilayer films made such research possible [10]. The classical quantitative measurements of sodium leakage across lipid bilayers were made by Dimitri Papahadjopoulos [2,3]. In 1971 he established the permeability of a wide variety of lipid bilayer vesicles to K^+, Na^+ and other common metal cations found in biological systems. His results have been confirmed by many investigators through the years [11–16]

Papahadjopoulos found that divalent cation leakage is several orders of magnitude lower than is that of the monovalent cations. Additionally, K^+ permeability is slightly greater than Na^+ permeability for anionic (PS or PG) lipid bilayers but not for PC bilayers. Nonetheless, the permeabilities are in a narrow range, $\sim 0.16\times10^{-12}$ cm s^{-1}. Equimolar cholesterol reduces the permeability of PS bilayers to Na^+, to about one third that of cholesterol-free lipids: from 0.16×10^{-12} to 0.05×10^{-12}. Equimolar cholesterol reduces K^+ permeability even more: from 0.91×10^{-12} to 0.047×10^{-12}. Thus, bilayers of these synthetic lipids are three times as permeable to sodium than are those containing cholesterol at the levels typically found in animal plasma membranes. In these studies he also established that there were two biologically relevant factors affecting the rate of cation leakage, the temperature and the concentration of cholesterol in the lipid bilayers. Although the bilayer permeability of K^+ has been examined [7] with consideration of the membrane potential and found to increase with increasing membrane potential, similar in vitro studies for Na^+ permeability are not available. The results of studies on K^+ permeability show significant increases as the membrane potential is increased. This increases the significance of the $\Delta\Psi$ as a cause of important Na^+ leakage at animal plasma membranes.

Studies of Na^+ leakage across rod cell membranes by Bonting et al. [16] showed that the permeability of cholesterol-free bilayers increased with the polyunsaturation of the phospholipids but was unaffected by the headgroup structure. Rod outer cell disk membranes are about 50 mol% docosahexaenoates, the most polynunsaturated chains found in mammals. Na^+ leaks through these membrane vesicles 7–11 times as fast as it does through bilayer vesicles with the same headgroup compositions but containing monounsaturated and saturated chains. They also found that the addition of 10 mol% cholesterol significantly inhibits Na^+ leakage.

Proton leakage became measurable across the bilayers of vesicles in the early 1980s [8,9,17–19]. The permeability is now well established and of the order of magnitude 10^{-5} cm/s. It shows little or no variation with changes in either the headgroups or the fatty acid chainlength [13] or unsaturation [17]. For a review see de Gier [20].

3. Sterols in membranes

Despite nearly a century of intensive research on cholesterol and the phytosterols their role in the plasma membranes of eukaryotes remains a mystery. Most authors, in introductory texts [21], advanced texts [22], research articles [23] and reviews [24] have explained cholesterol's role as affecting membrane rigidity or fluidity, although this has been questioned more recently [25]. Prokaryotes do not need sterols, albeit some contain a likely substitute — the hopanoids [26,27].

Finally, plant and eukaryote sterols have unexplained methyl and ethyl branches and double bonds on the side chain whereas cholesterol does not.

Certain qualities of cholesterol and phytosterols make them unique as lipids. These include: (1) their predominance in the plasma membrane of all eukaryotes, (2) that the deprivation of sterols is lethal to all eukaryote cells, (3) that cholesterol in animals is the one major lipid molecule that is not burned for energy regardless of need, (4) in humans and other mammals there are nearly 100 genes invested in the synthesis, degradation, regulation and transport of cholesterol. There are, for example, 18 enzymatic steps in the pathway from lanosterol to cholesterol [28] in addition to the many receptors, binding proteins and degradative steps in humans. Plants use a different pathway for the phytosterols, which includes cycloartenol in place of lanosterol [29] and is more extensive because of the addition of the side chain double bonds and branches.

Cholesterol is clearly not required for membrane integrity since prokaryotes, the endoplasmic reticulum and the inner membrane of mitochondrion lack the sterol or have only small amounts. Although cholesterol is largely synthesized in the endoplasmic reticulum one may ask, why it is there in only small amounts? An insightful proposal was made by Bretscher [25] who proposed that since the sterol can exchange into the cytosol (its off-rate, $T_{1/2}$, is $\sim$2h at 37°C) [30] then in 2 h about 50% of the plasma membrane cholesterol can diffuse into the cytosol. He thus attributed the location of the cholesterol in the cell to be an "equilibrium" issue. He proposed that it would be least prevalent in membranes rich in unsaturated fatty acids and membrane domains in which the transmembrane domains of the proteins are rich in hydrophobic amino acid residues. He presented evidence that the distribution of cholesterol in the Golgi comprises of a gradient in which the bilayer cholesterol concentration increases from the "*cis*" Golgi progressing toward the "*trans*" Golgi. He notes that this occurs simultaneously with the sphingolipids in the same bilayers. Sphingolipid synthesis begins in the "*cis*" Golgi and continues through the progression to "*trans*." Bretscher also gave evidence that the movement of cholesterol to the palsma membrane might be explained by a comparison of the location of the hydrophobic residues in the transmembrane domains of proteins derived from these two membranes. Thus the transmembrane domains of the 17 plasma membrane proteins had a longer cross-membrane stretch of hydrophobic residues than did the equivalent sample of 15 Golgi membrane proteins. The concept here is that the combination of sphingolipids with their greater saturation, taken together with the amino acid sequences of the proteins in these two membranes could explain the distribution of cholesterol among the organelle membranes and the plasma membrane of animal cells [25].

An early insight into the qualities of sterols in membranes was their remarkable ability to inhibit the flow of water through the membrane bilayer at the mole fraction of sterol that occurred in natural membranes [31–33]. Intensive research during the past 30 years has described the snug fit of the sterols to the aliphatic chains of the membrane lipids [25]. This is often attributed to the stiff region (fused ring domain) of the sterol which is oriented between the headgroup and $\Delta 9$ of the lipid chains which presumably squeezes out water.

In phospholipid bilayers, cholesterol reduces the passive water permeability in direct proportion to the level of cholesterol in the membrane [32]. At 50 mol% it eliminates passive glucose permeability in phosphatidyl choline bilayers [34]. It is the most effective of several sterols tested for reducing passive permeability through lipid bilayers. The constraints placed upon the motions of the fatty acid chains [35] suggest that an effect of cholesterol is to induce a tight lateral packing of the phospholipids as well as the sterol itself [36]. The observation is also consistent with the condensing

effect (the total area occupied by cholesterol plus phospholipids is less that the sum of the area occupied by each).

Over 80% of the sterols in yeast reside in the plasma membrane [37]. Approximately the same has been established for cultured fibroblasts [38]. Orci, Perrelet and coworkers [39–45] have used a freeze-fracture procedure for visualizing the distribution of cholesterol in a membrane by its filipin-cholesterol-produced pits. The technique has been used successfully to show that the cholesterol synthesized in the endoplasmic reticulum which contains 10–12 mol% of the cell's cholesterol, appears in increasing concentration as it proceeds through the Golgi stacks to the *trans*-Golgi, where the sphingolipids are synthesized. Its concentration is highest there and in the plasma membrane. This has been confirmed by Lange and others [46–48]. Furthermore, the pathway for ergosterol synthesis through the Golgi is similar in yeast [49].

There has been much research on the subject of whether the cholesterol in the plasma membrane is largely in the outer monolayer or if it is equally distributed between both monolayers. This has been difficult experimentally because cholesterol, unlike the phospholipids, flip-flops very rapidly from one monolayer in a bilayer to the other monolayer. Nes and coworkers [50] have explored the chemistry of many phytosterols and other sterols examining the parameters of the active sterol and its constraints in various biological sources. In this way they and others have established the minimal structural requirements for sterols in maintaining an efficient role in membranes. It must have a flat structure on the α-side of the sterol nucleus and the two methyls on its β-side [51]. This permits a snug fit of the sterol with one fatty acid chain facing the flat side and two chains on either side of the methyl groups on the β-side in bilayers of chain lipids. This triangular relationship of three chains to each cholesterol molecule is best demonstrated by the "condensing effect" in which a monolayer of cholesterol and phospholipid together occupies less monolayer area than would be expected by the sum of each. One approach has been to determine which lipids have a greater steady state affinity for cholesterol. Such measurements are conducted by exposing vesicles constituted of phospholipids or sphingolipids to cholesterol to measure the mole fraction of cholesterol in the steady state in the two vesicle preparation. Sphingolipids show a greater affinity for the sterol than do phospholipids. This is particularly apparent in the formation of rafts and caviolae. For a review see Barenholz and Thompson [52]. There is a specific affinity of cholesterol for sphingolipids in Niemann–Pick disease [53] and in spin labeled studies on their interactions [47]. Furthermore the biosynthesis of the sphingolipids is coordinated with the biosynthesis of cholesterol [48]. Cholesterol has a preference for saturated or at most monounsaturated chain lipids in contrast to polyunsaturated chain lipids in bilayers. Sphingolipids are saturated or monounsaturated and entirely on the outer monolayer of plasma membranes. Possibly another role for the structure of the sphingolipid chains is to sequester sterols into the outer monolayer of plasma membranes. Sterol binding proteins facilitate the dispersion of sterols to the membranes of the cell. The outer membrane of mammalian mitochondria contains cholesterol but the inner membrane does not. The inner membrane is rich in polyunsaturated phospholipid chains whereas the outer is rich in monounsaturated and saturated chains.

Many studies have been made on protein/cholesterol interactions in membranes [34]. Cholesterol has been shown to bind tightly to a variety of transmembrane membrane proteins. These include glycophorin [54] and band 3 [55,56], from the human red cell membrane [57]. Cholesterol seems to have binding affinity to and to affect the activity of proteins that are found in the plasma

membrane of eukaryote cells but not on proteins that are found in the intracellular membranes that lack cholesterol as a lipid component (Fig. 1).

Inhibition of cholesterol synthesis in vivo stimulates the Na+, K+ ATPase in rat brain [58] and in human erythrocytes and platelets where pravastitin was administered [59]. This is consistent with the present model in that reduced cholesterol in the membrane would be expected to increase the sodium concentration in the cell, an event that would stimulate the pump. However, high levels of cholesterol, above that found in the membranes, inhibit the Na+, K+ ATPase in reconstituted systems [60] and in the erythrocyte membranes [61]. These results are demonstrated by the data on bovine kidney membranes in Fig. 1a [62]. Levels of cholesterol below that found in membranes (<25–30 mol%) inhibits the Na+ pump but as the mol fraction of the cholesterol passes 35–40 mol% found in plasma membranes, the pump is inhibited. The stimulation of the pump at low levels in vitro might be explained by the fact that the Na^+, K^+ ATPase has evolved in animals with 30 mol% cholesterol in the membrane. Presumably that would stabilize the protein in its functional form. This notion is supported by the observation that the phytosterols, ergosterol and stigmasterol (and epicholesterol) at low levels have no stimulatory effect on the muscle plasma membrane Na^+, K^+ ATPase in vitro nor on the Na^+, Ca^{++} exchange protein, whereas 7-dehydrocholesterol, cholesterol and cholesterol did [63]. They also found that the Ca^{++} ATPase of the sarcoplasmic reticulum, a membrane that lacks cholesterol in vivo, was unaffected by any of the sterols. Additional data indicates that in the rabbit sarcoplasmic reticulum [64] cholesterol does not interact directly with the Ca^{++}ATPase [65]. The sterol is ineffective at modulating the activity of this enzyme [66]. These data show that the enzymes that are found in

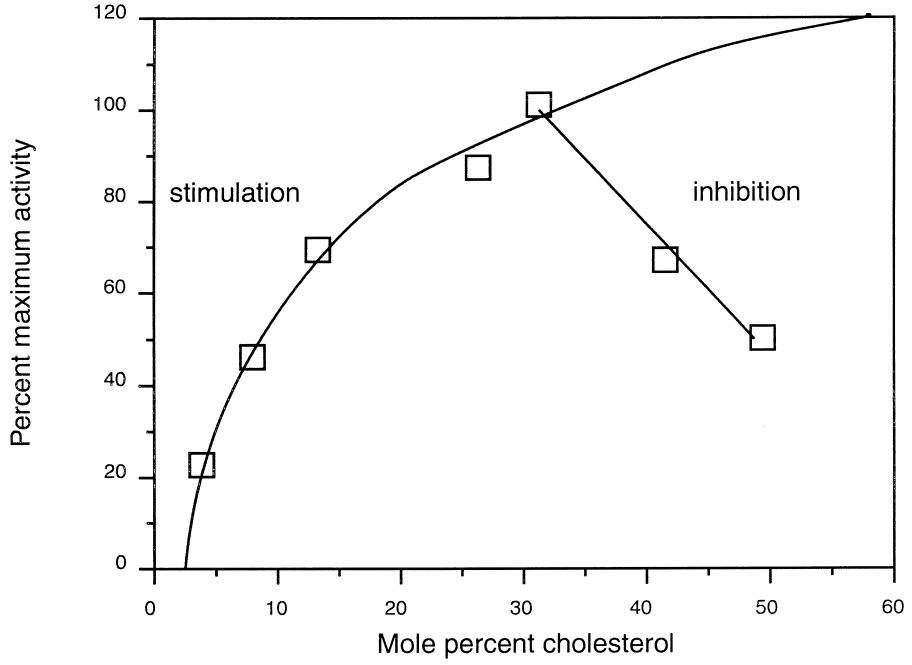


Fig. 1. (a) Effect of membrane cholesterol content on the enzymatic activity of the Na^+, K^+ ATPase in bovine kidney membrane vesicles. Cholesterol content was adjusted by incubation of the native membrane vesicles with bilayer lipid vesicles, with or without cholesterol [34].

membranes that contain cholesterol accommodate the sterol and visa versa. It is not surprising that the cooperativity of transmembrane proteins and lipids of a membrane protein is maximal when the bilayer lipids reflect its natural environment.

The inhibition at high levels of cholesterol can be rationalized by noting that the Na^+, K + ATPase is known to be stimulated by Na^+ on its cytosol face. As van Deenen has shown [35], passive transport through the lipids of bilayers of glucose, glycerol and Rb^+ is effectively halted at 50 mol% cholesterol in the bilayer. Thus, as cholesterol inhibits the leakage of sodium, the pumping is reduced.

It is unlikely that these small differences in enzymatic and/or transport activity can be ascribed to a biological role for cholesterol. They cannot explain why the absence of cholesterol is lethal for all animal cells.

3.1. Cholesterol and sodium

Living cells concentrate K^+ into their cytosol ($\sim$0.1M). Plants and prokaryotes have a wide variety of mechanisms to deal with osmolality, but for animals it is balanced with Na^+. Potassium is required for many cytosolic, e.g. glycolytic enzymes. Eukaryote cells, except for animal cells, contain a P-type H^+, ATPase, which establishes a proton gradient across the plasma membrane. The proton gradient results in a very large $\Delta\Psi$ (200–250 mv) which is used primarily for the uptake of cations and for the proton-associated co-transport of nutrients. In contrast, the $\Delta\Psi$ across the membranes of animals is generally well below 180 mv. The ion gradient and $\Delta\Psi$ in animal cells is maintained by the Na^+, K^+ ATPase which pumps a maximum of 3 Na^+ out and 2 K^+ in for each ATP consumed. In blood, the ratio of Na^+ to K^+ is more than 30:1; the exterior [Na^+] is 0.14 M [67]. Few cations are required to discharge a $\Delta\Psi$ [1] so that cations penetrating the lipid bilayer, driven by the $\Delta\Psi$, would dissipate the energy of the membrane potential.

3.2. Erythrocytes, cholesterol and sodium, a calculation

Red cells maintain a membrane potential of 10 mv, outside positive across their plasma membrane. The 10 mv potential is adjusted downward when the [Na^+] outside gets sufficiently in excess of 10 mv by leakage through K^+ channels (rectifiers) from inside the cell outward.

According to the measurements of Papahadjopoulos the leakage rate of both cations is about 10^{-11} or 10^{-12} mol- cm/s. Since the Na^+/K^+ ratio is 9:1 and since the positive side of the potential is on the outside of the cell, K^+ will not leak out of the cell through the lipids to any significent extent whereas Na^+ will leak into the cell. The ektolayer side of the membrane has a high density of negative charges; due to both the lipid headgroups and the ektolayer domains of the transmembrane proteins. This is important because of the Gouy–Chapman effect in which the bulk phase cations, largely sodium, are selectively concentrated close to, within 1.0 nm of, the membrane surface.

For the red blood cell, the total surface area is 10 μ^2 (10^{-4}cm^2). If one multiplies the 10^{-7} cm/s by 10^{-4} moles/cm^3 then the result is 10^{-11} moles/cm/s, the number of moles leaked into the cell. If this is divided by Avogadro's number (10^{-23}) then there are a total number of 10^{-12} sodium ions/cell/s leaked into the cell. We can assume that the bilayer is a capacitor and the charge on a sodium ion is 1.6×10^{-19} coulombs. $Q = c \times v$. Q coulombs $= 10^{-6}$ farads $\times 10^{-2}$ volts (across the

bilayer), therefore $Q = 10^{-8}$ coulombs/cm^2. Divide this by 10^{-4} cm^2 area/cell, thus it takes $\sim 10^5$ ions to discharge the cell's membrane potential.

Compare this to what the cell must expend in ATP to keep sodium out of the cell. The red cell contains $\sim$1000 copies of the Na^+, K^+ ATPase. Each pumps out one charge (3 Na^+ less 2 K^+) per ms. Thus 10^6 net ions are pumped out of the cell per second. Since approximately 10^{-12} ions leak inward and 10^6 ions are pumped out then there is a deficit of 10^6 ions/s. There appears not to be enough ATPase activity to maintain the 10 mv potential.

Although direct measurements of Na^+ leakage have not been made on the erythrocyte, it is clear that the leakage rate of sodium is substantial by the considerations above. Cholesterol's inhibition of it must surely save energy (ATP) for the cell. Saving energy in each animal cell is a useful explanation as to why the absence of cholesterol is lethal to the cell.

3.3. The cholesterol/sodium connection in animal membranes

In animals the cholesterol content of membranes has some correlation with extracellular [Na^+]. For example, in intestinal epithelial cells, cholesterol occurs in higher concentration at the basolateral membranes facing blood (>0.14 M $Na+$) than it does at the brush border membranes [<50 mM (Na^+)] [68,69]. The reverse is observed in renal epithelial cells [70] where the basolateral membrane is facing blood but the apical or bush border membrane is facing a slightly higher concentration of sodium to be excreted with the urine. The tight junctions of these cells act as a barrier preventing lateral diffusion of cholesterol between membrane domains [71]. This suggests that the concentration of cholesterol in membrane lipids may be regulated by the Na^+ concentration facing the membrane.

Cholesterol is excluded from the plasma membrane at gap junctions [72] where the [Na^+] is low. In the podocytes from kidney epithelial cells, the plasma membrane domain lacks cholesterol where it contacts the basal lamina, which lacks a Na^+, K^+ gradient [42]. The membrane domains of the same cells, in contact with the Na^+ K^+ gradient, are rich in cholesterol. Rod outer segment discs of the eye are made from plasma membranes near the inner segment. The discs are created as infoldings of the plasma membrane that mature into discs [73,74]. As they mature into intracellular membranes and are no longer in contact with sodium, they rapidly become depleted of cholesterol [75]. Here again the plasma membrane, rich in cholesterol, faces a Na^+, K^+ gradient but the internal disk does not.

When the protozoan *Crithidia fasciculata* is cultured on defined medium, with low levels of sodium, its membranes contain [76] the phytosterols typical of protozoa/algae. Under these circumstances the organism utilizes a proton gradient across its plasma membrane. Upon the addition of blood, a sodium gradient appears and the sterol composition shifts to include cholesterol as the major sterol. A similar result was obtained with *Trypanosoma cruzi* [77] wherein cholesterol and a sodium gradient replaced the protozoan sterols and the proton gradient when cultured in blood. In these examples the important consideration is not whether or not the cells synthesized the cholesterol (this was not measured) but that the sterol was incorporated into the membrane. The difference, albeit not the only difference, is that the cells selectively incorporated or synthesized phytosterols in the low sodium media and cholesterol in the high sodium media. The contrast is shown by experiments on yeast ergosterol auxotrophes, which can function with cholestanol, although they need small amounts of ergosterol [78]. If ergosterol is provided in the medium they

will take it up in exchange for the cholestanol. Yeast have a proton, not sodium, gradient across their plasma membrane.

The connection between cholesterol and sodium has an interesting implication for the regulation of cholesterol biosynthesis. The bilayer concentration of cholesterol increases within the Golgi from the "*cis*" through the "*trans*" domains to the plasma membrane. This was explained by Bretscher to be due to the accommodation of cholesterol by the sphingolipids and the sequences in the transmembrane proteins of the two membranes [25]. From the point of view of the sodium/cholesterol connection, one would expect that the biosynthesis of the sphingolipids would be somewhat regulated by sodium in the cytosol.

4. Protons leak across lipid bilayers

Experimental evidence that protons leak across simple lipid bilayers [8,9] at rates that are biologically relevant has been confirmed by many laboratories. Two molecular models have been proposed for proton leaks across lipid bilayers: the "defect" mechanism and the "water wire" mechanism. A third, the "cluster" mechanism will be proposed herein.

According to the "defect" mechanism [79] protons permeate bilayers via defects, or transient pores, as is widely presumed for monovalent metal cations. The pattern of the rate of proton permeability through membranes with increasing chain length lipids is distinctly different from that of monovalent cations such as Na^+ and K^+. A comparison of the kinetics [13] of metal cation leakage to that of protons showed that, if the defect model applied to metallic cations, proton permeation had a different mechanism.

Describing water diffusion through bilayers, Nagle and Morowitz [80] proposed a "water wire" mechanism. The model was later modified to explain proton leaks as a "proton wire" or a "hydrogen bonded chain" of water molecules [81,82]. More recently, support for a "water wire" mechanism has been derived from simulations and calculations [83]. A water wire has been found in the X-ray-derived structure of the photoreaction center of *Rhodobacter spheroides* wherein the "wire" is 14 water molecules in length [84]. The wire is, of course, in the protein crystal rather than in a bilayer above the Tm. Evidence against the water wire has been found by Krishnamoorthy [85] who measured the water in the bilayer using probes. The study also showed that cholesterol inhibited the leakage of protons across the bilayer.

The amount of water in the hydrophobic domain of the bilayer has been examined by Finkelstein [31] who compared the solubility of water in bilayers to its solubility in oil. His evidence suggests that water moves through bilayers by diffusion. Trauble [86] offered a molecular description of the diffusion model. He suggested that individual water molecules nested between the *g-t-g* kinks of lipid hydrocarbon chains as described by Flory [87] for polymers. Haines and Liebovitch [33,88] used these concepts to elaborate a detailed molecular model of water diffusion. Using a random walk calculation they showed that the measured lateral diffusion of the bilayer lipids could be used to predict the observed water diffusion across the bilayer. Because lipid head groups jump laterally at 10^6 s^{-1}, whereas the chain motions are 10^9 s^{-1}, their model predicted that the rate of water diffusion through a bilayer must be limited by the rate of the head group jump regardless of the lipid chain length or the bilayer thickness. These models describe diffusion as a mechanism that on the one hand gives water molecules freedom of motion within the low

dielectric but also assumes they are largely separated from each other. The mechanism received support from the experiments of Paula et al. [13], who measured water and cation diffusion across phosphatidylcholine preparations with chains containing from 14 to 24 carbons, each containing $\Delta 9$ double bonds to stabilize the liquid crystal state. The water diffusion was nearly identical for all of the bilayers, whereas the metal cation permeability decreased exponentially with increases in the bilayer thickness. This illustrates that the chainlength of the bilayer lipids (thickness of the bilayer) does not affect the water diffusion although it affected the rate of proton leakage.

4.1. The "cluster-contact" mechanism for proton leaks

What follows might be referred to as the "cluster contact" hypothesis for proton leakage. It is based on a few assumptions:

1. Water in the low dielectric forms small clusters due to hydrogen bonding in bilayers above the Tm.
2. Clusters may be protonated or deprotonated, in which case the cluster is stabilized by resonance of the charge within the cluster (Fig. 2).
3. The contact of a charged cluster in one monolayer with the oppositely charge cluster in the other monolayer constitutes a transfer of a proton across the lipid bilayer.

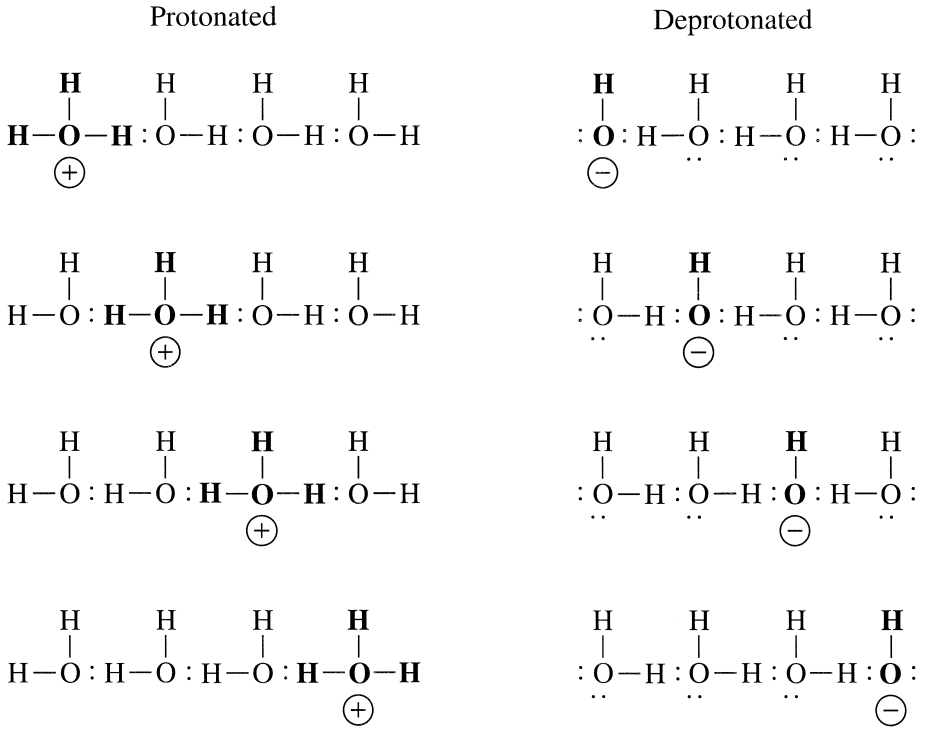


Fig. 2. Illustration of protonated (left) or deprotonated (right) clusters of water molecules. The resonance disperses the charge stabilizing the complex in the low dielectric. The charge also decreases the bondlength of the H-bonds while it increases their energy.

Despite the low level of water solubility in oil, water molecules in lipid bilayers of straight-chain lipids above the T_m may be expected to form clusters. This is because there is an energy balance between the enthalpy of hydrogen bonding and the entropy of diffusion of individual molecules. Crude calculations suggest there are up to 3 or 4 water molecules per cluster. The lowest energy is expended if the clusters are strings along the chains rather than clumps. Linear clusters will least disturb the chain motions and conformations.

It is well established that charges in a low dielectric medium are most stable if the charge is distributed by resonance. A classical example of that is dinitrophenol, the oxidative phosphorylation uncoupler, but there are many examples of it today. Thus a protonated cluster of water molecules is most stable if the positive charge resonates among the several oxygens of the cluster (Fig. 2). A deprotonated cluster would likewise be stabilized by resonance. The enthalpy of the H-bond would be enhanced by the resonant/charged cluster so that charged complexes may be larger than uncharged water clusters. Each monolayer of the bilayer would be expected to contain a ratio of the plus and minus charged water clusters, reflecting the pH of the water bathing that monolayer (Fig. 3).

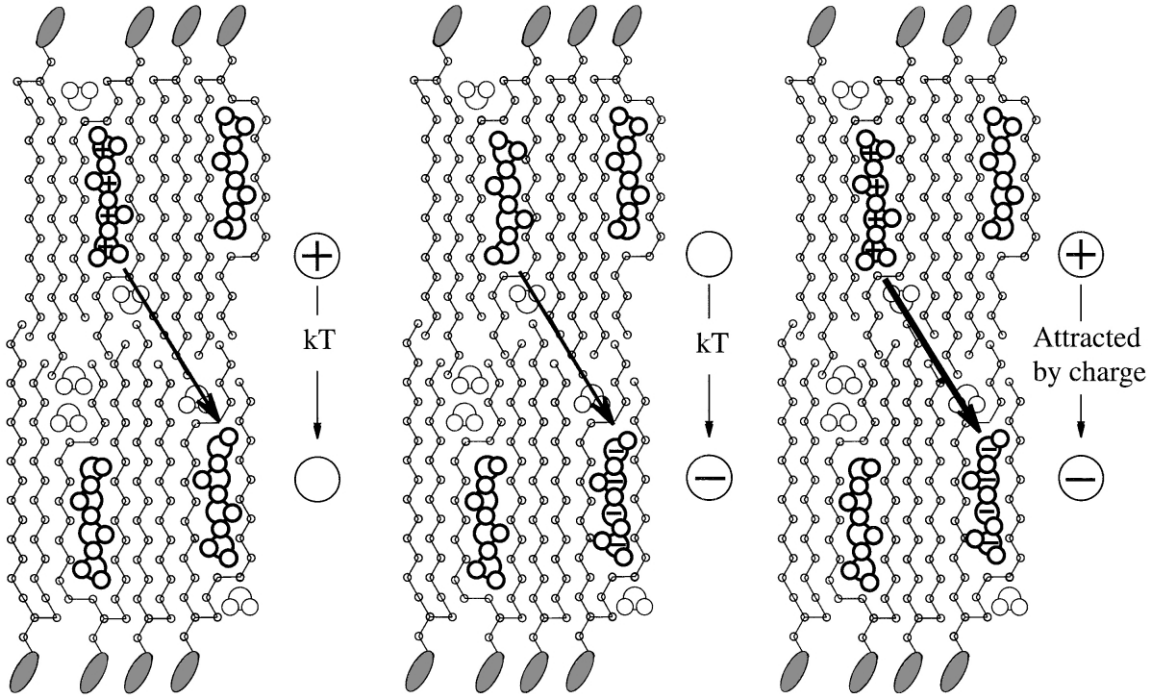


Fig. 3. Three mechanisms for transbilayer proton translocation. In the first two diagrams the string moves from one monolayer to the other by kinetic motion (kT). In the third diagram a protonated (positive) cluster is attracted to a deprotonated (negative) cluster across the bilayer center. In the low dielectric, a field can attract over a considerable distance. The model assumes the ratio of postively and negatively charged clusters in each monolayer reflects the pH in the water facing that monolayer. It predicts that the ΔpH across the bilayer, regardless of the absolute pH range, determines the rate of proton leaks.

In the low dielectric, the fields from such charged clusters would extend over a considerable distance (Fig. 3). Furthermore, the attractive force between them would result in collisions that transfer a proton from the positive (protonated) species to the negative (deprotonated) species (Fig. 3c). The kinetics of the reaction is favored by the field attraction between the two ions. The thermodynamics of the reaction are favored by the disappearance of charge in the low dielectric. Where such collisions occur between two charged clusters on the same side of the bilayer, there would be no observable effect. Where the collisions occur between a protonated cluster in one monolayer of a bilayer and a deprotonated cluster on the opposite monolayer, a proton is transferred from one side of the membrane to the other. Should there be excess hydrocarbon in the center of the bilayer the transbilayer leakage of protons would be inhibited (Fig. 4).

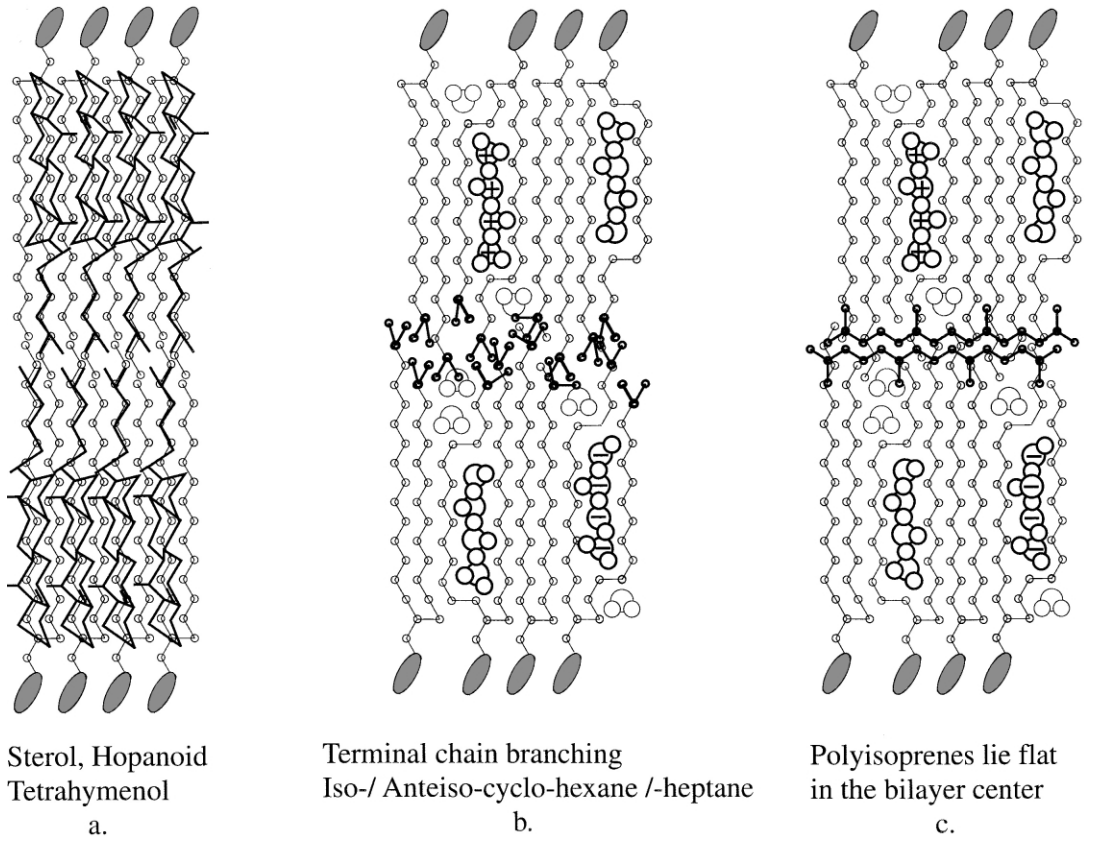


Fig. 4. Three experimental approaches for examining the hypothesis. The model assumes that cross bilayer cluster collisions can be inhibited by hydrocarbon that stuffs the center of the bilayer. There are three obvious ways to block contact between oppositely charged strings: (a) inhibit cluster forming by preventing the water from entering the bilayer close to the aqueous solution (sterols, hopanoids or tetrahymanol); (b) branch the ends of the lipid chains, sterically crowding the bilayer center (iso-/anteiso-lipid chains, cyclohexane/cycloheptane or plant sterols) or (c) place hydrocarbons in the center of the bilayer that are parallel to its plane (isoprenes: squalene, ubiquinone, plastoquinone, or dolichol).

Whether or not the details of the model are correct, it suggests several types of experiments to test it. Three experimental protocols could inhibit the movement of a proton across the center of the bilayer (Fig. 4). The first is to decrease the amount of water from entering the bilayer to form clusters. Sterols inhibit water transfer across bilayers that contain them [31]. Thus the sterol-like lipids such as phytosterols, tetrahymanol, or hopanoids would be expected to inhibit proton leaks (Fig. 4a).

Another experimental approach is to pack the center of the bilayer with hydrocarbon molecules. This would inhibit the oppositely charged clusters from colliding across the bilayer. In this experiment the hydrocarbon must be shown to reside in the center of the bilayer between the two monolayers. Two approaches are apparent: (1) branch the ends of the lipid chains of the bilayer lipids with methyl or ethyl groups (Fig. 4b), or (2) obtain a hydrocarbon that would selectively reside in the cleavage plane of the bilayer (Fig. 4c).

For the first experimental approach, the most appropriate chains would be the *iso-*/*anteiso*-lipids of Gram positive bacteria. These have methyl groups on the terminal ends of their chains: either ω-1 or ω-2. One would expect the branches to pack the center of the bilayer (cleavage plane) and inhibit the colliding of clusters from each of the two monolayers.

The second approach is to dope the bilayer with hydrocarbon isoprenes. With their multiple branches they would reside in the center of the bilayer rather than disturb the cooperativity of straight chain lipids in each monolayer. Squalene is a prime example.

4.2. Prokaryotes, prokaryote-like organelles and proton leaks

If protons leak through bioenergetic membranes, the energy stored in the proton gradient is dissipated. Prokaryotes and the "prokaryote-like" organelles, the mitochondrion and the chloroplast, use proton electrochemical gradients as energy transducers. The gradients may be maintained by redox and/or photon-driven pumps, or by the F_0,F_1-ATPase. Protons have been shown to leak through membranes engaged in oxidative phosphorylation in prokaryotes [89], and in mitochondria [7] and are sensitive to the membrane potential ($\Delta\Psi$).

Hinkle et al. [90] measured the energy lost due to the leakage of protons across the inner membrane of mitochondria during active oxidative phosphorylation. They found that the energy loss due to proton leakage is sensitive and proportional to the $\Delta\Psi$. Leaked protons were only 5–10% of the total $H+$ pumped, the remainder being used for ATP synthesis. These conclusions were confirmed and expanded by Brown and Brand [91] who found that the proton leakage through the mitochondrial membranes in hepatic cells [92] could be correlated with the body weight in mammals [93]. Their data imply that there are many biological uses made of the slips and leaks of the proton motive force in membranes that synthesize ATP [94]. The present proposal suggests that one way a cell can control the leakage of protons is to alter the mole fraction of ubiquinone in the inner membrane.

4.3. Chain lipids with branches at the enter of the bilayer

Blocking proton leaks is critical for acidophilic and alkaliphilic prokaryotes. These organisms, each in its own way, must maintain the proton motive force so that it can be used for ATP synthesis despite their hostile environment. One way to do this is to include lipids in their plasma

membranes that would inhibit such leaks. Experimentally blocking proton leaks by hydrocarbon in the bilayer center suggested the use of vesicles made of *iso*-/*anteiso*- lipids (Fig. 5a). These lipids are synthesized by elongating the fatty acid chain with acetate after starting the chain with the branched amino acids, valine, isoleucine [95,96] and also cyclohexane or cycloheptane. Details and references are available in an exhaustive review by Kaneda [97] who has described the phylogenetic distribution and content of *iso*-/*anteiso*- lipids. They appear to occur in all of the prokaryote acidophiles (except for the archaebacteria) and alkaliphiles that have straight-chain lipids. They also appear in organisms that are not in extreme environments, however they are dominant in the most extreme thermophilic acidophiles such as *Bacillus acidocaldarius* [98] and *Bacillus acidoterrestris* [99]. Further crowding the center of the bilayer with hydrocarbon, the lipids of thermoacidophiles sometimes terminate the ends of the otherwise straight chains with the alicyclics, cyclohexane and/or cycloheptane [100]. *Bacillus stearothermophilus* lipids were compared in cultures grown at 45°C and 65°C [7]. At the higher temperature the cultures increased the fraction of ante-iso lipid chains, and thereby increased the crowding in the bilayer center. *Iso*-/*anteiso*- chains have other roles including adjusting the T_m [101] at various temperatures.

Likewise alkaliphile membrane lipids are predominantly *iso*-/*anteiso*- chains [102–104]. A comparison of *Bacillus subtilis* lipids to those of two obligate alkaliphiles and two facultative alkaliphiles grown at pH 7.5 and 10.5 showed distinct lipid differences [103] that were related to the pH of the culture medium. All of the cultures, including *B. subtilis*, were rich in *iso*-/*anteiso*- chains. However only the organisms that could grow at high pH had squalene (Fig. 5b) and structurally similar but unidentified C40-, C50-isoprenoids [102]. The total isoprenoid consisted of $\sim$10% of the membrane lipids and increased to $\sim$15% as the pH of the medium was increased. Squalene, an extended hydrocarbon with methyls and isolated double bonds would be expected to reside in the cleavage plane of the bilayer. According to the present proposal, the alkaliphiles use both branched chains and a blocking hydrocarbon in the center of the bilayer [102]. The possible use of squalene to block proton leakage in prokaryotes is especially interesting because it is a key intermediate in the biosynthesis the sterols and sterol-like molecules. If these lipids block proton and sodium leakage their evolution is comprehensible.

4.4. Hopanoids and proton leaks

Hopanoids and tetrahymanol (Fig. 5) are made anaerobically from squalene. They have multiring systems and, like sterols, they pack the fused ring system in the domain of each monolayer close to the headgroups. These pentacyclic triterpenoids, discovered in the 1970s, are found primarily in prokaryotes and a few plants. They occur almost exclusively in membranes which contain large proton gradients [26,27]. The concentrations of hopanoids are greatest in acidophiles (*Acetobacter aceti*, *Acetobacter pasteurianus*, *Rhodopseudomonas acidophila*), and in organisms grown in $<$15% ethanol (*Zymomonas mobilis*, *Lactobacillus heterohiochii*). They are also found, however, in Cyanobacteria, Rhodospirillaceae, nitrogen-fixing bacteria and in obligate Methylotrophs. Lower levels of hopanoids are found in Gram positive acid bacteria that contain *iso*-/*anteiso*- chains, *B. acidocaldarius* and *B. acidoterrestris*. Evidence that they have a role inhibiting proton leaks is suggested by the observation that the hopanoid fraction of the membrane lipids of *B. acidocaldarius* increases in response to either a decrease of the pH to 3.0 or an increase of temperature [105].

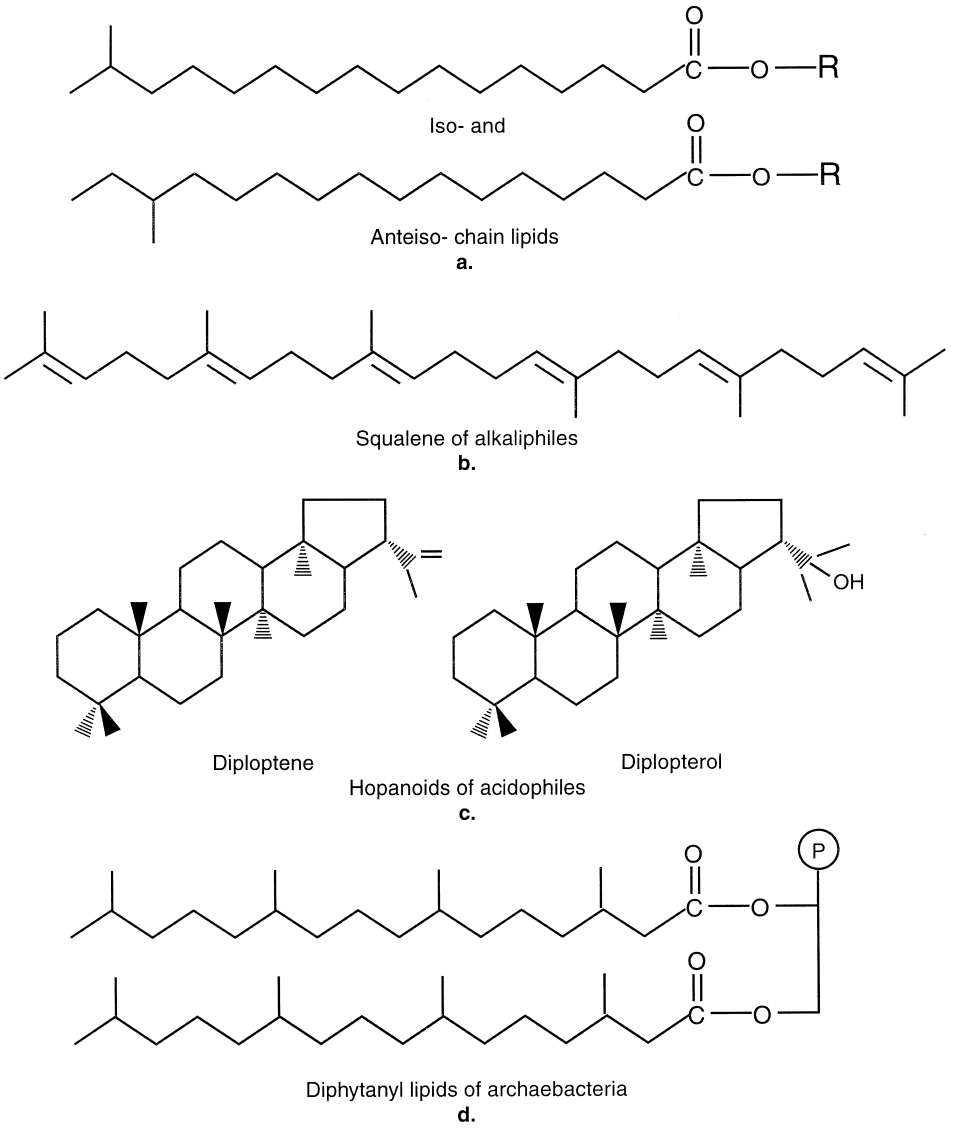


Fig. 5. Prokaryote lipids proposed to inhibit proton leaks across the bacterial membranes of acidophiles and alkaliphiles. (a) The *iso-*/*anteiso-* lipids are in both acidophiles and alkaliphiles as well as in many organisms that grow in the neutral pH range [97]. (b) Squalene, an isoprene that is found in alkaliphiles in addition to the *iso-*/*anteiso-* phospholipids. Its concentration in the membrane increases as the pH of the culture medium is increased to pH 9+ where it is 15% of the total lipids [120]. (c) Hopanoids are almost exclusively prokaryote products synthesized anaerobically by a bacterial squalene cyclase. These pentacyclic hydrocarbons are based on the two C_{30} skeletons, diploptene and diplopterol generally with a polyol sidechain. (d) Diphytanyl phospholipids are found exclusively in archaebacteria, some of which are found in extremely acidic environments [27]. They appear to have different molecular dynamics in lipid bilayers. They exhibit neither the lateral diffusion nor the proton leakage of chain lipids.

4.5. The diphytanyl chains of archaebacteria

Archaebacterial lipids are not straight chains but diphytanyl (isoprane) chains. The molecular dynamics of these lipids are radically different from those of straight-chain lipids as demonstrated by proton NMR [106]. The methyl hydrogens up and down the chain move rapidly whereas those on the methylenes that connect them move very slowly. The Haines–Liebovitch–Trauble model [33,88] for water transport for straight-chain lipid bilayers demonstrates in a calculation that the lateral mobility of chain lipids in a bilayer is locked to water transport across the bilayer for straight–chain lipids. In developing the model it was apparent that each water molecule that enters and leaves the low dielectric is accompanied by a lateral jump of the headgroup, which lateral jump is the rate-limiting step for water transport. An examination of the dynamics of the diphytanyl lipids implies that the water crosses archaebacterial membranes as isolated water molecules as they progress across the bilayer. Each water molecule is separated from the other by the methyl branches of the chains. This model predicted that such lipids would not demonstrate lateral mobility, a prediction independently demonstrated by Yamauchi et al. [107]. Water clusters are unlikely to form in these bilayers suggesting that proton leakage of diphytanyl bilayers is significantly lower than that of the straight-chain lipids. This suggests low levels of proton leaks will be observed in these membranes.

4.6. Phytosterols and proton leaks

Plant and microbial eukaryotes have a plasma membrane proton motive force (pmf) that consists of a higher exterior [H^+] (as much as 3–4 pH units below the pH 7 of the cytosol) and a large $\Delta\Psi$ (>200 mv). It arises from $H+$ pumped by a plasma membrane P-type H^+-ATPase using metabolic, mitochondrial or chloroplast-derived ATP. The very large $\Delta\Psi$ represents a strong driving force for the inward leakage of H^+ through the membrane lipids. Sterols with branches on their side chains are only found in the plasma membranes of organisms that use the $H+$ gradient in their plasma membranes. In addition to the ethyl or methyl group appended to the C24 of the sterol side chain, such sterols often have a double bond at C22. In their studies of the crystal structure of cholesterol, Duax et al. [108] noted considerable conformational flexibility in the side chain. *Gauche* conformers, which bring the terminal dimethyl away from the center of the bilayer (shortening the molecule), were found at each bond except for the one that is exo to the D ring (Fig. 6). In contrast, branched sterols have an ethyl or methyl group at C24 and often an *trans*-double bond at C22. The *trans*-double bond at C22 would stiffen the tail, thrusting the C24 branch and the terminal hydrocarbon *gem* dimethyl into the center of the bilayer. According to the cluster contact model, the branched sterol (phytosterol) inhibits proton leakage by two mechanisms: (1) the sterol ring system in the "stiff" region of the bilayer would inhibit water from entering the bilayer and therefore the formation of water clusters and (2) packing the center of the bilayer with hydrocarbon (methyl groups) would inhibit proton leaks by mechanically interfering with contact across the center of the bilayer.

Yeast, for example, can grow anaerobically on exogenous cholesterol if provided a small amount of ergosterol [109]. Sterol synthesis requires oxygen which, if provided the yeast, would replace the cholesterol with ergosterol. Yeast synthesize the ergosterol at considerable (energy) expense, refusing exogenous cholesterol. The role of ergosterol in yeast is not satisfied by cholesterol.

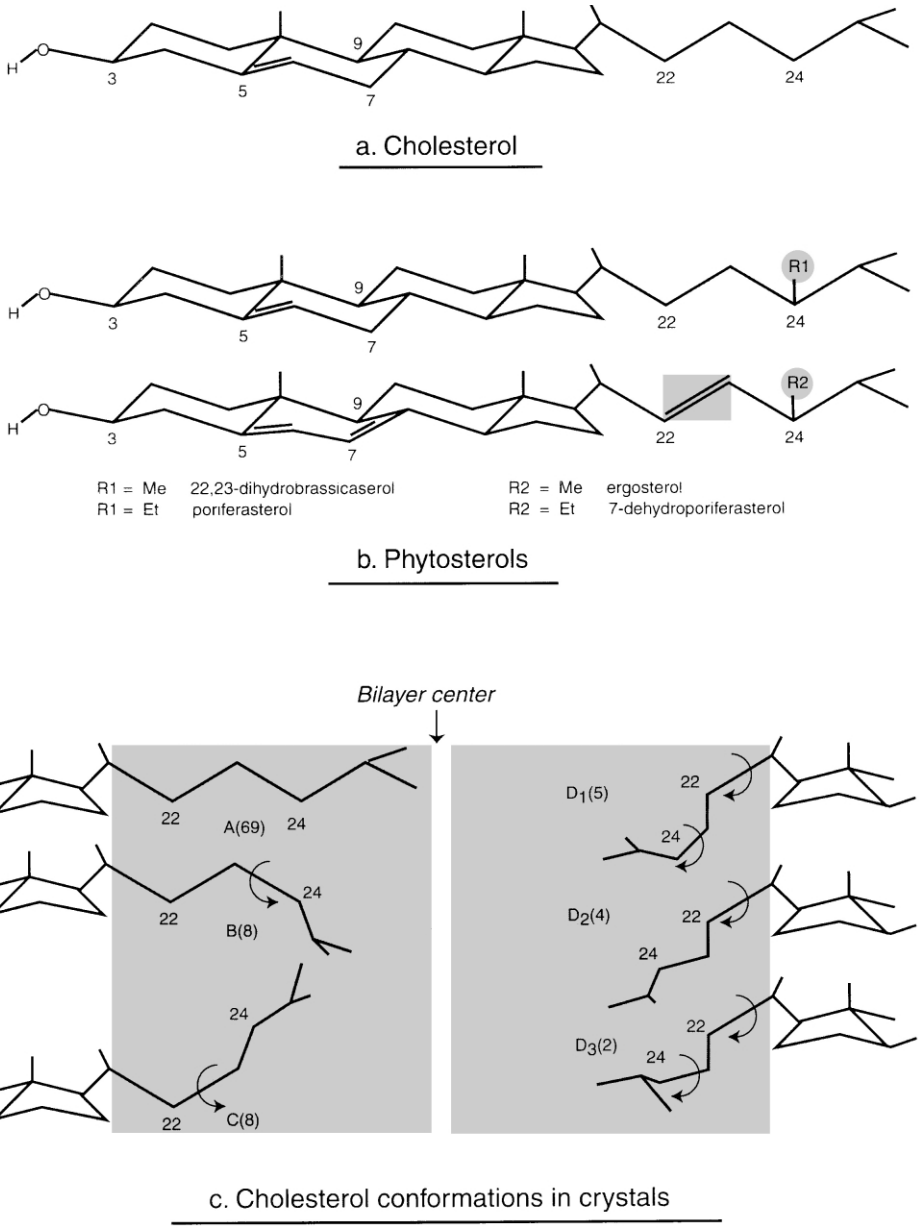


Fig. 6. (a) Structure of cholesterol which is found in membranes that have a sodium gradient and a membrane potential. (b) Some typical phytosterols which are present in eukaryote membranes that contain a proton motive force. They have a variety of structures always displaying a methyl or ethyl branch at C24. They may or may not have a *trans* double bond at C22. The double bond ensures that the hydrocarbon branches are inserted toward the center of the bilayer. (c) Conformations of cholesterol side-chain observed in crystal structures [108]. The numbers in the parentheses indicates the number of crystals for each side chain conformer. Curved arrows represent *gauche* conformations which contrast with the all-*trans* conformation of cholesterol. Each cholesterol comformer (B-D_3) effectively draws the terminal *gem* dimethyl away from the bilayer center. The C22 double bond in some phytosterols inhibits these *gauche* conformers from forming. The phytosterols appear to be designed to thrust hydrocarbon into the center of the bilayer.

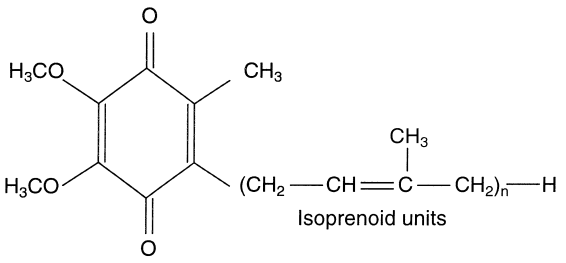
The two sterols presumably have the same role in the eukaryote. Here, it is suggested that the structure/function difference is that the yeast has a proton gradient across its plasma membrane whereas the animal cell has a sodium gradient. Insect herbivores, as they consume plant sterols, convert them to cholesterol. Here the side chains of the phytosterols are demethylated as they are incorporated into the insect, which has a sodium-rich lymph [110].

4.7. The eukaryote isoprenes as proton plugs

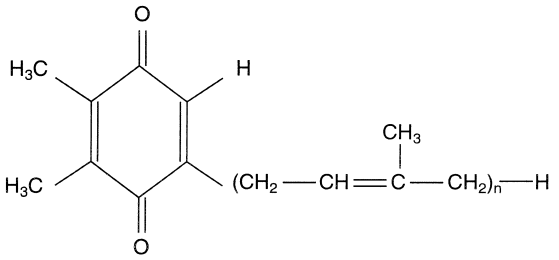
Eukaryotes contain surprisingly high levels of isoprene hydrocarbons in their organelle membranes. Each of these membranes contains a proton gradient across the membrane containing the isoprene. The most dominant eukaryote isoprenes (Fig. 7) are dolichols, ubiquinone, plastoquinone and very low quantities of vitamin cofactors such as Vitamin E and Vitamin A. In the energy-associated organelles, the mitochondrion (ubiquinone) and the chloroplast (plastoquinone), its isoprenes are present in very high concentrations compared to the amount needed for conducting the redox reactions for which they have been well described. The principal activity of the mitochondrion is the synthesis of ATP through its proton gradient across the inner membrane. The inhibition of ubiquinone down to very low levels had no impact on ATP synthesis (Dallner, G., personal communication). ATP synthesis is much lower in the chloroplast.

Dolichol is generally associated with the synthesis of glycoproteins. That activity is actually conducted by dolichol phosphate. However, the amount of dolichol phosphate in eukaryote organelles, mostly in the endoplasmic reticulum is dwarfed by the concentration of free dolichol found in the lysosome and the peroxisome. Dallner's group has made an extensive study of the distribution of phosphate-free dolichol and ubiquinone in the intracellular membranes of the liver [111]. A surprising portion of the dolichol is present as the fatty acid ester, which makes it very hydrophobic and a good candidate for lying parallel to the plane of the bilayer in the center. A role for the high concentrations of these isoprenes found in lysosomes, peroxisomes and the endoplasmic reticulum has been the subject of much speculation [112,113] and experimentation [114]. Dallner's group in cooperation with others has used direct NMR and neutron scattering data to establish that dolichol is localized in the center of synthetic phospholipid bilayers [115]. Thus dolichol and several of its analogues spread (thicken) the bilayer and yet do not alter the Tm or other features of each of the two monolayers it separates. They showed that the dolichol resides in the cleavage plane of the various synthetic phospholipid bilayers examined. Similarly, ubiquinone appears to be in the central hydrophobic core of membranes [116,117].

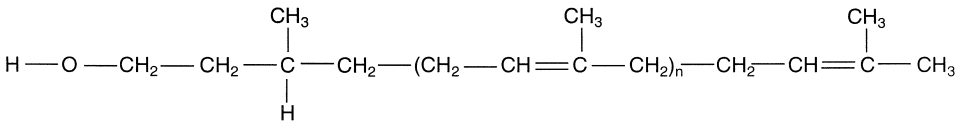
The pH of organelles varies from that in the cytosol [118]. This implies that there is a proton gradient across their membranes. A unique role is suggested by the current proposal in which the dolichol and ubiquinone inhibit proton leaks across these intracellular membranes. Where the pH of the lysosome is very low and the pH of the peroxisome is unusually high [119] the presence of the isoprenes in the center of the bilayer, separating the two monolayers, would serve to maintain the proton gradients for these organelles. In this connection it is interesting that the biosynthesis of dolichol in peroxysomes is different from that in the microsomes. Specifically it has a pH maximum of 8.0 [120]. A curious feature of the dolichol found associated with lysosomes and peroxysomes is that it occurs in small globules mixed with other lipids, particularly ubiquinone, outside the membranes in the cytosol or the lumen of these organelles [121]. Indeed, the volume of the peroxisomal compartment and the ubiquinone content in animal tissues are related [122].



Coenzyme Q (CoQ) or Ubiquinone
a.



Plastoquinone
b.



Dolichol (n = 9-22)
c.

Fig. 7. Isoprene and isoprane may inhibit proton leaks across the organelle membranes of eukaryotes by residing in the center of the membrane bilayer. (a) Ubiquinone (Coenzyme Q) ($n=$ 10 in mammals) is in all mitochondria at a concentration much greater than is needed for its role in oxidative phosphorylation. (b) Plastoquinone ($n=6$–8) is in all chloroplasts and in some biosynthetic prokaryotes. It is but one example of the mixture of the isoprenes in chloroplasts. Others including the hydrocarbons carotene and lutein generally constitute some 5% of the bilayer lipids. (c) Free dolichol ($n=9$–22) is present in large concentration (some 30 times the concentration of dolichol phosphate found in the endoplasmic reticulum) in lysosomes and peroxysomes along with ubiquinone. It is found mostly in globules of lipid in the lysosome and has been shown to reside in the center of the bilayer [115]. If it is inhibiting proton leakage as proposed, the dolichol in the globules may be incorporated into newly formed lysosomal membrane during the fusion of vesicles with the lysosome. It would thus aid in the maintenance of a pH gradient across the membrane.

Considering the role suggested here, these globules may serve as a reservoir of dolichol and ubiquinone to aid in the insulation of proton leakage as the budding and fusion of organelle membranes occurs. Thus plasma membrane, which is low in dolichol, may fuse with the lysosome and absorb dolichol/ubiquinone globules to help maintain the proton gradient across these membranes.

5. Sterols and energy

For aerobic prokaryotes, photosynthetic prokaryotes, mitochondria and chloroplasts the production of the proton gradient is remarkably efficient since it is direct. A transmembrane protein pumps the protons and the cell both produces and uses the pmf across that membrane to produce ATP. This is in contrast to the energy consumption required to maintain a membrane potential at the eukaryote plasma membrane. Here the production of ATP is either by mitochondria, etc., or by metabolic enzymes. The plasma and other organelle membranes are primarily consumers of the ATP wherein it is used to provide the chemiosmotic gradient. Plugging cation leaks through lipid bilayers conserves ATP. This applies as much to dolichol and ubiquinone in the lysosome as it does to sterols in the plasma membrane.

For example, the plasma membrane Na^+, K^+ ATPase is a major consumer of the cell's ATP. Racker [123] has estimated that 80% of the ATP of an erythrocyte is consumed by its Na^+, K^+ ATPase. Surely this is not the case for all cells, and not all sodium that enters the cell is due to leakage through the lipids of the membrane. Nonetheless, his calculation shows that the amount of Na^+ that must be pumped out cannot be explained by the co-transport of nutrients. That fraction of the sodium that does leak through the erythrocyte membrane must be pumped out at the expense of the ATP synthesized by glycolysis. The leakage fraction, whatever it is, would be tripled if cholesterol were not in the membrane. Thus cholesterol and the other lipids discussed are actually "energy savers." Pedersen and Amzel [124] have estimated that the ATP turnover of a human at rest may be as much as half an adult's body weight per day. The ATP saved may justify the elaborate genetic and energetic expense of synthesizing and managing cholesterol.

6. A note on sterol evolution

In a proposal on the evolution of sterols, Bloch noted [125] that sterol evolution stopped at squalene in prokaryotes, which lack sterols. This was prior to the appearance of eukaryotes and of oxygen in the atmosphere. The prokaryote hopanoids are cyclized from squalene in the absence of oxygen. Sterol synthesis from squalene on the other hand requires oxygen. If squalene inhibits proton leaks in alkaliphiles and other prokaryotes, then a cation leakage feedback control mechanism exists to control its synthesis. Using the model suggested herein, a cation feedback control mechanism applies to the many products of squalene metabolism. The model offers a role in energy metabolism for isoprenes and isopranes. It suggests that phytosterol synthesis is sensitive to pH in plants and those eukaryotes that have a proton gradient across the plasma membrane. It also suggests that some step in cholesterol metabolism is sensitive to sodium in the cytosol. An alternative to this proposal is that sterol synthesis has its own biosynthetic regulation

but that the presence of the sterol in the plasma membrane is actually regulated by the sphingolipids. If so, there may be a step in sphingolipid metabolism that is sensitive to sodium. In this view, the sterol is placed in the plasma membrane by its specific association with sphingolipids. This proposal is consistent with the recent recognition of a [48,126] special relationship between sphingolipid and cholesterol biosynthesis.

7. Summary and conclusions

A model is proposed for the leakage of protons across the lipids of cellular membranes. The model assumes water forms clusters in the low dielectric. It also assumes that certain clusters may be charged according to the pH of the water facing the bilayer. This model contrasts with the commonly accepted proton wire model. Either model implies that proton leaks may be inhibited by (1) extruding the water from the lipid bilayer or (2) blocking the contact between the clusters with hydrocarbon in the center of the bilayer although these two experimental and theoretical approaches derive readily from the cluster model. In examining the lipids found in cell membranes that have an extensive investment in a cation gradient it is seen that lipids having these properties are found in abundance in such membranes. Indeed, the proton leakage resistance offers a role for the unique design of many lipids that have heretofore been unknown as to function. That is, the proposal offers a coherent structure–function relationship *iso*- and *anteiso*-lipids, hopanoids, diphytanyl lipids, squalene, dolichol, the isoprenes, ubiquinone and plastoquinone, phytosterols and cholesterol. In addition to providing a functional use for structural features of the lipids, the proposal provides an explanation for their biological distribution.

For the most part, interference with the biosynthesis of most individual lipids is not lethal for the organism. Individual lipids are rarely essential or even specific for the functioning of a cellular activity. They primarily expand the options for the cell in its reponse to the environment. Yet it has long been noted that the absence of cholesterol is lethal to mammalian cells [34] as is the absence of phytosterols to plant and yeast cells [49].

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References

[1] Hille B. Ionic channels of excitable membranes. Sunderland, MA: Sinauer Associates, 1984. p. 19.
[2] Papahadjopoulos D. Biochim Biophys Acta 1971;241:254–9.
[3] Papahadjopoulos D, Nir S, Oki S. Biochim Biophys Acta 1972;266:561–83.

[4] Nicholls DG. Eur J Biochem 1974;50:305–15.
[5] Nicholls DG. Eur J Biochem 1977;77:349–56.
[6] Hinkle PC, Yu ML. J Biol Chem 1979;254:2450–5.
[7] Krishnamoorthy G, Hinkle PC. Biochemistry 1984;23:1640–5.
[8] Nichols JW, Deamer DW. Proc Natl Acad Sci USA 1980;77:2038–42.
[9] Nichols JW, Hill MW, Bangham AD, Deamer DW. Biochim Biophys Acta 1980;596:393–403.
[10] Bangham AD. Prog Biophys Mol Biol 1968;18:29–95.
[11] Brunner J, Graham DE, Hauser H, Semenza GJ. Membr Biol 1980;57:133–41.
[12] Mkheian EE, Sotskii OP, Akopov SE, Badzhinian SA. Biofizika 1981;26:137–8.
[13] Paula S, Volkov AG, Van Hoek AN, Haines TH, Deamer DW. Biophys J 1996;70:339–48.
[14] Singer MA, Jain MK. Can J Biochem 1980;58:815–21.
[15] Venema K, Gibrat R, Grouzis JP, Grignon C. Biochim Biophys Acta 1993;1146:87–96.
[16] Bonting SL, van Breugel PJ, Daemen FJ. Adv Exp Med Biol 1977;83:175–89.
[17] Cafiso DS, Hubbell WL. Biophys J 1983;44:49–57.
[18] Gutknecht J. Bioenerg Biomembr 1987;19:427–42.
[19] Gutknecht J. Proc Natl Acad Sci USA 1987;84:6443–6.
[20] de Gier J. Bioelectrochem Bioenerg 1987;27:1–10.
[21] Alberts B, Bray D, Lewis J, Roberts K, Watson JD. The molecular biology of the cell. New York: Garland Publishing, 1983.
[22] Gennis RB. Biomembranes, molecular structure and function. New York: Springer Verlag, 1989. p. 73–4.
[23] Slotte JP. Biochemistry 1992;31:5472–7.
[24] Yeagle PL. Biochim Biophys Acta 1985;822:267–87.
[25] Bretscher MS, Munro S. Science 1993;261:1280–1.
[26] Kleemann G, Kellner R, Poralla K. Biochim Biophys Acta 1994;1210:317–20.
[27] Sahm H, Rohmer M, Bringer-Meyer S, Sprenger GA, Welle R. Adv Microb Physiol 1993;35:247–73.
[28] Reinhart MP, Billheimer JT, Faust JR, Gaylor JL. J Biol Chem 1987;262:9649–55.
[29] Gershengorn MC, Smith AR, Goulston G, Goad LJ, Goodwin TW, Haines TH. Biochemistry 1968;7:1698–706.
[30] Lange Y, D'Alessandro JS. Biochemistry 1977;16:4339–43.
[31] Finkelstein A. Water movement through lipid bilayers, pores and plasma membranes. New York: Wiley, 1987. p. 102–5.
[32] Finkelstein A, Cass A. Nature 1967;216:717–8.
[33] Haines TH. FEBS Lett 1994;346:115–22.
[34] Yeagle PL. Biochimie 1991;73:1303–10.
[35] Demel RA, Bruckdorfer KR, Deenen Lv. Biochim Biophys Acta 1972;255:321–30.
[36] Gally HU, Seelig A, Seelig J, Hoppe Seylers Z. Physiol Chem 1976;357:1447–50.
[37] Parks LW, Bottema CD, Rodriguez RJ, Lewis TA. Methods Enzymol 1985;111:333–46.
[38] Lange Y, Ramos BV. J Biol Chem 1983;258:15130–4.
[39] Montesano R, Vassalli P, Perrelet A, Orci L. Cell Biol Int Rep 1980;4:975–84.
[40] Orci L, Miller RG, Montesano R, Perrelet A, Amherdt M, Vassalli P. Science 1980;210:1019–21.
[41] Orci L, Montesano R, Meda P, Malaisse-Lagae F, Brown D, Perrelet A, et al. Proc Natl Acad Sci USA 1981;78:293–7.
[42] Orci L, Singh A, Amherdt M, Brown D, Perrelet A. Nature 1981;293:646–7.
[43] Orci L, Montesano R, Meda P, Malaisse-Lagae F, Brown D, Perrelet A, et al. Proc Natl Acad Sci USA 1981;78:293–7.
[44] Orci L, Singh A, Amherdt M, Brown D, Perrelet A. Nature 1981;293:646–7.
[45] Montesano R, Vassalli P, Perrelet A, Orci L. Cell Biol Int Rep 1980;4:975–84.
[46] Lange Y. J Lipid Res 1991;32:329–39.
[47] Pincelli MM, Levstein PR, Fidelio GD, Gennaro AM. Chem Phys Lipids 2000;104:193–206.
[48] Ridgway ND, Byers DM, Cook HW, Storey MK. Prog Lipid Res 1999;38:337–60.
[49] Parks LW, Smith SJ, Crowley JH. Lipids 1995;30:227–30.
[50] Nes WR. Lipids 1974;9:596–612.

[51] Chan SI, Bocian DF, Petersen NO. Mol Biol Biochem Biophys 1981;31:1–50.
[52] Barenholz Y, Thompson TE. Chem Phys Lipids 1999;102:29–34.
[53] Lange Y, Ye J, Rigney M, Steck TJ. Biol Chem 2000;275:17468–75.
[54] Yeagle PL. J Membr Biol 1984;78:201–10.
[55] Klappauf E, Schubert D. FEBS Lett 1977;80:423–5.
[56] Schubert D, Boss K. FEBS Lett 1982;150:4–8.
[57] Yeagle PL, Albert AD, Boesze-Battaglia K, Young J, Frye J. Biophys, J 1990;57:413–24.
[58] Proverbio F, Rawlins FA. Mol Pharmacol 1978;14:911–9.
[59] Lijnen P, Celis H, Fagard R, Staessen J, Amery AJ. Hypertens 1994;12:59–64.
[60] Claret M, Garay R, Giraud FJ. Physiol (Lond) 1978;274:247–63.
[61] Sinensky M, Pinkerton F, Sutherland E, Simon FR. Proc Natl Acad Sci USA 1979;76:4893–7.
[62] Yeagle PL, Young J, Rice D. Biochemistry 1988;27:6449–52.
[63] Vemuri R, Philipson KD. J Biol Chem 1989;264:8680–5.
[64] Warren GB, Houslay MD, Metcalfe JC, Birdsall NJ. Nature 1975;255:684–7.
[65] London E, Feigenson GW. FEBS Lett 1978;96:51–4.
[66] Johannsson A, Keightley CA, Smith GA, Metcalfe JC. Biochem, J 1981;196:505–11.
[67] Pitts RF. Physiology of the kidney and body fluids. New York: Yearbook Medical Publishers, 1974. p. 19.
[68] Chapelle S, Gilles-Baillien M. Biochim Biophys Acta 1983;753:269–71.
[69] el Yandouzi EH, Zlatkine P, Moll G, Le Grimellec C. Biochemistry 1994;33:2329–34.
[70] Brasitus TA, Schachter D. Biochemistry 1980;19:2763–9.
[71] van Meer G, Simons K. EMBO J 1986;5:1455–64.
[72] Robenek H, Jung W, Gebhardt RJ. Ultrastruct Res 1982;78:95–106.
[73] Albert AD, Young JE, Yeagle PL. Biochim Biophys Acta 1996;1285:47–55.
[74] Papermaster DS, Dreyer WJ. Biochemistry 1974;13:2438–44.
[75] Andrews LD, Cohen AI. J Cell Biol 1979;81:215–28.
[76] Korn ED, Von Brand T, Tobie EJ. Comp Biochem Physiol 1969;30:601–10.
[77] Halevy S, Avivi L, Katan H. J Protozool 1966;13:480–3.
[78] Rodriguez RJ, Taylor FR, Parks LW. Biochem Biophys Res Commun 1982;106:435–41.
[79] Deamer DW, Volkov AG. Permeability and stability of lipid bilayers, chapter 8. In: Disalvo EA, Simon SA, editors. Boca Raton FL: CRC Press, 1995. p. 161–77.
[80] Nagle JF, Morowitz HJ. Proc Natl Acad Sci USA 1978;75:298–302.
[81] Nagle JF, Tristram-Nagle S. J Membr Biol 1983;74:1–14.
[82] Nagle JF. J Bioenerg Biomembr 1987;19:413–26.
[83] Pomes R, Roux B. Biophys J 1998;75:33–40.
[84] Ermler U, Fritzsch G, Buchanan SK, Michel H. Structure 1994;2:925–36.
[85] Krishnamoorthy I, Krishnamoorthy G. Journal of Physical Chemistry, B 2001;105:1484–8.
[86] Trauble H. J Membr Biol 1971;4:193–214.
[87] Flory PJ. Statistical mechanics of chain molecules. New York: Interscience Publishers, 1969.
[88] Haines TH, Liebovitch LS. Permeability and stability of lipid bilayers, chapter 6. Disalvo EA, Simon SA editors. Boca Raton, FL: CRC Press, 1995. p. 123–36.
[89] Garcia ML, Kitada M, Eisenstein HC, Krulwich TA. Biochim Biophys Acta 1984;766:109–15.
[90] Hinkle PC, Kumar MA, Resetar A, Harris DL. Biochemistry 1991;30:3576–82.
[91] Brown GC, Brand MD. Biochem J 1986;234:75–81.
[92] Nobes CD, Brown GC, Olive PN, Brand MDJ. Biol Chem 1990;265:12903–9.
[93] Porter RK, Brand MD. Nature 1993;362:628–30.
[94] Brown GC. FASEB J 1992;6:2961–5.
[95] Kaneda T. Biochim Biophys Acta 1966;125:43–54.
[96] Obermeier N, Poralla K. Arch Microbiol 1976;109:59–63.
[97] Kaneda T. Microbiol Rev 1991;55:288–302.
[98] Darland G, Brock TD, Samsonoff W, Conti SF. Science 1970;170:1416–8.
[99] Deinhard G, Blang P, Poralla K, Altan E. Syst Appl Microbiol 1987;10:47–53.

[100] Blume A, Dreher R, Poralla K. Biochim Biophys Acta 1978;512:489–94.
[101] Goldfine HJ. Lipid Res 1984;25:1501–7.
[102] Clejan S, Krulwich TA, Mondrus KR, Seto-Young D. J Bacteriol 1986;168:334–40.
[103] Amdur BH, Szabo EI, Socransky SS. J Bacteriol 1978;135:161–3.
[104] Koga Y, Nishihara M, Morii H. Biochim Biophys Acta 1984;793:86–94.
[105] Poralla K, Hartner T, Kannenberg E. FEMS Microbiol Lett 1984;113:107–18.
[106] Lindsey H, Petersen NO, Chan SI. Biochim Biophys Acta 1979;555:147–67.
[107] Yamauchi K, Doi K, Yoshida Y, Kinoshita M. Biochim Biophys Acta 1993;1146:178–82.
[108] Duax WL, Griffin JF, Rohrer DC, Weeks CM. Lipids 1980;15:783–92.
[109] Lorenz RT, Parks LW. Lipids 1991;26:598–603.
[110] Svoboda JA, Ross SA, Nes WD. Lipids 1995;30:91–4.
[111] Edlund C, Chojnacki T, Dallner G. Biochem Biophys Res Commun 1983;115:917–23.
[112] Chojnacki T, Dallner G. Biochem J 1988;251:1–9.
[113] Ernster L, Dallner G. Biochim Biophys Acta 1995;1271:195–204.
[114] Edlund C, Brunk U, Chojnacki T, Dallner G. Biosci Rep 1988;8:139–46.
[115] Valtersson C, van Duyn G, Verkleij AJ, Chojnacki T, de Kruijff B, Dallner GJ. Biol Chem 1985;260:2742–51.
[116] Katsikas H, Quinn PJ. Eur J Biochem 1983;131:607–12.
[117] Quinn PJ, Katsikas H. Coenzyme Q: Biochemistry, bioenergetics and clinical applications of ubiquinone. Lenaz G, editor. Salisbury: John Wiley, 1985.
[118] Grabe M, Oster GJ. Gen Physiol 2001;117:329–44.
[119] Dansen TB, Wirtz KW, Wanders RJ, Pap EH. Nat Cell Biol 2000;2:51–3.
[120] Appelkvist EL, Kalen A. Eur J Biochem 1989;185:503–9.
[121] Low P, Peterson E, Edlund C, Brunk U, Appelkvist EL. Lipids 1992;27:1–9.
[122] Aberg F, Zhang Y, Teclebrhan H, Appelkvist EL, Dallner G. Chem Biol Interact 1996;99:205–18.
[123] Racker E, A new look at mechanisms in bioenergetics. New York: Academic Press, 1976.
[124] Pedersen PL, Amzel LM. J Biol Chem 1993;268:9937–40.
[125] Bloch K. Reflections in biochemistry. In: Kornberg A, Horecker BL, Cornudella L, Orci J, editors. Oxford: Pergamon Press, 1976. p. 143–50.
[126] Ridgway ND. Biochim Biophys Acta 2000;1484:129–41.