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Anionic lipid headgroups as a proton-conducting pathway along the surface of membranes: A hypothesis

(proton pumps/proton translocation/chemiosmosis/acid membranes/acid–anions)

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ABSTRACT **Evidence has been gathering from several laboratories that protons in proton-pumping membranes move along or within the bilayer rather than exchange with the bulk phase. These experiments are typically conducted on the natural membrane *in vivo* or *in vitro* or on fragments of natural membrane. Anionic lipids are present in all proton-pumping membranes. Model studies on the protonation state of the fatty acids of liposomes containing entrapped water show that the bilayers always contain mixtures of protonated and deprotonated carboxylates. Protonated fatty acids form stable acid–anion pairs with deprotonated fatty acids through unusually strong hydrogen bonds. Such acid–anion dimers have a single negative charge, which is shared by the four negative oxygens of both headgroups. The two pK values of the resulting dimer will be significantly different from the pK of the monomeric species, so that the dimer will be stable over a wide pH range. It is proposed that anionic lipid headgroups in biological membranes share protons as acid–anion dimers and that anionic lipids thus trap and conduct protons along the headgroup domain of bilayers that contain such anionic lipids. Protons pumped from the other side of the membrane may enter and move within the headgroup sheet because the protonation rate of negatively charged proton acceptors is 5 orders of magnitude faster than that of water. Protons trapped in the acidic headgroup sheet need not leave this region in order to be utilized by a responsive proton-translocating pore (a transport protein using the proton gradient). Experiments suggest the proton concentration in the headgroup domain may vary widely and the anionic lipid headgroup sheet may therefore function as a proton buffer. Due to the Gouy–Chapman–Stern layer at polyanionic surfaces, anionic lipids will also sequester protons from the bulk solution at low and moderate ionic strengths. At high ionic strength metal cations may replace protons sequestered near the headgroups, but these cations cannot substitute for protons in the "proton-conducting pathway," which is based on hydrogen bonding.**

Recent experiments in several laboratories have suggested that protons that are translocated by proton-pumping membranes, such as those of mitochondria and halobacteria, need not enter the external bulk water phase in order to be utilized by proton-transporting proteins of the membrane, such as ATP synthetase. Notable among these experiments are those of Michel and Oesterhelt (1), who made careful measurements of proton-dependent ATP synthesis and pH changes in and near illuminated *Halobacterium halobium* cells. They found that the pH is not lowered in the bulk aqueous phase as the protons pumped by the purple patches of bacteriorhodopsin are utilized by ATP synthetase, which is not present in the purple patches but is found at some distance in red patches of the same plasma membrane. They concluded that the protons do not pass through the bulk aqueous phase but move very close to the membrane surface. This system is unique because the organism is cultured in 6 M NaCl, which implies that the Gouy–Chapman–Stern layer contains exclusively Na^+ and is not rich in H_3O^+.

Other reports (2–18) have described proton movements in restricted domains or in isolated open membrane sheets not capable of sustaining a transmembrane pH gradient (which nevertheless can be energized, ostensibly by generation of an electrochemical H^+ gradient for ATP synthesis) or have demonstrated that the pH may not account for the activities attributed to membrane "energization." Taken together, the observations suggest that there may be specific proton-conducting pathways in or on such energy-transducing membranes that allow them to carry out energy transduction without necessarily involving a pH gradient across the membrane.

Considerations developed in this paper suggest that the anionic headgroups of the lipids of energy-transducing membranes have the capacity to bind and conduct protons along the membrane surface. It is proposed that anionic groups, such as phosphodiester anions, form acid–anion dimers, two anions sharing a single proton via a hydrogen bond. Evidence is reviewed suggesting that such putative acid–anion dimers may be protonated and deprotonated at a high rate, permitting them to constitute a proton-conducting sheet or continuum, rapidly moving protons through the headgroups (along the surface) from H^+-pumping (e.g., electron transport chain protein) and H^+-utilizing systems (ATP synthetases) anywhere in the membrane. Protons trapped in such a proton-conducting continuum can, eventually, equilibrate with the bulk aqueous phase. This may account for the capacity of an imposed pH gradient to be used experimentally to generate ATP in proton-pumping membrane vesicles.

These suggestions came from experimental studies designed to explain the structure and dynamics of the flagellar membrane of the phytoflagellate *Ochromonas danica*. We had focused on the protonation state of the primary and secondary sulfates of the docosanediol 1,14-disulfate and the series of analogues containing one to six chloro groups on an otherwise saturated chain (19–28). This unusual natural membrane is devoid of phospholipids. This type of membrane occurs in many freshwater algae (29, 30) and may represent a diversion in the evolution of biological membranes (31). Natural membranes made of anionic detergents are surprising, more so when the detergent structure suggests that their anionic groups may be buried in the hydrophobic domain of the bilayer. Elemental analyses of such membrane preparations (unpublished data) excluded all potential counterions except for the proton. We therefore instituted studies on model bilayers containing alkyl sulfates or fatty acids in an attempt to understand how protons may stabilize such membranes and participate in their function.

Fatty Acid Liposomes. Unsaturated fatty acid liposomes that entrap aqueous compartments were described by Gebicki and Hicks 10 years ago (32). Liposomes may also be made of satu-

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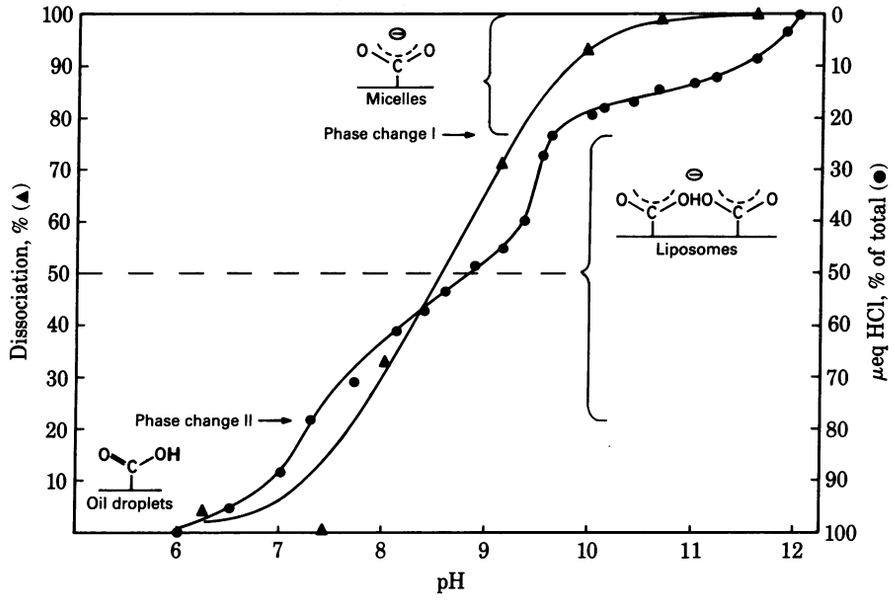

FIG. 1. Titration of a dispersion of 50 mM oleic acid and 10 mM [1-^{13}C]lauric acid from pH 12 to pH 6. ^{13}C NMR experiments were performed on a Varian CFT 20 20-MHz spectrometer in pulse-Fourier transform mode. Most spectra were obtained after 30,000 transients. The chemical shift of the labeled carbon is sensitive to the protonation state of the carboxylate. The percent of total chemical shift in ppm is directly proportional to the percent dissociation of carboxylate. The percent total shift is plotted against pH (▲). For comparison, a titration plotting the percent of total HCl required for complete protonation vs. the pH is shown (●). This titration displays two inflection points, at pH 9.45 and 7.15. The higher inflection point is associated with a transition from micelles to liposomes; the lower inflection point accompanies a phase change from liposomes to oil droplets. This titration is essentially that of Hargreaves and Deamer (33); they confirmed the presence of liposomes by interference microscopy. They and Gebicki and Hicks (32) measured the trapped volume of these liposomes. The pK of the fatty acid titration as estimated by NMR is 8.6. Stable liposomes are found between pH 9.5 and 7.2 and are oligolamellar.

rated fatty acids of short chain length (C_{14}) at room temperature or of longer chain lengths (C_{18}) at higher temperatures (33). Such membranes are stable only between pH 7.0 and 9.6. In collaboration with Michael Heller, the stability of such liposomes has been studied as a function of pH and of the protonation state of the carboxylate group as measured by ^{13}C NMR, using the chemical shift of the carboxyl carbon (Fig. 1). Two phase changes occur during a titration of fatty acids (50 mM) in water (33). Above pH 10 the fatty acids are exclusively in the micellar form; below pH 7 they form oil droplets. Between these pH values vesicular liposomes are formed; only in this pH range are both protonated and nonprotonated species simultaneously present. The liposomes are stable throughout a wide range of relative concentrations of each. The titration curve shows two apparent inflection points, presumably identified with the two phase changes.

Acid–Anion Dimers. That an acid–anion dimer in which one H^+ is shared by two carboxylate groups is a unique molecular species was shown by Westheimer and Benfey (34), who studied the pK values of various dicarboxylic acids in which the pK values of the two carboxylate groups were widely variant. A comparison of the pK values of maleic acid and fumaric acid shows the unique stability of the maleic acid–anion, which resists protonation down to pH 1.9 and resists deprotonation to pH 6.3 (Fig. 2). This ion has been extensively studied by x-ray crystallography (35) and Raman spectroscopy (36, 37). CNDO/2 (complete neglect of differential overlap) calculations (38) have been made (Fig. 3), and they agree with the Raman spectra. These data and calculations indicate that the single proton is shared and vibrates between the two carboxylates.

A simple argument, based on valence bond concepts, describing this species is as follows: If a protonated carboxylate is juxtaposed to an anionic carboxylate at the appropriate distance it will form a hydrogen bond with the anion. Hydrogen bonds involving an anion acceptor are unusually symmetrical and strong. The distance between the oxygens of the maleic acid–anion is short, 0.24 nm, compared to other O—H···O hydrogen bonds, which are usually 0.27 nm. The O—H covalent bond is about 0.1 nm, so that the distance from the anionic oxygen to the hydrogen is 0.14 nm. If the shared hydrogen moves 0.04 nm toward the anion it will switch partners; the protonated carboxylate becomes anionic and the anionic carboxylate becomes protonated. If the proton vibrates, then the single anionic

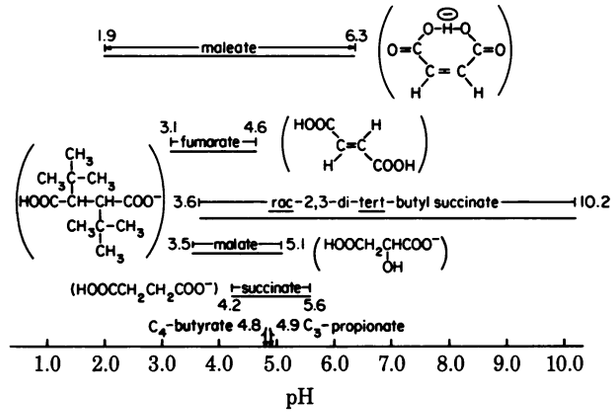

FIG. 2. pK values of selected di- and monocarboxylic acids are illustrated to emphasize the range of stability of the acid–anion, which is shown in the structures. Molecules in which the carboxylates are maintained in close proximity, either by molecular geometry (maleic acid) or by steric hindrance of flexibility (2,3-di-*tert*-butylsuccinate) are uniquely stable. The lower the first pK, the more resistant is the acid–anion to protonation. The higher the second pK, the more resistant the acid–anion is to giving up its proton. It is suggested that the alkyl chains of anionic lipids maintain the anions in such proximity as to stabilize acid–anion dimers over a wide pH range. Electrostatics widens this range because the polyanionic surface sequesters hydrogen ions from the solution.

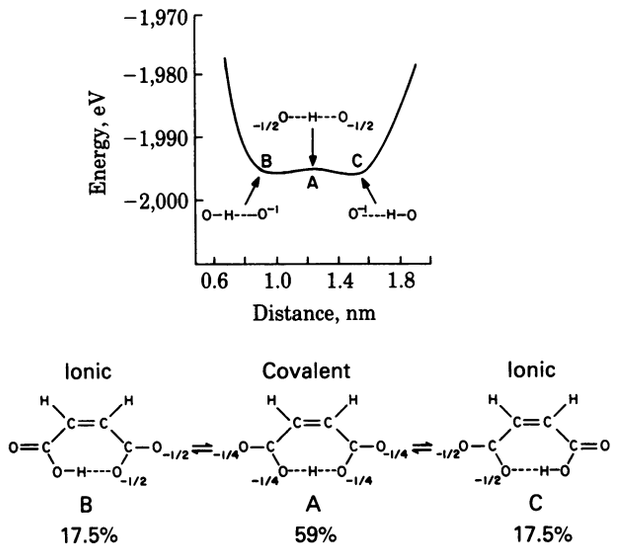

FIG. 3. Total energy for the hydrogen maleate anion calculated by CNDO/2 (complete neglect of differential overlap) (38). The calculation is in agreement with the Raman spectra and x-ray crystal structure data. The hydrogen bond distance (x-ray) is 0.2437 nm. A covalent O—H bond distance is about 0.1 nm. The calculation indicates that the hydrogen atom vibrates between just under 0.1 nm from one oxygen to just under 0.1 nm from the other. In these positions (B or C) one or the other of the carboxylates is ionic. Each of the two oxygens on the ionic carboxylate shares the negative charge. When the proton is halfway between the two oxygens the bond may be considered covalent (A). In this configuration each of the four oxygens retains only ¼ of the negative charge. The ion spends 59% of its time in this state. It is stabilized by the distribution of the charge by resonance. Most hydrogen bonds are of the order of 0.27 nm. This bond is unusually short (0.24 nm) because of the anion. In summary, the acid–anion hydrogen bond is unusually strong (stabilized by resonance) yet readily exchangeable because it spends some 30% of its time in ionic states.

charge becomes distributed over the four carboxylate oxygens. The hydrogen bond is symmetrical as a consequence of both resonance stabilization and tautomerism.

Fig. 2 demonstrates another unique feature about acid–anion dimers. Their characteristic pK values are derived not simply from the single protonated group (—COOH, —OPO_3H_2) but from forces that juxtapose the acid and anion. These forces may be bond orbitals (maleic acid–anion) or, in membranes, hydrophobic interactions. The formation of fatty acid acid–anion dimers has been known for many years. Titration of micellar soap solutions with KOH produces a precipitate containing two fatty acid chains to one K^+ (39). As Smith and Tanford have stated (40), "hydrogen bonds between R—COO^- and R—COOH are extraordinarily stable when carboxyl groups are attached to long alkyl chains." Acid–anion dimers have also been revealed by the x-ray analysis of several proteins. Lysozyme (41) and the acid proteases (42, 43), for example, contain such H^+-sharing acid–anions with unusual pK values.

Electrostatic Effect on the pK of Acid–Anions at Surfaces. The pK of simple carboxylates is around 4.0 (Fig. 2). It may seem surprising, therefore, that the pH range for stable liposome formation is pH 7 to 9.5. That the pH at a polyanionic surface is lower than the bulk pH was recognized and explained by Gouy in 1910. This property of anionic surface is now widely recognized and has been directly measured at bilayer and micelle surfaces by lipoid pH indicators (44). It is based on the capacity of the polyanionic surface to sequester cations within 1.0 nm of the surface (45). At low ionic strength the cation is predominantly the hydronium ion due to the ionization of water. At high ionic strength cations of the salt predominate. Knowledge of the charge density of anions at the surface and the ionic strength permits a direct calculation of the pH at the surface (45). The surface pH may be as much as 3.0 units below that of the bulk pH. The electrostatic effect at polyanionic surfaces explains why the fatty acids in Fig. 1 begin to be protonated at pH 10 on titration with acid and why they are fully protonated as oil droplets below pH 7, at which the surface pH is below 5. The electrostatic effect thus shifts the pH range expected for the pK values of anionic lipid headgroups in a bilayer phase.

Most membrane anionic lipids contain phosphate or sulfate groups, whereas phosphatidylserine, gangliosides, and certain other membrane lipids (46) contain carboxylate groups. In the case of phospholipids the anionic group is generally a phosphodiester. Like sulfate, which is always a monoester, the phosphate diester anion has an unusually low pK', below 3. It is therefore widely assumed that each lipid phosphate group in a membrane has a single negative charge at any pH above the observed pK'.

Trauble (47) conducted an elegant series of experiments describing the electrostatics at a membrane constituted of the synthetic phospholipid analogue 1,2-dialkyldiether glyceryl methyl phosphate. This glycerol diether is not as sensitive to acid hydrolysis as the diacyl derivative, allowing studies to be conducted at low pH. Bilayers formed from the calcium salt of this analogue were heated through the transition temperature. Although the calcium was quantitatively bound below the transition temperature, precisely half of it was released to the bulk solution above the transition temperature. Similarly, the protonated form when heated through the transition state ejected less than the total number of bound protons. Furthermore, a study of the pH jump conducted at a series of different pH values showed that the loss of protons from the fully protonated analogue bilayer occurred at a pK of 3.5. Maximum deprotonation occurred at pH 4.0. Surprisingly, above pH 4.5 protons were reabsorbed by the membrane, a property of the membrane not explained by electrostatic theory.

These data become more comprehensible when the membrane is assumed to contain acid–anion dimers of the anionic phospholipid analogue. At higher pH values the membrane firmly entraps protons, which may be important for its stability. At the first pK' phospholipid anions become protonated to form phospholipid acid–anion dimers. The second pK' would be expected at a much higher pH because the acid–anion is deprotonated to the anionic phospholipid. In the case of sulfate-containing lipids the acid–anion would gain unusual stability in comparison to the carboxylate or phosphate diester because the sulfate anion has three oxygen atoms sharing one negative charge whereas the acid–anion dimer has six.

Expected Properties of Anionic Lipid Bilayers Containing Acid–Anion Dimers. (*i*) Anionic lipid membranes constituted of a single species of lipid must exhibit two pK values. The lower pK' corresponds to the protonation of the acid–anion dimers to form fully protonated lipid headgroups. Fatty acid bilayers will be least stable, whereas phospholipid and sulfolipid anionic lipid membranes should be stable over a wider range of pH. Another implication of the dominance of acid–anions in membranes is that the observed pK' is not for the headgroup *per se* but for the entire headgroup sheet, particularly in mixtures of headgroup species.

(*ii*) The pK values of a lipid headgroup system would depend upon properties of the headgroup (carboxylate, sulfate, phosphate), the solution (ionic strength, affinity of the solution ions for the headgroups) and also on the nature of the forces that stabilize the juxtaposition of one headgroup with respect to another. These include the hydrophobic stabilization effect, the

transition temperature of the bilayer, the size of the headgroup, etc.

(*iii*) Electrostatic considerations as discussed above would predict that polyanionic membranes will bind protons differently at low or moderate ionic strengths on the one hand and at high ionic strength on the other. At low or moderate ionic strengths anionic lipid membranes would be expected to concentrate hydrogen ions in the Gouy–Chapman–Stern layer so that the surface water has a higher proton content than the bulk medium. At high ionic strength anionic lipid membranes would be expected to bind protons exclusively in the acid–anion (headgroup) domain. This may be important in proton-pumping membranes, because the surface provides a ready supply of protons even at high pH values.

(*iv*) Protons that are trapped by acid–anions on the surface of anionic lipid bilayers could move along the membrane surface at a significantly greater rate than they might move into the adjacent bulk aqueous phase. If a proton-pumping protein delivers a proton to the membrane surface then the anionic lipid sheet provides a negatively charged H^+ acceptor, A^-, whereas the bulk aqueous phase provides H_2O as H^+ acceptor. Eigen and de Maeyer (48) have shown that the rate constant of dissociation of HA for simple carboxylates (i.e., the transfer of H^+ from HA to water) is of the order of 10^5 sec^{-1}, whereas the rate of protonation of an anionic carboxylate group is around 10^{10} sec^{-1}. These measurements are based on solution kinetics and the latter value may be diffusion controlled. Nonetheless they emphasize that the headgroup anions are 10^5 better bases (proton acceptors) than is bulk water. Two features of the system would be expected to accentuate this difference. The first is the presence of a Stern layer of cations in the water just above the headgroups. At low ionic strength this layer is rich in hydronium ions. At high ionic strength the solution cations would further depress the rate of protonation of H_2O. A second feature of the anionic membrane surface is that its geometry would be expected to enhance the rate of proton transfer between its anionic lipids. Thus the steric behavior, the orientation of the anions, and the electrostatic interactions all favor a very high rate of proton transfer between headgroup anions. These features, combined with the capacity of the acid–anions to trap the protons in the membrane surface, would facilitate maximal rates of proton migration by protonation of anionic headgroups rather than protonation of bulk water. Protonation of an acid–anion dimer would be expected to destabilize it, resulting in a net movement of the entering proton along the surface of the membrane.

(*v*) The protonation state of lipid headgroups is independent of the ionic strength. As an example, it has been shown by Michel and Oesterhelt (1) that the movement of protons occurs in or on the membrane during proton pumping by intact *Halobacterium* cells. Such protons apparently do not alter the bulk phase pH and yet they are used for ATP synthesis. The membrane lipids of *Halobacterium halobium*, specifically the lipids of the purple patches (49, 50), are completely anionic. Because the organism is cultured in 6 M NaCl its Gouy–Chapman–Stern layer may be expected to be rich in Na^+ at the ectolayer and K^+ at the endolayer, but poor in H_3O^+. On the other hand, H^+ will be sequestered in the headgroup region underneath the Gouy–Chapman–Stern layer in acid–anion dimers. The anionic headgroup layer may thus function as a proton conductor, holding protons firmly yet allowing them to be conducted rapidly over some distance from bacteriorhodopsin to the H^+-driven ATP synthetase, without their exchanging with the bulk phase at high pH.

(*vi*) Another implication of the formation of acid–anions between negative lipid headgroups relates more generally to the nature of headgroup interactions. It is generally assumed (51) that headgroups of lipids are mutually repulsive. Rapid exchange of protons between headgroup acid–anions may tend to maintain a stable sheet so that if lipid molecules in a domain are pushed out of plane they will "snap back." Such a property of zwitterionic headgroups was utilized in a general theory of mechanical wave propagation in bilayers (52, 53), which emphasized compaction of the bilayer as an electrostriction (54) as an ion traverses it.

Mechanisms of Proton Cycling During ATP Synthesis. Mitchell (55) proposed in 1961 that protons translocated across the mitochondrial membrane from one aqueous phase to another could generate a pH gradient across the membrane, which could act to carry energy from electron transport to ATP synthesis or other energy-requiring activities of membranes. The term "chemiosmosis" was coined to describe the manner in which a bulk phase pH gradient could store and transmit osmotic energy. In the same year Williams (56) proposed that protons could be used to transfer energy, but by localized transfer of H^+ within the membrane rather than across it.

A major piece of evidence supporting the chemiosmotic hypothesis was the demonstration that experimental imposition of a bulk phase pH gradient across an energy-transducing membrane could be used to synthesize ATP (57). The bulk phase concept was especially emphasized by the observation that purple membrane patches of illuminated *Halobacterium* cells could pump protons (58, 59). The bacteriorhodopsin patches are physically separated from the ATP-synthesizing enzymes. Thus early experiments suggested that bacteriorhodopsin pumped protons into the bulk aqueous phase outside the cell and that the pH gradient so developed across the membrane was utilized to drive the biosynthesis of ATP elsewhere in the membrane. However, the recent observations of Michel and Oesterhelt (1) clearly indicate that when the protons are pumped by bacteriorhodopsin at very high ionic strength at pH 8.0 they do not enter the bulk phase, and yet they are used for ATP synthesis. The hypothesis herein proposed designates the anionic headgroups as carriers of protons laterally along the membrane headgroup sheet.

An important feature of such a two-dimensional network of acid–anions is that the concentration of the protonated species within the headgroup domain may vary considerably. This is illustrated in Fig. 1, in which the protonation state of the headgroups can vary from 80% protonated to 80% nonprotonated in the fatty acid liposomes. Thus proton pumping into the headgroup domain may produce a local "proton pressure" that can protonate amino acid anionic R groups of membrane proteins that utilize protons to carry out energy-dependent processes. The protons sequestered in the bilayer headgroup region of energy-transducing membranes will equilibrate with the bulk phase protons. The latter may, in the context of this hypothesis, act not only as a safety valve but also to store energy in a low intensity form as a pH gradient across the membrane. The headgroup proton conductor may be regarded as analogous to the multienzyme complexes, which generate and consume metabolic intermediates without permitting their diffusive loss into the bulk aqueous phase.

That anionic lipids are critical for the viability of cells has been established. Studies on mutants of *Escherichia coli* (60) and on mammalian cell lines (61) have shown that a fixed minimal fraction of the lipids must be anionic although one anionic lipid may be replaced by another. Many organisms have solely anionic lipids (and uncharged but not zwitterionic lipids). Anionic lipids constitute all photosynthetic membranes as well as membranes of the archaebacteria. The latter are believed to be among the earliest organisms in evolution (62). This author knows of no

organism that lacks anionic lipids.

Kell (10), Malpress (12), and Storey and Lee (15) also have presented arguments and data in favor of energized protons moving close to the membrane in proton-pumping membranes, but they have not considered the anionic lipids as a potential conductor. Freund and co-workers (63, 64) have observed a rapid movement of H^+ across the surface of metal hydroxides (Mg^{2+}, Ca^{2+}, Al^{3+}) in the solid state. This surface property, dependent upon fixed negative charges, is precisely analogous to the fast movement of H^+ along headgroup anions described here. The methods used by Freund to demonstrate this property have applicability to lipid membranes, perhaps most usefully in lipid monolayers.

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