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City University of New York PH.D. 1982

STUDIES ON THE SURFACE pH AND PROTONATION STATE OF MONOALKYL ANIONIC MEMBRANES AND LIPOSOMES

by

MICHAEL HELLER

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1982

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September 14, 1982
Date

Thomas H. Haines
Chairman of Examining Committee

September 14, 1982
Date

Executive Officer

Supervisory Committee

Abstract

STUDIES ON THE SURFACE pH AND PROTONATION STATE OF MONOALKYL ANIONIC MEMBRANES AND LIPOSOMES

by

Michael Heller

Advisor: Professor T. H. Haines

It has been shown that the flagellar membrane of the phytoflagellate, Ochromonas danica, lacks phospholipids. This novel membrane contains as its polar lipids 1, 14-docosanedisulfate (together with 1, 15-tetracosanedisulfate) with from 0 to 6 chloro groups replacing hydrogens on the chain (Chen, Pousada and Haines, J. Biol. Chem. 251, 1835-1842 (1976)). The single-chain amphiphiles represent 70% of the total lipids, with sterols (~12%) and free fatty acids (~17%) constituting the remainder. When this work was done it was not clear whether or not the free fatty acids were a structural component of the membrane or an artifact produced during isolation. It should be noted that O. danica is a unicellular alga that lacks an outer protective wall or membrane visible in the electron microscope. It extrudes vesicles of its plasma membrane in the log phase of growth and these "extracellular vesicles" have likewise been shown to consist of the above lipids in the same proportions.

As a model system with which to explore this unique

natural membrane, liposomes were prepared from a combination of single-chain amphiphiles such as oleic acid or oleyl sulfate (SOS) either in combination with cholesterol or with chain alcohols. Experiments were designed to seek explanations for the remarkable concentration of anions in the bilayer-most notably the secondary sulfate which appears to be in the low dielectric where it is presumably neutralized (uncharged). Examination of the protonation state of the anions (uncharged on protonation) was performed by ^{13}C-NMR. In the same vesicle and/or membrane system the pH at the surface (as opposed to that in the bulk aqueous phase) was measured directly by alkylated pH indicators hydrophobically bound to the membrane surface. The surface pH was expected to be 1 to 3 pH units below that of the bulk phase according to the Gouy-Chapman theory. This was found to be the case for both model vesicles and the natural membrane.

Oligolamellar vesicles that entrap water solubles (glucose, enzymes, etc.) are made by titrating oleic acid micelles from pH 12. The liposomes appear at pH 9.2 and are stable to pH 7.2, at which point they become oil droplets of protonated oleic acid. The two phase changes are accompanied by inflection points. A single carboxyl (oleate) exhibiting two inflection points in the titration led to the postulate that acid-anion dimers may act as the stabilizing feature of the fatty acid liposomes. An acid-anion dimer consists of a protonated and an unprotonated oleate pair bound by a hydrogen bond in which the hydrogen atom vibrates

allowing the charge to be shared by both carboxylates. Such species reduce the negative charge density at the surface and trap protons in the head-group domain of the anionic lipids. ^{13}C-NMR chemical shift studies using 1-^{13}C-laurate, showed that vesicle formation required that 20% of the headgroups be protonated and that oil droplets formed as 80% of the headgroups were protonated. The surface pH of the oleic acid vesicles was found to be 1.8 pH units below that of the bulk phase as the alkylated hydroxycoumarin indicator exhibited an apparent pK of 10.7 at a corrected bulk phase pH of 8.9.

A model system consisting of cholesterol, SOS and oleic acid was considered to be more closely analogous to the natural flagellar membrane and extracellular vesicles. A comparison of these three preparations showed that in the low pH range the surface pH of the model system and of both natural membranes is approximately the same as that of the bulk phase, whereas in the high pH range the surface pH is 1.5 pH units below that of the bulk pH. These data, in conjunction with the ^{13}C-NMR chemical shift data, are interpreted to mean that (1) the free fatty acids in the alkyl sulfate model membrane and in both natural membranes are fully protonated below pH 7.2 and fully deprotonated above pH 9.2, and (2) that there appears to be free fatty acids in the natural membrane which are fully protonated in the living organism which grows maximally at pH 4.5

Acknowledgements

This thesis is dedicated to all those beneficent beings, who, during the long course of this exercise in critical thinking, both gave their blessing and support.

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Thomas H. Haines, my mentor, who, despite all manner of uncontrollable obstacles, was able to direct my thesis, provide encouragement, and act as a loyal and true friend.

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And most importantly, Janet, my wife, who, besides providing love, continued to live with me in relative bliss despite my total preoccupation with this thesis.

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I. Introduction:

Anionic lipids are present in the membranes of all living cells. In most membranes they are in a mixture with zwitterionic and/or uncharged lipids. Where such mixtures occur the mole fraction of anionic lipids has been shown to be maintained at a fixed minimum in living cells (1, 2). Many natural membranes are made entirely of anionic lipids or consist of a combination of anionic and uncharged lipids. These latter are referred to as "acid membranes" because they exhibit a low pH at the membrane surface. The plasma membranes of the earliest known organisms, the archaebacteria (3, 4), are of this kind. This includes the halophiles (5). Photosynthetic membranes including both eukaryotic and prokaryotic, are also acid membranes (6, 7, 8).

The early ancestry of the acid membrane is not unusual when one realizes that they must have been the major bilayer form in the prebiotic sea. Along with amino acids (9) and purine and pyrimidine bases (10, 11) several types of lipids have been formed in experiments mimicking the prebiotic reducing atmosphere. Prominent among these are fatty acids (soaps) (12), single chain amphiphiles (anionic detergents), and phospholipids (13).

Hargreaves and Deamer (16) have presented evidence to support the contention that the formation of liposomes from detergent monomers such as fatty acids is entirely possible in the slightly basic ocean under the concentration conditions

postulated (pH8). Such liposomes could be the early entrapping forms for molecules necessary to the formation of life. Their experiments were conducted with single chain amphiphiles at 10mg/ml, which is below the critical micelle concentration for most of the lipids in this class. Although the lipid concentration of 10mg/ml (14) is not concentrated and might be below the critical micelle concentration (CMC) of some amphiphiles, a surface of fatty acids and oils (15) has been postulated to have existed in the early prebiotic sea.

Leaving the prebiotic sea, the earliest known bacteria, the archaebacteria (4) and surviving descendent lines, such as the halophiles, contain extensive acid membrane domains. For example, purple membrane of Halobacterium halobium consists of a protein, bacteriorhodopsin, surrounded by acidic lipids (17).

Other relatives of the early precursor prokaryote are the photosythetic bacteria (4) which are among the earliest known fossils (18). These bacterial membranes are composed of acidic lipids as are the thylakoid membranes of higher photosynthetic plants (19). These data suggest that there is an early connection between acid membranes and energy transduction, yet little has been done to relate these two phenomena. Any system as ancient and ubiquitous bears further scrutiny.

Liposomes and/or vesicles formed with various lipid compositions are excellent systems for probing the myriad interactions that occur on or within the natural membrane

surface. Phospholipid vesicles, introduced by Bangham et al., in 1965 (20) have been extensively investigated and characterized (21). Studies of the molecular dynamics of head groups (22), the hydrophobic domain (23), protein and lipid interaction (24) all have employed phospholipid vesicles as mimetic systems.

The simplest model membrane mimetic systems are the alkyl detergent type. Composed of anionic (i.e., fatty acids, alkyl sulfate or alkyl phosphate) or cationic (i.e. alkyl methylammonium), these liposomes can be defined as smectic mesophases of synthetic surfactant bilayers entrapping water (25).

In attempts to synthesize simply described liposomes for use in radiation studies Gebicki and Hicks (26) described the formation of liposomes that could only be made of unsaturated fatty acids. They named these structures "ufasomes".

Ekwall (27) had inferred a bilayer structure from phase diagram studies of sample surfactant dispersions. The potential use of these structures for model bilayer liposome studies, however was first noticed by Gebicki and Hicks (28). Shaking dispersions of unsaturated fatty acids between pH 8-9, they were able to form multilamellar structures (28). Experiments measuring the permiability of glucose and of enzymes (28) convinced them that these multilamellar structures behaved similarly to phospholipid liposomes albeit somewhat more fragile.

They concluded that slightly alkaline pH (8-9) and unsaturation of the acyl chain were requirements for liposome formation. Inclusion of cholesterol or an increase in unsaturation increased the permeability of the liposomes to glucose.

Hargreaves and Deamer (29) recognized and demonstrated that the transition temperature (Tm) and not the degree of unsaturation was the stabilizing factor (other than pH) for fatty acid liposomes. Experimenting with dispersions of saturated fatty acids between C-8 and C-18 they were able to demonstrate that titration from pH 12 to 7 results in the formation of crystals below the detergent transition temperature and liposomes above this temperature. All dispersions exhibited a critical concentration above which liposomes formed. This concentration was one half the critical micelle concentration (CMC). As chain length increased the pH at which liposomes formed at this critical concentration increased. For all classes of fatty acids the crystal form (below the Tm) precipitated at a higher pH than did liposome formation for the same class above the Tm. They further demonstrated two distinct pKa's for the titration curve of fatty acid liposomes or crystals. Beginning at pH 12 and titrating with acid the first pKa occurred around pH 9.0 and was accompanied by a phase change from micelles to liposomes. The second pKa occurred around pH 7 at which point a phase change from liposomes to

oil droplets occurred.

Hargreaves and Deamer also prepared dispersions of sodium dodecyl sulfate (SDS) and dodecanol. When dodecanol was added to solutions of SDS micelles above the transition temperature (42° C), dispersions of large oligomellar liposomes containing SDS:dodecanol were formed. Between 50 and 70 mole percent dodecanol, these stable liposomes exhibited an increased internal volume with increasing concentration of dodecanol. Interestingly, alkanols also converted fatty acid micelles at pH 11 or 12 into liposomes. The authors concluded that the alcohol "spacer" allowed closer chain packing in the SDS micelle while maintaining the proper charge separation between the headgroups. Previously x-ray crystallography of SDS crystals had demonstrated that the headgroup is too large to give the close packing of the lipid tails associated with bilayer membranes (30).

Tanford (31) provides a thermodynamic justification for bilayer formation when two alkyl chains are provided for each polar headgroup. Synthetic bilayer membranes produced by monoalkyl carboxylate, sulfonate (or sulfate) and phosphate headgroups have given experimental confirmation of this property (32, 33). Monoalkyl forms of the latter compounds do not spontaneously form bilayers unless uncharged single-chain spacers are provided in approximately equimolar amounts (50-70 mole percent) (29). Monoalkyl carboxylates (free fatty acids) have a sterically smaller headgroup. It

is not surprising therefore that fatty acid liposomes have rather different properties than their tetrahedral analogs (sulfate and phosphate). Before discussing the fatty acid liposome system it is first necessary to understand a principle that we have been forced to introduce in order to explain our data.

In the studies of Hargreaves and Deamer (29) the data implicated the transition temperature (chain length, chain packing, etc.) and pH as major factors in the stability of fatty acid liposomes.

The free energy upon which the stability either micelles or liposomes is based is often referred to as the hydrophobic effect. Described by Tanford (31), it can be summarized as follows:

Liquid water is ordered (on a very rapid time scale) by an extensive H-bonded network. The inclusion of methylene groups into liquid water both disrupts this network (enthalpy effect) and forces the water molecules to assume a ordered structure encapsulating the hydrocarbon (entropy effect). Each of these forces acting in the same direction increase the free energy of the system. In oil water mixtures the hydrocarbon may phase separate into emulsions or into separate layers. In low concentrations (50-100mM), anionic amphiphiles such as fatty acids or sulfate esters phase separate into micelles. A critical concentration, the critical micelle concentration (CMC) of a given amphiphile is necessary for the formation of micelles for that

amphiphile. Below this concentration the amphiphile is primarily present in the monomeric form although there is evidence that dimers and other aggregates are often present as well. Micelles are aggregates of amphiphiles with a lipoid interior and a hydrophilic hydrated exterior (34). The commonly accepted structure of micelles is that of a sphere (35, 36) or planar disk (37). Although there are some reservations about these structures (38), it is commonly accepted that the micelle diameter is no more than the combined length of two of its amphiphiles. The hydrophilic region, also known as the palisade layer, is often viewed as uneven and inundated with water molecules (39). Although the micelle is used for a good first approximation in establishing the dynamics of bilayer membrane systems, it has been superceded by the bilayer liposome which more closely mimics the bilayer membrane (25) (For a description of the controversy surrounding micelle structure see Menger (39)).

What are the phenomena that change the structure of the fatty acid micelle to the bilayer liposome? Why is this important in understanding natural anionic membranes such as the chlorosulfolipid membranes of Ochromonas danica?

Part of the answer comes from a theory first put forward by Gouy in 1910 (40) and later modified by Stern (41). The latter proposed a mathematical description of the effect of densely charged surface in buffered solutions.

Combining the effect of a net negatively (or positively) charged surface in which the charges are smeared over the surface, the field induced by such a surface, and the partition of counterions in solution, a method was found to calculate the increased concentration of counterions near the charged surface. In low ionic strength media the electrostatic field induced by a densely packed surface of anionic lipids will attract cations to within a distance of 9 nm (42). This layer rich in cations, is now called the Stern layer. The theory has been confirmed experimentally despite its many simplifications. A complete summary of this data and a clear statement of the theory has been made by McLaughlin (43).

One implication of this theory is that in low ionic strength buffers hydronium ions will collect in a proportionally higher concentration in the Stern layer as compared to the bulk solution. For example it may be calculated that a surface constituted solely of anionic lipid headgroups may be expected to exhibit a -200 mv surface potential. This potential would lower the pH at the surface 3 pH units (47). One can easily see then that the pKa for fatty acids, which is 8.6 - 9.0 (as measured in the bulk solution) is really 5.6 - 6 at the surface of the liposome, well within the pKa range of carboxylic acids.

It is clear from the above discussion that the pK of an anion at a negatively charged surface will not be the same as the anion in the bulk phase. For purposes of our discussion we will call this the "electrostatic effect" on

Figure 1.

The pK's 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 on 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.

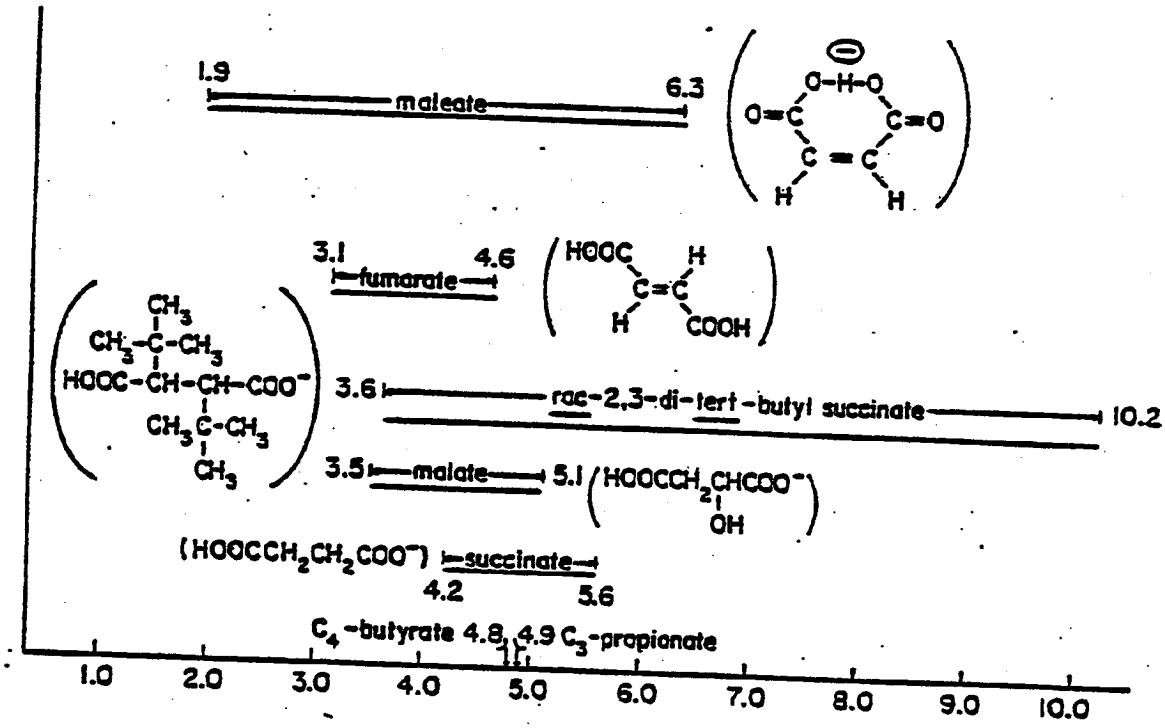
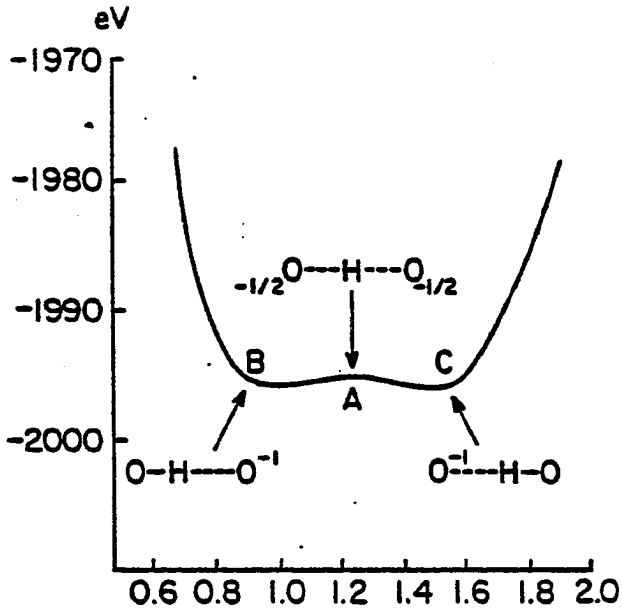
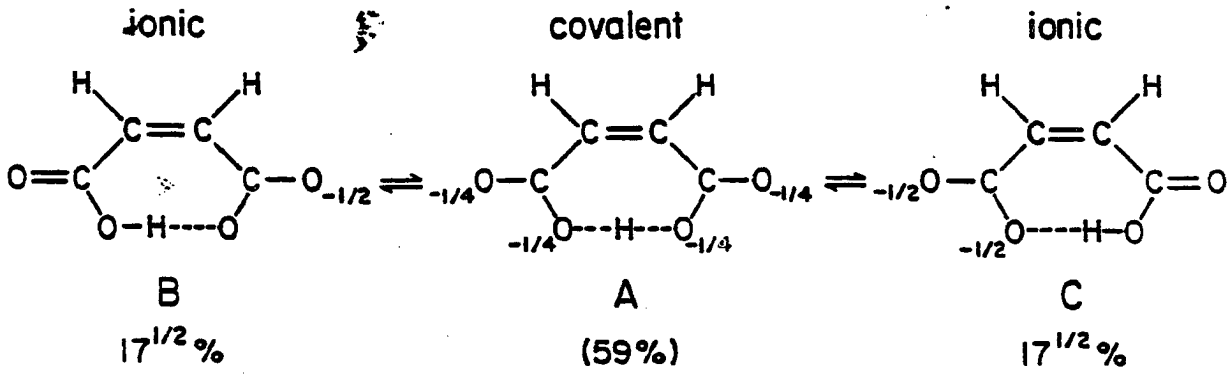


Figure 2.

Calculated (CNDO/2) total energy for the hydrogen maleate anion (53). The calculation is in agreement with the Raman spectra and x-ray crystal structure data. The H-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 (A or B) one or the other of the carboxylates is ionic. Each of the two oxygens on the ionic carboxylate share 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 retain only 1/4 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 H-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 H-bond is unusually strong (stabilized by resonance), yet readily exchangeable because it spends some 30% of its time in the ionic state.





the pK. We will now consider a second feature of lipoid anions which will alter their pK. This is acid-anion formation. It is well known in lipid chemistry that when titrated, fatty acids at their pKa may form a unique species, the acid-anion dimer. Dimerization between COO^- and COOH leads to extremely stable H-bonding when the carboxyls are attached to long alkyl chains (31).

Rosano et al. (44, 45), have isolated the acid-anion dimer of myristate. By a simple acid titration of potassium myristate from high pH he isolated a precipitate (below the transition temperature) which showed on analysis to consist of a single potassium associated with two myristate chains. In studies with isotherms of saturated C-16 to C-22 soap monolayers (46) it was shown that increased surface pressure or increased chain length (acting to decrease the surface area per headgroup due to an increase of van der Waals attraction) increased the binding of hydrogen ions over alkali-metal counterions to the monolayer. Remarkably, this effect was still seen at a pH of 12 where the fatty acids are ostensibly completely ionized.

The stability of the acid-anion complex has been studied using a series of dicarboxylic acids with various alkyl substituent groups replacing hydrogen on a succinate backbone (47-50). Figure one shows this series. Steric hindrance exerted by alkyl groups creates a difference between the pKa of the first carboxyl and that of the second.

The difference between the pKa's for each molecule is a measure of the stability of the acid anion hydrogen bond. When the second pKa is substantially higher than the pKa of propionic acid the acid-anion H-bond may be considered stable to deprotonation. The decrease of the first pKa below that of propionic acid indicates sufficent stability of the H-bond to protonation of the second carboxyl.

Maleic acid-anion dimer is probably the most extensively investigated. The O-O distance across the acid-anion H-bond of 0.2437nm was established by x-ray crystallography (51). The short H-bond length as compared with water-methanol (0.27nm) and formic acid dimer (0.274nm) (52) implies an unusually strong bond. An alternate appraisal of this bond-strength can be seen in the comparison of the pKa's of maleate to fumarate. The <u>trans</u> configuration of the fumarate precludes the formation of acid-anion dimers.

The total energy of the hydrogen maleate ion as a function of OH distance at a fixed O-O distance of 0.2437nm is seen in Figure two (53). This description is in agreement with a 1660 cm^{-1} fundamental frequency (54), and solution Raman studies (55). Figure 2 also shows the probability of the proton's localization at three points A, B or C. This delocalization effectively distributes the unit charge over four oxygens. This particular effect has been termed by Haines (56) as the vibrating proton or tautomeric resonance. In the case of sulfate ester's acid-anion structures, tau-

tomeric resonance would effectively distribute the positive charge over six carbons instead of four for the carboxylate. It must be kept in mind that the proton movement within the acid-anion dimer is on a very rapid time scale (10^9 - 10^{11} sec^{-1} is a typical timescale for protonation experiments). Tautomer formation of acetyl acetone (57) is more rapid that the lateral motion of phospholipids (58) which is already orders of magnitude faster than either ion transport or enzyme activities.

Two important features of the properties of membranes constituted of acidic (anionic) lipids emerges from the above discussion. The first is that the stability and therefore the pK's of the acid-anion is determined by the forces that hold the anions in close proximity. For maleic acid, it is the geometry of the covalent bonds (figure 1,2). For di-tert-butyl succinate (Figure 1) it is steric interactions. For the dimyristyl acid-anion isolated by Rosano et al. (44), it is the hydrophobic effect. In membranes made of anionic lipids the hydrophobic effect operates in tandem with the structure (and integrity) of the membrane.

The second important implication of the acid-anion in anionic lipid membranes is that it provides a mechanism for the entrapment of protons within the headgroup domains of such membranes. Thus hydrogen bonds between anionic lipid headgroups in bilayers that are in the gel state further stabilizes the gel state. Heating bilayers from the gel state through the transition temperature to the liquid

crystal state produces a pH jump in the bulk phase as protons involved in H-bonding are released from the bilayer.

Jacobson et al. (59) noted that the phase transition of various phosphatidic acid species occurred at higher temperature and lower transition enthalpies than that of the corresponding phosphatidyl choline (PC) bilayers. This deviation from a theoretical model involving only charge repulsion (60), implicates H-bonding below the transition temperature as the energy component most responsible for the increased temperature requirement (60, 61, 62). Although a pH jump occurs when protonated phosphatidic acids (63, 64) or phosphatidyl serine (65) are heated through the transition temperature, the number of protons released is only a fraction of the protons sequestered below the phase transition. Trauble (66) developed a solid theoretical framework for the electrostatics of this system. He found that heating the calcium salt of dihexadecylphosphatic acid (DMPA) through the phase Tm resulted in the quantitative ejection of calcium from the bilayers, whereas only partial ejection of protons (from the protonated form) was found under the same conditions. This could be explained now as the entrapment of protons within acid-anions.

Hauser et al. (67) has demonstrated that clustering of fatty acids occurs in lecithin membranes at pH 8-10, a point in the titration of fatty acid membranes at which they are partially protonated. The latter implies the simultaneous presence of protonated and non-protonated headgroups

which are therefore able to form H-bonded dimers or acid-anions.

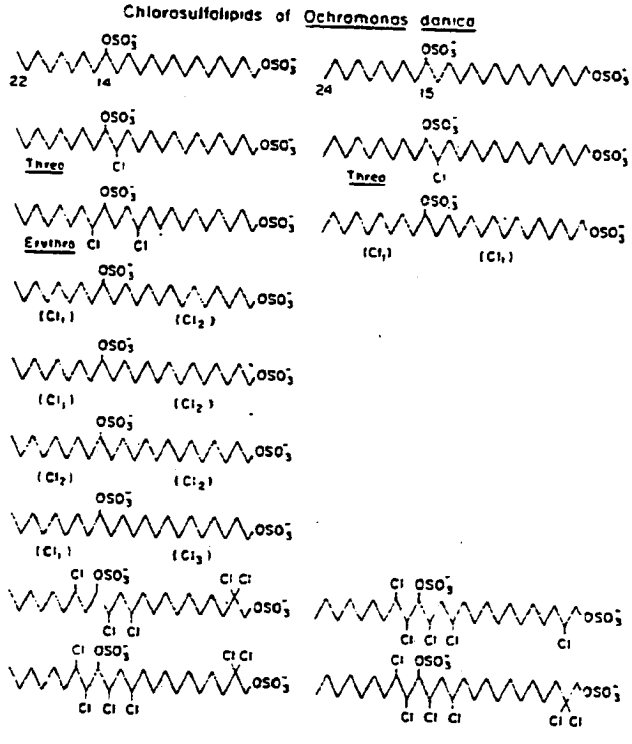
Several groups have tried to describe anionic head-group interaction in terms of charge repulsion. In this view charge repulsion between adjacent anions (without attractive interactions) dominates the dynamics of membrane lipids (68). Jahnig et al. (69) have described in detail, using DMPA, the dependence of phase transition upon charge. They showed that in such highly charged systems the phase transition occurs at a lower temperature due to chain tilt which is presumably caused by headgroup expansion.

Ochromonas danica is a unique alga in that it contains little phospholipid and is dominated in its lipid composition by alkyl disulfates. Although alkyl disulfates have been found in thirty species of algae (70, 71) and O. malhamensis only in O. danica have these lipids been shown to constitute the major polar lipid fraction of a membrane (72, 73). In O. danica the sulfates are docosanediol-1-14-disulfates with about five percent of the mixture occurring as tetracosanediol-1-15-disulfates (72). Up to six chlorine atoms may replace hydrogens in an otherwise saturated chain (74, 75). (See Figure 3.)

These compounds were discovered in 1962 (76) and have been characterized by several groups (72-78). For some years it was not clear that the compounds were membrane lipids (79, 80), most lipid chemists believing they could not be so because of their detergent properties.

Figure 3.

Disulfates of Ochromonas danica.



Flagellar membrane preparations were isolated allowing for a direct analysis of a pure membrane preparation uncontaminated with interior cell membranes of the organism (72). Surprisingly the purified preparation contained no phospholipid and ninety percent of the polar lipid was alkyl disulfate (73). With the exception of a small percentage of glycolipid the membrane consisted of two thirds chlorosulfolipid (73); the other was found to be composed of sterols (81) and fatty acids (82).

Partly fractionated O. danica yielded plasma membrane preparations enriched in chlorosulfolipids (83, 84). Recent experiments with a membrane vesicle excreted from all over the surface of the organism (85) reveals a lipid composition essentially the same as the flagellar membrane (86). This data and electron micrographs showing a continuous plasma membrane suggest that the organism contains an alkyl sulfate membrane. That a natural membrane could be constituted of single chain amphiphiles runs counter to the common experience of membrane lipid chemists.

The structure of the chlorosulfolipids of O. danica seems to preclude their consideration as components of a bilayer membrane with the secondary sulfate in the lipid interior, yet they have been established as the dominant lipid component of the bilayer membrane. The alternative situation which places the secondary sulfates away from the interior bilayer region is less plausible as can be demonstrated by the following argument. If the two sulfates are

at the interfacial surface then we would have ostensibly a "loop" between the sulfates and the methyl terminal strand that would be equivalent to three very short chains and two negatively charged headgroups. Theoretical arguments (31) and experimental results (29, 32) are in agreement that chains less than eight carbons (which would be exhibited by this folded conformation of the sulfatide) may not form bilayers.

A working model of the bilayer suggested by the data contains alkyl disulfates in their extended form, free fatty acids (87), sterols and negatively charged protein (88). This model must have some rather unusual chemistry.

The secondary sulfates are proposed to be in the center of each monolayer of the bilayer. Electrostatic considerations do not allow a charged sulfate to exist in such a low dielectric region ($\epsilon = 2$), therefore the sulfate must be neutralized. Stern et al. (88), in attempts to find such a counterion, conducted elemental analysis of purified flagellar membrane seeking both organic cations (nitrogen) and any alkali-metal cations in sufficient quantity to account for the neutralization of secondary sulfates.

The possibility of a salt bridge between protein amino groups and the sulfate was negated by the amino acid analysis that showed insufficient basic amino acids to serve as counterions (88). A membrane with apparently repulsive anionic groups everywhere must have a mechanism to prevent its disintegration. Refering to the earlier discussion of

electrostatics and acid-anion formation, a theory can be constructed accounting for the apparent membrane stability.

O. danica grows maximally at pH 4.5. Maximally packed anionic lipids can lower the surface pH to 1.5. This is precisely the pKa of a primary alkyl sulfate. Stability of putative acid-anion dimers is not only related to the forces that hold the headgroups proximal to the shared charge of the protonic species among the oxygens in the anion dimer. Alkyl sulfates would contain more resonance forms than the carboxylate because the charge is shared by six oxygens. These two effects would be expected to favor the formation of acid-anion tautomers in alkyl sulfate membranes. The large proton density at the surface makes the notion of a fully protonated secondary sulfate more acceptable. Acid-anion forms of the sulfates allow for a statistical moiety that has two alkyl chains per charged headgroup. The statistical caveat is necessary since the rapidity of lateral mobility in the bilayer prevents the isolation of any particular dimer.

Another aspect of the low pH of the surface of the O. danica alkyl sulfate membrane is that its free fatty acids must be fully protonated. Such fatty acids may thus act as hydrocarbon "spacer" molecules along with the sterols as has been proposed by Hargreaves and Deamer (29).

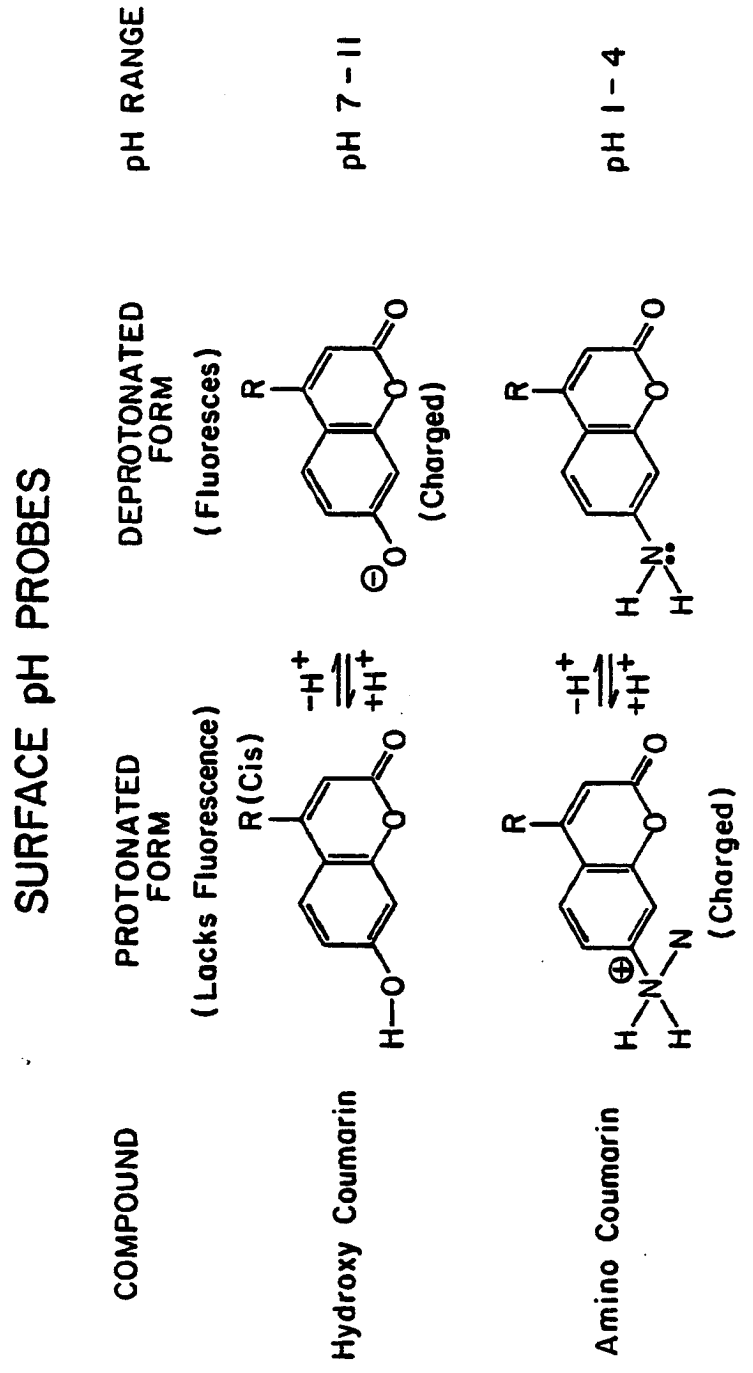
This model depends in its central features upon the protonation of anions at pH's that one would not expect. A reasonable approach to the exploration of the properties of

anionic lipid membranes is to make direct measurements of the two features of such systems that are described above: the pH at the surface (as opposed to that of the bulk phase) and the protonation state of the headgroups.

Early examinations of surface pH have had to rely on soluble (bulk phase) indicators. These indicators were assumed to bind to the surface of charged micelles. Originally soluble indicators with well defined spectra were used because their spectrophotometric equilibrium constants could be determined with high precision (89). Experimental verification of Gouy-Chapman theory using solubilized indicators and anionic detergent micelles was qualitatively informative, but fell short of quantitative agreement with the theory. Murkerjee and Banerjee (90) found that the dielectric constant at the surface, the charge density, and the mole fraction of various counterions all played a role in the determination of the surface potential. However, there was gross underestimation of the surface potential of SDS micelles in this paper and subsequent attempts where soluble indicators were used (91). Although experimentally it can be shown that aromatic type indicators are sequestered by charged (92) or uncharged (93) micelles, the partition into the bulk phase by the indicator creates an apparent decrease in the electrostatic potential of the surface (94).

In 1973, Fromherz (95) introduced a pair of alkylated pH sensitive fluorescent coumarin indicators. In a series of experiments using prepared monolayers of uncharged, zwit-

Figure 4.



terionic, anionic, cationic and various detergent mixtures he was able to demonstrate the sensitivity of these alkylated indicators to the hydrogen ion concentration near the surface (within 1.0nm) of the monolayer headgroups (95, 96).

Fernandez and Fromherz (94) (Figure 4), described the interaction of alkylated hydroxy coumarin and amino coumarin to the surface of uncharged and charged micelles. By using both uncharged acid (4-alkyl-7-hydroxycoumarin) and cationic acid (4-alkyl-7-aminocoumarin) indicators, they were able to differentiate between the charge and the polarity effects on the pKa of these indicators bound to hydrophobic surfaces (94). Pohl (97) has experimentally determined the orientations of these indicators along with several other alkylated indicators and is in agreement with Fromherz (94) that the orientation of the headgroup is ideal for the determination of surface pH phenomena. Recently, Fernandez (98) has used the hydroxy indicators to determine the interfacial potential of PC liposomes containing various mole fractions of phosphatidic acid producing several interfacial potentials.

Carbon 13-NMR is suitable for the investigation of the protonation state of fatty acids and other detergent amphiphiles (99). Previously carbon-13 resonance spectra have been used for conformational analysis (100) based on the changes in chemical shift of various carbons due to substituent effects.

Williamson et al. (101), have extensively analyzed carbon-13 nuclear resonance shifts of carboxylic acids,

their esters and anhydrides in dioxane with a view to develop some simple relationships between shielding and chemical shift. Hagan et al. (102), Batchelor (103), Sperling et al. (104), and Rabenstein (105) have examined the chemical shift dependence of carboxylic acids to the extent of protonation. Approximately a five ppm upfield shift for the carboxylate carbon occurs as a result of complete protonation. Ptak et al. (99) have exploited this property by employing a series of enriched carbon-13 probes to investigate the extent of ionization of fatty acids, amines and n-acylamino acids incorporated into PC vesicles.

We have used both alkylated fluorescent pH indicators and a series of carbon-13 enriched anionic single chain amphiphiles to investigate the surface pH and protonation state of fatty acid and alkyl sulfate liposomes. The results of these experiments are compared to similar experiments on two of the natural bilayer membranes of O. danica: the extra cellular vesicle and the flagellar membrane.

The aims of this thesis can be summarized with the following questions: One, can a model anionic-amphiphile liposome system be developed to study surface interactions due to pH? Two, what is the surface pH of membranes composed of anionic amphiphiles? (What is the relationship of the protonation state of the anionic amphiphile to the surface pH of the membrane?) Three, how do the changes in surface pH and amphiphile protonation state effect the stability

of the anionic membrane (natural or model)?

In order to answer question one, three classes of anionic liposomes (containing cholesterol) were developed: (1) fatty acid, (2) alkyl sulfate, (3) fatty acid/alkyl sulfate.

Our approach in answering question two was to test the suface pH with alkyl coumarin fluorescent indicators. Protonation state measurements were determined with ^{13}C-NMR chemical shift data obtained from synthetic amphiphile ^{13}C-NMR probes.

Question three was explored by examining the formation of model fatty acid liposomes at various pH's. Comparison of surface pH data obtained from the model systems and that data obtained on the natural membrane of O. danica showed that protonation of acidic species is important to the integrity of the membrane.

II. Experimental:

Sources and purity of materials: Cholesterol, O-methyl cholesterol, bromo-cholesterol, chloro-cholesterol, oleyl alcohol, and oleic acid were purchased from Sigma, St. Louis Mo., and are the purist grade. The above reagents were determined to be at least 99 percent pure by TLC and GLC. Thiourea, lithium aluminum hydride, pyridine:SO_3 complex (PST) and H_2O_2 (30 percent) were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, and are the finest grade. They were used without further purification. To avoid water contamination, an unopened bottle (25 g) of PST was used for each synthesis. Dodecanoic acid, 1-^{13}C- (90 atoms percent ^{13}C) was obtained from Merck and Co., Inc. Rahway, New Jersey, and used without further purification. Sodium fluorescein (Fisher Scientific, Pittsburgh, Pa.) was used as is. Coumarin 4 (CAS reg. 90-35-5), hydroxy coumarin and coumarin 311 (CAS reg. 87-01-4), amino coumarin (Eastman Kodak Company, Rochester, N.Y.) are laser grade and were used without further purification after checking with TLC. The alkyl coumarins (4-undecyl-7-hydroxy coumarin (11-HC), 4-pentadecyl-7-hydroxy coumarin (15-HC), and 4-heptadecyl-7-amino coumarin (17-AC) were gifts of P. Fromherz (Max-Planck-Institut für Biophysikalische Chemie, D 3400, Göttingen-Nikolausberg, West Germany). They were repurified by preparative TLC prior to use. All chromatographic and extraction solvents were redistilled and dried over

appropriate desiccants.

Thin layer chromatography: Thin layer chromatograms of alkanols and synthetic amphiphiles (oleyl alcohol, oleic acid etc.) were obtained on analytical plates (Brinkmann, Westbury, N.Y.) and developed in a non-polar (hexane:ether, 70:30) and polar (chloroform:methanol, 80:20) solvent system. Plates were visualized by spraying with 25 percent (W/V) sodium bisulfite in 3 percent (V/V) sulfuric acid followed by heating at 450° C.

Analysis of the TLC for the alkylated indicators suggested that the alkylated hydroxy coumarin (C-15) (R_f=0.65) contained an impurity co-chromatographing with hydroxy coumarin (coumarin 4) (R_f=0.35). Coumarins were visualized by their flourescence on the silica plate when excited by near UV. The 15-HC and the 17-AC were purified on preparative TLC (Brinkman non-fluorescent 25 mm silica plates). The hydroxy coumarin was developed in hexane:ether:methanol: acetic acid (50:50:2:1) and the streak furthest from the origin scraped and eluted in 10 ml of methanol. The amino coumarin was developed in hexane:ether:NH_3 (70:30:1) and the single streak eluted into 10 ml of methanol:chloroform (3:1).

Gas liquid chromatography: All GLC was performed on a Perkin Elmer 881 gas chromatograph equipped with a flame ionization detector. Alkanols and sterols were checked for purity using a 6' stainless steel column containing 10 percent DEGS PS on supelcoport, 80-100 mesh; oleic acid as its methyl ester after reaction with BF_3:methanol ampoule

(Supelco, Inc., Bellfonte, Pa.), was analyzed on a 6' stainless steel column containing 10 percent SP-2330 on supelcoport, 80-100 mesh.

Centrifugation: Centrifugation was performed on an International desk top centrifuge (International Equipment, Boston, Ma.) at 3000 x g for 20 minutes to remove titanium lost from the sonicator tip during sonication of liposomes.

Preparative centrifugation of liposomes, when needed, was performed on a Beckman L2-65B ultra-centrifuge at 40,000 x g for one hour. This generally resulted in the pelleting of precipitates and the formation of a thin liposome band above the aqueous phase.

Sonication of amphiphile dispersions: Dispersions were sonicated on a Branson sonifier (Branson Sonic Power Co., Danbury, Conn.) until no change in optical density at at 450 nm was seen. Optical density (OD_{450}) measurements were performed with a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, New York) Liposome dispersions were sonicated (without temperature control) for 15-20 minutes.

pH measurement of liposome dispersions: Hydrogen ion concentrations in bulk solution were determined with either a Corning digital 110 pH meter (Corning Medical and Scientific, Corning, N.Y.) or a Heath pH meter (Heath/Zenith Instruments Div., Heath Co., Benton Harbor, Minn.) equipped with a pencil combination electrode (Markson Science Inc., Delmar, Ca.)

Quantitative analysis: Cholesterol in liposomes was analyzed using the Lieberman-Burchard reagent (106) by adding the reagent (5 ml) directly to 0.1 ml of a liposome dispersion or column fraction. The total alkyl sulfate or sulfonic acid was determined using the procedure of Kean (107).

Samples prepared for elemental analysis were dried to constant weight over P_2O_5 (Aldrich Chemical Co.) at room temperature. Carbon hydrogen, by combustion at 900-1000° C under oxygen followed by gravimetric analysis, was carried out by Schwartzkopf Microanalytical Laboratories, Woodside, New York.

Natural membranes of O. danica: Extra cellular vesicles (ECV) were provided by E. El Maraghy (86). I. Winicov provided the flagellar membrane using the method of Stern et al. (88).

Interference microscopy: All dispersions were viewed with light optics utilizing an interference microscopy system. This consisted of a Zeiss photomicroscope equiped with Zeiss-Normarski differential interference equipment for transmitted light microscopy (Carl Zeiss, Inc., New York, N.Y.). Interference microscopy (IM) like phase contrast microscopy can be utilized to discriminate between various microscopic phases in a dispersion. David Deamer (Personal Communication) indicated that this system could discriminate between emulsions (phase-bright), oligomellar vesicles (low phase-contrast spheres), and multilamellar

vesicles (phase dark spheres) in essentially the same way as phase contrast. Samples of oleyl sulfate:cholesterol and fatty acid liposomes were compared with photographs of similar dispersions (29). Both systems gave equivalent and easily interpreted data.

Liposome size was determined by dynamic light scattering kindly performed by I. Winicov using the apparatus generously lent by H. Cummins.

Fluorescent spectroscopy: Fluorescent spectroscopy was performed on a Perkin-Elmer (Model MPF-24) fluorescent spectrometer equiped with a no. 43 filter (400 nm emission cutoff). To achieve the lowest baseline and maximum signal intensity the slit widths were varied during the experiment between 6 and 10 nm for the emission and excitation slits. The sensitivity was one. Excitation took place at 366 nm and the emission was recorded between 400-600 nm. An emission peak was seen at 454 nm and was used to measure the degree of dissociation of the indicators (amino and hydroxy coumarins).

^{13}C-NMR spectroscopy: Spectra were obtained with a Varian CFT-20 ^{13}C-NMR spectrometer (Varian Associates, Inc., Palo Alto, Ca.) at 20 Mhz using fourier transform mode and proton decoupling at 100 Mhz. Acquisition times were either 0.8 or 1.6 usec. For the best signal to noise ratio 1,000 to 30,000 transients were collected. Larger, slower rotating liposomes required more transients. The signal was locked to the internal D_2O solvent and all resonances are

downfield from the lock signal.

Synthesis of oleyl sulfate: The entire set-up (Figure 5) was dried and flushed with nitrogen several times. All procedures were carried out under nitrogen. Dry pyridine (100 ml) over BaO was distilled from flask a into flask b. Pyridine:sulfur trioxide complex (PST) (7.4 g) was added to flask b, the reactor. The reaction mixture was stirred and cooled in the reactor (4^{o} C) by immersion into an ice water bath. Oleyl alcohol (8.5 ml) was added to the stirring mixture. The reactants were stirred at 4^{o} for 60 minutes at which point two more grams of PST were added. The reaction was monitored by TLC and was completed in two hours (as demonstrated by a single spot from the reactor that co-chromatographed with SDS).

The slurry of pyridine, unreacted PST and product was poured into a 500 ml round bottomed flask. Chloroform (20 ml) was used to rinse the reactor. Additional chloroform (100 ml) was added to the suspension and warmed with stirring to 55^{o} for 20 minutes. The precipitate (insoluble PST) was filtered away from the amber solution containing the product (pyridinium oleyl sulfate). The solvent containing the product was evaporated in vacuo leaving a gelatinous red syrup. Water (150 ml) was added with gentle swirling; the creamy emulsion was neutralized with NaOH to pH 8.

The aqueous solution was extracted three times with 200 ml of dry butanol. The extracts were combined and

Figure 5. Apparatus for alkyl sulfate synthesis. a: distilling flask for pyridine; b: reaction flask; c: condenser finger; d: ice bath; e: outlet to $CaCl_2$ drying tube: f: 3-way valve for nitrogen or vacuum pump; g: thermometer.

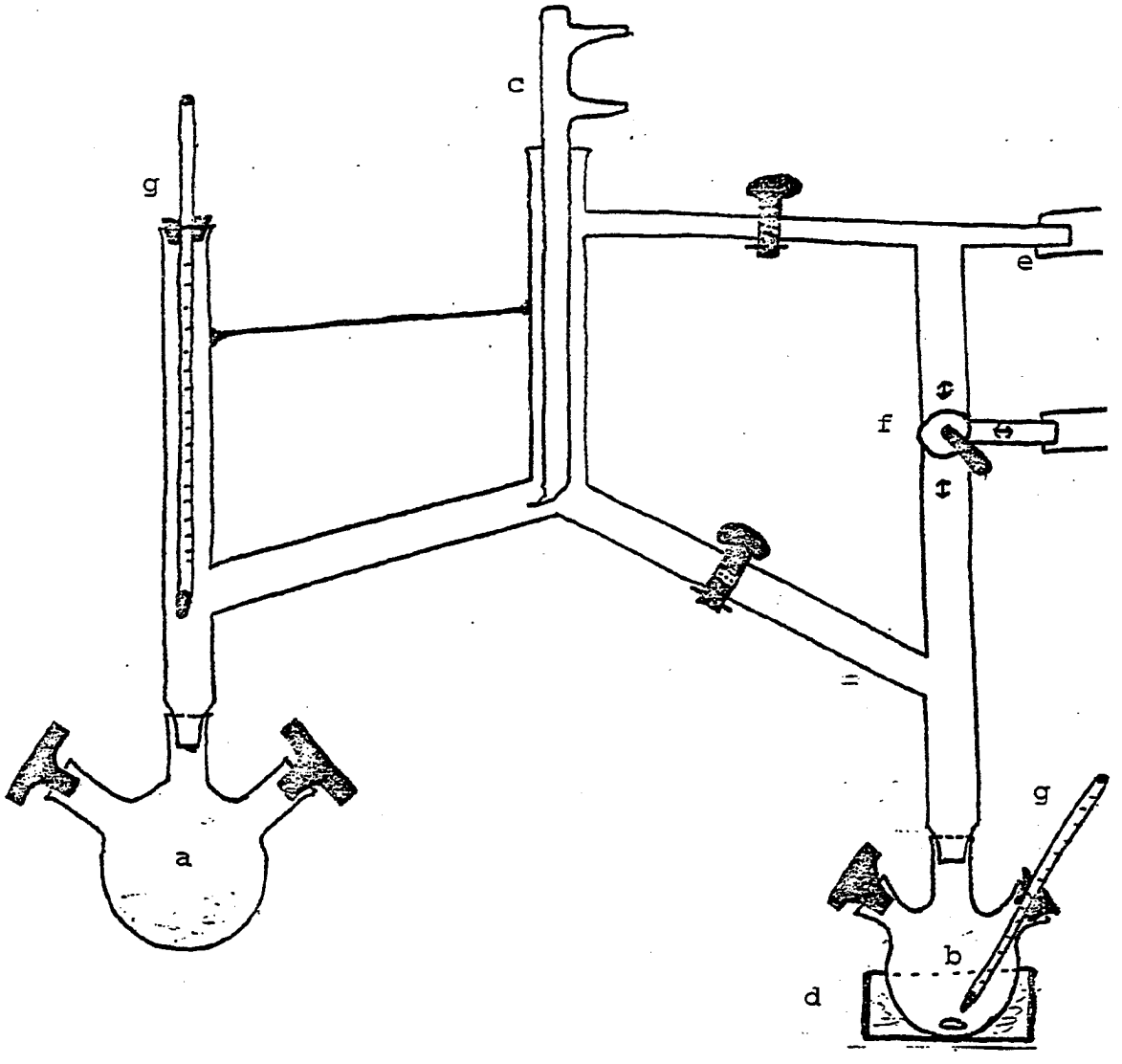


Figure 5.

Apparatus for alkyl sulfate synthesis.

dried (by simply azeotroping off the water) and removed *in vacuo* until crystals just separated out. The crystals were filtered from the mother liquor; the mother liquor was further concentrated *in vacuo* and another precipitate collected. The two were combined and adsorbed solvent was removed at 55° *in vacuo* yielding a crude tan precipitate weighing 10.7 grams. (93.2% yield)

A portion of the crude product (5.1 g) was recrystallized from butanol:water (100:10) after hot filtration. The filtrate was azeotropically concentrated to 20 ml. Another 20 ml of dry butanol was added and the solvent reduced by half *in vacuo*. After cooling to 4°, the white waxy precipitate was collected and dried *in vacuo* at 55° to constant weight, 4.18 g. The total yield for the reaction plus recrystallization was 77%.

Purity was determined with TLC, elemental analysis and quantitative sulfolipid determination (Kean assay (107)). The results of the elemental analysis and the sulfolipid determination are shown on Table I.

Synthesis of protonation probes: Starting material for all enriched carbon-13 probes was 1-^{13}C-laurate. This material was converted to 1-^{13}C-lauryl alcohol by $LiAlH_4$ reduction (108). The alcohol was then converted to either the sulfate or the sulfonic acid. Sulfate synthesis was through the reaction of PST with the alcohol (109). The sulfonic acid proceeded by a conversion of the alcohol to a mercaptan (110) and then oxidation (111) to the acid.

The results of the elemental analysis and the Kean assay are presented in Table I for these compounds.

Synthesis of 1-^{13}C-dodecyl alcohol: Synthesis of 1-^{13}C dodecyl alcohol from 1-^{13}C-dodecanoic acid was based on a procedure outlined by Nystrom et al. (108). All precautions against the involvement of moisture were taken.

A solution of 0.18 g of $LiAlH_4$ in 5.5 ml of ether was added to a 50 ml three necked flask supplied with a reflux condenser, dropping funnel, and mechanical teflon stir-bar. Protection from moisture was provided by flushing with N_2 and calcium chloride tubes. Through the dropping funnel, 0.5 g of lauric acid was added at a rate that produced a constant but gentle reflux. Twenty minutes after the addition had been completed and with continued stirring, two ml of distilled water were added cautiously and in a dropwise fashion to hydrolize any excess unreacted hydride. After addition of the water, 3 ml of ten percent sulfuric acid was added to the flask to decompose the alcoholate. The solution was then filtered and the product isolated by extracting four times with 10 ml of dry ether. After evaporation *in vacuo* of the ether 0.415 ml of lauryl alcohol were obtained. (Yield=83%)

Synthesis of 1-^{13}C-dodecyl sulfate: The same procedure, as outlined for the oleyl sulfate synthesis, was used. Dodecyl alcohol (0.4 g) was reacted with PST (0.8 g) for 2 hours. The unreacted PST was precipitated with chloroform. After removal of the solvent *in vacuo*, the pyridinium do-

decyl sulfate was neutralized with NaOH to pH 8. The aqueous sodium dodecyl sulfate solution was extracted with butanol and the solvent removed in vacuo. After recrystillization from butanol 0.51 g of a white flaky precipitate were obtained. (Yield=100%)

Synthesis of 1-^{13}C-dodecyl mercaptan: Conversion of 1-^{13}C-dodecyl alcohol to dodecyl mercaptan was based on the procedure of Frank and Smith (110).

Thiourea (173 mg), HBr (48%; 760 ul) and dodecyl alcohol (500 ul) were added to a 25 ml round bottomed flask equiped with a reflux condenser and a teflon stir bar. The mixture was refluxed at 100^{o} for 9 hours. Analysis of the reaction mixture at this point revealed that all the alcohol had reacted. The mixture was then flushed with nitrogen and ten percent NaOH (2.7 ml) was added to the flask. After stirring slowly at 100^{o} for 2 hours, the suspension was acidified with HCl to pH 4.

The pinkish oil was extracted three times with 10 ml of ether. The ether extracts were combined and dried over Na_2SO_4 for an hour and then filtered through a short (5 cm) silica column. The dried extract weighed 405 mg (90%). The identity of the compound was confirmed by IR (Hitachi IR spectrometer, Hitachi, Mountain View, Ca.). The purity was determined by TLC and elemental analysis.

Oxidation of 1-^{13}C-dodecyl mercaptan: Performic acid oxidation utilizing the procedure of Hirs (111) was used to convert the mercaptan to the sulfonate.

TABLE I

PURITY OF SYNTHETIC COMPOUNDS

Compound	Elememtal analysis Calculated (%) C	H	Found (%) C	H	Quantitative assay[b] Calculated (mM)	Found (mM)
$C_{18}H_{35}O_4SNa \cdot 3H_2O$[a]	50.94	8.25	49.44	8.01	3.3	3.2±.3
$C_{12}H_{25}O_3SK$	50.70	8.80	51.32	8.91	4.1	4.1±.4
$C_{12}H_{26}S$	71.28	12.87	71.95	12.49	-	-

Table comparing the elemental analysis of synthetic comounds with the calculated values. The sulfate and sulfonate detergents were also analyzed spectrophotometrically with the Kean assay (107). a: hydration assumed from a water content analysis of 6.56 % H_2O (110^{o}); b: aliquots of the detergent were assayed using SDS standards from 1 to 5 mM.

The mercaptan (400 mg) was added to 100 ml of the oxidizing solution (formic acid (88%):H_2O_2 (30%); 90:10). The solution was reacted for 30 minutes at room temperature. A flaky precipitate (tentatively identified as a oxidation product of the disulfide) was filtered. The remaining clear solution was removed in vacuo and a clear oil was obtained. This oil was dissolved in 5 ml of water and the amount of sulfonic acid in solution determined by the Kean assay (107). An equivalent amount of KOH was added to the solution. The potassium salt of dodecyl sulfonic acid was isolated by drying the solution over NaOH and H_2SO_4. Pure sulfonic acid, as determined by TLC and elemental analysis, weighing 284 mg was obtained (50%).

Fatty acid liposomes: Methods were derived from Gebicki and Hicks (28) and Hargreaves and Deamer (29).

Dispersions of fatty acids were prepared in double distilled deionized water in all cases or in specified concentrations of salts as indicated. Either 50 or 100 mM total lipids were used in water to which NaOH had previously been added to give a ten percent excess of hydroxide over fatty acids. This invariably gave pH's in the range of 12. The fatty acid was allowed to dissolve with stirring until a completely clear solution was obtained. The vesicles were produced by the titration of the solution with HCl. At pH 9.6 the solution began to take on a translucent cast. By pH 9.2 it was opalescent and it remained so through pH 8.2. Further titration resulted in a milky dispersion until pH 7

at which point the dispersion began to separate into oil and water phases.

Fatty acid and cholesterol liposomes: Preparations of these liposomes have appeared in the literature (28). Dispersions were made using the dissolved oleic acid at pH 12. Fifty mole percent cholesterol was added dry in aliquots to a rapidly mixing 10 ml vial of the oleic acid solution. The mixtures were then sonicated for 10 to 20 minutes at 50 W power with a titanium microtip in the sonicator. Without sonication (with only vortexing) some crystals of undissolved cholesterol remained. Sonication produced dispersions that were "smooth" to the eye.

All dispersions became opalescent even at high pH (to 12) when cholesterol was added. Sonicated dispersions were viewed by interference microscopy. Typical dispersions that were titrated to pH 7 showed no apparent change in the optical density.

Addition of other sterols to fatty acid solutions: Three sterols with groups substituted for the 3B-OH group of cholesterol (O-methyl-cholesterol, chlorocholesterol, and bromocholesterol) were used in an attempt to form liposome dispersions from fatty acid solutions at pH 12 through 7.

Between 10 and 50 mole percent of the above sterols were added to fatty acid solutions containing enough fatty acid to give a final concentration of 50 mM total lipid. The suspensions were then mixed for five minutes on a Vortex Genie (Scientific Industries, Inc., Springfield, Mass.) at

maximum power. Three pH ranges were studied: above 9.5, between 9.5 and 7.0, and below 7..

At high pH (above 9.5) preparations contained a flaky precipitate in a clear solution. Between pH 9.5 and 7 the mixture appeared opalescent with a flaky precipitate. Below pH 7 the solution was milky with precipitate.

Sonication at 50 W for 15 minutes (no temperature control) solubolized the precipitate at any point along the titration curve. Solutions became milky and contained no apparent precipitate. However, liposomes could only be seen in the dispersions between pH 7 and 9.5. The other systems contained only oil droplets when analyzed by interference optics.

Portions taken (after ultracentrifugation of each of the preparations) from the precipitate and the supernatent were analyzed by TLC for lipid content.

Sodium oleyl sulfate and cholesterol liposomes: Dispersions of 50 mole percent cholesterol containing 50 mM total lipid were prepared in unbuffered water. Sodium oleyl sulfate (SOS) was added to the appropriate amount of water in a glass vial and allowed to dissolve. When the solution was crystal clear, powdered cholesterol was added in small aliquots while the solution was stirred with a teflon stir bar. The dispersion became opalescent and was stirred at ambient temperature for 30 to 60 minutes until most of the cholesterol appeared to be dispersed. At this point a sample was viewed by interference microscopy to

determine if bilayer forms and liposomes were present. The pH was taken and the suspension was then sonicated. The dispersions were centrifuged to remove titanium (from the sonicator tip) before they were used in other experiments.

Fatty acid, cholesterol and SOS liposomes: Liposomes were formed containing 20 mM oleic acid, 20 mM cholesterol and 10 mM SOS according to the following procedure.

Cholesterol was added to SOS at pH 7. The dispersion was vortexed and sonicated. The dispersion was centrifuged to remove undissolved lipid and titanium (from the sonicator tip) and the upper phase was carefully pipetted away from the pellet and transferred to another vial. The dispersion was then titrated to pH 5.0 and the fatty acid added slowly while stirring the dispersion. The dispersion remained opalescent and the fatty acid was apparently incorporated into the SOS:cholesterol dispersion. The system was then titrated to pH 12 (with NaOH) without any noticable change in opalescence. Phase contrast microscopy revealed small oligomellar liposomes during various points along the titration; no oil droplets were seen. Back titration to pH 2 showed no change in OD_{450} until pH 2.5, at which point the dispersion became quite turbid (due to the formation of oil droplets).

Column chromatography of liposomes: Sonicated dispersions of SOS and cholesterol were chromatographed on a 30 x 1 cm Sephadex G-50 column in order to determine if the bulk ratios of cholesterol to SOS reflected the composi-

tion of the separated liposome fraction.

Dispersions of 50 mM total lipid containing 30, 50 and 70 mole percent cholesterol were prepared in 1 mM Tris buffer titrated with HCl to pH 7.4. Each dispersion was chromatographed by layering 0.5 ml of the dispersion on the column and eluting with Tris buffer. Thirty 1.8 ml fractions were collected and assayed for the presence of liposomes (optical density of the solution at 450 nm indicated the presence of liposomes) and the quantity of cholesterol and SOS present.

Volume trapping experiment: A solution containing 0.2 percent sodium fluorescein (NaF) was prepared in a buffer of 1 mM Tris and 100 mM NaOH. A test dispersion (50 mM total lipids; 50 mole percent cholesterol) of SOS:cholesterol liposomes was prepared in three ml of the NaF solution. This dispersion (0.5 ml) was chromatographed on a 30 x 1 cm G-50 Sephadex column. Fractions (1.8 ml) were collected by eluting the column with NaF free buffer corrected for osmolarity with 0.09 percent NaCl. Each fraction was monitored for liposomes with optical density measurements and for the presence of NaF. The presence of NaF was determined by measuring the total fluorescence with a Turner III fluorescent fluorometer (360 nm primary filter; 415 nm secondary filter) (Turner Designs, Mountain View, Ca.).

A control dispersion was prepared in the NaF free buffer. The dispersion was centrifuged at 50,000 x g for

one hour after which the aqueous phase was siphoned away from the upper liposome band. The NaF solution was added (3.0 ml) to the remaining liposomes and 0.5 ml of the dispersion chromatographed. Fractions were collected and tested for the presence of liposomes, SOS and cholesterol.

The same procedure was used to test the 30 and 70 mole percent cholesterol to SOS dispersions. Only the liposome fraction was tested for NaF fluorescence.

Surface pH measurements with the coumarin indicators: Descriptions of the experimental method for surface pH determination using these indicators are available (94, 95 96). Both the hydroxy and the amino coumarin indicators fluoresce when deprotonated. The intensity of emmission is directly proportional to the degree of deprotonation (94). A more complete discussion of their chemistry may be found in the results section.

Amino and hydroxy coumarins were treated as a pair and were used sequentially in each system unless otherwise specified. Non-alkylated indicators (300 nM) were titrated in water (3 ml) to determine the bulk phase pK's of the indicators. Three types of solutions (3 ml) containing the alkyl coumarin indicators were examined: one, micelles of SOS (50 mM) (indicator:lipid ratio, 1:30,000); two, model liposome dispersions (50 mM total lipid) (indicator:lipid ratio, 1:30,000) of either (a) oleic acid and cholesterol (1:1 mole ratio), (b) SOS:cholesterol (1:1 mole ratio), or (c) oleic acid, cholesterol and SOS (2:2:1 mole ratio);

three, the natural membranes of O. danica (200 ul of wet pellet suspended in 3.0 ml of water) (150 nM indicator) of either (a) the extra cellular vesicles (ECV) or (b) the flagellar membrane.

Solutions containing the hydroxy coumarin were titrated (with NaOH) to pH 12; solutions containing the amino coumarin were titrated to pH 7. Typically, measurement of the fluorescent intensity of the indicator was performed during the back titration of the solution (with HCl).

Carbon-13 NMR Spectra: ^{13}C-NMR titrations were performed on: one, acetic acid (0.5 M); two, 1-^{13}C-dodecanoic acid in oleic acid liposomes; three, 1-^{13}C-dodecanoic acid in cholesterol:SOS liposomes (1:1 mole ratio cholesterol to SOS); four, 1-^{13}C-dodecyl sulfate (5 mM, 50 mM); five, 1-^{13}C-dodecyl sulfonic acid (4 mM, 20 mM). Liposomes were 50 mM in unenriched lipid and 10 mM in the enriched laurate probe. All dispersions were formed in D_2O and titrated with either NaOD or DCl in the 10 mm quartz NMR tubes containing one ml of sample.

III. Results:

Fatty acid liposomes: A comprehensive examination of the experimental methods for the synthesis and the laboratory manipulation of these liposomes has already appeared in the literature (28). In summary, free fatty acids form, in general, oligolamellar, large liposomes containing entrapped water; they are semipermeable. They appear to be similar to PC vesicles with respect to freeze fracture or electron microscopy.

The free fatty acid liposomes we used were formed by titration of micellar solutions (25-50mM) of oleic acid with HCl. Three separate phases were noted during a titration from pH 12 to 6: the first, above pH 9.2, the clear solution contains micelles; the second, between pH 9.2 and 7.2 is an opalescent solution; the third, below pH 7.2 is a milky suspension. The second (opalescent) solution may be viewed with interference microscopy. It shows large oligolamellar liposomes approximately 1 to 10u in diameter. The third (milky) suspension shows a large number of smaller oil droplets (1.0nm dia.) when viewed with interference optics. Inflection points are noted at each of these two phase changes (between the first and second, and between the second and third phases). (See Figure 13). The apparent pKa between the micellar and liposome forms was 9.2; that between the liposome and the oil droplet suspension was 7.15. The association of a pKa with a phase change implies that a protonation (or deprotonation) is associated with the phase

change. Furthermore, the obsevation that two pK's are associated with a single carboxyl group is surprising.

Fatty acid and cholesterol liposomes: Hargreaves and Deamer (29) and Gebicki and Hicks (28) have described these dispersions in detail. Upon addition of cholesterol to a micellar solution of oleic acid at any pH above 9.5 immediately turns the solution opalescent. The opalescence is due to the formation of large oligomellar liposomes.

These liposomes form at pH 12 (higher pH's were not studied). Below pH 7 the solution abruptly changed from an opalescent dispersion to a milky solution. Interference microscopy revealed that below pH 7 the emulsion was entirely oil droplets.

The titration of oleic acid from pH 12 to pH 7 may be viewed as a single titration of the oleic acid carboxylate, despite the observation that two apparent pK's are seen as the liposomes form and are dissolved. When viewed as a single titration the pK is 8.5. This result is similar to that of Peters (112) in 1931 who examined the apparent pK's of fatty acids at benzene:water interfaces. Such a high pK (8.5) for a carboxylate which is normally between 4 and 5 is best explained in both systems by the polyanionic surface and the Gouy-Chapman theory. In contrast to the oleic acid liposome titration the oleic acid:cholesterol system does not exhibit inflection points at pK's 9.2 and 7.2. The absence of these inflection points is consistent with the absence of phase changes. The overall pK remains 8.5 in

this system.

Addition of other sterols to fatty acid solutions: The addition of O-methyl cholesterol, 3-chlorocholesterol or 3-bromocholesterol (to 50 mole percent) to fatty acid solutions at any pH between 7 through 12 did not produce liposomes. After stirring fatty acid with either of these sterols a flaky precipitate formed with a clear supernatant. Interference microscopy showed that the precipitate consisted of particles and crystals of undissolved sterol. Sonication of the suspension produced a milky dispersion at pH 12. This dispersion consisted of oil droplets when viewed immediately under interference optics. Titration from pH 12 to 8 produced liposomes. However, TLC of the liposome fraction showed that these liposomes consisted solely of fatty acids since it revealed no sterol in the liposome fraction.

Sodium oleyl sulfate (SOS) and cholesterol liposomes: Hargreaves and Deamer (29) described the formation of alkyl sulfate liposomes in the presence of alkanols. It was reasonable that sterols would be uniquely appropriate as alkanols. Thus addition of cholesterol to solutions of SOS above the CMC produced large oligomellar liposomes (1-5 μm dia.). Sonication of these liposomes produced a dispersion invisible to light microscopy. Although this implies that small (100nm dia.) liposomes or vesicles are formed, the preparation was not further investigated. Prolonged incubation at either room temperature or 45°C (a treatment that

provoked SDS:dodecanol dispersions to form larger (10μ dia.) liposomes) did not result in larger liposomes. This property of the liposomes appears to be characteristic of cholesterol as an alkanol. Indeed, the dispersions appeared stable for weeks and were routinely produced several days before specific experiments were conducted on them.

Experiments were conducted to determine: liposome size, the capacity of the liposomes to spontaneously alter the mole fraction composition during formation, and their capacity to trap dyes. The results of these experiments are summarized in Table II. Dynamic light scattering was employed to determine the size of the liposome dispersions to within 10 A. The 50 and 70 mole percent cholesterol dispersions had many characteristics in common including the same diameter, whereas the 30 mole percent differed (e.g. Column D) and was slightly smaller (Column E).

Chromatography revealed several properties of these dispersions. In general, the liposomes displayed the same mole fraction as that of the dispersed lipid from which they were formed except for the 30 mole percent liposomes. In the latter preparation the percentage cholesterol spontaneously increased slightly during the liposome formation. The total amount of SOS in each dispersion was not in the liposome fraction, but a significant amount (25 percent) was chromatographed as small molecular weight aggregates. The cholesterol was found only in the peak associated with the liposome fraction and not in any other eluted fraction.

Figure 6 shows the elution profiles of the liposomes and of the micelle fraction (+). Cholesterol was not found in any fraction after fraction 6. Figure 7 shows the relationship of the optical density (OD) (450nm) to the fraction number. The micelle peak is shown for comparison. Note that no OD 450nm is seen for the secondary peaks shown in Figure 6. This is due to the fact that the particle size is of the order of magnitude of micelles.

All dispersions trapped sodium fluorescein in the liposome fraction. Figure 8 shows a typical elution profile for fluorescein fluorescence for the 50 mole percent cholesterol dispersion. Dispersions to which the fluorescein was added after liposome formation did not show any dye entrapment or adsorbtion.

Fatty acid, cholesterol and SOS liposomes: Liposomes of this type have not previously been described. Their appearance is generally similar to fatty acid liposomes although they are stable to very low pH. Fatty acid:cholesterol:SOS liposomes were typically formed at pH 5.5. The absence of oil droplets at pH 5.5 demonstrated that the fatty acids were incorporated into the liposomes. The pH was then adjusted to 12 so that a titration could be performed that could be compared to the typical fatty acid liposome titration. The titration curve that was obtained was virtually identical to that seen for the fatty acid cholesterol liposomes. No phase change was seen however until pH 2.5. At this point a slight but observable inflec-

TABLE II

CHOLESTEROL:OLEYL SULFATE LIPOSOMES

A	B	C		D	E	F
Mole % cholesterol in original dispersion	Average mole % cholesterol[a] in fractions from peak I (void volume)	Percent total SOS[b] in fractions from: peak I Liposome	peak II Micelle	Ratio A/B x 100	Liposome diameter (Å)	Trapped volume[c]
37.4	45.2±3.5	65.0	26.1	120.8±9.4	744±10	+
54.0	52.1±4.1	60.0	25.4	96.5±7.5	818±5	+[d]
72.3	70.0±4.6	69.1	25.2	96.8±6.3	808±6	+

Table summarizing the composition, size and capacity to trap fluorescein of cholesterol:SOS liposomes after chromatography (G-50 Sephadex). Two peaks were observed. Peak I (void volume) consisted of liposomes (OD at 450 nm); peak II consisted primarily of small micelles.(See Figures 6,7.) Diameter measurements (Column E) were determined by dynamic light scattering. Column D is a measure of the capacity of liposomes to spontaneously alter the mole fraction composition during liposome formation. a: determined by Lieberman-Burchard reaction; b: determined by Kean (107) assay; c: determined by flourescence of trapped fluorescein; d: results of this experiment are shown in Figure 8.

Figure 6. Elution profile of liposomes from a G-50 sephadex column. Fractions (1.8 ml) were collected from a 30 x 1 cm column. A portion (0.5 ml) of a 30 (●), 50 (■) or 70 (▲) mole percent cholesterol (50 mM total lipids) of a cholesterol:sodium oleyl sulfate (SOS) dispersion was chromatographed. Twenty-five mM (0.5 ml) SOS (✱) was chromatographed as a micelle control. The fraction number is compared to the percent total SOS added to the column found in each fraction. Note that OD_{450} was only found for the liposome fractions 3-5, and not for the micelle fractions. The cholesterol content of these fractions was also measured but is not shown. The cholesterol remained totally within the liposomes and was not found beyond fraction 6.

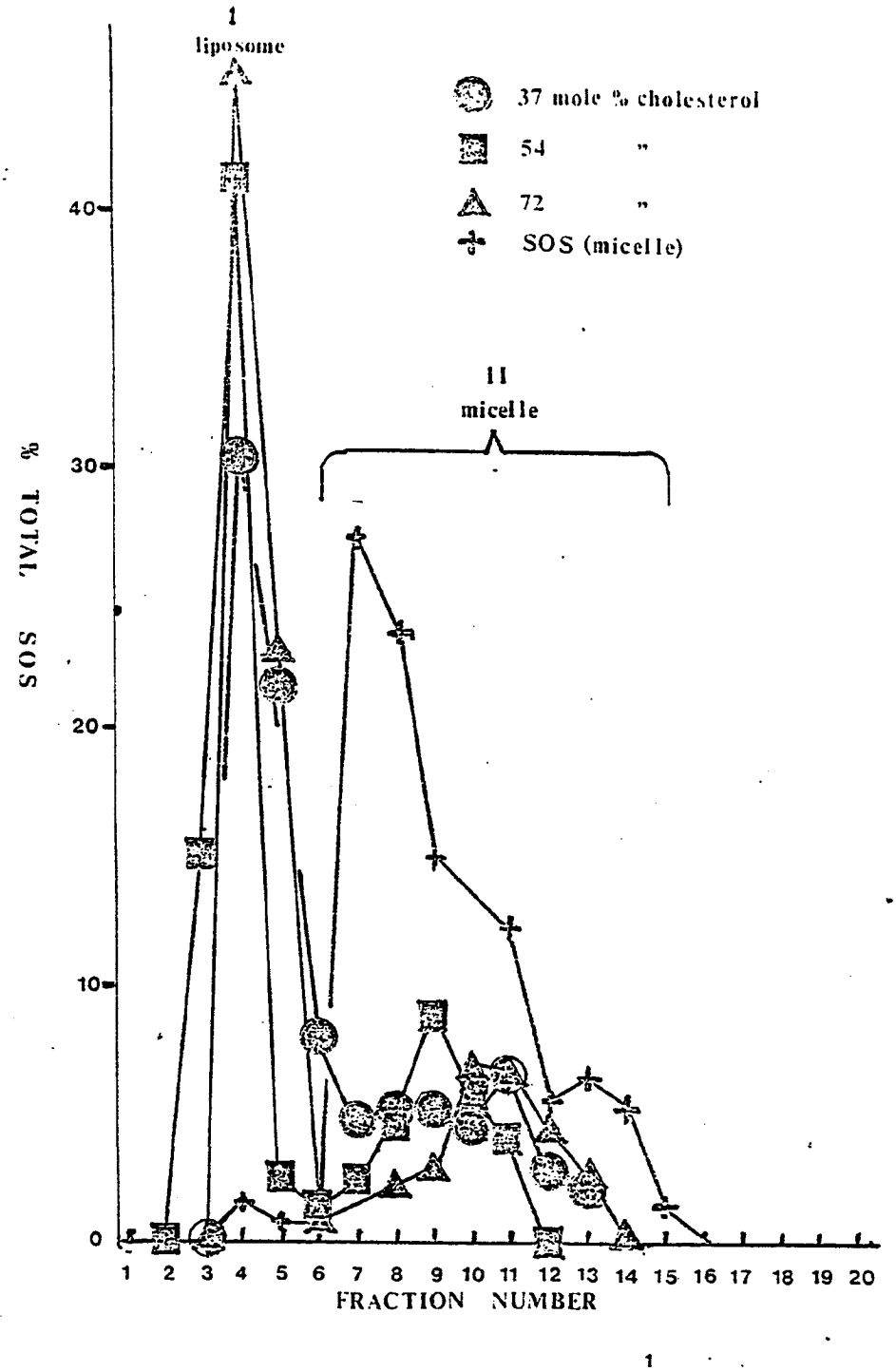


Figure 7. Elution profile of liposomes from a G-50 sephadex column. Fractions (1.8 ml) were collected from a 30 x 1 cm column. A portion (0.5 ml) of a 30 (●), 50 (■), or 70 (▲) mole percent cholesterol (50 mM total lipids) of a cholesterol:sodium oleyl sulfate (SOS) liposome dispersion was chromatographed. A profile of the OD 450 nm (left hand scale) versus the fraction number for the liposome dispersions is compared with 25 mM SOS (0.5 ml) micelle profile (+) (right hand scale).

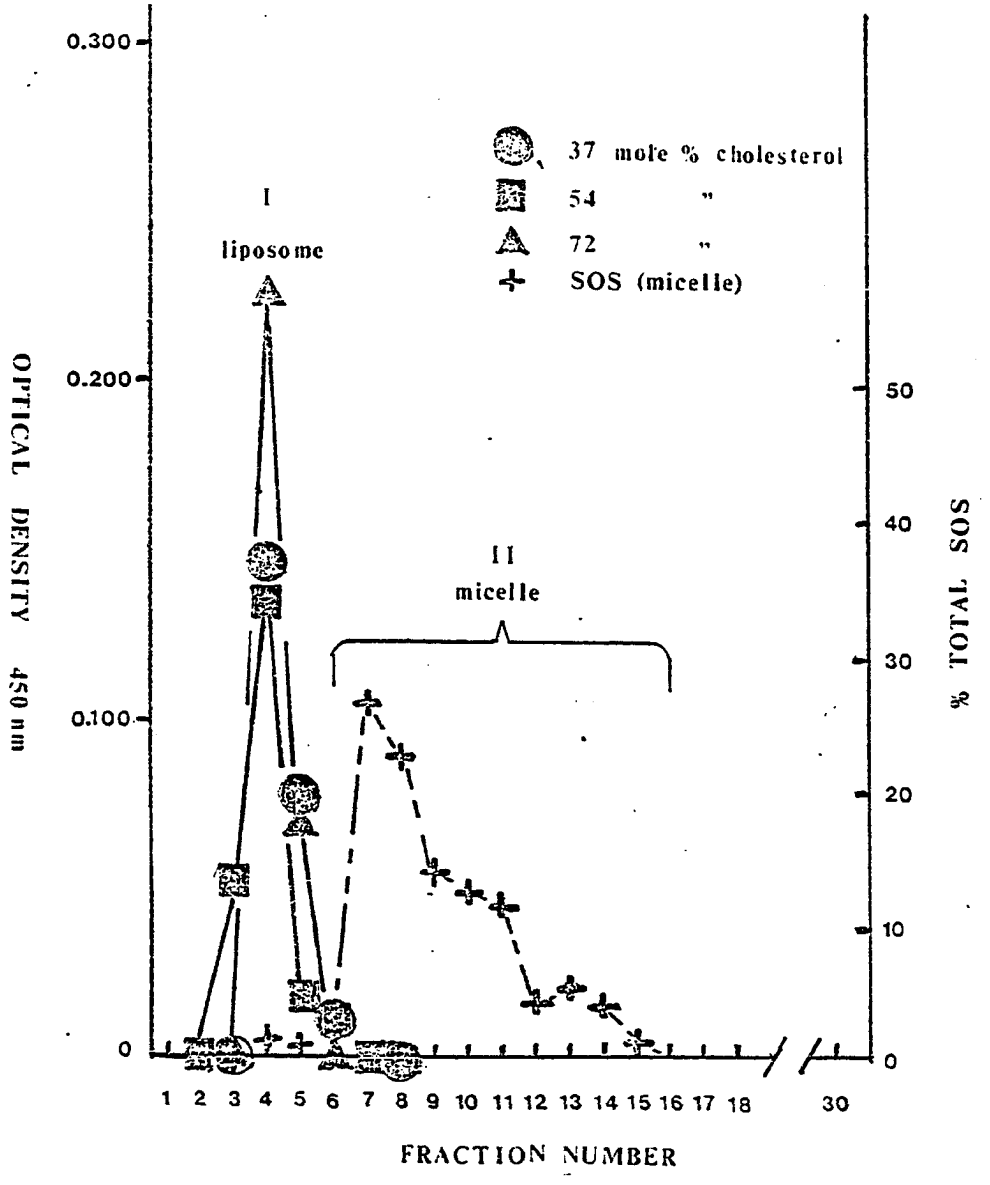
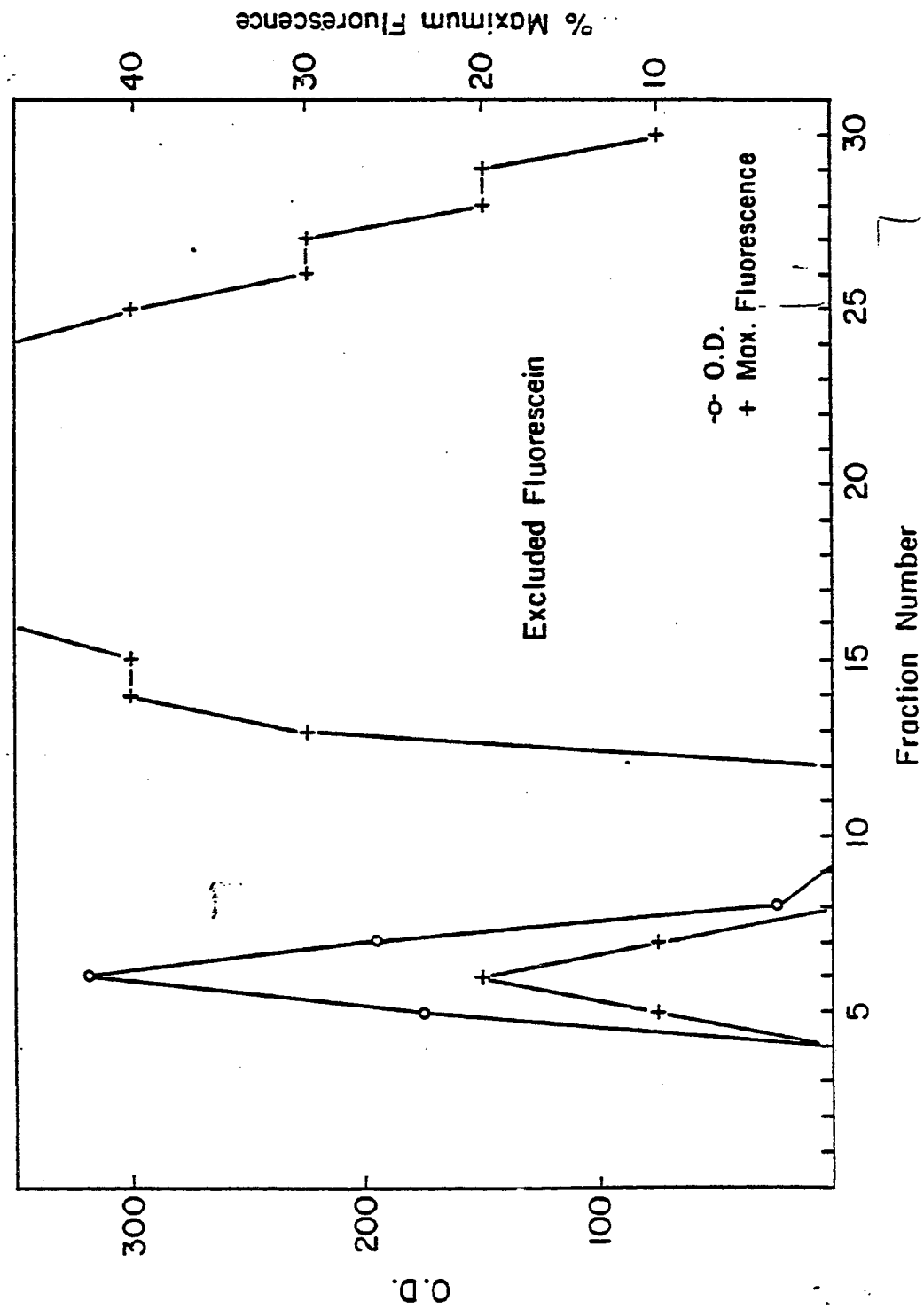


Figure 8. Elution profile of liposomes from a G-50 sephadex column. Liposomes were formed in a buffer containing 0.2 percent sodium fluorescein (NaF). The OD 450 nm (left hand scale, o) is compared with the total fluorescence of NaF (right hand scale, +) for each fraction. Fluorescence data is corrected for liposome absorbances.



tion was noted. Below pH 2.5, the dispersion became an oil droplet suspension as observed with interference microscopy.

Alkyl coumarin indicators: The alkylcoumarin indicators were the kind gift of P. Fromherz (95). Previously he had shown that these probes can be used to effectively measure the pH at the surface of the membrane and thereby the surface potential (within the Stern layer (94)). By using the indicator pair hydroxy coumarin (an uncharged acid) and amino coumarin (a cationic acid) (see Figure 4) he has been able to discriminate between two features of the system: the first is the pKa shift due to the charge on the alkylated probe and its interaction with the low dielectric at the interfacial region ($\epsilon = 32$) and the second is the electrostatic effect (pH at the surface or surface potential) which is due to the charges of the lipid headgroups that constitute the membrane (94). It was necessary to eliminate the former so that the latter could be productively exploited.

One can discriminate between these two effects because there is a difference in the free energy of binding to lipoid surfaces between the protonated and deprotonated forms of the indicators. A shift in pKa is exhibited by either indicator species in a neutral surface, which is directly related to this difference in free energy. The dissociation of the uncharged acid (hydroxy coumarin) produces a charged species (anionic) in the headgroup region. This is thermodynamically unfavorable. The dissociation of

the cationic acid (amino coumarin) produces a neutral species the binding of which is thermodynamically favorable at lipoid surfaces. In neutral or uncharged systems Fernandez and Fromherz (94) have shown that the pKa shifts of these two indicators are equal and in opposite directions. The hydroxy coumarin undergoes an apparent pKa shift toward higher pH's; while the amino coumarin undergoes an apparent pKa shift toward lower values. Each shift is compared to those of the soluble coumarins in water alone. (See Figure 9.) These shifts in the opposite direction are due to the interaction of the alkylated probe with the low dielectric.

The electrostatic effect, on the other hand, which reflects the surface pH (or surface potential), results in a pKa shift in SDS micelles, for example, that is in the same direction for both indicators; the pKa shifts toward higher values than that of the neutral Triton-X-100 system (94). By subtracting the pKa shift due to the neutral system (the low dielectric binding effect) from the total shift found for the charged lipid headgroup system it has been shown that the electrostatic effect on the pKa is equal and in the same direction for both indicators (94). We have verified the results of Fernandez and Fromherz (94) who used SDS micelles. For example, amino coumarin in aqueous solution displays a pKa of 2.35, whereas the pKa of the alkylated form in Triton X-100 is 1.25. It has thus shifted <u>downward</u> 1.1 pH units. In contrast the pKa of hydroxy coumarin in water is 7.75, whereas the alkylated

form of this indicator displays a pKa of 8.85 so that it has undergone an upward shift of 1.1 pH units due to its binding to the low dielectric. Since we will consider the electrostatic pKa shift for the indicator that is bound to the low dielectric, our calculation of the ΔpH at the membrane surface (as compared to that of the bulk phase) is based on the pKa of the indicator in the bound form. For amino coumarin we use 1.25; for hydroxy coumarin we use 8.85.

Negatively charged lipid surfaces will be expected to shift the pKa of each indicator upward provided the charge density at the surface and the ionic strength are the same at pH 1.25 and 8.85. Positively charged surfaces (which are unknown in natural membranes) shift the pKa symmetrically downward. In the case of SDS micelles the apparent pKa's of alkylated amino coumarin and hydroxy coumarin are 3.55 and 11.15 respectively. This implies that the surface of SDS micelles has a pH that is 2.30 units below that of the bulk phase both at low pH (3.55-1.25= 2.30) and at high pH (11.15-8.85=2.30).

Because the difference (ΔpH) between the pH in the bulk phase and the pH at the surface is due (in part) to the charge density at the surface, it is possible to convert the ΔpH directly to the surface electrostatic potential which is expressed in millivolts. This is done by using the relationship:

$$\psi = \frac{-2.3\ RT}{F} \cdot \Delta pH \qquad (1)$$

thus ψ (in millivolts) is the surface potential; R is the gas constant; T is the temperature (Kelvin) and F is Faraday's constant.

The results of a similar titration using SOS are shown in Figure 9. It may be seen that the ΔpH in the low pH range (amino coumarin) is 2.35 pH units whereas that of the upper pH range (hydroxy coumarin) is 1.65 units. This difference of 0.65 pH units in the SOS micelle as compared to no difference for the SDS micelle implies a difference in charge density at the surface since the ionic strength was the same as that of the SDS in both experiments. It is interesting to note (Table III) that the SOS-containing liposomes display the same anomaly.

Figure 10 displays the titration curves for Triton X-100, SOS:cholesterol liposomes, oleic acid:cholesterol:SOS liposomes, and the natural membrane extracellular vesicles (ECV) from O. danica. It has been noted above that the SOS:cholesterol vesicles give identical pKa shifts to those of the SOS micelles. All three systems exhibit pKa shifts toward higher pH's compared to those of Triton X-100 for the alkyl hydroxy coumarin indicator in the high pH range. It may be seen from this figure and also from Table III that the ΔpH for the fatty acid-containing SOS liposomes has a greater ΔpH at high pH than it does at low pH. Similar results are obtained for the natural membrane (ECV). The

TABLE III

SURFACE pH MEASUREMENTS

System	Indicator I (R-AC) pKn = 1.25[94]			Indicator II (R-OH) pKn = 8.85[94]		
	pKa	ΔpH	ψ(mV)	pKa	ΔpH	ψ(mV)
micelle (SDS)[94]	3.55	2.3	-134	11.15	2.3	-134
micelle (SOS)	3.60	2.35	-137	10.5	1.65	-95.9
liposome: SOS:cholesterol	3.50	2.25	-131	10.5	1.65	-95.9
liposome: oleate:SOS	-	-	-	10.7	1.8	-106.4
liposome: oleate:SOS: cholesterol	1.26	0.01	-0.6	10.3	1.45	-84.3
natural membrane: extra cellular vesicles	1.0	-0.25	+14.6	9.63	0.78	-45.3
natural membrane: flagellar	1.05	-0.20	+11.6	10.0	1.15	-66.8

Table comparing the shift of the alkyl coumarin pKa in a variety of model systems and the natural membrane of O. danica. The pKa refers to the apparent pK of the indicator in the membrane; the pKn refers to the apparent pK of the indicator in a uncharged (Triton X-100) membrane (polarity component). The pH is the difference, pKa - pKn, and is equivalent to the pH shift due to the surface charge (electrostatic component). Surface potential in mV is derived from the relation: $\psi = -2.3RT/F \times \Delta pH$.

Figure 9. The percentage dissociation of the pH indicator versus bulk pH. The graph compares titrations of the indicator pair hydroxy coumarin (closed markers) and amino coumarin (open markers) hydrophobically bound (alkylated indicators) to either neutral micelles of Triton X-100 (94) (broken line) or anionic liposomes (SOS:cholesterol; 1:1 mole ratio; 50 mM total lipid)(triangles) with non-alkylated, water soluble amino or hydroxy coumarin (circles).

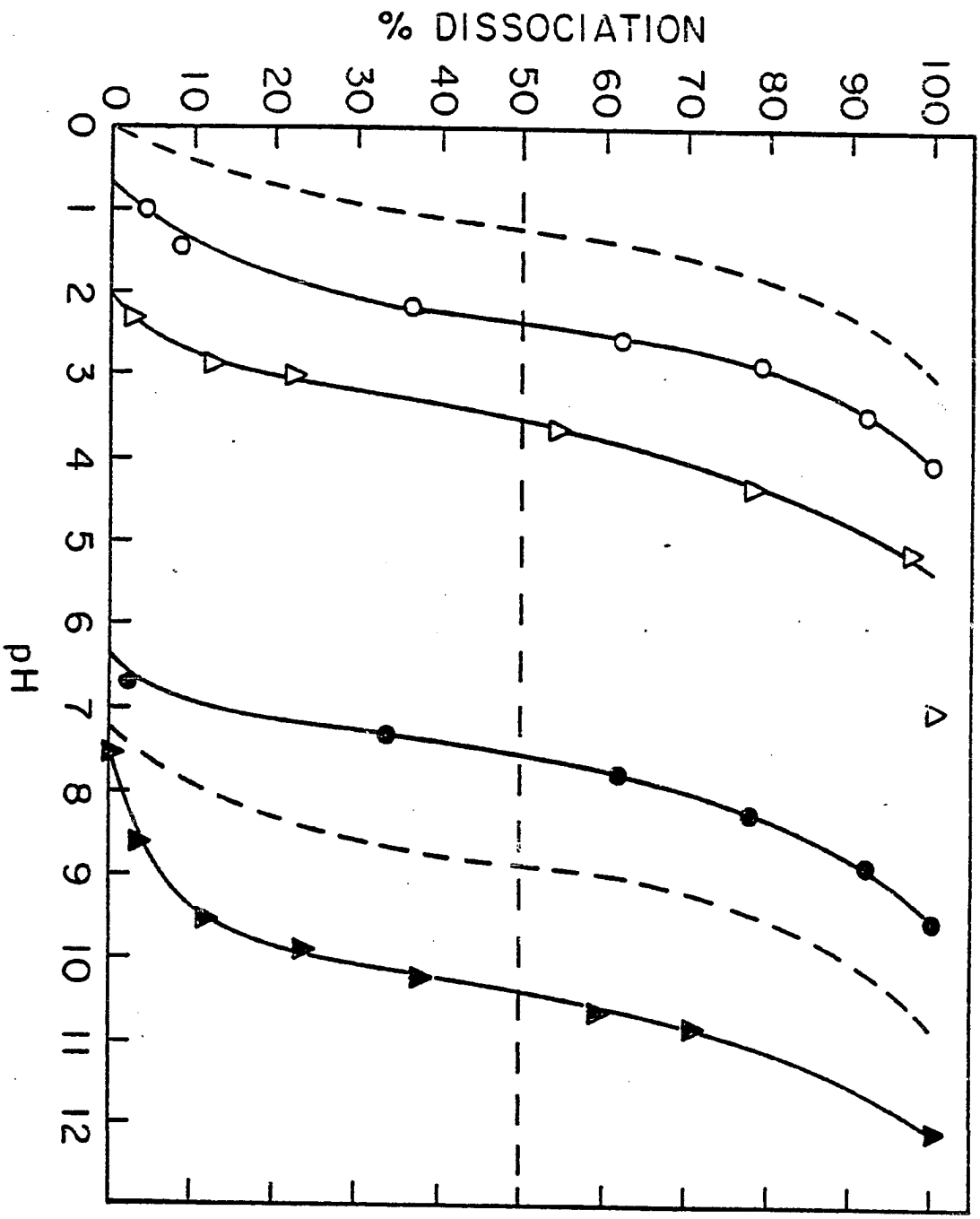


Figure 10. Percentage dissociation of pH indicator versus bulk phase pH. The graph compares the titrations of the indicator pair, alkylated hydroxy coumarin and amino coumarin bound to Triton X-100 (uncharged) micelles (94) (broken line); anionic liposomes containing sodium oleyl sulfate and cholesterol (1:1 mole ratio, 50 mM total lipid) (triangles); anionic liposomes containing oleic acid, cholesterol and sodium oleyl sulfate (2:2:1, 50 mM total lipid) (squares); and extra cellular vesicles (ECV) (circles). Open markers refer to amino coumarin, closed to hydroxy coumarin.

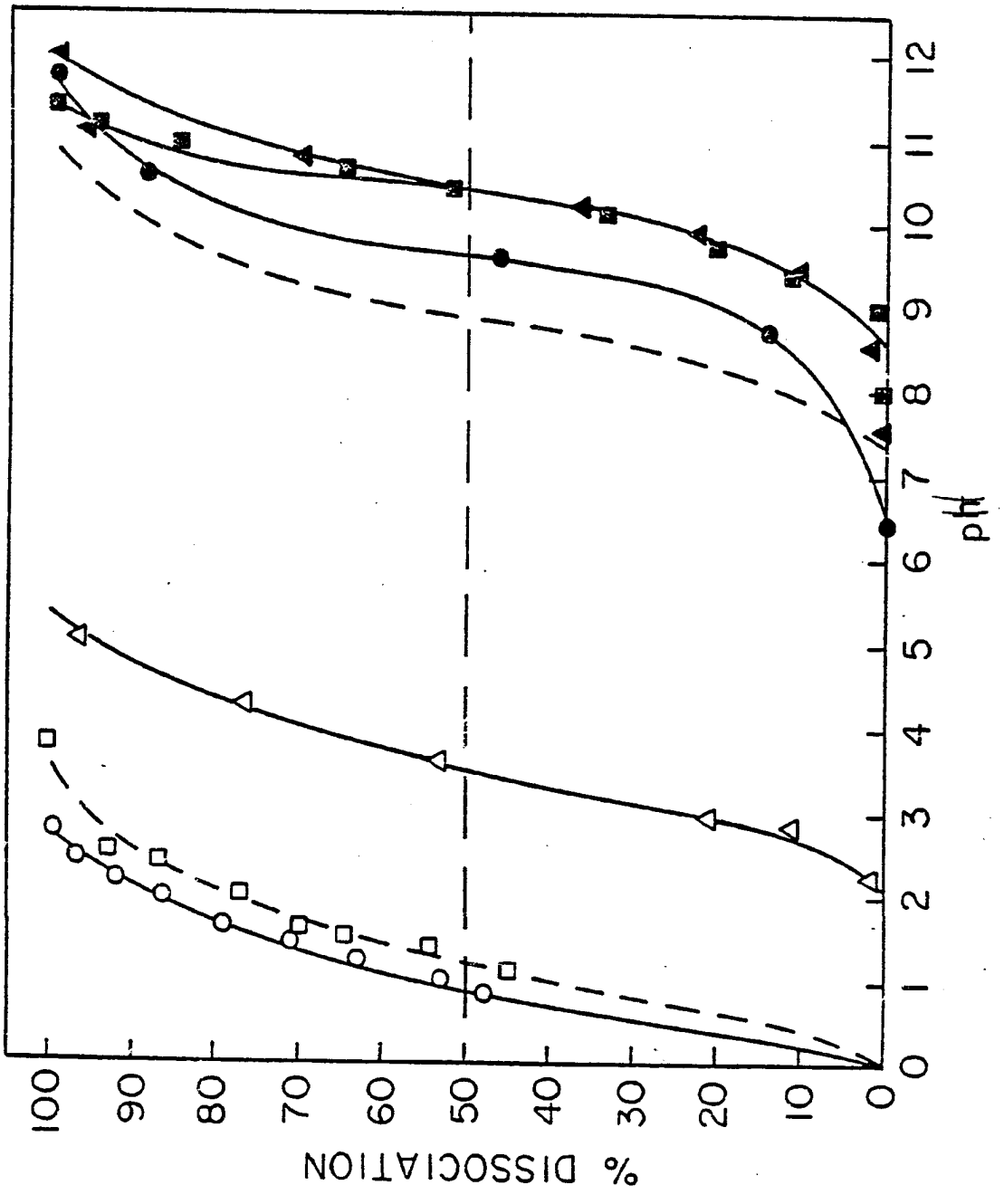
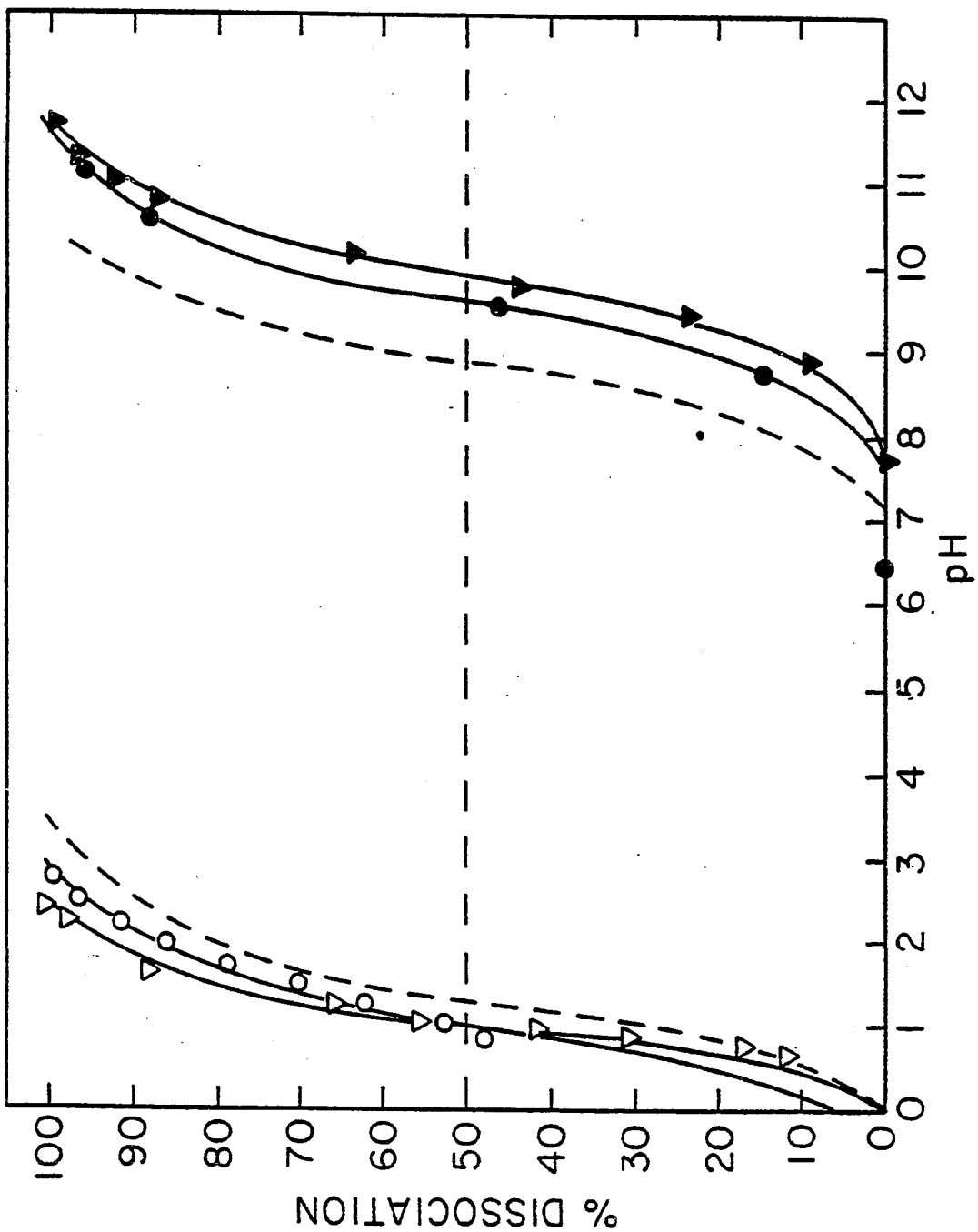


Figure 11. Percentage dissociation of the pH indicator versus bulk pH. The graph compares the indicator pair hydroxy coumarin and amino coumarin hydrophobically bound to uncharged micelles of Triton X-100 (94) (broken line); extra cellular vesicles (ECV) (circles); and flagellar membrane (triangles). Open markers refer to the amino coumarin, closed to the hydroxy coumarin.



difference in pK between these two is within the range of experimental error.

Figure 11 compares the two natural membrane preparations of O. danica, the ECV and the flagellar membrane (both alkyl sulfate membranes) with Triton X-100. Both of these natural membranes and the fatty acid-containing SOS liposomes exhibit the same pKa shift towards the pKa of the neutral membrane in the low pH region. The pKa shift of the flagellar membrane in the upper pH range is slightly greater than that of the ECV, a difference (0.38 pH units) which may be considered significant.

^{13}C-NMR titrations: Titrations are used to determine the change in pH that accompanies the addition of acid (or base). A pK is noted as an inflection point in which a protonation reaction can be followed. This occurrence of inflection points at both ends of our fatty acid liposome titration provoked a direct examination of the state of protonation of the carboxylate during liposome formation and dissolution.

^{13}C-NMR is uniquely useful for this kind of examination. Figure 12 compares an NMR titration of acetic acid to that of 1-^{13}C-laurate in oleic acid liposomes and oleyl sulfate: cholesterol liposomes. Pertinent chemical shift data and the derived pKa's for acetic acid and 1-^{13}C-laurate are displayed in Table IV. Acetic acid was used as a model for monomeric fatty acids. It displays a pKa of 5.0 which is over three pH units below those of the fatty acid aggre-

gates because it lacks a surface. The liposome dispersions, oleic acid and SOS:cholesterol gave pKa's for the laurate of 8.6 and 8.2 respectively.

Figure 13 compares the titration of oleic acid liposomes with the NMR titration of 1-^{13}C-laurate in oleate liposomes. The phase changes correspond to pKa inflections along the titration curve. The pKa of 8.6 corresponds to the midpoint of stability for fatty acid liposome formation. Note that liposome formation (phase change I) begins at 20 percent protonation and oil droplet formation (phase change II) at 80 percent protonation. The chemical shifts found on protonation of the carboxylate are approximately 5 ppm.

The NMR titration of 1-^{13}C-dodecyl sulfate above and below its CMC (7mM) is shown in Figure 14. The monomer form (5mM) exhibits a pKa of 1.9, whereas a pKa of 0.0 is found for the micelle form (50mM). Total chemical shifts were only 0.75 ppm for the monomer and 0.30 ppm for the micelle. The deprotonated chemical shift (downfield from the D_2O lock signal) of the monomer is 81.20 ppm and the micelle 80.71 ppm (pH 10). Titration data below 0.0 pH is unreliable because of destabilization of the D_2O lock signal. It should also be noted that 0.0 pH means 1 Molar DCl.

An NMR titration was attempted for 1-^{13}C-dodecyl sulfonic acid. No changes in chemical shift were seen to pH 0. Below pH 0, perturbations of the D_2O lock signal made evaluation impossible. The sulfonic acid exhibited chemical shifts of 62.64 (4mM) below the CMC (9mM), and 62.83 (20mM)

above the CMC.

TABLE IV

^{13}C-NMR CHEMICAL SHIFTS AND pKa'S OF CARBOXYLIC ACIDS

System	pH	Chemical shift (ppm)*	Δcs** (ppm)	pKa determined by this titration
acetic acid	12.50	193.0	-	
	5.20	191.0	2.0	5.0[a]
	1.44	188.2	4.8	
lauric acid in	12.35	195.2	-	
oleic acid	8.03	192.3	2.9	8.6[b]
liposomes	7.41	190.8	4.4	
lauric acid in	10.65	194.8	-	
SOS:cholesterol	7.7	192.7	2.1	8.2[c]
liposomes	2.47	188.1	6.7	

Table showing selected ^{13}C chemical shifts for acetic acid and lauric acid. The pH in the upper range was considered to be the deprotonated state, the lower pH is the protonated, and the mid-range pH was selected as one that was near the 50 percent dissociation of the acid. *chemical shift was calculated from the D_2O lock. **Δcs is equal to the change in chemical shift compared to that of the fully deprotonated carboxylate. a: determined from 6 points; b: determined from 8 points; c: determined from 3 points.

Figure 12. ^{13}C-NMR titration of acetic acid (500 mM) (open triangles); 1-^{13}C-laurate in oleic acid liposomes (50 mM oleate, 10 mM laurate) (open circles); and 1-^{13}C-laurate in sodium oleyl sulfate:cholesterol liposomes (25 mM SOS, 25 mM cholesterol, 10 mM laurate) (closed squares). The percent dissociation of the acid as a function of the portion of total chemical shift is plotted against the bulk pH.

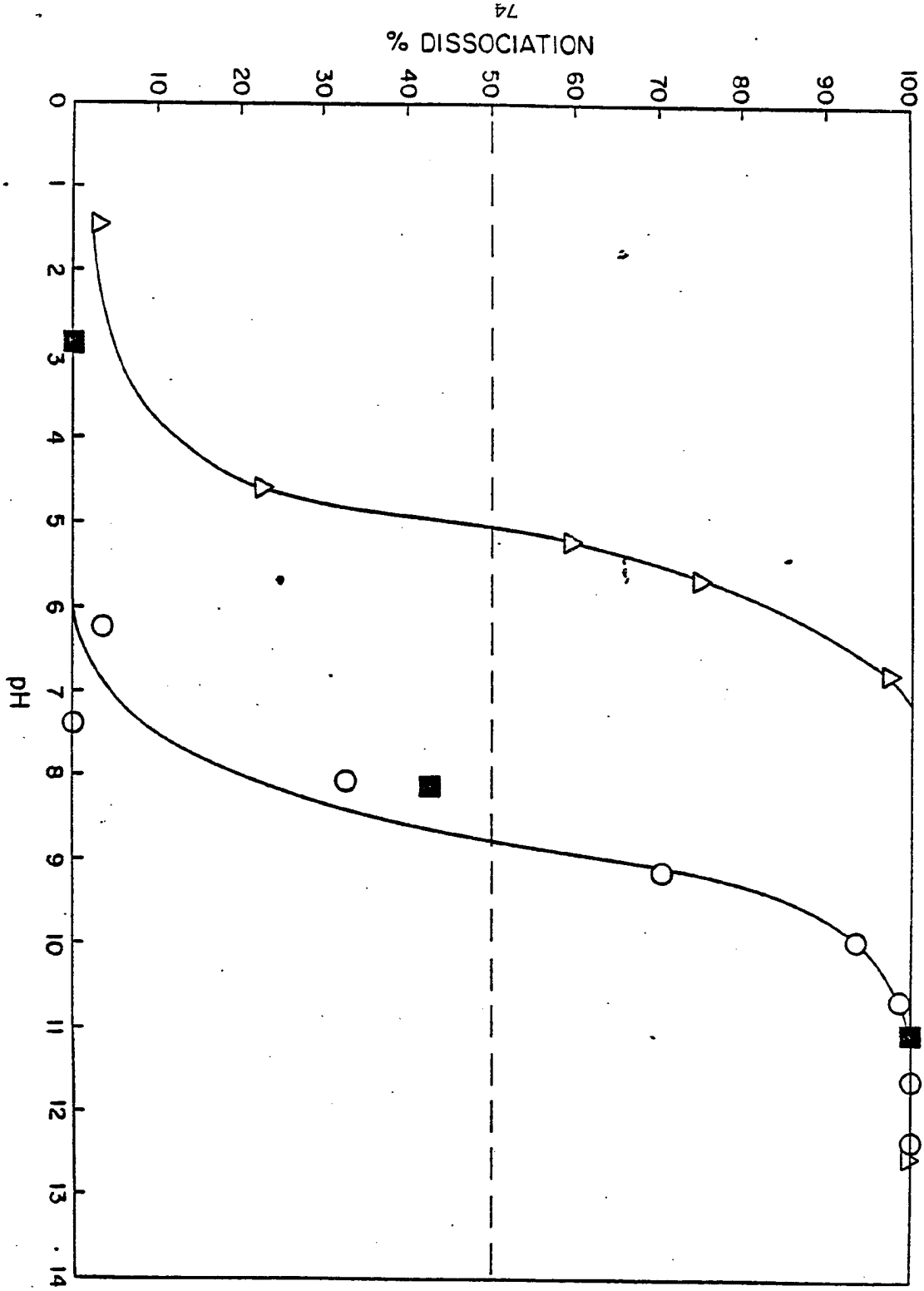


Figure 13. ^{13}C-NMR titration of a dispersion of 50 mM oleic acid and 10 mM 1-^{13}C-lauric acid from pH 12 to pH 6. The percent of total chemical shift (Δppm) is directly proportional to the percent dissociation of the carboxylate. The percent total shift is plotted against bulk pH (open triangles). For comparison, a titration plotting the percent of total HCl required for complete protonation versus 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. These inflections have also been shown for lauric acid liposomes (29). The pK of the fatty acid titration as estimated by NMR is 8.6. Stable liposomes are found between 9.2 and 7.2.

The titration curve was modeled (broken line) using the Henderson-Hasselbach relation for the pKa values shown above. The following relation was used to model the curves up to one pH unit away from the pKa.

$$(X) = \text{antilog}\ (pKa-pH)/1 + \text{antilog}\ (pKa - pH)$$

(X) represents the proportion of HA^- when the pKa is 9.45, or the propotion of H_2A when the pKa is 7.15. Deviations from the calculated curve probably derive from factors assoated with the phase transition from micelles to liposomes (phase change I).

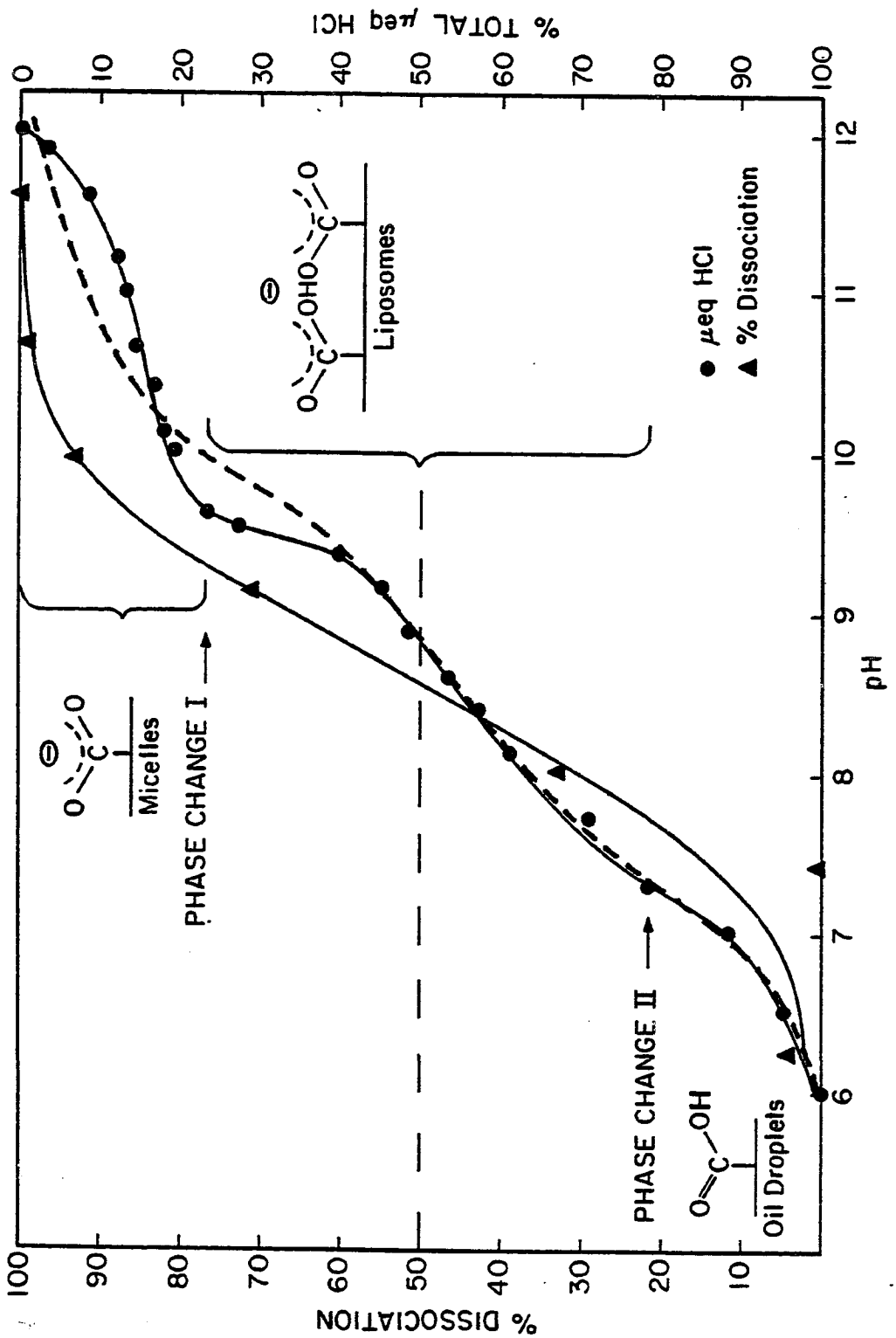


Figure 14. ^{13}C-NMR titration of SDS (1-^{13}C-dodecyl sulfate) above (50 mM) and below (5 mM) the CMC (7 mM). The percent dissociation of the acid as a function of the portion of total chemical shift is plotted against the bulk pH.

IV. Discussion:

Formation of anionic single-chain amphiphile-liposomes: The introduction of model bilayer systems to explore the structure and dynamics of biological membranes is one of the most significant advances in the membrane field in the past twenty years. These model systems have been used to show the wide variety of molecular aggregations that membrane lipids may assume. It has been assumed that single chain amphiphiles did not form liposomes that entrap an aqueous compartment, because all natural lipids that form liposomes contain two hydrocarbon chains per headgroup. Water-soluble amphiphiles (detergents or soaps) were considered incapable of such aggregation; they generally formed micelles above the critical micelle concentration. The finding of Gebicki and Hicks (26) in 1973, that unsaturated fatty acids readily formed liposomes by simple titration of a micellar solution was therefore unexpected. The requirement for an unsaturated chain provoked them to call these liposomes, "ufasomes". It was Hargreaves and Deamer (29) that placed this work in the context of the membrane bilayer with the observation that liposomes may be formed of any amphiphile if the aggregate is above the transition temperature (Tm).

Perhaps the most useful feature of single-chain amphiphile liposomes is their marginal stability. The fact that fatty acid liposomes form around pH 9 from micelles, and are destroyed (forming oil droplets) below approximately pH 7

allows the experimentalist to examine individual features of the membrane lipids (and other components) as stabilizing or destabilizing with respect to the liposome. Cholesterol and analogues of this sterol were studied with this in mind.

These liposome studies were conducted (1) to study amphiphile liposomes as experimental model systems, (2) to design a model system with which the Ochromonas danica chlorosulfolipid-membrane could be explained, and (3) to explore the state and role of anionic lipids in biological membranes.

Fatty acid liposomes: Previous work by Gebicki and Hicks (28) had shown that oleic acid liposomes could be formed from solutions of the acid at moderately alkaline pH's (between 8 and 9). They had been successful in demonstrating the impermeability of these liposomes to several hydrophilic molecules (proteins, sugars, and etc.) and had concluded that the unsaturation was responsible for the ability of fatty acids to form liposomes. Hargreaves and Deamer (29) conducted similar experiments with lauric acid liposomes. By adjusting the temperature of solution to slightly above that for the transition from crystal (gel) to liquid crystal lauric acid, they were able to form liposomes similar in all respects to those described by Gebicki and Hicks (26, 28).

Hargreaves and Deamer (29) suggested that formation of lauric acid liposomes (above the Tm) was dependent on the formation of a protonated carboxylate. They viewed this

formation as analogous to the addition of dodecanol as an uncharged alkane "spacer" molecule equivalent to the protonated carboxylate. Titrations of dodecanoate dispersions revealed inflections at approximately pH 8.4 and 6.4 (for the liposome above the Tm). Interestingly, titrations of crystals of dodecanoate (below the Tm, in the gel state) had inflections at higher pH's for both the upper (pH 9.6) and the lower (pH 7) plateaus.

In this work we have confirmed the formation of multilamellar liposomes from titration of oleic acid micellar solutions (at pH 12). Moreso, we have demonstrated that the titration curve of oleic acid (above its CMC) contains two inflection points. As in the laurate dispersion (29), the upper inflection (pH 9.5) corresponds to the formation of liposomes and the lower inflection (pH 7.2) to their disruption (as oil droplets).

The titration curve can also be viewed as one for a monoprotic acid and an average pKa of 8.5 can be estimated for oleic acid (above the CMC). The increase of the apparent pKa's found for oleate as compared to those for laurate are not surprising when one considers the difference in chain length (and therefore the strength of both the hydrophobic effect and van der Waals interaction) between the two fatty acids. Heikkila et al. (113) previously had shown that increasing chain length corresponded to an increase of apparent pKa for fatty acid monolayers. (He found an apparent pK of 8.3 for oleic acid.)

Additionally, by simultaneously monitoring the titration of oleic acid liposomes with ^{13}C-NMR we have demonstrated that these inflections correspond to 20 percent (pH 9.5) and 80 percent (pH 7.2) protonation of the carboxylate. This data is consistent with the formation of protonated species that can exchange protons rapidly in the form of acid-anion dimer complexes.

The introduction of sterols to fatty acid liposomes: Gebicki and Hicks (28) had shown that cholesterol could be added (up to 50 mole percent) to oleic acid liposome dispersions. Since they did not form liposomes from micelles (pH 12) of oleic acid by titration, the formation of liposomes from micelles by the addition of cholesterol went unnoticed. Hargreaves and Deamer (29) were able to demonstrate that addition of dodecanol to dodecanoate solutions at pH 12 (micelle) spontaneously induced the formation of dodecanol:dodecanoate liposomes. These liposomes appeared to be identical (by phase contrast microscopy) to the fatty acid liposomes formed solely by titration. They concluded that the alkanol acted as an uncharged chain "spacer" filling the gap left in chain packing due to the headgroup repulsion. This result is not surprising when one considers that the addition of stearyl alcohol to stearate monolayers (pH 12) produces a large condensation effect (114) despite the apparent charge repulsion due to the anionic headgroups. In fact, the decrease in molecular area found for the stearate chain after the addition of the alkanol was equivalent to

the monolayer at pH 9 (114) (approximately the pKa of stearic acid in the monolayer (115)).

A possible rationale for this behavior comes from a theory proposed by Haines (116) in which he suggests that the monolayers in liposome bilayers are stabilized by a balance between the repulsive (electrostatic repulsion of negative headgroups) and attractive (acid-anion, H-bonding) features of the amphiphiles. The stability of the headgroup region enhances the attractive interactions of the lipid tails by the exclusion of water and the enhanced ordering of the chains to carbon nine.

Although specific interactions involving the H-bonding of the B3-OH group of cholesterol to proton acceptors on adjacent lipids have been ruled out (117, 118 , 119) much evidence exists to suggest that it is essential for the stability of the bilayer (120, 121, 122). In order to create a liposome dispersion uninfluenced by a possible H-bonding (or other unspecified) interaction between the B3-OH of cholesterol and the carboxylate (of the fatty acid) we attempted to form liposome dispersions with non-hydroxy cholesterol analogs (chloro, bromo, and O-methyl). Experiments conducted showed that these sterols could not combine with fatty acid micelles to produce liposomes; nor could they be incorporated into preformed fatty acid liposomes. This was in sharp contrast to the ability of cholesterol to induce liposome formation.

This inability to stabilize bilayer formation was not

expected and further suggests that the specific orientation and presence of the B-OH (or an orientation forced on an alkanol) can stabilize the headgroup region of the fatty acid bilayers. This is consistent with previous studies that demonstrated that the a-hydroxy and keto forms of cholesterol do not condense PC monolayers (123).

The form of the titration curve for cholesterol:oleate or dodecanol:dodecanoate (29) which is that of a weak monoprotic acid (no inflections) also suggests that acid-anion formation (shown by the two pKa's for the free fatty acid dispersion) has been masked by H-bonding occurring between the alcohol and the acid. The increased hydrophobic interactions due to the insertion of the sterol nucleus into the bilayer may also segregate acid-anion dimers. This would remove the inflection due to acid-anion formation from micelles and only the second protonation would be seen. The complete protonation produces oil droplets below pH 7 and is not easily seen as an inflection upon titration.

Alkyl sulfate liposomes with cholesterol: Previously Hargreaves and Deamer (29) demonstrated that inclusion of dodecanol (up to 70 mole percent) into SDS micellar solutions results in the spontaneous formation of liposomes. Sonication of these dispersions, they believed, disrupts the large liposomes and creates mixed micelles which subsequently re-form into multilamellar liposomes. The average diameter of the liposomes increases with the mole percent of dodecanol.

Using a highly purified synthetic detergent, oleyl sulfate, and cholesterol (up to 70 mole percent) we have been able to form (after sonication) small (800 A dia.) liposomes containing a trapped volume. Larger liposomes did not spontaneously form after incubations of up to 4 weeks suggesting an unusually stable system.

We were able to determine that liposomes tended to reflect the mole ratios of lipids used to form them from the bulk solution. However, the dispersions tended to be in equilibrium with smaller aggregates of SOS (up to 25 percent of the SOS). A possible explanation is that mixed micelles of SOS and cholesterol are present. They are unstable and the process of chromatography destroys them, leaving the cholesterol on the column and chromatographing the SOS aggregates.

The introduction of fatty acids to alkyl sulfate liposomes: The Ochromonas danica cell membrane is composed of alkyl disulfates, sterol, and fatty acids (73, 86). In order to study the effect of pH on the natural system we developed a model liposome system containing fatty acids incorporated into the SOS:cholesterol dispersion. The inclusion of SOS into the fatty acid:cholesterol system remarkably reduced the pH (pH 2.5) at which the liposomes formed oil droplets. Contrast this with the destruction of oleate: cholesterol liposomes below pH 7. This enhancement of stability to lower pH is probably due to the presence of charged sulfate headgroups which become protonated at pH 2.5.

Studies on anionic single-chain amphiphile liposomes: The titrations of Hargreaves and Deamer (29) on fatty acids pointed out clearly the presence pf two limiting pH's that were associated with liposome formation and dissolution, respectively. Two apparent pK's associated with the single carboxylate is striking. Hargreaves and Deamer (29) explained the requirement of acid to convert micelles into liposomes by invoking "spacer" molecules to reduce the charge density at the anionic surface. The carboxylate anions in this view, are separated by protonated carboxylates so that a planar array of lipids can replace the spherical or cylindrical micelles. Likewise, it was proposed that protonation of the headgroup anions was accompanied by oil droplet formation. The first question to be asked in understanding this system (See Figure 13.) is, "Why are there two pK's?" The second question is, "Why are these pK's both above 7 when the pK of the sole carboxylate is less than 5?"

Fatty acid liposomes display two inflection points: formation and dissolution: Let us set the first question aside until later and consider the second. The answer to the second question is best found in electrostatics; the pH at the surface is below that in the bulk phase (measured pH). The carboxylates which are protonated at the surface in a range above their typical pK are in a local pH environment that is unexpectedly low. It is not known, however, just how low because direct measurement has not been possible. The use of pH indicators to examine the surface

pH makes this information accessible. McLaughlin (43) showed that the maximum theoretical pH difference between that of the bulk phase and that of the surface, at the greatest charge density possible, based on a single charge per chain, is three pH units. This calculation (See Figure 15) was consistent with the data of Peters (112), who in 1931 showed that the apparent pK of fatty acids in a benzene:water system is 8.5, just over three units above 4.8 for the same carboxylate in aqueous solution. The difference (one pH unit) between the 8.5 minus 3.0 (=5.5) and 4.8 is attributed to the tendency of fatty acids to form fully protonated dimers in benzene. The stability of these dimers would raise the "apparent" pK of the carboxylate, it was believed. The answer to the second question is simply that the surface pH is below that of the bulk phase pH.

It was surprising to find on measurement (Table III) that the pH at the surface of fatty acid liposomes as measured by the alkylated hydroxycoumarin was 1.8 pH units (10.7 -8.9) below that of the bulk phase. This is 1.2 pH units less than predicted theoretically and widens the difference between the apparent pK (8.5) and the bulk phase pK (4.8). It suggests that the tendency to form fully protonated dimers in the hydrocarbon (for Peters, the benzene) phase is greater than expected. It may account for about two pH units. It also implies that the charge density at the surface is considerably below the maximum. Indeed the charge density calculates (43) to less than one charge for

two hydrocarbon chains as may be seen in Figure 15.

The charge density calculation is thus consistent with for the presence of protonated carboxylates ("spacers") at the surface of the fatty acid liposomes. Experiments using the chemical shift of 1-^{13}C-laurate likewise showed directly that the carboxylates of the liposomes contained both protonated and deprotonated species as predicted by Hargreaves and Deamer (29). Indeed the NMR experiments demonstrate further that liposomes form from micelles when approximately 20 percent of the carboxylates are protonated and they are converted into oil droplets when approximately 80 percent of the carboxylates are protonated. Both lines of evidence (the charge density calculation based on surface pH measurements and the ^{13}C-NMR chemical shifts) indicate that these liposomes require both protonated and deprotonated headgroups for stability.

Finally, the titration itself (Figure 13) shows that both the formation of the liposomes at pH 9.2 and the dissolution of the liposomes at pH 7.2 are accompanied by inflection points. The former (at low ionic strength) is more prominent than the latter (at higher ionic strength).

The acid-anion as an explanation of the two inflection points: These inflection points demonstrate that the formation and dissolution of the liposomes are each associated with some sort of protonation event. Since the protonation must be of a carboxylate (the only funtional group present) it is reasonable to consider that the protonation and depro-

Figure 15. Graph comparing the ΔpH (pH at the surface as compared to the bulk phase) found for the alkyl coumarin indicators hydrophobically bound to the surface of various amphiphile systems with the theoretical calculated charge density (in charge per square angstrom). The charge density σ, is calculated from the derived surface potential using the Gouy-Chapman relationship:

$$A\sigma/\sqrt{c} = \sinh(ze\psi_o/2kT)$$

where

$$A = 1/(8N\epsilon_r\epsilon_o kT)^{\frac{1}{2}}$$

N = Avogadro's number; ϵ_r = the permeativity of the solution; ϵ_o = the permeativity of free space; k = Boltzman's constant; T = ^oK; z = charges per electrolyte; e = sign of the charge; ψ_o = the potential in mV; c = concentration of buffer in moles per liter (0.05 M).

The ΔpH is converted to potential (mV) using the relationship:

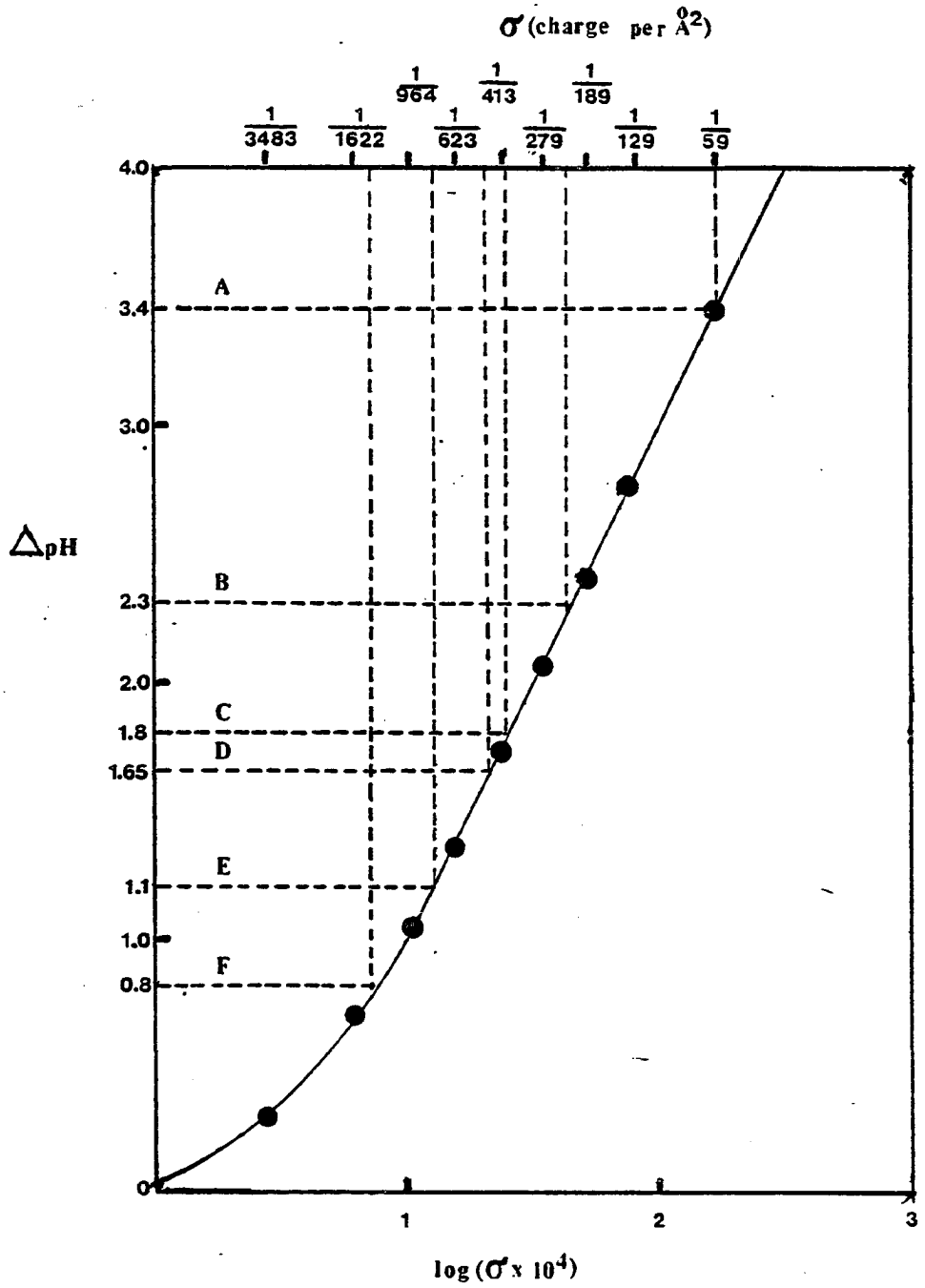
$$\psi_o = -2.3RT/F \times \Delta pH$$

R = the gas constant; T = ^oK; F = Faraday's constant.

The observed ΔpH's (from Table III) for various amphiphile systems are shown with their equivalent charge densities calculated from the Gouy-Chapman relationship: A, the maximum ΔpH (or surface potential) for a charge density equivalent to one charge per two alkyl chains; B, the calculated charge density at the maximum ΔpH measured for SOS or SDS micelles, and SOS:cholesterol liposomes; C, the cal-

culated charge density at the ΔpH found for cholesterol: oleate liposomes (at high pH); D, the calculated charge density at the ΔpH found for the SOS micelle or SOS:cholesterol liposome at high pH (hydroxy coumarin indicator); E, the calculated charge density at the maximum ΔpH (high pH) measured at the flagellar membrane surface; F, the calculated charge density at the maximum ΔpH measured at the ECV membrane surface (high pH).

Note that none of these highly charged systems exhibit the maximum theoretical ΔpH for a maximally packed dialkyl lipid which would occupy a 59 A^2 area. For example, the SDS system with a ΔpH of 2.3 is equivalent to a single charge per <u>five</u> monoalkyl uncharged amphiphiles. The implication is that the indicators are not measuring the total surface pH, but are neglecting the protons sequestered by the headgroup region.



tonation events are of an aggregated form of the carboxylate. The suggested form is the acid-anion dimer elaborated in the Introduction. Tanford (31) considered that such species are remarkably stable and Rosano et al. (44) were able to isolate and characterize it in a fatty acid sample that was prepared under similar conditions to those of Hargreaves and Deamer (29) but below the transition temperature of the membrane so that an acid-anion precipitate (rather than liposomes) was formed.

It is suggested that the two inflection points, also noticed without explanation by Hargreaves and Deamer (29) are due to acid-anion formation (at high pH) and protonation (at low pH). It is recognized that the acid-anion as a molecular species is very stable under such circumstances, but is also recognized that fleeting protonation of the species will result in <u>two</u> protonated monomers which may protonate other neighboring acid-anions. Such rapid exchange was used in a proposal by Haines (56) to explain the movement of protons along the surface of proton-pumping membranes through anionic lipid headgroups as putative acid-anion dimers that are rapidly exchanging the protons. The data shown in Figure 13 shows that the headgroups have a large capacity for trapping protons. The capacity ranges from 80 percent protonation down to 20 percent protonation. The data also shows that the protons trapped in the headgroup domain are in equilibrium with the hydronium ions in the bulk (aqueous) phase. Thus adjustments of the pH can be used to control

both the concentration of protons in the headgroup domain and also the charge density at the surface. Each of these are important in the activities of proton-pumping membranes.

A very important conclusion that is drawn from Figure 13 is that there are two species of hydrogen ions at the surface of fatty acid liposomes. The first is the hydronium ion (hydrated proton) that is in the bulk (aqueous) phase. This proton is recognized by the shift in the apparent pK of the carboxyl from 4.8 to 8.6. The concentration of hydronium ions is enhanced at the polyanionic surface because cations are sequestered by the surface and the ionic strength is relatively low. The second species of proton is trapped by the headgroups through their protonation. This proton is recognized by the ^{13}C-NMR estimate of the percent dissociation. The relative amounts of these two species shifts during the titration from all-hydronium-ion (at pH 10 or higher) to all-protonated-proton (at pH 7 or lower). The hydronium ions move into the membrane as the pH drops. This hypothesis may be tested in the future with (1) alkyl coumarin indicators positioned at varying heights from the bilayer, (2) indicators constructed to measure a series of pK's from high to low pH, and (3) an electrostatic field strength monitor such as the aminonapthalene sulfonates (ANS) (124). The first experiment can be used to determine the variation in pH as the indicator is moved away from the surface; the second can demonstrate the change in surface pH with protonation state; and the third can be used to correlate the mea-

sured field strength with that calculated from the pH measurements made with the indicators.

The entrapment of protons in the negative headgroups of lipids is potentially important for proton-pumping in membranes because all biological membranes contain anionic lipids and many membranes are constituted solely of anionic lipids. The latter includes all photosynthetic membranes (19) each of which pumps protons with the photon-derived energy.

Measurement of the pH at the surface of alkyl sulfate micelles and liposomes: The use of alkylated pH indicators was introduced by Fromherz (95) in order to examine the pH at the surface of charged amphiphile aggregates. In his application of these probes to anionic systems he was able to provide a convincing demonstration of the Gouy-Chapman-Stern theory. This work took advantage of the fact that hydroxy coumarin was charged in its deprotonated form whereas amino coumarin was charged in its protonated form. This feature of the matched pair allowed a direct estimate of the shift in pH that was observed when the uncharged form of the indicator bound more firmly to the surface (due to hydrophobic interactions) than the charged form. The effect led to an error that was equal for the two indicators but in opposite directions.

His application of these indicators to the SDS micelles showed that at high pH (9-11) there is a difference of pH at the micelle surface (-2.3 pH units) and that the same

difference is found in the low pH range (1-5). This means that SDS micelles have the same surface charge density in these two pH ranges.

That alkyl sulfates do not form bilayer membranes has always been attributed to the size of the sulfate headgroup and its charge (in relation to the single chain). This was dramatically demonstrated to be the case by the X-ray structure of SDS which was obtained by Sundell (30). He found that the sulfate bobbed up and down for closer packing in the dehydrated crystal. Hargreaves and Deamer (29) successfully made liposomes of alkyl sulfates and alkanols. The alkanol presumably serves as an uncharged hydrocarbon "spacer" reducing the surface charge density. Very likely it hydrogen bonds strongly with the sulfate relieving some of the charge on that anion as was found for sterols and alkanols (see above) in the fatty acid liposome system.

Liposomes made in this manner (SOS and a "spacer") were studied with the alkylated pH indicators. Alkylated pH indicators adsorbed to oleyl sulfate:cholesterol (1:1 mole ratio) liposomes displayed apparent pKa shifts that were essentially equal in magnitude to those of the oleyl sulfate micelles. (See Table III.) At low pH (amino coumarin indicators) the ΔpH (2.3 pH units) was the same for the SOS system as it was for the SDS micelles. In contrast, the high pH range indicator displayed a ΔpH (1.65 pH units) for the SOS system which represents a diminution of 0.65 pH units as compared to the SDS system. This difference, which

was the same in magnitude for the SOS micelle and the SOS: cholesterol liposome is attributed to the difference in charge density at the surface, not to any perturbation of the indicator. This difference may be attributed to an increased concentration of Na^+ ions at the surface over hydronium ions at high pH. Eisenmann has shown (125) that densly charged anionic surfaces have a high capacity for the binding of sodium ions from solution.

The most important finding, however, is that the ΔpH at the surface for the SOS micelle or the SOS:cholesterol liposome is maximally 2.3 pH units. This is 0.5 pH units greater than the 1.8 pH units found for the fatty acid liposome, yet it is still far below the theoretical maximum (3.4 pH units; see Figure 15) one would expect. Here too, we may invoke the theoretical description of two pools of protons: those that are bound in the headgroup region and those that exist as hydronium ions in the Stern layer (within 9 nm of the surface). Here, the sequestered protons contribute a difference of <u>one</u> pH unit as opposed to <u>two</u> units found in the carboxylate system.

When free fatty acids (40 mole percent) are introduced into the SOS:cholesterol liposome (20 mole percent SOS; 40 mole percent cholesterol) a dramatic difference between the charge density at the surface (measured in the form of pH units) of the liposome at high pH (7-12) and low pH (0-7) is observed (see Table III). In the upper pH range the ΔpH is 1.4 pH units but in the lower range no change in surface

pH (as compared to the uncharged system) is seen.

This phenomena is better understood when one realizes that below pH 7 (see Figure 12) the carboxylates in the SOS: cholesterol-lauric acid liposome are completely protonated. Ptak et al. (99) has also demonstrated that fatty acids incorporated into micelles of myristal sulfate exhibited a pKa of 7.75 and were completely protonated by pH 7. Recall also that both free fatty acids and fatty acid:alkanol liposomes formed oil droplets below pH 7 (completely protonated carboxylates) and that the SOS:cholesterol:oleic acid liposome dispersion phase separated into oil droplets below pH 2.5. The pKa of the indicator was found to be 1.25, a full unit below the dissolution pH of this model system. Reasonable conclusions drawn from this data are (1) the carboxylates are fully protonated below pH 7, (2) the sulfates are protonated below pH 2.5, and (3) the surface charge density is controlled by the protonation state of the amphiphiles.

The finding that fatty acids are protonated at pH 7 in acidic membranes has important implications for biological membranes at physiological pH's. For instance , at this pH one could expect easy partition across the bilayer for fatty acids as in mitochondrial uncoupling. But for O. danica the implications of this data are profound since the organism grows maximally at pH 4.5.

Implications to the membrane stability of Ochromonas danica: The unique lipid composition of the O. danica cell

membrane consists predominantly of monoalkyl disulfates (70 mole percent) (73), fatty acids (17 mole percent) (82), and sterols (12 mole percent) (81). The puzzling features of this membrane outlined in the Introduction prompted our exploration of the the surface pH and protonation state of model amphiphile liposome systems. Our sucess with the use of alkylated pH indicators as monitors of surface pH prompted our using them with two pure membrane preparations from the organism: extracellular vesicles and flagellar membrane.

The results of these investigations show that at high pH utilizing the hydroxy coumarin indicator a significant surface pH can be observed, but at low pH (amino coumarin indicator) there is no observable surface pH (see Figures 10, 11 and Table III). Recall that in the above discussion the same result was found for the model liposome dispersion containing oleyl sulfate, oleic acid and cholesterol. It was concluded that this effect was due to the protonation of the carboxylates below pH 7, and the protonation, in this system, of the sulfates below pH 2.5. (Note, the SOS system does not become completely protonated unless fatty acids are also present.) Therefore, the same result found for both the natural membrane preparations and the model system is direct evidence for the location of free fatty acids in the natural membrane. Protonated fatty acids are significant for the stability of the O. danica membrane since they can act as spacer molecules (29) and as proton doners for the putative acid-anion network suggested for the sulfate headgroups.

The strong evidence implicating protonated species as necessary components for the stability of fatty acid liposomes suggests that protonated sulfates are also important for the stability of the O. danica membrane. Although no direct evidence is available for this phenomena in the in the natural membrane, it is significant that the inclusion of fatty acids in the model system allowed for protonation of the sulfates below pH 2.5. Mixed sulfate carboxylate acid-anions formed from the protonated carboxylate and the anionic sulfate or sulfate dimers may be the species that is formed. It has also been shown that alkaline pH's destabilize the O. danica membrane (E. El Maraghy, unpublished results). The solubility of fatty acids in the bulk phase at such pH's may be the cause of this instability.

The protonated fatty acids and the putative sulfate acid-anions can provide a reservoir of protons at the surface of the membrane. This high concentration of protons would hinder the deprotonation of the secondary sulfates (which must remain completely protonated and therefore uncharged in the low dielectric region of the bilayer) located in the center of each monolayer of the bilayer.

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