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OF FATTY ACID MONOLAYERS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Richard Elmer Heikkila, B.A.

The Ohio State University
1969

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VITA

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Publications

"Red Blood Cell Lipids And The Plasma Membrane."

"Stability of Fatty Acid Monolayers And The Relationship Between Equilibrium Spreading Pressure, Phase Transformations, and Polymorphic Crystal Forms."
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The role of lipids as an integral part of membrane structure was first postulated by Overton in 1899 (1). He studied the osmotic properties of more than 500 different compounds and their effects on root hairs of *Hydrocharis Morsus ranae*. Overton found that these root hairs which had been grown in 7% sucrose showed plasmolysis when transferred to solutions of higher sucrose concentration and consequently higher osmotic strength, due to a loss of water from the cell. He then studied the effects of various compounds dissolved in sucrose solutions on the degree of plasmolysis. For example, root hairs grown in 7% sucrose showed no plasmolysis upon transfer to 7% sucrose solutions containing 3 w/v% methyl alcohol or ethyl alcohol. Overton attributed the lack of plasmolysis in these solutions of very high osmotic strength to a rapid entry of the compound into the cellular fluid and an equilibration between the cellular fluid and surrounding medium.
In general, Overton found that materials soluble in ether, fatty oils, or similar solvents entered the protoplast very rapidly and showed little plasmolysis. The higher the lipid-water partition coefficient, the greater was the permeability into the root hair and the less the plasmolysis.

Overton postulated that the osmotic properties of the cell would very likely be attributed to the end layers of protoplast, which he envisioned to be coated (impregnated) with substances whose solubility properties were similar to fatty oils. That these substances were not fatty oils was established by Overton as he saw no damage to the root hairs if they were left in sodium carbonate under conditions in which some of the fatty oil, if present, should have been saponified. Overton finally concluded, although without any direct experimental evidence, that cholesterol, lecithins, and in certain cases fatty oils were impregnated in the protoplasm layers. Cholesterol had been found in nearly all animal cells and since no biological function had been attributed to cholesterol at that time, Overton postulated that cholesterol was in the phase boundary. He suggested that the other compounds gave cholesterol a degree of fluidity. We know today from monolayer studies that the reverse is true since cholesterol liquifies
solid phospholipid monolayers (2).

The Plasma Membrane

Plowe, in 1931, using plasmolysis in conjunction with micromanipulation techniques, first showed that the plasma membrane was a distinct phase and not just the outer layer of the cytoplasm (3). Using the upper epidermis of bulb scales of Bermuda onions, Plowe concluded that the internal and external layers of protoplasm were distinct from the rest of the protoplasm, although the cells were indistinguishable microscopically. She found this membrane material to be protective in nature and highly elastic, but did not give any idea as to its chemical composition or molecular structure.

In a brief but monumental paper published in 1925, Gorter and Grendel gave the first evidence for the possible existence of a bimolecular layer of lipid and protein as the structural backbone of the cell membrane (4). They extracted the lipid from the washed erythrocytes of dog, sheep, rabbit, guinea pig, goat, and man with acetone and spread the lipid extract as a monomolecular film according to methods described by Langmuir (5) and Adam (6). They measured the surface area occupied by each erythrocyte using Knoll's formula (7), and they found the area occupied by the cells used for extraction by multiplying the number
of cells by the surface area of each cell. When they calculated their data, Gorter and Grendel found that the area occupied by the lipid extract was twice the area occupied by the erythrocyte surface. They thus postulated that the cell was covered by a bimolecular layer of lipid, with the polar groups directed to the inside and to the outside. They envisioned a priori that a watery solution of hemoglobin formed the boundary of one phase and that plasma formed the boundary of the other. A different structure was later proposed by Dervichian and Macheboeuf, who performed similar experiments and found that the ratio of film area to cell area was approximately one (8).

As pointed out by Bar, Deamer, and Cornwell (9), there were a number of deficiencies in the experimental design of both these groups. Gorter and Grendel used acetone for their lipid extraction and presented no data on their lipid recoveries. Dervichian and Macheboeuf used 10% ethanol in ether and extracted an arbitrarily defined "loosely bound" lipid fraction. Their extraction probably represented only 70-80% of the extracted lipid. New and better techniques for measuring surface area have shown, for example, that the surface area of the human erythrocyte is $145 \pm 8 \mu^2$ (10) as compared to the $99 \mu^2$ used by Gorter and Grendel. Dervichian and Macheboeuf also used the area occupied by
the monolayer at collapse while Gorter and Grendel used
the area at initial surface pressure, the area at collapse
being significantly smaller.

Using a better method of lipid extraction and more ac­
curate values for cell areas, Bar et al. have reinvesti­
gated this problem. They calculated the ratio of film area
to cell area at various surface pressures and indeed found
that at low surface pressures this ratio approached 2. It
appeared that the poor lipid recovery of Gorter and Grendel
was compensated for by their incomplete lipid extraction.
The value approached 1 at high surface pressures, which
indicated that Dervichian and Macheboeuf may too have been
correct.

Korn, in a recent review (11) using the data of Ways
and Hanahan on human erythrocyte extracts (12), and the
value of 70Å² as the space occupied by one cholesterol
molecule, and a cell area of 167μ², calculated the ratio
of film area to cell area to be 1.56. This 70Å² molecule
corresponded to a value of approximately 1.7 using the
data of Bar et al., but this difference may have been due
to the different value used for the cell area.

In 1935, Danielli and Davson, without any strong
experimental basis, postulated their model for the plasma
membrane (13). This model was formed with two layers of
lipid being covered with two layers of protein, the polar ends of the lipids being next to the protein and the non-polar portions of the lipids adjoining each other. This model was developed further over the next twenty years to include pores for the passage of polar materials, and to establish the amount of lipid material thereby defining the membrane thickness. The unit membrane model of Robertson (14) postulated in 1959 on the basis of microscopic studies is not much different from this basic concept of Danielli and Davson.

It is unusual that Danielli and Davson were unaware of the work of Gorter and Grendel who in 1925, ten years earlier, and with a much firmer experimental basis, postulated that the erythrocyte membrane was covered with a double layer of lipid. There seems to be no experimental basis to explain why Danielli and Davson in 1935 postulated a bimolecular leaflet. On the basis of their experimental data, a double layer was no more likely than a monolayer or a multilayer.

Danielli et al. (13, 15) measured the surface tension at the interface between aqueous egg material and egg oil from mackerel eggs. The tension at the interface between the two was found to be 0.8 dynes. The surface tension between egg oil and sea water was found to be approximately
7 dynes. Danielli thus concluded that the mackerel egg contained a substance which had a great surface activity and was responsible for lowering the tension at the surface of the egg oil. He concluded that this substance was protein and from this data postulated his model for the plasma membrane.

Again, as in the case with the work of Gorter and Grendel, the work of Danielli and Davson had serious problems in its experimental design. The composition of the mackerel oil used in the experiments was certainly not similar to the composition of membrane lipids. This oil was high in triglyceride while membrane lipids generally contain more polar species such as phospholipids in addition to cholesterol and in general contain little, if any, triglyceride. Danielli himself in 1937 showed that phospholipids were very effective in lowering interfacial surface tensions. Branton and Park in a collection of papers on biological membranes (16) doubt that Danielli and Davson would have proposed their model had they known two years earlier that polar lipids lower surface tensions and that the composition of mackerel oil was in no way like the composition of membrane lipid.

The Unit Membrane

Using refined physical techniques such as polarization
microscopy, X-ray diffraction, and electron microscopy, Robertson in 1959 postulated his unit membrane theory (14). This theory was actually only a refinement of the Danielli-Davson model, but Robertson's ideas seemed to clarify and unify a diverse body of knowledge and correlated the new ideas gained by physical methods with the older experiments and theoretical work.

Korn, in a review on the structure of biological membranes, severely criticized the ideas postulated by Robertson (11). Korn stressed in his argument that the unit membrane theory was not proved by the data which led to its formulation, that experimental data existed which did not bear out the theory, the theory was difficult to reconcile with concepts of molecular biology with regard to genetic control of membrane structure and physiological and biochemical regulation of membrane function, and most important of all, the data necessary to test the correctness of the theory were still not available.

Work by Luzzati and Husson (17) showed that the phospholipid from human brain could exist in two different liquid crystalline forms depending upon how much water was present. One of these was the lamellar arrangement of biomolecular leaflets formed in high water concentration, the other a hexagonal array of circular cylinders. In addition
Finean, Coleman, Green, and Limbrick (18), using low-angle X-ray diffraction in conjunction with electron microscopy found rat erythrocyte membranes to be in the order of 100 Å thick. Their electron micrograph data was consistent with the unit membrane theory and their membrane thickness similar to that of the unit membrane. The hexagonal array seen by Luzzati and Husson was favored at temperatures between 30°-40°C and at higher concentrations of phospholipids and lower concentrations of water. Lucy and Glauert (19) made mixtures of lecithin and cholesterol, closer in composition to a biological membrane, and have shown a variety of forms: lamellar, tubular, hexagonal, and helical. All systems of this type have one serious deficiency, they lack protein. It is difficult to extrapolate back to a living membrane from a model which chemically does not resemble the membrane. Another serious deficiency of the unit membrane theory was that there were problems in interpretation of electron micrographs. It is necessary, if unequivocal statements are to be made, to know what atoms are responsible for the microscopic image. Two common fixatives used in electron microscopy are osmium tetroxide (OsO₄) and potassium permanganate (KMnO₄). Dense lines seen in electron micrographs fixed with KMnO₄ have been attributed to the hydrophobic side chains of membrane lipids, but Korn and Weisman (20) have shown that under
conditions used in electron microscopy, lipids of amoeba were found to be unaffected by treatment with KMnO₄.

Claims have been made that OsO₄ reacts with polar ends of lipid molecules, this being the main if not only evidence that the bimolecular leaflet has its polar groups directed outward. Korn showed, however, using the fundamental work of Criegee (21) as a basis for his research that OsO₄ reacted stoichiometrically with the double bonds of olefinic compounds to form stable reaction products. Korn found no evidence that the OsO₄ first reacted with the double bond and then migrated to the polar ends of the molecules which was proposed by Stoeckenius (22). Furthermore, Fleischer et al. have shown that the unit membrane structure still could be seen after extraction of mitochondrial lipid (23), and Finean and Martonosi showed persistence of membrane structure in preparations of endoplasmic reticulum treated with phospholipase C (24). Since the unit membrane structure may still be seen with the lipid backbone of the membrane missing, in addition to Korn's objections, it appears unlikely that the unit membrane theory is a clear cut matter as believed by Danielli who said in 1962, "It now seems to be agreed that the basic structure of the plasma membrane is that which I suggested in 1934, and it is highly probable that the same structure
is present in many other intracellular membranes. So far as it is possible to predict at the present time, it is unlikely that the general picture will be substantially disturbed, and the focus of attention is likely to shift to other fields." (16)

The Lipoprotein Tripartite Membrane

Green and co-workers, in an extensive series of papers, postulated a new model for the mitochondrial membrane, a composite of nesting lipoprotein repeating units (25-29). Green and MacLennan envisioned (29) the bilayer theory as resting on three key assumptions: In biological membranes that phospholipid molecules were bonded hydrophobically to other phospholipid molecules; that protein molecules were bonded electrostatically to phospholipid molecules, and that phospholipid bilayer constituted the membrane continuum. They further stated that these three assumptions had all been disproved from a variety of sources, but they somehow forget to give references to these sources. The model for the mitochondrial membrane, as proposed by Green et al., using data from biochemical and electron microscopic experimentation envisioned as being one particle thick, with each particle being a repeating subunit. This membrane
could be dissociated into its monomeric repeating units, and these monomeric units could reassemble by hydrophobic recombination to form membranes (26,28). This fact that membranes formed from lipoprotein repeating subunits is, in Green's eyes, the greatest evidence against the Danielli-Davson hypothesis. Thompson in a discussion of a paper at a symposium on the plasma membrane (30) commented on this point, "I think one simple answer is that if one has a cake and cuts it into pieces, it is ridiculous to imagine that when the cake was made it was made out of the pieces that you cut it into. I think that this does not argue for the existence of a phospholipid bilayer in biological membrane but it does argue that subunits derived from a nonbonded system such as the cell membrane may not have any structural or functional significance in the intact membrane."

The repeating units of any membrane, according to the Green theory may have a different form and size and may also have different proteins and enzymic functions. The repeating unit of the mitochondrial membrane has a tripartite structure: a basepiece, a headpiece, and a stalk. Two essential features of these repeating units are the association of the phospholipid with the basepiece and the fact that the membrane protein is made up of an almost equal
mixture of catalytic and non-catalytic proteins.

Other membrane models basically very similar to the lipoprotein repeating subunit have been postulated (31,32). Benson (31) postulated a bilayer type membrane in which hydrophobic protein was bonded to the hydrophobic side chains of the lipids. The resulting lipoprotein aggregate has the polar groups of the phospholipid located on the surface. Using optical rotatory dispersion, Lenard and Singer (32) found that the erythrocyte ghost had between 20 to 50% of its protein present in the α-helix configuration. They found no protein in the β configuration, the remainder was assumed to be present as random coil. With this data, they suggested a new lipoprotein membrane model. This model placed the hydrophilic portions of both protein and lipid at the aqueous interface, with the hydrophobic portions of protein and lipid forming the interior core. It is interesting that this model differed from the Danielli-Davson hypothesis only in the placement of protein. With a vivid imagination, Danielli and Davson might have postulated this model themselves some thirty years early.

It is obvious that the problem of membrane structure is by no means solved. New theories of membrane structure continue to arise, even though Danielli and Green each feel that they have solved the problem. Engelmen, using data from lipid composition studies (33-35), X-ray diffraction
(36), and microscopic determinations of red cell size (10) has calculated that there is not enough lipid present for a bilayer to exist (37). He explains that the larger cell surface areas could be explained by a broadly conceived bilayer model in which proteins penetrated the lipid head-group layers to make hydrophobic contact with the hydrocarbon chains, thereby increasing the area per molecule. Alternatively, Engelman postulated that a bilayer with patches of protein could explain his calculations. However, this theory, or theories, appears no better than previous ideas. His theory is based on several assumptions on the structure of the lipid moiety of the membrane, the most questionable being that the hydrophobic region is of uniform thickness over the whole cell surface. Shah and Schulman (2), using molecular models, have shown that the cholesterol molecule is considerably shorter than the average phospholipid molecule. In addition, Engelman has made mathematical mistakes in his calculations of the molecular dimensions of phospholipids.

Until some novel experimental approach is used, it is unlikely that the problem of membrane structure will be solved. Such an experimental approach may involve the use of freeze-etch microscopy (38,39). This technique is advantageous in that it employs no chemical fixatives, embedding materials, or stains commonly used in electron microscopy.
Freeze-etch microscopy is based on the fracture of a frozen specimen upon a plane of weakness, demonstrated by Deamer and Branton (40) to be along planes of hydrophobic bonding. Freeze-etch microscopy has indicated the possibility of globular subunits as many particles on the order of \(85\) Å in diameter have been seen. Subsequent studies (41,42) have shown a broad spectrum of cellular structure by freeze-etching, among them the very smooth membrane faces seen in the myelin sheath and the highly particulate membranes of chloroplast lamellae. Thus, freeze-etching has given clues that biological membranes may have different structure according to their function. Obviously, new experimental techniques are necessary to substantiate these findings.

**The Bilayer Model**

The molecules which serve as the building blocks for membranes are generally amphipathic molecules, that is, they contain both hydrophobic and hydrophilic portions. Membranes are composed of cholesterol, gangliosides, other glycolipids, and phospholipids, as well as protein which may be either enzymatic or structural. While the lipid molecules which make up membranes are ideal for study as monomolecular films spread at an air-water interface using a Langmuir trough, Lucy (43) points to the shortcomings of this model system. "Although monolayers of lipid molecules at an air-water interface, or at an oil-
water interface, can be used to considerable advantage for the evaluation of the chemical and physical interactions between molecules, such films are less satisfactory as structural models for biological membranes. Some of the reasons for this are obvious enough: a monolayer consists of only one layer of lipid molecules which lies at the interface between air and water, or between oil and water, and it does not separate two aqueous phases. Hence, factors that affect the stability of lipid films in relation to the macromolecular arrangement of its constituent molecules are probably quite different in the monolayer from what they are in natural membranes." Lucy further points out that there are anomalies between the rates of water passage through monolayers and the rates through natural membranes.

Without actually having said it, Lucy pointed to his preference of a bilayer system as a model for the study of biological membranes. This model was developed by Mueller, Rudin, Tien and Westcott in 1962 (44) and it is described as being similar in structure to the bimolecular leaflet proposed by Danielli and Davson (13,15). Ordinarily these bilayer membranes (BLM) were formed by "painting" a lipid solution on a small hole in a plate separating two compartments of saline. The BLM consists of a double layer of lipid molecules with the polar ends oriented towards each saline phase and the hydrocarbon chain forming an organic phase. Typical data obtained from BLM studies are summarized
TABLE 1.
A COMPARISON OF PROPERTIES OBTAINED FROM BILAYER STUDIES WITH PROPERTIES OF NATURAL MEMBRANES.

<table>
<thead>
<tr>
<th>Lipid bilayers</th>
<th>Cell membranes</th>
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<tr>
<td>Refractive Index</td>
<td>1.66</td>
</tr>
<tr>
<td>Surface Tension</td>
<td>0.5-1 dyne/cm</td>
</tr>
<tr>
<td>Specific DC Resistance</td>
<td>0.2-4 \times 10^6 \text{ ohm/cm}^2</td>
</tr>
<tr>
<td>Capacitance</td>
<td>0.5-1 \mu F/cm$^2$</td>
</tr>
<tr>
<td>Water permeability (isotopic)</td>
<td>\sim 4 \mu /min/atm</td>
</tr>
</tbody>
</table>


(Table 1). In spite of these data, which correspond closely to data obtained from biological membranes, the bilayer system has disadvantages. The composition of lipids used for the formation of BLM, as tabulated by Tien and Diana (46), in no way resembles the composition of any natural membrane. Examples of agents, indispensable to the formation of bilayers, are silicone fluid, nujol oil, and in almost every case liquid hydrocarbons such as tetradecane. Until BLM can be formed from biological materials alone, without the necessity for other components, even
solvents, data obtained from bilayer studies will have to be accepted with reservation.

The Monolayer Model

In spite of the obvious shortcomings of the monolayer system and the doubts pertaining to its relevance for studying membrane systems, the monolayer technique is invaluable in studying molecular interactions in oriented systems. For example, monolayer techniques have proved useful in studying the interactions of polyene antibiotics with lipid monolayers (47), the interaction of psychoactive drugs with lipid monolayers (48), the interactions of proteins with lipids as exemplified by the hydrolysis of various phospholipids by phospholipases (49), the binding of various cations by negatively charged lipid monolayers (50,51), and the interaction of retinaldehyde with various phospholipid monolayers (52). Of particular interest and importance are the studies involving molecular interactions among the lipid constituents of the monolayers themselves.

Several studies have been carried out with cholesterol and phospholipids, as these compounds in general comprise a majority of membrane lipids (53-60). These studies all lead to the conclusion that there is an apparent condensing effect of cholesterol on phospholipids, that is, the cholesterol can effectively decrease the area occupied by a phospholipid molecule.
A mathematical relationship of the additivity rule for film areas for a two component system containing cholesterol and phospholipids is as follows:

$$A_F = A_{PL} M_{PL} + A_{Chol} M_{Chol}.$$  

where $A_F$ is the area of the mixed film at a specified surface pressure, $A_{PL}$ and $A_{Chol}$ are the areas of phospholipid and cholesterol, and $M_{PL}$ and $M_{Chol}$ are the molecules of phospholipid and cholesterol, respectively, in the film (60). Leathes (53) and later DeBernard (54) found that cholesterol, with molecular dimensions of $38\AA^2$/molecule, had a condensing effect on phospholipids. The $A_{PL}$ calculated for a mixed film by the above equation was lower than the $A_{PL}$ of the phospholipid fraction which was isolated by silicic acid column chromatography and determined directly (Table 2).

It is well known now that the degree of condensation differs significantly among different molecular species. However, the specific characteristics of the hydrocarbon chains or polar head groups necessary for condensation have not been unequivocally established. Furthermore, there are two major theories for this condensing effect, neither of which appears to have distinct advantages over the other (2,59).
Shah and Schulman (2) reported on the dual surface properties of cholesterol: its ability to liquify a monolayer as well as its capacity to occupy the molecular cavities caused by fatty acyl side chains. The introduction of cholesterol into a monolayer of 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine caused both a liquefaction of the previously solid film and a decrease in the apparent molecular area occupied by the phospholipid. Shah and Schulman, reasoning from film flow using talc, and molecular models of their compounds, interpret these phenomenon as being caused by a reduction in the cohesive forces of the fatty acyl side chains of the phospholipid and an insertion of the cholesterol molecule into space occupied by the phospholipid. The cholesterol molecule, being shorter than a phospholipid, can insert itself into spaces caused by unsaturation in phospholipids (Fig. 1).

An alternate theory has been proposed by Chapman, Owen Phillips, and Walker (59) who have related the monolayer properties of cholesterol-phospholipid monolayers to the thermotropic properties of the pure phospholipids. This study was very similar to that of Cadenhead and Phillips (55), who correlated the condensing effect of cholesterol with changes in phase transformations and liquid crystalline properties of various lipids. Chapman et al., have postulated that the effect of cholesterol is caused by an inhibition of
Fig. 1. Schematic drawing of fit of cholesterol molecule into space occupied by two phospholipid molecules. Source: Shah, D. O. and J. H. Schulman. 1967. J. Lipid Res. 8:215.
TABLE 2.

APPEARENT CONDENSING EFFECT OF CHOLESTEROL ON TOTAL ERYTHROCYTE PHOSPHOLIPIDS.

<table>
<thead>
<tr>
<th>dynes/cm</th>
<th>$A_{PL}(\AA^2)^a$</th>
<th>$A_{PL}(\AA^2)^b$</th>
<th>$\langle\AA^2\rangle^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>68</td>
<td>53</td>
<td>15</td>
</tr>
<tr>
<td>35</td>
<td>73</td>
<td>58</td>
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<tr>
<td>5</td>
<td>114</td>
<td>101</td>
<td>13</td>
</tr>
</tbody>
</table>


$^a$Pure phospholipids
$^b$Calculated from $A_{PL} = \frac{AF}{M_{PL}} - \frac{A_{Chol}M_{Chol}}{M_{PL}}$
$^cA_{PL} - A_{PL}^*$

motion of the fatty acyl side chains of the phospholipids. This effect is greater when the monolayer is close to its transition temperature for a change from expanded to condensed monolayers and inhibition of chain motion might cause the film to become more condensed. Chapman et al., classify phospholipids in relation to their critical temperature and the effects of cholesterol in accordance with the effective lowering of temperature to approach the critical temperature. This theory fails to account for the
fact, however, that cholesterol actually liquifies solid monolayers while a lowering of effective chain motion would actually solidify a monolayer. In addition, one major criticism of the theory of Chapman et al. (59) is that it fails to explain how cholesterol, by an effective decrease in temperature, could actually decrease the area of a phospholipid to an area smaller than that which it occupies at collapse (60). It is obvious that more work is needed in this area.

Formation and Stability of a Surface Film

During a study on the surface properties of mammalian erythrocytes (60), we found several anomalous π-A curves which were considerably more expanded than the typical π-A curves for cholesterol-phospholipid mixtures. We postulated that peroxidation had occurred in the unsaturated lipids resulting in an increase in surface area. Several experiments were carried out with hydrogen peroxide and indeed the π-A isotherms of oxidized erythrocyte lipids were expanded although substantial amounts of lipid were lost during the oxidation. At the same time that these preliminary data were obtained, Porter et al., (61) published a study which suggested that the oxidation of linoleic acid monolayers caused a contraction in molecular area. It was immediately apparent to us that a reinvestigation of the formation and stability of sur-
face films was required and we began a study on the metastable nature of fatty acid films and the effects of solution, evaporation and oxidation on film properties.

One stability parameter, the equilibrium spreading pressure ($\Pi_E$), is defined as the pressure where the monolayer is in equilibrium with the bulk phase, which may be either liquid or solid (62). Oleic acid is an example of a film forming compound whose bulk phase is a liquid while stearic acid is an example of a film forming compound whose bulk phase is a solid. The $\Pi_E$ value is difficult to obtain experimentally, due to anomalous results which may arise even from trace impurities and the fact that the crystal size is important in determining whether or not a film forming compound can be spread to form a film from the bulk phase (63). In general, the smaller the crystal size, the greater the ease of spreading. A compound, when placed on an appropriate subphase, will spread to form a film until the $\Pi_E$ value is obtained. If the area available to the film is too great, the $\Pi_E$ value may only be reached by subsequent compression. If there is an excess of the film forming material, the $\Pi_E$ will be reached and the excess molecules will form a bulk phase in equilibrium with the monolayer.

A second stability parameter, the collapse pressure ($\Pi_C$), is the highest pressure to which a monolayer can be
compressed without detectable expulsion of molecules to form a lens or bulk phase (64). In some instances, especially with liquids, the collapse pressure and the equilibrium spreading pressure are the same. In other instances, however, the collapse pressure is greater than the equilibrium spreading pressure and molecules may be over-compressed beyond the $P_E$ even though they are not in thermodynamic equilibrium. Adam (65) has suggested that this phenomenon is possible due to the absence of a stable crystal nuclei on which the collapsed film may build and form the bulk phase. The collapse pressure never has a lower value than the equilibrium spreading pressure.

The stability of lipid monolayers in relationship with the equilibrium spreading pressure and collapse pressure has not been studied in much detail. However, studies at low compression rate (66) or constant area (67) have shown apparent losses of molecules, as evidenced by the absence of transition points or a decrease in pressure. Ries and Kimball (68), however, have postulated a mechanism for collapse based on a technique utilizing electron microscopic observations of collapsed films. They envisioned that lipid molecules are forced up from the surface by compression and form a three-dimensional structure two molecules thick (Fig. 2).
There has been very little study on the processes of solution and evaporation from monolayers, although the loss of molecules by either of these processes would cause serious errors in surface measurements. Surface chemists tend to think that whatever amount of a compound they spread at the beginning of an experiment will remain throughout the course of the experiment, but this is certainly not the case. Fatty acid or fatty amine films held at constant area have shown decreases in surface pressure (69,70), while fatty acid films held at constant surface pressure have shown decreases in area (71,72). Fatty alcohol films held at constant surface pressure have also shown decreases in area (73). All these phenomena, which indicate the loss of molecules have been attributed to solution or evaporation or both, but the mechanism for film loss is in general poorly understood.
CHAPTER II

STATEMENT OF THE PROBLEM

Little attention has been paid to losses from monomolecular films. Four possible mechanisms for film loss have been postulated. They are: collapse into the bulk phase (66-68), autoxidation and cleavage of unsaturated compounds with subsequent solubilization of the reaction products (61), evaporation (73), and solubilization (69-72,74). In the present investigation, the mechanism of film loss of fatty acids spread at an air-water interface will be determined. The relationship between the equilibrium spreading pressure and collapse into the bulk phase will be studied. Correlations between the surface area at the equilibrium spreading pressure and the cross-sectional area of one polymorphic crystal of the fatty acid will be discussed. The effects of chain length, unsaturation, temperature, and the pH and ionic strength of the subphase on film solubility will be studied. The use of solubility data to determine apparent surface pH values and apparent pKa values for fatty acids will be described.
CHAPTER III
EXPERIMENTAL METHODS AND MATERIALS

Surface Pressure

Surface pressure, $\Pi$, and surface potential, $\Delta V$, are probably the two most common film properties studied by surface chemists. The surface pressure is considered to be equal to the reduction in surface tension of a pure liquid subphase caused by a film:

$$\Pi = \gamma^0 - \gamma$$

where $\gamma^0$ is the surface tension of the pure liquid and $\gamma$ the surface tension of the film covered surface.

Two common methods for measuring surface pressure are the Langmuir method and the Wilhelmy method. The Langmuir method utilizes a direct differential measurement between the film covered surface and the clean surface. A clean portion of the liquid surface is separated from the film covered surface by a partition, and the force acting on this partition is measured. In Langmuir's original balance, the partition consists of a movable float connected to a conventional balance with which the magnitude of the force is determined (75).
In the Wilhelmy method, a thin plate is suspended in the liquid surface. The forces acting on the plate are the gravitational and surface tension effects in one direction, and the buoyant effect due to the weight of liquid displaces, in the other direction. For a rectangular plate of dimensions $l$, $t$, and $w$ of a material of density $\rho$, immersed to a depth $h$ in a liquid of density $\rho_1$, the net downward force $F$ is:

$$F = \rho g l w t + 2\gamma (t+w) \cos \theta - \rho_1 g t w h$$

where $\gamma$ is the liquid surface tension, $\theta$ is the contact angle of the liquid subphase on the plate, and $g$ is the gravitational constant (76). A common procedure for the use of Wilhelmy balance is to maintain the plate completely wetted by the liquid and measure the change in $F$. Since the $\cos \theta = 1$, and the forces due to gravity and buoyancy remain constant, the equation simplifies to:

$$\Pi = - \frac{\Delta \gamma}{\Delta F} = \left[ \frac{\Delta F}{2 (t+w)} \right]$$

Thus all that is necessary to measure surface pressure by the Wilhelmy technique is an accurate balance connected to a dipping plate of known dimensions.

A combination of the Langmuir and Wilhelmy methods was used in the present study. The subphase liquid was contained in a Teflon trough with dimensions of 1.0 X 9.8 X 50.0 cm. The volume of the trough when filled just to the
edge was approximately 500 cc. A moving barrier 1 X 2.5 X 20 cm., also made of Teflon, separated the monolayer area from the clean subphase. The position of the barrier was noted by a steel rule attached to the side of the trough. The barrier was compressed in order to change the area available to the monolayer by a constant speed DC motor connected to a threaded brass bar. Excessive shock was eliminated from the system by a rubber coupling between the motor and the threaded bar. Force changes, used in calculating surface pressure, were measured by a Cahn R.G. recording balance utilizing a platinum dipping plate of negligible thickness with a height of 2 cm. and a width of 1 cm. 1.0 mg. corresponded to a change in surface pressure of 0.49 dynes/cm. The entire weighing assembly and trough were then enclosed in a plexiglass case with a wooden frame. \Pi-A isotherms generated were recorded on a Varian Aerograph Model 20 recorder, using a chart scale which corresponded to 100 mg., or 49 dynes/cm. The platinum plate was cleaned before each experiment by either immersing the plate in cleaning solution and rinsing it with distilled water or by heating the plate in a bunsen burner flame, or both. The subphase surface was cleaned by suction after each run and the surface tension of the pure subphase measured in order to avoid contamination. The trough was routinely cleaned with cleaning solution and rinsed with water to
maintain absolute cleanliness.

**Equilibrium Spreading Pressure**

Equilibrium spreading pressure was measured in two ways: by post-collapse and by direct spreading of excess bulk phase. In the post-collapse technique, monolayers were rapidly compressed past their collapse point and allowed to equilibrate overnight (generally 15-18 hours), or until the pressure remained constant with time. In the direct spreading technique, finely ground crystals of the compound to be studied were added directly to the surface and the pressure monitored again for 15-18 hours. In both cases at the conclusion of the experiment, \( \Pi \) was measured, the dipping plate was removed, cleaned, and the pressure measured again. This process was repeated until the pressure remained constant, often several times, and was done in order to ascertain that any material left on the plate was removed so that the contact angle remained zero and proper surface pressures would be obtained.

**Solution Into The Bulk Phase**

The disappearance of material into the subphase was studied with essentially the same system as described by Porter et al. (61). A Teflon trough, measuring 1 X 10 X 50 cm., which contained 500 cc. of subphase was partially immersed in a large refrigeration chest, which contained a heating unit and cooling unit. The chest was covered
with a plexiglass plate which had sliding doors which could be opened and closed to permit easy access to the trough. The temperature range was from 5°C to 40°C and could be controlled to ± 1°C. Due to the large volume of water in the chest, the air temperature could be kept at essentially the same temperature as that of the subphase.

The trough was filled with the subphase and a plexiglass floating barrier was placed on the surface. On one side of the barrier was placed an excess of castor oil to maintain the pressure of the system at the 16 dynes/cm. collapse pressure of castor oil (Fig. 3). Care was taken to observe that there were always small lenses of castor oil present which insured that the castor oil was present in sufficient quantity. On the other side of the floating barrier, sufficient fatty acid was placed so that the initial area of fatty acid present as a monomolecular film was 200-400 cm². The area was measured by use of a stainless steel rule mounted on one side of the trough. The disappearance of the film was determined by calculating the amount of film present at various times (Aₜ) and comparing it to the film area present at zero time (A₀). In some experiments the change in area (ΔA) was calculated from the difference between A₀ and Aₜ (See Fig. 10).

For anaerobic experiments the trough, all necessary equipment, and solutions were placed in a glove bag (12 R
Cheltenham, Pennsylvania). The subphase liquid had previously been boiled, then sparged with nitrogen during cooling to remove any oxygen which may have been dissolved. The glove bag was flushed with nitrogen five times and a positive nitrogen pressure maintained throughout the experiment. The experimental methods were the same as in the aerobic environment.

Materials

Water was redistilled from glass into polyethylene containers. Its conductivity was approximately 2.6 micromhos and pH from 5.5 to 6.8. 0.1N HCl was prepared by the reagent laboratory of The Ohio State University and standardized against NaOH. Various subphase solutions contained different ionic strengths of NaCl (Baker Chemical Co., Phillipsburgh, New Jersey) either 10.0 or 18.3 mM Tris buffer (Sigma Chemical Co., St. Louis, Missouri), and 0.1 mM EDTA (G. Frederick Smith, Columbus, Ohio) to complex with any divalent cations introduced with the other reagents. The solutions were brought to the desired pH using NaOH or HCl. In some anaerobic experiments, 0.2% hydroquinone was added to the subphase. Myristic (tetradecanoic), palmitic (hexadecanoic), stearic (octadecanoic), arachidic eicosanoic), behenic (docosanoic), oleic (octadecenoic), and linoleic (octadecadienoic) acids were purchased either from the Hormel Institute (Austin, Minnesota) or Applied Science
Fig. 3. Diagram of apparatus used to determine loss of molecules from a monolayer maintained at constant surface pressure.
CASTOR OIL

FATTY ACID

50 cm

10 cm

1 cm
Laboratories (State College, Pennsylvania). Castor oil was purchased from the E. R. Squibb Co. (New York, New York). Hexane, used as the spreading solvent was washed with concentrated sulfuric acid, refluxed with potassium permanganate, then washed successively with sodium bisulfite, sodium bicarbonate, and sodium chloride, then distilled at 69°C. All chemicals used for hexane purification were purchased from the J. T. Baker Chemical Co. (Phillipsburgh, New Jersey). Fatty acids were made up in hexane at a concentration of approximately 40 mg/cc and were added to form the monolayer by either a pipet or microsyringe. In anaerobic experiments fatty acids were sometimes added directly from a freshly-opened vial by means of a steel needle.
CHAPTER IV
RESULTS AND DISCUSSION

Monolayer Collapse

Force-area ($\Pi$-A) isotherms for hexadecanoic, octadecanoic, eicosanoic, and docosanoic acids were generated at a number of different compression rates. Continuous compression at rates which varied between 0.5 and 6.0 $\text{Å}^2$/molecule/minute had little effect on the transition point ($T$), the collapse point ($C$), and the characteristic shape of the $\Pi$-A isotherm in the pre-collapse region of the curve (Fig. 4). Very slow compression rates were achieved by intermittent compression and equilibration. This procedure has a profound effect on the shape of the $\Pi$-A isotherm for all saturated acids studied (Fig. 5). $\Pi$ increased during compression and $\Pi$ decreased during the equilibration period. Both the increase and decrease in $\Pi$ were directly proportional to the chain length of the fatty acid.

The shape of the $\Pi$-A isotherm in the post-collapse region of the curve depended upon both the compression rate and the chain length of the fatty acid. $\Pi$ decreased rapidly after collapse when an eicosanoic acid film was compressed rapidly (Fig. 4). Of the fatty acids studied, eicosanoic acid was a transition compound. The post-collapse $\Pi$ for
Fig. 4. Π-A isotherms for eicosanoic acid spread on 0.1N hydrochloric acid at 25-27°C. Monolayers were compressed at 0.62 Å²/molecule 1 minute (•) and 5.93 Å²/molecule/minute (○).
Fig. 5. II-A isotherms for hexadecanoic acid (0) and docosanoic acid (Δ) monolayers during intermittent compression and equilibration. Hexadecanoic acid was compressed for 2 min. at 0.46 Å²/molecule/min, and then equilibrated for 20 min. Docosanoic acid was compressed for 2 min. at 0.4 Å²/molecule/min, and then equilibrated for 20 min. Monolayers were spread on 0.1 N hydrochloric acid and the temperature was 31°C.
hexadecanoic and octadecanoic acid monolayers decreased rapidly when the film was compressed at either rapid or slow rates (Fig. 6). Conversely, the post-collapse $\Pi$ for docosanoic acid monolayers remained constant or even increased somewhat depending upon the speed of compression (Fig. 4).

When compression was stopped at any point beyond $C$, $\Pi$ decreased rapidly for a few minutes and then slowly over a 12 to 18 hour period until it reached a constant and reproducible value (Fig. 4 and 6). This $\Pi$ was specific for each fatty acid and was a function of chain length. It appeared that hexadecanoic and octadecanoic acid monolayers collapsed to $\Pi$ values that were nearly equal to the equilibrium spreading pressures that have been reported for these acids when they were spread from the excess bulk phase (77, 78). $\Pi_E$ values were then determined experimentally with the four acids used in this series of experiments. The monolayers were spread from bulk phases placed at the air-water interface of the Langmuir trough and $\Pi$ was monitored until equilibrium was attained. The $\Pi_E$ values were similar to the $\Pi$ values obtained after monolayer collapse and equilibration (Table 3).

Monolayer collapse at lower $\Pi$ values than those at point $C$ (Fig. 4 and 6) were noted when films were compressed
Fig. 6. Π-A isotherms for hexadecanoic acid (○) and docosanoic acid (□) spread on 0.1N hydrochloric acid at 25-27°C. Monolayers were compressed at rates which varied from 0.5 Å²/molecule/min. Data represent the mean values obtained for compression at different rates, since the shape of the curve in the post-collapse region was not affected by the compression rate.
### TABLE 3
SURFACE AREA AND SURFACE PRESSURE AT THE PHASE TRANSFORMATIONS OF SATURATED FATTY ACID MONOLAYERS SPREAD ON 0.1 N HYDROCHLORIC ACID AT 25-27°C

<table>
<thead>
<tr>
<th>Transformation Property</th>
<th>Fatty Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
</tr>
<tr>
<td><strong>A_I</strong></td>
<td>25.4±0.6</td>
</tr>
<tr>
<td><strong>A_E</strong></td>
<td>22.8±0.4</td>
</tr>
<tr>
<td><strong>Π_E</strong> (Collapse)</td>
<td>9.7</td>
</tr>
<tr>
<td><strong>Π_E</strong> (Spreading)</td>
<td>10.0</td>
</tr>
<tr>
<td><strong>Π_T</strong></td>
<td>20.6±0.3</td>
</tr>
<tr>
<td><strong>Π_C</strong></td>
<td>22.0±0.4</td>
</tr>
<tr>
<td><strong>AC</strong></td>
<td>19.9±0.2</td>
</tr>
<tr>
<td><strong>Π_C</strong></td>
<td>36.5±2.9</td>
</tr>
</tbody>
</table>

(A) is the area in Å²/molecule ± standard deviation. (Π) is the surface pressure in dynes/cm ± standard deviation. Subscripts indicate transformation points on the Π-A isotherm (Fig. 4 and 6).
at very slow rates (Fig. 5). Subsequent experiments showed that monolayers collapsed to $\Pi_E$ when compression was stopped at any pressure greater than $\Pi_E$, although the rate of collapse from films at pressures slightly greater than $\Pi_E$ was much slower than the rate of collapse from films in the high pressure or post-collapse regions of the isotherm. Thus $\Pi$ decreased about 35 dynes/cm in 1 minute when compressions were stopped in the post-collapse region of eicosanoic and docosanoic acid monolayers. (Fig. 4 and 6) while $\Pi$ decreased only 7.5 dynes/cm during the 20 minute equilibration sequence for the docosanoic acid film (Fig. 5).

Correlations between molecular structure in fatty acid monolayers and molecular structure in fatty acid crystals were first suggested by Müller (79), Lyons and Rideal (80), and Dervichian (81). Dervichian noted similarities between surface areas of specific monolayer phase transformations and the cross-sectional area reported for the B-form of stearic acid (76) and cross sectional areas which he calculated for the A and C forms of stearic acid. However, the surface area data obtained by Dervichian contained small errors and consequently his hypothesis was not generally accepted (82). Other hypotheses which involved intermolecular hydrogen bonding (83), the inhibition of chain rotation (84), and changes in the conformation of hydroxyl groups (85) were suggested as theoretical explanations for phase
transformations even though Stållberg-Stenhagen and Stenhagen (86), Stenhagen (87), and Lundquist (88) were able to relate different phases of a docosanoic acid monolayer with different packing modes for hydrocarbon crystals because of the similarities in their cross-sectional dimensions.

Fatty acid monolayers exhibit three phase transformation during their compression at room temperature (82, 86-88). In addition, complete phase diagrams can be constructed by doing monolayer studies at various temperatures. The method of assigning phases and denoting phase changes, however, is very complex, and often depends upon the detection of very minor changes in the compressibility (rate of change of $\Pi$ with change in area). Often experimental conditions are poorly defined and what one group of workers views as a phase transition may not appear as a phase transition to another group of workers. The $L_2$ phase classically appears at the initial or limiting area, point I on the $\Pi$-A isotherm, and this phase is transformed to the LS phase when the film is compressed to point T on the isotherm (Fig. 4 and 6). The LS to S phase transformation occurs between point T and C on the isotherm and the area at C is the smallest area at which the monolayer exists in the S phase. An additional low pressure phase, the $L_2'$ phase, exists in eicosanoic and docosanoic acid monolayers but this phase
is not observed unless the temperature of the monolayer is lowered below room temperature (86-88).

The S and LS phases are metastable states and may not even be seen if compression is carried out very slowly (Fig. 5). The L_2 phase has both stable and metastable regions. Monolayers in the S and LS phases are transformed spontaneously to the lower pressure and larger area L_2 phase which is stable at \( \Pi_E \). Brooks and Alexander (89), in a study of fatty alcohol monolayers predicted that monolayers were in a metastable state at pressures greater than \( \Pi_E \) and these monolayers would collapse to \( \Pi_E \). The effect of monolayer collapse on the shape of the compression isotherm has been described by Rabinovitch, Robertson and Mason (66). These investigators found that an octadecanoic acid monolayer could not be compressed to the classical collapse point when the film was compressed at a slow rate or when the film was repeatedly compressed and decompressed. These workers envisioned their phenomenon as being caused by the loss of molecules from the monolayer. This study shows that a phase transformation occurs at \( \Pi_E \) which is only visible when hexadecanoic acid is compressed at a very slow rate (Fig. 5), although the same phase transformation must certainly take place at faster compression rates. Fatty acids with longer hydrocarbon chains such as docosanoic acid
may be compressed somewhat beyond $\Pi_E$ and do not collapse to $\Pi_E$ during the equilibration period (Fig. 5). The absence of a visible transformation at $\Pi_E$ for docosanoic acid even at very slow compression suggests that the collapse rate for this fatty acid is much slower than the collapse rate for hexadecanoic acid. This transformation should be observed by a much slower compression rate or a longer equilibration period between intermittent compressions. The shape of the curve in the post-collapse region of the compression isotherm also suggests that the collapse rate depends on the chain length of the fatty acid. Thus hexadecanoic acid films always show a decrease in $\Pi$ at slow or fast compression rates (Fig. 6). It is apparent that the process of collapse of fatty acid monolayers is a dynamic time-dependent process which may be compensated by compression. This time-dependency compensation of collapse by compression explains why the low pressure phase transformation at $\Pi_E$ is not observed when monolayers are compressed at the usual rates.

Phase Transformation in Surface Films and Crystals

A relationship between molecular structure in monolayers and bulk phases was suggested by Stallberg-Stenhagen and Stenhagen (86), who showed that the surface area for docosanoic acid in the S phase at the collapse point was similar
to the cross-sectional area obtained by X-ray diffraction for hydrocarbons in the lower symmetry or triclinic phase (90, 91). They reasoned from these data that the lateral packing for the hydrocarbon chains of docosanoic acid in the S phase corresponded to the lateral packing for hydrocarbons in the triclinic crystal. In a later study, von Sydow (92), showed that the A-form of crystalline dodecanoic acid had the same structure as the triclinic hydrocarbon. Surface areas at the collapse point for all the fatty acids examined ($A_C$ in Table 3) are very similar to the cross-sectional areas for hydrocarbons in the triclinic phase, about 19.6 Å²/molecule. Thus all fatty acid monolayers in the S phase appear to have highly ordered structures which are closely related to their crystal structures in the A form.

Hydrocarbon chains for fatty acid monolayers are generally described as vertical and capable of both rotational and translational motion (86-88). A vertical orientation was suggested because the surface area of a molecule in the LS phase is only slightly larger than the surface area in the S phase. However, the surface areas of fatty acids at the point where they are transformed into the LS phase ($A_T$ in Table 3) are very similar to the cross-sectional areas obtained by x-ray diffraction for the tilted B-forms of these fatty acids. The cross-sectional areas obtained
by X-ray diffraction are 20.8 Å²/molecule for the B-form of hexadecanoic acid (93) and 20.5 to 20.7 Å²/molecule for the B-form of octadecanoic acid (79, 94). The correlations between monolayer surface area and the molecular area in a crystal lattice suggest that the fatty acid monolayers in the LS phase have highly ordered structures which are closely related to their tilted crystal structures in the B-form.

Both Stenhagen (87) and Lundquist (88) have suggested that hydrocarbon chains are tilted in the lower pressure and larger area L2' and L2 phases. The surface properties of enantionorphs and racemic mixtures in the L2 phase indicate that the phases have considerable molecular structure and liquid-to-solid transformations within these phases have been postulated (87, 88). The ΠE data support this hypothesis. Surface areas of fatty acids in ΠE (AE in Table 3) are very similar to the cross-sectional areas obtained by X-ray diffraction for the tilted C-forms of these fatty acids. Cross-sectional areas are 23.8 Å²/molecule for the C-form of hexadecanoic acid (90), 23.2 Å²/molecule for the C-form of docosanoic acid (96). The correlations between surface area at ΠE and the molecular area in a crystal form could be explained by a liquid-to-solid transformation at ΠE where the monolayer exists as a solid phase with a molecular structure of the C-form of the
fatty acid crystal. With rapid compression, the monolayer passes through the C, B, and A packing modes before it collapses into the stable C conformation. With slow or intermittent compression, sufficient time is available for the monolayer to collapse toward the C conformation during the compression experiment.

The data and explanations presented support the early hypotheses of Müller (78), Lyons and Rideal (80), and Dervichian (81) that molecular structure in saturated fatty acid monolayers is very similar to molecular structure in the different polymorphic forms of saturated fatty acid crystals. Saturated phospholipids exhibit polymorphism (96) and phase transformations have been observed in monolayers of saturated phospholipids (97-100), while unsaturated phospholipids with trans double bonds exhibit both polymorphism and a monolayer phase transformation (101). Membrane lipids are largely unsaturated and monolayers of these unsaturated fatty acids have large surface areas and do not exhibit phase transformations. Chapman, Owens, Phillips, and Walker (59), however, recently suggested that cholesterol reduced the molecular motion in the fatty acyl side chains of phospholipids. If cholesterol effectively reduces molecular motion, the phospholipid-cholesterol mixtures in membranes may have more highly ordered structures than has been previously supposed, and phase transformation
may be an important property of biological membrane.

**Monolayer Solution Into The Subphase**

The decrease in film area as a function of time for several fatty acid monolayers maintained at 16 dynes/cm is shown in Fig. 7. The rate of decrease in area was inversely proportional to chain length \((14:0 > 16:0 > 18:0)\) and directly proportional to the degree of unsaturation \((18:2 > 18:1 > 18:0)\). Since the \(\pi_E\) values for the unsaturated fatty acids were greater than 16 dynes/cm, it was obvious that monolayer stability was affected by other parameters besides collapse into the bulk phase. Fatty acids shorter than twelve carbon fatty acids could not be used in this study since they were too water soluble. The number of compounds used in this study was therefore limited. Highly fluid, higher molecular weight monolayers of lipids such as cholesterol or egg lecithin showed no film loss over extended periods of time (30 to 60 minutes).

Film losses were similar when octadecadienoic acid was spread on a trough exposed to the air and when octadecadienoic acid was spread on a trough enclosed in a nitrogen atmosphere (Fig. 8). In several experiments the octadecadienoic acid ampule was opened under nitrogen in the glove bag just before use. In other experiments octadecadienoic acid from an ampule that had been opened several weeks previously and stored under nitrogen was used. In other experiments,
Fig. 7. Effect of chain length and unsaturation on disappearance of fatty acids from a monolayer. The subphase contained 0.1M NaCl, 0.1mM EDTA and 0.01M tris-acetate buffered at pH 8.4. The temperature was 27°C.
Fig. 8. Effect of oxygen on the disappearance of octadecadienoic acid from a monolayer: 0, nitrogen atmosphere; Δ, air. The subphase is described in Fig. 7 except that the subphase for the anaerobic experiment also contained 0.2% hydroquinone. The temperature was 31°C.
the octadecadienoic acid was spread from a hexane solution. Similar results were obtained in all cases.

The effect of the hydrogen ion concentration in the subphase on film loss was studied by spreading fatty acid monolayers on subphases of varying pH (Fig. 9). The fraction of the initial film remaining at the time $t$, represented by $A_t/A_0$ was much less when the film was spread on alkaline subphases than when the film was spread on acid subphases. In all cases film loss was extensive on alkaline subphases and when the film loss was large, the rate of film loss decreased with time (see 14:0 at pH 8.4 in Fig. 9). The rate of film loss was therefore approximated from the relative change in monolayer area during the first minute of an experiment before other processes such as diffusion might control the rate of film loss. The initial rate of film loss was represented by $(\Delta A/A_0/\text{min.})$. These rates were low for all fatty acids when they were spread on acid subphases and increased markedly in a sigmoid shaped curve when the pH of the subphase was increased (Fig. 10).

The rate of film loss from monolayers was also studied as a function of temperature. (Fig. 11). Initial rates increased linearly for tetradecanoic, octadecanoic and octadecadienoic acids as the temperature was raised from $5^\circ C$ to $35^\circ C$. Hexadecanoic acid, however, had a definite
Fig. 9. Effect of subphase pH on disappearance of fatty acids from a monolayer. The subphase and temperature are described in Fig. 7.
At / Ao

1.0

MINUTES

14:0, pH 5.2

18:2, pH 5.2

18:2, pH 8.4

14:0, pH 8.4
Fig. 10. The effect of subphase pH on the initial rate of fatty acid disappearance from a monolayer. The subphase contained 0.1M NaCl, 0.1mM EDTA and 0.018M tris buffer. The temperature was 25°C.
Fig. 11. Effect of temperature on the initial rate of fatty acid disappearance from a monolayer. The subphase buffered at pH 8.0 is described in Fig. 10.
increase in the rate of solution between 20 and 25°C, at which point the slope of the curve increased considerably. The Arrhenius equation (102) allows the calculation of the energy of activation of a chemical reaction:

\[ E = \frac{2.303 (\log k_2 - \log k_1) R}{1/T_1 - 1/T_2} \]

where \( E \) is the energy of activation in calories per mole, \( T_1 \) and \( T_2 \) are the absolute temperatures at the two rates, and \( R \) is the gas constant in calories per mole. The energy of activation, which is a measure of the energy necessary to convert molecules to the reactive state, gave 1.5, 1.8, and 2.5 Kcal mole\(^{-1}\) respectively for tetradecanoic, octadecadienoic, and octadecenoic acids between 5 and 35°C, and 25 Kcal mole\(^{-1}\) for hexadecanoic between 20 and 35°C.

Several possible mechanisms have been proposed to explain contraction of monolayers. These include evaporation from the monolayer (73), collapse into a bulk phase (66, 68) which has been discussed in the previous section, solution into the subphase (69, 72, 74), and autoxidation of unsaturated compounds followed by solution of the short-chain reaction products (61).

Evaporation of fatty alcohols from monolayers has been described by Brooks and Alexander (73). These workers studied the losses of myristyl, cetyl, stearyl, and oleyl
alcohol at constant pH as a function of temperature. They theorized three possible mechanisms for film loss: collapse into the bulk phase, solubilization, and evaporation. Collapse was eliminated as rates of loss were similar regardless of whether the pressure was held above or below the equilibrium spreading pressures of the compounds. To test whether or not solution was occurring, they measured the rates of film loss on subphases of different ionic strength. When they found that the rate of loss of oleyl alcohol decreased with increasing subphase salt concentration, they concluded that oleyl alcohol was soluble in the subphase. With the other compounds tested, the rate of loss was the same regardless of the subphase salt concentration. They, therefore, postulated that the primary mechanism for film loss with saturated alcohols was by evaporation. In addition, they concluded that oleyl alcohol both evaporated and was solubilized at higher temperatures, due to the appreciable monolayer loss even at high subphase salt concentrations. To further substantiate their hypothesis of film evaporation, they measured the rates of film loss by blowing a stream of air across the surface of the film and finding that the rate of film loss increased. It was unlikely that evaporation contributed to the loss of fatty acids from monolayers in the present study since the rate of film loss increased
with increasing pH. Ionized carboxylate groups which were formed with increasing pH should interact strongly with the polar subphase. This increasing pH and increasing interaction, which should have retarded evaporation, or have had no effect on evaporation, actually enhanced film loss. In addition, the rate of film loss decreased with increasing ionic strength (Fig. 12,13), which should have no effect on evaporation.

As shown in the previous section, films were unstable and collapsed into the bulk phase with a decreased area at the air-water interface whenever they were maintained at surface pressures greater than their $\Pi_E$. The $\Pi_E$ for octadecanoic acid at 27°C was 7.3-7.8 dynes/cm (Table 3) and collapse probably could have explained the slow contraction of the monolayer when it was maintained at 16 dynes/cm on acid and alkaline subphases. The $\Pi_E$ values for octadecenoic acid and octadecadienoic acid at 27°C were 29 and 27 dynes/cm respectively. Since these values were greater than the 16 dynes/cm pressure generated by the piston oil, collapse into the bulk phase did not explain film loss with these unsaturated compounds. A similar argument could be extended to tetradecanoic and hexadecanoic acids which have $\Pi_E$ values at 27°C of 13-17 dynes/cm and 11-14 dynes/cm respectively (Table 3, 103). If collapse were responsible for the contraction
Fig. 12. Effect of pH and subphase ionic strength on the initial rate of tetradecanoic acid disappearance from a monolayer. The subphase contained 0.01M NaCl or 1.0M NaCl, 0.1M EDTA, and 0.01M tris buffer. The temperature was 25°C.
Fig. 13. Effect of ionic strength on the initial rate of tetradecanoic acid disappearance from a monolayer. The subphase contained NaCl, 0.1 mM EDTA, and 0.010 M tris buffer at pH 8.0. The temperature was 25°C.
Graph showing the relationship between NaCl concentration and (ΔA/A₀)/min.
of these monolayers, contraction would be relatively slow since their Π_E values are very similar to the Π_E of castor oil. Since saturated and unsaturated films with higher Π_E values contracted more rapidly than octadecanoic acid, a different mechanism besides collapse was necessary to explain film contraction.

Three results of the present study strongly supported the hypothesis that monolayer contraction occurred through fatty acid solution into the subphase. Film losses were directly proportional to the bulk phase solubilities of the saturated fatty acids in water (104). No data could be found on the solubilities of unsaturated fatty acids. Second, the rate of film loss was proportional to the hydrogen ion concentration in the subphase and the sigmoid function of rate vs. pH indicated that the contraction rate was proportional to carboxyl group ionization and the increased solubility of carboxylate anions. Similar results were obtained if different time intervals besides 0-1 minutes were used for these rate calculations although rates were much slower with other time intervals (Fig. 14-17). Third, the rate of film loss decreased with increasing ionic strength (Fig. 12,13). This is the familiar salting out effect used in organic chemistry to precipitate organic compounds. Fatty acid solubility in the aqueous subphase has been described by a number of different
Fig. 14. Effect of pH on the disappearance of tetradecanoic acid from a monolayer. The sub-phase and temperature are described in Fig. 10.
Fig. 15. Effect of pH on the disappearance of hexadecanoic acid from a monolayer. The sub-phase and temperature are described in Fig. 10.
Fig. 16. Effect of pH on the disappearance of octadecenoic acid from a monolayer. The subphase and temperature are described in Fig. 10.
Fig. 17. Effect of pH on the disappearance of octadecadienoic acid from a monolayer. The sub-phase and temperature are described in Fig. 10.
workers (69-72,74) Sebba and Briscoe (69) studied the decreases in surface pressure of palmitic and stearic acid held at constant areas. Because of the influence of pH on their results, they postulated the solubility of fatty acid films into the subphase. However, a number of their experiments were carried out considerably above the equilibrium spreading pressure and collapse into the bulk phase might possibly also have been a significant contributing factor in their studies. In a similar study (67), Gaines noticed decreases in the surface pressure of amines spread on acidic subphases. Gaines found distinct differences between rates of loss on hydrochloric acid and sulfuric acid subphases; the rate of solution was considerably greater on hydrochloric acid. Ter Minassian-Saraga (71) found that the rates of dodecanoic and tetradecanoic acid solution from films decreased with time, an observation that was confirmed in this study. She attributed this decrease to the fact that the contraction rate was dependent upon both the solution rate and the diffusion of the soluble species within the subphase. She postulated that the rate of film contraction became slower with time due to the build-up of fatty acid molecules in that portion of the subphase just beneath the interface and the formation of a pseudo-saturated solution in this area. To test her hypothesis, she agitated the subphase of a dodecanoic acid film and
found the rate of solubility was constant with time. Ter
Minassian-Saraga (71) furthermore found that the rate
of desorption was proportional to subphase temperature
as well as pH. In a similar study designed to show the
importance of ionic strength on surface potential (105),
Davies also noticed that the rate of desorption of
myristic acid was decreased with increasing ionic strength.
Gershfeld (72) has also used monolayer desorption studies
to calculate London-Vander Waals dispersion forces in
monolayers.

Recently a study appeared which showed the effects
of compression rate on the $\Pi$-$A$ isotherms of stearic acid
and myristic acid (74). With stearic acid on a 0.01 N
HCl subphase, there were no significant differences in
$\Pi$-$A$ isotherms with compression rates varying between
1.97 and 7.88 $\AA^2$/molecule/minute. With myristic acid
however, there were profound differences between $\Pi$-$A$
isotherms run from 1.97 to 7.88$\AA^2$/molecule/minute. With
decreasing compression speed, there was a shifting of the
$\Pi$-$A$ isotherms to smaller areas, which indicated a loss
of molecules. The authors attributed this effect to the
solubility of the myristic acid into the bulk phase. In
a similar study in our laboratory (106), the rate of
compression had a similar effect on monolayers of linoleic
acid spread on water. The higher the compression rate,
the more expanded was the $\Pi$-$A$ isotherm. When linoleic
Acid was run on 0.1 N HCl, however, the compression rate had no effect on the shape of the Π-A isotherm and the isotherms on HCl were similar to the most expanded curve on water. All these data were consistent with the solution of linoleic acid from the film.

The loss of unsaturated materials from the air-water interface has been investigated by Porter, Henick, and Clifford (61), who studied the kinetics of autoxidation of a linoleic acid film spread at the air-water interface. Their system which employed a freely floating barrier upon an aqueous subphase and a castor oil piston was the technique adopted in the present study. They suggested that the rate of oxidation could be determined by measuring the rate of movement of the barrier as the linoleic acid disappeared from the interface since there was no barrier movement in a nitrogen atmosphere. Barrier movement depended on pH and they attributed this effect to the formation of short-chain oxidation products which became increasingly soluble in the subphase with increasing pH. When the rate of oxidation of linoleic acid was plotted as a function of pH, the resulting curve appeared very similar to a titration curve with the half-maximum rate of oxidation at approximately pH 8. Porter et al. studied the effects of the well known antioxidants hydroquinone in the subphase and a-tocopherol spread with the
linoleic acid but found no apparent antioxidant effects. Because of their data on linoleic acid, Porter et al. speculated that all surface studies done with unsaturated compounds may be in error and indicated the necessity of reinvestigation.

However, the data of Kwong (106) on Π-A isotherms obtained at different compression rates and the data from the present study that film contraction took place at similar rates in both anaerobic and aerobic environments (Fig. 8) indicated that simple solution and not oxidation was primarily responsible for the disappearance of octadecadienoic films. Furthermore, film losses occurred with saturated fatty acids which are not susceptible to autooxidation and with octadecenoic acid which is less susceptible to oxidation than octadecadienoic acid. The hydrogen ion concentration in the subphase had the same general effect on all fatty acids studied. Porter et al. attributed this effect of hydrogen ion concentration as making the reaction products of the autooxidation more soluble, but failed to take into account the solubility of octadecadienoic acid itself. It is unfortunate that Porter et al. did not choose a saturated fatty acid which could not be oxidized as a control. If they had done so, they might not have been drawn into an incorrect interpretation of data from their very unique model system.
Surface pH

Measurements of film contraction rates may be used to investigate the effects of changes in monolayer ionization properties. The rate of film loss was a sigmoid function of bulk phase pH which resembled a titration curve (Fig. 10). If the solution rate were related to the number of carboxylate ions in the monolayer, as was likely, the pH at which the fatty acid was half-ionized could be estimated from rate data by determining the pH at which the rate was half maximum. By this method, the apparent pKa of hexadecanoic acid was 9.7, octadecenoic acid 8.3, octadecadienoic acid 8.0, and tetradecanoic acid 7.9. This point was several pH units to the alkaline side of the point where soluble fatty acids were half-ionized in aqueous solutions, although the pKa for oleic acid in an emulsion has been reported to be higher (107, 108).

The concept of surface pH was first described by Peters in 1931 (109) when he studied the interfacial tension of fatty acids adsorbed at a benzene-water interface. Peters found the half-maximum change in surface tension, which he attributed to the fatty acid being half-ionized, about 3 pH units to the alkaline side of the point where fatty acids were half ionized in bulk solution. Peters postulated that these changes could be attributed to
different pH values between the bulk phase and interfacial region. He suggested that shifts in the pKa of the acids were unlikely, and to this day the conjectures of Peters on surface pH and pKa are accepted as dogma. Danielli, in 1937 (110) found similar pH shifts in interfacial tension studies with palmitic acid. He attributed these shifts to the ionization of the fatty acid and he showed that this ionization was dependent upon the ionic strength of the aqueous phase. Similar pH changes have also been found for indicator dyes in solution (111). These lipid soluble dyes were adsorbed at interfaces and seemed to indicate that the pH values of bulk solutions and interfaces were quite different.

Similar pH shifts could be interpreted from the data of Goddard and Ackilli (112). These workers showed that II-A isotherms for stearic acid on 0.01 M NaCl were identical from pH 3-8.5, but curves shifted to smaller areas above pH 8.5. This indicated that stearic acid was un-ionized until the pH was raised above pH 8.5, where ionization began. The decrease in molecular area at pH values above pH 8.5 may have been caused by solution into the subphase.

Surface potential studies have also been used to demonstrate changes in surface pH. Christodoulou and Rosano (113) recently studied the effects of pH on the surface potentials of various saturated fatty acids. This
procedure depended upon the fact that surface potential (ΔV) for fatty acid monolayers decreased with an increase in subphase pH. According to Schulman and Hughes (114), the surface potential for ionized monolayers was as follows:

$$\Delta V = 12 \frac{\mu}{A} + \psi_{AB}$$

where ΔV was expressed in millivolts, A in Å²/molecule, \(\mu\) the vertical dipole moment in millidebyes and \(\psi_{AB}\) the potential drop between the charged surface AB and the bulk phase in millivolts. Under the conditions of the experiment, Christodoulou and Rosano showed decreases in ΔV with increases in pH, which would be attributed to decreases in \(\psi_{AB}\). When their data was plotted, Christodoulou and Rosano showed an apparent shift in the pKa 3-4 units to the alkaline side of the bulk pKa value for short chain fatty acids in solution. However, these results must be accepted with reservation due to the fact that the ionized fatty acid molecules may have left the monolayer at high pH values and introduced errors.

Davies and Rideal (115) have also suggested those pKa shifts may be explained by the difference between surface and bulk phase pH values. These differences in pH arise because of the attraction for positively charged hydrogen ions by the negatively charged fatty acid monolayer. This attraction would neutralize the excess
negative charge present at the interface and would
decrease the charge repulsion and help stabilize the mono-
layer. This increase in the number of hydrogen ions at
the interface would cause the surface pH to be consider-
ably lower than the bulk phase pH. An alternative ex-
planation would be that the ionization of the carboxyl
groups at the interface was affected and the surface and
bulk pH values were actually the same.

In 1910 Gouy (116) proposed his theory of the diffuse
double layer when he solved Boltzman equations for the
distribution of ions in terms of a potential difference
Ψ near the surface relative to the bulk of the liquid.
The Boltzman equations are:

$$C_{\text{surface}} = C_{\text{bulk}} \exp(-e \Psi/kT)$$

where C refers to hydrogen ion concentrations in the sur-
fase and bulk phases, e is the charge on the electron,
Ψ is the potential difference between the subphase and the
surface, K the Boltzman constant, and T the absolute
temperature (111). Since by definition, the pH equals the
negative log of the hydrogen ion concentration, it follows:

$$\text{pH}_{\text{surface}} = \text{pH}_{\text{bulk}} + e\Psi/2.3kT$$

According to these formulas derived by Hartley and Roe
(111), the large pH shifts as seen in the present study
indicate that there would be large potential differences
between the surface and the bulk phases (Fig. 10,12). For a positively charged surface this potential (\(\Psi\)) would be positive and for a negative surface this potential would be negative. Davies (105) and Crisp (117) showed that this potential difference (\(\Psi\)) was proportional to subphase salt concentration but did not relate their findings to possible differences in surface pH phenomena. Davies showed that \(\Psi\) decreased 57mv with each ten-fold increase in salt concentration for amines while Crisp showed that \(\Psi\) increased by 57mv for each ten-fold increase in salt concentration for acids. If these mathematical relationships relating surface pH to bulk pH are correct, then an increase in salt concentration should cause increases in surface pH or decreases in the apparent pKa, which is the opposite of the observations in this study (Fig. 12). The results of Danielli (110) and Mattson and Volpenheim (107) do however, indicate that increases in salt concentration cause decreases in surface pKa. These anomalies may be interpreted by assuming either that these equations do not hold for a monolayer system, or that the system used in this study is not applicable for studying surface pH phenomena. Since the data arrived at with this system tend to agree with data obtained by other monolayer techniques such as surface pressure (112) and surface potential (113), the validity of the equations must be questioned.
Charged groups on surfaces sometimes exhibit anomalous behavior. Quarles and Dawson (49,118) found that the addition of an anionic detergent to an aqueous suspension shifted the pH optimum of phospholipase hydrolisis. Although the explanation for this shift is unknown, quite possibly the addition of the detergent caused the reaction to take place on the surface of the detergent, which may have had a different pH from the bulk phase. In addition, Papahadjopoulos and Weiss (119) found that liquid crystalline vesicles of phosphatidyl ethanolamine did not react with 2,4,6,-trinitrobenzene sulfonic acid as was expected by normal condensation with free amino groups. They suggested that reactivity was affected more by salt linkages than by surface pH effects, since the surface pH changes calculated by the Hartley-Roe equation (111) were not large. However, early studies by Peters (109) and Danielli (113), in addition to the data reported in this study, show that surface pH charges may indeed be large and these changes could account for altered reactivity. In addition, these large pH effects and subsequent potential differences could account for the passing of protons in biological systems.

Phase transitions in fatty acid monolayers have been studied by a number of investigators and phase diagrams for saturated fatty acids have been prepared (86-88). All fatty acids studied showed increases in solubility with
increases in temperature (Fig. 18-21), but only hexadecanoic acid appeared to undergo a phase change as measured by initial solution rates in the temperature range used (Fig. 11). This would be expected, since tetradecanoic, octadecenoic, and octadecadienoic acids form liquid monolayers between 5 and 35°C. This, the increase in solution rate between 15 and 25°C for hexadecanoic acid may be related to the CS → L₂ phases transition (84). The high activation energy of solutions indicated that solution is occurring from a different phase than from the other fatty acid films.
Fig. 18. Effect of temperature on the disappearance of tetradecanoic acid from a monolayer. The subphase contained 0.1M NaCl, 0.1mM EDTA, and 0.0183M tris buffer at pH 8.0.
Fig. 19. Effect of temperature on the disappearance of hexadecanoic acid from a monolayer. The sub-phase is described in Fig. 18.
Fig. 20. Effect of temperature on the disappearance of octadecenoic acid from a monolayer. The subphase is described in Fig. 18.
Fig. 21. Effect of temperature on the disappearance of octadecadienoic acid from a monolayer. The sub-phase is described in Fig. 18.
CHAPTER V
SUMMARY

Force-area isotherms were obtained for hexadecanoic, octadecanoic, eicosanic and docosanoic acid monolayers at different compression rates. Equilibrium spreading pressures were determined both by monolayer collapse and spreading from the bulk phase. Monolayers formed metastable phases at all pressures above their equilibrium spreading pressures and at all surface areas smaller than the surface areas at their equilibrium spreading pressures. These metastable phases collapsed to stable phases at the equilibrium spreading pressures of the fatty acids. Collapse phenomena and compression experiments at very slow compression rates suggested that a previously unrecognized phase transformation occurred at the equilibrium spreading pressure. The surface area at this phase transformation corresponded to the cross-sectional area of the C-form of the fatty acid crystal. Fatty acid monolayers at the phase transformations previously described by other workers had surface areas which were closely related to molecular areas in their A and B polymorphic crystal forms. These
correlations indicated that molecular structure in saturated fatty acid monolayers was similar to molecular structure in fatty acid crystals.

The contraction or decrease in area of fatty acid monolayers maintained at a constant surface pressure of 16 dyne/cm was studied as a function of fatty acid chain length, unsaturation, temperature, and the hydrogen ion concentration in the subphase. The data were consistent with the hypothesis that fatty acid solution from the monolayer into the subphase was the mechanism for film loss. Autoxidative reactions did not contribute significantly to film loss since contraction occurred with saturated fatty acid monolayers and with unsaturated fatty acid monolayers in an anaerobic environment. The decrease in area per unit time or the solution rate was inversely proportional to chain length and directly proportional to the degree of unsaturation. Arrhenius plots showed activation energies of 1.5-2.5 Kcal mole\(^{-1}\) for tetradecanoic, octadecenoic, and octadecadienoic acids and 25 Kcal mole\(^{-1}\) for hexadecanoic acid. The solution rate from the monolayer increased in a sigmoidal fashion with an increase in subphase pH and the apparent surface pKa was estimated as the point where the solution rate was half-maximum. Apparent surface pKa values were
hexadecanoic acid 9.7, octadecenoic acid 8.3, tetradecanoic acid 7.9, and octadecadienoic acid 8.0.

Further work must be done to determine the effects of ionic strength on the apparent pKa of fatty acids spread as monolayers. The discrepancies between pKa shifts caused by changes in ionic strength as seen in this study compared to the opposite shifts seen in other studies must be resolved. In addition, surface pH studies must be done with amines, which are positively charged, so that the ionization properties of these monolayers may be better understood in terms of the ionization potential (Ψ) which has an opposite sign for amine and acid monolayers.
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